„Signalerkennungs- und Signalwandlungsprozesse bei der Auslösung von nichtkultivarspezifischen Pathogenabwehrmechanismen in Pflanzen“

H A B I L I T A T I O N S S C H R I F T

zur Erlangung des akademischen Grades

Doctor rerum naturalium habilitatus (Dr. rer. nat. habil.)

vorgelegt der

Mathematisch-Naturwissenschaftlich-Technischen Fakultät
der Martin-Luther-Universität Halle-Wittenberg

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Geb. am 07. April 1962 in Dresden

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urn:nbn:de:gbv:3-000005837
[http://nbn-resolving.de/urn/resolver.pl?urn=nbn%3Ade%3Agbv%3A3-000005837]

Inhalt Seite

Vorbemerkung

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1. Die Grundlagen der pflanzlichen Pathogenresistenz

Die Fähigkeit zur Unterscheidung zwischen „Selbst“ und „Nicht-selbst“ ist eine grundlegende Eigenschaft aller Lebensformen. In Pflanzen ist sie beispielsweise von entscheidender Bedeutung für die Aufrechterhaltung der Selbstinkompatibilität während der Reproduktion (Abwehr von „selbst“) oder für die Aktivierung von Abwehrreaktionen gegen mikrobielle Infektionen (Abwehr von „nicht-selbst“).

Das Spektrum pflanzlicher Abwehrreaktionen gegen mikrobielle Infektionen ist komplex und in vielen Pflanze/Pathogen-Interaktionen sehr ähnlich (Boller, 1995; Ebel und Scheel, 1997; Yang et al., 1997; Heath, 2000; Dangl und Jones, 2001; Nürnberger und Scheel, 2001). Die Auslösung eines Teils dieser Reaktionen beruht auf der transkriptionellen Aktivierung von Genen, die für Enzyme der Biosynthese antimikrobieller Phytoalexine, für Abwehrproteine unbekannter molekularer Funktion („*pathogenesis-related proteins*“, PR-Proteine) oder für lytische Enzyme (Chitinasen, Glukanasen, Proteasen) kodieren (Kombrink und Somssich, 1995; Scheel, 1998). Andere Abwehrmechanismen, wie z. B. die oxidative Quervernetzung der Zellwände, die Einlagerung von Kallose und Lignin in die Zellwände sowie die Produktion antimikrobieller reaktiver Sauerstoffspezies (H_2O_2, O_2^-), basieren dagegen auf der Aktivierung spezifischer Enzyme (Yang et al., 1997; Scheel, 1998; Alvarez, 2000; Bolwell et al., 2002). Eine sehr häufige, aber nicht immer beobachtete Abwehrreaktion stellt der hypersensitive Zelltod (hypersensitive Reaktion, HR) dar, der durch das lokal begrenzte Absterben pflanzlicher Zellen am Infektionsort charakterisiert ist (Yang et al., 1997; Alvarez, 2000). Dieser „programmierte Zelltod“ zeigt auf zellbiologischer Ebene Ähnlichkeiten zum apoptotischen Zelltod tierischer Zellen (Lam et al., 2001). Aufgrund fehlender Kenntnisse über die molekulare Basis der pflanzlichen HR lassen sich solche Parallelen momentan jedoch nicht zufriedenstellend belegen. Abwehrreaktionen im die Infektionsstelle umgebenden („lokalen“) Gewebe werden in vielen Pflanzen begleitet durch eine Aktivierung von Resistenzmechanismen in nichtinfizierten Teilen der Pflanze (Dong, 2001). Für die Ausprägung dieser pathogenunspezifischen, systemischen Abwehrreaktionen („systemic acquired resistance“, SAR), welche Pflanzen für eine längere Zeit gegen Folgeinfektionen durch ein weites Spektrum verschiedener Erreger schützen, scheint Salizylsäure von herausragender Bedeutung zu sein (Dong, 1998). Die Pflanzenhormone Ethylen und Jasmonsäure scheinen hingegen notwendig für die Aktivierung einer anderen Form der systemischen Resistenz (induzierte systemische Resistenz, ISR) zu sein, die u.a. durch Kontakt mit nichtpathogenen Bodenbakterien wie *Pseudomonas fluorescens* hervorgerufen wird (Dong, 1998; Pieterse und van Loon, 1999).

Allgemein wird angenommen, daß das Zusammenspiel konstitutiver und induzierbarer Barrieren zur pflanzlichen Resistenz gegen mikrobielle Invasoren führt. Diese Sicht wird durch die Tatsache unterstützt, daß es in keinem der bearbeiteten Modellsysteme zum Studium von Pflanze/Pathogen-Interaktionen bislang gelungen ist, die Abwehrreaktion zu definieren, die letztlich entscheidend für die Unterdrückung oder den Abbruch einer Infektion ist.

Grundsätzlich unterscheidet man zwischen zwei Typen der pflanzlichen Pathogenresistenz. Die Tatsache, daß die meisten Pflanzenarten in ihrer natürlichen Umgebung resistent gegen die meisten potentiell phytopathogenen Mikroorganismen sind, wird als Spezies- oder Nichtwirtsresistenz bezeichnet. Diese Resistenzform, die auch als Basisresistenz oder Basisinkompatibilität bekannt ist, beschreibt ganz allgemein die Unfähigkeit einer mikrobiellen Spezies, bestimmte Pflanzenarten kolonisieren und als Nährstoffquelle zur Komplettierung ihres Lebenszyklus erschließen zu können. Die Immunität ganzer Pflanzenspezies gegen phytopathogene Mikroorganismen stellt die vorherrschende Form der pflanzlichen Krankheitsresistenz dar (Heath, 2000). Sie ist gekennzeichnet durch eine relativ hohe genetische Stabilität, welche sich in nur selten vorkommenden Veränderungen im Wirtsspektrum pflanzlicher mikrobieller Pathogene manifestiert (Heath, 2000; Kamoun, 2001). In Fällen, in denen Mikroorganismen nicht in der Lage sind, die pflanzlichen Abschlußgewebe zu überwinden und präformierte Abwehrstoffe (Saponine, zyanogene Glykoside, Phenole, Laktone) zu tolerieren, findet keine Erkennung des Pathogens durch die Pflanze statt, und es werden keine Abwehrreaktionen ausgelöst (Abb. 1). Neben konstitutiven Mechanismen tragen jedoch auch induzierbare Abwehrstrategien zur Ausprägung der Nichtwirtsresistenz bei. Die Initiation aktiver Pathogenabwehr erfordert die Wahrnehmung pathogenspezifischer Strukturen durch pflanzliche Perzeptionssysteme. Eine Vielzahl von Pathogen-assoziierten Oberflächenstrukturen, sogenannte „generelle Elicitoren“, sind als Auslöser von sorten- oder nichtkultivarpezifischen Abwehrreaktionen in Pflanzen beschrieben worden (Boller, 1995; Ebel und Scheel, 1997; Nürnberger, 1999). Außerdem ist bekannt, daß von Pathogenen stammende

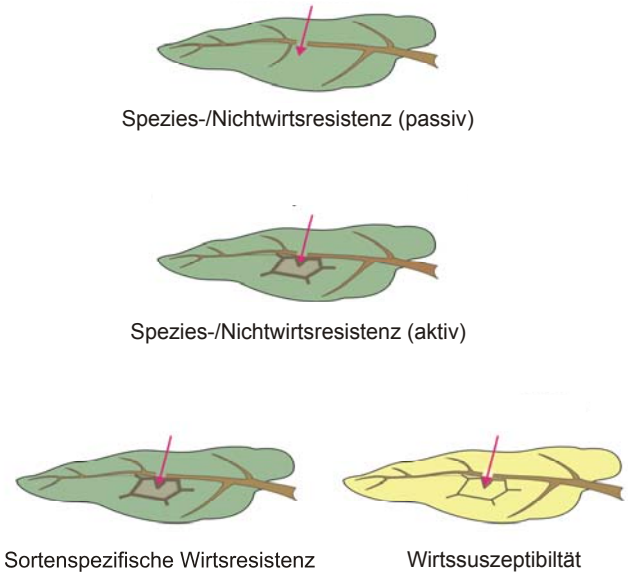

Abb. 1: Übersicht über die verschiedenen Formen der pflanzlichen Pathogenresistenz

lytische Enzyme in der Lage sind, insbesondere Kohlenhydratfragmente aus der pflanzlichen Zellwand freizusetzen, die ihrerseits pflanzliche Abwehrreaktionen auslösen können.

Im Verlaufe der Evolution haben Mikroorganismen Pathogenizitätsfaktoren erworben, die es ihnen ermöglichen, bestimmte (im Vergleich zur Zahl der Nichtwirtspflanzenspezies jedoch wenige) Pflanzenarten zu kolonisieren und diese in Wirtspflanzen zu verwandeln (Heath, 2000; Cohn et al., 2001). Interaktionen zwischen virulenten Pathogenen und suszeptiblen Pflanzenspezies werden mit den Begriffen Wirtssuszeptibilität bzw. Basiskompatibilität beschrieben. Der auf suszeptiblen Wirtspflanzen lastende Selektionsdruck hat in bestimmten Sorten einer an sich suszeptiblen Pflanzenspezies jedoch zur Ausprägung von Resistenzen geführt (sorten- oder kultivarspezifische Form der Wirtsresistenz). Ein Merkmal dieser sogenannten Wirtsresistenz ist es, daß sie oft nur gegen eine oder einige wenige (avirulente) Rassen oder Stämme einer Pathogenspezies gerichtet ist. Deshalb wird in diesem Zusammenhang oft auch von rassen-/kultivarspezifischer Wirtsresistenz oder inkompatiblen Pflanze/Pathogen-Interaktionen gesprochen (Abb. 1) (Cohn et al., 2001; Dangl und Jones, 2001).

Die „Gen-für-Gen-Hypothese“ beschreibt die genetische Basis der pflanzenkultivar-spezifischen Krankheitsresistenz (Flor, 1955; Flor, 1971; De Wit, 1996). Danach kodieren Avirulenzgene (*AVR*) des Pathogens und pflanzliche Resistenzgene (*R*) für Produkte, deren direkte oder indirekte Interaktion zur Auslösung von Abwehrreaktionen und letztlich zur Resistenz führen (van der Biezen und Jones, 1998; Takken und Joosten, 2000; Dangl und Jones, 2001; Schneider, 2002). Die Abwesenheit eines der beiden einander komplementären Gene oder die nichtfunktionelle Expression der Genprodukte führt dabei zur Krankheit der infizierten Pflanze. Obwohl die physiologische Funktion der bislang identifizierten AVR-Proteine häufig unklar ist, wird davon ausgegangen, daß sie als Virulenzfaktoren (z.B. als Toxine) zur Kolonisation des Wirtes beitragen, und nur in resistenten, das komplementäre *R*-Gen exprimierenden Pflanzenkultivaren als „spezifische Elicitoren“ von Abwehrreaktionen wirken. Die einfachste biochemische Interpretation der „Gen-für-Gen-Hypothese“ geht davon aus, daß AVR-Proteine Liganden sind, die direkt an *R*-Gen-kodierte Rezeptoren binden. Obwohl solche Ligand/Rezeptor-Paare identifiziert und ihre direkte Bindung aneinander belegt worden ist (Tang et al., 1996; Jia et al., 2000), scheint die molekulare Basis der Pathogenerkennung in anderen Pflanze/Pathogen-Interaktionen weitaus komplexer zu sein. Bislang sind mehr als 30 *R*-Gene identifiziert und charakterisiert worden, die pflanzenkultivarspezifische Resistenz gegen phytopathogene Viren, Bakterien, Oomyzeten, Pilzen, Nematoden und Insekten vermitteln (Takken und Joosten, 2000; Hulbert et al., 2001; Bonas und Lahaye, 2002). Dabei wurden leuzinreiche Sequenzwiederholungen („leucine-rich repeats“, LRR) und „coiled-coil“-Domänen als sehr häufig auftretende Strukturmerkmale beschrieben, die in der Lage sind, Protein/Protein-Wechselwirkungen einzugehen (Kobe und Deisenhofer, 1994). In einigen untersuchten Fällen hat sich jedoch gezeigt, daß *R*-Gen-kodierte LRR-Proteine nicht direkt mit AVR-Proteinen interagieren, sondern höchstwahrscheinlich Komponenten größerer Signalperzeptionskomplexe sind. In Verbindung mit dem experimentellen Nachweis von AVR-Protein-Bindeproteinen in suszeptiblen

Pflanzenkultivaren bildeten diese Ergebnisse die Grundlage für die sogenannte „Guard"-Hypothese (Dixon et al., 2000; Cohn et al., 2001; Dangl und Jones, 2001; van der Hoorn et al., 2002). Dieses Modell besagt, daß AVR-Proteine als Virulenzfaktoren zuerst mit Bindungsstellen („virulence targets") interagieren, über die mikrobielle Pathogene versuchen, den Wirtszellmetabolismus zu ihrem Vorteil zu manipulieren. Virulenzfaktoren werden erst dann zu Elicitoren sortenspezifischer Resistenzreaktionen und damit zu Avirulenz-bestimmenden Faktoren, wenn Resistenzgenprodukte als Teil des Signalperzeptionsmechanismus („guards") fungieren. Die physiologische Funktion von R-Proteinen ist mit anderen Worten also die Wahrnehmung der Komplexbildung zwischen AVR-Proteinen und ihren pflanzlichen Zielproteinen und die damit verbundene Störung von Wirtszellfunktionen. Dieses Modell wird durch experimentelle Befunde aus verschiedenen Pflanze/Pathogen-Interaktionen gestützt (Kooman-Gersmann et al., 1996; Ji et al., 1998; Ren et al., 2000; Luderer et al., 2001; Krüger et al., 2002; Mackey et al., 2002). Letztere Arbeiten zeigen z.B., daß einige Pathovare der Bakterienspezies *Pseudomonas syringae* Effektorproteine produzieren (AvrRPM1, AvrB), deren Funktion es ist, durch Bindung an das *Arabidopsis*-Protein RIN4 (ein Regulator der Pflanzenabwehr) pflanzliche Abwehrreaktionen zu unterdrücken. Nur in solchen Ökotypen (Kultivaren) von *Arabidopsis*, die das Resistenzgen *RPM1* (Grant et al., 1995) exprimieren, kann diese Störung der Wirtszellhomöostase wahrgenommen und damit die Aktivierung der Pflanzenabwehr eingeleitet werden (Mackey et al., 2002).

2. Die molekulare Basis der Nichtwirtsresistenz und das Konzept der „angeborenen Immunität"

2.1. Generelle Elicitoren als Auslöser der nichtkultivarspezifischen Pflanzenabwehr

Unabhängig von der Art der pflanzlichen Resistenz, die einer aktiven Pathogenabwehr zugrunde liegt, erfordert deren Auslösung eine sehr sensitive Wahrnehmung pathogenspezifischer Strukturen. Nichtkultivarspezifische, pflanzliche Abwehrreaktionen können durch sogenannte Elicitoren induziert werden. Dieser Begriff, der ursprünglich für Stoffe geprägt wurde, die in Pflanzen die Akkumulation von antimikrobiellen Phytoalexinen auslösen (Keen und Bruegger, 1977; Darvill und Albersheim, 1984), wird heute ganz allgemein zur Bezeichnung von Signalmolekülen verwendet, die die Pathogenabwehr (oder Teilreaktionen davon) stimulieren (Boller, 1995; Ebel und Scheel, 1997; Nürnberger, 1999). Diese Signale umfassen (Glyko)proteine, Peptide, Kohlehydrate und Lipide aus nahezu allen bekannten Gattungen mikrobieller Krankheitserreger (Boller, 1995; Ebel und Scheel, 1997; Nürnberger, 1999; Heath, 2000). Die eigentlichen molekularen und physiologischen Funktionen dieser Elicitoren sind jedoch häufig ungeklärt. Aufgrund ihrer oft konstitutiven Expression scheinen sie entweder Strukturkomponenten der Zellwand, Toxine oder Enzyme zu sein. Mikrobielle Toxine mit der Fähigkeit, Abwehrreaktionen in tierischen oder pflanzlichen Zellen auszulösen, sind in der Literatur vielfach beschrieben worden (van 't Slot und Knogge,

2002). Beispiele für Enzyme mit Elicitoraktivität sind z.B. Xylanasen aus *Trichoderma spp*. (Enkerli et al., 1999; Furman-Matarasso et al., 1999) oder Transglutaminasen aus *Phytophthora* (Brunner et al., 2002). Im Gegensatz zu Endopolygalakturonasen von *Erwinia carotovora* (Davis et al., 1984; Davis und Hahlbrock, 1987) entfalten diese Enzyme ihre Elicitoraktivität jedoch nicht durch die hydrolytische Freisetzung von elicitoraktiven Abbauprodukten aus der pflanzlichen Zellwand.

Die Isolierung und strukturelle Charakterisierung einer nahezu unüberschaubaren Zahl von Elicitoren in den letzten 15-20 Jahren läßt vermuten, daß Pflanzen über sehr große molekulare Kapazitäten zur Erkennung von potentiellen Mikroorganismen verfügen. Generell gilt jedoch, daß sich einzelne Elicitoren durchaus wesentlich in ihrer Wirkung auf verschiedene Pflanzen unterscheiden. Es ist bis heute nicht verstanden, weshalb bestimmte Pflanzen Perzeptionssysteme für bestimmte generelle Elicitoren besitzen und ob bzw. welchen Beitrag die durch diese Signale ausgelösten Abwehrreaktionen zur Speziesresistenz leisten (Heath, 2000; Nürnberger und Brunner, 2002). Ein besseres Verständnis für die Tatsache, weshalb Pflanzen derartige „antigene" Strukturen auf Pathogenoberflächen zur Nichtselbsterkennung und zur Aktivierung ihres Abwehrsystems ausnutzen können, eröffnet ein Blick auf die Mechanismen der Aktivierung angeborener Immunreaktionen („innate immunity") in tierischen Systemen. Im Jahre 1997 erstellten Medzhitov & Janeway eine Terminologie zur Beschreibung dieser ersten und direkt aktivierbaren „Verteidigungslinie" gegen mikrobielle Infektionen in Tieren (Medzhitov und Janeway, 1997). Danach sind charakteristische mikrobielle Strukturen sogenannte Pathogen-assoziierte molekulare Muster („pathogen-associated molecular patterns", PAMP), die an spezielle Rezeptoren („pattern recognition receptors") potentieller Wirte binden und die Expression von Abwehrgenen sowie die Produktion antimikrobieller Proteine und Peptide auslösen. Diese Immunreaktionen sind in verschiedenen tierischen Systemen (Mensch, Maus, Fische, Krustentiere, Insekten) sehr intensiv bearbeitet worden, und es hat sich gezeigt, daß das Konzept der Nichtselbsterkennung evolutionär erstaunlich konserviert zu sein scheint (Medzhitov und Janeway, 1997; Aderem und Ulevitch, 2000; Khush und Lemaitre, 2000; Imler und Hoffmann, 2001; Underhill und Ozinsky, 2002). Sehr gut untersuchte mikrobielle Auslöser tierischer Abwehrreaktionen sind z.B. die Lipopolysaccharidhülle (LPS) Gram-negativer Bakterien, Peptidoglykane Gram-positiver Bakterien, eubakterielles Flagellin sowie Glukane, Chitine, Mannane und verschiedene Proteine pilzlicher Herkunft. Interessanterweise sind eine Reihe dieser Substanzen zum Teil seit langer Zeit als Auslöser nichtkultivarspezifischer Abwehrreaktionen in verschiedenen Pflanzen bekannt (Boller, 1995; Ebel und Scheel, 1997; Nürnberger, 1999; Dow et al., 2000). So sind beispielsweise Komponenten des bakteriellen LPS oder Glukan- und Chitinfragmente der Zellwand phytopathogener Oomyzeten potente Induktoren der Pflanzenabwehr. Ein weiterer, sehr detailliert untersuchter Elicitor von Abwehrreaktionen in so verschiedenen Pflanzen wie *Arabidopsis* und Tomate ist ein N-terminales Fragment (flg22) aus bakteriellem Flagellin (Felix et al., 1999). Diese Befunde sind deshalb bedeutsam, da sie andeuten, daß Pflanzen ganz ähnlich wie Tiere die Fähigkeit erworben haben, Pathogene anhand typischer mikrobieller Oberflächensignaturen

wahrzunehmen, und diese Erkennung in Abwehrreaktionen umzusetzen. Es sollte hier jedoch auch erwähnt werden, daß die Strukturepitope von etwa Flagellin, die zur Erkennung durch Tier- und Pflanzenzellen notwendig sind, variieren. So ist das flg22-Motiv zur Auslösung von Immunreaktionen in tierischen Zellen nicht vonnöten (Donnelly und Steiner, 2002). Außerdem scheinen Unterschiede in der Erkennung von flg22 bereits zwischen verschiedenen Pflanzenspezies zu bestehen, da die Responsivität von Reiszellen auf Flagellin unabhängig von flg22 zu sein scheint (Felix et al., 1999; Che et al., 2000). In ähnlicher Weise reagieren Sojabohnenzellen, nicht aber Reis, auf Hepta-ß-glukoside aus den Zellwänden phytopathogener Oomyzeten der Gattung *Phytophthora* mit der Synthese von Phytoalexinen, während Tetraglukosylgluzitol (ein dem Hepta-ß-glukosid strukturell nicht ähnliches ß-Glukan aus *Pyricolaria oryzae*) Abwehrreaktionen in Reis, jedoch nicht in Soja, induziert (Klarzynski et al., 2000; Mithöfer et al., 2000; Yamaguchi et al., 2000).

Die Auslöser der angeborenen Immunantwort in tierischen Systemen (PAMP) zeichnen sich dadurch aus, daß sie typische Merkmale ganzer Pathogengattungen sind, jedoch in potentiellen Wirtsorganismen nicht vorkommen. Außerdem sind sie bedeutsam für bestimmte Lebensfunktionen des Pathogens und deshalb evolutionär stark konserviert (Medzhitov und Janeway, 1997; Aderem und Ulevitch, 2000). Sind Elicitoren nichtkultivarspezifischer pflanzlicher Abwehrreaktionen (wie z. B. der Synthese antimikrobieller Phytoalexine) nun möglicherweise funktionelle Äquivalente von PAMPs, die u.a. die Synthese antimikrobieller Substanzen in tierischen Systemen auslösen? Und weisen Ähnlichkeiten in der molekularen Organisation von Nichtselbsterkennungssystemen in verschiedenen eukaryotischen Reichen möglicherweise auf einen gemeinsamen Ursprung oder eine konvergierende Evolution dieses Prinzips hin?

Im Rahmen der vorliegenden Arbeit wurde ein 13 Aminosäuren umfassendes Peptidfragment (Pep-13) in einer Zellwandtransglutaminase von *Phytophthora sojae* identifiziert, das komplexe Abwehrreaktionen in Petersilie und Kartoffel stimuliert (Nürnberger et al., 1994; Brunner et al., 2002). Pep-13 ist nahezu vollständig konserviert in Transglutaminasen der Gattung *Phytophthora*, scheint jedoch in Pflanzen nicht vorzukommen. Außerdem konnte gezeigt werden, daß das „antigene Epitop“, d.h. die für die Initiation der Pflanzenabwehr notwendige Struktur, auch essentiell für die Transglutaminaseaktivität, d.h. für die endogene Aktivität des Proteins ist (Brunner et al., 2002). Damit erfüllt dieser Elicitor die klassischen Kriterien eines PAMPs der angeborenen Immunantwort in Tieren (s. auch Kap. 3) ebenso wie ein in nahezu allen Bakterien vorkommendes RNA-Bindungsprotein, für das in einer ähnlich konzipierten Studie gleiche Eigenschaften nachgewiesen werden konnten (Felix und Boller, 2003). Desweiteren ist davon auszugehen, daß pilzliches Chitin, Glukane von Oomyzeten oder Bestandteile des bakteriellen Flagellins (flg22) ebenfalls Strukturen sind, die unverzichtbar für das jeweilige mikrobielle Pathogen sind, und damit ebenfalls als PAMPs anzusehen sind.

2.2. Erkennung komplexer Oberflächenmuster mikrobieller Pathogene durch pflanzliche Zellen

Pflanzenzellen werden bei der Interaktion mit mikrobiellen Pathogenen mit einer Vielzahl „antigener" Oberflächenstrukturen, d.h. potentieller Elicitoren, konfrontiert. So enthalten pilzliche Zellwände beispielsweise Chitinfragmente, N-mannosylierte Glykopeptide sowie Ergosterol (Basse et al., 1993; Baureithel et al., 1994; Granado et al., 1995; Ito et al., 1997), und phytopathogene Oomyzeten der Gattungen *Phytophthora* und *Pythium* tragen Heptaglukanstrukturen (Cosio et al., 1988; Cheong et al., 1991) sowie Elicitine (Ricci et al., 1989) und andere (Glyko)Proteine mit Pflanzenabwehr induzierendem Potential (Nürnberger et al., 1994; Baillieul et al., 1995; Veit et al., 2001; Fellbrich et al., 2002; Qutob et al., 2002). Dazu vergleichbar besitzen verschiedene Pathovare von *Pseudomonas syringae* LPS-Hüllen (Dow et al., 2000; Coventry und Dubery, 2001; Meyer et al., 2001) und produzieren proteinogene Elicitoren wie Flagellin (Felix et al., 1999) und Harpin (He et al., 1993; Lee et al., 2001a). Auch wenn nicht alle Pflanzenspezies alle diese Signale erkennen und zur Abwehrinduktion nutzen, so ist die Erkennung verschiedener Elicitoren von ein und demselben Mikroorganismus durch ein und dieselbe Pflanze mehrfach beschrieben worden. So binden sowohl Tabak- als auch *Arabidopsis*-Zellen bakterielles Harpin (He et al., 1993; Desikan et al., 1999; Lee et al., 2001a) und Flagellin (Felix et al., 1999), während Tomatenzellen sowohl pilzliche Chitinfragmente als auch Ergosterol perzipieren können (Basse et al., 1993; Baureithel et al., 1994; Granado et al., 1995). Tabakzellen sind in der Lage, sekretierte Proteine von *Phytophthora* wie z. B. Elicitine und das im Rahmen dieser Arbeit charakterisierte Protein NPP1 (Kap. 3.5) wahrzunehmen (Ricci et al., 1989; Fellbrich et al., 2002; Qutob et al., 2002), während in Suspension kultivierte Petersiliezellen sowohl NPP1 als auch Pep-13 erkennen können (Nürnberger et al., 1994; Fellbrich et al., 2002). Obwohl synergistische Effekte in den meisten Fällen experimentell nicht belegt sind, wird dennoch angenommen, daß die Effizienz der Pathogenabwehr ganz maßgeblich durch die Fähigkeit der Pflanze bestimmt wird, nicht nur einzelne, sondern komplexe Pathogenstrukturen wahrzunehmen (Boller, 1995; Ebel und Scheel, 1997; Nürnberger und Brunner, 2002). Interessanterweise ist die Fähigkeit zur komplexen Mustererkennung auch ein Charakteristikum der Aktivierung angeborener Immunreaktionen in tierischen Systemen und somit eine weitere Parallele zwischen pflanzlichen und tierischen Abwehrsystemen (Aderem und Ulevitch, 2000; Imler und Hoffmann, 2001; Underhill und Ozinsky, 2002). So werden Entzündungsreaktionen in humanen Geweben nach Infektion mit Gram-negativen Bakterien durch die Perzeption von LPS, Flagellin und bakterienspezifischen, nichtmethylierten CpG-Dinukleotidmotiven initiiert.

2.3. Perzeption von Elicitoren der nichtkultivarspezifischen Pflanzenabwehr

Pflanzliche Bindungsproteine für generelle Elicitoren und ihre Ligandenbindungseigenschaften sind sehr ausführlich charakterisiert worden (Boller, 1995; Ebel und Scheel, 1997; Scheel, 1998; Nürnberger und Scheel, 2001). Ein aus diesen Analysen resultierendes, übereinstimmendes Merkmal ist die hohe Affinität der Ligand/Rezeptor-Interaktionen, die eine hohe Empfindlichkeit pflanzlicher Perzeptionssysteme für Elicitoren mikrobieller Herkunft widerspiegelt. Ungleich schwerer als ihre Identifikation und Charakterisierung erwies sich die Isolation von Elicitorrezeptoren aus pflanzlichen Membranpräparationen. Pionierarbeit wurde bei der chromatographischen (Umemoto et al., 1997) bzw. bei der eleganteren ligandenaffinitätschromatographischen Aufreinigung (Mithöfer et al., 2000) des 75-kDa Hepta-ß-glukosidrezeptors aus Sojabohne geleistet. Das Fehlen erkennbarer Domänen für die Signalübertragung über die Plasmamembran (Mithöfer et al., 2000) und die bereits vorher in Photoaffinitätsmarkierungsexperimenten festgestellte Detektion mehrerer Proteinspezies (Cosio et al., 1992) legen dabei die Vermutung nahe, daß das Elicitorbindeprotein Teil eines Multikomponentenkomplexes ist, welcher für die Signalerkennung und -wandlung verantwortlich ist.

Der Elicitinrezeptor aus Tabak stellt ein weiteres Beispiel für die Existenz von Elicitorperzeptionskomplexen in Pflanzen dar. Elicitine aus verschiedenen *Phytophthora*- bzw. *Pythium*-Spezies aktivieren Abwehrreaktionen in Tabak (Ricci et al., 1989; Kamoun, 2001) nach Bindung an einen aus zwei Glykoproteinen (162 und 50 kDa) bestehenden Rezeptorkomplex (Bourque et al., 1999). Elicitine sind Sterolcarrierproteine, die wahrscheinlich für die Aquisition pflanzlicher Sterole durch die selbst keine Sterole produzierenden Oomyzeten verantwortlich sind (Osman et al., 2001a). Erst kürzlich konnte gezeigt werden, daß Elicitine tatsächlich Sterole binden können und daß die Bindung von Elicitinen an den Elicitorrezeptor durch vorherige Sterolbeladung deutlich verstärkt wird (Osman et al., 2001b). Dies läßt vermuten, daß es der Elicitinrezeptor Pflanzen ermöglicht, sich gegen Pathogene zur Wehr zu setzen, die die pflanzliche Sterolhomöostase stören. Diese Interpretation entspricht interessanterweise der bereits erwähnten „guard“-Hypothese (Dixon et al., 2000; van der Hoorn et al., 2002), die den Mechanismus der Aktivierung von AVR/R-Protein-vermittelten Abwehrreaktionen in resistenten Kultivaren von Wirtspflanzen beschreibt.

Die Perzeption pilzlicher Chitinfragmente ist eine in Pflanzen weit verbreitete Fähigkeit (Baureithel et al., 1994; Boller, 1995; Ito et al., 1997; Day et al., 2001). Kürzlich wurde eine Rezeptorkinase aus Tabak isoliert, die eine extrazelluläre Domäne mit hoher Ähnlichkeit zu Chitinasen aufweist (Kim et al., 2000). Da ein für die Chitinaseaktivität essentieller Aminosäurerest in dieser Region mutiert ist, ist es möglich, daß dieses putative Rezeptorprotein Chitin zwar binden, jedoch nicht hydrolytisch spalten kann. Obwohl der experimentelle Nachweis der Bindungsaktivität aussteht, legt die Akkumulation des

kodierenden Transkripts in mit *Phytophthora nicotianae* infizierten Tabakpflanzen eine Beteiligung des Genproduktes an der Wahrnehmung des Pathogens nahe.

Einer der wesentlichsten Beiträge zu unserem Verständnis über die Perzeption von generellen Elicitoren wurde durch die Klonierung des Flagellinrezeptors aus *Arabidopsis* geleistet. Boller und Mitarbeiter nutzten flg22 aus *Pseudomonas syringae* pv. *tabaci* zur Suche nach Flagellin-insensitiven Mutanten in einer mit Ethylmethansulfonat mutagenisierten Population von *A. thaliana* Ökotyp *Landsberg erecta* (Felix et al., 1999; Gomez-Gomez et al., 1999). Dieser Screen ergab zwei unabhängige Mutationen in dem Gen *FLS2* (für *flagellin-sensing2*) (Gomez-Gomez und Boller, 2000; Gomez-Gomez und Boller, 2002).

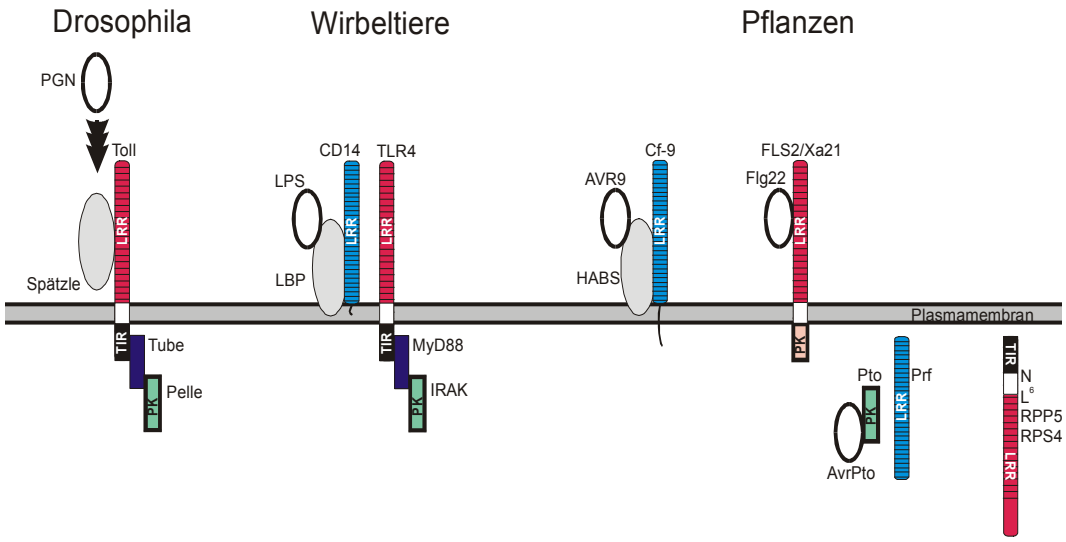

Abb. 2: Strukturelle Ähnlichkeiten von Signalperzeptionskomplexen zur Auslösung von Immunabwehrreaktionen in Insekten, Wirbeltieren und Pflanzen. In *Drosophila* initiieren z.B. Peptidoglykane (PGN) Gram-positiver Bakterien eine proteolytische Kaskade, die zur Generierung von Spätzle, einem Liganden des Toll-Rezeptors führt. Die Lipopolysaccharidhülle Gram-negativer Bakterien (LPS) stimuliert die angeborene Immunabwehr in Wirbeltieren. Nach Bindung an ein lösliches LPS-Bindungsprotein (LBP) kommt es zu einer Komplexbildung mit dem leuzinreiche Sequenzwiederholungen (LRR) tragenden Protein CD14, welches wiederum mit dem Transmembranprotein TLR4 (Toll-like receptor) interagiert. Sowohl in *Drosophila* als auch in Wirbeltieren vermitteln Adaptorproteine (Pelle und MyD88, myeloid differentiation factor) die Signaltransduktion von der intrazellulären TIR (Toll-interleukin 1 receptor)-Domäne von Toll bzw. TLR4 zu den zytoplasmatischen Proteinkinasen Pelle und IRAK (interleukin 1 receptor-associated kinase). In Pflanzen existieren verschiedene LRR-Proteine mit Ähnlichkeiten zu Toll, CD14 und TLR4. AVR9 (*Cladosporium fulvum*), das strukturell ähnlich zu Spätzle ist, bindet in Tomate an eine hochaffine Bindungsstelle ohne direkte Plasmamembranverankerung (HABS). Dieser Komplex interagiert entweder direkt oder indirekt mit Cf9, einem Protein mit struktureller Homologie zu CD14. *Arabidopsis* FLS2 und das Resistenzgen Xa21 aus Reis (determiniert Resistenz gegen *Xanthomonas oryzae* pv. *oryzae*/AvrXa21) vermitteln das Rezeptorsignal vermutlich direkt durch ihre zytoplasmatische Proteinkinasedomäne. Dabei scheint Flagellin von *Pseudomonas syringae* direkt mit FLS2 zu interagieren (Bauer et al., 2001). Gram-negative phytopathogene Bakterien benutzen ein Typ III-Sekretionssystem, um Effektorproteine wie z.B. AvrPto in die Wirtszellen zu translozieren (Büttner und Bonas, 2002). AvrPto bindet direkt an die Proteinkinase Pto, die Ähnlichkeiten zu Pelle und IRAK aufweist. Ein weiterer Interaktionspartner von Pto ist das zytoplasmatische LRR-Protein Prf. Verschiedene pflanzliche, zytoplamatische Resistenzgene mit LRR- und TIR-Domänen vermitteln kultivarspezifische Resistenz gegen Viren (*N* in Tabak), Bakterien (*RPS4* in *Arabidopsis*), Oomyzeten (*RPP5* in *Arabidopsis*) und Pilze (L^6 in Flachs) (aus Nürnberger und Scheel, 2001).

Dieses Gen kodiert für eine Transmembranrezeptorkinase, welche eine extrazelluläre LRR-Domäne trägt (Gomez-Gomez und Boller, 2000). Eine direkte Korrelation zwischen Flagellinsensitivität verschiedener Ökotypen bzw. funktionell aktiven *FLS2*-Allelen und der Präsenz von flg22-Bindungsproteinen macht es sehr wahrscheinlich, daß diese Rezeptorkinase entweder direkt oder als Teil eines größeren Rezeptorkomplexes für die Erkennung von bakteriellem Flagellin verantwortlich ist (Bauer et al., 2001). Außerdem zeigten chemische Vernetzungsexperimente mit radioaktiv markiertem flg22 die Bindung des Radioliganden an ein Protein, welches dasselbe Molekulargewicht wie FLS2 (~120 kDa) aufweist (Gomez-Gomez und Boller, 2002). Ein weiteres, sehr bedeutsames Ergebnis dieser Arbeiten ist, daß ein Sequenzvergleich von FLS2 mit anderen Proteinsequenzen hohe Ähnlichkeiten mit dem Toll-Rezeptor aus *Drosophila melanogaster* sowie menschlichen TLR-Rezeptoren (Toll-like receptors) ergab (Abb. 2) (Gomez-Gomez und Boller, 2000). So ist TLR5, ein Rezeptor, der die Erkennung von bakteriellem Flagellin in menschlichen Zellen ermöglicht, in seiner extrazellulären Struktur sehr ähnlich zu FLS2 (Hayashi et al., 2001).
Daraus läßt sich ersehen, daß die Ähnlichkeiten in der molekularen Organisation angeborener Abwehrsysteme in Tieren und Pflanzen nicht nur die Erkennung ähnlicher mikrobieller Oberflächenstrukturen einschließen, sondern sich offensichtlich auch auf den Modus der Signalperzeption erstrecken. Strukturell noch deutlicher ausgeprägte Ähnlichkeiten zeigen sich zwischen FLS2 und einer Reihe von pflanzlichen R-Genprodukten (Abb. 2) wie z. B. der LRR-Rezeptorkinase Xa21 aus Reis, die Resistenz gegen avirulente Stämme von *Xanthomonas oryzae* pv. *oryzae* vermittelt (Song et al., 1995; Nürnberger und Scheel, 2001; Gomez-Gomez und Boller, 2002). Weitere strukturelle Ähnlichkeiten in der TIR(Toll-Interleukin1-Rezeptor)-Domäne von pflanzlichen R-Proteinen und tierischen TLR-Rezeptoren unterstützen die These von einem evolutionären Grundkonzept eukaryotischer Nichtselbsterkennungs- und Pathogenabwehrsysteme (Cohn et al., 2001; Dangl und Jones, 2001; Gomez-Gomez und Boller, 2002; Nürnberger und Brunner, 2002).

2.4. Signalwandlungsprozesse in der Ausprägung pflanzlicher Abwehrreaktionen

Die Bindung von Liganden an Rezeptoren resultiert in der Aktivierung intrazellulärer Signaltransduktionskaskaden, die letztlich die Auslösung stimulusspezifischer Adaptations- oder Abwehrreaktionen vermitteln. Umfassende Forschungsaktivitäten dokumentieren eine starke molekulare Konservierung elicitor- oder pathogenaktivierter Signalkaskaden, die relativ unabhängig ist vom Typ der einer Pflanze/Pathogen-Interaktion zugrunde liegenden Resistenz. Nicht überraschend ist es deshalb, daß das Spektrum der durch generelle Elicitoren bzw. durch AVR-Proteine (spezielle Elicitoren) ausgelösten Abwehrreaktionen in Pflanzen sehr ähnlich ist (Yang et al., 1997; Nürnberger und Scheel, 2001).
An der Elicitorsignaltransduktion sind Komponenten wie beispielsweise Änderungen des zytoplasmatischen Kalziumspiegels, reaktive Sauerstoffspezies (ROS) und Stickstoffmonoxid

(NO) sowie die posttranslationale Aktivierung von Mitogen-aktivierten Proteinkinasen (MAPK) ganz wesentlich beteiligt (Yang et al., 1997; Scheel, 1998; Nürnberger und Scheel, 2001; Jonak et al., 2002), denen ebenfalls eine zentrale Funktion bei der Auslösung angeborener Immunreaktionen in tierischen Systemen zugeschrieben wird. Aus diesem Grunde beschäftigt sich das nachfolgende Kapitel lediglich mit ausgewählten Aspekten der elicitor- bzw. pathogenaktivierten Signaltransduktion in Pflanzen, und erhebt ausdrücklich keinen Anspruch auf Vollständigkeit.

Veränderungen in der Ionenpermeabilität der Plasmamembran (Einstrom von Ca^{2+} und H^+, Ausstrom von K^+ und Cl^-) gehören zu den frühesten Reaktionen pflanzlicher Zellen auf Elicitorbehandlung oder mikrobielle Infektionen (Atkinson et al., 1996; Gelli et al., 1997; Jabs et al., 1997; Zimmermann et al., 1997; Blatt et al., 1999; Klüsener und Weiler, 1999). Die rezeptorvermittelte Aktivierung von Ionenkanälen mit geringer Ionenspezifität scheint dabei nicht nur für die Auslösung dieser in zahlreichen Experimentalsystemen beobachteten Ionenflüsse verantwortlich zu sein, sondern erwies sich auch als notwendig für die Aktivierung elicitorinduzierter Abwehrreaktionen (Zimmermann et al., 1997; Blatt et al., 1999). In elektrophysiologischen („patch-clamp“) Analysen an Tabakzellen, die das Resistenzgen *Cf-9* aus Tomate exprimierten und mit dem komplementären Avirulenzfaktor AVR9 aus *Cladosporium fulvum* behandelt wurden, konnten die Aktivierung eines auswärts gerichteten K^+-Stromes und die gleichzeitige Inaktivierung eines K^+-Einwärtsstromes nachgewiesen werden (Blatt et al., 1999). Es ist daher anzunehmen, daß eine differentielle Regulation von K^+-Kanälen zum für elicitierte Pflanzenzellen typischen K^+-Ausstrom führt. In einem verwandten Experimentalsystem wurde außerdem ein AVR5-induzierter, einwärtsgerichteter Ca^{2+}-Kanal in Plasmamembranen von Protoplasten der Tomate detektiert (Gelli et al., 1997). Unsere Studien zur Pep-13-induzierten Signaltransduktion in Petersilie erbrachten den Nachweis einer rezeptorvermittelten Aktivierung eines Ionenkanals sehr hoher Ionenleitfähigkeit, der in der Lage ist, sowohl den Einstrom von Ca^{2+} als auch den Ausstrom von K^+ zu ermöglichen (Zimmermann et al., 1997). Nachfolgende Arbeiten zeigten, daß dieser Ca^{2+}-Einstrom zu einer länger anhaltenden Erhöhung der zytoplasmatischen Kalziumkonzentration [Ca^{2+}_{cyt}] führte, welche sich wiederum als notwendig für die Auslösung der Phytoalexinbiosynthese erwies (Blume et al., 2000). Ähnliche, relativ dauerhafte Veränderungen der zytoplasmatischen Kalziumsignatur konnten in mit avirulenten Bakterien infizierten *Arabidopsis*-Pflanzen nachgewiesen und in kausalen Zusammenhang mit der Auslösung kultivarspezifischer Abwehrreaktionen gestellt werden (Grant et al., 2000). Interessanterweise zeigen mit Hepta-ß-glukan elicitierte Sojabohnenzellen eine völlig andere, nämlich stark transiente Erhöhung der zytoplasmatischen Kalziumkonzentration, was auf speziesspezifische Unterschiede in der Regulation von Ca^{2+}-abhängigen Signalkaskaden hindeutet (Mithöfer et al., 1999).

Erhöhte [Ca^{2+}_{cyt}]-Spiegel sind ein essentieller Bestandteil von Signalkaskaden zur Auslösung der Immunantwort in tierischen Zellen (Tedder et al., 2002). Ein wesentlicher Unterschied zu pflanzlichen Systemen scheint indes darin zu bestehen, daß diese Anstiege insbesondere auf eine Inositol-3-phosphat (IP_3)- und Ryanodinrezeptor-vermittelte Freisetzung von Ca^{2+} aus

internen Speichern zurückzuführen sind (Galione und Churchill, 2002), während in Pflanzen extrazelluläres Kalzium die hauptsächliche, wenn auch nicht notwendigerweise die einzige Quelle dafür darstellt (Mithöfer et al., 1999; Blume et al., 2000; Grant et al., 2000; Lecourieux et al., 2002).

Neben der Aktivierung von kalziumabhängigen Proteinkinasen (CDPK) (Romeis et al., 2001) und Calmodulin (Heo et al., 1999; Kim et al., 2002), welche beide essentiell für die Aktivierung pflanzlicher Abwehrreaktionen sind, ist die Produktion reaktiver Sauerstoffspezies (ROS) in elicitierten bzw. infizierten Pflanzenzellen scheinbar generell abhängig von extrazellulärem Ca^{2+} (Nürnberger und Scheel, 2001; Talarczyk und Hennig, 2001; Yang et al., 2001). Die pflanzliche ROS-Synthese („oxidative burst“) scheint der Katalyse dieser Substanzen in humanen Phagozyten („respiratory burst“) mechanistisch zu ähneln (Babior et al., 1997). Im Gegensatz zu einem aus fünf Untereinheiten bestehenden NADPH-Oxidasekomplex tierischer Zellen scheint die pflanzliche NADPH-Oxidase jedoch lediglich aus einer der tierischen gp91-Untereinheit vergleichbaren Komponente zu bestehen (Groom et al., 1996; Keller et al., 1998; Torres et al., 1998; The *Arabidopsis* Genome Initiative, 2000). In Tabakzellen konnte eine direkte Aktivierung von NADPH-Oxidaseaktivität durch erhöhte Ca^{2+}-Spiegel gezeigt werden (Sagi und Fluhr, 2001). Desweiteren resultierte die Expression von *antisense*-Konstrukten der in der Plasmamembran von Tabakzellen lokalisierten NADPH-Oxidase in einer vollständigen Inhibierung der durch den *Phytophthora cryptogea*-Elicitor Cryptogein induzierbaren Produktion von ROS (Simon-Plas et al., 2002). In ähnlicher Weise zeigten Mutanten von *A. thaliana*, die in der Expression von zwei Genen einer acht Mitglieder umfassenden Familie von NADPH-Oxidasegenen gestört waren, eine starke Verringerung in der ROS-Produktion nach Infektion mit einer avirulenten Rasse von *Peronospora parasitica* (Torres et al., 2002). Es werden verschiedene physiologische Funktionen reaktiver Sauerstoffspezies in der Pathogenabwehr diskutiert (Yang et al., 1997; Scheel, 1998; Talarczyk und Hennig, 2001). So sind diese Substanzen akut toxisch und können der direkten Abwehr eines Invasoren dienen. Außerdem kann eine durch ROS katalysierte oxidative, lokale Vernetzung der Zellwände das weitere Eindringen eines Pathogens verhindern (Bradley et al., 1992). Eine dritte Funktion von ROS in elicitierten bzw. pathogeninfizierten Zellen scheint direkt in der intrazellulären Signaltransduktion begründet zu sein. So haben Arbeiten in verschiedenen Systemen gezeigt, daß entweder O_2^- (das direkte Produkt der NADPH-Oxidase) oder H_2O_2 (aus O_2^- gebildet durch Superoxiddismutaseaktivität) eingebunden sind in die Auslösung verschiedener pflanzlicher Abwehrreaktionen wie der Phytoalexinbiosynthese in Petersilie oder der HR in *A. thaliana* (Jabs et al., 1996; Jabs et al., 1997; Alvarez et al., 1998).

Im Zusammenspiel mit ROS scheint Stickstoffmonoxid (NO) eine wichtige Rolle bei der Auslösung pflanzlicher und tierischer Abwehrreaktionen gegen Pathogene zu spielen (Delledonne et al., 1998; Klessig et al., 2000; Wendehenne et al., 2001). Die gesteigerte Synthese von NO wurde sowohl in pathogeninfizierten Pflanzen als auch in elicitorbehandelten Pflanzenzellen nachgewiesen. Ein potentieller Partner für die Perzeption von NO könnte das Enzym Aconitase sein, welches in die Aktivierung der HR einbezogen zu sein scheint (Navarre et al., 2000; Wendehenne et al., 2001). In Säugerzellen bindet NO an

die zytosolische Aconitase und konvertiert dieses Protein damit in ein regulatorisches Eisenprotein, das mRNA binden kann, damit die Translation spezifischer mRNAs beeinflußt und zu einem erhöhten zytosolischen Eisenspiegel beiträgt (Wendehenne et al., 2001). In Tabak wurde eine Modulation der zytosolischen Aconitaseaktivität durch NO nachgewiesen, und es wurde eine strukturelle Ähnlichkeit der für die regulatorische Aktivität gegen mRNA verantwortlichen Domänen zum Säugerenzym gefunden (Navarre et al., 2000). Es wird daher vermutet, daß NO-vermittelte erhöhte Eisenspiegel die Fenton-Reaktion katalysieren können, in deren Ergebnis aus NADPH-Oxidase-abhängig produzierten ROS extrem toxische Hydroxylradikale entstehen, die Zelltod auslösend oder - verstärkend wirken. In tierischen Systemen ist NO außerdem maßgeblich an der Aktivierung des Transkriptionsfaktors NF-κB beteiligt, der wiederum direkt in die Expression von Immunabwehrgenen involviert ist (Aderem und Ulevitch, 2000; Underhill und Ozinsky, 2002). In ähnlicher Weise ist die NO-vermittelte transkriptionelle Aktivierung pflanzlicher Abwehrgene beschrieben worden (Durner et al., 1998).

MAP-Kinasekaskaden stellen zentrale Elemente in der pflanzlichen Streßsignaltransduktion dar (Zhang und Klessig, 2001; Jonak et al., 2002). MAPK sind die terminalen Elemente dieser Ketten, deren einzelne Mitglieder durch Transphosphorylierungen aktiviert werden. Im Genom von *Arabidopsis* sind insgesamt 20 für MAPK kodierende Gene gefunden worden, denen 10 MAPK-Kinase-Gene und ca. 60 MAPKK-Kinase-Gene gegenüberstehen (The *Arabidopsis* Genome Initiative, 2000). Mit dieser Vielfalt an kombinatorischen Möglichkeiten läßt sich die Schaffung und/oder Aufrechterhaltung der Signalspezifität in verschiedenen Signalkaskaden erklären, ungeachtet der Tatsache, daß veränderte MAPK-Aktivitäten in zahllose Entwicklungs- und Anpassungsprozesse involviert zu sein scheinen. Insbesondere AtMPK6 aus A*rabidopsis* sowie orthologe Aktivitäten aus Tabak, Luzerne und Petersilie werden durch Infektion mit avirulenten Pathogenen bzw. nach Elicitorbehandlung posttranslational aktiviert (Nühse et al., 2000; Zhang und Klessig, 2001; Jonak et al., 2002). Eine weitere Klasse von elicitor- bzw. pathogenresponsiven MAPK bilden orthologe Enzyme zu AtMPK3, deren eigene Aktivierung durch eines der genannten Stimuli bislang jedoch nicht beschrieben worden ist (Zhang und Klessig, 2001; Jonak et al., 2002). Die Aktivierung von MAPK in elicitierten Zellen ist Ca^{2+}-abhängig, scheint aber unabhängig von ROS zu sein (Ligterink et al., 1997; Romeis et al., 1999; Yang et al., 2001). In Pep-13-behandelten Petersiliezellen konnte außerdem gezeigt werden, daß eine von drei aktivierten MAPK in den Zellkern transloziert wird, wo sie zur ROS-unabhängigen Expression von *PR*-Genen beiträgt, die wiederum direkt von Transkriptionsfaktoren des WRKY-Typs abhängig ist (Ligterink et al., 1997; Kroj et al., 2003). In diesem Zusammenhang ist bedeutsam, daß kürzlich eine komplette MAPK-Kaskade in *Arabidopsis* identifiziert worden ist, die durch bakterielles Flagellin aktivierbar ist und die die vom Transkriptionsfaktor WRKY29 abhängige Expression von *PR*-Genen und Pathogenresistenz vermittelt (Asai et al., 2002). In Experimenten mit Tabakpflanzen, in denen MAPK oder deren regulatorische MAPKK konstitutiv aktiv exprimiert wurden, konnte ebenfalls ein funktionaler Zusammenhang zwischen erhöhter MAPK-Aktivität und der Expression von

PR-Genen und hypersensitivem Zelltod hergestellt werden (Yang et al., 2001; Ren et al., 2002).

Andere pflanzliche MAPK scheinen in die negative Regulation von Abwehrreaktionen einbezogen zu sein (Petersen et al., 2000; Frye et al., 2001). Mutationen in *AtMPK4* führten in den betroffenen Pflanzen zu erhöhter Resistenz gegen virulente Pathogene bei gleichzeitig erhöhtem Salizylatgehalt und konstitutiver *PR*-Genexpression (Petersen et al., 2000). Der Zwergphänotyp dieser Mutante deutet aber auch an, daß AtMPK4 ebenfalls wichtig für eine normale Entwicklung der Pflanze zu sein scheint. Mutanten mit einem Defekt in *EDR1*, einem für eine Raf-MAPKKK kodierendem Gen, zeigten ebenso erhöhte Pathogenresistenz wie *mpk4*-Pflanzen, jedoch keinen Anstieg im Salizylatspiegel und auch keine konstitutive *PR*-Genexpression (Frye et al., 2001). Daraus ist zu schließen, daß EDR1 wahrscheinlich nicht in die Aktivierung von AtMPK4 involviert ist, und daß in *Arabidopsis* mindestens zwei MAPK-Kaskaden zur Repression von Abwehrreaktionen unter Normalbedingungen beitragen.

Da MAPK-Kaskaden nicht nur bei der pflanzlichen Pathogenabwehr sondern auch bei der Ausprägung von Immunantworten in tierischen Systemen eine wichtige regulatorische Funktion besitzen, stellt dies eine weitere Parallele in der molekularen Organisation eukaryotischer Abwehrsysteme dar (Asai et al., 2002; Dong et al., 2002; Jonak et al., 2002). Ähnliches trifft auch auf die Rolle von zytoplasmatischem Ca^{2+}, ROS und NO zu (Nürnberger und Scheel, 2001). Es sei jedoch ausdrücklich betont, daß eine ähnliche funktionelle Einbindung dieser Signaltransduktionskomponenten in verschiedenen eukaryotischen Systemen nicht automatisch auf eine hohe Konservierung der gesamten Signalnetzwerke hindeutet. Es ist vielmehr davon auszugehen, daß die molekulare Architektur solcher Signalnetze nicht nur sehr unterschiedlich zwischen Pflanzen und Tieren sein kann, sondern auch relativ große Unterschiede zwischen verschiedenen Pflanzenspezies möglich sind.

3. Die Interaktion von Petersilie mit verschiedenen Spezies der Gattung *Phytophthora* – ein Modellsystem zum Studium der molekularen Grundlagen der pflanzlichen Nichtwirtsresistenz

Petersilie ist eine klassische Nichtwirtspflanze für das Sojabohnenpathogen *Phytophthora sojae*. Zoosporen des Oomyzeten sind zwar in der Lage, auf der Blattoberfläche von jungen Petersiliepflanzen zu keimen und primäre Infektionsstrukturen (Appressorien) zu bilden, jedoch wird diese Infektion in allen bekannten Genotypen der Petersilie durch eine multifaktorielle Abwehrreaktion abgebrochen (Abb. 3) (Jahnen und Hahlbrock, 1988). Befallene Zellen reagieren dabei mit der Bildung von Zellwandauflagerungen aus Kallose und der Produktion phenolischer Substanzen. Etwas später zeigen sich an der Infektionsstelle typische Symptome des hypersensitiven Zelltodes. Eine weitere charakteristische Reaktion der die Infektionsstelle umgebenden Zellen ist die Produktion und Sekretion von Phytoalexinen, die zur Stoffklasse der Furanocoumarine gehören (Hahlbrock und Scheel, 1989).

In Suspension kultivierte Zellen und Protoplasten der Petersilie reagieren auf Behandlung mit Elicitorenpräparationen aus den Zellwänden verschiedener *Phytophthora*-Spezies in sehr ähnlicher Weise wie Zellen infizierter Blätter. Dies trifft insbesondere auf die Produktion autofluoreszierender Furancoumarine und die transkriptionelle Aktivierung einer Reihe von elicitorresponsiven Genen zu (Kombrink und Hahlbrock, 1986; Dangl et al., 1987; Parker et al., 1988).

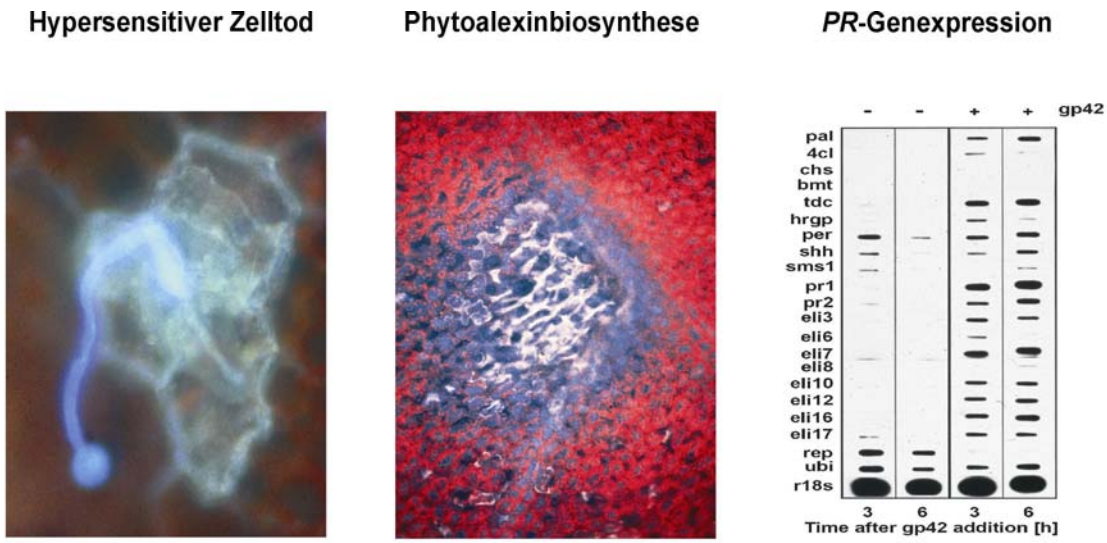

Abb. 3: Aktivierung von Abwehrreaktionen in der Nichtwirtspflanze Petersilie nach Infektion mit Zoosporen von *P. sojae* (hypersensitiver Zelltod und Phytoalexinbiosynthese) bzw. nach Behandlung von Petersilieprotoplasten mit dem Proteinelicitor GP42 (*PR*-Genexpression).

Dazu zählen solche Gene, die für Enzyme des generellen Phenylpropanstoffwechsels und für PR-Proteine kodieren (Somssich et al., 1989). Ein unschätzbarer Vorteil dieses Experimentalsystems ist, daß Petersilieprotoplasten relativ insensitiv gegenüber den während der Zellwandmazeration freigesetzten Kohlehydratfragmenten sind, die in vielen anderen Pflanzen als starke Elicitoren der Pathogenabwehr wirken. Petersilieprotoplasten eignen sich daher in idealer Weise zur Quantifizierung von Abwehrreaktionen sowie als transientes Genexpressionssystem, und sind damit von hohem Wert für Studien zur elicitorvermittelten Signalerkennung und -wandlung (Hahlbrock et al., 1995).

3.1. Die Identifizierung von Pep-13

Signalperzeptionsstudien in Pflanze/Pathogen-Interaktionen erfordern homogene und molekular definierte Elicitorpräparationen. Dieser Arbeit vorausgegangene Analysen hatten gezeigt, daß submers kultivierte Isolate von *P. sojae* proteinogene Elicitoren in das Kulturmedium sezernieren, die die Phytoalexinbiosynthese in Petersilieprotoplasten stimulieren (Kombrink und Hahlbrock, 1986; Parker et al., 1988). Aus dem Kulturfiltrat des

Oomyzeten konnte daraufhin ein 42-kDa Glykoprotein (GP42) gereinigt werden, dessen Elicitoraktivität dem Proteinbestandteil zugeschrieben werden konnte (Parker et al., 1991). Präparationen dieses Proteins dienten als Startmaterial für den in Kapitel 3 dargestellten Teil der experimentellen Arbeiten.

In einem Versuch, ein für die Elicitoraktivität von GP42 verantwortliches minimales Strukturmotiv zu identifizieren, wurden verschiedene Proteasen auf ihre Fähigkeit getestet, elicitoraktive Peptide aus dem intakten Protein freizusetzen (Nürnberger et al., 1994). Durch Endoproteinase Glu-C-Verdau des Proteins und nachfolgende HPLC-gestützte Separation der Proteolyseprodukte gelang die Isolation eines 13 Aminosäuren umfassenden Peptides (Pep-13) mit hoher Elicitoraktivität (Abb. 4).

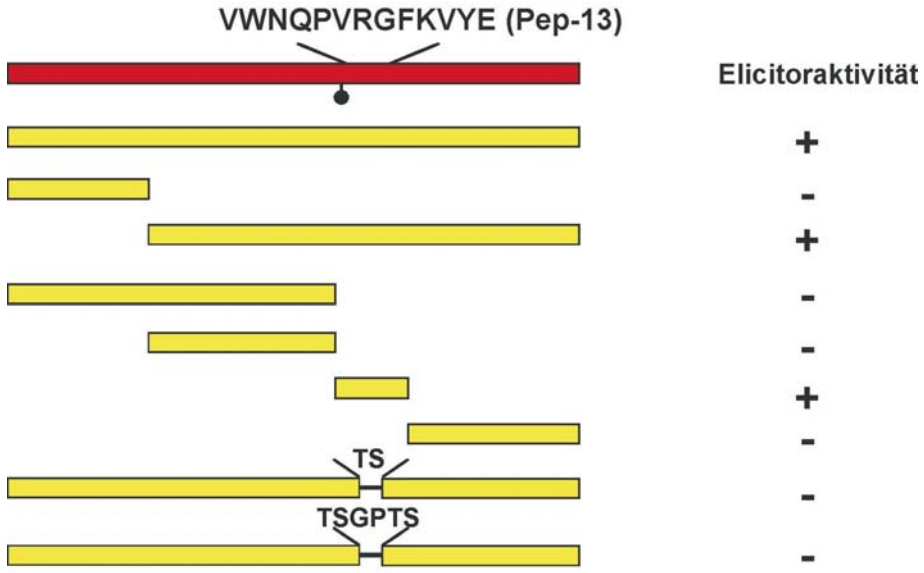

Abb. 4: Elicitoraktivität von Deletionsmutanten von GP42. Rekombinantes GP42 bzw. verschiedene GP42-Fragmente (gelbe Markierung) wurden durch Expression der kodierenden DNA-Fragmente in *E. coli* erzeugt und nachfolgend als Elicitoren der Phytoalexinbiosynthese in Petersilieprotoplasten eingesetzt (Nürnberger et al., 1994). Die Lokalisation von Pep-13 in GP42 sowie die Position der einzigen N-Glykosylierungsstelle in GP42 ist im obersten Balken (rote Markierung) dargestellt. +, Elicitoraktivität; -, keine Elicitoraktivität

Alle weiteren elicitoraktiven Peptide erwiesen sich nach Sequenzierung als unvollständige Abbauprodukte von GP42, die jedoch alle das Pep-13-Motiv enthielten. Anhand der Sequenz einer für GP42 kodierenden cDNA konnte Pep-13 als ein Bestandteil des C-Terminus des Proteins identifiziert werden (Sacks et al., 1995). Die heterologe Expression der vollständigen cDNA sowie einer Reihe von Deletionsfragmenten von GP42 in *E. coli* und die Bestimmung der Elicitoraktivität der Expressionsprodukte ließ vermuten, daß Pep-13 offensichtlich das einzige elicitoraktive Strukturmotiv innerhalb des intakten Proteins ist (Nürnberger et al., 1994). In einem komplementären Versuchsansatz, in dem die Pep-13 repräsentierenden Aminosäuren deletiert oder durch sechs nichtverwandte Reste ersetzt wurden, konnte bestätigt werden, daß Pep-13 nicht nur hinreichend sondern auch notwendig für die Elicitoraktivität von GP42 ist (Abb. 4). Struktur-Aktivitätsstudien mit einer Serie von chemisch

synthetisierten Derivaten von Pep-13 zeigten, daß ein Minimalpeptid bestehend aus elf Aminosäuren (WNQPVRGFKVY) die kürzeste Struktur mit signifikanter Elicitoraktivität darstellt. Durch individuellen Austausch aller Aminosäuren gegen Alanin wurden lediglich die Aminosäuren Tryptophan (Trp-2) und Prolin (Pro-5) von Pep-13 als essentiell für die Elicitoraktivität des Peptides identifiziert, während alle anderen Substitutionen keine vergleichbaren Aktivitätsverluste zur Folge hatten (Nürnberger et al., 1994). Neuere Struktur-Funktionsstudien zeigen überdies, daß nur der Austausch von Trp-2 gegen Phenylalanin sowie von Pro-5 gegen Hydroxyprolin ohne starken Aktivitätsverlust möglich ist. Austausche dieser Reste gegen andere Aminosäuren führten dagegen zu einer drastischen Reduktion der Elicitoraktivität der entsprechenden Pep-13-Derivate. Interessanterweise resultierten sowohl die Variation der Zahl der Aminosäuren zwischen Trp-2 und Pro-5 als auch multiple Mutationen in Pep-13 in starken Verlusten der Elictoraktivität, sodaß die strukturelle Basis der Elicitoraktivität des Peptides wahrscheinlich komplexer ist als ursprünglich angenommen (Hahlbrock et al., 2003).

Pep-13 stimuliert die Phytoalexinbiosynthese in Petersilieprotoplasten in Konzentrationen (EC_{50}=31nM), die typisch sind für die Auslösung pflanzlicher Abwehrreaktionen durch generelle Elicitoren (Nürnberger et al., 1994). Das Spektrum der in Pep-13-behandelten Petersiliezellen oder -protoplasten gebildeten Furanocoumarine unterscheidet sich dabei qualitativ nicht von dem, welches durch die Behandlung mit intaktem GP42 oder einer Zellwandpräparation von *P. sojae* ausgelöst wird. Ähnlich wie letztere Elicitoren stimuliert Pep-13 ebenfalls die transkriptionelle Aktivierung von Genen, die für Enzyme der Furanocoumarinbiosynthese (Phenylalanin-Ammoniak-Lyase, 4-Coumarat:CoA-Ligase) oder für PR-Proteine kodieren.

Nach Zugabe zu Petersiliezellkulturen oder nach Infiltration von Pep-13 in Petersilieblätter lassen sich keine Anzeichen von Zelltod oder Nekrosen beobachten. Es ist daher davon auszugehen, daß die durch Zoosporen von *P. sojae* auf Petersilienblättern ausgelösten Läsionen von anderen Oberflächenstrukturen des Oomyzeten hervorgerufen werden.

Zu den sehr frühen, d. h. bereits 2-10 Minuten nach Zugabe von Pep-13 zu beobachtenden Reaktionen von Petersiliezellen gehören die Aktivierung von Ionenkanälen in der Plasmamembran (Einstrom von H^+ und Ca^{2+}, Ausstrom von K^+ und Cl^-), die Produktion von Superoxidanionen und H_2O_2 (ROS) sowie die posttranslationale Aktivierung von mindestens drei verschiedenen MAP-Kinasen (Nürnberger et al., 1994; Jabs et al., 1997; Ligterink et al., 1997; Kroj et al., 2003). Wie in Kap. 2 dargestellt, sind diese frühen Reaktionen Teile eines Signaltransduktionsnetzwerkes, welches letztlich die Aktivierung der Phytoalexinbiosynthese und die Expression von *PR*-Genen vermittelt. Weitere von Pep-13 induzierte, jedoch nicht ursächlich mit der Transduktion des Elicitorsignals in Verbindung zu bringende zelluläre Reaktionen sind die Stimulierung der Biosynthese von Ethylen bzw. Jasmonat (Nürnberger et al., 1994; Scheel, 1998).

Die Responsivität von Pflanzen gegenüber Pep-13 scheint nicht auf Petersilie beschränkt zu sein. Kürzlich konnte nach Zugabe von Pep-13 zu Suspensionskulturen der Kartoffel die transkriptionelle Aktivierung von Genen, die für eine Lipoxygenase, für PR-1 und für

Phenylalanin-Ammoniak-Lyase kodieren, nachgewiesen werden (Brunner et al., 2002). Interessanterweise waren Strukturderivate von Pep-13 mit stark reduzierter Elicitoraktivität in Petersilie ebenfalls schwache Induktoren der *PR*-Genexpression in Kartoffel, was auf die Existenz ähnlicher Pep-13-Bindungsstellen in beiden Pflanzen hindeutet.

3.2. Pep-13 als Teil einer extrazellulären Transglutaminase in Oomyzeten

In Datenbanken konnten keine Sequenzen mit signifikanter Ähnlichkeit zu GP42 gefunden werden. Einen Hinweis auf die mögliche Funktion des Proteins lieferte deshalb die Mitteilung von Henrik Dalbøge und Sakari Kauppinen (Novozymes A/S, Bagsvaerd, Dänemark), daß eine von ihnen gereinigte Transglutaminase (TGase) aus *Phytophthora cactorum* starke strukturelle Ähnlichkeit mit GP42 zeigte. In der Tat wiesen sowohl gereinigtes als auch rekombinantes GP42 TGase-Aktivität (*R*-Glutaminylpeptidamino-γ-Glutamyltransferase, EC 2.3.2.13) auf (Brunner et al., 2002). Die Aktivität von GP42 war dabei strikt Ca^{2+}-abhängig und sensitiv gegenüber Inhibitoren cysteinabhängiger Enzyme. Durch die irreversible Bindung von radioaktiv markiertem Iodacetamid (ein Inhibitor cysteinabhängiger tierischer TGasen) an GP42, nachfolgende proteolytische Spaltung und Radio-HPLC-gestützte Separation der Spaltprodukte konnte Cys-128 als ein Teil der katalytischen Triade von TGasen identifiziert werden (nicht publiziert).

Die Aufklärung der molekularen Funktion von GP42 versetzte uns in die Lage, insbesondere die Frage zu beantworten, inwiefern generelle Elicitoren grundsätzlich ähnliche Charakteristika wie PAMPs aufweisen, die die angeborene Immunabwehr in tierischen Systemen stimulieren. PAMPs sind in der Regel oberflächenexponierte Strukturen, welche in mikrobiellen Organismen sehr weit verbreitet sind, nicht jedoch in potentiellen Wirtsorganismen auftreten. Außerdem zeichnen sie sich dadurch aus, daß sie funktionell bedeutsam für den mikrobiellen Organismus und daher evolutionär stark konserviert sind (Medzhitov und Janeway, 1997; Nürnberger und Brunner, 2002). Immunzytochemische Arbeiten zeigten, daß GP42 ein konstitutiver Bestandteil keimender Hyphen von *P. sojae* ist (Hahlbrock et al., 1995). Aus detaillierten Studien zur Tertiärstruktur des Proteins resultierte die Erkenntnis, daß die Pep-13 umfassende Region Teil eines hydrophilen, oberflächenexponierten Loops ohne ausgeprägte Sekundärstruktur ist (Brunner et al., 2002). Für TGasen kodierende Gene, Transkripte sowie TGase-Proteine und -aktivitäten konnten in insgesamt zehn getesteten Spezies der Gattung *Phytophthora*, nicht jedoch in der verwandten Gattung *Pythium* nachgewiesen werden (Brunner et al., 2002). Aus dem Vergleich der Sequenzen aller TGase-Transkripte ging außerdem hervor, daß die Pep-13-Domäne in allen Spezies nahezu vollständig konserviert war. Der einzige in zwei Spezies festzustellende Unterschied betraf einen Austausch eines Tyrosinrestes gegen einen Phenylalaninrest, der jedoch keinen Einfluß auf die Elicitoraktivität eines solchen synthetisierten Pep-13-Derivates hatte. Die gezielte Einführung von Aminosäureaustauschen in das Pep-13-Motiv der intakten TGase diente nachfolgend der Analyse, inwiefern Mutationen, die die Elicitoraktivität des

intakten Proteins beinträchtigen (Trp-231-Ala und Pro-234-Ala), auch die TGase-Aktivität beeinflussen. Dabei zeigte sich, daß beide Mutationen sowohl die Elicitor- als auch die Enzymaktivität in nahezu gleichem Ausmaß reduzierten, während der Austausch von Tyr-241-Ala keinen nennenswerten Einfluß auf beide Aktivitäten hatte (Brunner et al., 2002). Desweiteren sollte durch Inaktivierung des für die TGase kodierenden Gens in *P. infestans* eine mögliche Beeinträchtigung des Lebenszyklus des Oomyzeten ermittelt werden. Diese Studien wurden in Zusammenarbeit mit Francine Govers (Landwirtschaftliche Universität Wageningen, Niederlande) durchgeführt. Obwohl insgesamt 14 stabil mit einem „silencing"-Konstrukt transformierte Linien von *P. infestans* hergestellt wurden, zeigte keines der transgenen Isolate reduzierte TGase-Aktivität. Da die Regenierbarkeit dieser Isolate zudem sehr wenig effizient war, könnte man vermuten, daß der (partielle) Verlust des Enzyms möglicherweise schwerwiegende physiologische Konsequenzen für *Phytophthora* hat. Aufgrund der Tatsache, daß die gezielte Inaktivierung von Genen in Oomyzeten und die Regeneration transgener Isolate keine Routineanwendung ist, können jedoch auch technische Gründe für dieses unbefriedigende Ergebnis verantwortlich sein (van West et al., 1999; Tyler, 2002).

Zusammenfassend läßt sich festhalten, daß Pep-13 wesentliche Charakteristika klassischer PAMPs der Immunabwehr in Tieren aufweist. Somit stützen unsere Ergebnisse die Hypothese, daß generelle Elicitoren der nichtkultivarspezifischen Pathogenabwehr in Pflanzen möglicherweise als physiologische Äquivalente zu PAMPs anzusehen sind (Brunner et al., 2002; Gomez-Gomez und Boller, 2002; Nürnberger und Brunner, 2002; Felix und Boller, 2003).

3.3. Der Pep-13-Rezeptor

Fluoreszenzmikroskopische Analysen unter Verwendung von fluoresceinyliertem Pep-13 belegten unzweifelhaft die Existenz von Bindungsstellen in der Plasmamembran von Petersilieprotoplasten (Diekmann et al., 1994). Zur exakten kinetischen Charaktierisierung der Ligandenbindung wurde ein am Tyrosinrest radioaktiv jodiertes Derivat von Pep-13 ([^{125}I-Tyr]-Pep-13) eingesetzt. Als Rezeptorpräparationen wurden entweder die mikrosomale Membranfraktion von Petersiliezellen oder Petersilieprotoplasten verwendet. In diesen Experimenten konnte die Existenz einer Klasse hochaffiner Plasmamembranbindungsstellen für [^{125}I-Tyr]-Pep-13 mit einer Dissoziationskonstante von 2.4 (in Mikrosomen) bzw. 11.4 nM (in Protoplasten) nachgewiesen werden (Nürnberger et al., 1994). Die Ligandenbindung war saturierbar und reversibel. Außerdem konnte eine klare quantitative und qualitative Korrelation zwischen der Elicitoraktivität von Pep-13-Derivaten (Aktivierung von Ionenflüssen und MAPK-Aktivität, Produktion von ROS und Phytoalexinen) und ihrer Fähigkeit zur Kompetition der Radioligandenbindung festgestellt werden. Daraus läßt sich schließen, daß die Pep-13-Bindungsstelle mit hoher Wahrscheinlichkeit die Aktivierung aller genannten zellulären Reaktionen vermittelt und deshalb als Rezeptor oder Teil eines Rezeptorkomplexes

angesehen werden muß. Mit Hilfe homobifunktioneller chemischer Quervernetzer gelang es, ein 100-kDa Protein in Petersilieplasmamembranen als primäre Pep-13-Bindungsstelle zu identifizieren (Nürnberger et al., 1995). Eine zusätzlich auftretende, jedoch reproduzierbar schwächere Markierung eines 144-kDa-Proteins in diesen Experimenten könnte als Hinweis auf die Existenz eines heterooligomeren Rezeptorkomplexes interpretiert werden. Da letztere Bande jedoch nicht mehr detektiert werden konnte, wenn die Membranen vor der Quervernetzung mit nichtionischen Detergenzien solubilisiert wurden, stellt das 100-kDa-Protein mit hoher Wahrscheinlichkeit die eigentliche Pep-13-Bindungsstelle dar (Nennstiel et al., 1998).

Die ligandenaffinitätschromatographische Anreicherung des Pep-13-Rezeptors an immobilisiertem Pep-13 erwies sich zwar als prinzipiell möglich (Nennstiel et al., 1998), war jedoch wesentlich weniger effizient als das von Ebel und Kollegen zur Reinigung des Hepta-ß-glukanrezeptors aus Sojabohne etablierte Protokoll (Mithöfer et al., 1996). Dafür verantwortlich war insbesondere die relativ geringe Stabilität der Ligandenbindungsaktivität des solubilisierten Rezeptors. Als experimentelle Alternative wurde deshalb eine Kombination aus chemischer Quervernetzung des Rezeptor/Ligand-Komplexes und ligandenaffinitätschromatographischer Anreicherung gewählt (Nennstiel et al., 1998). Dazu wurde biotinyliertes [^{125}I-Tyr]-Pep-13 an mikrosomale Membranen aus Petersilie gebunden und nachfolgend chemisch mit der Pep-13-Bindungsstelle vernetzt. Der Solubilisierung von Membranprotein folgte die Fixierung des Rezeptor/Ligand-Komplexes an Avidin-Agarose, bevor durch Biotin eluierbare Proteine einer präparativen SDS-Polyacrylamidgelelektrophorese zugeführt wurden. Aufgrund der radioaktiven Markierung konnte dann die Position des Rezeptor/Ligand-Komplexes exakt lokalisiert und die entsprechende Bande aus dem Gel isoliert werden. Dieses Protokoll hat bislang zur geschätzten Isolation von ca. 10 pmol Rezeptorprotein geführt, welches nach tryptischem Verdau und LC-MS-chromatographischer Auftrennung zur Proteinsequenzierung eingesetzt werden soll.

3.4. Die Rolle von Kalzium bei der Pep-13-vermittelten Pathogenabwehr

Die bislang früheste nachgewiesene Reaktion von Pep-13-behandelten Petersiliezellen ist die Aktivierung von Ionenflüssen über die Plasmamembran (Nürnberger et al., 1994). Experimente unter Nutzung pharmakologischer Effektoren haben dabei insbesondere die Bedeutung des Einstroms von extrazellulärem Kalzium für die Transduktion des Elicitorsignals sowie die Auslösung aller bislang beschriebenen Abwehrreaktionen in Petersilie erkennen lassen (Jabs et al., 1997; Ligterink et al., 1997; Zimmermann et al., 1997; Blume et al., 2000). „Patch-clamp“-Analysen an Petersilieprotoplasten wurden deshalb mit dem Ziel durchgeführt, einen oder mehrere elicitorresponsive Kalziumkanäle in der Plasmamembran zu detektieren. Bei einer extrazellulären Ca^{2+}-Konzentration von 1 mM, die repräsentativ für die tatsächliche Konzentration dieses Ions in der pflanzlichen Zellwand ist, konnte ein einwärts gerichteter,

La^{3+}- und Gd^{3+}-sensitiver Ca^{2+}-Kanal mit einer Einzelkanalleitfähigkeit von 80 pS detektiert werden (Zimmermann et al., 1997). Dieser Kanal erwies sich jedoch nicht als Ca^{2+}-spezifisch sondern transportierte mono- und divalente Kationen, jedoch keine Anionen. Bedeutsamer war jedoch, daß die Öffnungswahrscheinlichkeit des Kanals, nicht aber dessen Leitfähigkeit, nach Zugabe von Pep-13 oder einer Rohpräparation aus der Zellwand von P. *sojae* stark anstieg (Zimmermann et al., 1997). Diese Aktivierung konnte indes nur beobachtet werden, wenn in der Ganzzellkonformation gemessen wurde. Hingegen konnte in isolierten Membranstücken keine Kanalaktivierung durch Elicitor nachgewiesen werden. Daraus läßt sich schließen, daß die Wirkung von Pep-13 auf den Ionenkanal nicht durch direkte Bindung, d.h. analog zur Ligandenaktivierung tierischer Ionenkanäle, sondern indirekt zustande kommt. Vermutlich sind darin zytoplasmatische Komponenten involviert, die bei der Präparation der Membranstücke verloren gegangen waren. Eine rezeptorvermittelte Stimulierung dieses Kanals wird auch durch die Tatsache belegt, daß ausschließlich elicitoraktive Strukturderivate von Pep-13 in der Lage waren, aktivierend zu wirken (Zimmermann et al., 1997). Aufgrund der Ionenspezifität und der Elicitorresponsivität dieses Ionenkanals läßt sich vermuten, daß zumindest ein Teil der in elicitierten Petersiliezellen detektierten Ionenströme (Ca^{2+}-Einstrom/K^+-Ausstrom) über einen Kanal verlaufen. Es ist jedoch nicht auszuschließen, daß sich der Nettoflux für beide Ionen aus den Aktivitäten mehrerer Ionenkanäle zusammensetzt.

Zytoplasmatisches Kalzium fungiert als „second messenger“ in so verschiedenen physiologischen Prozessen wie der Wurzelknöllchenbildung, der Phototransduktion durch Phytochrome, der Stomataöffnung, des Geotropismus, des Pollenschlauchwachstums und der Streßadaptation (Trewavas und Malho, 1998; Sanders et al., 2002). Aufgrund der Bedeutung von extrazellulärem Kalzium für die Elicitorsignaltransduktion in Petersilie war es deshalb von Interesse festzustellen, ob sich die Ca^{2+}-Aufnahme direkt in einer Erhöhung der zytoplasmatischen Ca^{2+}-Konzentration [Ca^{2+}_{cyt}] niederschlagen würde. Zur Quantifizierung von [Ca^{2+}_{cyt}] wurden Zellinien der Petersilie angelegt, die den Ca^{2+}-Indikator Apoaequorin stabil im Zytoplasma exprimierten. Elicitierung dieser Zellen mit Pep-13 resultierte innerhalb von etwa 120 s in einem steilen Anstieg der [Ca^{2+}_{cyt}] von 70 nM auf ca 1 µM, bevor diese auf ein länger anhaltendes Niveau von ca. 300 nM abfiel (Blume et al., 2000). Erstaunlicherweise erwies sich der erste Teil dieser zweiphasigen [Ca^{2+}_{cyt}]-Signatur als nicht notwendig für die elicitorinduzierte Produktion von ROS und Phytoalexinen. Bei niedrigen, für eine Elicitierung der Phytoalexinbiosynthese aber ausreichenden Konzentrationen von Pep-13 oder durch Verwendung von Elicitoren (GP42, Harpin), die ausschließlich einen langanhaltenden Anstieg von [Ca^{2+}_{cyt}] induzierten, konnte der [Ca^{2+}_{cyt}]-Peak nicht mehr beobachtet werden (Blume et al., 2000). Durch Einsatz pharmakologischer Effektoren konnte außerdem gezeigt werden, daß nicht die Freisetzung von Ca^{2+} aus internen Speichern (Vakuole) sondern der Einstrom von extrazellulärem Ca^{2+} für den Pep-13-vermittelten, länger anhaltenden Anstieg der [Ca^{2+}_{cyt}] verantwortlich ist. Desweiteren ging aus diesen Studien hervor, daß alle Inhibitoren, die diese Erhöhung blockierten, ebenfalls die Synthese von ROS und Phytoalexinen unterbanden. Komplementär dazu löste das Ionophor Amphotericin einen Anstieg von [Ca^{2+}_{cyt}] aus und stimulierte die genannten Abwehrreaktionen in der Abwesenheit von Pep-13. Deshalb und

aufgrund einer klaren Korrelation zwischen der Fähigkeit von Pep-13-Derivaten zur Rezeptorbindung, zur Erhöhung von [Ca^{2+}_{cyt}] und zur Auslösung von Abwehrreaktionen scheint ein funktionaler Zusammenhang zwischen diesen Prozessen zu bestehen (Blume et al., 2000).

3.5. NPP1 und Pep-13 – Komponenten eines Oomyzeten-spezifischen Pathogenmusters

Infektionen durch *P. sojae* führen auf Petersilieblättern zu hypersensitivem Zelltod an der Infektionsstelle (Jahnen und Hahlbrock, 1988). Da Pep-13 keine HR induziert, müssen für die Auslösung dieser Abwehrreaktion andere Elicitoren verantwortlich sein. Zusätzlich dazu war bereits bekannt, daß Kulturfiltrate von verschiedenen in Flüssigkultur gewachsenen *Phytophthora*-Spezies proteinogene Elicitoren der Pathogenabwehr in Petersilie enthielten, die nicht identisch mit der vorher beschriebenen TGase waren (Kombrink und Hahlbrock, 1986; Parker et al., 1991; Fellbrich et al., 2000). Zur umfassenden Charakterisierung des „antigenen Potentials" der Oberfläche von *Phytophthora* wurden deshalb Anstrengungen unternommen, weitere Elicitoren pflanzlicher Abwehrreaktionen zu isolieren. Aus dem Kulturfiltrat von *P. parasitica*, welches eine besonders starke, nicht mit Pep-13 assoziierte Elicitoraktivität aufwies, wurde ein 24-kDa-Protein gereinigt (Fellbrich et al., 2000; Fellbrich et al., 2002). Dieses Protein (NPP1, für Nekrosen-induzierendes Protein aus *Phytophthora*) sowie für NPP1 kodierende Gene konnten in fünf weiteren *Phytophthora*-Spezies und zwei Spezies der Gattung *Pythium* (*Py. vexans* und *Py. aphanidermatum*) nachgewiesen werden (Veit et al., 2001; Fellbrich et al., 2002; Qutob et al., 2002). NPP1 scheint jedoch nicht nur ein molekulares Merkmal von Oomyzeten zu sein, denn verwandte Gene ließen sich auch im Genom echter Pilze (*F. oxysporum*) oder in Bakterien (*B. halodurans*, *Vibrio* spp.) finden (Nelson et al., 1998; Takami et al., 2000). Aufgrund von Sequenzvergleichen ließen sich keine Rückschlüsse auf die physiologische oder biochemische Funktion des Proteins ziehen. Allerdings konnten Qutob et al. zeigen, daß NPP1-Transkripte in *P. sojae* während des Überganges von der biotrophen in die nekrotrophe Wachstumsphase stark akkumulierten, was auf eine Toxinaktivität des Proteins hindeutet (Qutob et al., 2002). Mikrobielle Toxine mit der Fähigkeit, Abwehrreaktionen in tierischen oder pflanzlichen Zellen zu stimulieren, sind hinlänglich beschrieben worden (Knogge, 1998). Ein klassisches Beispiel (obwohl nicht unbedingt ein physiologisches Toxin) ist das auch als LPS bekannte bakterielle Endotoxin, welches in niedrigen Konzentrationen als Elicitor wirkt, oberhalb bestimmter Schwellwerte jedoch zellulytische Aktivität aufweist (Dow et al., 2000; Coventry und Dubery, 2001).

NPP1 induziert in Petersiliezellkulturen nicht nur die Phytoalexinbiosynthese sondern auch Zelltod (Fellbrich et al., 2002). Nach Infiltration in Blätter verschiedener zweikeimblättriger Pflanzen (Petersilie, *A. thaliana*, Tabak) konnte außerdem eine starke Nekrosebildung beobachtet werden. Wie für die homologen Proteine aus *Py. aphanidermatum* und *F. oxysporum* berichtet, tritt dieser Phänotyp jedoch nicht in einkeimblättrigen Pflanzen auf

(Bailey, 1995; Veit et al., 2001). Eine umfassende Analyse der NPP1-Elicitoraktivität in *A. thaliana* zeigte, daß NPP1 nicht nur hypersensitiven Zelltod in zehn getesteten Ökotypen hervorrief, sondern ebenfalls die Biosynthese von Kallose, Ethylen und H_2O_2 stimulierte (Fellbrich et al., 2002). Wie in Kap. 3.6. ausführlicher beschrieben, resultierte die Infiltration von NPP1 in Blätter zusätzlich in einer gesteigerten Transkriptakkumulation von ca. 600 Genen, unter denen sich auch eine Reihe von *PR*-Genen befanden (nicht publiziert). Aufgrund der Fähigkeit von NPP1, eine sehr komplexe Abwehrreaktion in der für genetische Analysen hervorragend geeigneten Pflanze *A. thaliana* hervorzurufen (Fellbrich et al., 2002; Varet et al., 2002), soll dieses System in Zukunft zur Isolierung NPP1-insensitiver Mutanten genutzt werden. Der Vorteil eines solchen genetischen Experiments gegenüber konventionellen Versuchen zur biochemischen Anreicherung von Rezeptorproteinen ist sehr eindrucksvoll durch die Isolierung des Flagellinrezeptors FLS2 demonstriert worden (Gomez-Gomez und Boller, 2000).

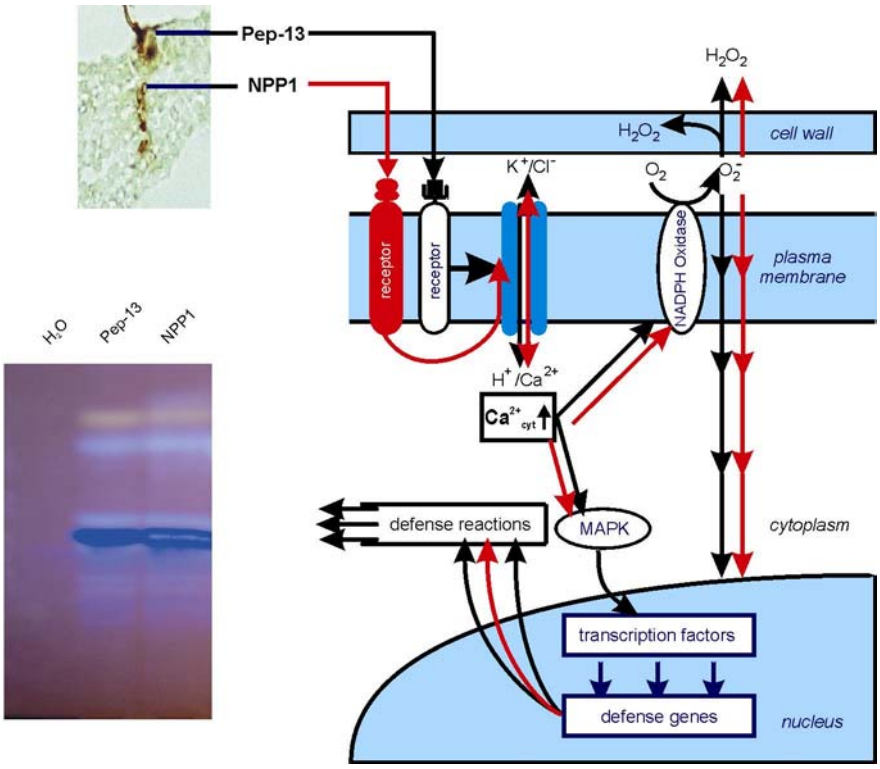

Abb. 5: Hypothetisches Modell der Signalperzeption und -transduktion bei der Interaktion von Petersilie und *Phytophthora*. Pep-13 und NPP1 initiieren durch separate Perzeptionssysteme rasch konvergierende Signalketten, die zur Aktivierung der Phytoalexinbiosynthese führen. Die zelltodauslösende Aktivität von NPP1 ist hier nicht dargestellt. Im linken oberen Bild ist eine Infektion eines Petersilieblattes durch *P. sojae* (Querschnitt) zu sehen. Links unten ist eine dünnschichtchromatographische Auftrennung von aus dem Kulturmedium von Petersiliezellen extrahierten Furanocoumarinen gezeigt, welche für 24 h mit Wasser, Pep-13 oder NPP1 behandelt wurden (Nürnberger et al., 1994; Fellbrich et al., 2002).

Vergleichende Analysen der Elicitoraktivität von Pep-13 und NPP1 zeigten, daß die Stimulierung der Phytoalexinbiosynthese in mit NPP1 behandelten Petersiliezellen nicht durch den Pep-13-Rezeptor vermittelt wird (Fellbrich et al., 2002). Beide Elicitoren stimulierten jedoch dasselbe Muster aller nachfolgenden zellulären Reaktionen und damit ganz offensichtlich sehr rasch konvergierende Signalketten in diesem System (Abb. 5).
NPP1 und Pep-13 stellen damit Elemente eines komplexen Oberflächenmusters von *Phytophthora* dar, die zum einen funktionell redundant zur Erkennung des Pathogens beitragen können, sich in ihrem Wirkungsspektrum allerdings auch ergänzen. So können durch die Perzeption beider Elicitoren Abwehrreaktionen ausgelöst werden, die dem in der natürlichen Pflanze/Pathogen-Interaktion beobachteten Spektrum entsprechen. Potentielle, durch die gleichzeitige Erkennung von zwei Elicitoren hervorgerufene synergistische Effekte auf die Aktivierung der Pathogenabwehr in Petersilie werden gegenwärtig untersucht.

3.6. Generelle Elicitoren und pflanzliche Pathogenresistenz

Die Vermutung ist naheliegend, daß die Aktivierung nichtkultivarspezifischer Abwehrreaktionen in Pflanzen das Ergebnis der Erkennung genereller Elicitoren ist, und daß die Perzeption komplexer mikrobieller Oberflächenmuster durch mehrere Rezeptoren die molekulare Basis für die relativ stabile Nichtwirtsresistenz von Pflanzen bildet (Heath, 2000; Nürnberger und Brunner, 2002). Obwohl eine Reihe korrelativer Daten diese Annahme zu stützen scheint, gibt es bislang keinen Nachweis eines kausalen Zusammenhangs zwischen der Aktivierung pflanzlicher Abwehrreaktionen durch generelle Elicitoren und nichtkultivarspezifischer Pathogenresistenz in Pflanzen. Es ist unumstritten, daß verschiedene Elicitoren in der Lage ist, in einer Vielzahl von Pflanzenspezies Abwehrreaktionen zu stimulieren. Dies ist insbesondere durch Zugabe von Elicitoren zu pflanzlichen Zellsuspensionskulturen oder durch Infiltration in intakte pflanzliche Gewebe demonstriert worden. Schwieriger zu beantworten ist jedoch die Frage, ob die Erkennung genereller Elicitoren in echten Pflanze/Pathogen-Interaktionen überhaupt oder in jedem Falle stattfindet.
Zur Klärung dieses Sachverhaltes haben wir *Arabidopsis*-Pflanzen mit avirulenten Bakterien (*P. syringae* pv. *tomato* DC3000/*AvrRPM1*) infiziert (nicht publiziert). In einem Parallelexperiment wurden Pflanzen mit ursprünglich virulenten Bakterien (*P. syringae* pv. *tomato* DC3000/hrcC$^-$) behandelt, die aufgrund eines Blocks in ihrem Typ III-Sekretionssystem (TTSS) ebenfalls nicht pathogen waren. Viele Gram-negative, phytopathogene Bakterien benötigen solch ein Sekretionssystem zum Export und zur Translokation von Virulenzfaktoren (einschließlich Avirulenzfaktoren, s. Kap. 4) in das Innere pflanzlicher Wirtszellen (Bonas und Lahaye, 2002; Collmer et al., 2002). Durch Nutzung des TTSS-defizienten Stammes wurde sichergestellt, daß diese Bakterien pflanzliche Abwehrreaktionen nur unabhängig von ihrem eigentlichen Infektionsinstrumentarium induzieren konnten, d. h. also durch Oberflächenstrukturen wie z.B. Flagellin und LPS (Dow et al., 2000; Gomez-Gomez und Boller, 2002). Biotinmarkierte cRNA aus infizierten Pflanzen wurde zur Hybridisierung von

DNA-Mikroarrays (Affymetrix®) eingesetzt, die Proben für ca. 8.300 *Arabidopsis*-Gene trugen (Kooperation mit Torrey Mesa Research Institute, Syngenta Corp., San Diego, USA). Aus diesem Experiment ging hervor, daß die Zahl der in ihrer Expression veränderten Gene in beiden Experimenten quantitativ vergleichbar war. Auch qualitativ zeigten sich erstaunliche Übereinstimmungen, auf die aus patentrechtlichen Gründen hier jedoch nicht näher eingegangen werden kann. Grundsätzlich läßt sich jedoch feststellen, daß das „antigene Potential" mikrobieller Oberflächen allein in der Lage ist, in intakten Pflanze/Pathogen-Interaktionen komplexe Änderungen des Genexpressionsmusters zu bewirken.
In einem weiteren Experiment (nicht publiziert, Abb. 6) konnten wir zeigen, daß sogar einzelne Elicitoren (NPP1) in der Lage sind, komplexe Veränderungen im Transkriptom von *Arabidopsis* hervorzurufen, die denen durch intakte Organismen stimulierten Effekten quantitativ vergleichbar waren.

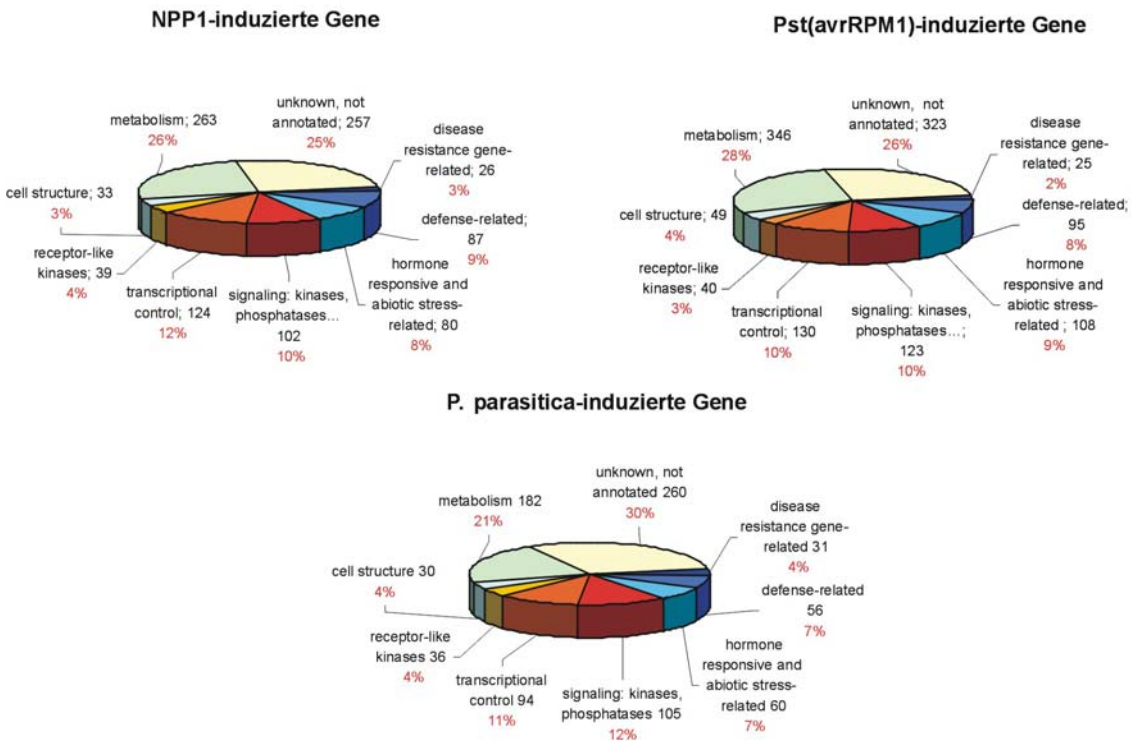

Abb. 6: DNA-Mikroarrayanalyse in *Arabidopsis thaliana*. Pflanzen des Ökotyps Col-0 wurden für 6 h mit 2 µM NPP1, mit 5 x 10^7 Kolonien/ml *P. syringae* pv. *tomato* (avrRPM1) oder für 24 h mit 1 x 10^5 Sporen/ml *Peronospora parasitica* Cala2 (avirulent) infiltriert. Aus diesen Pflanzen gewonnene RNA wurde zur Hybridisierung von *Arabidopsis*-Chips (8.300 Gene) der Firma Affymetrix® eingesetzt. Dargestellt sind die Gesamtzahl und eine Klassifizierung der Gene, deren Transkriptmenge sich bei der jeweiligen Behandlung um mindestens das 2-fache erhöhte.

Eine ganz ähnliche Erkenntnis resultierte aus Experimenten, in denen mononukleäre, periphäre Blutzellen des Menschen entweder mit hitzedenaturierten Bakterien oder nur mit der LPS-Fraktion Gram-negativer Bakterien behandelt wurden (Boldrick et al., 2002). Auch dies

mag als weiterer Beleg für Ähnlichkeiten in der biologischen Aktivität genereller Elicitoren und von PAMPs, die für die Auslösung der angeborenen Immunität tierischer Zellen verantwortlich sind, dienen.

Aus den geschilderten Experimenten geht hervor, daß die Erkennung genereller Elicitoren in echten Pflanze/Pathogen-Interaktionen stattfindet und damit durchaus zur aktiven Nichtwirtsresistenz beitragen kann. Es ist desweiteren nicht auszuschließen, daß die Erkennung von generellen Elicitoren durch Pflanzen ebenfalls von Bedeutung für die effiziente Ausprägung kultivarspezifischer Abwehrreaktionen in Wirtspflanzen ist. So ist durchaus vorstellbar, daß unter für ein Pathogen suboptimalen Infektionsbedingungen (definiert durch Luftfeuchte, Temperatur, Inokulumsdichte) eine erfolgreiche Infektion von Wirtspflanzen bereits durch die Auslösung der nichtkultivarspezifischen Basisresistenz verhindert werden kann (Tyler, 2002). Auch die Auslösung von ISR und SAR, beides Prozesse, die durch einzelne generelle Elicitoren (LPS, Harpin) aktiviert werden können (Pieterse und van Loon, 1999; Dong, 2001), stellt eine denkbare Konsequenz der Erkennung genereller Elicitoren in Pflanze/Pathogen-Interaktionen dar. Es gilt jedoch festzuhalten, daß momentan keine experimentellen Belege dafür existieren, daß die durch Elicitoren ausgelösten Abwehrmechanismen tatsächlich zur pflanzlichen Pathogenresistenz beitragen.

In tierischen Systemen hingegen konnte insbesondere durch genetische Analysen klar gezeigt werden, daß die Erkennung von PAMPs nicht nur für die Aktivierung der angeborenen Immunantwort in *Drosophila* (Lemaitre et al., 1996; Khush und Lemaitre, 2000; Imler und Hoffmann, 2001) oder dem Menschen (Medzhitov und Janeway, 1997; Aderem und Ulevitch, 2000; Underhill und Ozinsky, 2002), sondern nachfolgend auch für die Auslösung adaptiver, d.h. auf der Produktion spezifischer Antikörper gegen pathogentypische Antigene beruhender, Immunreaktionen in Vertebraten notwendig ist (Medzhitov et al., 1997). So konnte beispielsweise eine deutlich erhöhte Empfindlichkeit gegen bakterielle Infektionen in Mäusen nachgewiesen werden, in denen beide Allele des für die Perzeption von LPS verantwortlichen TLR4-Rezeptors inaktiviert waren (Poltorak et al., 1998; Hoshino et al., 1999).

Eine große experimentelle Herausforderung besteht zukünftig darin, den Beitrag zu bestimmen, den die Erkennung einzelner genereller Elicitoren zur Pathogenresistenz in Pflanzen leistet. Die genetische Inaktivierung von mikrobiellen Genen, die für (die Produktion von) Elicitoren kodieren, verbunden mit gleichzeitigen Bemühungen zur Ausschaltung von Elicitorrezeptoren auf der Pflanzenseite schaffen dafür geeignete Voraussetzungen. Das Gelingen solcher Experimente ist allerdings davon abhängig, daß sowohl die Mikroorganismen als auch die entsprechenden Pflanzen genetischer Manipulation zugänglich sind. Da die Auswahl experimenteller Systeme in der Vergangenheit nicht von dieser Prämisse ausgegangen ist, ergeben sich natürlich Schwierigkeiten insbesondere bei Systemen wie *Phytophthora* oder Petersilie. Es war deshalb ein Anliegen dieser Arbeit, ein Modellsystem (Arabidopsis/NPP1) zu etablieren, das solche genetischen Ansätze ermöglicht (s. Kap. 3.5.). Erschwerend auf eine genetische Beweisführung wird sich allerdings die Tatsache auswirken, daß die Nichtwirtsresistenz offensichtlich das Ergebnis einer komplexen Interaktion zwischen Pflanzen und potentiellen mikrobiellen Pathogenen ist (Heath, 2000;

Nürnberger und Brunner, 2002; Felix und Boller, 2003). Da Pflanzen häufig multiple Oberflächenstrukturen von Pathogenen mittels distinkter Rezeptoren erkennen können, ist deshalb zu erwarten, daß die genetische Inaktivierung einzelner Erkennungsereignisse nicht notwendigerweise zu einem Bruch der Nichtwirtsresistenz führen muß. Funktionelle Beweisführungen werden daher u. U. schwieriger als im Falle von monogen determinierten Rasse/Kultivar-spezifischen Pflanze/Pathogen-Interaktionen sein.

4. Harpin$_{Psph}$ – ein Effektorprotein aus *Pseudomonas syringae* pv. *phaseolicola*

4.1. Die Rolle von Harpin$_{Psph}$ bei der bakteriellen Pathogenese

Die *Hrp*-Gene phytopathogener, Gram-negativer Bakterien kodieren für Proteine, die sowohl für die mikrobielle Pathogenizität als auch für deren Fähigkeit, auf resistenten Wirtspflanzen oder auf Nichtwirtspflanzen hypersensitiven Zelltod und andere Abwehreaktionen zu induzieren, verantwortlich sind (Bogdanove et al., 1996; Galan und Collmer, 1999). Einige dieser Genprodukte sind Komponenten des bakteriellen Typ III-Sekretionssystems (TTSS), mit dessen Hilfe Effektorproteine entweder exportiert oder in das Zytoplasma pflanzlicher Wirtszellen translozíert werden können (Büttner und Bonas, 2002; Collmer et al., 2002). Obwohl die molekulare Funktion nahezu aller Virulenzfaktoren phytopathogener Bakterien unbekannt ist, wird in Analogie zu detaillierter untersuchten Proteinen tierischer Krankheitserreger vermutet, daß diese zur Umprogrammierung des Wirtsstoffwechsels und zur Suppression der Wirtsabwehr dienen. Das unter Infektionsbedingungen massiv produzierte Effektorprotein Harpin von *Pseudomonas syringae* wird von dem Gen *HrpZ* kodiert (He et al., 1993; Lee et al., 2001b). Orthologe Gene finden sich ebenfalls in Vertretern der Gattungen *Ralstonia* (Arlat et al., 1994) und *Erwinia* (Wei et al., 1992). Im Gegensatz zur umfangreichen Literatur über die Wirkung von Harpin als Auslöser nichtkultivarspezifischer pflanzlicher Abwehrreaktionen, ist dessen Funktion bei der bakteriellen Infektion von Pflanzen nur unzureichend bekannt. Dies ist insbesondere der Tatsache zuzuschreiben, daß nichtpolare Mutationen im *HrpZ*-Gen keine nennenswerte Reduktion der Pathogenizität der Mutanten zur Folge hatten (Charkowski et al., 1998; Lee et al., 2001b).

Teilsequenzen von Harpin aus *P. syringae* pv. *phaseolicola* (Harpin$_{Psph}$) ließen Ähnlichkeiten mit Strukturdomänen des Effektorproteins YopB aus *Yersinia enterocolitica* erkennen, welches als Teil eines Proteinkanals in die Membran von Wirtszellen eingelagert wird (Tardy et al., 1999; Lee et al., 2001b). Obwohl ektopisch exprimiertes *HrpZ*$_{Psph}$ durch das Typ III-Sekretionssystem von *Y. enterocolitica* korrekt sekretiert werden konnte, war es nicht in der Lage, eine Mutation des *YopB*-Gens funktionell zu komplementieren (Lee et al., 2001b). Assoziationsstudien an mit Lipiddoppelmembranen überzogenen Silikatperlen zeigten jedoch, daß das Protein dennoch zur Membranbindung befähigt ist. Desweiteren konnte in elektrophysiologischen Experimenten belegt werden, daß das Protein nicht nur mit Membranen assoziieren konnte, sondern Kationenflüsse durch planare Lipiddoppelschichten ermöglichte (Lee et al., 2001b). Diese Aktivität von Harpin$_{Psph}$ konnte auch für die

entsprechenden Proteine aus *P. syringae* pv. *tomato* und *P. s.* pv. *syringae* nachgewiesen werden. Die durch Harpin geformte Ionenpore ist nicht selektiv für mono- und divalente Kationen, verhindert jedoch den Transport von Anionen, was auf ein elektronegatives Porenlumen schließen läßt (Lee et al., 2001b). In einem weiteren Assay, der auf der Aktivierung eines in Liposomen eingeschlossenen Na^+-sensitiven Fluoreszenzfarbstoffes beruhte, konnte die porenbildende Aktivität sowohl von $Harpin_{Psph}$ als auch von einem funktionell verwandten Protein aus *Erwinia amylovora* (HrpN) nachgewiesen werden (nicht publiziert). Die in Experimenten mit chemischen Quervernetzern demonstrierte Fähigkeit von $Harpin_{Psph}$ zur Homooligomerisation deutet darauf hin, daß, ähnlich wie für andere bakterielle porenbildende Proteine beschrieben, mehrere Untereinheiten zum Aufbau einer funktionellen Struktur beitragen könnten. Gegenwärtig ist offen, welche Substrate unter physiologischen Bedingungen durch die von $Harpin_{Psph}$ gebildete Pore transportiert werden. Es ist vorstellbar, daß sie sowohl dem Transport von Effektormolekülen in die Wirtszelle als auch der Ernährung der sich apoplastidär ernährenden und reproduzierenden Bakterien dient.

4.2. $Harpin_{Psph}$ als Elicitor der pflanzlichen Pathogenabwehr

Wie in Kap. 4.1. erwähnt, sind Harpine verschiedener phytopathogener Bakterien in der Lage, nichtkultivarspezifische pflanzliche Abwehrreaktionen zu stimulieren (Wei et al., 1992; He et al., 1993; Arlat et al., 1994; He, 1996; Desikan et al., 1999; Lee et al., 2001a). Dazu zählen der hypersensitive Zelltod, die Aktivierung von Ionenkanälen in der Plasmamembran, die Produktion reaktiver Sauerstoffspezies, die posttranslationale Aktivierung von MAPK, die Expression von *PR*-Genen und die Auslösung von systemisch erworbener Resistenz. Neben AVR-Proteinen und einigen generellen Elicitoren ist Harpin damit ein weiteres Beispiel dafür, daß Pflanzen im Laufe ihrer Evolution die Fähigkeit erworben haben, potentielle Virulenzfaktoren mikrobieller Pathogene zu erkennen und als Auslöser für das eigene Verteidigungsarsenal zu nutzen.

Wegen seines die Pflanzenabwehr induzierenden Potentials in vielen Kulturpflanzen wird Harpin aus *E. amylovora* unter dem Namen Messenger® als Resistenzinduktor in der Landwirtschaft eingesetzt. Allerdings ist über die molekulare Basis der Erkennung von Harpin an der pflanzlichen Plasmamembran nur sehr wenig bekannt. Aufgrund der Tatsache, daß synthetische Ionophore in der Lage sind, pflanzliche Abwehrreaktionen in Abwesenheit mikrobieller Elicitoren auszulösen (Jabs et al., 1997; Scheel, 1998), und aufgrund der Erkenntnis, daß $Harpin_{Psph}$ ionenpermeable Poren in Lipiddoppelmembranen formen kann (Lee et al., 2001b), war es daher vorstellbar, daß die Ionenkanalaktivität des Proteins auch die molekulare Basis für die Aktivierung der Pathogenabwehr in Pflanzen bildet. Um diese These zu testen, wurden Teile des Proteins rekombinant hergestellt und hinsichtlich ihrer Fähigkeit zur Ionenkanalbildung und zur Auslösung verschiedener Abwehrreaktionen in Tabak und Petersilie untersucht. Dabei zeigte sich, daß lediglich intaktes $Harpin_{Psph}$ zur Porenbildung in

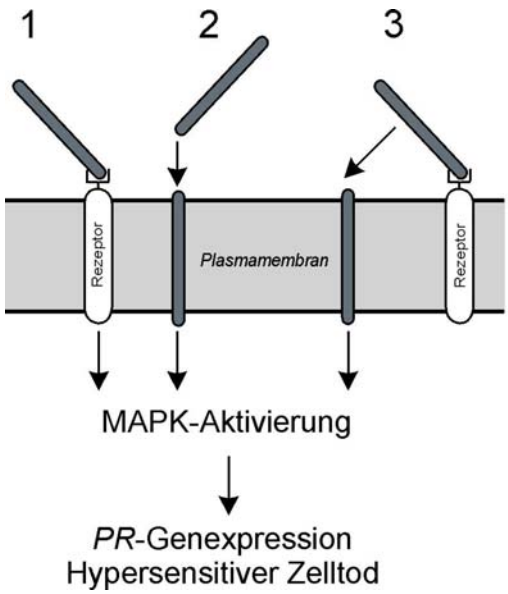

Abb. 7: Modell zur Beschreibung der Interaktion von $Harpin_{Psph}$ mit Plasmamembranen von Tabak. (1) rezeptorvermittelte Aktivierung der Pathogenabwehr. (2) Porenbildung. Es ist nicht auszuschließen, daß einfache Porenbildung durch intaktes $Harpin_{Psph}$ zur Aktivierung von Abwehrreaktionen führt. Dieser Mechanismus kann jedoch nicht hinreichend die Elicitoraktivität von solchen $Harpin_{Psph}$ -Fragmenten erklären, die unfähig zur Porenbildung sind. (3) Durch ein Bindungsprotein vermittelte Insertion in die Plasmamembran und damit verbundene Abwehrinduktion.

der Lage war (nicht publiziert), während sowohl das vollständige Protein als auch verschiedene C-terminale Fragmente abwehrinduzierend wirkten (Lee et al., 2001a)

Da diese Teilproteine ebenfalls die Bindung von radioaktiv jodiertem $Harpin_{Psph}$ an eine in der Plasmamembran von Tabak- bzw. Petersiliezellen lokalisierte hochaffine Bindungsstelle kompetierten, kann geschlossen werden, daß (i) die Porenbildung nicht hinreichend für die Elicitoraktivität ist und daß (ii) die Stimulierung der Pflanzenabwehr vermutlich durch einen Rezeptor vermittelt wird (Abb. 7) (Lee et al., 2001a). Dies bedeutet, daß nach diesem Modell beide Prozesse unabhängig voneinander parallel verlaufen, und so die mögliche Virulenzfunktion von $Harpin_{Psph}$ (Porenbildung) und die Fähigkeit von Pflanzen zur rezeptorvermittelten, elicitorinduzierten Abwehr widerspiegeln. Es ist jedoch auch vorstellbar, daß die Bindung von $Harpin_{Psph}$ an eine einfache Bindungsstelle Voraussetzung für die Insertion des Proteins in die Plasmamembran ist und diese Kanalbildung letztlich zur Auslösung pflanzlicher Abwehrreaktionen führt. Beispiele für eine derartige unterstützte Einlagerung von Proteinen in Lipiddoppelmembranen, die im Liposomenassay zur Porenbildung nicht notwendigerweise nachweisbar ist, finden sich in der Literatur (Breukink et al., 1999; Thevissen et al., 2000a; Thevissen et al., 2000b).

5. Ausblick

Neuere Publikationen belegen erstaunliche Ähnlichkeiten in der molekularen Organisation des angeborenen Immunsystems in Wirbeltieren, Insekten und Pflanzen. Wesentliche Übereinstimmungen lassen sich so in den Eigenschaften von PAMPs und generellen Elicitoren sowie in der Struktur der Signalperzeptions- und -transduktionssysteme finden. Pflanzliche und tierische Abwehrsysteme gleichen sich desweiteren in ihren Fähigkeiten, komplexe Muster mikrobieller Oberflächen zu erkennen, wobei einzelne Signalerkennungsereignisse bereits sehr komplexe Abwehrreaktionen in eukaryotischen Systemen auslösen können.

Vergleichende Analysen der Genome von Tieren und Pflanzen zeigen, daß sich wesentliche Aspekte der evolutionären Entwicklung von Tieren und Pflanzen getrennt vollzogen haben (Meyerowitz, 2002). Die genannten Parallelen zwischen tierischer und pflanzlicher Immunabwehr (dabei insbesondere strukturelle Ähnlichkeiten von TLR, Toll und dem pflanzlichen Flagellinrezeptor, Gomez-Gomez und Boller, 2000; Meyerowitz, 2002) lassen jedoch vermuten, daß der letzte gemeinsame Vertreter beider Linien bereits ein molekulares Abwehrsystem besaß, welches sich im Zuge einer divergent verlaufenden Evolution bis in die heute anzutreffenden Formen weiterentwickelt hat (Cohn et al., 2001; Gomez-Gomez und Boller, 2002; Nürnberger und Brunner, 2002). Andererseits kann aufgrund des gegenwärtigen, limitierten Erkenntnisstandes aber auch nicht ausgeschlossen werden, daß sich beide Abwehrsysteme getrennt voneinander herausgebildet haben, und daß die offensichtlichen Parallelen das Ergebnis konvergierender Evolution bzw. das Resultat der Selektion auf die effizienteste Form der Abwehr gegen mikrobielle Infektionen sind (Gomez-Gomez und Boller, 2002).

Die intensive Forschungstätigkeit auf dem Gebiet der Pathogenabwehr in Pflanzen läßt in den kommenden Jahren einen raschen Informationszuwachs erwarten, der insbesondere der verstärkten Nutzung des genetischen Modellsystems *Arabidopsis thaliana* zuzuschreiben sein dürfte. Im Zusammenspiel mit verbesserten biochemischen und zellbiologischen Techniken werden solche Ansätze die Zahl isolierter Elicitorrezeptoren und Elicitorsignaltransduktionselemente derart erhöhen, daß eine fundiertere Analyse der Genese und Evolution eukaryotischer Abwehrsysteme möglich sein wird.

6. Literatur

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7. Anhang

Liste der dieser Habilitation zugrunde liegenden und beigefügten Publikationen

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Danksagung

Ich danke allen Mitarbeitern der Abteilung Biochemie des Max-Planck-Institutes für Züchtungsforschung Köln und der Abteilung Streß- und Entwicklungsbiologie des Institutes für Pflanzenbiochemie Halle für ihre stets gewährte wissenschaftliche und intellektuelle Unterstützung und die angenehme Arbeitsatmosphäre.

Ein besonderer Dank gilt jenen Mitarbeitern, die maßgeblich zum Gelingen der vorliegenden Arbeit beigetragen haben. Insbesondere sind dies Wendy Sacks, Thorsten Jabs, Dirk Nennstiel, Wolfgang Wirtz, Justin Lee, Annette Romanski, Guido Fellbrich, Beatrix Blume, Frédéric Brunner, Birgit Kemmerling und Yvonne Gäbler.

Meinen Mentoren Klaus Hahlbrock und Dierk Scheel, die in ihrer kameradschaftlichen Art und mit ihrer wissenschaftliche Ausstrahlung für mich stets Vorbild und Ansporn waren und meine berufliche Entwicklung ganz wesentlich mitgeprägt haben, bin ich zutiefst verbunden.
In Dierk für mehr als elf Jahre nicht nur einen Chef sondern auch einen Freund gehabt zu haben, erfüllt mich dabei mit besonderer Freude.

Meinen Freunden, Eltern und insbesondere Birgit danke ich für ihre vielfältige Unterstützung und Hilfsbereitschaft, auf die ich mich auch in schwierigen Momenten verlassen konnte.

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Erklärung

Hiermit erkläre ich an Eides statt, daß ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfaßt, andere als die angegebenen Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Diese Arbeit wurde nur an der Mathematisch-Naturwissenschaftlich-Technischen Fakultät der Martin-Luther-Universität Halle-Wittenberg vorgelegt.

Halle, den 27. Februar 2003

Cell, Vol. 78, 449–460, August 12, 1994, Copyright © 1994 by Cell Press

High Affinity Binding of a Fungal Oligopeptide Elicitor to Parsley Plasma Membranes Triggers Multiple Defense Responses

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Summary

An oligopeptide of 13 amino acids (Pep-13) identified within a 42 kDa glycoprotein elicitor from P. megasperma was shown to be necessary and sufficient to stimulate a complex defense response in parsley cells comprising H^+/Ca^{2+} influxes, K^+/Cl^- effluxes, an oxidative burst, defense-related gene activation, and phytoalexin formation. Binding of radiolabeled Pep-13 to parsley microsomes and protoplasts was specific, reversible, and saturable. Identical structural features of Pep-13 were found to be responsible for specific binding and initiation of all plant responses analyzed. The high affinity binding site recognizing the peptide ligand (K_D = 2.4 nM) may therefore represent a novel class of receptors in plants, and the rapidly induced ion fluxes may constitute elements of the signal transduction cascade triggering pathogen defense in plants.

Introduction

Plants mount a complex array of defense reactions in response to invading fungal pathogens. These inducible reactions result either from transcriptional activation of defense-related genes encoding, for example, enzymes of the general phenylpropanoid pathway (Hahlbrock and Scheel, 1989; Dixon and Lamb, 1990) or from specific enzyme activation initiating cell wall reinforcement (Kauss et al., 1989; Bradley et al., 1992) and the oxidative burst (Sutherland, 1991). These reactions are frequently associated with a highly localized response, hypersensitive cell death, although there is no evidence at present for a causal link (Atkinson, 1993; Jakobek and Lindgren, 1993).

Initiation of these events requires perception of either plant-derived (endogenous) or fungus-derived (exogenous) signals, collectively referred to as elicitors. A widely accepted hypothesis assumes that plant resistance occurs at the cultivar or species level after recognition of either race-specific or general elicitors (Knogge, 1991). Elicitors of diverse chemical nature, including carbohydrates, lipids, and proteins, have been isolated from a variety of phytopathogenic and nonpathogenic fungi and have been shown to trigger plant defense responses (Ebel and Cosio, 1994). The cultivar or species specificity of purified elicitors, their ability to induce various plant responses at nanomolar concentrations, and the high degree of signal specificity required for the elicitor-mediated activation of plant defense responses are characteristics that strongly suggest the involvement of receptors in elicitor recognition and subsequent intracellular signal generation (Ebel and Cosio, 1994).

The mechanisms underlying both elicitor perception at the plant cell surface and subsequent intracellular transmission of this signal to target sites are still not well understood. Accumulating evidence indicates that high affinity receptors for fungal elicitors do reside in plant plasma membranes (Cosio et al., 1988; Cheong and Hahn, 1991a; Basse et al., 1993; Shibuya et al., 1993). Elicitor-induced changes in ion fluxes across the plasma membrane and in protein phosphorylation have been described (Farmer et al., 1989; Dietrich et al., 1990; Felix et al., 1991; Mathieu et al., 1991; Renelt et al., 1993) that precede induction of defense responses and are likely to be involved in signaling. Moreover, cis-acting elements in the promoter regions of defense-related genes have been found to be essential for elicitor-stimulated transcription (Dron et al., 1988; Lois et al., 1989), and trans-acting factors binding to these elements have been characterized (da Costa e Silva et al., 1993; Yu et al., 1993; Korfhage et al., 1994).

Parsley leaves develop a typical species resistance reaction against the soybean pathogen Phytophthora megasperma comprising hypersensitive cell death, defense-related gene activation, and phytoalexin formation (Jahnen and Hahlbrock, 1988; Schmelzer et al., 1989). We have been investigating signaling mechanisms that trigger defense-related gene activation and furanocoumarin phytoalexin formation in suspension-cultured parsley cells or protoplasts treated with an elicitor preparation derived from the fungal cell wall. A set of 21 arbitrarily selected elicitor-responsive genes has been described for parsley (Somssich et al., 1989) that includes genes encoding enzymes of general phenylpropanoid metabolism, such as phenylalanine ammonia-lyase (Lois et al., 1989) and 4-coumarate:coenzyme A ligase (Douglas et al., 1987). The signal transduction pathway leading to the transcriptional activation of these genes appears to involve rapid and transient changes in the permeability of the plasma membrane to Ca^{2+}, K^+, H^+, Cl^- and phosphorylation/dephosphorylation of specific proteins (Dietrich et al., 1990; Renelt et al., 1993). To study in detail the molecular mechanisms underlying elicitor recognition and subsequent signal transduction, homogeneous elicitor-active compounds from different fungal sources were isolated. A 42 kDa glycoprotein elicitor was consequently purified from fungal culture filtrate whose elicitor activity resides in the protein moiety (Parker et al., 1991).

Here we report the existence of a high affinity binding site in parsley microsomal membranes and protoplasts for an elicitor-active oligopeptide proteolytically released from the glycoprotein elicitor. Using a series of structurally re-

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lated peptides, a functional link was established between the binding of this ligand at the plasma membrane, the stimulation of ion fluxes across the plasma membrane, and the occurrence of an oxidative burst as well as more ensuing plant responses based on transcriptional activation of defense-related genes, such as phytoalexin biosynthesis.

Results

Active Site Identification within a Fungal Glycoprotein Elicitor

Several endo- and exopeptidases were tested for their ability to release peptides retaining elicitor activity from the 42 kDa glycoprotein elicitor. Endoproteinase Glu-C yielded elicitor-active peptides (Sacks et al., 1993), which were subsequently purified by reverse-phase high pressure liquid chromatography (HPLC) and sequenced by automated Edman degradation. The peptide sequences obtained matched unequivocally amino acid sequences deduced from a cDNA encoding the fungal glycoprotein, which was recently cloned and characterized (Sacks et al., 1994). The smallest elicitor-active peptide obtained was found to consist of 13 amino acid residues with the sequence H_2N–VWNQPVRGFKVYE–COOH (Pep-13). All of the other elicitor-active peptides were longer versions of this peptide and represented partially digested glycoprotein.

To ascertain whether Pep-13 was the sole determinant of elicitor activity within the mature glycoprotein, portions of the polypeptide were expressed in Escherichia coli. Clones in which the 13 amino acids representing Pep-13 were replaced by either two or six amino acids unrelated to the peptide (Figure 1A) were obtained by ligation of polymerase chain reaction products spanning the N- and C-terminal parts of the glycoprotein. The full-length cDNA as well as these deletion constructs were cloned into a modified version of the prokaryotic expression vector, pGEX-2T (Kawalleck et al., 1993a). Expressed fusion proteins were solubilized from inclusion bodies (Figure 1B) and tested for elicitor activity. Only the mature protein containing Pep-13 was elicitor active, whereas the deletion products failed to display elicitor activity (Figure 1A). In similar experiments, all other domains of the protein did not elicit phytoalexin production in parsley (Sacks et al., 1994). This oligopeptide sequence is therefore necessary and sufficient for the elicitor activity of the glycoprotein.

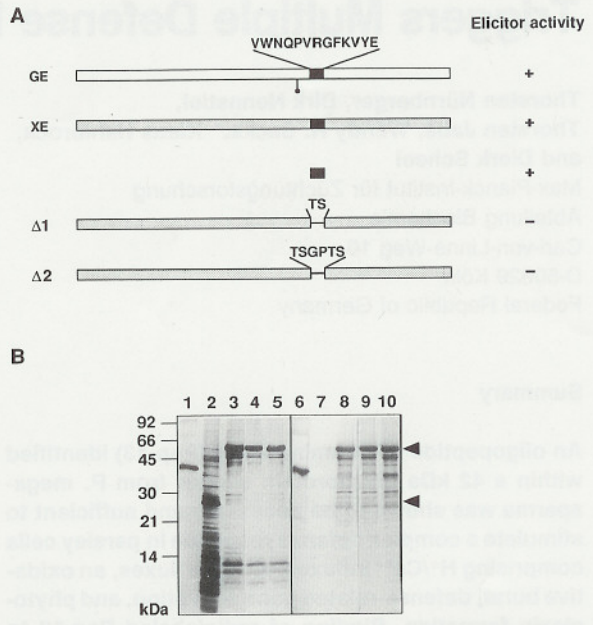

Figure 1. Structural and Functional Comparison of the Glycoprotein Elicitor with Various Deletion Derivatives Expressed in E. coli

(A) cDNAs encoding the protein portion of the 42 kDa elicitor (XE) and deletion derivatives Δ1 and Δ2, in which the Pep-13-encoding sequence was replaced with nucleotide sequences coding for TS or TSGPTS, respectively, were constructed in a modified pGEX-2T vector and transformed into E. coli. After IPTG treatment, proteins accumulating in inclusion bodies were solubilized and assayed for their ability to stimulate phytoalexin formation in parsley protoplasts (plus sign, full activity relative to that of the native glycoprotein elicitor; minus sign, no activity detectable). The open bar represents the mature 42 kDa elicitor (glycoprotein elicitor [GE]) with its glycosylation site indicated (closed circle attached by a solid line to the open bar). The stippled bars represent the expressed portions of the elicitor polypeptide, with the N-terminus on the left. The closed bars denote the 13 amino acid region representing Pep-13.

(B) Analysis of total silver-stained proteins on SDS–polyacrylamide gels (lanes 1–5), and immunodetection with a polyclonal antiserum raised against the deglycosylated 42 kDa glycoprotein of these proteins after transfer to nitrocellulose (lanes 6–10). Protein was prepared from IPTG-treated E. coli cells transformed with either the modified pGEX-2T vector alone (lanes 2 and 7), with the vector containing the full-length cDNA encoding the elicitor polypeptide (lanes 3 and 8), or with deletion derivatives Δ1 (lanes 4 and 9) or Δ2 (lanes 5 and 10). Lanes 1 and 6 contained the mature glycoprotein elicitor purified from the fungal culture medium. Arrowheads at the right indicate the positions of glutathione-S-transferase (lower) and the glutathione-S-transferase–fusion proteins (upper). The molecular masses, in kDa, of marker proteins are indicated on the left.

Plant Cell Responses to Treatment with Peptide Elicitor

Elicitor concentrations necessary for half-maximal stimulation of phytoalexin formation in parsley protoplasts (EC_{50}) were similar for Pep-13 and the glycoprotein elicitor, although Pep-13 appeared to be slightly less active than was the intact glycoprotein (EC_{50} = 31 nM versus EC_{50} = 9 nM). To determine whether suboptimal information content of the peptide elicitor was responsible for the difference detected, a longer version of this peptide (H_2N–DVTAGAE–Pep-13–QTEMT–COOH) was chemically synthesized (Pep-25). This peptide, however, exhibited the same elicitor activity as did Pep-13 (EC_{50} = 36 nM), suggesting that reduced elicitor stability rather than incomplete sequence information affected the elicitor activity of Pep-13.

To examine whether the peptide elicitors activate the same defense-related genes as do the crude cell wall (P. megasperma [Pmg] elicitor) and the 42 kDa glycoprotein elicitors, total RNA was extracted from parsley protoplasts harvested 2 hr after the addition of different elicitor preparations. RNA–slot blot hybridization was performed with ^{32}P-labeled cDNAs encoding phenylalanine ammonia-

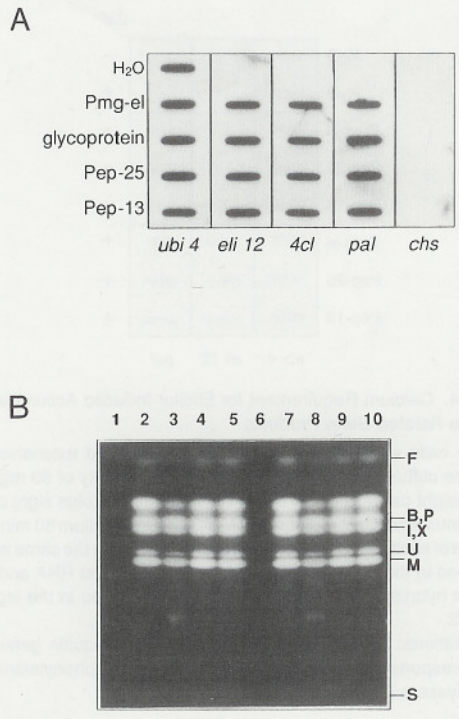

Figure 2. Elicitor-Induced Accumulation of Defense-Related Gene Products and Phytoalexin Formation

(A) Accumulation of defense-related gene products. Parsley protoplasts (5 × 10^6/ml) were treated for 2 hr with the elicitors indicated (50 μg/ml Pmg elicitor (Pmg-el), 1 μg/ml 42 kDa glycoprotein, or 100 ng/ml Pep-25 or Pep-13) or with water. Isolation of total RNA, slot blot transfer, and hybridization with ^{32}P-labeled cDNAs complementary to mRNAs encoding polyubiquitin (*ubi4*), elicitor-responsive *eli12*, 4-coumarate:coenzyme A ligase (*4cl*), phenylalanine ammonia-lyase (*pal*), and chalcone synthase (*chs*) from parsley were performed as described in Experimental Procedures.

(B) Phytoalexin formation. Suspension-cultured parsley cells (60 mg of fresh weight per milliliter, lanes 1–5) and protoplasts (5 × 10^6/ml, lanes 6–10) were treated for 24 hr with water (lanes 1 and 6), Pmg elicitor (lanes 2 and 7), the purified glycoprotein elicitor (lanes 3 and 8), or the peptide elicitors Pep-25 (lanes 4 and 9) and Pep-13 (lanes 5 and 10). Elicitor concentrations were the same as those used in lyase and 4-coumarate:coenzyme A ligase as well as the product of *eli12*, an elicitor-responsive gene of unknown function (Somssich et al., 1989). Figure 2A shows that both peptides induced the expression of these genes in the same manner as the other elicitors did. High levels of mRNA of a constitutively expressed polyubiquitin gene from parsley (Kawalleck et al. 1993b), but no mRNA of the UV light–responsive chalcone synthase, accumulated in protoplasts treated with any of the elicitors tested.

Thin-layer chromatographic analysis of furanocoumarins secreted by parsley cells and protoplasts 24 hr after addition of different elicitor preparations revealed that both peptides elicited the full complement of parsley phytoalexins (Figure 2B). No significant differences could be detected among the furanocoumarin products generated by treatment with the Pmg elicitor and peptide elicitors, suggesting that the overall expression patterns of defense-related genes found after treatment with these elicitors were similar.

Treatment of parsley cells with the Pmg elicitor (Chappell et al., 1984) or Pep-13 stimulated ethylene biosynthesis 4-fold within 8 hr relative to cells treated with water (data not shown). The enzymes, 1-aminocyclopropane-1-carboxylate synthase and ethylene-forming enzyme, involved in ethylene biosynthesis were both activated by these elicitors.

The oxidative burst, a rapid release of H_2O_2, was investigated by challenging cultured parsley cells with either peptide elicitor. Concentrations of 6.9 nM (Pep-13) and 8.5 nM (Pep-25) were found to induce H_2O_2 formation half maximally, beginning within 2–5 min after addition of the elicitor and approaching a maximum after 20 min (Pep-13) and 30 min (Pep-25) (Figure 3A).

(A). Coumarin derivatives were extracted from the culture media and analyzed by thin-layer chromatography. Cochromatography of standard compounds is indicated (B, bergapten; P, psoralen; I, isopimpinellin; X, xanthotoxin; U, umbelliferone; M, marmesin; S, origin; and F, solvent front).

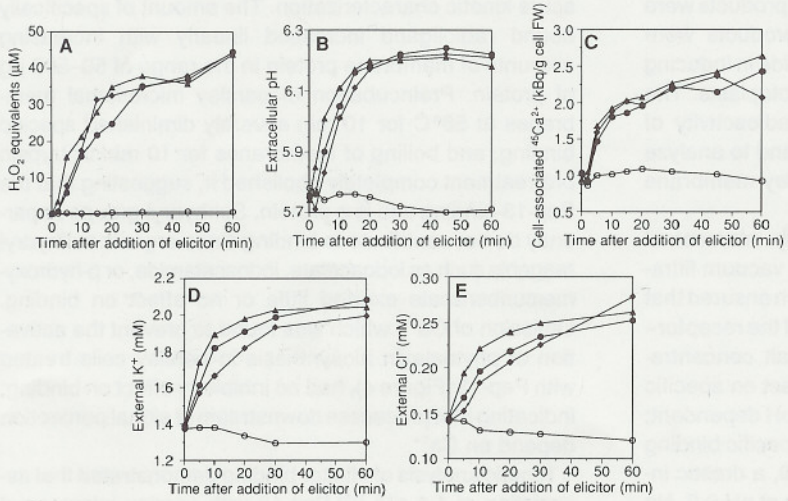

Figure 3. Time Courses of Elicitor-Stimulated H_2O_2 Formation and Ion Fluxes across the Plasma Membrane in Suspension-Cultured Parsley Cells

(A) H_2O_2 formation, (B) extracellular alkalinization, (C) Ca^{2+} influx, (D) K^+ efflux, and (E) Cl^- efflux. Elicitor concentrations used were those given in the legend to Figure 2. Each data point represents the average of duplicates.

Abbreviations: open circle, untreated; closed diamond, Pmg elicitor; closed circle, Pep-25; closed triangle, Pep-13; and kBq/g cell FW, kilobecquerel per gram of cell fresh weight.

Changes in permeability of the plasma membrane to H^+, Ca^{2+}, K^+, and Cl^- were among the earliest events detectable after treatment of parsley cells with various elicitors (Figures 3B–3E). Ca^{2+} uptake into parsley cells was stimulated within 2–5 min and lasted for about 30 min (Figure 3C). Furthermore, elicitor treatment caused rapid extracellular alkalinization and increases in extracellular K^+ and Cl^- concentrations (Figures 3B, 3D, and 3E). Again, within 2–5 min, flux stimulation took place and a maximum was reached 20 min (H^+) or 30 min (K^+, Cl^-) after addition of elicitor. No differences were detectable between Pep-13 and Pep-25 as inducers of the ion fluxes with respect to time course and EC_{50} values. Peptide concentrations required for half-maximal induction of the fluxes were 1.9 nM (H^+), 4.9 nM (Ca^{2+}), 3.3 nM (K^+), and 4.4 nM (Cl^-) for Pep-13 and 2.6 nM (H^+), 3.2 nM (Ca^{2+}), 6.1 nM (K^+), and 9.0 nM (Cl^-) for Pep-25.

To investigate whether Ca^{2+} is required for the transcriptional activation of defense-related genes and subsequent furanocoumarin biosynthesis, thoroughly washed parsley cells were treated either with Pep-13, Pep-25, or with the Pmg elicitor in the absence or presence of extracellular Ca^{2+}.

Hybridization of total RNA with cDNAs representing the elicitor-responsive phenylalanine ammonia-lyase and *eli12* genes revealed that in the absence of Ca^{2+} in the medium, little or no mRNA accumulated in parsley cells treated with Pmg elicitor or peptide elicitors (Figure 4). This response closely corresponded to the levels of furanocoumarins detectable 24 hr after elicitor treatment. Residual amounts of furanocoumarins in Ca^{2+}-free cell suspensions were 2.1% (Pep-13, and Pep-25) and 23.0% (Pmg elicitor) of the levels that accumulated in response to the respective elicitors in the presence of Ca^{2+}. No significant differences in cell viability were observed among parsley cells cultivated in the absence or presence of Ca^{2+} within 24 hr.

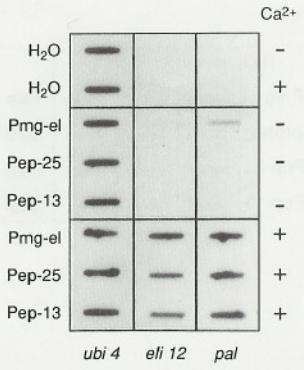

Figure 4. Calcium Requirement for Elicitor-Induced Accumulation of Defense-Related Gene Products

Parsley cells were harvested by filtration, washed extensively with Ca^{2+}-free culture medium, and adjusted to a density of 60 mg of cell fresh weight per milliliter in either Ca^{2+}-containing (plus sign) or Ca^{2+}-free (minus sign; Ca^{2+} replaced by Mg^{2+}) culture medium 30 min before addition of elicitor. Elicitor concentrations used were the same as those described in the legend to Figure 2. Isolation of total RNA and RNA–slot blot hybridization were carried out as described in the legend to Figure 2.

Abbreviations: Pmg-el, Pmg elicitor; *ubi4*, polyubiquitin gene; *eli12*, elicitor-responsive protein encoding gene; and *pal*, phenylalanine ammonia lyase encoding gene.

Binding of Radiolabeled Pep-13 Elicitor to Parsley Microsomes and Protoplasts

Pep-13 was labeled for binding assays by introducing radioactive iodine into metapositions of the phenoxyl ring of the Tyr-12 residue. A mixture of mono- and disubstituted derivatives was obtained, and the individual products were purified by reverse-phase HPLC. Both products were found to be as active as the unmodified peptide in inducing furanocoumarin biosynthesis in parsley protoplasts. The monoiodinated peptide, with a specific radioactivity of 2200 Ci/mmol, was chosen as the radioligand to analyze binding of the oligopeptide elicitor to parsley membrane preparations.

Binding of ^{125}I-Pep-13 elicitor to parsley microsomal membranes was investigated at 0°C using vacuum filtration to separate free from bound ligand, which ensured that loss of ligand caused by rapid dissociation of the receptor–ligand complex was negligible. Varying salt concentrations (0.01–2 M KCl) had no significant effect on specific binding. Binding was, however, found to be pH dependent; at acidic and neutral pH values, very little specific binding was detectable, but at values above pH 7.0, a drastic increase was observed, reaching a maximum at pH 9.0. No significant decline was detectable at higher pH values, indicating the existence of an alkali-stable binding site. Binding assays were routinely performed at pH 8.0, since elicitor-induced phytoalexin accumulation at this pH was found to be similar (20% reduction) to that obtained under standard cell cultivation conditions (pH 5.5). The stability of the ligand under binding assay conditions was confirmed by thin-layer chromatography and autoradiography of aliquots taken from the binding assay mixture after various times of incubation.

Specific binding of the radiolabeled ligand to parsley microsomes represented about 90% of total binding at an initial ligand concentration of 1.1 nM. No more than 5% of the initially applied ligand became bound to microsomal membranes, ensuring that ligand depletion did not obscure kinetic characterization. The amount of specifically bound radioligand increased linearly with increasing amounts of membrane protein in the range of 50–500 μg of protein. Preincubation of parsley microsomal membranes at 56°C for 10 min severely diminished specific binding, and boiling of membranes for 10 min or trypsin pretreatment completely abolished it, suggesting that the Pep-13-binding site is a protein. Sulfhydryl groups apparently do not contribute to binding since organic sulfhydryl reagents such as iodoacetate, iodoacetamide, or p-hydroxymercuribenzoate exerted little or no effect on binding. Omission of Ca^{2+}, which was found to prevent the activation of phytoalexin biosynthesis in parsley cells treated with Pep-13 (Figure 4), had no inhibitory effect on binding, indicating that processes downstream of signal perception depend on Ca^{2+}.

Kinetic analysis of elicitor binding demonstrated that association of 1.1 nM ^{125}I-Pep-13 with parsley microsomal

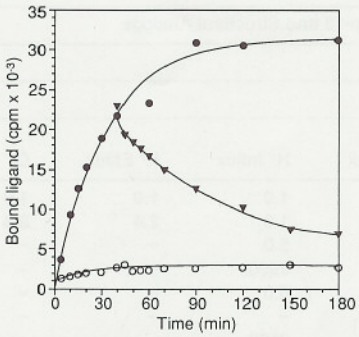

Figure 5. Time Courses of Binding and Displacement of ^{125}I-Pep-13

Assays were initiated by adding 1.1 nM radioligand to parsley microsomes. Specific binding (closed circle), nonspecific binding (open circle), and displacement of bound ^{125}I-Pep-13 initiated 40 min after addition of the radioligand (closed inverted triangle) were monitored at the times indicated. Specific binding was obtained by subtracting nonspecific binding from total binding. Nonspecific binding and displacement of bound ^{125}I-Pep-13 were determined in the presence of 15 μM unlabeled Pep-13. Each data point represents the average of duplicates.

membranes was initially faster than dissociation. Half-maximal binding was achieved within 20 min after addition of the ligand, and equilibrium between association and dissociation was reached after 90 min (Figure 5). Nonspecific binding remained constant during this time. Addition of a 10,000-fold molar excess of unlabeled elicitor 40 min after addition of the radioligand to parsley microsomes led to a rapid dissociation of bound label, demonstrating the reversible nature of this binding event. The K_{off} rates for the dissociation of the receptor–ligand complex found either during equilibrium or during displacement by unlabeled ligand were 0.022/min and 0.010/min, respectively. The slower dissociation observed during displacement could be due to inefficient competitor distribution after addition to the dense microsomal membrane preparation, as has been observed in other systems as well (Basse et al., 1993).

In saturation analyses, microsomal preparations were incubated with increasing concentrations (0.5–100 nM) of the radioligand (Figure 6A). Saturation of specific binding of Pep-13 to its binding site was achieved at a ligand concentration of approximately 20 nM, whereas total binding further increased solely owing to an increase in nonspecific binding (Figure 6A, inset). Linearization of the data in a Scatchard plot (Figure 6B) indicated the existence of a high affinity, single class binding site for the oligopeptide in parsley microsomal membranes (K_D = 2.4 nM). A Woolf plot, which specifically emphasizes higher ligand concentrations used in saturation experiments, also revealed a K_D of 2.4 nM. The apparent concentration of binding sites was 88 fmol/mg of protein. Assuming that one receptor molecule binds one ligand molecule, this value corresponds to approximately 1600 binding sites per cell. A Hill plot of the binding data shown in Figure 6A yielded a Hill coefficient of 1, excluding cooperativity in binding of Pep-13 to parsley microsomes (Figure 6C). At a K_D of 2.4 nM, a 30- to 40-fold shorter incubation time is predicted to be required to achieve binding equilibrium at room temperature relative to that required at 0°C (Hulme and Birdsall, 1992). This suggests that complete equilibrium of binding of Pep-13 to its putative receptor is achieved within 2–3 min after addition of the elicitor to parsley microsomal membranes at room temperature.

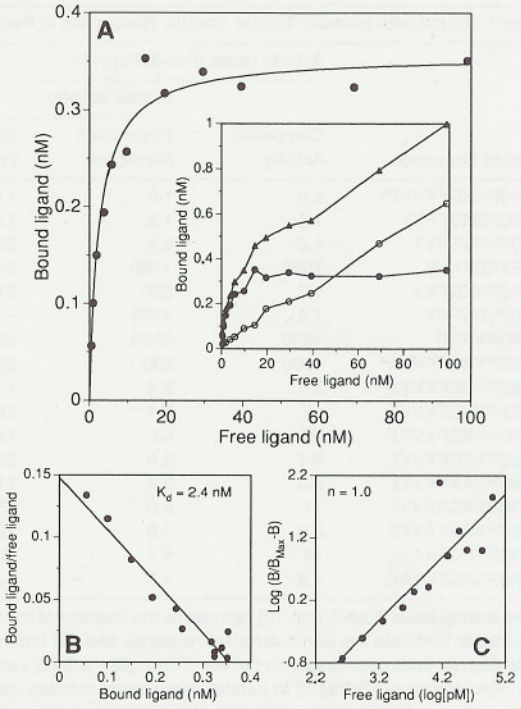

Figure 6. Saturability of ^{125}I-Pep-13 Binding

Parsley microsomes were incubated with increasing amounts of ^{125}I-Pep-13.

(A) Total binding (closed triangle) and specific binding (closed circle) calculated as described in the legend to Figure 5 are shown, and nonspecific binding (open circle) determined in the presence of 15 μM unlabeled Pep-13 is shown. The free ligand concentration was calculated by subtracting the amount of bound ligand from the total amount of ligand initially used in the assay.

(B) Scatchard plot and (C) Hill plot of the binding data shown in (A). The binding constant (given in [B]) and the Hill coefficient (given in [C]) were determined according to Hulme and Birdsall (1992). Each data point represents the average of duplicates.

Abbreviation: K_d, K_D.

To examine binding of the peptide elicitor to its binding site under in vivo conditions, parsley protoplasts were incubated with 1.1 nM ^{125}I-Pep-13 at 0°C at pH 8.0. Specific binding increased with increasing numbers of protoplasts and was found to be competable as well as saturable. In saturation analyses using increasing amounts of ^{125}I-Pep-13 (Figure 7), an apparent K_D of 11.4 nM was determined in a Scatchard plot (Figure 7, inset), in good agreement with the value obtained with microsomes (see Figure 6B). The estimated number of binding sites was found to be 4.7 fmol/10^6 protoplasts, corresponding to approximately 2900 binding sites per protoplast. Taken together, these results indicate that the Pep-13-binding site is a protein of relatively low abundance and is localized in the plasma membrane.

Table 1. Correlation between Elicitor-Specific Responses in Parsley Challenged with Pep-13 and Structural Analogs

Peptide Sequence	Activity Index (Pep-X/Pep-13)[a]						
		Elicitor Activity					
	Competitor Activity	Phytoalexin Formation	H_2O_2 Formation	Ca^{2+} Influx	H^+ Influx	K^+ Efflux	Cl^- Efflux
VWNQPVRGFKVYE[b]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
VWNQPVRGFKVY	1.2	1.5	1.8	1.8	1.8	2.4	3.3
WNQPVRGFKVY	1.6	2.5	22	–[c]	5.0	–	–
NQPVRGFKVY	7000	1100	3400	–	2900	–	–
VWNQPVRGFKV	1.7	250	21	–	6.0	–	–
WNQPVRGFKV	7.6	1100	–	–	–	–	–
VWNQPVRGF	1500	2500	6000	–	8100	–	–
VANQPVRGFKVYE[d]	1500	230	70	390[e]	150	270[e]	280[e]
VWAQPVRGFKVYE	2.1	2.4	1.5	0.8	1.7	1.4	1.0
VWNAPVRGFKVYE	12	8.1	28	8.7	26	25	16
VWNQAVRGFKVYE	50	63	120	110	37	120	83
VWNQPARGFKVYE	5.1	3.6	22	14	18	13	23
VWNQPVAAFKVYE	2.0	2.8	11	–	7.9	–	–
VWNQPVRGAKVYE	11	6.0	35	–	26	–	–
VWNQPVRGFAVYE	2.9	3.8	28	–	12	–	–
VWNQPVRGFKAYE	16	6.1	(335	230	87	200	69[f])
VWNQPVRGFKVAE	1.3	1.6	1.3	1.7	0.8	1.7	0.9

[a] The activity index (Pep-X/Pep-13) represents the quotient of the half-maximal concentration of the respective Pep-13 structural derivative (Pep-X) required to stimulate the particular plant response and the half-maximal effector concentration of Pep-13. Pep-13 is presented first. EC_{50} values were derived from dose-response curves. Competitor activity represents the half-maximal concentration (IC_{50}) of the respective peptide required to inhibit binding of ^{125}I-Pep13 to parsley microsomal membranes in competition assays, as described in Hulme and Birdsall (1992). Each value is the average of at least two replicates obtained from independent experiments.

[b] The absolute EC_{50} (IC_{50}) values of Pep-13 for the respective responses are (in the order given in the table) 4.7 nM, 31 nM, 6.9 nM, 4.4 nM, 1.9 nM, 3.3 nM, and 4.4 nM.

[c] Not determined.

[d] Bold letters represent Ala substitution sites within Pep-13.

[e] Data were obtained from experiments using a Thr substitution analog. This peptide was as inactive as the Ala substitution analog in stimulating phytoalexin formation in parsley protoplasts.

[f] This peptide exhibited unusually low activity as an elicitor of phytoalexin accumulation in cultured cells compared with protoplasts, presumably owing to its instability in cell culture medium. EC_{50} values obtained from experiments performed in cell suspension (shown in parentheses) might therefore not be directly comparable to those obtained from experiments using microsomes and protoplasts, i.e. binding and phytoalexin formation, respectively.

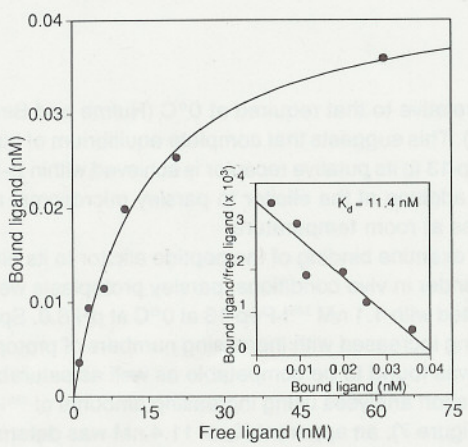

Figure 7. Saturability of the Pep-13-Binding-Site In Vivo

Parsley protoplasts were incubated with increasing amounts of ^{125}I-Pep-13. Specific binding (closed circle) and the free ligand concentration were calculated as described in the legends to Figures 5 and 6, respectively. The inset shows a Scatchard plot of the binding data. The binding constant was determined according to Hulme and Birdsall (1992). Each data point represents the average of duplicates.
Abbreviation: K_d, K_D.

Functional Link between Elicitor Binding and Elicitor-Induced Plant Responses

To analyze structural determinants of elicitor activity as well as to obtain information about the biological significance of Pep-13 binding to parsley membranes, primary structure/activity relationships were investigated with respect to binding affinity, individual ion fluxes, the formation of H_2O_2, and phytoalexin accumulation. Derivatives of Pep-13 with progressive deletions of amino acid residues from both termini were used either as competitors of binding of ^{125}I-Pep-13 to parsley microsomes or as elicitors of the responses described above.

Both Pep-13 (IC_{50} = 4.7 nM, EC_{50} = 31 nM) and the intact fungal glycoprotein elicitor (IC_{50} = 10 nM, EC_{50} = 9 nM) were powerful competitors of the binding of ^{125}I-Pep-13 to parsley microsomes. As shown in Table 1, considerable differences were observed among the individual deletion derivatives in their effectiveness as competitors or as elicitors. Derivatives of Pep-13 lacking either the C-terminal Glu-13 or both this residue and the N-terminal Val-1 retained almost full competitor and elicitor activities. Elimination of either one of the adjacent aromatic amino acids, Trp-2 or Tyr-12, had drastic effects. Deletion of Tyr-

12 led to a considerable reduction in the levels of H_2O_2 and phytoalexins synthesized, and removal of Trp-2 dramatically reduced all plant reactions. Smaller fragments were even less active; an octamer (N-terminal deletion) and a heptamer (C-terminal deletion) were found to be completely inactive with respect to all elicitor-induced reactions tested (data not shown). Moreover, treatment of parsley protoplasts with combined proteolytic cleavage products of Pep-13 (trypsin treatment yielded one hepta- and two tripeptides, and Lys-C treatment yielded one deca- and one tripeptide) did not restore elicitor activity, indicating that the contiguity of the sequence is required for proper recognition of the elicitor at its putative plasma membrane receptor and for subsequent signal generation.

The importance of individual amino acid residues within the oligopeptide for recognition of the ligand at its binding site and for elicitor activity was investigated by use of a series of Ala substitution analogs of Pep-13 (Table 1). Ala was chosen as a substitute since it would not impose extreme electrostatic or steric effects. Replacement of Trp-2 or Pro-5 severely decreased the ability of these analogs to elicit the six plant responses tested (70- to 390-fold and 37- to 120-fold, respectively) and rendered them similarly inactive as competitors of binding (1500- and 50-fold, respectively). Replacement of Tyr-12 left the biological activity of this analog completely intact, suggesting that the reduced activity of the corresponding deletion product was due to insufficient length, insufficient stability, or both. Substitution of all other amino acid residues had either little (Asn-3, Arg-7, Gly-8, Lys-10) or moderate impact on the elicitor-specific plant responses analyzed. In contrast with all other Pep-13 derivatives, however, the peptide in which Val-11 was replaced by Ala exhibited unusually low activity as an elicitor of phytoalexin accumulation in cultured cells compared with protoplasts. This may have been due to particular instability of this peptide in the cell culture medium and therefore invalidates the EC_{50} values obtained for responses investigated in cell suspensions, such as the ion fluxes and H_2O_2 production. When this is taken into account, the elicitor activitity as well as the displacement activity of this derivative was only moderately affected by this particular amino acid substitution.

The same structural requirements were generally shown to be responsible for both the efficient inhibition of the binding of ^{125}I-Pep-13 to parsley microsomal membranes and for the stimulation of early and late plant cell responses to elicitor treatment. Furthermore, the magnitudes of the EC_{50} and IC_{50} values obtained with the various peptides in the reactions investigated were consistently found to be similar.

Discussion

An Oligopeptide Elicitor Necessary and Sufficient To Induce a Complex Plant Defense Response

We have identified and isolated from a fungal 42 kDa glycoprotein elicitor a 13 amino acid oligopeptide that acts as a potent elicitor in parsley. This peptide induces the same reactions as are stimulated by a crude cell wall preparation from the fungus, including both short-term events, such as changes in plant plasma membrane permeability to H^+, Ca^{2+}, K^+, and Cl^- and production of H_2O_2, and long-term events, such as defense-related gene activation, accumulation of furanocoumarin phytoalexins, stimulation of ethylene biosynthesis, and extracellular accumulation of chitinase activity (the latter not shown). Additionally, simultaneous treatment of parsley cells with UV light and Pep-13 resulted in the abolition of light-induced vacuolar accumulation of flavonoids and a significant reduction in elicitor-induced phytoalexin production (data not shown), as was also found previously for the fungal cell wall elicitor (Lozoya et al., 1991). The fact that a single, small molecule elicits the same uniquely complex plant response as a crude fungal cell wall preparation clearly facilitates studies directed towards dissection of elicitor-specific signal transduction pathways. The Pep-13/parsley cell system therefore provides a valuable tool to study the functions of the individual elicitor-activated reactions which are part of a multifacetted plant defense response.

The recent isolation and analysis of a cDNA encoding the glycoprotein (Sacks et al., 1994) allowed the localization of this peptide to the C-terminal portion of the glycoprotein. A comparison of the peptide sequence with sequences contained in several databases did not reveal significant similarity to known proteins, as was also found for the glycoprotein elicitor at the amino acid and the nucleotide levels (Sacks et al., 1994). Pep-13 represents 3.5% of the mature glycoprotein, is not associated with the single glycosylation site, is not particularly hydrophobic, and was demonstrated to be necessary and sufficient for elicitor activity of the intact glycoprotein. A minimal length of 11 amino acids (Trp-2–Tyr-12) was required to efficiently stimulate the various reactions of parsley cells. By Ala substitution scanning, single amino acid residues (Trp-2 and Pro-5) were identified within the peptide that are essential for elicitor activity, whereas other exchanges caused only moderate or negligible reductions in elicitor activity. Similar observations regarding biological activity were made when the polypeptide systemin, which is likely to be a systemic signal involved in wound-induced accumulation of proteinase inhibitors in tomato leaves, was analyzed by a comparable experimental approach (Pearce et al., 1993).

A number of (glyco)protein and peptide elicitors have been purified from phytopathogenic microorganisms representing a variety of structures to which plant cells are capable of responding in an apparently species-specific manner (Ebel and Cosio, 1994). In the few cases where efforts have been undertaken to pinpoint elicitor activity to specific structural properties, small elements, such as short amino acid sequences (He et al., 1993), single Cys residues (Joosten et al., 1994), or a branched trisaccharide at the nonreducing end of an oligosaccharide (Cheong et al. 1991b) appeared to be responsible for loss or gain of function. The cultivar or species specificity of purified elicitors, their ability to induce various plant responses at nanomolar concentrations, and the precisely defined signal structures required for elicitor-mediated activation of plant defense responses are indicative of the involvement of

receptors in elicitor perception and subsequent signal generation.

A Novel Class of High Affinity Binding Sites in Plant Plasma Membranes

Parsley membranes contain a high affinity binding site for the oligopeptide elicitor Pep-13 that appears to be localized in the plasma membrane. The Pep-13-binding site clearly fulfills a number of criteria expected of an authentic receptor (Hulme and Birdsall, 1992) as binding of the ligand was shown to be competable, reversible, saturable, and highly specific with respect to structural properties of the signal required for binding.

Ligand saturation analysis performed with parsley microsomal membranes revealed the existence of a single class of binding sites for the oligopeptide ligand. Use of radioligand concentrations in the range of 100–2000 nM did not result in a further increase in specific binding (data not shown), ruling out the presence of a second class of binding sites with significantly lower affinity. The K_D of 2.4 nM indicates that plants possess peptide elicitor-binding sites with an affinity very similar to that found for carbohydrate elicitors of phytoalexin biosynthesis (Cosio et al., 1990; Cheong and Hahn, 1991a; Shibuya et al., 1993). In addition, the affinity of this oligopeptide for its binding site in parsley is similar to that found for numerous peptide ligands and their corresponding receptors in mammalian systems.

Binding of ^{125}I-Pep-13 to parsley protoplasts exhibited a K_D very similar to that obtained with microsomes, indicating that the elicitor-binding site resides in the plasma membrane. Further support for plasma membrane localization was provided by the similar numbers of binding sites per cell calculated from binding assays performed with microsomes and protoplasts. Additional evidence for the localization of the putative Pep-13 receptor in the parsley plasma membrane was provided by Diekmann et al. (1994), who were able to visualize microscopically binding sites for the 42 kDa glycoprotein elicitor and for Pep-13 on parsley protoplasts by means of silver-enhanced immunogold labeling.

The few elicitors for which high affinity binding sites in plant membranes have been convincingly demonstrated are either carbohydrates (Cosio et al., 1990; Cheong and Hahn, 1991a; Shibuya et al., 1993) or glycopeptides, of which the carbohydrate moiety has been found to be sufficient for the interaction between the ligand and its binding site (Basse et al., 1993). Our findings therefore reveal the existence of a novel class of putative elicitor receptors: those which interact with oligopeptide ligands. More generally, we also demonstrate that plants do possess acceptor sites for oligopeptide signal molecules that mediate the transcriptional activation of defense-related genes. The existence of such binding sites had been postulated since the polypeptide hormone systemin was isolated from tomato plants, in which it was found to be involved in systemic wound signaling (Pearce et al., 1991). The finding that plants respond to peptide signals, such as the first report of the involvement of an oligosaccharide signal in the interaction of a parasite with its animal host (Velupillai and Harn, 1994), contributes to the expanding list of analogies between plant and animal signaling systems, as recently noted by Ryan (1994).

Functional Link between Elicitor Binding and Plant Responses

Identical structural elements within Pep-13 were required for binding to parsley membranes and for induction of phytoalexin accumulation, indicating that the binding site indeed functions as a receptor in transmission of extracellular signals leading to nuclear gene activation. In addition to a minimal length of 11 amino acids, 2 amino acid residues within Pep-13, Trp-2, and Pro-5 were found to be of particular importance for both elicitor binding and activity. Furthermore, similar concentrations of the individual peptides were required to activate phytoalexin production half maximally and to inhibit ^{125}I-Pep-13 binding. A comparably high degree of correlation between elicitor and displacement activities has also been reported for the oligo-β-glucan (Cheong and Hahn, 1991a) and the yeast invertase-derived glycopeptide elicitors (Basse et al., 1993).

The effects of elicitors on ion fluxes across the plasma membrane have been considered to be part of elicitor-specific signal transduction leading to the induction of plant defense responses in several plant–pathogen interactions (Ebel and Cosio, 1994). Four lines of correlative evidence strongly suggest that oligopeptide-stimulated ion fluxes and phytoalexin formation in parsley cells are, indeed, functionally inseparable. First, omission of Ca^{2+} from the culture medium blocked defense-related gene activation and phytoalexin formation, as was also shown for the Pmg elicitor at the level of transcript accumulation (Dietrich et al., 1990) and transcript synthesis (Ebel and Scheel, 1992). Furthermore, elicitor treatment of cultured parsley cells in the presence of Ca^{2+} channel blockers such as flunarizine significantly inhibited both the targeted ion flux and phytoalexin production (Nürnberger et al., 1994). Second, identical structural properties of the oligopeptide were found to be required for efficient stimulation of all four ion fluxes and furanocoumarin synthesis as well as for binding of the elicitor to its binding site. Third, similar elicitor concentrations were needed for half-maximal stimulation of the various plant responses. Fourth, initial changes in ion fluxes were detectable within 2–5 min after addition of the elicitor and peaked after 20–30 min, thereby clearly preceding transcriptional activation of defense-related genes and phytoalexin formation. Similar structural requirements of oligogalacturonides were found to be important for rapid stimulation of ion fluxes through plasma membranes of cultured tobacco cells and for induction of morphogenetic events, such as flower formation in tobacco explants (Mathieu et al., 1991).

The rapid release of H_2O_2, referred to as the oxidative burst, has been reported to occur in several plants upon their treatment with elicitor (Apostol et al., 1989; Schwacke and Hager, 1992; Devlin and Gustine, 1992). Use of Pep-13 and derivatives as elicitors of this reaction in parsley again revealed the same signal specificity as was observed for the other reactions, indicating that a single elicitor triggers the oxidative burst, ion fluxes, and defense-

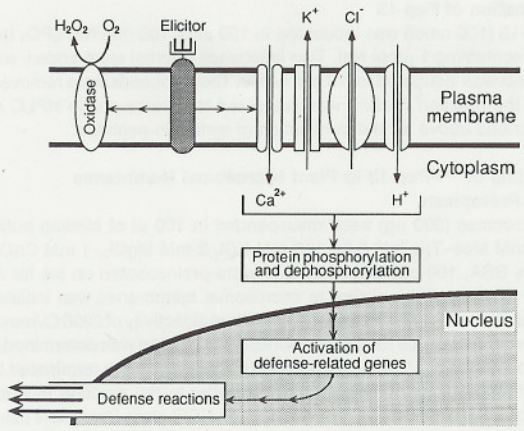

Figure 8. Hypothetical Model for Elicitor Signal Transduction in Parsley

related gene activation apparently by binding to a single class of plasma membrane receptors. Since degradation of H_2O_2 by catalase did not affect elicitor-stimulated phytoalexin formation in parsley (data not shown), we propose that extracellular H_2O_2, at least, is not a necessary element of the signaling system triggering furanocoumarin production. Assuming, however, that related active oxygen species such as O_2^- are biologically active, the rapid initiation and signal specificity of the oxidative burst in parsley may argue for their involvement in the signaling process investigated. That active oxygen species function as signal transduction components in plant cells is supported by Chen et al. (1993), who propose that H_2O_2 or related active oxygen species play a role in the establishment of systemic acquired resistance in tobacco. The molecular mechanism through which H_2O_2 could mediate the expression of defense-related genes might, furthermore, be similar to the H_2O_2-dependent activation of transcription factors in human cells regulating genes involved in inflammatory, immune, and acute phase responses (Schreck et al., 1991). However, whether the oxidative burst plays a part in the defense response of parsley, either by directly killing invading pathogens or by mediating a stiffening of the plant cell wall, or whether it is indeed involved in the transduction of the elicitor signal remains to be elucidated.

The model shown in Figure 8 summarizes current knowledge of elicitor signal perception and transduction in parsley. The earliest reactions of parsley cells observed after binding of a fungal oligopeptide elicitor to its high affinity binding site in the plant plasma membrane appear to be changes in the activities of plasma membrane–localized ion channels and a rapid formation of H_2O_2. The Pep-13-binding site appeared to be fully saturated within 3 min, and changes in H^+, Ca^{2+}, K^+, and Cl^- fluxes occurred within 2–5 min upon addition of peptide elicitor. Specific inhibitors of slow-type Ca^{2+} channels inhibited both elicitor-stimulated Ca^{2+} fluxes and phytoalexin synthesis, indicating that specific Ca^{2+} channels are involved in elicitor-mediated signal transduction (Nürnberger et al., 1994). Moreover, an increase in the cytosolic Ca^{2+} concentration has been detected using the fluorescent indicator, indo-1, in parsley cells treated with the Pmg elicitor (Sacks et al., 1993). Elicitor-specific, Ca^{2+}-dependent in vivo phosphorylation of several proteins has been demonstrated in parsley (Dietrich et al., 1990). These proteins may then be involved directly or indirectly in regulation and expression of defense-related genes in plants.

In summary, conclusive evidence is presented here that a novel oligopeptide-binding site in the parsley plasma membrane represents a receptor for a fungal peptide elicitor. Since most bacterial and fungal elicitors purified to date are proteinaceous in nature (Ebel and Cosio, 1994) and since oligopeptides appear to play a role as endogenous signals in plants as well (Pearce et al., 1991), the existence of multiple types of such receptors can be postulated. Peptide signaling in plants may therefore be of similar importance as in animals. The tools now available in the parsley system are well suited to isolate the oligopeptide receptor and the corresponding gene in order to further investigate this signaling cascade.

Experimental Procedures

Materials

Peptide synthesis was carried out by Kem-En-Tec A/S (Copenhagen, Denmark). Synthesized peptides were purified to 80%–95% homogeneity as described below. Radioiodination of Pep-13 was performed by Anawa Laboratorien Aktiengesellschaft (Wangen, Switzerland). Proteases were from Boehringer Mannheim (Federal Republic of Germany).

Elicitors

A crude elicitor was prepared from the mycelium of P. megasperma f. sp. glycinea, race1, a glycoprotein elicitor purified from the fungal culture filtrate, and elicitor activity was quantified as described (Parker et al., 1991). Furanocoumarin phytoalexins were analyzed according to Dangl et al. (1987).

Plant Cell Culture and Protoplast Preparation

Cell suspension cultures of parsley (Petroselinum crispum) were maintained as described (Kombrink and Hahlbrock, 1986). Cell viability was determined using fluorescein diacetate (Renelt et al., 1993). Protoplasts were prepared from cultured cells five days after inoculation, according to Dangl et al. (1987).

Isolation of Elicitor-Active Peptides and Protease Treatment of Pep-13

The purified glycoprotein (75 μg) was dissolved in 200 μl of 25 mM NH_4HCO_3 (pH 7.9) containing 5 μg of endoproteinase Glu-C and was incubated at 25°C for 6 hr. After a second addition of 5 μg of the protease and continued incubation for 18 hr, the reaction was stopped by boiling the mixture for 5 min. The digestion mix was subjected to reverse-phase HPLC (Hewlett-Packard HP1090 [Waldbronn, Federal Republic of Germany]) using a Vydac C_4 column (300 Å, 5 μm, 4.6 × 250 mm; Separations Group [Hesperia, California]) at a flow rate of 0.5 ml/min. Bound peptides were eluted with a biphasic linear gradient of 0%–70% acetonitrile in 0.07% ammonium acetate for 105 min (0%–35% for 65 min, and 35%–70% for 40 min). Absorbance was monitored at 214 and 280 nm. Fractions collected were lyophilized, redissolved in water, and assessed for elicitor activity. Active samples were rechromatographed with the same column/gradient system, using 0.1% trifluoroacetic acid instead of 0.07% ammonium acetate. Peaks were collected, the solvent removed in a Speed Vac concentrator, the peptides redissolved in water, and aliquots tested for elicitor activity. Synthetic peptides were purified using the same reverse-phase HPLC system with a linear gradient of 0%–35% isopropanol in 0.1% trifluoroacetic acid for 65 min and a flow rate of 0.5 ml/min. Protease treatment of Pep-13 was performed according to the instructions of the supplier. Peptide sequencing was performed by automated

Edman degradation using an Applied Biosystems 477A Protein Sequencer and an Applied Biosystems 120A PTH Analyzer.

RNA Isolation and Hybridization

Total RNA from parsley cells or protoplasts were prepared as described by Dangl et al. (1987). Heat-denatured (at 65°C for 20 min) RNA (10 μg in 100 μl of 6 × SSC and 7.5% formaldehyde) was loaded into slots of a Minifold Filtration Unit (Schleicher and Schuell [Dassel, Federal Republic of Germany]) and was vacuum-transferred to nitrocellulose. UV cross-linking of RNA to the filter, prehybridization, hybridization, and ^{32}P-labeling of cDNA probes were carried out as described (Kawalleck et al., 1993a).

Prokaryotic Expression

A modified version (Kawalleck et al., 1993a) of the vector pGEX-2T (Pharmacia [Freiburg, Federal Republic of Germany]) was used to express the full-length cDNA encoding the 42 kDa glycoprotein elicitor as well as deletions derived from it. Inserts were synthesized by polymerase chain reaction using the cDNA as template and appropriate primers as described (Sacks et al., 1994) and were cloned into the vector as EcoRI fragments. The full-length construct, XE, utilized an EcoRI site at the 3′ end of the cDNA and one introduced at the 5′ end by polymerase chain reaction (Sacks et al., 1994). Δ1 was constructed by introducing SpeI sites at the 5′ and 3′ borders of the Pep-13 coding sequence. Two fragments were thereby synthesized: an EcoRI–SpeI fragment encoding elicitor sequences N-terminal to Pep-13 and a SpeI–EcoRI fragment encoding elicitor sequences C-terminal to Pep-13. The fragments were digested with SpeI, ligated, digested with EcoRI, and cloned into the vector, thereby replacing the sequences encoding Pep-13 with a SpeI site encoding Thr and Ser. Δ2 represents a cloning artifact in which were found, instead of a single SpeI site, two such sites separated by the sequence GGACCT. Expression, solubilization, analysis of elicitor activity of the fusion proteins, SDS–polyacrylamide gel electrophoresis, protein transfer to nitrocellulose, and immunodetection of the mature glycoprotein elicitor and fusion proteins were performed according to Sacks et al. (1994).

Ion Flux Measurements and H_2O_2 and Ethylene Determination

Changes in the concentrations of H^+, K^+, and Ca^{2+} were determined according to Conrath et al. (1991) using ion-selective electrodes, such as a pH microelectrode (Metrohm [Herisau, Switzerland]) or the K^+-selective electrode K500 with reference electrode R502 for K^+ (inner electrolyte ELY/IN/502 and the bridge electrolyte 2% 1.2 M NaCl [Wissenschaftlich-Technische Werkstätten or WTW {Weilheim, Federal Republic of Germany}]), or by monitoring the uptake of [^{45}Ca]Cl_2 into parsley cells. Cl^- concentrations were determined as described for K^+ by using the Cl^--selective electrode, Cl500, with reference electrode R502 for Cl^- (inner electrolyte ELY/IN/502, bridge electrolyte ELY/BR/5023 [WTW {Weilheim, Federal Republic of Germany}]) and 5 M $NaNO_3$ instead of NaCl as the ionic strength adjuster. The accumulation of active oxygen species in the culture medium of elicitor-treated parsley cells was determined as described (Schwacke and Hager, 1992). For K^+, Cl^-, Ca^{2+}, and H_2O_2 measurements, parsley cells were resuspended in 4% v/v B5 medium with 3% w/v sucrose and 10 mM Mes, adjusted to pH 5.7 with Bis–Tris. Ethylene was determined according to Chappell et al. (1984).

Preparation of Microsomal Membranes

Cultured parsley cells (6 days) were harvested by filtration and frozen in liquid nitrogen. Frozen cells (150 g) were thawed in 150 ml of 50 mM Mes–Tris (pH 7.5), 200 mM KCl, 0.25 M sucrose, 15 mM EDTA, 6 mM DTT, and 2 mM PMSF containing 10 g of DOWEX 1X2–400 equilibrated with Tris–HCl (pH 7.5). Cells were homogenized (six times, for 3 min each time) on ice using a polytron (Kinematica [Kriens, Switzerland]). The homogenate was centrifuged at 4°C for 20 min at 10,000 × g. The fraction containing microsomal membranes was obtained by ultracentrifugation of the supernatant at 4°C for 60 min at 141,000 × g. The pellet was rinsed with 1 ml of 20 mM Mes–Tris (pH 8.0), 100 mM KCl, 2 mM $MgCl_2$, and 1 mM $CaCl_2$, and was resuspended in 5 ml of this buffer using a Potter-Elvehjem homogenizer. The protein content of the microsomal membrane preparation was determined with the BCA Protein Assay Kit (Pierce [BA Oud Beijerland, The Netherlands]) using bovine serum albumin as standard.

Iodination of Pep-13

Pep-13 (100 nmol) was incubated in 100 μl of 100 mM NaH_2PO_4 (pH 6.5) containing 1 μmol NaI. Two iodobeads (Pierce) were added, and the mix was incubated for 10 min on ice. The iodobeads were removed, and the reaction mixture was subjected to reverse-phase HPLC as described above for the purification of synthetic peptides.

Binding of ^{125}I-Pep-13 to Plant Microsomal Membranes and Protoplasts

Microsomes (300 μg) were resuspended in 100 μl of binding buffer (20 mM Mes–Tris [pH 8.0], 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA, 100 μM leupeptin) and were preincubated on ice for 30 min. Binding of the elicitor to microsomal membranes was initiated by addition of 1.1 nM ^{125}I-Pep-13 (specific radioactivity of 2200 Ci/mmol) and was kept on ice for 90 min. Nonspecific binding was determined in the presence of 15 μM unlabeled Pep-13. Assays were terminated by addition of 10 ml of ice-cold binding buffer to the reaction mixture, and filtration was carried out on Whatman GF/B glass fiber filters using a Schleicher and Schuell Manifold Filtration Unit (Dassel, Federal Republic of Germany). Filters were washed twice with 10 ml and 5 ml of the same solution and transferred to scintillation vials containing 5 ml of Aquasol-2 (DuPont de Nemours [Dreieich, Federal Republic of Germany]), and radioactivity was determined by scintillation spectrometry.

Binding to parsley protoplasts (3 × 10^6 in 250 μl of B5 medium [Gamborg et al., 1968] containing 0.4 M sucrose and 0.1% BSA, adjusted to pH 8.0 with 20 mM Mes–Tris) was initiated by adding ^{125}I-Pep-13 to the reaction mixture. Conditions for binding were those used for microsomes. Incubations were terminated by adding 10 ml of ice-cold wash buffer (0.24 M $CaCl_2$ and 0.1% BSA) to the assay mixture, pelleting the protoplasts at 4200 × g (4°C) for 5 min, washing with the same volume of wash solution, and determining bound radioactivity as described for microsomes.

Acknowledgments

The technical assistance of Daja Reinhardt, Katharina Adamitza, and Magdalena Jung is gratefully acknowledged. We are indebted to Prof. Thomas Boller and Dr. Jürg Felix for their help with ethylene measurements. We thank Dr. Klaus Palme and Elisabeth Luley for peptide sequencing. T. N. was supported by a postdoctoral fellowship from Bayer Aktiengesellschaft of Leverkusen, Federal Republic of Germany. This work was supported by the Deutsche Forschungsgemeinschaft (Sche 235/3–1) and the European Economic Community (CHRX-CT93–0168).

Received May 4, 1994; revised June 10, 1994.

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Planta (1994) 195:126–137

Planta

Visualization of elicitor-binding loci at the plant cell surface

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Received: 9 March 1994 / Accepted: 15 April 1994

Abstract. We describe a method which allows the visualization of elicitor-binding loci at the surface of plant protoplasts. Prerequisites for this method are the preparation of protoplasts under conditions of minimal proteolysis, and the availability of antibodies against either the elicitor itself or against the fluorescein portion of elicitor-fluorescein conjugates. Silver enhancement is used to amplify the visibility of 5-nm gold particles which are attached to an appropriate secondary antibody. Bound elicitor can then be visualized by epipolarization microscopy. This method, designated SEIG-EPOM (for *s*ilver *e*nhanced *i*mmunogold as viewed by *epi*polarization *m*icroscopy), has been applied to protoplasts of parsley (*Petroselineum cirspum* L.), using the *Phytophthora megasperma* elicitor, and soybean (*Glycine max* Merr.) using polygalacturonic acid elicitor isolated from citrus pectin. We have been able to estimate the number of specific binding loci as being less than 100 per protoplast. Such loci possibly represent clusters of individual elicitor-receptor complexes. Structurally related elicitors have been shown to compete effectively for binding sites. The latter are sensitive to proteolysis, as is the elicitation response of protoplasts.

Key words: Binding loci (elicitor) – Elicitor – Epipolarization microscopy – Protoplast (parsley, soybean)

Dedicated to Professor Peter Sitte on the occasion of his 65th birthday

Abbreviations: BSA = bovine serum albumin; IgG = immunoglobulin G; PEP-13(15) = 13(15) amino-acid-containing oligopeptides of Pmg-elicitor; PGA = polygalacturonic acid; PM = plasma membrane; Pmg = *Phytophthora megasperma* f.sp. *glycinea*; SEIG-EPOM = silver enhanced immunogold as viewed by epipolarization microscopy

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Introduction

The plant cell can perceive both physical (light, gravity, temperature, humidity) and chemical (hormones, toxins, elicitors) stimuli. As might be expected, the site of perception for the latter group is generally regarded to be the plasma membrane (PM; for hormones see for example Jones and Herman 1993; for toxins, see for example Oecking and Weiler 1991; for elicitors, see for example Ebel and Scheel 1992). In the case of elicitors, recognition at the cell surface triggers a chain of events, collectively termed "signal transduction" (Palme 1992; Renelt et al. 1993; Sacks et al. 1993a). The effects of elicitor perception at the PM may be rapid, e.g. a stimulation of callose synthesis (Kauss et al. 1989) or an oxidative burst (Legendre et al. 1993). There are also long-term responses involving activation of genes responsible for the production of a number of defence-related substances which include cell-wall-associated phenylpropanoids and hydroxyproline-rich glycoproteins, as well as vacuolar-based and secreted enzymes such as chitinases and glucanases (reviewed by Ebel and Scheel 1992). Parsley and soybean cells represent well-studied model systems for the analysis of such plant defence responses. A glycoprotein elicitor has been isolated from *Phytophthora megasperma* f.sp. *glycinea* (Pmg elicitor) that induces phytoalexin accumulation and activates defence-related genes both in cells and protoplasts of parsley (Parker et al. 1991). One oligopeptide fragment in the C-terminal half of this glycoprotein has been found to be necessary and sufficient for elicitor activity (Nürnberger et al. 1994). Polygalacturonides, which are released from plant cell walls by pathogen-derived enzymes, also stimulate phytoalexin synthesis and have been shown to initiate an oxidative burst in suspension-cultured soybean cells (Nothnagel et al. 1983; Legendre et al. 1993).

Elicitors can be effective at very low (nM) concentrations, and evidence from in-vitro binding experiments (e.g. Cheong and Hahn 1991; Cosio et al. 1992; Renelt et al. 1993) indicates a relatively low abundance of putative binding sites per cell. The availability of antibodies

against elicitors makes the microscopical detection of bound elicitors a feasible proposition; however, when a cell wall is present visualization is restricted to postembedding methods on sections. The fixation steps which are a prerequisite for such procedures, together with the fact that single sections represent only a random fraction of the total structural information in a cell, will automatically preclude a quantitative assessment of elicitor binding when carried out in this way. Such reservations do not apply to protoplasts, provided they can be isolated without degradation of the binding sites at the surface of the PM. In this paper we describe an immunological method for the visualization of elicitor-binding loci at the PM of parsley and soybean protoplasts which have been prepared under conditions which minimize proteolysis. It allows observations to be made in parallel with both light and electron microscopes and also enables the number of binding loci per cell to be quantified.

Material and methods

Plant material

Parsley (*Petroselineum crispum* L.; Hahlbrock 1975) and soybean (*Glycine max* Merr cv. Kent; Low and Heinstein 1986) suspension cultures were cultivated on modified Gamborg's B-5 (Kombrink and Hahlbrock 1986) and a Murashige-Skoog (Hasegawa et al. 1980) medium, respectively. Logarithmic-phase cells were removed, washed in fresh culture medium, and then protoplasted. Leaves from four-week-old, greenhouse-grown bean plants (*Vicia faba* L. from the local market of Osnabrück) were removed and cut into vein-free pieces roughly 20 mm^2 in size.

Preparation of protoplasts

(*i*) *Parsley cells.* The protoplast-releasing enzyme mixture contained 0.5% (w/v) cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan), 0.05% (w/v) pectolyase Y-23 (Seishin, Tokyo, Japan), 1 mM $CaCl_2$, 2% (w/v) bovine serum albumin (BSA; fraction 5; Biomol, Hamburg, Germany) in 0.4 M mannitol pH 5.6. This medium was heated to 45°C for 10 min in order to inactivate proteases (Van der Valk 1984). Cells were resuspended in the protoplast-releasing medium, transferred to Erlenmeyer flasks and incubated for 1 h at 25°C in a shaker-bath. Protoplasts were washed by centrifugation (100·g, 5 min) and resuspended in 0.4 M mannitol containing 1 mM $CaCl_2$.

(*ii*) *Soybean cells.* The protoplast-releasing enzyme mixture contained 1.7% (w/v) cellulase Onozuka RS, 0.05% (w/v) pectolyase Y-23, 0.05% (w/v) mazerozyme Onozuka R10, 10 mM sodium ascorbate, 2% (w/v) BSA (fraction 5), and 0.49 M mannitol, which were dissolved in Murashige-Skoog-medium (pH 5.6) and then heat-treated as above. Cells were resuspended in this medium (osmolality approx. 600 mOsm), transferred to Erlenmeyer flasks and incubated for 1 h at 25°C in a shaker-bath. After centrifugation (80·g, 2 min) the cells were resuspended in a hypotonic medium containing 90 mM mannitol and 2 mM $CaCl_2$ dissolved in Murashige-Skoog-medium (osmolality approx. 200 mOsm). Under these conditions the protoplasts swell and slip out of the predigested cell walls (Elzenga et al. 1991). The protoplasts were harvested by centrifugation (80·g, 2 min) and were resuspended in an incubation medium containing 90 mM mannitol, 100 mM sucrose, and 2 mM $CaCl_2$ dissolved in Murashige-Skoog-medium.

(*iii*) *Bean leaves.* Leaf segments were vacuum-infiltrated with a protoplast-releasing enzyme mixture (heat-treated before use, as above) containing 0.5% (w/v) cellulase Onozuka RS, 0.05% (w/v) pectolyase Y-23, 0.5% (w/v) BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ in 0.5 M mannitol (pH 6). After incubating for 15 min at 25°C mesophyll protoplasts were washed by centrifugation (100·g, 5 min) and resuspended in 0.5 M mannitol containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

(*iv*) *Surface proteolysis of protoplasts.* Freshly isolated protoplasts were subjected to proteolysis by adding either 0.1% (w/v) carboxypeptidase A or a mixture of 0.05% thermolysin + 0.05% chymotrypsin to a suspension of the protoplasts. After incubation for 30 min at 25°C or for 2 h at 4°C the protoplasts were washed five times by centrifugation and resuspension in 0.4 M mannitol + 1 mM $CaCl_2$.

Elicitors and elicitation competitors

(*i*) *Parsley protoplasts.* A 42-kDa glycoprotein elicitor was isolated and purified from *Phytophthora megasperma* f.sp. *glycinea* (Pmg-elicitor) as previously given (Parker et al. 1991). In addition two oligopeptide fragments, PEP-13 and PEP-25, covering the elicitor active site of the Pmg-elicitor were chemically synthesized according to their previously determined sequences (data not shown). As shown by their ability to induce the synthesis of furanocoumarins in parsley cells and protoplasts (Parker et al. 1991) both oligopeptides were elicitor-active, but neither could be recognized by the antiserum generated against the intact Pmg-elicitor (see below). A fluorescein derivative of PEP-13 was prepared by incubating 1 mg of the oligopeptide with 3 mg NHS-fluorescein (Pierce, BA Oud Beijerland, The Netherlands) in 50 M Hepes (pH 7.4) for 2 h on ice. After centrifuging for 10 min at 10000·g the supernatant was subjected to reverse-phase HPLC using a VYDAC C_4 semipreparative column (30 nm, 5 µm, 250 mm long, 4.6 mm i.d., The Separations Group, Mesperia, Cal., USA) at a flow rate of 0.5 ml·min^{-1} and 0–70% *iso*propanol:water:0.1% TFA (by vol.) gradient. Elicitor-active fractions were collected and lyophilized. All steps in this procedure were performed under dim red light; the product was stored in the dark. Since two primary amino groups are present in PEP-13, the conjugate contained maximally two fluoresceins per molecule, one at the NH_2-terminus and one at the lysine residue (residue 10). Since five to six sugar or amino-acid residues are necessary to constitute an antigenic determinant on a polysaccharide or polypeptide, and the same number of amino-acid residues are present in the antigenic binding site of an immunoglobulin G (IgG) molecule, it is highly unlikely, considering the relative sizes of PEP-13 and IgG that two antibodies could bind to a single PEP-13 molecule.

(*ii*) *Soybean protoplasts.* A polygalacturonide elicitor (PGA-elicitor) was isolated from a partial acid hydrolysate of citrus pectin as given by Nothnagel et al. (1983). Polygalacturonic acid fractions collected during the chromatography steps were selected for their ability to induce an oxidative burst in suspension-cultured soybean cells (see below). The final purified product had a DP of approx. 14, as measured by gel filtration using proteins of known molecular weights as standards. The resulting PGA was labelled with fluorescein by reaction with an excess fluorescein thiosemicarbazide in dimethyl formamide for 4 h in the dark to form the thiosemicarbazone. Free fluorescein thiosemicarbazide was removed by extensive dialysis against distilled water. We have estimated that the resulting conjugate contained one fluorescein per PGA molecule, located at the former reducing end of the PGA.

Measurement of elicitor-induced phytoalexin and active-oxygen production

Phytoalexin synthesis by parsley protoplasts was determined by resuspending protoplasts ($5 \cdot 10^5$ per assay) in elicitor-incubation medium (see below) and allowing the mixture to stand in Petri

dishes with occasional agitation for 24 h at 25°C. Protoplasts were separated from the medium by centrifugation and the levels of secreted furanocoumarins in the medium determined in a spectrofluorometer (excitation at 350 nm, fluorescence at 410 nm; Parker et al. 1991). Fluorescence was expressed in relative units. Active-oxygen production by soybean protoplasts in response to crude and purified PGA-elicitor preparation was measured according to the luminol procedure as given in Schwacke and Hager (1992).

Elicitor binding and incubation with antibodies

Protoplasts ($3 \cdot 10^6$ ml^{-1} in buffered 0.4 M mannitol + 1 mM $CaCl_2$) were exposed for 30 min at 4°C to either the Pmg- or PGA-elicitor (1 µg·ml^{-1}). After washing four times by centrifugation and resuspension in elicitor-free medium the protoplasts were preincubated for 30 min at 4°C in 20 mM potassium phosphate buffer (pH 7) containing 0.4 M mannitol, 1 mM $CaCl_2$ and 1% (w/v) BSA, before exposing to the primary antibody solution for 60 min at 4°C. The protoplasts were then washed four times by centrifugation and resuspension in phosphate/mannitol buffer containing 0.5% BSA before exposing to the secondary antibody solution. After incubating at 4°C for 60 min the protoplasts were washed again four times, but in 0.5% BSA-C (acetylated BSA; Biotrend, Köln, Germany) in 0.4 M mannitol.

Primary and secondary antibodies and their dilutions

The primary antibodies used in this study were: (i) polyclonal antibodies (rabbit IgGs) raised against the intact Pmg-elicitor (Parker et al. 1991), diluted 1:250; (ii) polyclonal antibodies (rabbit IgGs) raised against fluorescein (A-889; Molecular Probes, Eugene Oreg., USA), diluted 1:250; (iii) monoclonal antibodies (mouse IgG subclass 1) prepared against a PM epitope from *Vicia faba* mesophyll designated G23-7-D6 (Key and Weiler 1988), diluted 1:1000.

Depending on the primary antibody type, the secondary antibodies were either goat-anti rabbit IgGs or goat-anti mouse IgGs coupled to 5-nm gold particles (Biocell, Plano, Marburg, Germany). Primary and secondary antibodies were presented to protoplasts in 50 mM potassium phosphate-buffer (pH 7.4) containing 0.4 M mannitol, 1% BSA and 0.02% BSA-C. The dilution factor for all secondary antibodies was 1:250.

Protoplast fixation (for light and electron microscopy)

Protoplasts decorated with antibody-gold conjugates were resuspended in a primary fixative containing 2% (w/v) glutaraldehyde, 1 mM $CaCl_2$, 0.4 M mannitol and 50 mM potassium phosphate pH 7.0 and allowed to stand for 30 min at room temperature. After washing 4 × 10 min in 0.4 M manitol the protoplasts were immersed in 2% (w/v) OsO_4 in 50 mM phosphate pH 7.0 again for 30 min at room temperature. For light microscopy the concentration of osmium tetroxide in the secondary fixative was 0.01% (w/v).

Silver enhancement for light microscopy

Double-fixed protoplasts were washed 4 × 10 min in distilled water before exposing to silver solutions for 10 min at 25°C. Silver enhancement was performed for 5–15 min exactly according to the instructions in the maker's kit (Biogenzia Lemania, Bochum, Germany). Protoplasts were then investigated by reflection (epi)polarization microscopy using an Axiovert 35 microscope (Zeiss, Oberkochen, Germany) equipped with a Plan-Neofluar 63x / 1,25 oil Ph3 Antiflex objective (Zeiss). Specimens were observed either under aqueous conditions or after embedding in plastic (permanent preparations were prepared by polymerizing drops of plastic containing protoplasts on light-microscope slides under coverslips).

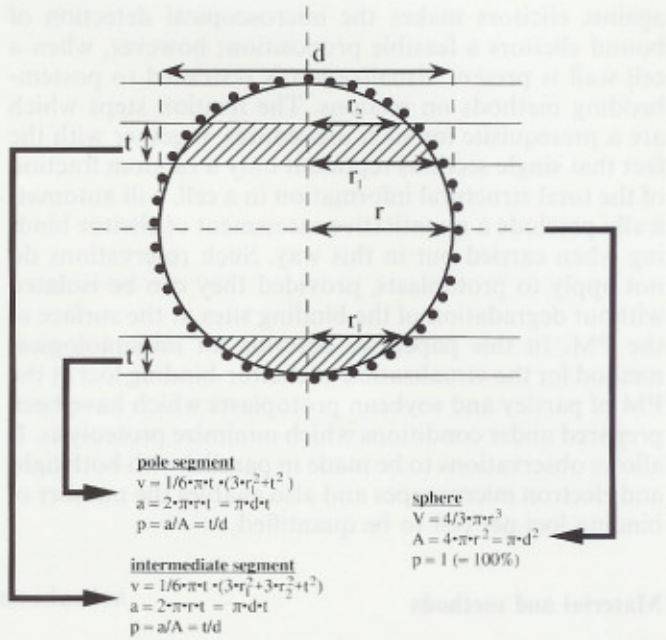


Fig. 1. Determination of the number of elicitor-binding loci on the surface of a protoplast. Derivation from thin sections visualized in the electron microscope. Assuming a homogeneous distribution of elicitor-binding loci one can calculate their total number by extrapolating from their number in individual segments (≡ sections) using the formulae:

$$p = a/A = n/N;\ p = t/d;\ N = d/t \cdot n$$

where n is the number of binding loci, v the volume, and a the surface area of a segment. p represents the relative portion or surface area per segment (expressed in %). t is the thickness of the segment (100 nm), d the protoplast diameter, A the surface area of the entire protoplast and V its volume

Silver enhancement for electron microscopy

Fixed, washed protoplasts were dehydrated through a graded acetone series and embedded in plastic (Spurr's low viscosity resin). Thin sections were exposed to silver-enhancing solution for 5 min and then double-stained with uranyl acetate and lead citrate before observing in a CM 10 electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV.

Quantitation of elicitor-binding loci

Two methods of estimation have been applied: (i) from thin sections visualized in the electron microscope (Fig. 1), (ii) from individual focal planes visualized by epipolarization microscopy (Fig. 2). Whereas the former derivation involves a simple extrapolation from the number of silver-enhanced gold complexes visible at the surface of a sectioned protoplast, it was necessary to introduce two glass discs into the ocular in order to obtain the necessary data for the second derivation. The first disc was a conventional graduated eyepiece, the second was aperture-like in that it had a circle etched in its surface whereby the depth of focus is larger than the depth of the pole segment. A suitable protoplast was brought into the centre of the optical axis and focussed at the equatorial plane in order to

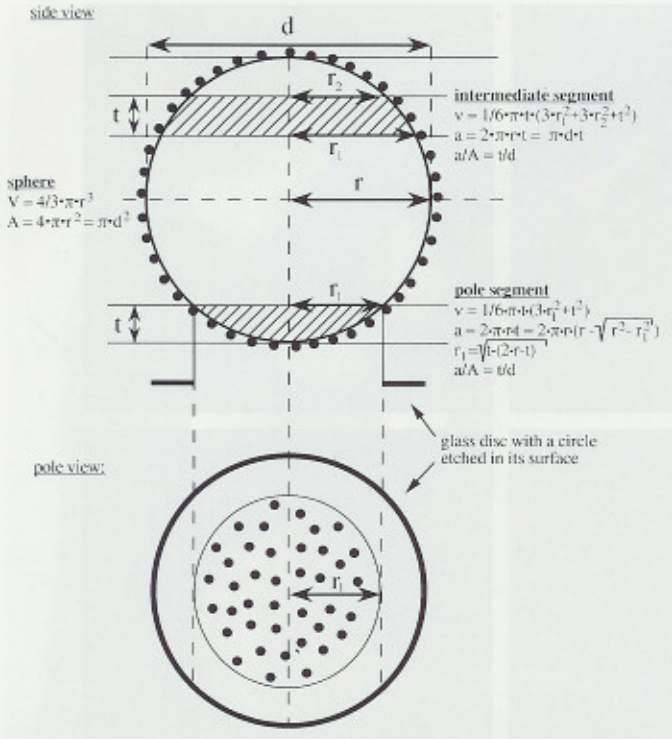


Fig. 2. Determination of the number of elicitor-binding loci on the surface of a protoplast. Derivation from individual focal planes visualized by epipolarization microscopy. Assuming a protoplast is a perfect sphere one can calculate the total number of bound elicitor molecules from the formula

$N = d/t \cdot N$ (see Fig. 1)

where n is the number of point light sources visible within the etched circle of the aperture. However, since t cannot easily be measured, we have estimated it using the relationship

$t = r - \sqrt{r^2 - r_1^2}$

where r is the radius of the protoplast and r_1 the radius of the etched circle in the inserted aperture. The density of elicitor-binding loci x can be determined from the formula

$x = N/A$ where A is the surface area of the protoplast

measure its diameter. The protoplast was then refocussed at its pole with the etched circle symmetrically placed around it. The number of point light sources which were visible within the etched circle were counted. Measurements of protoplast (from normal light optics) and etched circle diameters in relative units were converted to µm by using a calibrated glass slide.

Results

Binding of elicitors to the PM

In order to visualize the binding of elicitors to the PM two prerequisites have to be fulfilled: (i) the production of protoplasts under conditions in which the (proteolytic) degradation of binding sites is minimized, (ii) maintaining protoplasts under condition(s) which do not lead to a reduction in the number of binding sites, e.g. through endocytosis (Goldstein et al. 1985).

It is well known that commercial protoplast-releasing enzyme preparations also have high protease contents. We therefore embarked upon a series of experiments designed to minimize this proteolytic activity. In this respect we found that a combination of inhibitors against proteases (aprotinin, bestatin, E-64, leupeptin, pepstatin) were of little value, at least at the concentrations usually used when these substances are included in homogenizing media (e.g. Demmer et al. 1993). On the basis of the azocaseinase assay (Hamano et al. 1984) we found instead that a combination of two manipulations were very effective in reducing the proteolytic activity in protoplast-releasing enzyme mixtures. The first was the inclusion of high concentrations of BSA (at least 1–2% w/v) as an alternative substrate for protease action. By this means proteolytic activity was reduced to roughly one-third (data not shown). Secondly we have found that a heat treatment, as suggested by Van der Valk (1984) was capable of further reducing proteolytic activity in the protoplast-releasing enzyme mixture to a level where it could no longer be detected. We have also lowered the times for protoplast release to an hour or less, thereby minimizing even further the dangers of cell surface proteolysis.

Endocytosis, which could lead to the internalization of cell surface-bound ligands, does not take place in animal cells at 4°C (e.g. Pastan and Willingham 1985). To show that this would also hold true for plants we incubated soybean cells and protoplasts (prepared under conditions of minimal proteolysis) with a fluorescent PGA-elicitor conjugate at 4°C and 25°C (Fig. 3). Using the confocal laser scan microscope the label is seen to be restricted to the PM-cell wall interface at 4°C (Fig. 3b, d, e), whereas at 25°C label accumulates in the vacuole, as previously described by Horn et al. (1989) using conventional fluorescence microscopy.

Elicitor binding at the surface of the PM at 4°C can also be demonstrated by pre-embedding immunogold labelling (Figs. 4a–d). Here the bound elicitor was revealed by incubating protoplasts sequentially with an appropriate primary (elicitor) antibody followed by a gold-tagged secondary antibody before fixation and embedding. In thin sections label is sparse, rarely present as single gold particles (Fig. 4a), but more frequently occurring in small groups (Fig. 4b). After silver-enhancement, electron-opaque globules of a uniform size (approx. 0.02 µm in diameter) were observed exclusively at the surface of the PM (Figs. 4c, d).

SEIG-EPOM: a method for visualizing elicitor-binding with the light microscope

When parsley and soybean protoplasts are exposed to their respective elicitor (for 30 min at 4°C) and then thoroughly washed, the residual bound elicitor at the surface of the PM can be visualized by a procedure involving four steps: (i) incubation with a primary antibody directed against either the elicitor itself (Pmg-elicitor), or the non-eliciting portion of an elicitor-conjugate (fluorescein-PGA-elicitor), (ii) incubation with 5-nm-gold-conju-

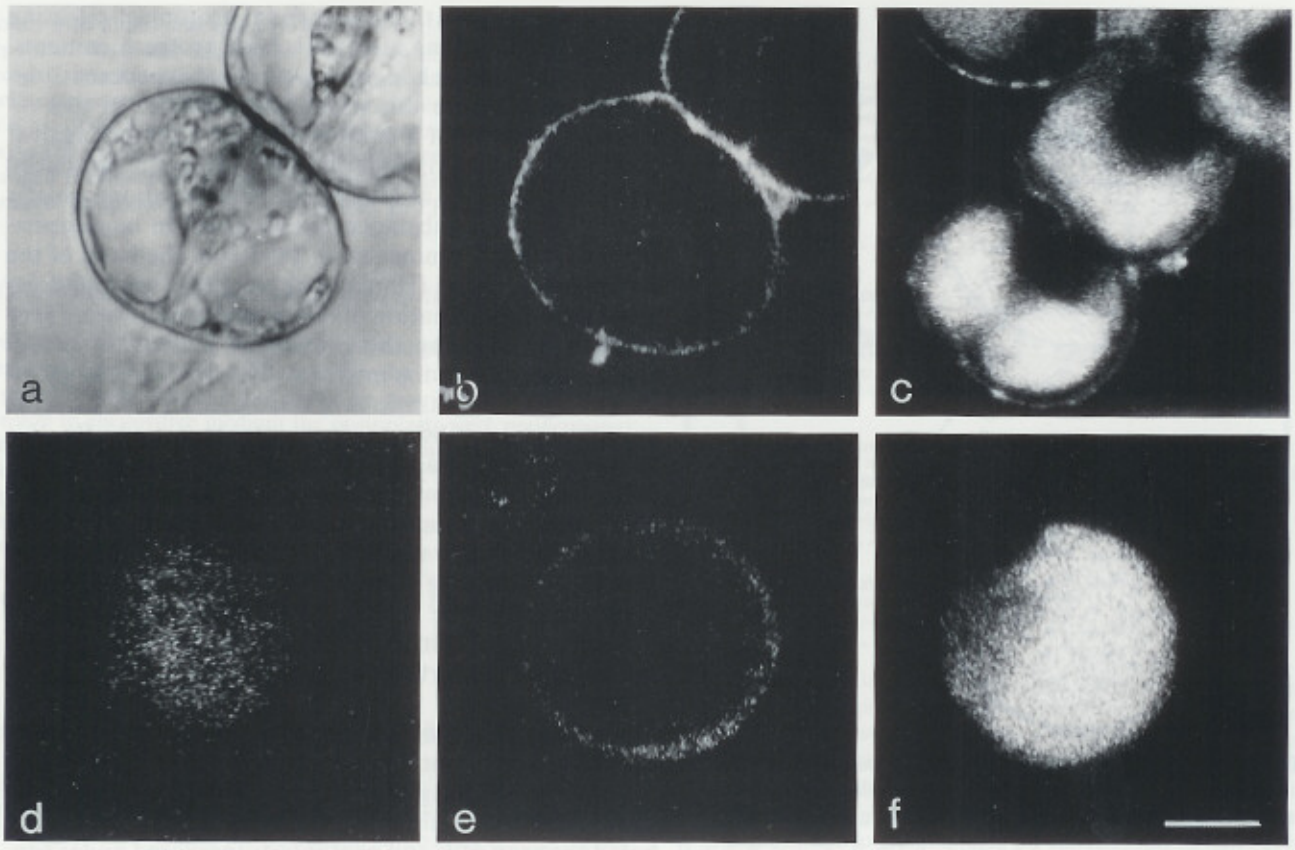


Fig. 3a–f. Surface binding and subsequent internalization of PGA-fluorescein by soybean cells and protoplasts as visualized by confocal laser scan microscopy. **a–c** cells; **d–f** protoplasts; **a**, **b**, **d**, **e** incubations in elicitor-conjugate for 30 min at 4°C; **c**, **f** as for **a**, **b**, **d**, **e** but after a further incubation for 2 h at 25°C in the presence of PGA-fluorescein; **a** normal light, **b–f** fluorescence micrographs. All micrographs, except for **d** which represents a cap view, depict equatorial focal planes. Bars = 10 μm; ×1300

gated secondary antibodies, (iii) silver enhancement of the bound gold particles following fixation, (iv) observation in an epipolarization microscope. We have named this method "silver *e*nhanced *i*mmunogold as viewed by *epi*polarization *m*icroscopy" (SEIG-EPOM).

Figure 5a–f shows the typical appearance of parsley and soybean protoplasts prepared in this way and photographed at different focal planes. In equatorial images, refractile light sources are present as an intermittent circle (Fig. 5c, f) confirming that the protoplasts were intact and had not internalized the elicitor. When focussed at their poles ("cap images") individual, randomly distributed point light sources are seen at the surface of the protoplasts. We have ruled out the possibility that the refractile light sources might represent unspecific binding of the gold-conjugated secondary antibody by performing control incubations: minus elicitor, minus primary antibody (Fig. 5g, i); minus elicitor plus primary antibody (Fig. 5h, j). In both cases, subsequent incubation in the gold-tagged second antibody solution led to only extremely low numbers of point light sources. That the punctate light sources reflect the unspecific binding of colloidal gold aggregates can also be ruled out. This is possible at low pH (5.5 or below; Behnke et al. 1986; Geoghegan 1988), which causes a desorption of the antibodies from the gold particles, but is unlikely at the pH used here for the secondary incubation (pH 7.4). Moreover, the negative results obtained with the controls just described are not compatible with such an effect.

We have performed SEIG-EPOM on parsley protoplasts incubated with the Pmg-elicitor using enhancement times varying from 2 to 15 min (Fig. 6a). Although there is considerable variance in numbers of visualized punctate sources at each particular time point, a fact reflecting the heterogeneity of the protoplast population, a significant increase in their number after 10 min was not observed. An enhancement time of 10 min also appears to be optimal for other surface antigens, for example an unspecified antigen in the PM of bean leaf protoplasts which can be identified using the monoclonal antibody G23-7-D6 of Key and Weiler (1988). After 2 min of silver enhancement the point light sources are variable in size and the smallest are difficult to recognize (Fig. 6b). At 10 min the punctate sources are more uniform in size and can be easily counted. Longer enhancement periods lead to larger punctate sources whose visualization, because of the intensity of light emitted, is not practical.

Estimation of number of elicitor-binding loci per cell

By counting the number of point light sources in a polar focal plane (e.g. Fig. 5a, d), and extrapolating to the whole surface of the PM (Fig. 1, see *Materials and meth-*

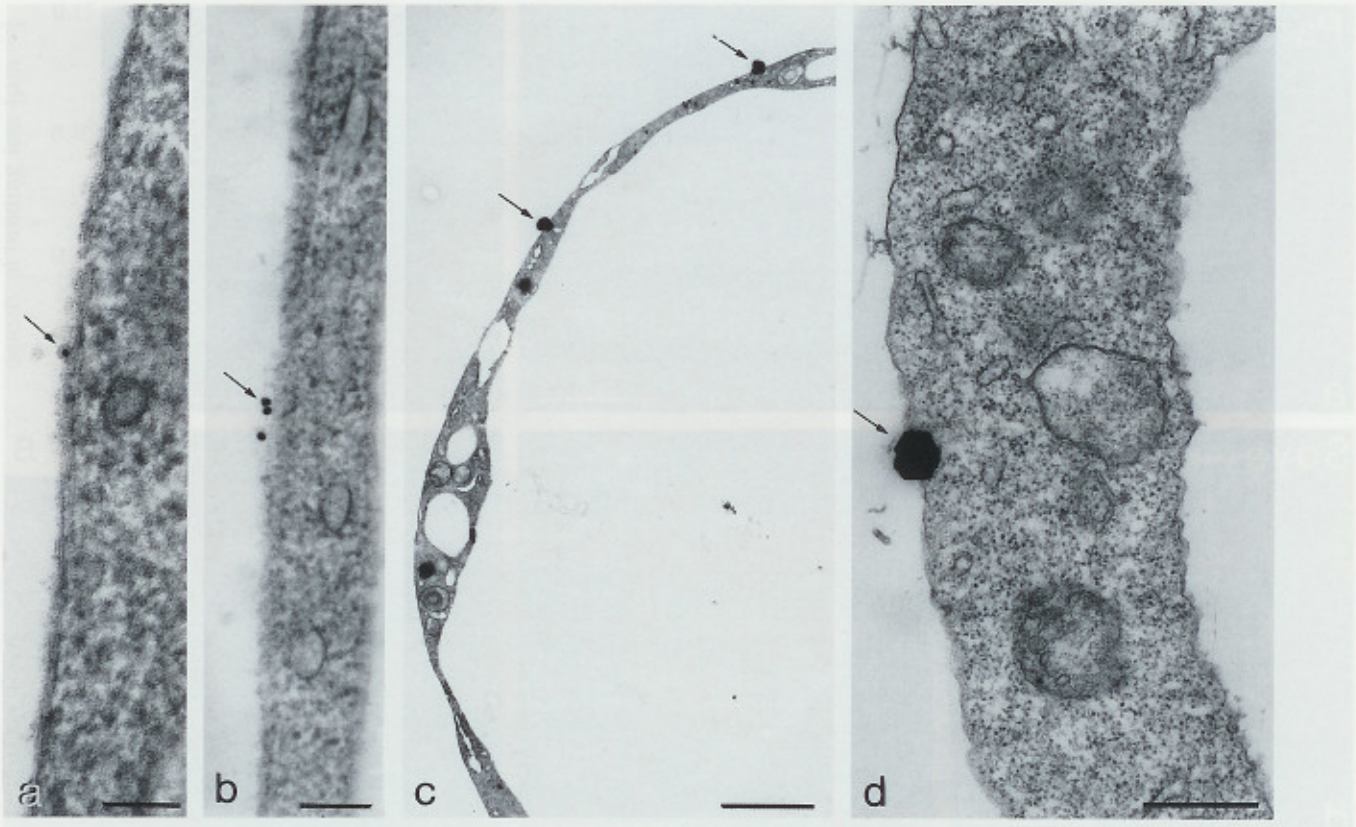


Fig. 4a–d. Demonstration of Pmg-elicitor binding at the surface of parsley protoplasts by pre-embedding immunogold labelling followed by electron microscopy. Protoplasts were exposed to the elicitor for 30 min at 4°C and then incubated in primary antibody, followed by gold-conjugated secondary antibody, before fixing, embedding and sectioning. In **c** and **d** the protoplasts were subjected to silver enhancement (10 min) before embedding. *Arrows* point to individual (**a**) or clustered (**b**) gold particles. Bars = 0.1 μm (**a**, **b**), 2 μm (**c**), 0.5 μm (**d**); × 100 000 (**a**, **b**), × 6500 (**c**), × 30 300 (**d**)

Table 1. Quantitation of binding loci as visualized by epipolarization of silver-enhanced IgG-gold conjugates. Polygalacturonide elicitor from citrus pectin applied to protoplasts from suspension-cultured cells of *Glycine max*

Treatment	Diameter of protoplasts (μm)	Total number of binding loci	Density of binding loci (μm^{-2})	Probability[3]
+elicitor[1] −competitor[2]	24 ± 6	131 ± 115	0.05 ± 0.03	
+elicitor[1] +competitor[2] (10 000-fold, simultaneously) 1 h at 4° C	26 ± 6	48 ± 38	0.02 ± 0.01	(1–2) $P = 0.0001$

[1] Fluorescein-labelled PGA elicitor (Nothnagel et al. 1983; Horn et al. 1989)
[2] Crude, partial hydrolysate of citrus pectin (see *Materials and methods*)
[3] Statistical analysis (Mann and Whitney 1947)

ods) we have been able to determine quantitatively the number and density of elicitor-binding loci per protoplast. Because the protoplasts are not of uniform size there is considerable variance in the values obtained (at least 50 cells were counted per treatment); however, on the average we can say that parsley protoplasts have 200, and soybean protoplasts 130 detectable elicitor-binding loci at their surfaces (Tables 1, 2). Given their low numbers and random distribution, this relates to a maximum of 2–3 visualizable loci when 100-nm-thick sections are observed in the electron microscope: a value which we have confirmed experimentally (Fig. 4).

The values given for the numbers of elicitor-binding loci were obtained by incubating protoplasts and elicitors at pH values corresponding to the pH of the media in which the cells were growing before protoplast release (pH 5.5 for parsley, pH 5.6 for soybean). Incubation at alkaline pHs (pH 7–8) gives rise to somewhat higher numbers of punctate light sources (roughly 30% in each case) but it is questionable whether this is a physiologically significant observation, since, even in tissues, apoplasmic pH is nearly always acidic.

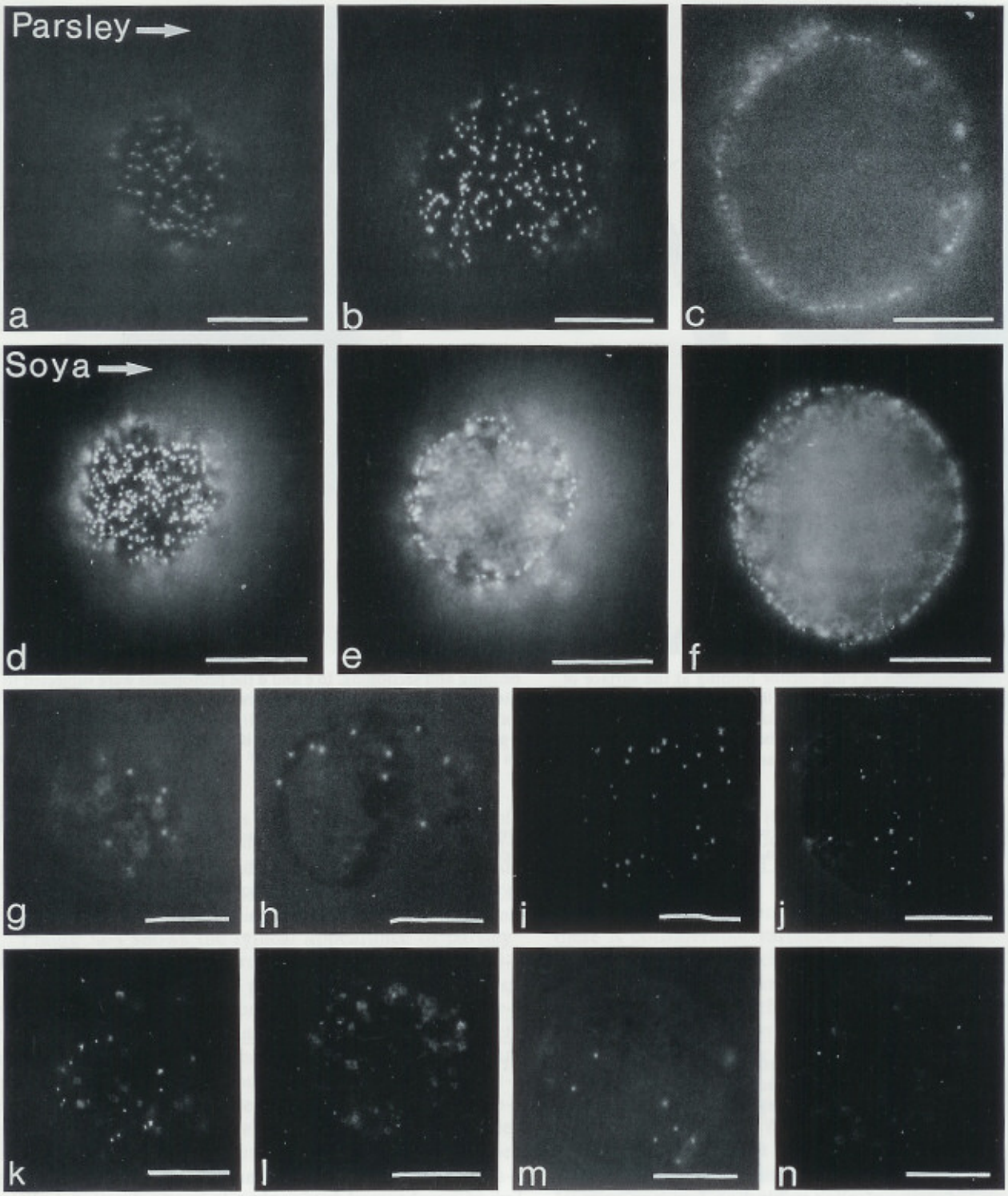
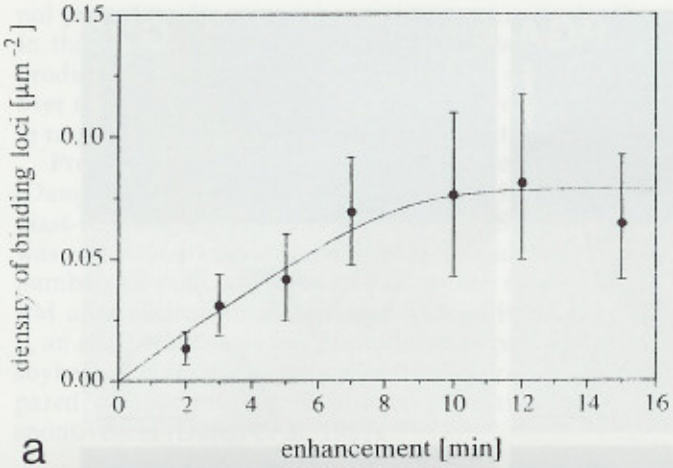
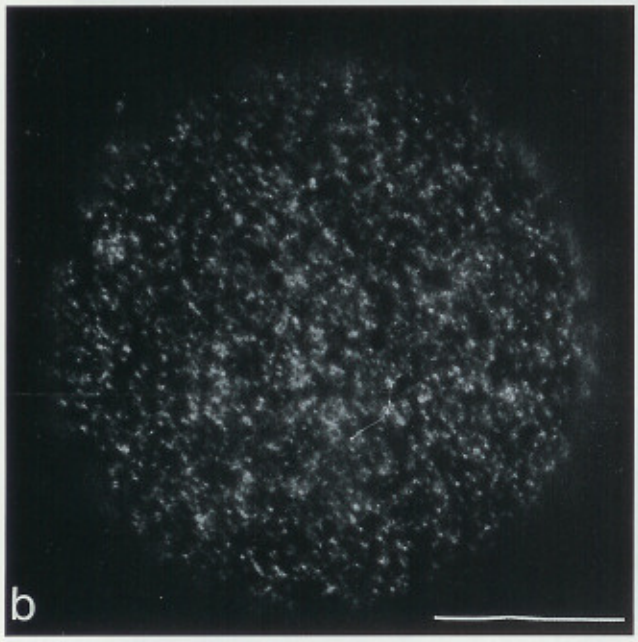


Fig. 5a–n. Elicitor-binding loci at the surface of protoplasts as visualized by SEIG-EPOM. **a–f** Protoplasts incubated with elicitor at 4°C for 30 min followed by sequential incubations in primary and gold-conjugated secondary antibodies. Silver enhancement was carried out on fixed protoplasts. **a–c** Parsley protoplasts exposed to the Pmg-elicitor; **d–f** soybean protoplasts exposed to the PGA-elicitor. In each case three different focal planes are presented. **g–j** Control incubations of parsley (**g**, **h**) and soybean (**i**, **j**) protoplasts: incubation without elicitor and without primary antibody (**g**, **i**); incubation without elicitor but with primary antibody (**h**, **j**). **k**, **l** Parsley (**k**), and soybean (**l**) protoplasts prepared without protection against proteolysis and then incubated as in **a–f**. **m**, **n** Parsley protoplasts prepared as in **a–c** and subsequently incubated for 30 min at 25°C with 0.1% (w/v) trypsin + 0.1% (w/v) thermolysin (**m**) or 0.1% (w/v) carboxypeptidase A (**n**) before addition of elicitors and visualization of binding sites. Bars = 10 μm; ×1600 (**a–f**), ×1400 (**g–n**)





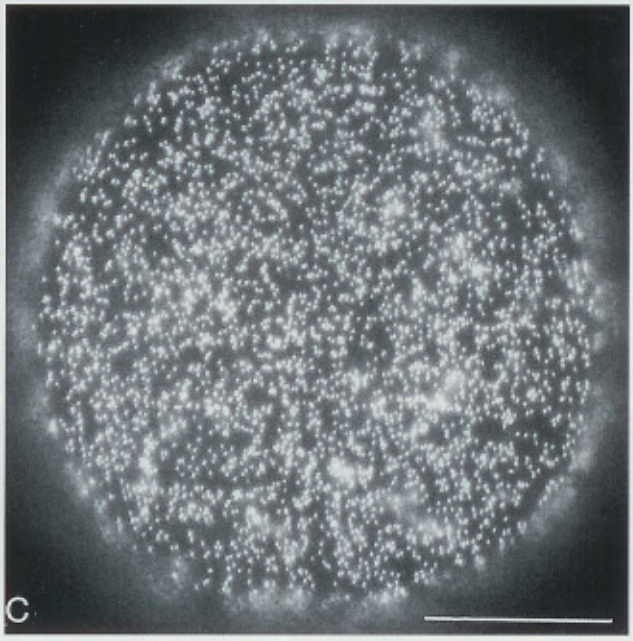


Fig. 6a–c. The effect of time of exposure to the silver enhancement solution on the number and size of punctate light sources as visualized by the SEIG-EPOM method. **a** Development of surface labelling of parsley protoplasts which had been exposed to the Pmg-elicitor for 30 min at 4°C. **b**, **c** Detection of an epitope in the PM of bean leaf protoplasts using the monoclonal antibody G23-7-D6. Silver enhancement for 2 min (**b**), and 10 min (**c**). Bars = 10 µm; ×2500

Table 2. Quantitation of binding loci as visualized by epipolarization of silver-enhanced IgG-gold conjugates. The 42-kDa glycoprotein elicitor from *Phytophthora megasperma* was applied to protoplasts from suspension-cultured cells of *Petroselinum crispum*

Treatment	Diameter of protoplasts (µm)	Total number of binding loci	Density of binding loci (μm^{-2})	Probability[5]
−elicitor[1] 1 h at 4° C	31 ± 8	58 ± 52	0.02 ± 0.02	(1–2, 3) $P = 0.0001$
+elicitor[1] −competitor[2] 1 h at 4° C	27 ± 5	193 ± 106	0.08 ± 0.03	
+elicitor[1] +competitor[2] (10000-fold, simultaneously) 1 h at 4° C	26 ± 6	175 ± 122	0.08 ± 0.03	(2–3) $P = 0.236$
+elicitor[3] −competitor[4] 1 h at 4° C	29 ± 8	187 ± 94	0.07 ± 0.05	
+elicitor[3] +competitor[4] (10000-fold, simultaneously) 1 h at 4° C	30 ± 8	73 ± 39	0.03 ± 0.02	(4–5) $P = 0.0001$

[1] Intact glycoprotein elicitor from *Phytophthora megasperma* (Parker et al. 1991)
[2] Elicitor-active, 25-amino-acid-containing peptide (PEP-25) from *Phytophthora megasperma* (Sacks et al. 1993 a)
[3] Fluorescein-conjugated PEP-13
[4] Elicitor-active, 13-amino-acid-containing peptide (PEP-13) from *Phytophthora megasperma* (Sacks et al. 1993 a)
[5] see Table 1

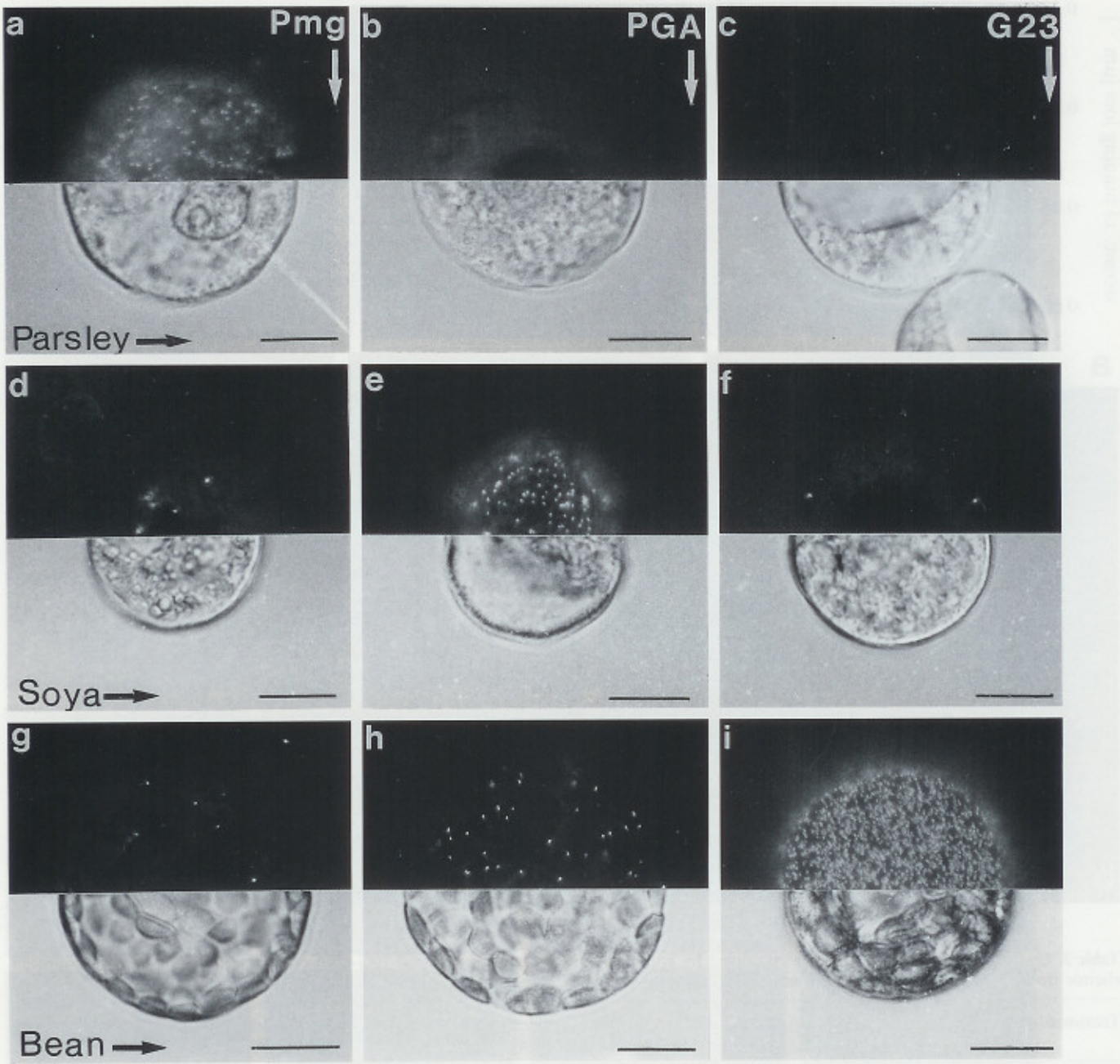


Fig. 7a–i. Specificity of surface elicitor binding as demonstrated by the SEIG-EPOM method. Protoplasts from suspension-cultured parsley, soybean, and bean leaves were exposed to the Pmg- and PGA-elicitors and surface labelling visualized with SEIG-EPOM (**a**, **b**, **d**, **e**, **g**, **h**). In addition the monoclonal antibody G23-7-D6 was used to determine the presence of a bean leaf PM epitope at the surface of parsley (**c**) and soybean (**f**) protoplasts. In order to document those cases with negligible, or low surface labelling, individual protoplasts were photographed by epipolarization (upper portion of each figure) and normal light (lower portion) microscopy. Bars = 10 μm; × 1200

Characterization of elicitor-binding loci

Proteolytic sensitivity. The degree of surface labelling as monitored by SEIG-EPOM is virtually eliminated when protoplasts, which have been isolated as above, are subjected to treatment with proteases (trypsin + thermolysin, carboxypeptidase A) before being exposed to elicitor preparations (Fig. 5m, n). Surface proteolysis also reduced the capability of parsley protoplasts to synthesize and secrete furanocoumarins in response to the Pmg-elicitor. Thus parsley protoplasts treated for 1 h with 0.1% carboxypeptidase A at 4°C (a treatment which does not lead to hydrolysis of the Pmg-elicitor; Sacks et al. 1993a) and then exposed to the Pmg-elicitor for 24 h released only 10% of the amount of furanocoumarins in comparison with untreated controls during the same time period (data not shown). Similar experiments were also performed with soybean protoplasts using the lumi-

nol procedure for measuring active-oxygen production. In this case protease-treated protoplasts were found to produce only 30–50% of the amount of active oxygen over a 1-h period in comparison with untreated controls in response to exogenous PGA-elicitor (data not shown).

Protoplasts which were prepared as previously given (Dangl et al. 1987), i.e. incubated overnight in a protoplast-releasing enzyme mixture lacking BSA and which was not heat-treated, also revealed considerably lower numbers of punctate light sources at the surface of the PM after elicitor incubation and SEIG-EPOM (Fig. 5k, l), an effect which was less pronounced in parsley than in soybean. This might explain why parsley protoplasts prepared without proteolysis protection retain elicitor responsiveness (Dangl et al. 1987).

Specificity. In order to determine whether the elicitor-binding loci, which were visualized by SEIG-EPOM, are species-specific we have exposed parsley protoplasts to the PGA-elicitor, soybean protoplasts to the Pmg-elicitor, and bean leaf protoplasts to both elicitors. As a control we have examined parsley and soybean protoplasts for the presence of the G23-7-D6 bean leaf PM epitope. The results of these heterologous incubations are presented in Fig. 7. Binding-loci for the Pmg-elicitor were almost absent from broadbean leaf and soybean protoplasts but abundant on parsley protoplasts (compare Fig. 7d, e with a). Whereas parsley protoplasts had no PGA-elicitor-binding loci (Fig. 7b), substantial numbers of PGA-binding loci were visualized in the case of bean leaf protoplasts (Fig. 7h). The bean leaf PM epitope detected by the monoclonal antibody G23-7-D6 is either occluded or absent from the surface of parsley and soybean protoplasts.

Competition through structural analogues. The results of competition experiments give further support to our contention that the punctate light sources at the protoplast surface do indeed represent elicitor-binding loci. Here we have used structurally related elicitor compounds which are not recognized by the primary antibodies. These compounds were applied in 10 000-fold excess simultaneous with the addition of the elicitors. Competition was partially successful, to the extent of a 50% reduction of visualized binding loci when unlabelled PGA and fluorescein-PGA were added simultaneously (Table 1). On the other hand, when PEP-25 was added together with the intact Pmg-elicitor only a small, statistically insignificant, reduction in surface labelling was obtained. Competition was, however, much more successful when the Pmg-elicitor oligopeptide PEP-13-fluorescein (Table 2) was used. We have not attempted to carry out competition experiments using the SEIG-EPOM procedure between PEP-13 and completely inactive PEP-13 derivatives, since the latter had already been shown to be ineffective as competitors in [^{125}I]PEP-13 microsomal binding studies (data not shown). Moreover, since SEIG-EPOM is a very time-conserving method we also decided against performing competition experiments with partially active PEP-13 derivatives at the cytological level.

Discussion

Silver-enhanced immunogold methods for light microscopy are in regular use in animal cell biology (reviewed by Hacker 1989). One of the possibilities of visualizing the silver is by epipolarization microscopy, which has now become a standard procedure (Ellis et al. 1988; Cornelese-ten Velde et al. 1990), especially for the detection of cell surface antigens (reviewed by De Waele 1989). By comparison these methods have been much less frequently employed by plant cell biologists, and, to our knowledge, there is only once case in which epipolarization optics were involved (Villanueva et al. 1993). Although protoplasts were also used in this latter study, the PM was not visualized in surface view. Instead a laser scanning microscope was used to follow the uptake of colloidal gold conjugates of BSA.

Visualization of bound elicitor was achieved either directly by gold-conjugated antibodies directed against the elicitor itself (Pmg-elicitor), or indirectly by gold-conjugated antibodies recognizing the fluorochrome of a fluorescein-conjugated elicitor (PGA-elicitor, Pmg-PEP13-elicitor). The binding loci are susceptible to proteolysis, as is the elicitation response of the protoplasts. On the basis of control experiments we have shown that the majority of the punctate sources of light seen at the surface of elicitor-treated protoplasts by the SEIG-EPOM method are not artefacts. For example, specific surface labelling is not seen on protoplasts which have not been exposed to elicitors, and this labelling can only be visualized with the right combination of elicitor and protoplast types. The validity of the SEIG-EPOM procedure is further supported by competition experiments.

The PGA competed binding of fluorescein-PGA to soybean protoplasts and PEP-13 that of fluorescein-PEP-13 to parsley protoplasts, whereas PEP-25 was not capable of reducing the number of binding loci detectable with the Pmg-elicitor. Therefore, the intact Pmg-elicitor appears to have a higher affinity to its binding site than the PEP-25 oligopeptide. In addition, binding of iodinated intact Pmg-elicitor could only be reduced by 27% in the presence of excess unlabelled Pmg-elicitor (Renelt et al. 1993). This high degree of unspecific binding may be due to the extreme hydrophobicity of the Pmg-elicitor (Sacks et al. 1993a, b). In competition experiments with a glucan elicitor and soybean protoplasts, as well as with glycopeptides and tomato cells, levels of unspecific binding amounted to 25–30% of total binding (Cosio et al. 1988; Basse et al. 1993). We therefore regard competition as having been successful although 50% (soybean) or 40% (parsley) of the surface labelling remained visible. The origin of the unspecific labelling is unclear but our control experiments with and without the primary antibody (Fig. 7g–j, Table 1) suggest that non-elicitor or non-fluorescein IgGs in the antisera used might in part be responsible. Unfortunately preimmune sera were not available so that a rigorous examination of this possibility could not be undertaken.

The concentration of binding sites for different elicitors on plant microsomal membrane preparations has been found to lie between 19 and 1200 fMol·mg protein^{-1}

(Cosio et al. 1988; Cheong and Hahn 1991; Cosio et al. 1992; Basse et al. 1993; Shibuya et al. 1993). Although these values cannot be directly compared to the number of binding loci on single protoplasts as detected by SEIG-EPOM, it is obvious that these binding sites are of very low abundance. In contrast to in-vitro binding experiments, SEIG-EPOM allows direct quantitation only of those binding loci at the cell surface. Although it is possible that one luminescent point at the surface of a protoplast may represent the binding of a single elicitor molecule, we wish to point out that silver enhancement of neighbouring immunogold complexes could lead to the production of a common point light source. In many cases in animal cells, cell surface receptors are preclustered in large numbers even before ligand binding (Rothberg et al. 1990). On the other hand, it is also very common for ligand binding to induce the clustering of receptors (Braun and Unanue 1983; Heffetz and Zick 1986; Hurwitz et al. 1991; Metzger 1992), even at 4°C (Pastan and Willingham 1981). Because labelling with primary/secondary antibodies might cause additional aggregation, it is difficult to extrapolate the number of elicitor receptors from analyses of the number of binding loci on the protoplast surface. In support of our contention that the punctate light sources at the surface of elicitor-treated parsley and soybean protoplasts represent multiple binding sites is the clear difference in their size and number when compared to an epitope on the PM of bean leaf protoplasts when visualized by SEIG-EPOM under the same conditions. The latter are much smaller and at least 10 times as frequent.

Although introduced here to detect elicitor-binding loci, SEIG-EPOM is clearly a method which can be used to visualize any component at the surface of plant cells against which the appropriate antibodies are available. It may, therefore, also have potential in terms of research into endocytosis. In animal cells, many receptor-ligand interactions at the PM result in a clathrin-coated vesicle-mediated endocytosis of the receptor-ligand complex (see Goldstein et al. 1985; Van Deurs et al. 1989 for reviews). This "down regulation" is necessary in order to recycle ligand-free receptors back to the PM. Whilst we do not know much about the nature of putative receptors for elicitors (the current status on this has been recently reviewed by Ebel and Scheel 1992), preliminary evidence for a receptor-mediated internalization of elicitors is now at hand (Low et al. 1993). Since endocytosis, but neither ligand-binding nor antibody-antigen interactions, is temperature dependent, it is conceivable that warming up protoplasts, which have been labelled at cold temperature with ligand, could lead to a reduction in the number of binding loci which can subsequently be visualized. Experiments along these lines are now in progress, but have the inherent caveat that cell wall regeneration, which sets in more or less immediately upon returning protoplasts to fresh growth medium, may interfere with antibody binding. On the other hand, re-release of protoplasts, at least in the case of a polysaccharide elicitor, would lead to ligand destruction. Another cautionary aspect for endocytosis studies which can be inferred from the present work, is that the low densities of binding loci at the cell surface will make it extremely difficult to detect internalized receptor-ligand complexes by post-embedding immunogold labelling on thin sections. We come to this conclusion on the assumption that there will be, at any one time during a down-regulation experiment as described above, a number of internalized receptor-ligand complexes maximally equivalent to the original complement at the cell surface at the beginning of the experiment. The demonstration of only two to five silver-grain deposits at the surface of protoplasts in thin section (Figs. 6, 7) succinctly underlines this problem of immunodetection.

We thank Professor Elmar Weiler (Lehrstuhl Pflanzenphysiologie, Universität Bochum, Germany) for sending us the monoclonals against bean leaf plasma membrane. We also are indebted to Dr. A. Hille-Rehfeld (Institut für Biochemie und Molekulare Zellbiologie, Universität Göttingen, Germany) for the Confocal laser scan micrographs. We also thank Renate Hagemann and Heike Freundt for typing the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (to D.G.R. and D.S.) and the National Science Foundation (to P.S.L.). W. Diekmann was a recipient of a stipend from the Hanns-Seidel-Stiftung (München, Germany).

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Mol Gen Genet (1995) 246:45–55

ORIGINAL PAPER

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Molecular characterization of nucleotide sequences encoding the extracellular glycoprotein elicitor from *Phytophthora megasperma*

Received: 5 April 1994 / Accepted: 4 July 1994

Abstract cDNA sequences encoding the 42 kDa glycoprotein elicitor from the oomycete, *Phytophthora megasperma*, that induces the defense response in parsley have been cloned and sequenced. The 5′ end of the mRNA matches a consensus derived from sequences surrounding the transcription initiation sites of seven other oomycete genes. The major transcript of 1802 nucleotides contains a 529-codon open reading frame, which was predicted to encode a 57 kDa precursor protein. On the basis of peptide sequencing, the N-terminus of the mature protein is at position 163, suggesting that proteolytic processing events, in addition to signal peptide cleavage, generate the protein purified from the fungal culture filtrate. Expression studies in *Escherichia coli* with the cDNA as well as smaller subfragments demonstrated that a region of 47 amino acids located in the C-terminal third of the protein was sufficient to confer elicitor activity. The gene encoding the elicitor was found to be a member of a multigene family in *P. megasperma*. Homologous families of differing sizes were found in all eight other *Phytophthora* species tested, but not in other filamentous fungi including other Oomycetes. No significant similarity of the elicitor preprotein to sequences present in the databases has yet been detected.

Key words *Phytophthora megasperma* · Oomycetes
Elicitor · Cell wall protein · Parsley

Communicated by J. Schell

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Introduction

Plants are resistant to the vast majority of potentially pathogenic microorganisms they encounter (basic or non-host resistance). Only in a minority of cases have pathogens developed effective mechanisms for circumventing the defense responses of a particular plant species, allowing them successfully to colonize the species (basic susceptibility). Particular cultivars of these species, however, possess the ability to recognize particular races of the pathogen species and, consequently, to mount a successful resistance response (cultivar resistance; Heath 1991). The spectrum of reactions elicited in plants undergoing either type of resistance is similar and includes a variety of processes that require specific gene activation, such as phytoalexin biosynthesis. The event(s) triggering these responses is generally thought to be the binding of pathogen- or plant-derived signal molecules to target sites on the plant plasma membrane. A large number of chemically diverse molecules derived from phytopathogenic fungi have been identified that stimulate defense responses in intact plants or cultured plant cells – the so-called elicitors (reviewed in Ebel and Scheel 1992). However, with the exception of the avirulence gene products from *Cladosporium fulvum* which act as cultivar-specific elicitors in tomato (Joosten et al. 1994; van den Ackerveken et al. 1992), it is not known if any of these compounds serve as recognition signals in the corresponding plant/pathogen interaction. Moreover, only for one such elicitor, a β-glucan from *Phytophthora megasperma* f. sp. *glycinea*, have binding sites been convincingly demonstrated in plasma membranes from the corresponding host plant, soybean (Cheong and Hahn 1991; Cosio et al. 1988). Although a 70 kDa protein has been implicated as a component of this binding site (Cosio et al. 1992), it has yet to be further purified or characterized in greater detail. High affinity binding has also been demonstrated in cell membranes of tomato and rice for a glycopeptide elicitor derived from yeast invertase, and a purified chito-oligosaccharide, respectively (Basse et al. 1993; Shibuya et al. 1993),

but the nature of the binding sites remains to be elucidated.

We have been using the non-host interaction of *P. megasperma* with parsley (*Petroselinum crispum*) as a model system for studying signal perception and transduction leading to defense gene activation. Cultured parsley cells or freshly prepared protoplasts treated with elicitor preparations from the fungus respond with a spectrum of reactions very similar to that evoked in the interaction between the intact organisms (Dangl et al. 1987; Scheel et al. 1986). One component of the overall response is the biosynthesis of furanocoumarins, the phytoalexins of parsley. We reported previously that fungal proteins rather than carbohydrates function as elicitors in this system (Parker et al. 1988) and subsequently purified a 42 kDa glycoprotein from the fungal culture filtrate with potent elicitor activity (Parker et al. 1991). Attempts to use the glycoprotein as a ligand in binding studies were ultimately unsuccessful, presumably due to the hydrophobicity it exhibited under most experimental conditions, which translated into high levels of nonspecific binding (Renelt et al. 1993). In order to gain further insight into this signalling process and to facilitate the identification of a more suitable ligand for binding studies, we have cloned fungal nucleotide sequences encoding this protein and have begun to localize the portion of the molecule conferring elicitor activity.

Materials and methods

Fungal culture and elicitor preparation

Race 1 of *P. megasperma* f. sp. *glycinea* was maintained as described previously (Kombrink and Hahlbrock 1986). Liquid cultures were harvested after 3–4 weeks of growth on asparagine medium (Keen 1975). Mycelium was processed to prepare the crude fungal wall elicitor as described by Ayers et al. (1976) and the 42 kDa glycoprotein elicitor was purified from culture filtrate as described by Parker et al. (1991). N-terminal, tryptic and endoproteinase Glu-C-generated peptides were sequenced using an Applied Biosystems 477A Sequenator.

P. infestans (race 1) was cultured either on vegetable juice agar (Rohwer et al. 1987) or in liquid Henninger medium (Henninger 1963). *P. cactorum* and *P. palmivora* were cultured on vegetable juice agar while *P. cryptogea*, *Pythium aphernidermatum* and *Pythium ultimum* were grown on malt agar (20 g/l malt extract, 20 g/l agar). These three *Phytophthora* and two *Pythium* species were kindly provided by Dr. Heinz-Wilhelm Dehne (Bayer AG, Leverkusen, Germany).

Fungal DNA preparation

Frozen *P. megasperma* mycelium from liquid cultures was ground to a fine powder with quartz sand in liquid nitrogen and added to 2 ml/g TES (100 mM TRIS-HCl, pH 8.0, 10 mM EDTA, 2% SDS). Proteinase K was added to a final concentration of 200 µg/ml and the mixture incubated for 30 min at 56°C. After one extraction with phenol, several with phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) and one with chloroform/isoamylalcohol (24:1, v/v), the DNA was precipitated with 0.04 volume of 5 M NaCl and 0.8 volume of 2-propanol and resuspended in TE (10 mM TRIS-HCl, pH 8.0, 1 mM EDTA).

Genomic DNAs from *P. infestans*, *P. cactorum*, *P. cryptogea*, *P. palmivora*, *P. aphernidermatum* and *P. ultimum* were prepared from mycelia grown over cellophane on solid medium. The method of Pieterse et al. (1991) was used with the exceptions that EGTA was replaced with EDTA in the extraction buffer and the DNAs were not gradient, purified. DNAs from *P. capsici*, *P. cinnamomi*, *P. nicotianae* and *P. parasitica* were kindly provided by Dr. Pierre Ricci (Station de Pathologie Végétale INRA, Antibes, France); DNA from *Bremia lactucae*, by Dr. Richard Michelmore (University of California, Davis, USA); and DNAs from *Claviceps purpurea* and *Gibberella pulicaris*, by Dr. Klaus-Michael Weltring (Institut für Botanik und Botanischer Garten, Münster, Germany).

Standard cloning and sequencing techniques

Standard cloning techniques including electrophoresis and blotting of DNA and RNA were carried out essentially as described (Sambrook et al. 1989). Dideoxy chain-termination sequencing (Sanger et al. 1977) was carried out using the T7 Sequencing Kit (Pharmacia Biosystems, Freiburg, Germany) according to the manufacturer's instructions. Sequence compilation and analysis were performed using the University of Wisconsin Genetics Computer Group (GCG) Software Package, version 6.2 (Devereux et al. 1984).

Polymerase chain reaction (PCR) amplification

(1) Amplification of genomic *P. megasperma* DNA with the degenerate primers indicated in Table 1 was carried out using 100 ng/ml *Sal*I-digested template, 5 µM primers and 25 U/ml *Taq* polymerase (Perkin-Elmer, Weiterstadt, Germany) in 10 mM TRIS–HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 240 µM each dNTP and 0.01% glycerol. The incubation conditions were as follows: 4 min, 94°C; 29 cycles (1 min, 94°C; 5 min, 50°C; 2 min, 72°C); 1 cycle (1 min, 94°C; 5 min, 50°C; 10 min, 72°C). The 566 bp product obtained was cloned using *Bam*HI and *Xba*I sites introduced via the 5′ ends of the primers into pBS+ (Stratagene, Heidelberg, Germany) and one clone sequenced in its entirety.

(2) Routine PCR with plasmid templates and non-degenerate primers was carried out with 2 ng/ml linearized template, 1 µg/ml each primer, 240 µM each dNTP, 25 U/ml *Taq* polymerase (Amersham Buchler, Braunschweig, Germany or Life Technologies, Eggenstein, Germany) with commercially supplied buffer. The following conditions were used: 4 min, 94°C; 29–39 cycles (1 min, 94°C; 1 min, 50°C; 1 min, 72°C); 1 cycle (1 min, 94°C; 1 min, 50°C; 10 min, 72°C).

Labelling and hybridization

Probes for hybridization were synthesized by PCR using appropriate primers and comprised the following nucleotide sequences (see Fig. 3): A, positions 572–1704; B, 20–320; C, 572–841; D, 1209–1350. Random-primed digoxigenin (DIG) labelling of probes was carried out using the DIG-DNA Labelling and Detection Kit (Boehringer Mannheim, Germany) according to the supplier's instructions as was subsequent hybridization, washing and detection. Screening of the cDNA library described below was carried out on nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) at a hybridization temperature of 68°C, while hybridization of Southern and Northern blots was carried out on Hybond N (Amersham Buchler) at 42°C in the presence of 50% formamide. The following 2 × 15 min washes were in 2 X SSC (1 X SSC is: 0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS at room temperature; washing conditions were stringent (2 × 30 min; 68°C; 0.1 X SSC, 0.1% SDS) except where noted. Moderately stringent washing conditions were 2 × 30 min; 55°C; 2 X SSC, 0.1% SDS. Positive cDNA clones were detected using a colour

Table 1 Amino acid sequences of peptides derived from the purified 42 kDa elicitor and degenerate oligonucleotides used for polymerase chain reaction (PCR)

Peptide	Origin	Sequence
		163[a] 186
1	N-terminus (mature protein)	Glu Ala Asn Gly Asn Gln Asp Ile Ala Lys Leu Glu Ala Tyr Phe Gly Thr Lys Met Glu Met ? Leu Lys
		(Glu)[b]
		5′ AAC GGA AAC CAA GAC ATA GC 3′
		T C T G G T C
		G T
		183 193
2	Glu-C digest	Met Thr Leu Lys Asp Leu Pro Thr Val Gly Val
		309 320
3	Trypsin digest	Gln[c] Asn Gly Val Thr Phe ? Pro Met Asp Leu Lys
		(Ser)
		341 352
4	Trypsin digest	Phe Asn Gly Gly Thr Asp Thr Thr Asp Glu Tyr Gly
		3′ CTA TGA TGA CTA CTC ATA CC 5′
		G C C G T G
		G G
		T T
		394 404
5	Glu-C digest	Asn Gln Pro Val Arg Gly Phe Lys Val Tyr Glu
		402 417
6	Trypsin digest	Ser Tyr Glu Gln (Trp) Glu Met Thr Leu Glu (Trp) Gly Ala Gln (Thr) Phe
		(Val) (Ile) (Glu)
		(Ala)
		422 431
7	Glu-C digest	Ala Tyr Pro Trp Asn Ala Ala Ala Lys Ser
		445 464
8	Glu-C digest	Thr Tyr Thr Asp Gly Gly Leu Val ? ? Gly Gln ? Asp Lys Phe ? ? Gly Gln

[a] Numbers above ends of peptides refer to the coding sequence predicted in Fig. 3
[b] Amino acids enclosed in parentheses indicate uncertain positions
[c] Underlined amino acids differed from the corresponding amino acids predicted in Fig. 3

reaction while signals on blots were detected by chemiluminescence according to the manufacturer's instructions.

Fungal RNA preparation

Freshly harvested *P. megasperma* mycelium was ground as described above and extracted for 15 min at room temperature with 4 ml/g homogenization buffer comprising equal parts (v/v) of 0.15 M sodium acetate, 1 mM EDTA, 4% SDS, pH 5.0: phenol-chloroform-isoamylalcohol (25:24:1, v/v/v). The aqueous phase was further extracted several times with phenol-chloroform-isoamylalcohol (25:24:1, v/v/v) once with chloroform-isoamylalcohol (24:1, v/v) then sequentially precipitated with ethanol, 2 M LiCl and again with ethanol. Poly(A)$^+$-RNA for cDNA library construction was isolated by affinity chromatography on oligo(dT)-cellulose (Pharmacia Biosystems), and for Northern blot hybridization and rapid amplification of cDNA ends (RACE) by batch affinity purification on Dynabeads Oligo $(dT)_{25}$ (Dynal, Hamburg, Germany).

cDNA library construction and screening

An oligo(dT)-primed λZAPII cDNA library was prepared from 600 ng poly(A)$^+$ RNA from *P. megasperma* using the ZAP-cDNA Synthesis Kit (Stratagene) according to the manufacturer's instructions. The 566 bp PCR product described above was used to screen 10^5 plaques, which had been amplified from 10^5 original recombinants. Of 22 positive clones retained following three rounds of plaque purification and in vivo excision of the pSK(–)-based recombinants, nine were examined in greater detail. Five groups could be distinguished on the basis of restriction profiles, two of which yielded complex patterns and were not studied further. One member of each of the three remaining groups was partially or completely sequenced.

RACE experiments

The RACE protocol for obtaining 5′ ends of mRNAs as described by Frohman (1990) was followed with some modifications. In the first experiment, cDNA was synthesized from 200 ng fungal poly(A)$^+$ RNA using primer A (nucleotides 1272–1255, Fig. 3) and Superscript reverse transcriptase (Life Technologies) at 37°C for 1 h. Following Sephacryl S-400 (Pharmacia Biosystems) spin-column chromatography, ethanol precipitation and poly(dA)-tailing with Terminal dTransferase (Life Technologies), 0.02 volume of the sample was amplified in 50 µl using approximately 6 pmol modified SK-dT_{17} primer (Kawalleck et al. 1993b), 6 pmol SK primer, 13 pmol primer B (nucleotides 1051–1032 in Fig. 3, specific for the elicitor-encoding cDNA) and the incubation conditions of Frohman (1990), except that the annealing temperature was 50°C instead of 55°C. The products of this reaction were digested with *Spe*I, which cuts within the SK primer sequences and *Not*I, which cuts within the cDNA (Fig. 1), and cloned into

pKS(–) (Stratagene). Of a large number of clones hybridizing with probe A (Fig. 1) and containing a *Hind*III site (Fig. 1), which is contained in only one of the other cDNA clones, three with larger inserts were sequenced. On the basis of the poly(dA) tail lenghts and the exchange mentioned in the text, these clones were independent.

The second RACE experiment was carried out as above except that primer A comprised nucleotides 515–499 and primer B, nucleotides 415–399 (Fig. 3). Additionally, a second round of amplification was carried out using 0.05 volume of this pool with the SK primer, primer C (nucleotides 320–303, Fig. 3) and the conditions described above (PCR amplification 2). The products of this reaction were then digested with *Spe*I and *Pst*I and cloned into pKS(–). Seven clones hybridizing to probe B (Fig. 1) were sequenced and found to be independent on the basis of their poly-(dA) tail lenghts.

Prokaryotic expression

All of the expression clones represented in Fig. 4 were constructed in modified versions of pGEX-2T (Pharmacia Biosystems; Kawalleck et al. 1993a) which were kindly provided by Dr. Imre Somssich (Max-Planck-Institut für Züchtungsforschung, Köln, Germany). The template for all PCR reactions described below was the elicitor cDNA clone linearized with *Pvu*I. The inserts were generated as follows: XE, XN and XB were cloned as *Eco*RI fragments following introduction of an *Eco*RI site immediately 5′ to position 571 (Fig. 3) by PCR. The XE insert extends to the *Eco*RI site lying 25 bp downstream from nucleotide 1786. The 3′ *Eco*RI sites in XN and XB were introduced by PCR 3′ to positions 841 and 1210, respectively. NB extends from the *Not*I site at position 839 to the 3′ *Eco*RI site of XB. BD was cloned by introducing a *Kpn*I site 5′ to position 1209 and an *Eco*RI site 3′ to position 1350. DE extends from a *Kpn*I site introduced 5′ to position 1349, to the 3′ *Eco*RI site of XE. NE extends from the *Not*I site at 839 to the same 3′ *Eco*RI site. The integrity of all inserts was confirmed by sequencing.

All constructs were transformed into *Escherichia coli* strain DH5a (Life Technologies). Fresh overnight cultures were diluted 1:50–1:100 in LB broth containing 75 µg/ml ampicillin and grown at 37°C to an A_{600} of ~0.7. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 2 mM and incubation continued for a further 2–3 h. Bacteria were pelleted, frozen at −20°C and thawed. Lysis, isolation and washing of inclusion bodies was carried out as described (Sambrook et al. 1989, pp 17.38–17.39) except that 10 mM $MgCl_2$ was included in the buffer during DNase I digestion. Soluble fractions represented supernatants obtained following centrifugation of inclusion bodies isolated after this step. Inclusion bodies from 10 ml of IPTG-treated culture were resuspended in 0.5 ml NET (100 mM NaCl, 1 mM EDTA, 50 mM TRIS-HCl pH 8.0) containing 6 M guanidine chloride (NET/GuCl) and 0.1 mM phenylmethylsulphonyl fluoride. After agitation for 1 h at room temperature, insoluble material was pelleted and the protein content of the supernatant adjusted to 0.5 mg/ml with NET/GuCl. This preparation was then dialysed once against PBS (8.0 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.4) containing 0.1% SDS and twice against PBS containing 0.01% SDS. Insoluble material was removed by pelleting. Assessment of the elicitor activity of serial 10-fold dilutions of these preparations was carried out in microtitre assays with parsley protoplasts as described (Parker et al. 1991).

Protein analysis

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described (Laemmli 1970) as was Western transfer of electrophoresed proteins to nitrocellulose filters (Burnette 1981). Filters were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature and probed with a polyclonal rabbit antiserum raised against the deglycosylated, purified elicitor (Parker et al. 1991) at a 1:5000 dilution in 1% BSA/PBS overnight at 4°C. Following 3 × 5 min washes with PBS at room temperature, filters were incubated with peroxidase-conjugated goat anti-rabbit IgG antiserum (Sigma) at a 1:8000 dilution in 1% BSA/PBS for 1 h at room temperature. After washing as above, signals were visualized using 0.02% 4-chloronaphthol, 0.01% H_2O_2 in PBS containing 10% methanol.

Results

Isolation and analysis of elicitor-encoding nucleotide sequences

The previously reported purification of a 42 kDa glycoprotein elicitor from *P. megasperma* (Parker et al. 1991) provided us with material from which we were able to obtain sequence information for the N-terminus as well as several internal tryptic peptides (Table 1). On this basis, degenerate oligonucleotides were designed (Table 1) and synthesized for use as primers in PCR with fungal genomic DNA as template. The resultant 566 bp PCR product was then cloned and used to screen a cDNA library prepared from mycelial RNA. Three classes of partial cDNAs were subsequently isolated, which, on sequencing, were found to represent related genes which were distinct from one another and from the PCR product used for screening (67–93% similarity at the amino acid level in the regions sequenced). The largest of these clones (1.2 kb) contained an open reading frame (ORF) of 367 codons, which begins at the third base pair with the N-terminal amino acid sequence of the purified elicitor. It contained all of the other sequenced tryptic peptides as well as four peptides generated subsequently by endoproteinase Glu-C digestion (Table 1; Fig. 1). One discrepancy each with respect to the amino acid sequences of peptides 3 and 4 (Table 1; Fig. 3) was found, but both related to amino acids whose identities were uncertain. The predicted coding sequences of the other clones, on the other hand, contained several amino acid differences in the regions sequenced.

The protein encoded by the 1.2 kb cDNA was predicted to possess a molecular mass of 40202 and a pI of 5.03, in excellent agreement with the values determined experimentally for the purified glycoprotein (42 kDa and 5.2–5.4, respectively; Parker et al. 1991). A 109 bp 3′-untranslated region and a 16 bp poly(dA) stretch followed the large ORF.

Since the elicitor is a secreted protein (Parker et al. 1991), the N-terminal amino acid of which is glutamic acid, it must be synthesized as a larger precursor. Moreover, the mRNA detected using the coding sequence of the elicitor as a probe (probe A, Fig. 1), was found to be approximately 1.8 kb in length (Fig. 2), 0.6 kb longer than the cloned cDNA. RACE experiments (Frohman 1990) were therefore undertaken to obtain information on the 5′ end of the mRNA and, consequently, the N-terminus of the primary translation product. Using two gene-specific, nested primers and an internal *Not*I site, clones hybridizing with probe A were isolated, the in-

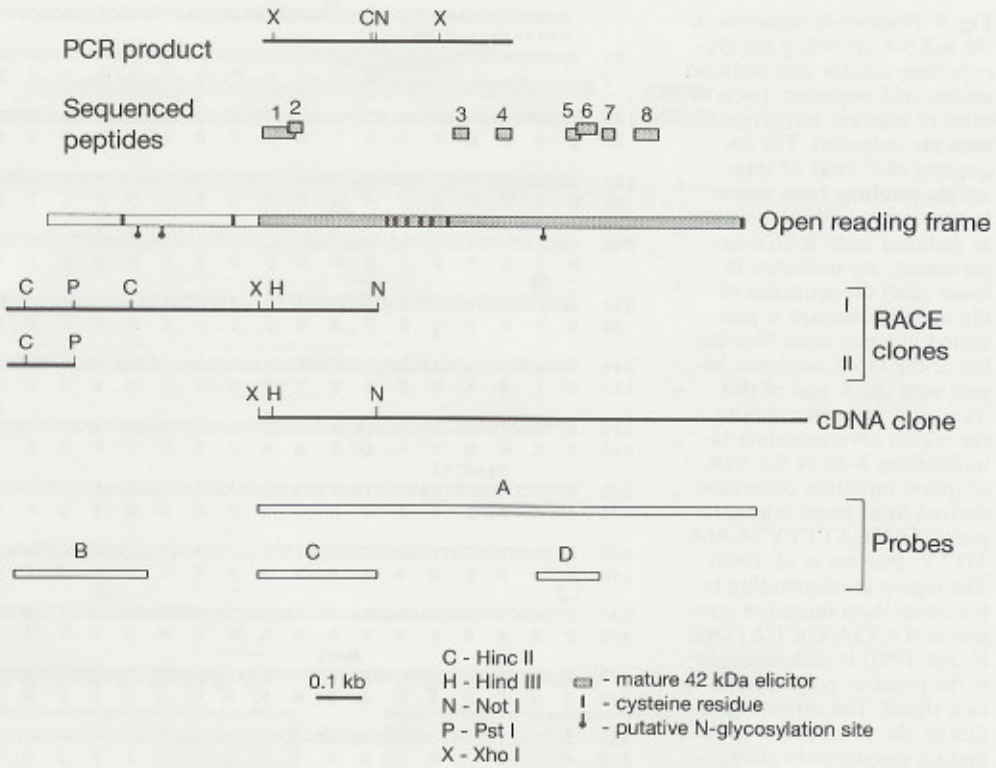

Fig. 1 Restriction map of RACE (random amplified cDNA ends) and cDNA clones encoding the glycoprotein elicitor. *Above* these maps is a schematic representation of the predicted open reading frame (ORF). Sequenced peptides derived from the purified 42 kDa glycoprotein by proteolytic digestion are *numbered* in accordance with Table 1, and their relative positions within the mature protein indicated above the ORF. On the *uppermost line* are indicated the restriction map and relative position of the cloned fragment obtained by polymerase chain reaction (PCR) with genomic fungal DNA and degenerate oligonucleotide primers, as described in the text. *Below* the map of the cDNA clone are indicated the relative locations of hybridization probes used throughout this study

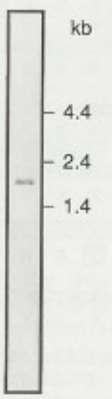

Fig. 2 Northern blot analysis of elicitor mRNA. Poly(A)$^+$ RNA (600 ng) was electrophoresed on a 1.3% formaldehyde/agarose gel, blotted onto a nylon membrane, and hybridized with digoxigenin-labelled Probe A (Fig. 1). The positions of RNA markers are indicated in kb

serts of three of which were sequenced (RACE clones I, Fig. 1). Except for a single difference in one of the clones, the three sequences were identical. All three clones displayed complete identity with the cDNA clone in the 270 bp region of overlap except for a single discrepancy at position 730. A probe derived from the 5′ portion of one of these clones (probe B, Fig. 1), however, detected an mRNA indistinguishable in size from that detected by the cDNA clone (data not shown). Likewise, genomic Southern blots probed with this fragment yielded profiles that were nearly identical in qualitative terms to those obtained with probe A (Fig. 5), confirming the authenticity of these RACE clones.

In order to verify further the sequence at the 5′ end of the mRNA, a second series of RACE experiments was conducted using independently isolated RNA from a different batch of mycelium. For these experiments, the gene-specific primers were derived from the sequence of the RACE clones described above in order to initiate reverse transcription closer to the 5′ end. Seven independent clones containing inserts hybridizing to probe B were thereby isolated and sequenced (RACE clones II, Fig. 1). The 5′ ends of five of these were found to be identical to those of the RACE clones described above. Two, however, contained an additional 4 or 5 bp at their 5′ ends (Fig. 3), probably representing the products of minor transcriptional start sites. With these exceptions, as well as a single base exchange in one of the clones, the sequences were identical to the corresponding portions of the RACE clones obtained earlier.

The entire nucleotide sequence, as deduced from the cDNA cloning and RACE experiments described above, is shown in Fig. 3. The predicted size of this transcript, including the poly(A) tail, of 1802 bp corresponds precisely to that determined on Northern blots. The first in-frame ATG occurs at position 88 with respect to the major transcriptional start site. No in-frame termination codons lie between these points. This ORF extends that encoding the mature elicitor by 162 N-terminal amino acids, predicting a primary translation product of M_r 57360. The N-terminus has the hallmarks of a signal sequence (von Heijne and Abrahmsén 1989) with an 11-amino acid stretch of hydrophobic residues beginning at position 8 and a string of small uncharged amino acids, which could serve as signal peptidase cleavage sites, at positions 26–29. As indicated in Figs. 1 and 3, the N-terminal portion of the protein is predicted to contain two N-glycosylation sites, while the mature elicitor contains one. Of particular note is a cluster of six

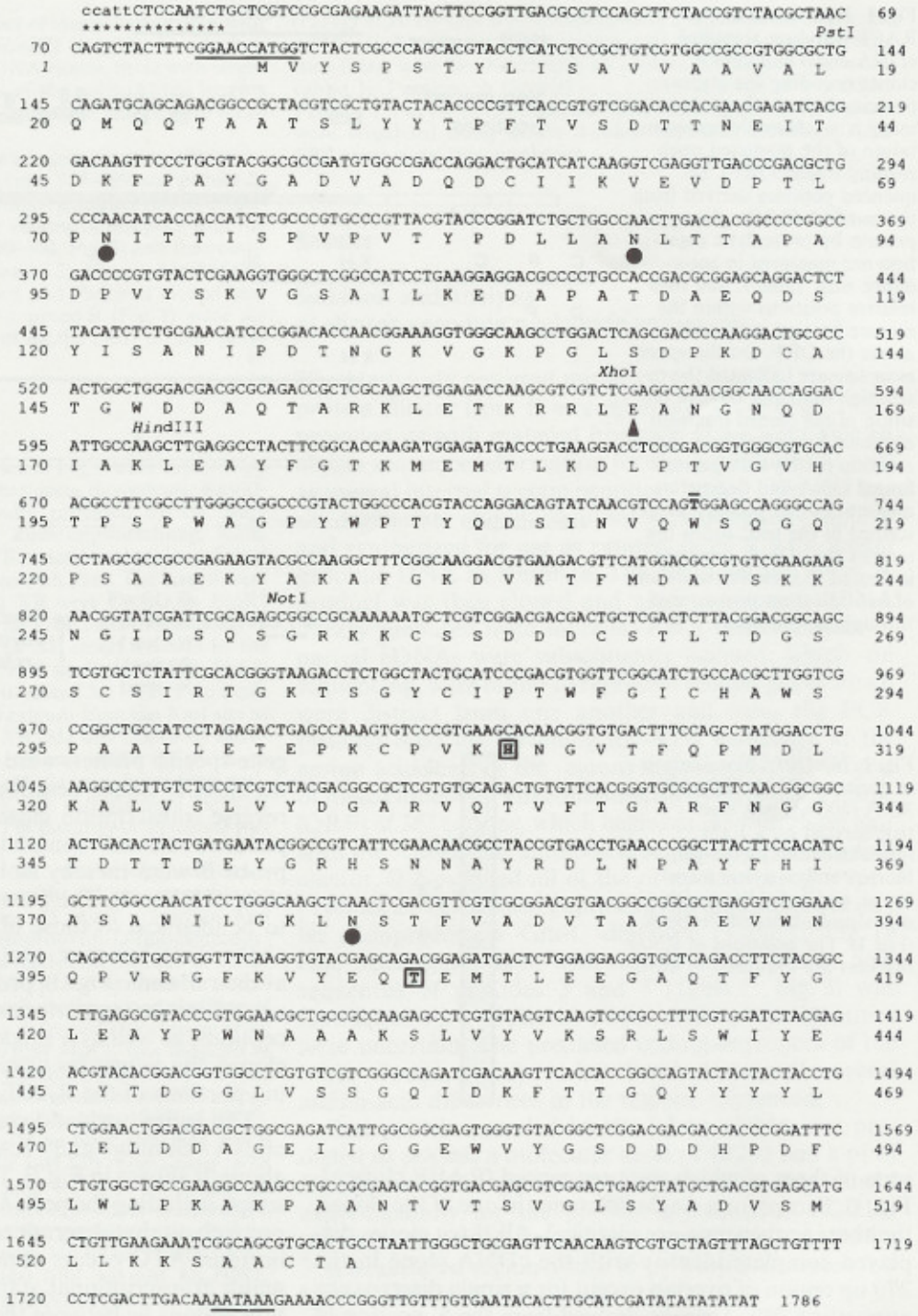

Fig. 3 Nucleotide sequence of the mRNA encoding the glycoprotein elicitor and deduced amino acid sequence. Locations of relevant restriction sites are indicated. The sequences of 5′ ends of transcripts resulting from minor transcription initiation events, as deduced from RACE experiments, are indicated in *lower case*; the sequence of the major transcript is presented in *upper case*. Numbering of the DNA sequence begins with the 5′ end of this transcript. *Asterisks* denote the region corresponding to nucleotides 3–16 of the transcription initiation consensus derived from seven oomycete genes (GCTCATTYYNCA(A/T)TTT; Pieterse et al. 1994). The region corresponding to the translation initiation consensus (GCC(A/G)CCATGG; Kozak 1991) is *underlined*, as is the putative polyadenylation signal. The *arrowhead* indicates the N-terminus of the mature glycoprotein elicitor, as determined by amino acid sequencing; *closed circles* denote predicted glycosylation sites. The *overlined* nucleotide at position 730 is the nucleotide found in the RACE clones, which differed from the G residue found in the cDNA clone at this position. *Boxed* amino acids (positions 309 and 406) are those that differed at the corresponding positions in the sequenced peptides (Table 1). The nucleotide and amino acid sequences reported here have been assigned GenBank accession number U10471

fairly regularly spaced cysteine residues (Fig. 1) lying in the central portion of the protein (amino acids 256–305, Fig. 3). Two additional cysteines lie in the N-terminal portion and one at the C-terminus. Contrary to its observed behaviour under experimental conditions, the mature elicitor is not predicted to be particularly hydrophobic, containing 30% hydrophobic amino acid residues. These are, however, asymmetrically distributed, falling predominantly in the C-terminal half of the molecule. No significant similarity of this sequence to any sequence present in the data bases has yet been found.

Prokaryotic expression of cloned elicitor cDNA

In order to localize the region(s) of the fungal protein conferring elicitor activity, studies were initiated in

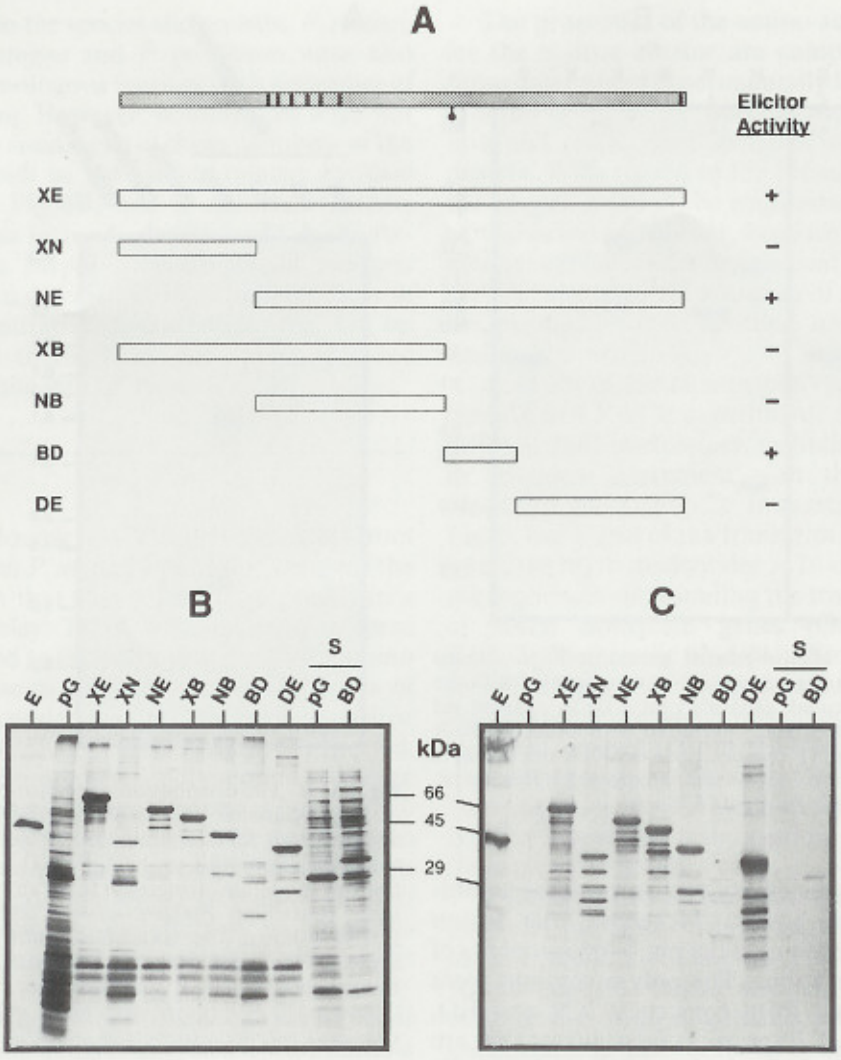

Fig. 4A Elicitor activities of portions of the *Phytophthora megasperma* elicitor expressed as fusion proteins with glutathione S-transferase in *Escherichia coli*. Beneath a representation of the ORF encoding the mature elicitor (see Fig. 1 for key) are shown the prokaryotic expression constructs with the relative positions and lengths of the portions of the mature elicitor they are predicted to encode. Fusion proteins from each construct, as well as the empty vector, were expressed at least twice and solubilized from inclusion bodies. Elicitor activity was tested by adding serial tenfold dilutions of the preparations to parsley protoplasts and measuring furanocoumarin fluorescence in the medium after 24 h. Maximal induction (100%) was obtained with crude fungal cell wall elicitor at 3 µg/ml or purified glycoprotein at 30 ng/ml. All fusion proteins designated as elicitor-active induced, on average, 100% furanocoumarin levels at total protein concentrations of 100–200 ng/ml. Those designated as inactive yielded values equivalent to buffer controls ($\leq 5\%$) at these concentrations, as did glutathione S-transferase expressed from the empty vector; at concentrations up to 10 µg/ml, values $<20\%$ were obtained. **B** Silver-stained SDS-polyacrylamide gel (15%) of fusion proteins harvested from inclusion bodies or soluble fractions (S) of the indicated bacterial lysates (1 µg total protein/lane). pG, Empty expression vector. E; pure 42 kDa glycoprotein elicitor (0.3 µg). **C** Western blot of gel identical to that shown in **B**, probed with an antiserum raised against the purified, deglycosylated elicitor (Parker et al. 1991). The positions of molecular mass markers are indicated in kDa

which the cDNA encoding the mature elicitor was overexpressed in a prokaryotic expression system. Constructs were made using a modified version of pGEX-2T (Pharmacia; Kawalleck et al. 1993a), in which proteins expressed as fusions with glutathione S-transferase at the N-terminus can then be readily purified by affinity methods. Since previous work had shown that the elicitor, when deglycosylated, was extremely insoluble (Parker et al. 1991), it was hoped that the glutathione S-transferase would also confer some degree of solubility. This was not found to be the case, as the fusion product accumulated exclusively in inclusion bodies. These could be solubilized using 6 M guanidine chloride and following dialysis, could be maintained in buffers such as PBS in the presence of 0.01% SDS. Under these conditions, the fusion protein did not bind to glutathione-Sepharose and could therefore not be easily purified. The crude, solubilized preparation, however, was found to be fully active as an elicitor of phytoalexin synthesis when added to parsley protoplasts (Fig. 4A).

A series of subfragments spanning the cDNA were cloned into this vector and similarly overexpressed. As shown in Fig. 4B, proteins of the sizes expected for all

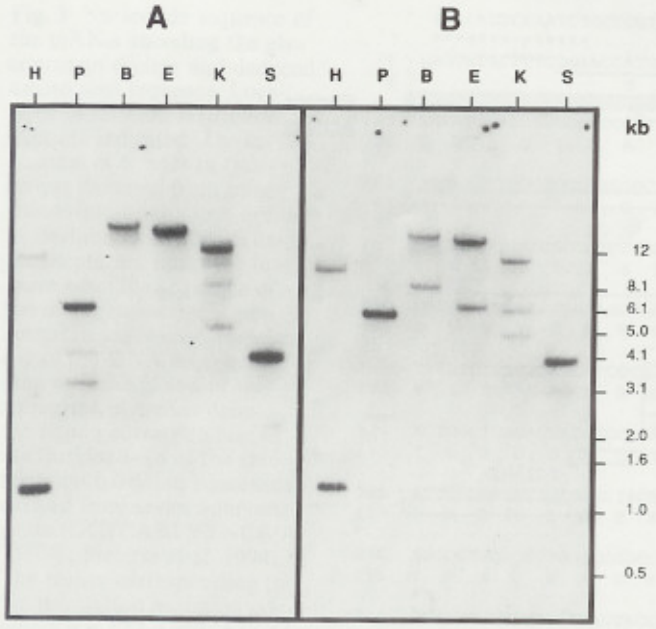

Fig. 5A, B Analysis of elicitor-related genes in *Phytophthora megasperma*. Genomic *P. megasperma* DNA was digested with the restriction enzymes indicated, separated on a 0.8% agarose gel (2 µg per lane), then blotted and hybridized with either probe A (**A**) or probe B (**B**, see Fig. 1). The positions of DNA size markers are shown in kb. Restriction enzyme abbreviations: H, *Hin*dIII; P, *Pst*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sac*I

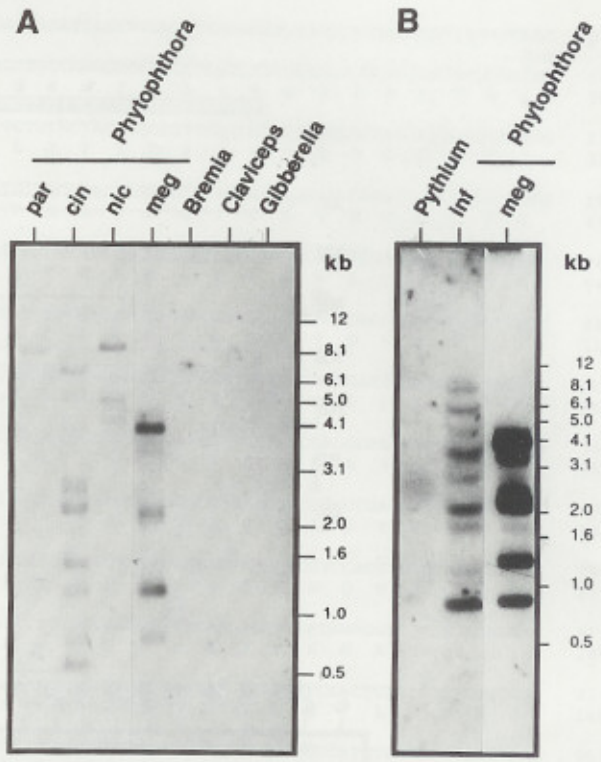

Fig. 6A, B The distribution of elicitor-related genes in a number of fungi. Genomic DNAs (2 µg) from the indicated fungi were digested with *Sac*I, separated on 0.7% (**A**) or 0.8% (**B**) gels, blotted, hybridized with probe A (Fig. 1) and washed under conditions of moderate stringency. In **B**, an overnight exposure of the lanes containing *Pythium ultimum* and *Phytophthora infestans* DNAs is shown, whereas a 3 h exposure of the *P. megasperma* lane is presented. The positions of DNA size markers are shown in kb. Fungal species: par, *P. parasitica*; cin, *P. cinnamomi*; nic, *P. nicotianae*; meg, *P. megasperma*; inf, *P. infestans*; *Bremia lactucaea, Claviceps purpurea, Gibberella pulicaris, Pythium ultimum*

constructs, including the empty vector, could be solubilized from inclusion bodies. Moreover, the fusion proteins generally represented the major components of the solubilized preparations. The only exceptions were the proteins expressed from constructs XN and BD, both representing small fragments of the elicitor that are predicted to be relatively hydrophilic. Indeed, the protein expressed from construct BD was found to accumulate predominately in the soluble fraction of bacterial lysates, as was a significant amount of glutathione S-transferase expressed from the empty vector (Fig. 4B). A Western blot of an identical gel (Fig. 4C) probed with an antiserum raised against the deglycosylated purified elicitor (Parker et al. 1991) confirmed that the fusion products contained portions of the fungal protein. The preparations, in addition, contained smaller versions of the fusion proteins that were probably generated either by proteolytic degradation or premature termination of translation. The only product that did not display significant immunoreactivity was that expressed from construct BD; this was not unexpected since the antiserum did not recognize a smaller purified peptide also derived from this portion of the mature elicitor (not shown).

All of these constructs were expressed at least twice and the expressed proteins processed and assessed for elicitor activity in microtitre assays with parsley protoplasts, as decribed in the legend to Fig. 4A. The data of Fig. 4A clearly show that only those constructs encompassing the region of the elicitor cDNA contained in construct BD, expressed proteins displaying elicitor activity. Proteins expressed from construct BD were fully active at 100–200 ng/ml when derived from solubilized inclusion bodies and at tenfold lower concentrations when prepared from the supernatant fraction of bacterial lysates; hence they are roughly as active on a molar basis as those derived from the full-length construct. These results demonstrate that the 47 elicitor-derived amino acids encoded by construct BD alone are sufficient to confer elicitor activity.

Southern blot analysis

Figure 5A shows the results obtained when genomic fungal DNA was hybridized with probe A. Consistent with the isolation of the four distinct cDNA and PCR clones described above, the gene encoding the elicitor was found to be a member of a gene family. Longer exposures of this and similar blots revealed as many as seven hybridizing bands of varying intensities (not shown). As shown in Fig. 6, the presence of this gene family seems to be a general property of *Phytophthora*

species. In addition to the species shown here, *P. capsici*, *P. cactorum*, *P. cryptogea* and *P. palmivora* were also found to possess homologous families with a number of members (not shown). However, homologues were not detected under these conditions in other members of the Oomycete family, such as the closely related *Pythium* species (*P. ultimum*, Fig. 6B; and *P. aphernidermatum*, not shown) or *Bremia lactucae*, and also not in the Ascomycetes *Claviceps purpurea* or *Gibberella pulicaris* (Fig. 6A). The pattern detected in Fig. 6 differed little in qualitative or quantitative terms when the 157 bp probe D (equivalent to the insert in expression clone BD) was used as probe (not shown).

Discussion

We report here the cloning of nucleotide sequences from the soybean pathogen *P. megasperma*, which encode the 42 kDa glycoprotein that elicits defense responses in a non-host plant, parsley. The ORF contained in these sequences is predicted to encode a protein of 529 amino acids with a pI value of 4.6 and a molecular mass of 57 kDa in the unglycosylated state. The purified elicitor is a secreted protein (Parker et al. 1991) and, as expected, the N-terminus of the predicted polypeptide sequence fulfils many of the criteria of signal peptides (von Heijne and Abrahmsén 1989) within the first 29 residues. On the basis of peptide sequencing, the mature glycoprotein is predicted to begin at amino acid 163, implying that one or more additional proteolytic processing events must take place to generate the protein purified from culture filtrate.

Multi-step processing of elicitors is known to occur in the interaction of *Cladosporium fulvum* with its host plant, tomato. The race-specific elicitor encoded by the fungal avirulence gene, *avr*9, is initially secreted into the extracellular space of its host as a preprotein that is further processed by plant proteases to yield the stable peptide elicitor (van den Ackerveken et al. 1993b), as also appears to be the case for the products of the *avr*4 gene and two putative pathogenicity genes from the same fungus (Joosten et al. 1994; van den Ackerveken et al. 1993a). In the case of the *P. megasperma* elicitor, however, a fungal enzyme(s) must be involved since the processed protein is isolated from the medium of fungus cultured in vitro. Alternatively, the 42 kDa glycoprotein may represent a particularly stable proteolytic degradation product generated as an artefact of in vitro culture. On the one hand, however, in addition to being found in the culture medium, the 42 kDa protein was found in large amounts tightly associated with fungal cell wall preparations (Parker et al. 1991). On the other hand, several of these wall preparations (Parker et al. 1991) contained a minor band of the mobility expected for the precursor form of the protein, which was also recognized by the anti-elicitor antiserum. Which of these forms predominates during the normal life cycle of the fungus, however, remains unknown.

The properties of the amino acid sequence predicted for the mature elicitor are completely consistent with those determined experimentally for the purified protein in terms of molecular mass, isoelectric point, glycosylation and amino acid sequence of 27% of the mature protein. With regard to the presence of related genes in the fungus, it should be emphasized that those portions of the other genes that were isolated were all readily distinguishable at the amino acid level from the elicitor cDNA. Moreover, sequencing of peptides derived from the purified protein revealed no heterogeneity at the amino acid level.

Analysis of the elicitor cDNA by combined cDNA cloning and RACE experiments revealed a major transcript of 1802 nucleotides, including a 16 nt poly(A) tail, in complete agreement with the size estimated on Northern blots (Fig. 2). Interestingly, as indicated in Fig. 3, the 5′ end of the transcript showed high homology (12/14 nt) to nucleotides 3–16 of a consensus found in the sequences surrounding the transcriptional start sites of seven oomycete genes (GCTCATTYYNCA(A/T)TTT; Pieterse et al. 1994). Single or multiple transcriptional start sites were found to be located throughout this sequence which was invariably positioned within 100 nt upstream from the translational start sites, as also appears to be the case for the elicitor transcript. This sequence motif, therefore, appears to represent an oomycete-specific transcription initiation consensus (Pieterse et al. 1994).

It should be noted here that the initiation site, particularly of the major transcript, should be taken with a certain amount of caution. The RACE procedure involved poly(dA) tailing after cDNA synthesis, permitting the use of an oligonucleotide containing $(dT)_{17}$ as the initial 5′ primer for PCR. In the RACE clones, therefore, it is impossible to distinguish between T residues contributed by the 5′ end of the cDNA and those contributed by the tailing and subsequent amplification. The major transcript could therefore initiate at either of the two T residues immediately 5′ to the C denoted as position 1 in Fig. 3. Additional features of the nucleotide sequence include good conservation of the consensus sequence surrounding the translation initiation codon (5/7 nt) including the critical purine at position −3 and G at position +4 (Kozak 1991). A putative polyadenylation signal (AATAAA) lies 55 nt upstream from the polyA tail.

The results of the prokaryotic expression studies described here confirm and extend the results obtained previously with the purified fungal glycoprotein (Parker et al. 1991). Consistent with the finding that elicitor activity is insensitive to deglycosylation treatments, the protein expressed in *E. coli* was active as an elicitor. Elicitor activity has also been shown to be heat-stable, suggesting that integrity of three-dimensional structure was not important for this activity. The finding that the protein retained elicitor activity, as described here, in a fusion with glutathione S-transferase and following solubilization from inclusion bodies using guanidine chlo-

ride, also supports this conclusion. Localization of elicitor activity to a 47-amino acid stretch in the C-terminal half of the molecule extends this observation and, additionally, provides an explanation for the potent elicitor activity of the crude fungal wall preparation in which no discrete protein species were detectable on SDS gels (Parker et al. 1991). In view of the likelihood that such a fungal protein would encounter plant degradative enzymes before interacting with the plant plasma membrane, this result is not surprising. Indeed, most of the other well characterized, heat-stable fungal elicitors are small macromolecules, such as peptides or small proteins, carbohydrate fragments or glycopeptides (reviewed in Ebel and Scheel 1992). A detailed examination of the requirements for elicitor activity in our system is currently in progress.

The genomic Southern blot shown in Fig. 5 demonstrated that the gene encoding the *P. megasperma* elicitor is part of a gene family consisting of itself and several other related, but distinctly different genes, consistent with results obtained in PCR and cDNA cloning experiments. The presence of this gene family appeared to be a property common to *Phytophthora* species (Fig. 6), although the size of the family was variable, ranging from approximately 6 (*P. parasitica*, visible on longer exposures) to possibly 12 (*P. cinnamomi*) hybridizing *Sac*I bands. Similar results were also obtained when fragment D (Fig. 1) encoding the elicitor-active portion of the protein was used as probe (not shown). Moreover, *P. nicotianae* and *P. parasitica* were previously found to express proteins that cross-reacted with anti-elicitor antiserum (Parker et al. 1991). The proteins were slightly smaller in apparent molecular mass than the *P. megasperma* elicitor but were also found in culture filtrates and mycelial wall preparations. Although no such protein was detected in similar preparations from *P. infestans*, approximately 9 bands were, in fact, detected with probe A (Fig. 6B).

A very similar profile was recently reported for a gene from *P. parasitica* encoding an elicitor of the hypersensitive response in a number of plant species (Kamoun et al. 1993). Gene families ranging in size from 2–10 members were detected in all eight *Phytophthora* species tested. In contrast, fungal genes encoding cultivar-specific elicitors have thus far proven to be single-copy genes (Joosten et al. 1994; van Kan et al. 1991; W. Knogge, personal communication).

Under the conditions used for the Southern blotting experiments described here (medium stringency washes), homologous genes were not detected in the other Oomycetes tested. These consisted of two members of the closely related genus, *Pythium*, which like *Phytophthora* is a member of the Pythiaceae family, as well as *Bremia lactucae*, which belongs to the Peronosporaceae family. Not surprisingly, then, homologues were not detected in two Ascomycete phytopathogens, nor were immunologically cross-reactive proteins detected in four additional non-Oomycete pathogens (Parker et al. 1991).

The function of the *P. megasperma* elicitor in the fungus remains unknown. The amino acid sequence predicted from the RACE and cDNA clones displayed no significant similarity to any sequence present in the databases. Its constitutive expression during culture in vitro, the presence of multiple gene homologues and localization in the mycelial wall may be indicative of an important function in saprophytic growth. This is further supported by the detection of the protein by immunohistochemical methods exclusively in the cell wall of hyphae growing within parsley leaf tissue (E. Schmelzer, personal communication). The proposed function, however, must distinguish the *Phytophthora* species from the *Pythium* species to which they are extremely similar in terms of morphology, life cycle, disease route and symptoms produced (Agrios 1988). One difference lies in host range: *Pythium* species infect predominantly monocots, whereas *Phytophthora* species infect dicots (Brasier and Hansen 1992). Differences in the properties of the mycelial wall might easily be imagined that would contribute to host-range determination, just as such a component of the wall would be an appropriate signal to the plant to initiate the defense response.

However, neither this glycoprotein nor smaller portions derived from it serve as general pathogen recognition signals in plants: proteinaceous cell wall and culture filtrate constituents from *P. megasperma* are inactive as elicitors in its host plant, soybean, as well as in potato and Arabidopsis (Parker et al. 1988; Scheel et al. 1989; our unpublished results). On the other hand, the response of parsley cells to this protein is specific, since neither protein fractions from *E. coli* lysates containing glutathione S-transferase (Fig. 4B) nor a mixture of protein molecular weight markers (not shown) elicit the defense response in this system. Parsley leaves, moreover, do not respond to infiltration with *E. coli* suspensions (F. Van Gijsegem, I. Somssich and D. Scheel, manuscript submitted), which initiate defense responses in other plants (Jakobek and Lindgren 1993). These findings suggest that parsley is able to discriminate between phytopathogenic and non-pathogenic microorganisms, thereby avoiding unnecessary initiation of defense reactions. The peptide fragment from *P. megasperma* that is specifically recognized by parsley cells is likely to contain sequence elements characteristic of hyphal wall proteins of *Phytophthora* species and possibly also of other phytopathogenic fungi. This feature probably accounts for the evolution of a mechanism for its perception as a signal of pathogen attack.

Acknowledgements We are grateful to Klaus Palme and Joachim Feldwisch for amino acid sequencing. Many thanks are due to Matthias Rohe, Elmon Schmelzer and Imre Somssich for critical reading of the manuscript as well as helpful discussions. The technical assistance of Magdalena Jung and Katharina Adamitza are also gratefully acknowledged. This work was supported by a postdoctoral fellowship from Bayer Chemical Company, Leverkusen, to T. N.

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Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2338–2342, March 1995
Biochemistry

Covalent cross-linking of the *Phytophthora megasperma* oligopeptide elicitor to its receptor in parsley membranes

(defense gene activation/*Petroselinum crispum*/phytoalexins/pathogen defense/signal transduction)

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Contributed by Klaus Hahlbrock, November 28, 1994

ABSTRACT **An oligopeptide elicitor from *Phytophthora megasperma* f.sp. *glycinea* (Pep-13) that induces phytoalexin accumulation in cultured parsley cells was radioiodinated and chemically cross-linked to its binding site in microsomal and plasma membrane preparations with each of three homobifunctional reagents. Analysis by SDS/PAGE and autoradiography of solubilized membrane proteins demonstrated labeling of a 91-kDa protein, regardless of which reagent was used. Cross-linking of this protein was prevented by addition of excess unlabeled Pep-13. The competitor concentration found to half-maximally reduce the intensity of the cross-linked band was 6 nM, which is in good agreement with the IC_{50} value of 4.7 nM, obtained from ligand binding assays. No cross-linking of ^{125}I-labeled Pep-13 was observed by using microsomal membranes from three other plant species, indicating species-specific occurrence of the binding site. Coupling of ^{125}I-Pep-13 to the parsley 91-kDa protein required the same structural elements within the ligand as was recently reported for binding of ^{125}I-Pep-13 to parsley microsomes, elicitor-induced stimulation of ion fluxes across the plasma membrane, the oxidative burst, the expression of defense-related genes, and phytoalexin production. These findings suggest that the 91-kDa protein identified in parsley membranes is the oligopeptide elicitor receptor mediating activation of a multicomponent defense response.**

Plants have evolved diverse defense mechanisms to protect themselves against potential fungal pathogens, including hypersensitive cell death (1), phytoalexin biosynthesis (2), expression of pathogenesis-related proteins such as chitinases or 1,3-β-glucanases (3, 4), the oxidative burst (5), and local cell wall reinforcement (6, 7). Induction of these defense responses requires recognition by the plant of either fungus- or plant-derived signals, collectively referred to as elicitors. This is believed to be mediated by receptors that specifically bind these signal molecules and thereby initiate intracellular signal transduction. The high degree of signal specificity required for the elicitor-mediated activation of plant defense responses, as observed in several systems, strongly suggests the involvement of highly specialized receptors in elicitor perception and subsequent intracellular signal generation (8).

Binding sites for highly purified carbohydrate or peptide elicitors of fungal origin have been shown to reside in the plasma membranes of soybean, tomato, and parsley cells (9–13). Ligand saturation analysis using radiolabeled elicitor preparations revealed the existence of single-class binding sites with high ligand affinities in the respective plant species (9–12). At present, however, the molecular structures of elicitor binding sites remain rather unclear. This may be due mainly to the low abundance of these sites, which has severely hampered their isolation. The first report on the identification of an elicitor binding site implicated a 70-kDa protein from soybean microsomal membranes to be a constituent of a heptaglucan elicitor receptor, as demonstrated by means of photoaffinity cross-linking (14).

We recently identified a 13-mer oligopeptide within a 42-kDa glycoprotein from *Phytophthora megasperma*, which was found to be necessary and sufficient to initiate ion fluxes across the plasma membrane, an oxidative burst, the expression of defense-related genes, and phytoalexin biosynthesis in cultured parsley cells. The structural specificity of the oligopeptide elicitor for the induction of these plant responses was identical to that found for binding of the radiolabeled elicitor to parsley membranes, indicating a functional link between signal perception and plant reactions (12). Here we report the identification, by chemical cross-linking, of a 91-kDa parsley plasma membrane protein that very likely represents the oligopeptide elicitor receptor or an integral part of it.

MATERIALS AND METHODS

Materials. Peptide synthesis was carried out by Kem-En-Tec (Copenhagen). Synthesized peptides were purified as described (12). Radioiodination of Pep-13 (specific radioactivity, 2200 Ci/mmol; 1 Ci = 37 GBq) was performed by Anawa AG (Wangen, Switzerland). Cross-linking reagents 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP), bis(sulfosuccinimidyl) suberate (BS^3), disuccinimidyl suberate (DSS), and *N*-hydroxysulfosuccinimidyl-4-azidobenzoate (S-HSAB) were from Pierce (Oud Beijerland, The Netherlands).

Plant Cell Culture/Elicitor Treatment. Cell suspension cultures of parsley (*Petroselinum crispum*) were maintained as described (15). Protoplasts prepared from 5-day-old cultured cells were treated with various peptide elicitors (12) and elicitor activity was quantified by fluorescence spectroscopy (16). *Glycine max* cell suspension cultures were propagated as described (17). *Arabidopsis thaliana* cell suspensions were initiated from surface-sterilized seeds and maintained on Murashige and Skoog medium (18) in continuous darkness. *Daucus carota* calli were initiated from root tissue and cultivated on agar plates containing B5 medium (19) and 1 mg of 2,4-dichlorophenoxyacetic acid per liter.

Microsomal and Plasma Membrane Preparation. Parsley microsomal membranes were prepared as described (12) except that microsomal pellets were subsequently resuspended in 50 mM sodium phosphate, pH 8.0/0.15 M NaCl (PBS) instead of Mes/Tris. Microsomal membranes from other plant sources were obtained by following the same protocol with 15 g of 6-day-old *Arabidopsis* cells, 11 g of 6-day-old soybean cells, and 11 g of carrot calli as starting material. For preparation of parsley plasma membranes, 6-day-old cells were harvested by filtration and frozen in liquid nitrogen. Frozen cells (150 g) were ground with quartz sand and the powder was resuspended

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Abbreviations: DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); BS^3, bis(sulfosuccinimidyl) suberate; DSS, disuccinimidyl suberate; S-HSAB, *N*-hydroxysulfosuccinimidyl-4-azidobenzoate.

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in 275 ml of 50 mM Hepes·KOH (pH 7.5) containing 0.5 M sucrose, 5 mM ascorbic acid, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.6% polyvinylpyrrolidone. The homogenate was filtered through a 240-μm nylon cloth and centrifuged at 4°C for 15 min at 10,000 × *g*; the supernatant was centrifuged at 4°C for 30 min at 50,000 × *g*. Plasma membrane vesicles were enriched from the microsomal fraction by two-phase partitioning (20). The final upper phase (10 ml) was diluted to a total vol of 210 ml, and plasma membranes were obtained after ultracentrifugation at 4°C for 60 min at 100,000 × *g*. The pellets were resuspended in ligand binding buffer (12).

Ligand Binding Assays. Ligand binding assays were performed in PBS (pH 8.0) containing 0.1% bovine serum albumin and 100 μM leupeptin under the conditions described (12). Competition studies were carried out in the presence of increasing amounts of unlabeled competitors.

Chemical Cross-Linking. Ligand association to either microsomal (400 μg of protein) or plasma (100 μg of protein) membranes was initiated by addition of 2.5 nM ^{125}I-labeled Pep-13 under the same conditions as described for ligand binding assays. Samples were kept on ice for 2 h; microsomes were pelleted by centrifugation (90 sec at 10,000 × *g*), washed with 1 ml of PBS (pH 8.0), and made up to the original volume (120 μl) with PBS (pH 8.0) containing 8 mM cross-linking reagent DTSSP, BS^3, DSS, or S-HSAB. When ^{125}I-Pep-13 was cross-linked to parsley plasma membranes, DTSSP was added directly to the ligand binding mixture. All cross-linking reactions were allowed to proceed for 30 min and were terminated by the addition of 100 mM Tris·HCl (pH 8.0) except when S-HSAB was used. Reaction mixtures containing S-HSAB were placed on ice and irradiated for 30 min with UV light (254 nm) at a distance of 3 cm from the light source. Cross-linked proteins were solubilized (15 min in 62.5 mM Tris·HCl, pH 6.8/2% SDS), aliquots were subjected to SDS/PAGE under either reducing (5 min at 95°C in 5% 2-mercaptoethanol) or nonreducing (5 min at 95°C) conditions, and gels were dried and subsequently analyzed by autoradiography. Laser densitometry was performed on the autoradiograms.

***N*-Glycosidase F Treatment.** Parsley microsomal membranes covalently labeled with ^{125}I-Pep-13 were incubated with *N*-glycosidase F (2 units; Boehringer Mannheim) in 50 mM sodium phosphate, pH 7.5/25 mM EDTA for 12 h at 37°C. Aliquots of the digest were analyzed by SDS/PAGE as described above.

RESULTS

The cleavable, homobifunctional cross-linking reagent DTSSP was used to identify the binding site(s) of the oligopeptide elicitor Pep-13 in parsley membrane preparations. Microsomal membranes were preincubated with ^{125}I-Pep-13 in the presence or absence of excess unlabeled Pep-13, and bound ligand was separated from free ligand by centrifugation prior to addition of the cross-linker. ^{125}I-Pep-13 was found to be as active as the unmodified Pep-13 in inducing furanocoumarin biosynthesis in parsley protoplasts. Cross-linked proteins were solubilized, subjected to SDS/PAGE, and subsequently analyzed by autoradiography. As shown in Fig. 1, DTSSP treatment resulted in the appearance of one major band corresponding to a ^{125}I-Pep-13–protein complex with an apparent molecular mass of 93 kDa, which was absent when 10 μM unlabeled Pep-13 was included in the assay. This 93-kDa complex could be visualized as well in parsley plasma membranes (Fig. 1) or protoplasts (data not shown).

When separation of free from bound label was omitted from the protocol (cross-linker added directly to the binding reaction mixture), the same protein band was found to be specifically cross-linked to the radioligand (Fig. 1). Cross-linking with ^{125}I-Pep-13 of two other bands with molecular masses of

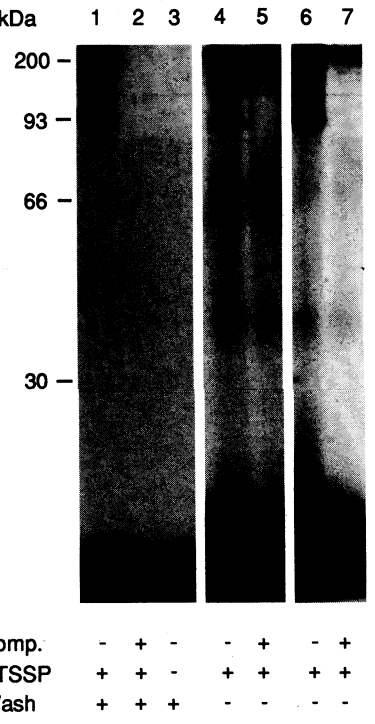

FIG. 1. Chemical cross-linking of ^{125}I-Pep-13 to parsley membranes with DTSSP. Microsomal membranes (lanes 1–5) or plasma membranes (lanes 6 and 7) were subjected to chemical cross-linking in the absence (−) or presence (+) of DTSSP and a 4000-fold molar excess of unlabeled Pep-13 as competitor (Comp.). When separation of free from bound label (Wash) was omitted from the protocol (lanes 4–7), DTSSP was added directly to the binding reaction mixture. Solubilized membrane proteins were separated by SDS/PAGE and analyzed by autoradiography. Sizes of molecular mass standard proteins are indicated on the left.

67 and 35 kDa, respectively, was not blocked by competition with a large excess of unlabeled Pep-13 and, in the first case, appeared to be attributable to bovine serum albumin used in the assay.

The actual amount of ^{125}I-Pep-13 covalently cross-linked was 1–2% of the specifically bound radioligand, as determined by measuring radioactivity in gel slices containing the 93-kDa band. Labeling of this protein was not observed when (*i*) DTSSP was omitted from the assay mixture (Fig. 1); (*ii*) the reagent quench, Tris (100 mM), was added together with DTSSP; or (*iii*) DTSSP-treated membranes were incubated with 2-mercaptoethanol (20 min at 95°C), which reduced the disulfide bond within the cross-linker. Incorporation of ^{125}I-Pep-13 into the 93-kDa band was rapid, approaching a maximum after 10 min, and found to be half-maximal at a reagent concentration of 0.8 mM. Cross-linking reagent concentrations higher than 8 mM were required to obtain maximal incorporation of the radioligand.

The capacity of two other homobifunctional reagents, BS^3 and DSS, as well as the heterobifunctional photoaffinity reagent S-HSAB to mediate cross-linking of ^{125}I-Pep-13 to parsley membranes was assessed. While S-HSAB, which has a significantly shorter chain length than all other reagents used, was virtually ineffective, BS^3 and DSS were highly effective in mediating the formation of the 93-kDa band, as revealed by SDS/PAGE under nonreducing as well as reducing conditions (Fig. 2). This suggests that the binding site does not contain interchain disulfide bonds. Assuming that the Pep-13 molecule binds to the receptor molecule at a 1:1 ratio, the molecular mass of the Pep-13 binding site was calculated to be 91 kDa. In addition, a second, much weaker band with an apparent molecular mass of 135 kDa was detected under both electro-

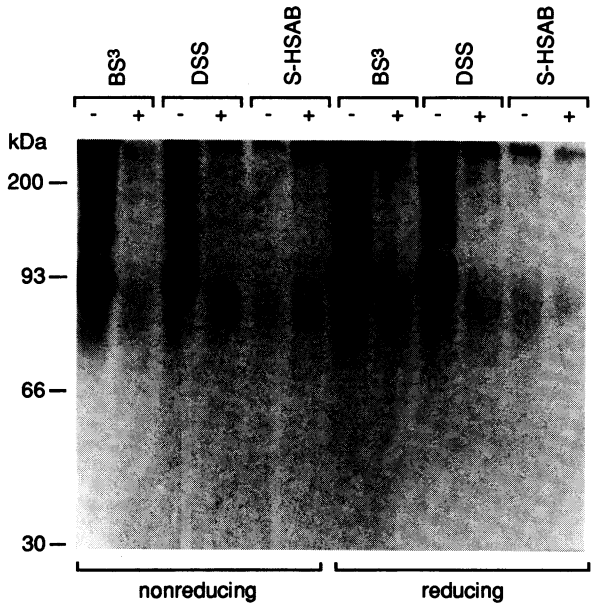

FIG. 2. Detection of the 91-kDa protein by use of different cross-linking reagents. ^{125}I-Pep-13 was cross-linked to parsley microsomes with 8 mM each BS^3, DSS, and S-HSAB in the absence (−) or presence (+) of excess unlabeled Pep-13 as described in the legend to Fig. 1. Solubilized membrane proteins were analyzed on SDS/polyacrylamide gel under reducing as well as nonreducing conditions.

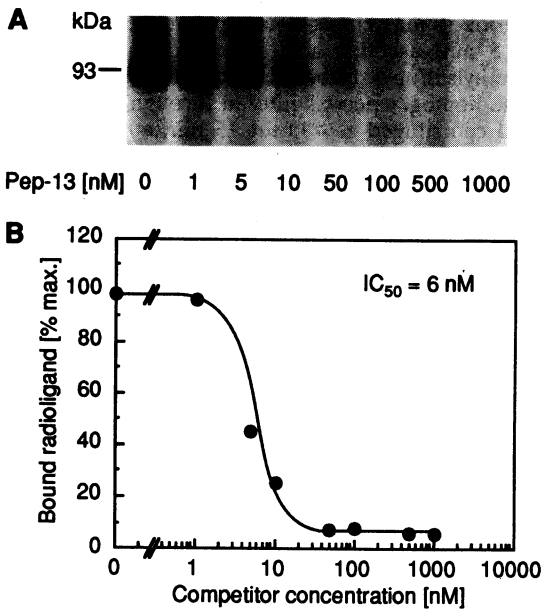

FIG. 3. Competition of ^{125}I-Pep-13 cross-linking to parsley microsomes with unlabeled Pep-13. (*A*) Autoradiogram of the 93-kDa complex as visualized after DTSSP-mediated cross-linking of ^{125}I-Pep-13 to parsley microsomes in the presence of increasing amounts of unlabeled Pep-13. (*B*) Intensity of the radiolabeled protein band was analyzed by laser densitometry of the autoradiogram. Integrated peak areas were plotted vs. competitor concentrations and a curve fit was performed by computerized nonlinear regression analysis.

phoresis conditions. Further cross-linked proteins could not be observed.

The BS^3 cross-linked Pep-13 receptor was incubated with *N*-glycosidase F for 12 h at 37°C after solubilization of the membranes with *N*-octyl β-glucoside and SDS. This treatment did not result in a reduction of the apparent molecular mass of the 91-kDa protein, suggesting that the receptor did not bear sizable carbohydrate moieties linked to asparagine through an N-glycosidic bond.

Attempts to detect specific, high-affinity binding of ^{125}I-Pep-13 to microsomal membrane preparations from another umbellifer, carrot, or from soybean and *Arabidopsis* in ligand binding assays, as well as to cross-link ^{125}I-Pep-13 to these membranes, were unsuccessful, indicating species specificity of the elicitor binding site.

To further verify the specificity of the interaction of ^{125}I-Pep-13 with its binding site, parsley microsomal membranes were incubated with the radioligand in the presence of increasing concentrations of unlabeled Pep-13. Cross-linking with DTSSP revealed a dose-dependent reduction in the label intensity of the 91-kDa protein (Fig. 3*A*). The autoradiogram was scanned by laser densitometry, and the peak areas were plotted as a function of Pep-13 concentration (Fig. 3*B*). The ligand concentration required to half-maximally displace ^{125}I-Pep-13 from the binding site was 6 nM, which is in good agreement with the IC_{50} value observed in standard ligand binding assays without cross-linker (4.7 nM; Table 1).

The ability of peptides structurally related to Pep-13 to inhibit ^{125}I-Pep-13 cross-linking to parsley microsomal membranes was evaluated. A 40-fold and a 4000-fold molar excess of the individual peptides listed in Table 1 was added concomitantly with the radioligand to the assay mixture, and the samples were processed as described above. Results from a representative experiment are shown in Fig. 4. A deletion derivative of Pep-13 lacking both the N-terminal valine (Val-1) and the C-terminal glutamic acid (Glu-13) residues retained full competitor activity as was also found for a substitution analog in which the tyrosine residue at position 12 (Tyr-12) was replaced by alanine. In contrast, either additional deletion of Trp-2 from the otherwise active 11-mer peptide analog or replacement of Trp-2 or Pro-5 by alanine in the native Pep-13 severely decreased the ability of these derivatives to compete for cross-linking of ^{125}I-Pep-13 to parsley microsomal membranes. These findings closely correspond to the IC_{50} values (Table 1) obtained in ligand binding assays using increasing concentrations of each analog (Fig. 5). In addition, Pep-13 derivatives, which were poor competitors of cross-linking of ^{125}I-Pep-13 to parsley microsomal membranes, were weak

Table 1. Structure–activity relationships of Pep-13 derivatives

Peptide sequence	Competitor activity (IC_{50}), nM	Elicitor activity (EC_{50}), nM
VWNQPVRGFKVYE (Pep-13)	4.7	31
WNQPVRGFKVY (Pep-11)	19	41
NQPVRGFKVY (Pep-10)	33,000	33,000
V**A**NQPVRGFKVYE (Pep-13/A2)	7,000	7,000
VWNQ**A**VRGFKVYE (Pep-13/A5)	240	1,900
VWNQPVRGFKV**A**E (Pep-13/A12)	6.3	50

IC_{50} values represent concentrations of peptides required to half-maximally inhibit binding of ^{125}I-Pep-13 to parsley microsomes as determined in the ligand binding assays shown in Fig. 5. EC_{50} values refer to peptide concentrations required to yield 50% stimulation of phytoalexin production in parsley protoplasts. Underlined boldface letters represent alanine substitution sites within Pep-13.

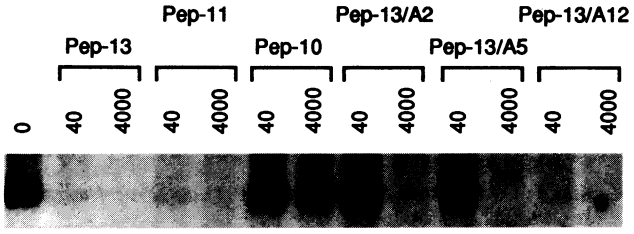

FIG. 4. Ligand specificity of ^{125}I-Pep-13 cross-linking to parsley microsomes. Autoradiogram of the 93-kDa complex as obtained after DTSSP-mediated cross-linking performed in the presence of a 40-fold or 4000-fold molar excess of Pep-13 structural analogs. Peptide sequences of the various Pep-13 derivatives and their acronyms are shown in Table 1.

elicitors of phytoalexin production in parsley protoplasts as well (Table 1).

DISCUSSION

Chemical cross-linking of radiolabeled peptide ligands to their binding sites has proven to be widely applicable for identification of a variety of receptors in different systems, including receptors for insulin, angiotensin, tumor necrosis factor, and interferon γ (21–24). In contrast to most photoaffinity labeling procedures that have been preferentially used to identify ligand binding sites in plants, this labeling technique does not require derivatization of the peptide. Furthermore, chemical cross-linking often results in less nonspecific labeling in comparison to photoaffinity techniques, most likely due to the need for appropriate spacing and chemical accessibility of reactive groups on both receptor and ligand.

We used three different homobifunctional cross-linking reagents to covalently attach ^{125}I-Pep-13 to its binding site in parsley microsomal and plasma membrane preparations. Each of these reagents recognized a predominant protein of 91 kDa, which apparently does not bear N-linked glycans. The proteinaceous nature of the binding site was established by the demonstration that trypsin pretreatment of parsley microsomal membranes completely abolished binding of ^{125}I-Pep-13 (12) and cross-linking to the 91-kDa band (data not shown). The apparent size of this protein was unaltered by the reducing agent 2-mercaptoethanol, suggesting a lack of disulfide bonds to other protein subunits. This finding makes it tempting to speculate on a homo(oligo)meric structure of the Pep-13 binding site. With two of the reagents used, however, an additional protein of ≈135 kDa was labeled with the same specificity (data not shown) as the 91-kDa protein. Whether

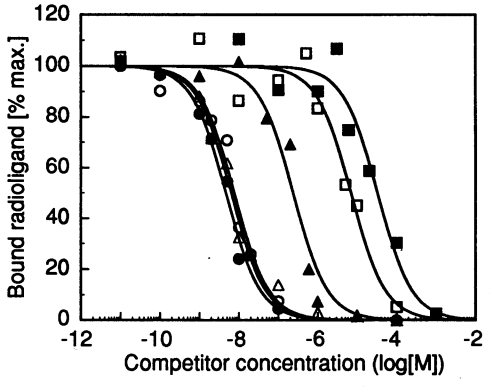

FIG. 5. Competitive inhibition of ^{125}I-Pep-13 binding to parsley microsomes with Pep-13 structural analogs. The IC_{50} values of the respective competitors were calculated by computerized nonlinear regression analysis and are given in Table 1. ●, Pep-13; ○, Pep-11; ■, Pep-10; □, Pep-13/A2; ▲, Pep-13/A5; △, Pep-13/A12.

this still reflects a heteromeric subunit structure of the binding site rather than reaction of the protruding cross-linker with neighboring but functionally unrelated membrane proteins cannot yet be conclusively answered.

Four lines of evidence indicate that the 91-kDa protein and the oligopeptide elicitor binding site characterized in ligand binding assays (12) represent identical molecules. (*i*) Labeling of a single predominant band is consistent with the single-class binding site for Pep-13 found in parsley membranes (12). (*ii*) The 91-kDa protein is a constituent of the plasma membrane of parsley protoplasts as is the Pep-13 binding site, which was demonstrated by means of silver-enhanced ImmunoGold labeling (13) and ligand binding assays (12). (*iii*) Covalent coupling of ^{125}I-Pep-13 to the 91-kDa protein is inhibited by the same concentrations of native Pep-13 that are effective in competing for binding of the radioligand to its receptor (Fig. 4). (*iv*) The ability of Pep-13 structural derivatives to compete for binding of ^{125}I-Pep-13 to parsley microsomes parallels their effectiveness in inhibiting cross-linking of the radioligand to the 91-kDa protein. This is relevant to a role of this protein as (part of) the oligopeptide elicitor receptor. Moreover, cross-linking of ^{125}I-Pep-13 was not only highly specific with respect to structural properties of the ligand but also to plant species that recognized this ligand. Interestingly, even another umbellifer, carrot, does not possess a protein functionally homologous to the parsley Pep-13 binding site. In contrast, the *P. megasperma* heptaglucan elicitor of phytoalexin accumulation in soybean is recognized by other legume species such as lupin and pea (E. Cosio, personal communication).

Most importantly, we have demonstrated that the same criteria apply to specific cross-linking of ^{125}I-Pep-13 to a 91-kDa parsley plasma membrane protein and, as recently reported (12, 25), to the stimulation of a multifaceted plant defense response comprising both early reactions—such as influxes of H^+ and Ca^{2+}, effluxes of K^+ and Cl^-, and the oxidative burst—and late reactions—such as ethylene biosynthesis, defense-related gene activation, and phytoalexin formation. Our data strongly support the idea that this binding site represents the oligopeptide elicitor receptor mediating the activation of defense-related genes and subsequent phytoalexin production.

Isolation of elicitor receptors by affinity chromatography or expression cloning of the respective genes in eukaryotic cells will provide further important information on the molecular structure and mode of action of these proteins. In this connection, it is expected that functional analysis of plant disease resistance genes, which are likely to encode receptors for pathogen-derived signals, will substantially contribute to our understanding of the mechanisms underlying signal perception and signal generation at the plant cell surface.

We are grateful to Drs. I. Dubery and W. Sacks for critical reading of the manuscript. The technical assistance of L. Bollenbach is gratefully acknowledged. This work was supported by Bayer AG, Leverkusen (T.N.), the European Community (D.S.; CHRX-CT93-0168), and Fonds der Chemischen Industrie (K.H.).

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Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4150–4157, May 1995
Colloquium Paper

This paper was presented at a colloquium entitled "Self-Defense by Plants: Induction and Signalling Pathways," organized by Clarence A. Ryan, Christopher J. Lamb, André T. Jagendorf, and Pappachan E. Kolattukudy, held September 15–17, 1994, by the National Academy of Sciences, in Irvine, CA.

Oligopeptide elicitor-mediated defense gene activation in cultured parsley cells

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ABSTRACT **We have used suspension-cultured parsley cells (*Petroselinum crispum*) and an oligopeptide elicitor derived from a surface glycoprotein of the phytopathogenic fungus *Phytophthora megasperma* f.sp. *glycinea* to study the signaling pathway from elicitor recognition to defense gene activation. Immediately after specific binding of the elicitor by a receptor in the plasma membrane, large and transient increases in several inorganic ion fluxes (Ca^{2+}, H^+, K^+, Cl^-) and H_2O_2 formation are the first detectable plant cell responses. These are rapidly followed by transient changes in the phosphorylation status of various proteins and by the activation of numerous defense-related genes, concomitant with the inactivation of several other, non-defense-related genes. A great diversity of cis-acting elements and trans-acting factors appears to be involved in elicitor-mediated gene regulation, similar to the apparently complex nature of the signal transduced intracellularly. With few exceptions, all individual defense responses analyzed in fungus-infected parsley leaves have been found to be closely mimicked in elicitor-treated, cultured parsley cells, thus validating the use of the elicitor/cell culture system as a valuable model system for these types of study.**

A crucial and rapidly expanding area of research concerns the chemical communication within and among organisms. This basic level of intercellular and interorganismic communication plays a critical role in determining the composition and dynamic behavior of ecosystems through such processes as the discrimination between self and nonself and the determination of symbiotic and pathogenic relationships. In functional terms, the chain of molecular events comprising these pathways can be divided into three parts: (*i*) generation and recognition of extracellular signals, (*ii*) intracellular signal conversion and/or transduction, and (*iii*) signal-specific responses of target cells.

We are studying elements of all three functionally interconnected parts of such a signal–response chain, exploiting the fact that many details of the non-host-resistance response of parsley leaves (*Petroselinum crispum*) to infection with the soybean pathogen *Phytophthora megasperma* f.sp. *glycinea* (*Pmg*) can be mimicked by treatment of suspension-cultured parsley cells with an elicitor preparation from this fungus. We have focused our interest on the following elements:

- the nature and mechanism of action of a *Pmg*-derived molecule with high elicitor activity on parsley cells;
- rapid cell membrane- and cell wall-associated changes, as well as intracellular changes, in metabolic activity; and
- the elicitor-mediated activation and inactivation of numerous defense-related and non-defense-related genes.

Prior to a discussion of some mechanistic details of the responses of cultured parsley cells to treatment with *Pmg* elicitor, the present state of knowledge of the whole-plant interaction of parsley leaves with the intact fungus will be briefly summarized.

Responses of Parsley Leaves to *Pmg* Infection

Young, primary parsley leaves were used for most of our studies (1–4), since a higher rate of infection with *Pmg* was obtained than with old leaves under the conditions used (1). Combining all of the results obtained so far with *Pmg*-infected parsley leaves, three major steps in the overall defense response can be distinguished (Fig. 1).

Following leaf inoculation with fungal zoospores, cyst formation, germination, and formation of appressoria and infection vesicles (≈4 hr postinoculation), the first microscopically visible sign of a plant defense response is hypersensitive (very rapid and highly localized) cell death. This hypersensitive response is associated with reinforcement of the affected cell wall—for example, by apposition of callose and incorporation of phenolics. The newly incorporated phenolics are readily detectable without histochemical staining by their autofluorescence under blue/UV light (1–4). Results obtained recently with a similar system, potato (*Solanum tuberosum* L.) leaves infected with *Phytophthora infestans*, indicate that the penetrating fungal infection vesicle is killed concomitantly with hypersensitive plant cell death (5). In both the parsley and potato systems, as well as in many others, hypersensitive cell death appears to be a particular early-plant-defense response initiated by those cells that are invaded by the fungus or in direct contact with fungal structures. Available evidence suggests a close correlation between the frequency of hypersensitive cell death (provided it occurs at all) and the degree of resistance (5).

The second line of defense consists of numerous rapidly accumulated enzymes, structural proteins, and metabolites, at least some of which possess antifungal activity. Among these antifungal compounds are the so-called phytoalexins, plant species- or family-specific classes of broad-range antibiotics. In parsley, the phytoalexins are a mixture of linear furanocoumarins (6–8), which are easily detectable by their blue autofluorescence under UV light. Many of these second-line defense reactions are activated transcriptionally, as demon-

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Abbreviations: *Pmg, Phytophthora megasperma*; PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate:CoA ligase; C4H, cinnamate 4-hydroxylase; PR proteins, pathogenesis-related proteins; PRH, homeodomain-containing PR protein; BPF, box P-binding factor; BIF, box I-binding factor; CPRF, common plant regulatory factors.

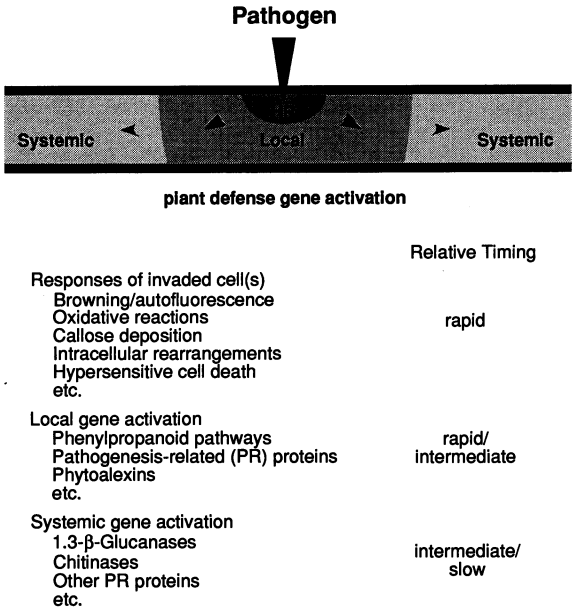

FIG. 1. Schematic representation of three major types of plant defense response to fungal infection. HR, hypersensitive response.

strated by *in situ* mRNA hybridization in *Pmg*-infected parsley leaves (3, 4, 9). The affected tissue is depicted in Fig. 1 as the area undergoing "local gene activation" and is restricted to a defined area surrounding the fungal penetration site. Relative to the small number of cells displaying the hypersensitive response, however, the number of cells exhibiting local gene activation is large.

In contrast, the third line of defense is induced systemically throughout the entire infected leaf, and often the entire organism as well, and comprises yet another set of antifungal activities. This systemic response, which in potato (10), tobacco (11), and various other plants has been shown to include the systemic activation of genes encoding several chitinase and 1,3-β-glucanase isoforms, is most likely to occur also in *Pmg*-infected parsley leaves.

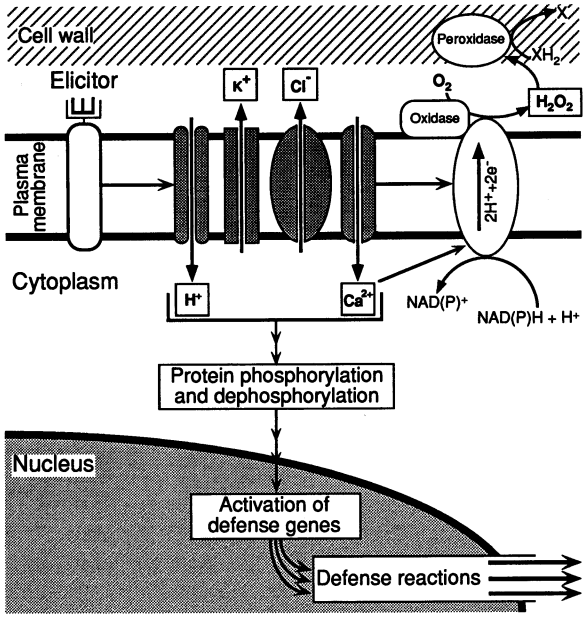

FIG. 2. Schematic illustration of major, partly hypothetical components of the signaling cascade from elicitor recognition to defense gene activation.

Systemic gene activation appears to be slow relative to hypersensitive cell death and local gene activation but gradually leads to the accumulation of large amounts of protein and enzyme activity (12). A particularly interesting aspect of this third-line systemic response is the phenomenon of "systemic acquired resistance," which renders a previously infected plant more resistant to a second infection, even by a different type of pathogen (13).

Thus, the complex, multicomponent defense response of parsley leaves to *Pmg* infection can be subdivided into three distinct stages: (*i*) hypersensitive cell death; (*ii*) concurrent but physically distinct local-defense gene activation in the surrounding tissue; and (*iii*) subsequent systemic activation of additional defense genes. It remains open at the present time whether the two successive steps of local and systemic gene activation are causally linked by sequential signaling events. In contrast, we have recently obtained evidence that cell death is not a prerequisite for concomitant local gene activation to occur (14), suggesting that a fungus-derived signal triggers local gene activation. The following results support this notion.

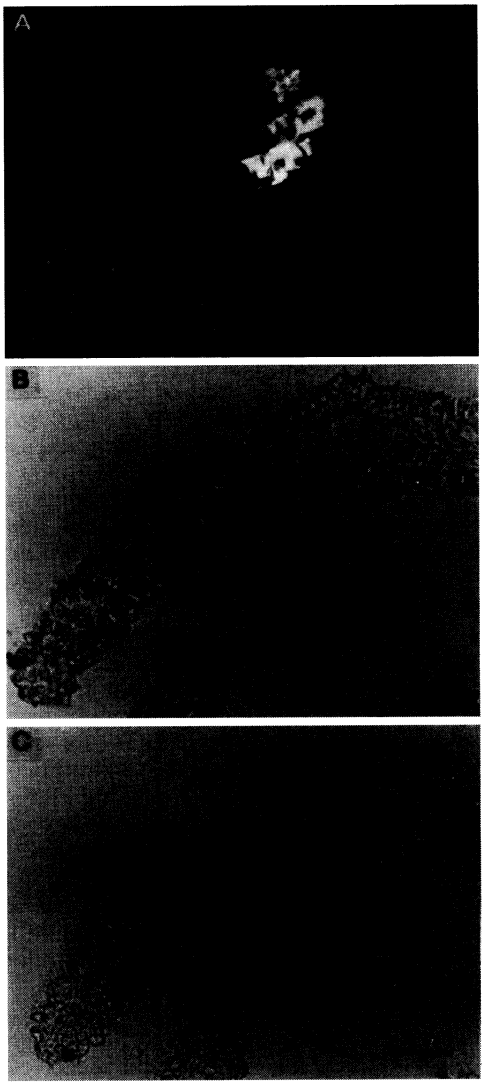

FIG. 3. Immunohistochemical localization of elicitor in a cross section of a *Pmg*-infected parsley leaf bud. Methods were the same as described (1), except that a polyclonal antiserum raised against the purified, deglycosylated elicitor protein (21) was used. (*A*) Autofluorescence under UV-epifluorescent light, indicating hypersensitive cell death. (*B*) Same section under visible light, showing indirect immunoperoxidase staining of elicitor. (*C*) Adjacent section used for the control reaction with preimmune serum. (Bar = 40 μm.)

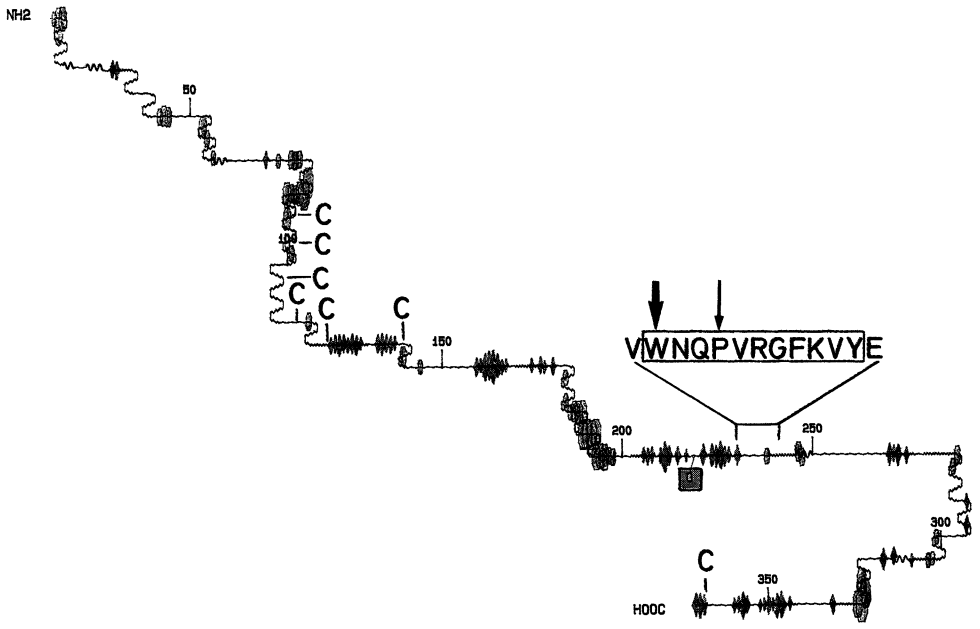

FIG. 4. Chou–Fasman prediction of the structure of the 42-kDa elicitor protein as determined by the PLOTSTRUCTURE program of the GCG (Genetics Computer Group) package (24). The amino acid sequences of the 13-meric oligopeptide elicitor and the smallest elicitor-active derivative (box) as well as their positions within the full-length protein predicted from cDNA sequence information (23) are indicated. Arrows indicate positions critical for activity as determined by alanine substitution mutation analysis (18).

The Oligopeptide Elicitor from *Pmg*: Molecular Characterization and Mode of Perception

Treatment of suspension-cultured parsley cells with a crude *Pmg* mycelial wall preparation (crude *Pmg* elicitor) induces most or all of the defense responses associated with local gene activation in *Pmg*-infected leaf tissue (7–9, 15–17). Numerous rapid and transient metabolic changes have been shown to occur in parsley cells treated with the crude *Pmg* elicitor, including inorganic ion fluxes across the plasma membrane, a release of active oxygen species referred to as the oxidative burst (18, 19), alterations in the phosphorylation status of soluble and membrane-associated proteins (20), activation of defense-related genes and inactivation of other genes (16, 21, 22), and accumulation of furanocoumarin phytoalexins (7). These responses and the more recently identified elicitor receptor (see below) are summarized schematically in Fig. 2 together with their possible causal interrelationships.

To study in detail the molecular mechanisms underlying elicitor recognition and subsequent signal transduction, efforts were undertaken to isolate homogeneous elicitor-active compounds from various fungal sources. A 42-kDa glycoprotein elicitor was purified from fungal culture filtrate whose elicitor activity was found to reside solely in the protein moiety (21). A monospecific polyclonal antiserum raised against the deglycosylated elicitor protein identified the antigen in the cell walls of a few selected *Phytophthora* species but not in several other phytopathogenic fungi (21). Moreover, use of the same antiserum has localized the elicitor *in situ* to hyphal cell walls of *Pmg* growing in or on parsley leaves (Fig. 3).

A peptide consisting of 13 amino acids was identified within the intact glycoprotein to be sufficient to stimulate the same responses as the crude *Pmg* elicitor and the glycoprotein elicitor (18). The isolation of a cDNA encoding the glycoprotein (23) allowed the localization of this oligopeptide to a hydrophilic region within the C-terminal portion of this protein as shown in Fig. 4. A comparison of the peptide sequence with sequences contained in several data bases did not reveal significant similarity to known proteins, as was also found for the glycoprotein elicitor at the amino acid and the nucleotide levels (23). Deletion of one N- and C-terminal amino acid yielded the minimum peptide with full elicitor activity (18) (boxed sequence in Fig. 4). Substitution analysis, in which each individual amino acid of this 11-meric peptide was replaced by alanine, identified two residues critical for activity (arrows in Fig. 4). All other exchanges exerted little or no effect on the elicitor activity of the oligopeptide (18).

With use of the radioiodinated oligopeptide as a ligand in binding assays, a single-class binding site with high affinity (K_d = 2.4 nM) could be detected in parsley microsomal membranes and protoplasts (18). The number of binding sites per protoplast was estimated to be ≈2900. Binding of the peptide elicitor was competable, reversible, saturable, and highly specific with respect to both structural properties of the signal required for binding and plant species that recognized the ligand. The oligopeptide binding site therefore meets a number of criteria expected of an authentic receptor. A series of peptide elicitor derivatives was tested for their ability to compete for binding of the radioligand to parsley microsomes and their effect on ion fluxes, oxidative burst, and phytoalexin formation. The findings show a functional link between the various plant responses to elicitor treatment, which in part may constitute elements of the signal transduction chain leading to defense-related gene activation and subsequent phytoalexin production in parsley.

Intracellular Signal Transduction

The most rapid responses of parsley cells to elicitor treatment detected so far are ion fluxes across the plasma membrane (18, 19). Within 2–5 min upon addition of elicitor, a transient influx of Ca^{2+} and H^+ and an efflux of K^+ and Cl^- are initiated. Immediately thereafter, the concentration of H_2O_2 in the cell culture medium increases greatly (oxidative burst), returning to background levels several hours later (18). Within 5–30 min after elicitation, phosphorylation of several proteins was observed *in vivo* (20), and run-on transcription of the most rapidly activated defense-related genes was detectable (16). Accumulation of the respective mRNAs and increases in activity of the corresponding enzymes were found to occur later but before the formation of phytoalexins (7, 16).

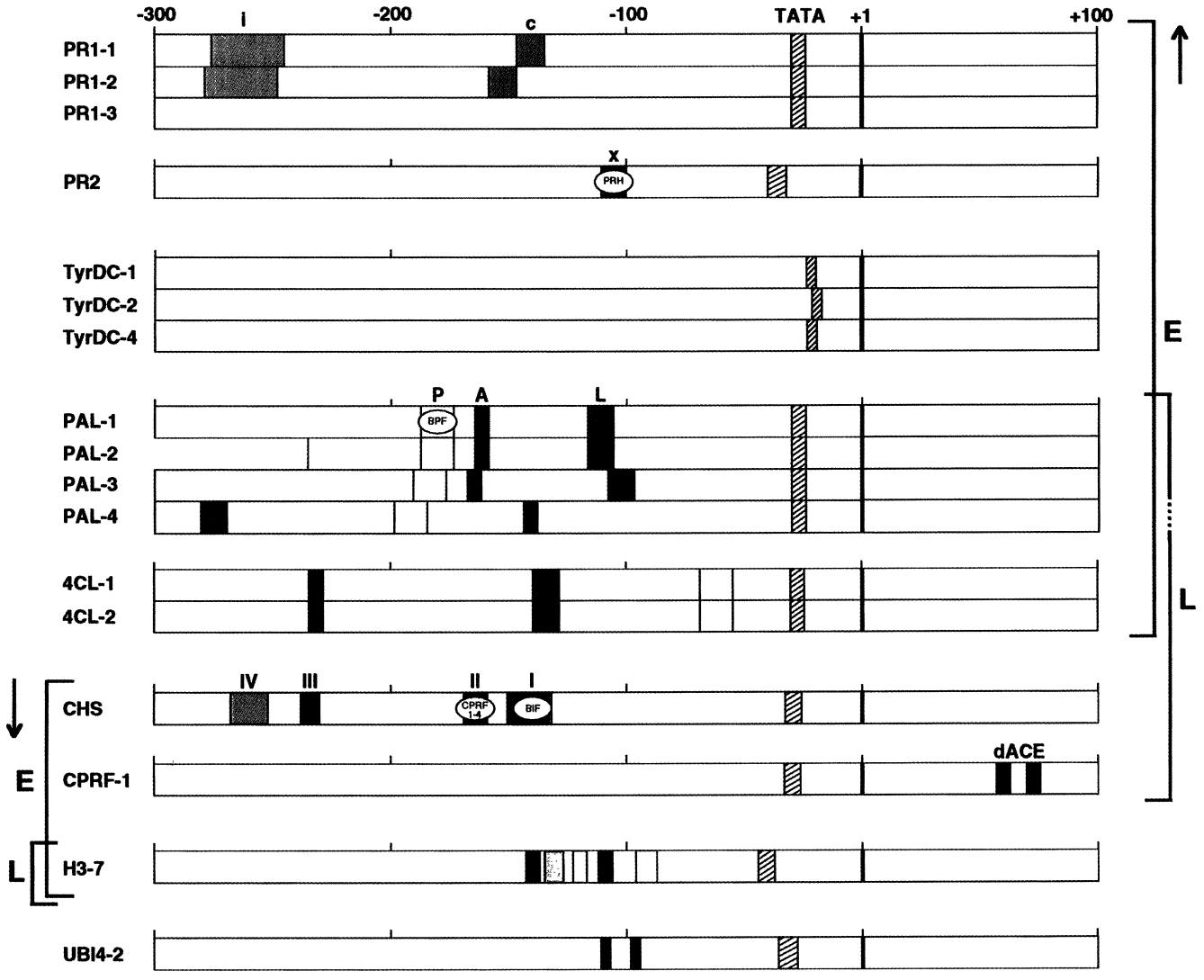

FIG. 5. Schematic representation of TATA-proximal regions of genes shown to be elicitor-responsive (E brackets) in cultured parsley cells. Positions −300 to +100 are represented, except for the PAL-2 gene, for which sequence is available only to position −242. Elicitor-activated genes are indicated by an arrow directed upwards; elicitor-repressed genes, by an arrow directed downwards. For comparison, genes responding to UV light (L brackets) and one control gene responding neither to elicitor nor to light (UBI4-2; ref. 39) are also shown. Boxes represent cis-acting elements, with sequence similarity indicated by color or shading. Letters or numbers indicate elements identified functionally. DNA-binding proteins are depicted as ovals located on the fragment used for their identification. See text for descriptions of these proteins. CHS, chalcone synthase; TyrDC, tyrosine decarboxylase; H3-7, histone H3 subclass; PRH, homeodomain-containing PR protein; BPF, box P-binding factor; BIF, box I-binding factor; dACE, duplicated ACGT-containing element.

Loss- and gain-of-function experiments demonstrated that all of these elicitor responses were strictly dependent on the presence of Ca^{2+} in the cell culture medium (19, 25, 26), were prevented by ion-channel inhibitors that suppress elicitor-mediated fluxes of one or more ions (26), and were initiated by compounds that stimulate all elicitor-induced ion fluxes in the absence of elicitor (25, 26). Therefore, transient activation of plasma membrane-located ion channels appears to be an early component of elicitor signal transduction.

While both the oxidative burst and protein phosphorylation/dephosphorylation could be located downstream of ion channel activation, their possible causal interconnections with other signaling components remain to be elucidated. Circumstantial evidence suggests that the intracellular signal transduction chain involves inositol phosphates but not GTP-binding proteins or cAMP (27).

The scheme shown in Fig. 2 includes the most clearly demonstrated components of the signal transduction chain together with their potential causal relationships. A major target of the signaling cascade is the up- or down-regulation of numerous elicitor-responsive genes.

Regulation of Elicitor-Responsive Genes

The list of genes responding to elicitor treatment of cultured parsley cells with rapid, transient activation is large. It includes the genes or gene families encoding phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL), the first and last steps of general phenylpropanoid metabolism (28, 29); tyrosine decarboxylase (TyrDC) (30); *S*-adenosyl-L-methionine synthetase (SMS) and *S*-adenosyl-L-homocysteine hydrolase (SHH), two enzymes of the activated methyl-group cycle (31); one particular isoform of chitinase (32); anionic peroxidase (POX) and a hydroxyproline-rich glycoprotein (HRGP), two cell wall-associated proteins (33); and two intracellular "pathogenesis-related" (PR) proteins, PR1 and PR2 (9, 34). In addition, several functionally unidentified, elicitor-responsive genes have been shown to behave similarly (16). The genes encoding *S*-adenosyl-L-methionine:bergaptol *O*-methyltransferase [(BMT); a specific enzyme of furanocoumarin biosynthesis (7, 35)] and another chitinase isoform (32) were activated more slowly than the other genes mentioned.

Fewer genes are known to be down-regulated by elicitor, although many such genes may exist. Among the well-established cases are the genes encoding chalcone synthase (CHS), a light-regulated enzyme of the flavonoid pathway (22); a putative transcription factor, common plant regulatory factor 1 (CPRF-1), possibly involved in light-dependent chalcone synthase gene activation (refs. 36 and 37; and A. Block, personal communication); and the histone H3 subclass H3-7 (unpublished data).

It is generally believed that the stimulus-dependent signal transduction pathway modulating gene expression involves transcription factors binding to stimulus-specific cis-acting elements. A comparison of elicitor-responsive genes for which TATA-proximal sequences are available is presented schematically in Fig. 5. Cis-acting elements identified either experimentally or by homology to previously identified elements are also presented, together with the locations of sequences shown to bind characterized DNA-binding proteins. These proteins include: PRH, binding to box X of the PR2 gene promoter (40); BPF, binding to box P of the PAL-1 gene promoter (4); CPRF-1 through CPRF-4, binding to box II of the chalcone synthase gene promoter (36, 37); and Myb-like BIF, binding to box I of the chalcone synthase gene promoter (M. Feldbrügge, M.P., K.H., and B. Weisshaar, unpublished results). It is noteworthy that each type of DNA-binding protein belongs to a different class of putative transcription factors (PRH, homeodomain-containing; BPF, novel class; CPRF, bZIP (basic/leucine zipper); BIF, Myb-like).

In parsley, there is an intimate metabolic connection between elicitor- (pathogen-) and UV light-triggered responses (see below). This is reflected in particular by a partial overlap in the involvement of cis-acting elements in elicitor- and light-mediated PAL-1 gene activation (boxes P, A, and L in elicitor responsiveness and boxes P and L in UV light responsiveness; ref. 28) and in general by the complexity of up- and down-regulatory effects exerted by these two stimuli (brackets in Fig. 5). With respect to elicitor-regulated gene activity, several major conclusions can be drawn from the results obtained so far.

- No common stimulus-specific cis-acting elements have been detected among the various elicitor- and UV light-responsive gene promoters.
- The combinations of those cis-acting elements that have been shown functionally to be involved in elicitor-mediated gene activation (boxes i, X, P, A, and L in Fig. 5) may be similar within gene families or between particularly closely interconnected gene families, such as PAL and 4CL, but generally differ greatly among gene families.
- Notable exceptions appear to be ACGT-containing elements (ACE; green boxes in Fig. 5), which occur frequently in plant gene promoters (37). These elements are present in all promoters (or functional units thereof; refs. 37 and 41) tested that are either down-regulated (CHS, CPRF-1, and H3-7 gene promoters among others) or unaffected (UBI4-2) by elicitor (ref. 22 and unpublished data).
- These ACEs and dACE versions thereof (see Fig. 5) are likely to be involved in both gene activation and gene repression, depending on the sequence context (36) and metabolic conditions (unpublished data).
- The three boxes (P, A, and L) originally identified in the PAL-1 promoter by *in vivo* DNA footprinting (28) are present in similar form in all known PAL and 4CL gene promoters from numerous plant species, strongly indicating their functional importance.

Further evidence for the functional relevance of box P was provided by the formation of specific DNA–protein complexes in mobility-shift assays with nuclear extracts from parsley cells and by cloning and characterization of one such protein, BPF-1 (4). The interaction of BPF-1 with its target sequence box P could be shown to occur *in vivo*: in cotransfection experiments, a chimeric protein containing the DNA-binding domain of BPF-1 fused to the transcriptional activation domain of viral protein VP16 (42) could specifically activate transcription from an artificial promoter containing a tetramer of box P (M.P. and K.H., unpublished results). Box L has also been found to interact with nuclear proteins. The formation of one of the DNA–protein complexes exhibits the property of being stimulus (elicitor)-inducible (Fig. 6). The same nucleotide sequence that was previously shown to display an elicitor-inducible footprint was now found to form elicitor-inducible interactions *in vitro*. Such inducibility by elicitor was observed neither for the complexes formed with box P, although the BPF-1 mRNA level in elicitor-treated parsley cells is to a large extent transcriptionally regulated (4), nor for a recently reported complex formed specifically between box X of the PR2 gene promoter (Fig. 5) and a homeodomain-containing protein, PRH (40). Further strong evidence for the functional importance of box L is provided by the observation that a tetramer of box L activates transcription when fused to a heterologous promoter (M.P. and K.H., unpublished results).

The biochemical functions of PR1, PR2, and some products of elicitor-responsive genes and the overall physiological significance of elicitor-mediated accumulation of enzymes of the general phenylpropanoid pathway [PAL, cinnamate 4-hydroxylase (C4H), and 4CL; refs. 43 and 44] have yet to be fully elucidated. Fig. 7 outlines those functional relationships that have been established to date for elicitor-stimulated genes or gene families associated directly or indirectly with phenylpropanoid metabolism.

Regulation of UV Light-Responsive Genes

The extensive overlap in the regulation of secondary metabolism by elicitor and UV light is manifest in the responsiveness

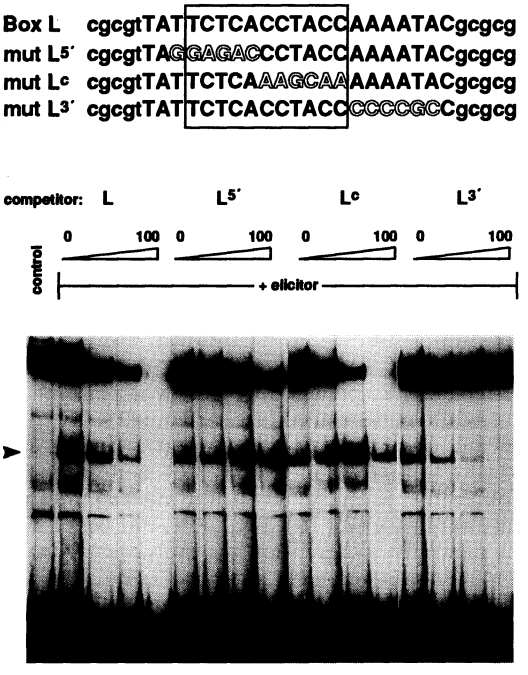

FIG. 6. Gel mobility-shift assays performed with nuclear extracts from untreated (control) or 3-hr elicitor-treated parsley cells and a box L-containing labeled probe (*Upper*, top line; box L enframed). Unlabeled competitors were either the same box L-containing probe or mutated versions (*Upper*) as indicated, used at 0-, 25-, 50- and 100-fold molar excess (*Lower*). By this criterion, the elicitor-inducible shift (arrowhead in *Lower*) is specific for intact box L. Methods were essentially the same as those described previously for box P-binding proteins (4).

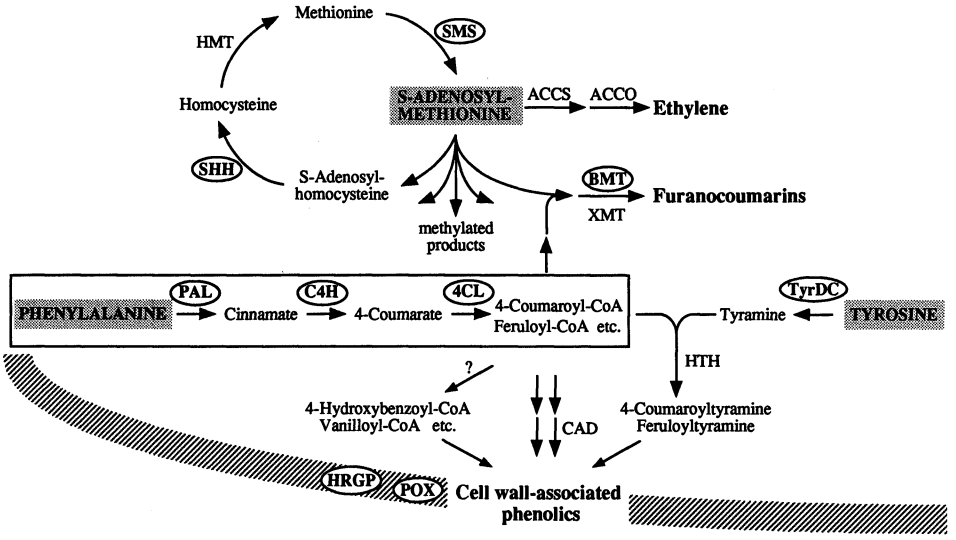

FIG. 7. Metabolic interconnections among various selected elicitor-inducible reactions in cultured parsley cells. Ovals indicate enzymes for which elicitor-mediated gene activation has been demonstrated with the corresponding cDNAs. CAD, cinnamyl alcohol dehydrogenase; HTH, hydroxycinnamoyl-CoA:tyramine hydroxycinnamoyltransferase; HMT, homocysteine *S*-methyltransferase; ACCS and ACCO, 1-aminocyclopropane-1-carboxylic acid synthase and oxidase; XMT, *S*-adenosyl-L-methionine:xanthotoxol *O*-methyltransferase; SHH, *S*-adenosyl-L-homocysteine hydrolase; SMS, *S*-adenosyl-L-methionine synthetase; BMT, *S*-adenosyl-L-methionine:bergaptol *O*-methyltransferase; TyrDC, tyrosine decarboxylase; HRGP, hydroxyproline-rich glycoprotein; POX, anionic peroxidase.

of the three steps of general phenylpropanoid metabolism to both stimuli (Fig. 8). While the synthesis of the three enzymes PAL, C4H, and 4CL is strongly and coordinately induced by both fungal elicitor and UV-containing white light, the subsequent phenylpropanoid branch pathways are regulated in a more narrow, stimulus-specific manner (44). Thus, induction of the flavonoid branch pathway is strongly dependent on specific light conditions (45, 46) and is repressed by elicitor (22). On the other hand, formation of wall-bound phenolics (1), furanocoumarin phytoalexins (6, 7), and phenolic esters (47) is induced by elicitor, whereas light exerts little, if any, effect (22).

This differential response of parsley cells to light and elicitor raised the question of whether the individual members of the PAL and 4CL gene families respond differentially to the two stimuli as well. Although certain methodological difficulties precluded a definite answer for 4CL (48), all available evidence suggests that the two 4CL genes respond similarly to light and elicitor. In contrast, recent studies using gene-specific probes for the four PAL genes (ref. 48; E.L. and K.H., unpublished results) demonstrated clear-cut differential behavior of PAL-1, PAL-2, and PAL-3 on the one hand and PAL-4 on the other. In cultured parsley cells, all four PAL genes responded to elicitor, whereas only the first three, but not PAL-4, were responsive to light (see also Fig. 5). Surprisingly, the enzyme kinetic properties of all four PAL proteins (49), as well as those of the two 4CL proteins (50), are almost indistinguishable, making the need for differential induction of isoenzymes difficult to explain.

PAL Gene Expression Patterns in Parsley Plants

These unexplained findings obtained in cultured parsley cells prompted us to investigate the mode of PAL gene expression in intact plants as well. Through use of RNA probes for mRNA localization *in situ*, total PAL gene expression was detected predominantly in three cell types of every organ tested, including stem and flower (Fig. 9 *A* and *B*): epidermal cells, where PAL is probably involved in flavonoid biosynthesis (51); oil-duct epithelial cells, the site of furanocoumarin production (8); and collenchyma and lignifying cells of the vascular bundles (38). At certain stages of flower development, anthers (not shown) and ovaries (Fig. 9*B*) are additional sites of abundant PAL mRNA accumulation.

Examination of the expression of individual PAL gene family members with gene-specific probes for *in situ* hybrid-

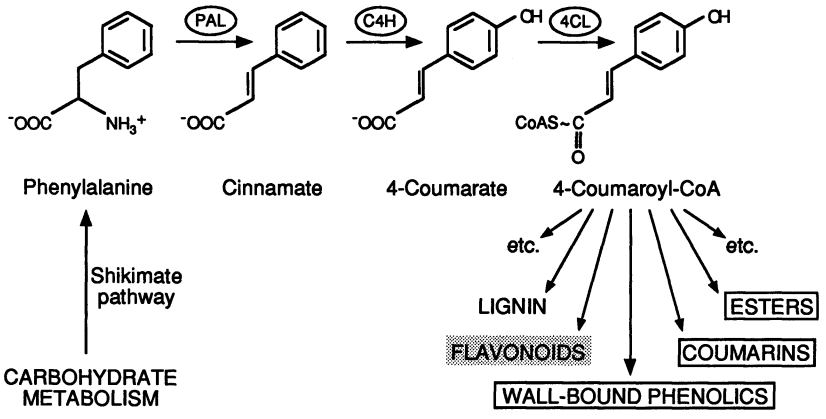

FIG. 8. Biosynthetic relationship of general phenylpropanoid metabolism with various branch pathways, one of which (stippled) is light-induced and at least three of which (boxed) are elicitor-induced in cultured parsley cells.

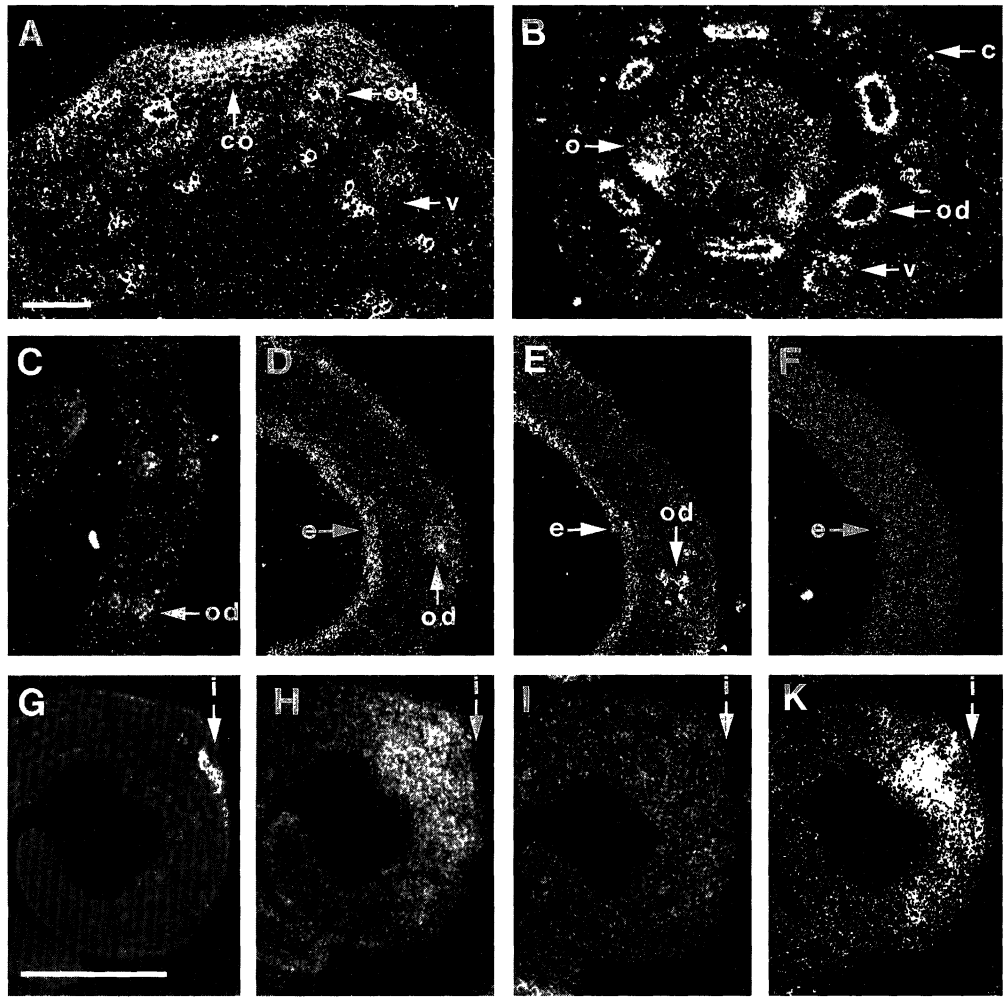

FIG. 9. PAL gene expression patterns in various parsley tissues. Cross sections of stem (*A*), gyneceum (*B*), and primary leaf buds that were 24-hr dark-adapted (*C*), 16-hr reilluminated (*D–F*), or 6-hr *Pmg*-infected (*G–K*) were hybridized *in situ* (3, 4) with PAL-1 antisense RNA (hybridizing with all PAL mRNA species) (*A–D* and *H*) or with PAL-1/PAL-2-specific (*E* and *I*) or PAL-4-specific (*F* and *K*) antisense RNA probes derived from the 5′ untranslated regions of the respective mRNAs. *C–F* and *H–K* are serial sections, respectively. *G* is identical to *H* but is shown under UV-epifluorescent light for identification of the infection site (i) (arrow). c, Carpel; co, collenchyma; e, epidermis; o, ovary; od, oil duct; v, vascular bundle. (Bars in *A* for *A* and *B* and in *G* for *C–K* = 100 μm.)

ization has so far been successful for PAL-4 and for PAL-1 and PAL-2 in combination (PAL-1/PAL-2). The results obtained for PAL induction by light and *Pmg* infection are shown in Fig. 9 *C–K*. Total PAL mRNA was present in oil ducts in both etiolated and illuminated leaf buds (Fig. 9 *C* and *D*) but accumulated preferentially in the upper and lower epidermis only in the latter. The same expression pattern was found with the PAL-1/PAL-2-specific probe (Fig. 9*E*), whereas PAL-4 mRNA (Fig. 9*F*) was neither induced by light nor present in detectable amounts in oil ducts. The opposite induction behavior was observed at *Pmg*-infection sites (Fig. 9*G*), where PAL mRNA had accumulated strongly by 6 hr postinoculation with fungal spores (Fig. 9*H*). While PAL-1 mRNA (Fig. 9*I*) was not induced to detectable levels, PAL-4 mRNA (Fig. 9*K*) exhibited the same accumulation pattern as that of total PAL mRNA (see local gene activation in Fig. 1). Thus, in whole-plant tissue as in cultured cells, induction of PAL-1/PAL-2 but not PAL-4 mRNA was triggered by light, while PAL-4 mRNA accumulated strongly at fungal infection sites. In contrast to cultured cells, however, PAL-1/PAL-2 mRNA was not detected in response to infection.

This discrepancy represents the first example of a major difference in induction behavior of parsley cells in culture and in infected tissue, although a large number of properties have been examined. We tentatively assume that this apparent discrepancy is associated with a particularly strict mode of cell type-specific gene expression at infection sites, an intriguing possibility for further investigation.

Conclusions

Suspension-cultured cells have proven to be an excellent model system for studies of the various aspects of elicitor recognition, signal transduction, and defense gene activation. In particular, the proteinaceous nature of the *Pmg* elicitor that acts on parsley cells enabled us to perform functional analysis by expressing the corresponding gene and mutated versions in *Escherichia coli* (23). Furthermore, the identification of an oligopeptide as the elicitor-active determinant within the protein allowed binding studies to be carried out, leading to the establishment of a functional link between signal perception via a peptide receptor on the cell surface, transient stimulation of plasma membrane-located ion channels, and activation of defense-related nuclear genes (18). Although several components of elicitor signal transduction have now been identified and interconnected, the present picture is still not complete. In particular, a question that remains to be answered is how the high specificity of the incoming elicitor signal is maintained and transduced through the entire chain of events from extracellular recognition to the activation and inactivation of

numerous defense-related and non-defense-related genes. We expect, however, that the combination of defined elicitors and cultured cells will continue to serve as useful model systems for expanding our knowledge about this particular type of signaling between and within organisms.

An additional advantage of this cell culture system is the fact that single cells or cell aggregates can be infected by the intact fungus. This infection system represents an intermediate stage between elicitor-treated cultured cells and fungus-infected, intact plant tissue (14). Since infection stimulates all responses analyzed thus far in both intact tissue and cultured cells (3, 5, 14) while elicitor treatment fails to stimulate hypersensitive cell death and callose accumulation (19), cultured cells can be used for micromanipulation, replacing the invading fungus by local application of the elicitor. In particular, it will be interesting to determine whether the physical force exerted by the fungal penetration peg contributes to the recognition and signaling mechanisms between the two organisms. In summary, we are beginning to appreciate how the great diversity of organismic interactions in ecosystems is reflected by an enormous complexity of integrated molecular networks within individual, affected cells.

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Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2751–2755, March 1997
Plant Biology

Receptor-mediated activation of a plant Ca^{2+}-permeable ion channel involved in pathogen defense

(patch-clamp/peptide elicitor/*Petroselinum crispum*/phytoalexins/signal transduction)

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Communicated by Klaus Hahlbrock, Max Planck Institute for Breeding Research, Cologne, Germany, December 26, 1996 (received for review November 15, 1996)

ABSTRACT **Pathogen recognition at the plant cell surface typically results in the initiation of a multicomponent defense response. Transient influx of Ca^{2+} across the plasma membrane is postulated to be part of the signaling chain leading to pathogen resistance. Patch-clamp analysis of parsley protoplasts revealed a novel Ca^{2+}-permeable, La^{3+}-sensitive plasma membrane ion channel of large conductance (309 pS in 240 mM $CaCl_2$). At an extracellular Ca^{2+} concentration of 1 mM, which is representative of the plant cell apoplast, unitary channel conductance was determined to be 80 pS. This ion channel (LEAC, for large conductance elicitor-activated ion channel) is reversibly activated upon treatment of parsley protoplasts with an oligopeptide elicitor derived from a cell wall protein of *Phytophthora sojae*. Structural features of the elicitor found previously to be essential for receptor binding, induction of defense-related gene expression, and phytoalexin formation are identical to those required for activation of LEAC. Thus, receptor-mediated stimulation of this channel appears to be causally involved in the signaling cascade triggering pathogen defense in parsley.**

Plants use a large arsenal of defense reactions to resist invading microbial pathogens (1–4). The molecular basis of pathogen recognition at the plant cell surface and of signaling cascades leading to the initiation of plant defense responses, however, is largely unknown. Perception of fungal pathogen-derived signals, referred to as elicitors, is believed to be mediated by specific receptors residing in the plant plasma membrane (5–8). Intracellular signal conversion and transduction include changes in the ion permeability of the plasma membrane, generation of reactive oxygen species, and alterations in the phosphorylation status of various proteins, giving rise to signal-specific responses of the plant (9–12).

The nonhost resistance response of parsley (*Petroselinum crispum*) leaves to infection with zoospores of the phytopathogenic fungus, *P. sojae*, has been found to be closely mimicked in parsley cell cultures upon treatment with fungus-derived elicitors (13, 14). An oligopeptide (Pep-13) originating from a cell wall glycoprotein of the fungus induces transcriptional activation of defense-related genes and phytoalexin production in parsley cells and protoplasts (15, 16). Recognition of the elicitor by its receptor, a 91-kDa plasma membrane protein, rapidly stimulates large, transient influxes of Ca^{2+} and H^+ and effluxes of K^+ and Cl^- (15, 17, 18). Pharmacological studies revealed that this pattern of ion fluxes, and in particular extracellular Ca^{2+}, is necessary for the production of reactive oxygen species (oxidative burst), defense-related gene activation, and phytoalexin production (18). Both omission of Ca^{2+} from the extracellular medium and inhibitors of animal slow-type Ca^{2+} channels abolished these plant responses (15, 19). Furthermore, identical structural features of the elicitor were found to be essential for receptor binding and initiation of all plant responses analyzed, indicating a sequence of events that may constitute part of a signaling cascade triggering pathogen defense in plants (15).

Ca^{2+} channels in the plasma membrane have been suggested to provide a major pathway for Ca^{2+} influx into higher plant cells (20–22). To identify plasma membrane ion channels that mediate Ca^{2+} influxes and thereby contribute to elevated levels of cytosolic Ca^{2+} in elicitor-treated parsley cells (23), we applied the patch-clamp technique to parsley protoplasts. Here we report the electrophysiological identification of a large conductance Ca^{2+}-permeable ion channel, which was specifically activated upon addition of elicitor.

MATERIALS AND METHODS

Plant Cell Culture/Protoplast Preparation. Cell suspension cultures of parsley (*P. crispum*) were maintained as described (24). Parsley protoplasts were isolated from 5-day-old cultured cells (25).

Elicitor Treatment/Inhibitor Studies. Elicitors and inhibitors were applied as stock solutions at concentrations given in the text. Elicitor-stimulated production of furanocoumarin phytoalexins was routinely tested (25) for each protoplast preparation used in patch-clamp experiments. The elicitor-induced oxidative burst and phytoalexin production in cultured parsley cells were quantified as described in ref. 15. Viability of cultured parsley cells was checked 24 hr after addition of inhibitor (11).

Patch-Clamp Experiments. Patch-clamp experiments with freshly prepared parsley protoplasts were performed using standard protocols (26) and the experimental setup as described (27). Digitized data (VR10, Instrutech, Elmont, NY) were stored on videotape and analyzed using a TL-1 DMA interface and patch-clamp software PCLAMP 5.5.1 (Axon Instruments, Foster City, CA). Unless stated otherwise, the bath solution was 240 mM $CaCl_2$, 10 mM Mes/Tris (pH 5.5) and the pipette solution was 150 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 10 mM Mes/Tris (pH 6.8). In both solutions osmolality was adjusted to 640 mosmol with D-sorbitol as was done in

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Abbreviation: LEAC, large conductance elicitor-activated ion channel.

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experiments with reduced extracellular Ca^{2+} concentrations. Removal of elicitor or inhibitor from the bath solution was achieved by perfusing the recording chamber with 5 ml of fresh bath solution (10-fold chamber volume, flow rate 1–2 ml/min). To assure establishment of whole-cell configuration, the accessibility of the protoplast interior (series resistance, whole-cell capacitance) was regularly checked throughout each experiment. Membrane voltage values were corrected for the liquid junction potential as described (28). Channel activity was quantified as $Np_O = \Sigma_{n=1}^{N} np_n$, where p_O is the open probability of the single channel, p_n is the probability that n channels are open simultaneously, and N is the apparent number of channels. The value of p_n was calculated according to ref. 29. Np_O was calculated from 60 sec of recording obtained between 5 and 6 min after onset of any treatment. Elicitor-induced increase in large conductance elicitor-activated ion channel (LEAC) activity (Np_O) was expressed as the ratio between the activities in the elicited and in the nonelicited state for each individual protoplast analyzed. Mechanosensitive ion channel activity was evoked as described (30).

RESULTS AND DISCUSSION

To identify plasma membrane ion channels that may contribute to macroscopic Ca^{2+} influxes observed in elicitor-treated parsley cells (23), we applied the patch-clamp technique to parsley protoplasts. Under asymmetric ionic conditions designed to resolve Ca^{2+}-inward currents, we were able to detect a channel (LEAC) that exhibited openings often lasting for some hundred milliseconds or even seconds (Fig. 1*A*). The high Ca^{2+} concentration in the bath solution, corresponding to the ion concentration used during protoplast isolation (25), greatly facilitated resolution of single channels mediating Ca^{2+}-inward currents. In addition, long open times, a limited number of channels that opened simultaneously, and a large current amplitude of this channel enabled us to detect single channel openings in whole-cell configuration (Fig. 1*A*). The single channel conductance determined in whole-cell configuration and in excised outside–out membrane patches was 309 ± 24 pS and 325 ± 35 pS, respectively (Fig. 1*B*). Expectedly, the number of active channels in excised membrane patches was much lower than that observed in whole-cell configuration (not shown). The activity of this channel did not significantly depend on the membrane potential within the physiologically relevant voltage range (−30 to −150 mV). Current amplitudes smaller than those mediated by LEAC were detected at higher membrane potentials (see $V_m = -110$ mV, Fig. 1*A*), which may either represent different channel activities or sublevels of LEAC (Fig. 1*A*).

Reduction of the extracellular concentration of $CaCl_2$ from 240 mM to 5 mM (the minimum concentration at which unitary LEAC currents could be resolved in whole-cell configuration) resulted in both a shift of the reversal potential toward more negative voltages (Δ−34 mV, Fig. 2*A*) and a decrease in single channel conductance (Fig. 2*B*). Single channel conductances determined from mean current amplitudes at 240 mM, 50 mM, 10 mM, and 5 mM $CaCl_2$ were 309 pS ($n = 15$), 245 pS ($n = 5$), 216 pS ($n = 4$), and 186 pS ($n = 3$), respectively. A negative shift of the reversal potential (against a positive shift of the reversal potential for Cl^-) indicates a preferential cation permeability of LEAC. Therefore, under our experimental conditions, Ca^{2+} (influx) and K^+ (efflux) rather than Cl^- represent major charge carriers of this channel. To explicitly rule out the existence of Cl^- efflux, KCl in the pipette solution was substituted by K^+-gluconate for which the anion is considered to be incapable of permeating ion channels. Channel amplitude and channel open probability remained unchanged under these conditions, demonstrating that LEAC did not mediate Cl^- efflux. The reversal potential

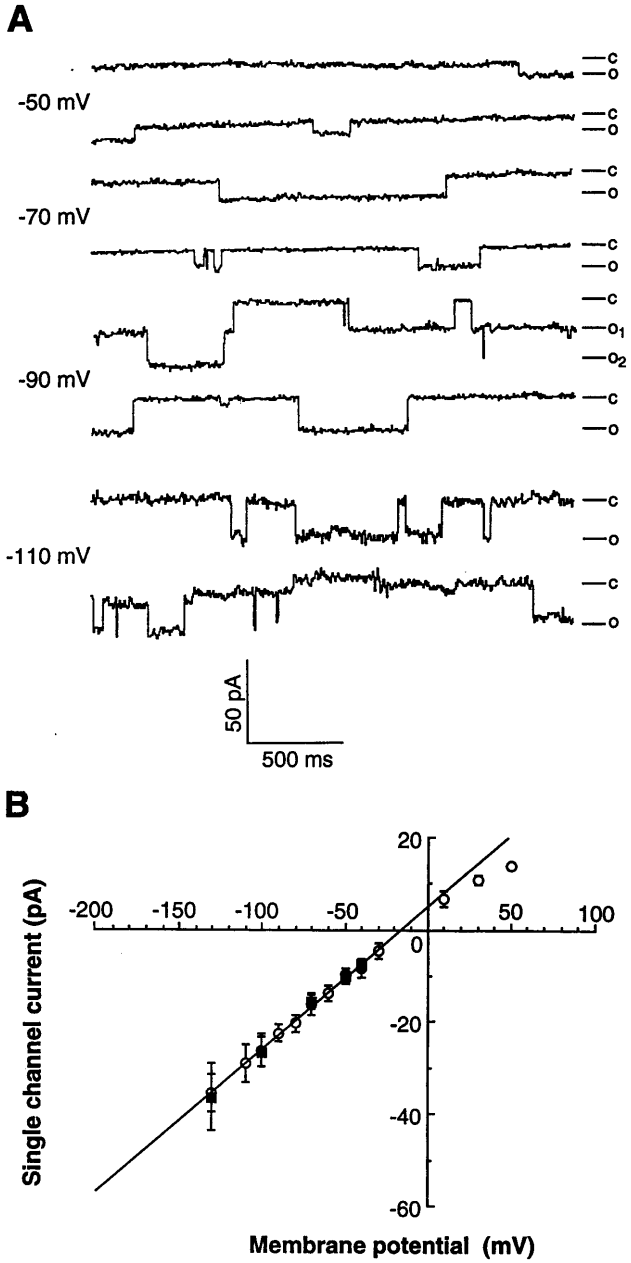

FIG. 1. Activity of a large conductance ion channel in the plasma membrane of parsley protoplasts. (*A*) Single channel recordings in the whole-cell configuration with the membrane potential (V_m) clamped to −50, −70, −90, and −110 mV. (*B*) *I–V* plot of unitary currents from recordings in whole-cell (○, $n = 15$) and outside–out configuration (■, $n = 6$), respectively. Freshly prepared parsley protoplasts were used for patch-clamp analyses under the conditions described in *Materials and Methods*.

of the channel, as determined by linear regression (Fig. 2*A*), did not correspond to that of one particular cation, suggesting that both Ca^{2+} and K^+ were transported. Under biionic conditions (given that LEAC is impermeable to anions and disregarding alterations of the internal Ca^{2+} concentration) the relative permeability ratio for Ca^{2+} to K^+ (P_{Ca2+}/P_{K+}) at 240 mM extracellular $CaCl_2$ was 0.16 as calculated by the Goldman–Hodgkin–Katz equation ($E_{rev} = RT/2F \ln 4P_{Ca2+}[Ca^{2+}]_{out}/P_{K+}[K^+]_{in}$) (29). Assuming constant permeability for the K^+-outward current, as well as regarding variable Ca^{2+}-inward currents at varying extracellular Ca^{2+} concentrations, nonlinear fits of both currents revealed increasing P_{Ca2+}/P_{K+} at decreasing extracellular Ca^{2+} concen-

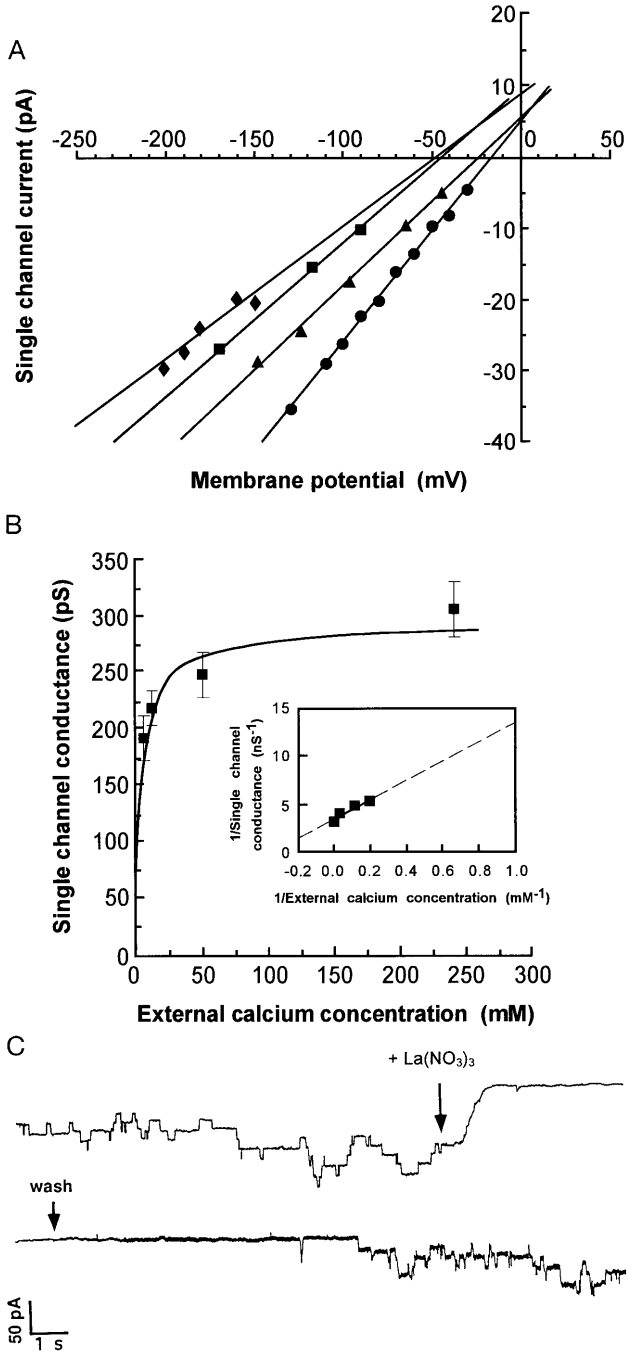

FIG. 2. Cation permeability of LEAC. (*A*) *I–V* plot of unitary currents recorded in whole-cell mode at various extracellular Ca^{2+} concentrations [240 mM (●), 50 mM (▲), 10 mM (■), and 5 mM (♦), respectively]. (*B*) Single channel conductance of LEAC as a function of extracellular Ca^{2+} concentration. Single channel conductances from *A* were plotted vs. extracellular Ca^{2+} concentration. The graph was fitted to Michaelis–Menten kinetics according to $\gamma = \gamma_{max}Ca^{2+}{}_{out}/(K_d + Ca^{2+}{}_{out})$ with γ being LEAC conductance. (*Inset*) Lineweaver–Burk plot of the data shown in *B*. The broken line may facilitate determination of single channel conductances at lower external Ca^{2+} concentrations than those tested. (*C*) Inhibition of LEAC by the Ca^{2+} channel inhibitor $La(NO_3)_3$. Channel activity was recorded in whole-cell mode ($V_m = -50$ mV) in standard bath solution either in the absence or presence of 1.5 mM $La(NO_3)_3$. Arrows indicate time points of addition of inhibitor [$La(NO_3)_3$] and removal of inhibitor (wash) by perfusion of the recording chamber.

trations (not shown). Thus, a reduction of the external Ca^{2+} concentration toward physiological concentrations would result in an increased Ca^{2+} permeability of LEAC. In addition, in the physiological range of plant membrane potentials (more negative than the reversal potential of LEAC), currents mediated by LEAC would largely correspond to Ca^{2+} influx.

LEAC unitary conductance was saturated at higher external Ca^{2+} concentrations (Fig. 2*B*). A Lineweaver–Burk plot of the Michaelis–Menten kinetics (Fig. 2*B Inset*) revealed a unitary channel conductance of 80 pS at an extracellular Ca^{2+} concentration of 1 mM, which is representative of the plant cell apoplast (31). Taken together, an unusually large Ca^{2+} conductance associated with the particular gating behavior characterize LEAC as a new type of plant Ca^{2+}-permeable ion channel. To our knowledge, there is no report yet on a plant ion channel with comparably large Ca^{2+} conductance.

LEAC activity could be completely and reversibly inhibited within seconds upon the addition of $La(NO_3)_3$ (1.5 mM, $n = 5$) (Fig. 2*C*) and $GdCl_3$ (1 mM, $n = 6$) (not shown), which are inhibitors of a wide range of Ca^{2+} channels (31, 32). Similar inhibitory effects of both $La(NO_3)_3$ and $GdCl_3$ were also observed at concentrations of 125 μM ($n = 8$) and 50 μM ($n = 4$), respectively. Both inhibitors also blocked the elicitor-induced production of reactive oxygen species and phytoalexins in parsley cells when added at these concentrations (not shown), whereas viability of parsley cells was not significantly affected by treatment with either inhibitor.

LEAC could be activated by *P. sojae*-derived elicitors, such as the peptide elicitor Pep-13 (Figs. 3*A* and 4) and a fungal cell wall preparation (Fig. 4). This activation was solely attributable to an increased channel activity (Np_O, ref. 29), since the unitary channel conductance remained virtually unchanged. Conductances were 343 ± 32 pS ($n = 4$) and 365 ± 45 pS ($n = 4$) for Pep-13 and the fungal cell wall elicitor, respectively, and 309 ± 24 pS ($n = 15$) in nonelicited protoplasts (Fig. 3*B*). Addition of water did not activate LEAC (not shown).

A heterogeneous ligand sensitivity of LEAC was observed because only 70% of the protoplasts analyzed were elicitor-responsive, a situation comparable to the abscisic acid-mediated activation of Ca^{2+} and K^+ permeable channels in *Vicia faba* guard cells (33). In addition, relatively large variations of LEAC elicitor responsiveness in individual protoplasts (ranging for example from 2- to 45-fold for Pep-13) were observed, which is typical for single-cell analyses with cells from a nonsynchronously growing cell culture (see SD values in Fig. 4). Both findings, however, may as well reflect differences in the physiological fitness of individual protoplasts caused by protoplast preparation.

Activation of LEAC by either elicitor could be observed only in whole-cell configuration but not in membrane patches in outside–out configuration (not shown, $n = 7$). This, as well as a delay in channel activation of 2–5 min with respect to addition of elicitor, suggests that the elicitor did not activate LEAC directly but through components mediating signal transfer between the elicitor receptor and the ion channel. Removal of the elicitor from the bath solution resulted in a decline of channel activity, indicating that LEAC activation is reversible (Fig. 3*A*).

As summarized in Fig. 4, LEAC could be efficiently activated by those elicitors that were previously shown to strongly induce macroscopic Ca^{2+} influx and phytoalexin production in parsley cells (15). A structural derivative of Pep-13, in which the tyrosine residue at position 12 was replaced by alanine (Pep-13/A12), retained its capacity to efficiently stimulate all three responses. In contrast, another single amino acid exchange within Pep-13 (tryptophan by alanine at position 2, Pep-13/A2) rendered this analog largely inactive with respect to LEAC activation, corresponding to observed losses of stimulation of Ca^{2+} uptake and phytoalexin formation (15), even when this derivative was applied at 10-fold higher concentrations than Pep-13. Similarly, deletion of one C-terminal and two N-terminal amino acid residues from Pep-13 (Pep-10) completely abolished both the ability of this derivative to

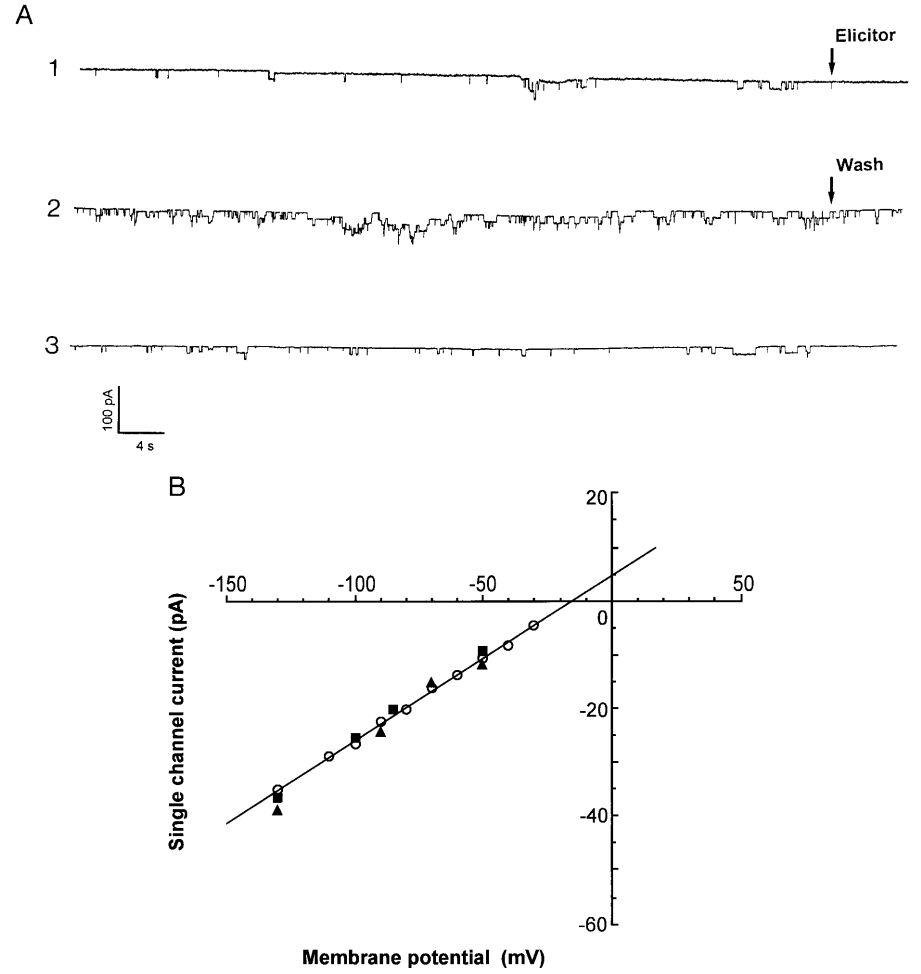

FIG. 3. Activation of LEAC by the oligopeptide elicitor Pep-13. (*A*) Whole-cell recordings of LEAC activity (V_m = −50 mV) under standard conditions before addition (trace 1), 5 min after addition of 100 nM Pep-13 (trace 2), and 5 min after removal of Pep-13 by perfusion (trace 3). Arrows indicate time points of addition of elicitor (trace 1, Elicitor) and start of perfusion (trace 2, Wash). Traces represent recordings of reversible activation by elicitor of LEAC for an individual protoplast (n = 4). Stimulation by elicitor of LEAC activity was 15-fold in this particular experiment. Channel activity was quantified as described in *Materials and Methods*. (*B*) *I–V* plot of unitary currents recorded in whole-cell mode without elicitor (○, taken from Fig. 1*B*, n = 15), and after addition of 50 μg/ml of the fungal cell wall elicitor (▲, n = 4) or 100 nM Pep-13 (■, n = 4), respectively.

stimulate LEAC and phytoalexin production. Since identical structural characteristics of the elicitor were also found to be responsible for specific interaction of Pep-13 with its receptor (15), our findings provide strong evidence that LEAC activation by fungal elicitor is a receptor-mediated process. Furthermore, stimulation with the same signal specificity of a multifaceted plant defense response comprising ion fluxes, oxidative burst, ethylene biosynthesis, activation of defense-related genes, and phytoalexin formation (15) indicates that LEAC constitutes a key element of the signal transduction chain initiating pathogen defense in parsley. This is further substantiated by the fact that Ca^{2+} channel inhibitors, which efficiently inhibit LEAC, block elicitor-induced oxidative burst and phytoalexin production as well.

A mechanosensitive ion channel activity in the parsley plasma membrane was evoked upon suction, which exhibited very similar gating behavior, single channel conductance (320 ± 24 pS in 240 mM $CaCl_2$), and sensitivity toward $La(NO_3)_3$ and $GdCl_3$ as LEAC (not shown). However, whether LEAC and the mechanosensitive ion channel are identical or represent distinct channels remains to be elucidated. Stretch-operated channels have been identified on the plasma membrane of several plants and found to be not highly selective for Ca^{2+}, but also allow K^+ to permeate (30, 31). Complex behaviors of adaptation and linkage have been ascribed to these channels, which suggest multiple pathways of regulation, e.g., in mediating the plant response to pathogen infection (31, 34). Furthermore, a mechanically activated oxidative burst, which may be mediated by stretch-sensitive channels has been reported from plant cells (35).

LEAC, the first plant plasma membrane ion channel shown to be activated by a phytopathogen-derived signal, is very likely to contribute to the elicitor-induced macroscopic ion fluxes observed in parsley (15, 18). Long open times of LEAC, its cation permeability, a large unitary Ca^{2+} conductance at physiological extracellular Ca^{2+} concentrations, and the elicitor inducibility of LEAC may account for the significant increase in cytoplasmic Ca^{2+} concentration (23) in elicitor-treated parsley cells. Alternatively, membrane depolarization by Ca^{2+} influx through LEAC could activate Ca^{2+} and/or voltage-dependent anion channels as well as outward-rectifying K^+ channels (36). Since similar macroscopic ion fluxes have been detected in other plants upon elicitor treatment (10, 37–41), the existence of functional homologs of LEAC in these species can be anticipated. A Ca^{2+}-permeable ion channel with similar unitary conductance and gating behavior was found to reside in the plasma membrane of tobacco protoplasts (unpublished results). However, activation of this channel by fungal elicitor remains to be analyzed.

Activation of LEAC by a fungal elicitor exemplifies the hypothesis that the plasma membrane of higher plants harbors a number of Ca^{2+}-permeable ion channels, of which a major

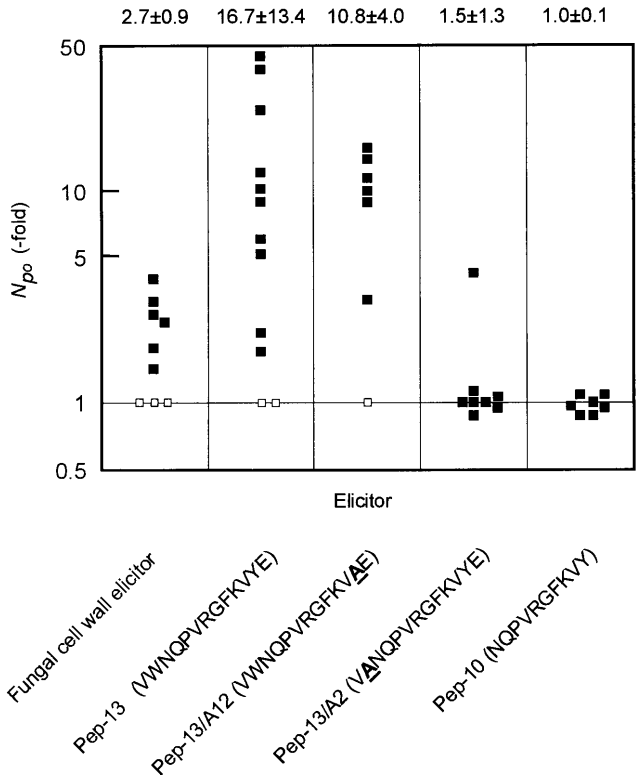

FIG. 4. Increase in LEAC open probability (Np_O) induced by fungal cell wall elicitor (n = 6), Pep-13 (n = 10), and structural derivatives of Pep-13 (n = 6, Pep-13A/12; n = 8, Pep-13/A2; n = 7, Pep-10, respectively). Np_O was calculated from 60 s of recording obtained between 5 and 6 min after addition of elicitor as described in *Materials and Methods* and normalized to background activity recorded before addition of elicitor. Note the logarithmic scale used to plot increase in LEAC open probability. Data obtained from nonresponsive parsley protoplasts (□, see text for explanation) are included. Mean values ± SD, excluding those for nonresponsive protoplasts, are given. Elicitor concentrations used in patch-clamp experiments were 50 μg/ml fungal cell wall elicitor, 100 nM (Pep-13, Pep-13/A12), and 1 μM (Pep-13/A2, Pep-10), respectively. Amino acid sequences of Pep-13 and its structural derivatives are given in one-letter code. Underlined boldface letters represent alanine substitution sites within Pep-13.

part are quiescent but can be rapidly recruited for Ca^{2+}-dependent signal transduction. Complementary to the functional analysis of plant disease resistance genes and of plant mutants impaired in pathogen defense, structural analysis of signal transduction intermediates, such as plant ion channels, will substantially broaden our knowledge on the organization of signaling cascades involved in plant pathogen defense.

We are grateful to Drs. H. Barbier-Brygoo, J. L. Dangl, W. Knogge, I. E. Somssich, and S. Thomine for critical reading of the manuscript. We thank the Max-Planck-Society for substantial support. This work was supported by Grants Sche 235/3-1 and Sche 235/3-2 to D.S. and He 1640/1-3 to R.H. from the Deutsche Forschungsgemeinschaft, by Grant CHRX-CT93-0168 from the European Community Human Capital and Mobility Program to D.S., and by a grant from the European Community Biotech Program to J.G. J.-M.F. received an Alexander-von Humboldt-Stiftung postdoctoral fellowship.

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Characterization and partial purification of an oligopeptide elicitor receptor from parsley (*Petroselinum crispum*)

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Received 14 May 1998; revised version received 8 June 1998

Abstract Parsley cells recognize the fungal phytopathogen *Phytophthora sojae* through a plasma membrane receptor. A 13 amino acid oligopeptide fragment (Pep-13) of a 42 kDa fungal cell wall glycoprotein was shown to bind to the receptor and stimulate a complex defense response in cultured parsley cells. The Pep-13 binding site solubilized from parsley microsomal membranes by non-ionic detergents exhibited the same ligand affinity and ligand specificity as the membrane-bound receptor. Chemical crosslinking and photoaffinity labeling assays with [^{125}I]Pep-13 revealed that a monomeric 100 kDa integral plasma membrane protein is sufficient for ligand binding and may thus constitute the ligand binding domain of the receptor. Ligand affinity chromatography of solubilized microsomal membrane protein on immobilized Pep-13 yielded a 5000-fold enrichment of specific receptor activity.

Key words: Ligand affinity chromatography; Phytoalexin; *Phytophthora sojae*; Signal transduction

1. Introduction

Plant receptors for pathogen-derived elicitors are instrumental to pathogen recognition and subsequent activation of the plant's surveillance system [1–3]. A number of receptors for fungal elicitors have been reported to reside in the plasma membrane of plant cells. Ligand binding is believed to mediate generation of an intracellular signal and subsequent stimulation of plant defense reactions through activation of an intracellular signal transduction cascade. Kinetic properties of elicitor binding proteins, such as high affinity, saturability, and reversibility of ligand binding, together with a direct correlation between the binding affinities and the elicitor activities of the respective ligands indicate that such proteins function as physiological receptors.

Insight into the precise molecular mechanisms underlying non-self recognition and intracellular signal generation requires isolation of elicitor receptors and the encoding genes. In addition, these genes are considered to be most valuable tools for fungal disease control in transgenic crop plants. Isolation of a number of these genes is therefore attempted worldwide. Unfortunately, low abundance of elicitor receptors and the apparent lack of specialized cell types in which elicitor receptors are strongly expressed have severely hampered isolation of these proteins by means of ligand affinity chromatography and other chromatographic techniques.

Very recently, purification of a 75 kDa soybean plasma membrane protein was reported [4,5]. This protein interacts with elicitors of phytoalexin production such as a *Phytophthora sojae*-derived mixture of structural isomers of β-glucans or a synthetic hepta-β-glucoside. A cDNA encoding this protein was isolated and used for production of recombinant receptor in *Escherichia coli* [5]. This protein recognized large glucan fragments with high affinity, but only barely interacted with the hepta-β-glucan elicitor.

An oligopeptide fragment (Pep-13) from a cell wall protein of the same fungus, *P. sojae*, induces transcriptional activation of defense-related genes and phytoalexin production in cultured parsley cells and protoplasts [6]. Recognition of the elicitor by its plasma membrane receptor rapidly stimulates large, transient influxes of H^+ and Ca^{2+}, effluxes of K^+ and Cl^-, production of reactive oxygen species and activation of a MAP-kinase pathway [6–10]. Pharmacological studies and use of structural derivatives of Pep-13 with differing elicitor activities revealed that induction of macroscopic ion fluxes and, subsequently, superoxide anions are necessary for defense-related gene activation and phytoalexin production in elicitor-treated parsley cells. Thus, recognition of Pep-13 by its receptor initiates a signaling cascade through which activation of plant defense responses is mediated. Here we report the molecular characterization of the Pep-13 receptor and partial purification of the functionally intact receptor protein by ligand affinity chromatography.

2. Materials and methods

2.1. Materials

Solid-phase peptide synthesis was performed on Economy Peptide Synthesizer EPS 221 (ABIMED, Langenfeld, Germany) according to the manufacturer's instructions. Synthesized peptides were purified to homogeneity as described [6]. N-terminal acetylation of Pep-13 was achieved prior to cleavage from the solid phase by incubation with a 10-fold molar excess of 98% acetic acid for 1 h. Radioiodination of Pep-13 and its derivatives (specific radioactivity 2200 Ci/mmol) was performed by Anawa AG (Wangen, Switzerland). Bis(sulfosuccinimidyl) suberate (BS^3), *N*-hydroxysulfosuccinimidyl-4-azidobenzoate (S-HSAB), sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-Biotin), UltraLink AB1, and the BCA reagent for determination of protein concentrations were from Pierce (Oud Beijerland, The Netherlands). Detergents were obtained from Calbiochem (Bad Soden, Germany). Sepharose 4B and avidin agarose were from Pharmacia (Freiburg, Germany) and Toyopearl AF-Tresyl-650M from Tosohaas (Stuttgart, Germany).

2.2. Preparation and solubilization of parsley microsomal membranes

Parsley (*Petroselinum crispum*) microsomal membranes were prepared as described [6]. Solubilization of microsomal membranes (5 mg/ml protein) was performed on ice by consecutively adding detergent stock solutions (150 mg/ml) up to final detergent concentrations given in the text. After 60 min insoluble material was removed by ultracentrifugation (140 000 × *g*, 1 h, 4°C) and the supernatant was used without intermittent storage.

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2.3. Ligand binding assay, chemical crosslinking and photoaffinity labeling

Ligand binding assays, BS^3-mediated chemical crosslinking of [^{125}I]Pep-13, and analysis of crosslinked proteins were performed as described [11] with the following modifications. Whatman GF/B glass fiber filters saturated with 0.3% polyethyleneimine were utilized for ligand binding assays with solubilized protein. Chemical crosslinking of [^{125}I]Pep-13 to solubilized proteins was achieved by directly adding the crosslinker to the reaction mixture.

For photoaffinity labeling 5 pmol of N-terminally acetylated [^{125}I]Pep-13 (Nac-[^{125}I]Pep-13) was incubated with 1 mM S-HSAB for 60 min at room temperature (RT). Excess S-HSAB was inactivated by addition of 1 M Tris/HCl, pH 8.0 (12 h, RT). 200 fmol of photoaffinity-labeled Nac-[^{125}I]Pep-13 was incubated with 400 μg microsomal membrane protein under ligand binding assay conditions. Subsequently, microsomes were pelleted (90 s at 10 000 × *g*), washed (1 ml PBS, pH 8.0), and made up to the original volume with PBS, pH 8.0. Samples were irradiated for 15 min with UV light (254 nm). Labeled proteins were analyzed by SDS-PAGE/autoradiography as described before. In contrast to our previous work [11] radiolabeled broad-range molecular size markers from Bio-Rad (Munich, Germany) were utilized for formula weight determination. This may explain the discrepancy in the size of the Pep-13 receptor determined in this and the previous study.

2.4. Ligand affinity chromatography

For immobilization 10 μmol Pep-13 was dissolved in 2 ml PBS (pH 8.0) and incubated with either 1 ml Sepharose 4B, UltraLink AB1 or Toyopearl AF-Tresyl-650M for 1 h at RT with continuous stirring. In the case of Sepharose 4B the matrix was washed with 100 ml 10 mM HCl before coupling. After immobilization any remaining reactive groups on the matrix were inactivated with 10 ml 3 M ethanolamine, pH 9.0. The amount of immobilized peptide was estimated by determination of the difference between integrated HPLC peaks corresponding to Pep-13 [6] before and after coupling.

Detergent-solubilized microsomal membranes (1 ml) were incubated with 50 μl Pep-13 matrix for 3 h at 4°C. The matrix was washed with 5 × 1 ml 100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1% octyl glucoside, and Pep-13 binding proteins eluted by incubating the matrix in 200 μl of the same buffer containing 10 mg/ml Pep-13/A2 for 3 h at 4°C. For ligand binding assays Pep-13/A2 was removed by addition of 20% PEG-4000 (1 h, 4°C), proteins were pelleted by ultracentrifugation (1 h, 140 000 × *g*, 4°C) and redissolved in ligand binding assay buffer.

Pep-13 (10 mg) was biotinylated with NHS-LC-Biotin according to the supplier's instructions, and subsequently purified to homogeneity by HPLC [6]. Upon radioiodination 2 nM of biotinylated [^{125}I]Pep-13 was crosslinked to microsomes with 10 mM BS^3 as described before. Triton X-100-solubilized material (2%, 1 h) was cleared by centrifugation (1 h, 10 000 × *g*, 4°C) and 1 ml of the supernatant incubated with 100 μl avidin agarose for 30 min at RT. The matrix was washed with 3 × 1 ml 100 mM Tris-HCl, pH 6.8, 100 mM NaCl, 2% Triton X-100 and bound proteins eluted with 10 × 50 μl 100 mM Tris-HCl, pH 6.8, 5% glycerol, 5% SDS, 100 mM biotin. Eluted proteins were analyzed by chemical crosslinking/SDS-PAGE/autoradiography as described before.

3. Results

Solubilization in a functionally active form is a prerequisite for the purification of membrane receptors. We have tested a series of structurally diverse detergents for their ability to release the Pep-13 receptor from parsley microsomal membranes. As shown in Table 1, non-ionic detergents such as *n*-octyl-β-D-glucopyranoside (octyl glucoside), Triton X-100, Lubrol PX, MEGA-10, and digitonin were most efficient, while zwitterionic (CHAPS, Zwittergent 3-12) and ionic detergents (cholic acid, deoxycholic acid) were much less efficient or failed to solubilize this protein. Treatment of microsomal membranes with increasing concentrations of NaCl (up to 1.5 M) removed 20% of the total membrane protein, but did not solubilize the Pep-13 binding site. Thus, the Pep-13 receptor appears to be an integral component of the membrane rather than merely attached to it. Using octyl glucoside (1.5%), Triton X-100 (1.5%) or digitonin (1%), 80% of the total binding activity could be solubilized by either detergent. A detergent-to-protein ratio of about 6:1 (w/w) was determined to be necessary for optimum recovery of the elicitor binding site by octyl glucoside (not shown). Maximum amounts of elicitor binding protein were solubilized with detergent concentrations corresponding to the critical micelle concentration (CMC) of the particular detergent or higher concentrations. Routinely, solubilization of membrane protein by either detergent resulted in a two-fold enrichment of specific Pep-13 binding activity. The solubilized receptor could be stored at −20°C for 5 days without any significant loss of binding activity. However, storage at 4°C reduced binding activity by 30% within 24 h, and after 72 h elicitor binding activity was no longer detectable. Receptor activity could only be maintained at CMC or higher detergent concentrations suggesting that

Table 1
Solubilization of the Pep-13 receptor from parsley microsomal membranes with various detergents

Detergent	Detergent concentration (%)	Specific binding solubilized (%)	Protein solubilized (%)
None	0	0	0
Lubrol PX	0.5, 1, 1.5, 2 [v/v]	40–60	45
Triton X-100	0.5, 1, 1.5, 2 [v/v]	50–80	59
Tween 20	1.5 [v/v]	6	29
Digitonin	0.5, 1, 1.5, 2 [w/v]	50–70	38
n-Hexyl-β-D-glucopyranoside	1.5 [w/v]	0	21
n-Heptyl-β-D-glucopyranoside	1.5 [w/v]	0	28
n-Octyl-β-D-glucopyranoside	0.5, 1, 1.5, 2 [w/v]	50–80	40
n-Octyl-β-D-thioglucopyranoside	1.5 [w/v]	80	n.d.
n-Octanoyl sucrose	1.5 [w/v]	1	n.d.
MEGA-8	1.5 [w/v]	5	19
MEGA-9	1.5 [w/v]	60	n.d.
MEGA-10	1.5 [w/v]	70	35
Cholic acid	1.5 [w/v]	16	45
Deoxycholic acid	1.5 [w/v]	15	42
CHAPS	1.5 [w/v]	5	37
Zwittergent 3-12	1.5 [w/v]	10	61
Zwittergent 3-08	1.5 [w/v]	0	10

Parsley microsomal membranes were solubilized as described in Section 2 with the detergent concentrations indicated. Specific binding of [^{125}I]Pep-13 to and protein content of the solubilized material is given in percent corresponding to a 100% binding and protein content determined in microsomal membranes in the presence of detergent. n.d., not determined.

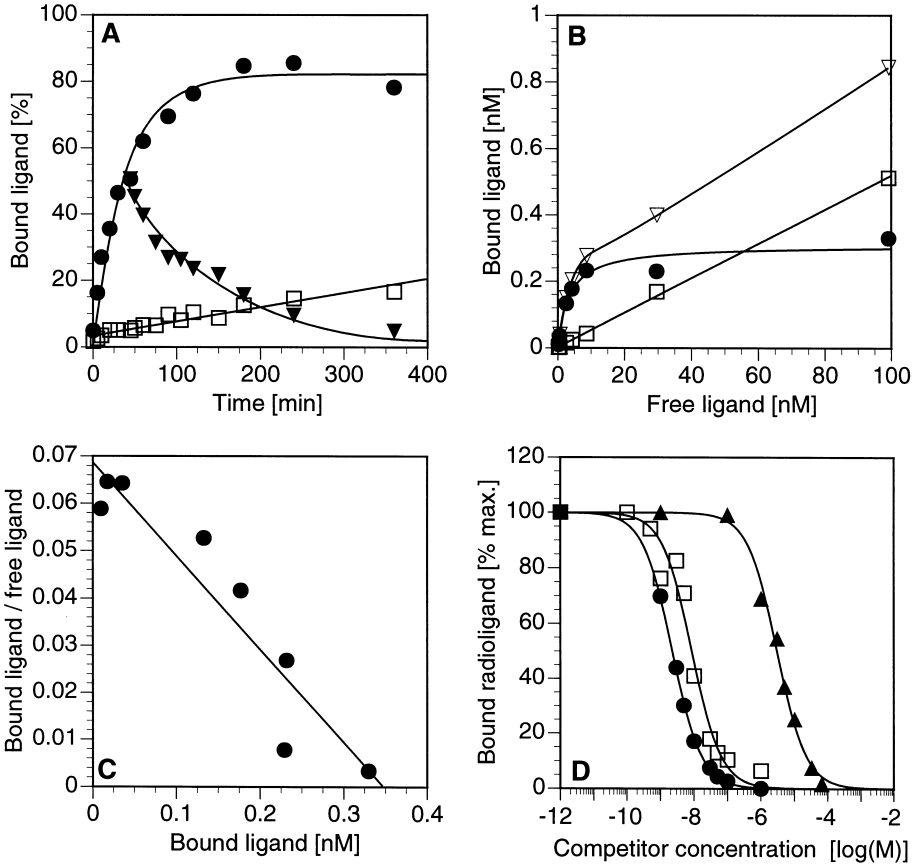

Fig. 1. Binding of [^{125}I]Pep-13 to detergent-solubilized parsley microsomal membrane proteins. Parsley microsomal membranes were solubilized with 1.5% octyl glucoside and solubilized material was used in binding assays as described in Section 2. A: Kinetics of specific binding (circles), non-specific binding (squares) and displacement (triangles) of [^{125}I]Pep-13. Specific binding represents the difference between total binding and non-specific binding determined in the presence of 15 μM Pep-13. Displacement of [^{125}I]Pep-13 was initiated by adding 15 μM Pep-13 to the assay mixture 40 min after addition of the radioligand. B: Saturability of [^{125}I]Pep-13 binding. Increasing concentrations of [^{125}I]Pep-13 were incubated with solubilized parsley microsomal membrane protein, and total binding (triangles), specific binding (circles) and non-specific binding (squares) was determined. C: Scatchard plot of specific binding from B. D: Competitive inhibition of binding of [^{125}I]Pep-13 to solubilized parsley microsomal membrane protein by Pep-13 structural derivatives. Binding assays were performed with increasing concentrations of Pep-13 (circles), Pep-13/A12 (squares) or Pep-13/A2 (triangles), and specific binding of [^{125}I]Pep-13 was determined. See text for details on Pep-13/A12 and Pep-13/A2.

incorporation into micelle structure is essential for biological activity of the protein.

Since solubilization of receptors can alter kinetic properties of the ligand/receptor interaction ligand binding assays with [^{125}I]Pep-13 and solubilized parsley microsomal membrane protein as receptor source were performed. Kinetic analysis of elicitor binding demonstrated that half-maximal association of the radioligand and octyl glucoside-solubilized receptor was achieved within 30 min, while equilibrium between association and dissociation was reached after 100 min (Fig. 1A). Addition of a 10 000-fold molar excess of unlabeled elicitor 40 min after addition of the radioligand resulted in a marked decline of bound radioactivity, demonstrating that interaction between Pep-13 and its binding site was fully reversible. In saturation analyses, octyl glucoside-solubilized membrane protein was incubated with increasing concentrations (0.1–100 nM) of the radioligand (Fig. 1B). Saturation of specific binding of Pep-13 was achieved at a ligand concentration of approximately 20 nM. Scatchard analysis of these data (Fig. 1C) suggested the presence of a single class of binding sites with an affinity constant (K_d) of 5.0 nM and an apparent receptor concentration of 100 fmol/mg solubilized membrane protein. A Hill plot of the data shown in Fig. 1B yielded a Hill coefficient of 0.98, excluding cooperativity in binding of Pep-13 to its receptor (not shown). Both ligand binding kinetics and affinity constant closely correspond to those obtained from experiments with microsomal membranes and intact protoplasts (K_d = 2.4 nM and 11.4 nM, respectively [6]). Thus, solubilization does not negatively affect ligand binding properties of the Pep-13 receptor. To examine whether the solubilized Pep-13 binding site exhibited the same ligand specificity as the membrane-localized binding site, structural derivatives of Pep-13 were tested in ligand competition assays. Binding of [^{125}I]Pep-13 could be efficiently inhibited by those analogs that were previously shown to efficiently compete for binding of the radioligand to parsley microsomal membranes (Fig. 1D) as well as to strongly activate macroscopic Ca^{2+} influx, to enhance open probability of a Ca^{2+}-permeable plasma membrane ion channel, and to stimulate oxidative burst and phytoalexin production in parsley cells [6,9,10]. A structural derivative of Pep-13 in which the tyrosine residue at position 12 was replaced by alanine (Pep-13/A12) retained its competitor activity in assays with detergent-solubilized membrane protein, and also efficiently stimulated all four responses

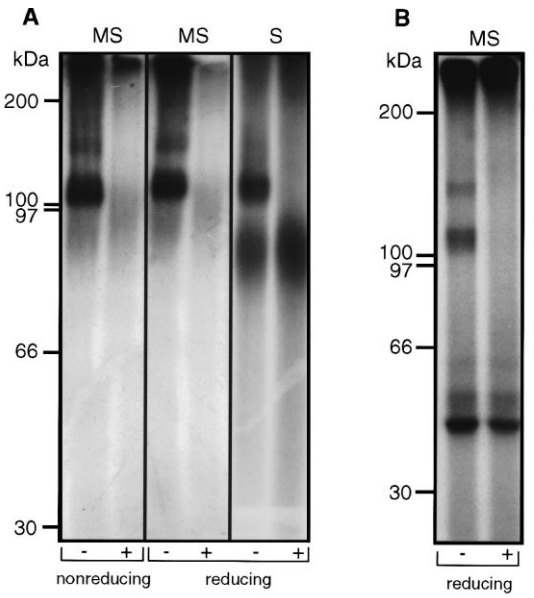

Fig. 2. Identification of the Pep-13 receptor by chemical crosslinking (A) and photoaffinity labeling (B). Parsley microsomal membranes (MS) or solubilized microsomal membranes (S) were incubated with [^{125}I]Pep-13 and subsequently subjected to BS^3-mediated chemical crosslinking. For photoaffinity labeling N-terminally acetylated [^{125}I]Pep-13 was labeled with S-HSAB, incubated with microsomal membranes and covalently attached to protein by irradiation with UV light. Binding of the radioligand was performed either in the absence (−) or in the presence (+) of 15 μM Pep-13. Radiolabeled proteins were separated by SDS-PAGE under reducing or non-reducing conditions and analyzed by autoradiography. Sizes of formula weight markers are indicated on the left.

(IC_{50}=8.6 nM with solubilized protein, IC_{50}=6.3 nM with microsomal membranes). In contrast, another single amino acid exchange within Pep-13 (tryptophan by alanine at position 2, Pep-13/A2) rendered this analog largely inactive as competitor of binding (IC_{50}=3 μM with solubilized protein, IC_{50} = 7 μM with microsomal membranes), corresponding to observed losses of stimulation of all other responses [6,10]. Taken together, ligand affinity as well as ligand specificity of the detergent-solubilized binding site remained unaltered as compared to the membrane-bound receptor.

Covalent attachment of [^{125}I]Pep-13 to its binding site in parsley microsomal membrane preparations and in octyl glucoside-solubilized membrane protein preparations was performed using either the homobifunctional chemical crosslinker, BS^3 [11], or the photoaffinity reagent, S-HSAB. For uniform photoaffinity labeling of [^{125}I]Pep-13 at the sole lysine residue N-terminally acetylated [^{125}I]Pep-13 (Nac-[^{125}I]Pep-13) was utilized. Analysis by SDS-PAGE and autoradiography of membrane proteins revealed labeling of a 100 kDa protein in either type of experiment (Fig. 2). Labeling of this protein was prevented by addition of a 10 000-fold molar excess of unlabeled Pep-13 as competitor. A second, significantly weaker band with an apparent molecular mass of 145 kDa was solely detectable in microsomal membrane preparations, but not in detergent-solubilized membrane protein preparations. No larger bands corresponding to oligomerized complexes of the receptor could be detected. In addition, electrophoretic separation of crosslinked proteins under denaturing, non-reducing conditions also revealed labeling of the 100 kDa band (Fig. 2). An oligomeric subunit structure of the receptor protein due to disulfide bridges is therefore unlikely. The 100 kDa

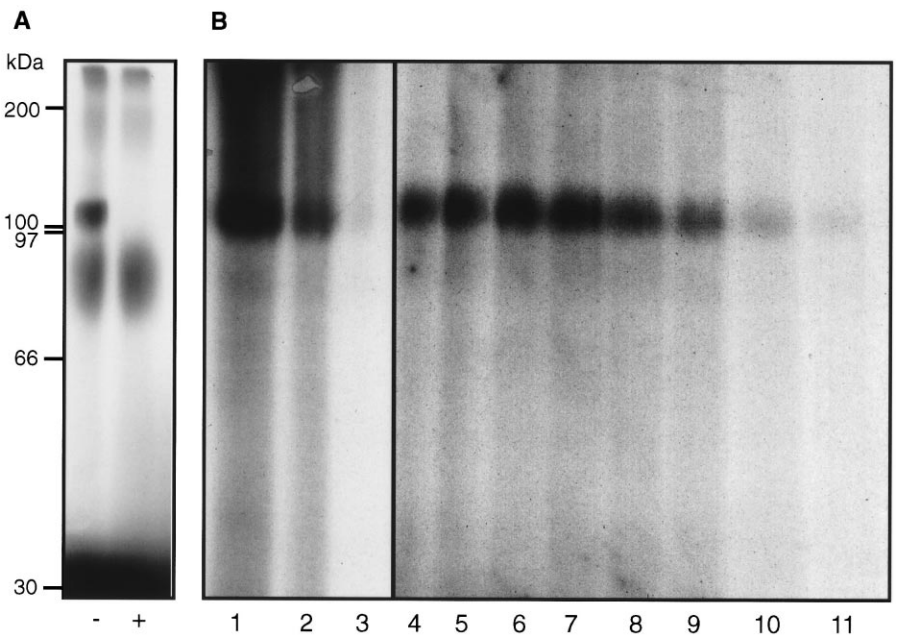

Fig. 3. Ligand affinity chromatography of detergent-solubilized parsley microsomal membrane protein. A: Octyl glucoside-solubilized parsley microsomal membrane proteins were subjected to ligand affinity chromatography on Sepharose 4B. Protein eluted from the matrix with Pep-13/A2 (for details see text) was used in chemical crosslinking assays with BS^3 and analyzed as described in legend to Fig. 2. Binding of the radioligand was performed either in the absence (−) or in the presence (+) of 15 μM Pep-13. Sizes of formula weight markers are indicated on the left. B: Biotinylated [^{125}I]Pep-13 was crosslinked to parsley microsomal membranes by BS^3. Microsomal membrane protein solubilized by 1% Triton X-100 was subjected to ligand affinity chromatography on avidin agarose as described in Section 2. Aliquots of solubilized microsomal membrane protein (lane 1), of detergent-insoluble membrane protein (lane 2), of protein which was not retained on avidin agarose (lane 3), and of protein eluted from the matrix by 100 mM biotin (lanes 4–11) were analyzed by SDS-PAGE/autoradiography. Note that aliquots analyzed in lanes 1–3 represent one fourth of the material analyzed in lanes 4–11.

polypeptide solubilized from parsley microsomal membranes may thus represent the ligand binding domain of the Pep-13 receptor.

Instability of the detergent-solubilized receptor severely confines purification protocols for this protein to only a limited number of steps. Although gel permeation and anion exchange chromatography proved successful in removing contaminating protein there was no enrichment in specific binding activity detectable in receptor-containing fractions (not shown). This was most likely due to concomitant inactivation of the receptor protein. We therefore attempted to adopt a recently reported ligand affinity chromatography-based one-step purification protocol for the soybean hepta-β-glucan receptor [4]. Three matrices (Sepharose 4B, UltraLink AB1, Toyopearl AF-Tresyl-650M) differing in hydrophobicity, spacer length, and ligand binding capacity were employed for immobilization of Pep-13. In all cases Pep-13 was coupled through its N-terminal primary amino group to the matrix. The amount of immobilized Pep-13 was approximately 6 mM regardless of the matrix used. The derivatized matrices were tested for their ability to retain the Pep-13 binding site from octyl glucoside-solubilized parsley microsomal membrane protein. Incubation of solubilized membrane protein with either matrix resulted in retention of 85–95% of the binding activity applied. Matrices without immobilized Pep-13 neither retained the binding site nor impaired binding activity of the solubilized receptor. Approximately 20% of the Pep-13 binding activity retained on the matrix could reproducibly be eluted with 6 μM Pep-13/A2, thereby yielding 5000-fold enrichment of specific receptor activity. Pep-13/A2, a structural derivative of Pep-13, is significantly less active than Pep-13 as competitor of binding of [^{125}I]Pep-13 to its receptor [6]. Unlike Pep-13 this peptide could be completely removed from eluted proteins by PEG-4000-mediated precipitation, which allowed subsequent quantification of receptor activity. Ligand binding assays performed with ligand affinity chromatography-purified receptor revealed no significant changes in the kinetics of ligand association/dissociation and IC_{50} value for Pep-13 in comparison to experiments performed with detergent-solubilized receptor. BS^3-mediated chemical crosslinking of [^{125}I]Pep-13 to proteins from fractions containing receptor activity resulted in labeling of the 100 kDa protein as viewed by autoradiography (Fig. 3A). Alternatively, biotinylated [^{125}I]Pep-13 was crosslinked to parsley microsomal membranes and membrane proteins solubilized with 1% Triton X-100 were subjected to ligand affinity chromatography on avidin agarose (Fig. 3B). After thoroughly washing the matrix bound proteins were eluted in the presence of 100 mM biotin. Again, upon autoradiographic analysis labeling of a 100kDa-band corresponding to the receptor/ligand complex could be visualized.

4. Discussion

The ligand binding domain of the previously identified parsley plasma membrane elicitor receptor [6,7,11] very likely constitutes a monomeric 100 kDa integral plasma membrane protein. Solubilization of the receptor by several structurally very diverse non-ionic, amphipathic detergents, but not by high salt concentrations is indicative of integration of the protein into the membrane. Moreover, all detergents which solubilized the Pep-13 receptor form large micelles with high aggregation numbers [12,13], suggesting that receptor activity depends on integration into complex structures. This is further corroborated by the finding that activity of the solubilized receptor declined significantly at detergent concentrations below CMC. Receptors for fungal elicitors from soybean, rice and tomato have also been shown to be integral plasma membrane proteins [5,14–17].

Chemical crosslinking studies using [^{125}I]Pep-13 and parsley microsomal membranes, solubilized membrane protein, or protein eluted from immobilized Pep-13 consistently yielded labeling of a 100 kDa band. Crosslinking of biotinylated [^{125}I]Pep-13 to parsley membranes and subsequent ligand affinity chromatography of solubilized membrane protein on avidin agarose substantiated the latter experiment. Labeling of this protein band was detectable upon gel electrophoretic separation of solubilized membrane protein under either reducing or non-reducing conditions, suggesting a monomeric nature of the 100 kDa protein. Specific labeling of bands corresponding to oligomers of the 100 kDa protein could never be detected in crosslinking assays. In contrast to chemical crosslinking experiments, which may result in covalent attachment of several subunits of a receptor complex, photoaffinity labeling studies with radioligands containing one photolabile azide moiety preferentially leads to labeling of individual protein species. Since such experiments also revealed a 100 kDa protein that interacted specifically with Nac-[^{125}I]Pep-13, this polypeptide is assumed to be the monomeric ligand binding site of the Pep-13 receptor. It is yet not possible to say whether the 140 kDa band, which was detectable only in crosslinking assays with intact membranes, represents a functional subunit of a then heterooligomeric receptor complex or is merely a neighboring but functionally unrelated protein.

Ligand affinity and ligand specificity of the Pep-13 binding site remained unaffected upon detergent solubilization. Thus, the protein is amenable to purification by ligand affinity chromatography. We have developed a protocol for retention of the protein on immobilized Pep-13 and subsequent ligand-specific desorption. This protocol resulted in a 5000-fold enrichment of specific receptor activity, which is typical for this technique [18]. A 9000-fold enrichment was recently reported for the purification of the soybean hepta-β-glucan elicitor receptor by means of ligand affinity chromatography [4]. In order to purify this very low abundant receptor protein future experiments will focus on upscaling of this protocol. Information on the molecular weight and subunit structure of the ligand binding domain of this receptor will thereby facilitate direct isolation of the protein by electroelution upon gel electrophoretic separation of affinity-purified protein.

In addition, molecular characterization of the Pep-13 receptor has enabled an alternative experimental strategy for obtaining sequence information on this protein. Ligand affinity screening of mammalian COS cells transfected with size-enriched cDNA has proven successful for isolation of many receptor cDNAs [19,20], and may therefore be suitable for isolation of a cDNA encoding the 100 kDa ligand elicitor binding site from parsley.

Acknowledgements: We thank Helga Nixdorf for excellent technical assistance and Dr. Beatrix Blume for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (NU 70/2-1) and Fonds der Chemischen Industrie.

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CMLS, Cell. Mol. Life Sci. 55 (1999) 167–182
1420-682X/99/020167-16 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Signal perception in plant pathogen defense

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Abstract. Highly sensitive and specific recognition systems for microbial pathogens are essential for disease resistance in plants. Structurally diverse elicitors from various pathogens have been identified and shown to trigger plant defense mechanisms. Elicitor recognition by the plant is assumed to be mediated by receptors. Plant receptors for fungus-derived elicitors appear to reside preferentially in the plasma membrane, whereas viral and bacterial elicitors may enter the plant cell and are perceived intracellularly. Receptor activation initiates an intracellular signal transduction cascade leading to stimulation of a characteristic set of plant defense responses. Isolation of plant elicitor receptors and their encoding genes is expected to provide significant information on the molecular basis of signal perception and intracellular signal generation in plant-pathogen interactions.

Key words. Avirulence gene; elicitor; elicitor receptor; pathogen defense; resistance gene; signal transduction.

Introduction

Adaptation to environmental conditions is a general feature of all living organisms. In particular, plants as sessile organisms have evolved sophisticated and unique mechanisms to tolerate nonoptimal life situations or to resist microbial pathogen attack. To trigger appropriate protective measures against invading pathogens plants need to distinguish between 'self' and 'nonself'. In contrast to antigen recognition and defense activation by the immune system of vertebrates, which is essentially based on the circulation and interaction of highly specialized cells throughout the whole organism, each plant cell is autonomously capable of sensing the presence of potential phytopathogens as well as mounting defense. Conceptual similarities between the immune response of vertebrates and activation of plant pathogen defenses have repeatedly been proposed [1, 2]. The structural basis of defense systems in organisms from either kingdom may, however, be quite different.

In their natural environment plants encounter a vast array of potential phytopathogens, such as viruses, bacteria, fungi and nematodes. Nevertheless, in the majority of cases plants withstand pathogen attack, and successful colonization of the plant is the exception rather than the rule in plant-pathogen interactions (nonhost resistance, species resistance). The reasons why a plant may be an inappropriate host for most potential phytopathogens are several [3]: (i) the plant does not support the lifestyle of an invading pathogen and thus does not serve as a substrate for microbial growth; (ii) plants possess preformed structural barriers or are equipped with antimicrobial compounds that prevent pathogen ingress and spread; or (iii) the plant may recognize the pathogen and initiate its endogenous multicomponent defense system. Attempted infection of a nonhost plant by a particular pathogen may thus not necessarily be counteracted by an active plant defense response. It is yet unknown to what extent induced defense responses contribute to nonhost resistance in plants. Infrequent changes in the host range of phytopathogens over recorded history [4] indicate relative genetic stability of nonhost resistance. This is likely due to functionally redundant signal perception and plant defense mechanisms, which may constitute the molecular basis of this particular type of resistance. Generally, the genetic determination of nonhost resistance is poorly understood.

Only in the minority of cases have pathogens developed effective mechanisms for circumventing the defense machinery of a particular plant species, allowing them to successfully colonize the species (basic or host susceptibility). Particular cultivars of these species, however, possess the ability to recognize certain strains or races

of the pathogen species and, consequently, mount an efficient resistance response (host resistance, race/cultivar-specific resistance) [4–6]. Race-specific pathogen recognition is determined by the action of complementary pairs of (semi)dominant resistance (*R*) genes in the host plant and (semi)dominant avirulence (*avr*) genes in the pathogen [5, 6]. Lack or nonfunctional products of either gene would result in colonization of the plant. Genetic evidence for this gene-for-gene relationship was first provided by Flor's [7] pioneering work on the interaction between flax and the causal agent of flax rust, the phytopathogenic fungus *Melampsora lini*. A biochemical interpretation of this gene-for-gene concept implies a receptor/ligand-like interaction between plant *R* gene products and the corresponding *avr* gene products from the pathogen [6, 8].

The spectrum of reactions elicited in plants undergoing either type of resistance is complex, but nevertheless strikingly similar. Plant defense mechanisms include processes that result from transcriptional activation of defense-related genes, such as production of lytic enzymes, phytoalexin biosynthesis and systemic acquired resistance [3, 9]. Other plant responses associated with pathogen defense result from allosteric enzyme activation initiating cell wall reinforcement by oxidative cross-linking of cell wall components, apposition of callose and lignins, and production of reactive oxygen intermediates [3, 9–11]. The molecular basis for a very frequently observed highly localized response, hypersensitive cell death, is still uncertain. A fundamental requirement for the activation of the plant's surveillance system are highly sensitive and specific perception mechanisms for microbial pathogens. A widely accepted hypothesis assumes that activation of plant defense in incompatible plant-microbe interactions results from recognition by the plant of either cell surface constituents of the pathogen or factors that are produced and secreted by the pathogen upon contact with the host plant. Plant-derived elicitors released from the plant cell wall by fungal hydrolytic enzymes are thought to act in a way similar to pathogen-derived elicitors [9, 12]. Accumulating evidence indicates that high-affinity receptors for pathogen-derived signals do function either at the plant cell surface or intracellularly [6, 9, 13], mediating conversion of an extracellular signal into an intracellular signal. Subsequently, an intracellular signal transduction cascade is initiated, triggering activation of the defense arsenal of the challenged host plant cell [3, 9].

Although still fragmentary, our knowledge of the precise molecular mechanisms underlying nonself recognition and intracellular signal transduction in plant-pathogen interactions is rapidly expanding. Instead of attempting comprehensive coverage of the whole field I therefore focus on recent discoveries in selected well-studied experimental systems which are representative of a much larger number of antagonistic plant-microbe interactions. Further information on topics which receive limited attention here can be obtained from excellent reviews published recently [2, 3, 6, 9–11, 13–18].

Elicitors of plant pathogen defense

The term 'elicitor', originally coined for compounds that induce accumulation of antimicrobial phytoalexins in plants, is now commonly applied to agents stimulating any type of defense response [9, 19]. Elicitors of diverse chemical nature and from a variety of different plant pathogenic microbes have been characterized and shown to trigger defense responses in intact plants or cultured plant cells. These elicitors include (poly)peptides, glycoproteins, lipids and oligosaccharides (table 1). While the first elicitors characterized were predominantly oligosaccharides [20], research over recent years has revealed a multitude of viral, bacterial or fungal (poly)peptides, respectively, which trigger initiation of plant pathogen defense [9, 21–23]. The tremendous structural diversity of purified elicitors rules out the existence of a universal structural motif as a general signal for initiation of plant pathogen defense [9, 24].

Plant pathogen resistance occurs at the cultivar or species level, and is believed to be mediated by recognition of race-specific or race-nonspecific (general) elicitors, respectively [6, 8, 9]. General elicitors stimulate defense responses in all cultivars of at least one plant species or even at the plant family level. In contrast, according to the gene-for-gene hypothesis, race-specific elicitors are considered to be either direct or indirect products of avirulence genes, conferring resistance only to host plant cultivars carrying the corresponding resistance gene [4–6]. Race-specific elicitors have indeed very often been found to be direct products of *avr* genes [6, 15, 21]. An exception to this rule are syringolides, which are glycolipid elicitors produced by gram-negative bacteria expressing *Pseudomonas syringae* pv. *tomato* avirulence gene *D* (*avrD*) [25, 26]. The *avrD* gene product directs a genotype-specific hypersensitive response (HR) in soybean plants carrying the *R* gene *Rpg4*. It likely encodes an enzyme involved in syringolide biosynthesis.

The intrinsic function of elicitors in the life cycle of phytopathogenic microorganisms often remains elusive. Fungal elicitors of the general type appear to be constitutively present in the cell wall, for example as structural components. In contrast, harpins, a class of bacterial elicitors of the hypersensitive response in nonhost plants as well as in resistant genotypes of some host plants, are produced and secreted only upon con-

Table 1. Elicitors of plant defense responses and their putative receptors.

Source of elicitor	Elicitor, elicitor type	Biological response stimulated	Characteristics of the elicitor receptor	Reference
Cladosporium fulvum	28-mer polypeptide AVR9, race/cultivar-specific	leaf necrosis in resistant tomato cultivars (*CF-9*)	$K_d = 70$ pM	108
Phytophthora cryptogea	10.2-kDa β-elicitin cryptogein, general	leaf necrosis in tobacco leaves and SAR	$K_d = 2$ nM	121
Phytophthora sojae	hepta-β-glucan, general	phytoalexin production in soybean	$K_d = 3$ nM; $K_d = 0.7$ nM	118, 119
Phytophthora sojae	42-kDa glycoprotein, 13-mer fragment Pep-13, general	furanocoumarin phytoalexin production in parsley	$K_d = 2.4$ nM $M_r = 91$ kDa	94
Pseudomonas syringae pv. *glycinea*	syringolide 1, indirect product of *avrD*, race/cultivar-specific	HR in resistant soybean cultivars (*Rpg4*)	$K_d = 8.7$ nM	115
Pseudomonas syringae pv. *syringae*	$harpin_{Pss}$, HrpZ, general	HR in tobacco leaves	cell wall binding site	117
Pseudomonas syringae pv. *tomato*	Avr Pto	HR in resistant tomato cultivars (*Pto*)	cytoplasmic serine/threonine kinase Pto	51, 52
Puccinia graminis f. sp. *tritici*	67-kDa peptidoglycan, general	lignification in wheat leaves	$K_d = 2$ μM $M_r = 30$ kDa	139
Trichoderma viride	22-kDa endoxylanase, general	leaf necrosis in tobacco and tomato, phytoalexin synthesis	$K_d = 6.2$ nM $M_r = 44$ kDa	122
Yeast	8-mer glycopeptide fragment from invertase (gp8 c), general	ethylene production, PR gene activation	$K_d = 3.3$ nM	123
Various fungi	fungal chitin fragments, general	alkalinization response in tomato cells	$K_d = 1.4$ nM	105
	N-acetylchitooligosacharides, general	phytoalexin production in rice	$K_d = 5.4$ nM	124

tact with the plant or under experimental conditions mimicking the apoplastic space of plants [13, 21].

Race-specific elicitors are very often synthesized and secreted only upon infection of the host plant. Therefore a role as pathogenicity factor has been ascribed to race-specific elicitors. A number of bacterial *avr* genes were shown to be indispensible for full virulence in compatible host plants [21, 27–29]. The *avrBs2* gene from *Xanthomonas campestris* pv. *vesicatoria* appears to be important for the pathogen to cause disease on its host, pepper, as deletion of the gene resulted in reduced pathogenicity [30]. Sequence similarity of the *avrBs2* gene product to *Agrobacterium tumefaciens* agrocinopine synthase suggests a role of this protein in pathogen nutrition during infection [31].

Similarly, the *avrNIP1* gene from the barley leaf scald-causing fungus, *Rhynchosporium secalis*, was shown to contribute to virulence of the pathogen on susceptible barley cultivars [32]. A function in pathogenicity may, however, not hold true for all fungal *avr* genes, as deletion did not always reduce virulence of the transformants. A possible role of fungal avirulence genes for development, reproduction or general fitness under field conditions has instead been suggested [33]. Obviously, the role of *avr* genes and their gene products as avirulence determinants is coincidental and disadvantageous for the pathogen. This situation may reflect rather intriguingly the plant's ability to recognize a pathogen and activate its pathogen defense machinery through components that are important (if not indispensible) for the life cycle of the pathogen [6, 15].

In the few cases investigated elicitor activity was found to be determined by small fragments of the intact elicitor molecule, suggesting recognition of 'epitope'-like structures by receptors at the plant cell surface. The cultivar or species specificity of elicitors, their ability to induce plant defense responses at trace amounts as well as data from structure-activity relationship studies performed with race-specific or general elicitors, respectively, support the idea that highly specific plant receptors mediate nonself recognition in plant-pathogen interactions [3, 9].

Products of avirulence genes

Viral coat protein (CP), replicase as well as movement protein have all been shown to be determinants of avirulence in the interaction of tobacco mosaic virus

(TMV) with its host plants, tomato and tobacco [34–36]. Based on the three-dimensional structure of the TMV coat protein specific amino acid substitutions were introduced into different structural areas of the elicitor. This allowed identification of elicitor domains essential for host recognition and subsequent activation of HR in *Nicotiana sylvestris* cultivars carrying the *N′* resistance gene [36, 37]. All mutations that impaired the ability of the mutant protein to cause HR could be located to the right face of the CP's α-helical bundle. The amino acid composition of this region is characteristic of recognition surfaces known from various proteins, which suggests binding of the *avr* gene product to a host-encoded receptor. Despite overall sequence divergence among different alleles of CP, this motif was found to be highly conserved in a number of avirulent viral strains tested [38].

More than 30 bacterial avirulence genes, mostly from the genera *Pseudomonas* and *Xanthomonas*, have been cloned and proven to be determinants of incompatibility in the interaction between bacteria and resistant host plant cultivars [39]. Comparison of *avr* gene sequences with sequences present in databases has failed to deduce the biochemical function of the encoded gene products. Bacterial *avr* genes encode hydrophilic, soluble proteins which lack an N-terminal leader sequence. Immunolocalization and biochemical fractionation studies demonstrated cytoplasmic localization of the *X. campestris* pv. *vesicatoria* AvrBs3 and *X. oryzae* pv. *oryzae* AvrXa10 proteins [40, 41]. In addition, when injected into the intercellular space of resistant host plants, these proteins as well as AvrB from *P. syringae* pv. *syringae*, did not induce HR [39, 42, 43]. The site of action of bacterial *avr* gene products has therefore remained uncertain for a long time.

The ability of bacteria to elicit a hypersensitive response in nonhost plants and to cause pathogenicity in susceptible host plant cultivars is controlled by clustered *hrp* genes [44]. HR induction in resistant host plant cultivars not only requires living bacteria [39] as well as bacterial *avr* genes matching particular host plant *R* genes but was also found to be dependent on *hrp* genes [22, 45]. For example, constitutive expression *of X. campestris* AvrBs3 or hyperexpression of *P. syringae* AvrB, respectively, in *hrp*-deficient bacteria did not result in HR induction in resistant host plant cultivars [43, 46]. Sequencing a large number of *hrp* genes from different phytopathogenic bacteria revealed significant homologies of some of these genes to components of a type III protein secretion pathway, recently discovered in animal pathogens of the genera *Yersinia*, *Shigella* and *Salmonella* [22, 47, 48]. In addition, colinearity in the genomic organization of these so-called *hrc* genes (a particular subset of *hrp* genes) [49] and of *Yersinia* genes (*ysc*), encoding components of this protein secretion apparatus, was observed [50]. Our understanding of the mechanism of bacterial phytopathogenicity has since then substantially profited from the discovery that mammalian pathogenic bacteria translocate pathogenicity factors directly into the host cell, employing this *ysc*-encoded protein secretion and delivery apparatus [22]. To prove whether phytopathogenic bacteria would be able to induce resistance upon expression of *avr* genes *in planta* suddenly became the experiment to perform. *Agrobacterium*-mediated transient or stable expression of bacterial *avr* genes in resistant host plant cultivars as well as biolistic transient expression of *avr* genes *in planta* was carried out [13, 22]. Indeed, induction of HR could be demonstrated when *avrPto*, an avirulence gene from *P. syringae* pv. *tomato*, which causes HR in tomato as well as tobacco plants carrying the corresponding *R* gene (*Pto*), and *avrBs3* from *X. campestris*, the determinant of HR induction on pepper cultivars carrying *R* gene *Bs3*, were expressed inside the host plant [42, 51, 52]. Importantly, transgenic *Agrobacterium*-mediated HR induction in host plants was T-DNA-dependent, indicating that expression and recognition by the host occurred inside the infected plant. Stable expression of *avrB* from *P. syringae* pv. *glycinea* and *avrRpt2* from *P. syringae* pv. *tomato*, respectively, confirmed the hypothesis of intracellular recognition of bacterial *avr* genes in host plants [43, 53]. This is intriguing evidence for the plant's ability to trigger endogenous defense mechanisms upon recognition of a part of the aggressive arsenal of attacking pathogens. Translocation of *avr* gene products into host cells also suggests that bacterial avirulence determinants may in the first place act as pathogenicity factors which utilize a sophisticated translocation system to invade host cells.

Bacterial avirulence gene products have not been scrutinized to the same extent as viral or fungal elicitors, respectively, for amino acids essential for recognition by target proteins within the host. However, motifs have been identified within these products that determine specificity in the interaction with host plants. A prominent example is *X. campestris* AvrBs3, which carries an internal region consisting of 17.5 nearly identical 34-amino acid repeats [54]. Deletions of repeats not only rendered *X. campestris* strains virulent on pepper lines carrying *Bs3* but revealed a new resistance specificitiy for an *avrBs3* deletion mutant on a pepper line susceptible to wild-type *avrBs3* [54]. The number of structural repeats may therefore determine specificity in this particular interaction. Furthermore, functional nuclear localization signals have been identified within members of the *avrBs3* family, suggesting specific interaction of the corresponding gene products with constituents of the nuclear import machinery [42, 55].

While bacterial *avr* gene products are believed to act predominantly intracellularly, fungal *avr* gene products appear to be perceived at the host plant cell surface. In contrast to similar experiments performed with purified bacterial *avr* gene products or total bacterial protein extracts, infiltration of purified fungal race-specific elicitors into the host plant intercellular space resulted in activation of plant defense [22, 32, 56, 57]. It is thus conceivable that plant receptors for race-specific fungal elicitors may preferentially reside in the plasma membrane of host plant cells.

Two *avr* gene products from the tomato pathogen *Cladosporium fulvum* (AVR4 and AVR9, respectively) act as elicitors of hypersensitive cell death on tomato cultivars carrying the matching resistance genes *Cf-4* and *Cf-9*, respectively [56, 57]. Gene complementation and disruption experiments have unequivocally proven the role of the *avr4* and *avr9* gene products as determinants of race/cultivar-specific resistance in this interaction [56–58]. Expression of the *avr4* and *avr9* genes is specifically induced during pathogenesis [56, 57]. However, *avr4* and *avr9* gene products appear to be dispensible for pathogenicity of the fungus [59].

Fungal strains virulent on tomato *Cf-9* cultivars completely lack the *avr9* gene [57], whereas virulence on *Cf-4* cultivars results from single point mutations in the coding region of the *avr4* gene [60]. Transcripts of mutant *avr4* alleles were found in all virulent fungal strains tested upon infection of *Cf-4* tomato cultivars, but elicitor protein was immunologically undetectable. However, infection of *Cf-4* plants with potato virus X hyperexpressing mutant *avr4* alleles resulted in HR induction. Thus, these alleles appear to encode potentially active elicitors. It is thus concluded that instability of AVR4 isoforms produced by virulent *C. fulvum* races *in planta* are crucial factors in circumvention of *Cf-4*-mediated resistance [60].

AVR4 and AVR9 are synthesized as larger precursors [56, 61]. Upon secretion both peptides are proteolytically processed by either fungal or plant proteases, yielding 105-mer and 28-mer polypeptides, respectively. Elicitor activity of both peptides was shown to depend on disulfide bridge formation between cysteine residues. Two-dimensional (2D) ^{1}H-NMR studies on the secondary structure and global fold of AVR9 revealed a rigid, barrel-like structure containing three antiparallel β-sheets connected by two loops and three disulfide bridges linking all six cysteine residues in a cystine knot [62]. This structural motif is also found in proteinase inhibitors or animal growth factors which are known to interact with specific target proteins, such as enzymes or receptors. Substitution of single amino acids within AVR9 revealed residues that are essential for elicitor activity. Particularly, the hydrophobic β-loop of AVR9 appears to be crucial for necrosis-inducing activity in *Cf-9* tomato cultivars [63].

Certain races of the barley pathogen *Rhynchosporium secalis* secrete a small protein, NIP1, that acts as a race-specific elicitor of defense gene activation in barley cultivars carrying the resistance gene *Rrs1* [32, 64]. Proof of avirulence gene function of the *nip1* gene was provided by gene disruption and gene complementation experiments [15, 32]. Replacement of the *nip1* gene by a nonfunctional gene in an avirulent race yielded virulent transformants (W. Knogge, personal communication). Transformation of virulent races of the fungus with the *nip1* gene rendered the transformants avirulent only on barley cultivars carrying the *Rrs1* gene. This was further substantiated by experiments in which purified NIP1 protected a barley cultivar carrying *Rrs1* against infection by a virulent fungal race lacking a functional *nip1* allele [32]. Avirulence of fungal races on *Rrs1* plants consistently correlated with the production of elicitor-active NIP1. In contrast, virulent races either lack the *nip1* gene or possess a *nip1* allele in which single nucleotide exchanges rendered the corresponding gene product elicitor-inactive [32].

On susceptible barley cultivars, fungal *nip1* disruption transformants exhibited reduced levels of virulence compared with NIP1-expressing wild-type races, suggesting a role of NIP1 as virulence factor [15]. This is corroborated by the nonhost-specific necrosis-inducing activity of this peptide on all barley cultivars as well as on various mono- and dicotyledonous plants [15, 65]. NIP1 exerts its toxic activity partially through indirect activation of the plasma membrane H^+-ATPase [65]. Thus, NIP1 may simultaneously act as a general virulence factor and, additionally, as an avirulence factor in resistant barley cultivars. The quantities of NIP1 required to trigger necrosis in barley leaves appear to be substantially higher than those required for defense gene activation in resistant barley cultivars (W. Knogge, personal communication). Intriguingly, resistant host plant cultivars may have acquired the ability to recognize a pathogen through tolerable nontoxic amounts of a fungal virulence factor. At the molecular level, this would be consistent with the existence of two distinct NIP1 receptors differing largely in their ligand affinities. The 82-amino acid product of the *nip1* gene is processed to yield a 60-amino acid mature protein [32]. NIP1 contains ten cysteine residues whose distribution within the complete amino acid sequence is reminiscent of fungal hydrophobins [66]. These cysteines form disulfide bridges which are required for both the elicitor and toxin activity of this peptide (V. Li and W. Knogge, personal communication).

Two elicitor peptides from the rust fungus *Uromyces vignae* that induce hypersensitive cell death in resistant cowpea cultivars have been purified to homogeneity [67]. These peptides are the first race-specific elicitors to be isolated from an obligate biotrophic fungus. The

heat-stable, acidic and hydrophobic peptides did not show significant sequence similarity to any known protein. Unlike other fungal race-specific elicitors, these peptides lack cysteine residues. A striking feature of these elicitors is the presence of proline-rich regions, which may define a rapid and strong protein-binding capacity.

Particular races of the rice blast pathogen *Magnaporthe grisea* possess the *avr* gene *AVR2-YAMO*, which renders these races avirulent on rice cultivars carrying the corresponding *R* gene. *AVR2-YAMO* encodes a 223-amino acid protein with homology to neutral Zn^{2+}-proteases [15, 68]. Point mutations in the putative protease active site were found in virulent isolates of the fungus. It is therefore tempting to propose a role of AVR2-YAMO in the generation of an active elicitor rather than being itself an elicitor [6]. AVR2-YAMO may therefore resemble AvrD from *P. syringae* pv. *tomato* [25].

General elicitors

Gram-negative phytopathogenic bacteria of the genera *Erwinia*, *Pseudomonas* and *Ralstonia* produce and secrete proteinaceous elicitors, collectively termed harpins, in an *hrp*-dependent manner. Harpins have been identified from *E. amylovora* ($harpin_{Ea}$) [69], *E. chrysanthemi* ($harpin_{Ech}$) [70], *P. syringae* pv. *syringae*, *tomato* and *glycinea* ($harpin_{Pss, Pst, Psg}$) [71, 72], and from *R. solanacearum* (PopA1 and PopA3) [73]. Although quite dissimilar in sequence, harpins commonly induce a hypersensitive response upon infiltration into nonhost plants, such as tobacco or in some cases in resistant cultivars of host plants [13]. Harpins are therefore most likely not determinants of host range in plant-bacteria interactions. $Harpin_{Pss}$ has also been shown to trigger systemic acquired resistance (SAR) in cucumber [74] as well as expression of a number of *hrp*-dependently induced HR-associated tobacco genes (*hin* genes) [75]. Harpin-induced HR in tobacco appears to be an active plant response that depends on gene transcription and protein synthesis [71, 76]. At present, the endogenous function of harpins remains unclear [13]. Mutations in the genes encoding $harpin_{Ea}$ or $harpin_{Ech}$, respectively, significantly reduced pathogenicity of the mutant strains, whereas in similar experiments with PopA1 no reduction in pathogenicity was observed [69, 70, 73]. It remains to be seen to which extent structurally unrelated harpins contribute either directly or indirectly to host colonization in compatible plant-pathogen interactions. Undoubtedly, phytopathogenic bacteria may take selective advantage of harpins. Otherwise, the genes encoding harpins most likely would have been eliminated during evolution.

When infiltrated into tobacco leaves, $harpin_{Pss}$ elicits necrosis which is indistiguishable from the HR elicited by bacteria [71]. Furthermore, HR-inducing activity of various harpins is heat-stable and may therefore not be due to enzyme activity [13]. This rules out generation by harpins of plant-derived elicitors such as oligogalacturonides, which have been shown to trigger a series of defense responses in tobacco cells [77]. Harpins are highly hydrophilic proteins, and thus are unlikely to cross the plant plasma membrane. In addition, harpins activate a number of rapidly induced defense responses that are known to be receptor-mediated in other systems. Taken together, these arguments strongly suggest a direct action of harpins at the plant cell surface. The carboxy-terminal 148-amino acid portion of $harpin_{Pss}$ was identified to be neccessary and sufficient for elicitor activity [71]. Within this moiety two directly repeated sequences were defined which, when individually deleted, rendered the recombinant product elicitor-inactive. This apparent signal specificity suggested a receptor/ligand-like interaction of harpin with its putative plasma membrane target site. In a similar approach nonoverlapping, His-tagged portions (N-terminal 109 amino acids and C-terminal 216 amino acids, respectively) of the $harpin_{Pss}$-encoding gene, *hrpZ*, were expressed in *E. coli* [78]. Surprisingly, infiltration of the purified recombinant proteins in tobacco plants resulted in HR induction with either product. This led the authors to conclude that elicitor activity resides in multiple regions of HrpZ, a concept that is rather difficult to reconcile with a true ligand/receptor interaction.

Since the first barrier invading fungi have to overcome is the plant cell wall, fungal endohydrolytic enzymes have been suggested to act as elicitors of the general type [12]. However, elicitor activity of a *Trichoderma viride* endoxylanase stimulating HR, ethylene and phytoalexin production in tobacco and tomato was found to be independent of enzyme activity [79]. On the other hand, research on fungal endopolygalacturonases has revealed that these enzymes release elicitor-active oligogalacturonide fragments from the plant cell wall, rather than being elicitors of defense themselves. This intriguing concept of plant-derived (endogenous) elicitors activating pathogen defense is very likely to function in many plant-pathogen interactions. Most plants possess a cell wall polygalacturonase-inhibiting protein (PGIP) which can physically interact with fungal cell wall endopolygalacturonases. This interaction may favor release of elicitor-active oligogalacturonides from the plant cell wall over complete depolymerization of cell wall polygalacturonides [12].

Elicitins constitute a family of highly conserved, nonglycosylated 10-kDa proteins that are present in the entire *Phytophthora* genus (except in some highly virulent isolates of the tobacco pathogen *P. parasitica* var.

nicotianae, *Ppn*) as well as some *Pythium* species [80–82]. Elicitins stimulate HR-like leaf necrosis in tobacco, other *Nicotiana* spp. and apparently in a cultivar-specific manner in some radish and turnip cultivars [80, 83–85]. HR-like necrosis is accompanied by systemic protection of the plant against subsequent infection with virulent *Ppn* isolates or the unrelated pathogen, *Sclerotinia sclerotiorum* (SAR) [85, 86]. Induction of SAR, however, is not dependent on the presence of elicitins in leaves remote from the site of elicitor application [86]. Recently, a low-molecular weight diffusible signal was found to be released from cultured tobacco cells treated with the *P. cryptogea* elicitin, cryptogein [87]. This compound is capable of triggering activation of the same defense genes as cryptogein, but in cells which are not in intimate contact with this elicitor. Thus, cells directly stimulated by fungal elicitors appear to secrete secondary signal molecules that activate defense responses in neighboring cells, thereby amplifying the overall response of challenged plants.

The virulence of *Ppn* on tobacco is inversely correlated with elicitin secretion, implying that elicitins are avirulence factors acting as genus-specific determinants in this plant [83, 88]. This has recently been demonstrated by an elegant approach to inhibit elicitin production in *P. infestans* by gene silencing. Fungal mutants incapable of producing elicitin became highly virulent on *N. benthamiana*, a nonhost plant to wild-type *P. infestans* (S. Kamoun and F. Govers, personal communication).

Elicitins fall into two classes according to their leaf necrosis-inducing activity. Acidic α-elicitins, such as capsicein (from *P. capsici*), are 100-fold less toxic than basic β-elicitins, such as cryptogein. Similarly, basic elicitins are 10–50-fold more active than acidic elicitins in inducing SAR in tobacco [85]. Use of recombinant structural derivatives of cryptogein revealed that point mutations consistently affected both HR- and SAR-inducing activity in the same way (I. Penot and P. Ricci, personal communication). Elicitation of necrosis and SAR appears therefore to be mediated by a single elicitin receptor. To identify domains within elicitins that are sufficient for elicitor activity, Perez et al. [89] used synthetic 10- to 18-mer peptides covering different parts of capsicein and cryptogein, respectively, as elicitors of HR and PR gene expression. This study, however, concludes that two different defense pathways are independently induced by different domains of elicitins.

The 10 elicitins sequenced so far share more than 60% sequence homology at the amino acid level [89]. Only very few residues were identified as key determinants accounting for much of the observed difference in necrotic activity of the two elicitin types [90]. Six conserved cysteine residues form three disulfide bridges crucial for necrotic activity [80, 91]. X-ray crystallography of cryptogein revealed a complex structure of six α-helices, an antiparallel two-stranded β-sheet, and an Ω-loop. This motif is assumed to be a recognition site for a putative receptor [92].

A 42-kDa cell wall glycoprotein of *Phytophthora sojae* induces transcriptional activation of defense genes and accumulation of furanocoumarin phytoalexins in parsley cell cultures and protoplasts [93, 94]. This response can be also observed in parsley seedlings upon infection with zoospores of the fungus [95]. HR induction and callose apposition observed in fungus-infected parsley seedlings could not be detected in elicitor-treated cell cultures. Single plant cells may therefore utilize different signals as well as nonoverlapping signal transduction pathways to trigger activation of subsets of the overall defense response.

Characterization of corresponding complementary DNA (cDNA) clones revealed that the gene encodes a 57-kDa precursor protein [96]. This suggests proteolytic processing of the gene product into the mature 42-kDa protein. An internal peptide of 13 amino acids (Pep-13) was found to be necessary and sufficient for elicitor activity of the intact glycoprotein [94–96]. The amino acid sequences of the oligopeptide and the intact protein elicitor did not show any significant homology to known sequences [96]. Substitution analysis, in which individual amino acids of Pep-13 were progressively replaced by alanine, identified only two residues critical for activity. ^{1}H-nuclear magnetic resonance (NMR) studies revealed a random-coil-like structure of the oligopeptide in aqueous solution (J. Vervoort, personal communication).

Branched (1-3, 1-6)-β-glucans from the mycelial wall of the same fungus, *P. sojae*, were identified to stimulate phytoalexin production in the host plant soybean in a non-race/cultivar-specific manner [9, 97]. Unlike the cell wall of other fungi the cell wall of *Phytophthora* species consists largely of β-glucans instead of chitin [98]. In a search to elucidate the minimal structural motif required for elicitor activity a branched hexa(β-D-glucopyranosyl)-D-glucitol was identified [99, 100]. Within that molecule the branched trisaccharide at the nonreducing end as well as a characteristic spacing between two branch points were found to be essential for elicitor activity [101].

Interestingly, phytoalexin production in parsley cells could exclusively be induced by proteinaceous components of a crude elicitor preparation from this fungus. In contrast, glucan fractions of the same elicitor preparation, but not proteinaceous components, activated the same response in soybean plants [102]. In both plants elicitor treatment triggers transcriptional activation of genes encoding enzymes of phytoalexin biosynthesis [103]. To test whether different signals from one pathogen may trigger similar signaling cascades in both plants, a reporter gene was fused to the promoter of a

glucan elicitor-inducible soybean gene, encoding such an enzyme. Protein elicitor treatment of parsley protoplasts transiently expressing this construct strongly stimulated reporter gene activity [104]. This finding points towards the existence of highly conserved signaling cascades operational in both plants.

Other oligosaccharide elicitors of various plant defense responses comprise fungal oligochitin fragments, oligochitosan and plant-derived oligogalacturonides [9]. Precisely defined chitooligosaccharide structures were again required for elicitor-mediated activation of plant defense responses, suggesting interaction of the ligand with a specific receptor site [105, 106].

Plant resistance genes: structure and function

Highly sensitive perception systems for either pathogen-derived (exogenous) or plant-derived (endogenous) elicitors are the key to successful plant pathogen defense. Plant receptors are instrumental for signal recognition and initiation of an intracellular signal transduction cascade mediating activation of multifaceted defense reactions, both in host and nonhost incompatible plant-pathogen interactions. Numerous plant elicitor receptors have been biochemically characterized, and kinetic parameters of ligand/receptor interactions have been determined (table 1).

It is hypothesized that host plant resistance genes encode receptors for ligands encoded either directly or indirectly by *avr* genes [6, 8]. Fundamental mechanistic differences in the site (and mode?) of recognition of viral/bacterial and fungal pathogens appear to exist. Phytopathogenic viruses enter host plants directly through wounds, and intercellular spread is mediated through trafficking of viral components via plasmodesmata [107]. A contact-dependent transfer mechanism appears to mediate delivery of *avr* gene products from phytopathogenic bacteria directly into host plant cells, where av*r* gene products are assumed to interact directly with corresponding plant *R* gene products [13, 22]. The molecular architecture of such perception systems for fungal *avr* gene products, however, appears to be different [59, 108]. Race-specific elicitors encoded by fungal *avr* genes appear to interact with receptor-like structures at the host cell surface [6, 15]. There is yet no evidence for internalization of fungal signals. Signal perception may instead trigger subsequent generation of an intracellular signal that initiates pathogen defense [6]. Recognition of fungal *avr* gene products may thus be mediated by integral plasma membrane receptors, whereas viral and bacterial elicitors are perceived intracellularly by possibly soluble receptors.

A key question remains whether plant *R* genes indeed encode receptors for pathogen-derived signals. There is overwhelming genetic evidence for plant *R* genes being specificity determinants in plant-pathogen interactions, but the biochemical function of the encoded proteins is often uncertain. Plant genes conferring resistance to the major classes of plant pathogens have now been isolated from various, taxonomically unrelated plant species by either positional cloning or transposon mutagenesis [2, 14, 16]. According to common structural motifs plant *R* genes can be divided into six classes [14]. A compilation of cloned plant resistance genes and their most prominent structural features is given in table 2. Domain similarities are observed in plant *R* genes conferring resistance against pathogens as diverse as viruses, bacteria, fungi and root-parasitizing nematodes. Thus, plant pathogen defense against a broad spectrum of pathogens may depend on common molecular mechanisms of signal perception and signal transduction. Moreover, it appears likely that signal transduction pathways triggered by different *R* gene products converge at some point downstream of signal perception. This possibility has recently been proven by the isolation of an *Arabidopsis* gene, *ndr1*, that is required for resistance against both bacterial and fungal pathogens, and may thus integrate different signals to activate a common defense response [109]. Several of these genes have been demonstrated to exist in plants and to be important for disease resistance [14, 18].

Sequence homology of *R* gene products to yeast and animal proteins of known function suggests a role for these proteins in signal perception and subsequent signal transduction. Isolated plant *R* genes share common sequence motifs such as transmembrane domains, nucleotide binding sites and imperfect repetitions of approximately 25 amino acids, termed leucine-rich repeats (LRR) [2, 14]. In particular, LRRs are thought to mediate protein-protein interactions and ligand binding in eukaryotic signal-transducing proteins [110]. LRRs are found in various proteins that differ in their function and cellular location. *R* genes carrying LRR but no membrane anchor (myristoylation site) or transmembrane domain may encode intracellular, soluble receptors (for examples see table 2). In contrast, *R* genes harboring LRR and transmembrane domains, such as tomato *Cf*-genes and the rice gene *Xa21* (which confers resistance against all strains of the phytopathogenic bacterium *X. oryzae* pv. *oryzae*), may encode (plasma) membrane receptors. A role of the Xa21 protein as plasma membrane receptor, however, is hard to reconcile with *hrp*-dependent injection of the avrXa21 protein into the host plant cell. Xa21 may therefore represent an intracellular membrane receptor. In general, determination of the cellular location of *R* gene products will definitely be crucial to elucidating the role of *R* gene products in signal perception and intracellular signal transduction. *Xa21* as well as the tomato *Pto* gene

Table 2. Structural features of isolated plant resistance genes. Classification of *R* genes was adapted from ref. 14.

Class	Plant *R* gene	Pathogen *avr* gene	Predicted functions, structural features and location of the plant *R* gene product	Reference
I	maize *HM1*	*Cochliobolus carbonum* race1	NADPH reductase	112
II	tomato *Pto*	*Pseudomonas syringae* pv. *tomato avrPto*	intracellular serine/threonine kinase	140
III a	*Arabidopsis RPS2*	*Pseudomonas syringae* pv. *tomato avrRpt2*	LZ/LRR/NBS, intracellular	141, 142
	Arabidopsis RPM1	*Pseudomonas syringae pv. maculicola avrRpm1/avrB*	LZ/LRR/NBS, intracellular	143
	tomato I_2	*Fusarium oxysporum* f.sp. *lycopersicon*	LZ/LRR/NBS, intracellular	144
III b	tobacco *N*	tobacco mosaic virus replicase (?)	LRR/NBS/Toll, intracellular	2, 145
	flax *L6*	*Melampsora lini AL6*	LRR/NBS/Toll, intracellular	146
	flax *M*	*Melampsora lini AM*	LRR/NBS/Toll, intracellular	147
	Arabidopsis RPP5	*Peronospora parasitica*	LRR/NBS/Toll, intracellular	148
IV	tomato *Cf-9*	*Cladosporium fulvum avr9*	intracellular LRR/TM	149
	tomato *Cf-2*	*Cladosporium fulvum avr2*	intracellular LRR/TM	150
	tomato *Cf-4*	*Cladosporium fulvum avr4*	intracellular LRR/TM	151
	tomato *Cf-5*	*Cladosporium fulvum avr5*	intracellular LRR/TM	14
V	rice *Xa-21*	*Xanthomonas oryzae* pv. *oryzae* (all races)	extracellular LRR/TM, cytoplasmic kinase domain	136
VI	barley *mlo* (recessive)	*Erysiphe graminis* f.sp. *hordei* (all races)	six membrane-spanning helices	114

Abbreviations used are: LZ, leucine zipper domain; LRR, leucine-rich repeat; NBS, nucleotide binding site; Toll, proteins with sequence homology to *Drosophila* Toll protein; TM, transmembrane domain.

(conferring resistance against *P. syringae* pv. *tomato*) possess a structural motif that is characteristic of serine/threonine protein kinases. This suggests a role of Xa21 in activating an intracellular signal transduction cascade in a receptor kinase-like manner. In contrast, Pto appears to be a soluble, cytoplasmic serine/threonine kinase with proven autophosphorylating as well as substrate-phosphorylating activity [111].

As stated earlier, biochemical evidence for receptor function of plant *R* genes has very rarely been presented. A reason for that may be that only in some systems have the corresponding *avr* genes and/or *avr* gene products been isolated [6]. The only plant/pathogen system in which physical interaction of the products of an *avr* gene and a plant *R* gene has been demonstrated is the *P. syringae* pv. *tomato*/tomato interaction [51, 52]. Taking advantage of the yeast two-hybrid system, the authors proved direct interaction of the *avrPto* and *Pto* gene products. Importantly, gene products of susceptible *pto* alleles lacked both the ability to induce pathogen defense and to bind to AvrPto protein, thus validating the specificity of this interaction. Since AvrPto is delivered into the host plant cell in an *hrp*-dependent manner [52], intracellular interaction of AvrPto and Pto may reflect the physiological situation.

However, unequivocal, direct evidence for the receptor function of a plant *R* gene product has yet to be presented. Alternative functions of plant *R* gene products in signal perception and intracellular signal transduction are discussed in the following section.

Two notable exceptions exist among isolated plant *R* genes, suggesting functions of the encoded proteins apart from signal perception and transduction. The maize *HM1* gene encodes an NADPH reductase which catalyzes degradation of a host-selective toxin produced by the phytopathogenic fungus *Cochliobolus carbonum* [112]. Detoxification of this pathogenicity factor renders the interaction of the fungus and otherwise susceptible maize cultivars incompatible. Recessive alleles of the barley *mlo* gene confer nonrace-specific resistance to the fungus *Erysiphe graminis* f. sp. *hordei* [113]. This finding is compatible with a negative control function of the Mlo protein in the onset of pathogen defense; absence of Mlo primes the responsiveness for the onset of multiple defense functions. The only sequence motif deducible from the gene sequence suggests formation of six membrane-spanning helices [114].

Elicitor receptors

Tremendous experimental effort has been undertaken to characterize the interaction between AVR9, the product of the *C. fulvum avr9* gene, and tomato cultivars with various resistance specificities. A saturable high-affinity binding site for AVR9 ($K_d = 0.07$ nM) was indistinguishably detectable in all tomato cultivars, including those lacking a functional *Cf-9* resistance gene [108]. Moreover, all solanaceous plant species tested possessed this binding site, whereas nonsolanaceous plants did

not. The presence of the AVR9 binding site correlated with the presence of members of the *Cf-9* gene family, but apparently not with the presence of a functional allele of this *R* gene [108]. Membrane preparations from transgenic *Arabidopsis* plants expressing the *Cf-9* gene did not show detectable binding of ^{125}I-AVR9, suggesting that under these conditions (expression and proper assembly of Cf-9 were not investigated in these transgenics) Cf-9 does not bind AVR9 [6]. Furthermore, synthetic mutant AVR9 peptides as well as AVR9 mutant peptides purified from PVX::AVR9-infected tobacco plants were used as competitors in binding assays and as elicitors of HR in resistant tomato cultivars (P. De Wit, personal communication). Since binding activity of these peptides always correlated with their HR-inducing activity, receptor function of the AVR9 binding site seems plausible. Taken together, these data appear to argue against the *Cf-9* gene product representing the receptor for the race-specific elicitor AVR9. Instead, the *Cf-9* gene product may be recruited by the AVR9-bound-receptor into a heteromeric complex to facilitate subsequent signal transduction and *Cf-9*-specific induction of plant defense, or may function downstream of signal perception.

It may, however, be possible that nonfunctional *Cf-9* homologs are still capable of binding AVR9 but lack the ability to initiate an intracellular signaling cascade. This would be consistent with the above-described findings, and propose a role of the Cf-9 protein as the AVR9 receptor. Alternatively, two types of AVR9 receptors with differing ligand affinities may exist [6]. A low-affinity binding site encoded by *Cf-9* would mediate cultivar-specific induction of plant defense, whereas the high-affinity binding site detected in kinetic studies [108] would serve another so far unknown purpose. To date, it remains open whether Cf-9 is implicated in AVR9 recognition or may constitute a downstream signaling element at an early rate-limiting step in the signal transduction cascade [6].

A similar situation can be envisaged in the case of syringolides which are glycolipid elicitors produced by *P. syringae* pv. *glycinea* expressing avirulence gene *avrD* [25]. The syringolides induce pathogen defense only on soybean cultivars carrying the *Rpg4* resistance gene. A proteinaceous, soluble, high-affinity binding site for ^{125}I-syringolide 1 ($K_d = 8.7$ nM) was detected in all soybean cultivars tested irrespective of their resistance specificities [115]. Using a series of structural derivatives of syringolide 1 in binding assays demonstrated a direct correlation between binding affinity to the soluble fraction and elicitor activity. Thus, the binding site fulfills several criteria of a bona fide receptor, but may not be the product of the *Rpg4* gene. Recently, a 34-kDa protein that accounted for syringolide 1-binding activity was isolated and shown to share homology with thiol proteases [116].

Harpin$_{Pss}$ from *P. syringae* pv. *syringae* elicits HR in the nonhost, tobacco [21]. Binding of this molecule to the periphery of cultured tobacco cells was detected but was undetectable in tobacco protoplasts [117]. Binding was dependent on extracellular Ca^{2+}, as EGTA-treatment of cultured cells abolished binding. However, specificity of the binding by competition experiments could not be demonstrated under the experimental conditions used. These results suggest that the cell wall is crucial for induction of HR in tobacco cells.

High-affinity receptors for fungal elicitors of the general type have been reported to reside in the plasma membrane of plant cells [9, 24]. A single class, high-affinity receptor for the *P. sojae*-derived oligopeptide Pep-13 has recently been identified in parsley membrane preparations and protoplasts ($K_d = 2.4$ nM) [94]. A binding site recognizing a hepta-β-glucan elicitor from the same fungus has been characterized in plasma membranes from soybean [118, 119] and other fabaceae [120]. Membrane binding sites of very similar ligand affinities have been reported for the *P. cryptogea* β-elicitin cryptogein [121] and for the *T. viride* endoxylanase [122] in tobacco, for yeast invertase glycopeptide fragment (gp8c) and chitin fragments in tomato [123], and *N*-acetylchitooligosaccharides in rice cells [124]. While significantly different from the K_d of the AVR9 receptor, these affinity constants appear to be characteristic for receptors of proteinaceous as well as nonproteinaceous general elicitors [9]. Another common feature of all elicitor binding sites is their low abundance, e.g. parsley cells harbor approximately 1600 Pep-13 binding sites per cell [94]. In general, kinetic properties of these elicitor-binding proteins, such as high affinity, saturability and reversibility of ligand binding together with a direct correlation between the binding affinities and the elicitor activities of the respective ligands, indicate that such elicitor binding sites function as physiological receptors. Use of structural derivatives of Pep-13 or gp8c, respectively, allowed demonstration of a functional link between signal perception and elicitation of a physiological response in parsley or tomato cells, respectively [94, 123]. The dual function of receptors, that is perception of an extracellular signal on the one hand and initiation of an intracellular signal transduction cascade on the other hand, is nicely exemplified by the tomato gp8c-glycopeptide receptor. While the carbohydrate moiety of gp8c was found to compete binding of intact gp8c to its receptor, ethylene production-inducing activity of gp8c was dependent on the intact ligand [123]. Thus, ligand domains responsible for signal recognition and intracellular signal generation can be structurally separated from each other in this molecule.

Chemical cross-linking experiments with homobifunctional or photoaffinity reagents, respectively, as well as labeled ligands have been performed to elucidate the

subunit structure of elicitor receptors. For example, a 91-kDa monomeric parsley plasma membrane protein was identified that most likely represents the Pep-13 receptor [125]. Similarly, a 75-kDa soybean plasma membrane protein representing (part of ?) the receptor for the *P. sojae* hepta-β-glucan elicitor was detected by means of photoaffinity labelling [126]. Very recently, purification of this soybean plasma membrane protein was reported [127, 128]. A cDNA encoding this protein was isolated and used for production of a recombinant receptor in *E. coli*, cultured tobacco cells [127] or baculovirus-infected insect cells (A. Mithöfer and J. Ebel, personal communication). Evidence for the recombinant protein representing the functionally intact hepta-β-glucan elicitor, however, is lacking. Current efforts are directed towards isolating elicitor receptors and cloning cDNAs encoding these molecules. Unfortunately, the apparent lack of specific plant target cells or tissues, in which the respective elicitor receptor is abundantly expressed, significantly hampers isolation of elicitor receptors.

At present no elicitor receptor has been isolated nor has receptor function directly been proven for any of the cloned plant resistance genes conferring resistance to microbial pathogens. In addition, molecular genetic analysis of various plant mutants impaired in pathogen defense has not revealed genes whose products may function as elicitor receptors [14].

Elicitor receptor-mediated intracellular signal generation

Different molecular genetic and biochemical strategies are currently pursued to identify and characterize signal transduction components mediating activation of plant pathogen defense [3, 14]. Targeted loss of function by constitutive sense or antisense suppression, or transposon tagging of particular genes, may eliminate individual elements of the multicomponent defense response of infected plants. This will help determine the contribution of these factors to overall resistance. Alternatively, random mutagenesis of resistant plants and screening for disease-sensitive mutants will lead to the identification of genes other than *R* genes, important for plant disease resistance. A general limitation to loss-of-function experiments, however, is functional redundancy in the targeted pathways or lethal effects of mutations. Negative regulators of plant defense responses may be identified by re-mutagenesis of mutants impaired in disease resistance (gain-of-function). T-DNA-activation tagging may prove similarly effective in identifying potential elements of signal transduction cascades in plant defense as in hormone signaling [129, 130]. Mutagenesis of transgenics homozygous for defense gene promoter-driven reporter genes may yield both mutants insensitive to pathogen-derived signals as well as mutants expressing reporter gene activity constitutively in the absence of any stimulus. Numerous genes encoding putative signal transduction elements have been identified this way, which are now being analyzed for their biochemical function. Experimental systems of reduced complexity, such as plant cell suspensions, have proven extremely valuable in characterizing very rapidly induced defense-associated plant responses. In addition, pharmacological dissection of signaling pathways involved in plant defense has extensively been carried out using cell cultures. This would not have been possible to the same extent in intact plants. Nevertheless, results obtained with cell cultures should be verified using intact plant tissue wherever possible [3].

Receptor function comprises signal perception and subsequent activation of downstream elements of signal transduction. Ligand binding to cell surface receptors is assumed to impose a conformational change on the receptor, resulting in initiation of an intracellular signal transduction cascade and finally in activation of a specific cellular response. Defined by the transduction mechanism involved, most cell-surface receptors of eukaryotic cells belong to either the class of ion channel-linked receptors, G protein-linked receptors or enzyme-linked receptors (e.g. receptor protein kinases). Use of specific antibodies and various pharmacological effectors has provided evidence for the involvement of heterotrimeric as well as small GTP-binding proteins, a number of serine/threonine protein kinases, elements of the MAP-kinase pathway and protein phosphatases in elicitor-mediated plant defense responses [3, 9, 131–134]. These elements are constituents of numerous well-defined signal transduction cascades in animal cells and are activated upon ligand binding to either G protein-linked receptors or receptor protein kinases. Plant signal transduction chains may therefore be connected to similar receptor types mediating extracellular signal perception and generation of an intracellular signal.

Plant cells carry cell surface proteins with intrinsic protein kinase activity. These plant receptor-like kinases (RLKs) structurally resemble receptor protein kinases of animal cells containing large extracytoplasmic domains, single transmembrane spanning segments and cytoplasmic kinase domains [135]. While animal receptor kinases are predominantly tyrosine kinases recognizing exclusively peptide ligands, plant receptor-like kinases are serine/threonine kinases whose cognate ligands are very often unknown. Plant RLKs are involved in many physiological processes, such as sporophytic self-incompatibility, plant growth and organogenesis, and cell differentiation [135]. The rice gene *Xa21* conferring resistance to the bacterial pathogen *Xanthomonas oryzae* encodes a receptor-like protein kinase [136]. It remains to be seen whether plant cells employ

functional RLKs to recognize phytopathogen-derived signals and trigger pathogen defense.

Cytoplasmic receptors for strain-specific bacterial elicitors have been identified for *P. syringae* pv. *tomato* AvrPto and *P. syringae* pv. *glycinea* syringolides, respectively [51, 115]. Pto, a cytoplasmic serine/threonine protein kinase, has been shown to physically interact with AvrPto [51], suggesting activation of an intracellular signaling cascade via protein phosphorylation of specific substrates. Using the yeast two-hybrid system, different putative substrates for Pto have been identified (Ptis), one of them, Pti1, being itself a serine/threonine kinase [137, 138]. Expression of a *Pti1* transgene in tobacco plants enhanced the hypersensitive response to a *P. syringae* pv. *tabaci* strain carrying the avirulence gene *avrPto*. These findings indicate that Pti1 is involved in a Pto-mediated signaling pathway, probably by acting as a component downstream of Pto in a phosphorylation cascade. Another three putative substrates of Pto identified in yeast two-hybrid screenings comprise proteins resembling transcription factors with homology to the tobacco ethylene-responsive element-binding proteins (EREBPs) [138]. Using a gel mobility-shift assay, these newly identified proteins were shown to specifically recognize and bind to a DNA sequence that is present in the promoter region of a large number of genes encoding PR proteins. Since expression of several *PR* genes and a tobacco *EREBP* gene is specifically enhanced upon AvrPto-Pto recognition in tobacco, these observations establish a direct connection between a disease resistance gene and the specific activation of plant defense genes. Intriguingly, Pto-mediated phosphorylation of transcription factors that trigger transcriptional activation of *PR* genes may thus represent a complete signal transduction cascade involved in activation of a subset of the plant defense arsenal.

Acknowledgments. I thank all my colleagues who authorized me to refer to their unpublished data, particularly Pierre De Wit, Jürgen Ebel, Francine Govers, Sophien Kamoun, Wolfgang Knogge, Volkhard Li, Axel Mithöfer, Isabelle Penot, Pierre Ricci and Jacques Vervoort. I do apologize to all my colleagues whose work could not explicitly be considered here due to limited space available. This work was supported by the Deutsche Forschungsgemeinschaft (grant NU 70/2-1).

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Plant Cell Physiol. 41(6): 692–701 (2000)

Phytophthora parasitica Elicitor-Induced Reactions in Cells of *Petroselinum crispum*

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Cultured parsley (*Petroselinum crispum*) cells respond to treatment with elicitors derived from different species of the genus *Phytophthora* with transcript accumulation of defense-associated genes and the production of furanocoumarin phytoalexins. Pep-25, an oligopeptide fragment of a *Phytophthora sojae* 42-kDa cell wall protein, and a cell wall elicitor preparation derived from *Phytophthora parasitica* (Pp-elicitor) stimulate accumulation of the same gene transcripts and formation of the same pattern of furanocoumarins. Treatment of cultured cells and protoplasts with proteinase-digested Pp-elicitor identified proteinaceous constituents as active eliciting compounds in parsley. Similar to Pep- 25, Pp-elicitor induced effluxes of K^+ and Cl^- and influxes of protons and Ca^{2+}. Concomitantly, as monitored in aequorin-transgenic parsley cell lines both elicitors induced an immediate increase in the cytoplasmic Ca^{2+} concentration up to sustained levels of 175 nM (Pp-elicitor) or 300 nM (Pep-25), respectively. The signature of the Ca^{2+} response differed greatly between the two elicitors tested. Extracellular Ca^{2+} proved essential for activation of an oxidative burst, MAP kinase activity and phytoalexin production by either elicitor. While Pp-elicitor induced a qualitatively similar spectrum of defense responses as did Pep-25, elicitor-specific quantitative differences in response intensity and kinetics suggest activation of a conserved signaling cascade through separate ligand binding sites.

Key words: Calcium — Oomycete — Oxidative burst — Parsley — Pathogen defense.

Activation of the plant defense arsenal is believed to be receptor-mediated through recognition of pathogen-derived elicitors (Yang et al. 1997, Scheel 1998). Receptor activation initiates an intracellular signal transduction cascade which leads to stimulation of a characteristic pattern of plant defense responses, comprising hypersensitive cell death, transcriptional activation of defense-related genes, local cell wall reinforcement, production of reactive oxygen intermediates, lytic enzymes, and antimicrobial phytoalexins as well as establishment of systemic acquired resistance (Hammond-Kosack and Jones 1996, Somssich and Hahlbrock 1998).

Structurally diverse fungus-derived elicitors comprising (glyco)proteins, peptides, and oligosaccharides have been shown to trigger defense responses in intact plants or cultured plant cells. Plant receptors for such elicitors appear to reside preferentially in the plasma membrane (Nürnberger 1999). Different experimental systems have been used to comprehensively study early elicitor-induced plant cell responses. Cell suspensions of tobacco (Bourque et al. 1998, Lebrun-Garcia et al. 1998, Yano et al. 1998), tomato (Felix et al. 1993, 1994, Xing et al. 1996), rice (Kuchitsu et al. 1997), soybean (Levine et al. 1994, 1996, Chandra and Low 1997, Delledonne et al. 1998, Mithöfer et al. 1999), carrot (Schwacke and Hager 1992, Bach et al. 1993) and parsley (Nürnberger et al. 1994, Kauss and Jeblick 1995, Jabs et al. 1997, Ligterink et al. 1997) have been employed to analyze immediate responses of plant cells to treatment with non-race-specific elicitors. In addition, tobacco plants transformed with the tomato resistance gene, *Cf-9*, and cell cultures established from these transgenic plants were used to analyze early elicitor-induced reactions of plant cells to the race-specific elicitor, AVR9, from *Cladosporium fulvum* (May et al. 1996, Piedras et al. 1998).

In most of the experimental systems investigated elicitor-stimulated ion fluxes concurringly comprise influxes of Ca^{2+} and H^+ as well as effluxes of K^+ and Cl^- (Scheel 1998). Accordingly, elevated levels of cytoplasmic Ca^{2+} (Knight et al. 1991, Chandra and Low 1997, Mithöfer et al. 1999) and cytoplasmic acidification (Mathieu et al. 1996, He et al. 1998) have been monitored in challenged cells. Extracellular Ca^{2+} appears to be crucial to induction of plant pathogen defense as absence of extracellular Ca^{2+}, or

Abbreviations: A-9-C, anthracene-9-carboxylic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; DDC, sodium diethyldithiocarbamate; DPI, diphenylene iodonium; IC_{50} value, inhibitor concentration causing half-maximal inhibition; IDP, diphenyl iodonium; MAP kinase, mitogen-activated protein kinase; ROS, reactive oxygen species.

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use of Ca^{2+} channel blockers did not only inhibit the targeted ion flux but also plant defense reactions in cultured cells of tobacco or parsley (Nürnberger et al. 1994, Tavernier et al. 1995, Jabs et al. 1997, Pugin et al. 1997, Zimmermann et al. 1997). Further constituents of signaling cascades mediating activation of plant pathogen reactions comprise reactive oxygen species (ROS) (Lamb and Dixon 1997), nitric oxide (Delledonne et al. 1998), lipid-derived metabolites (Chandra et al. 1996, May et al. 1996), GTP-binding proteins (Bischoff et al. 1999), serine/threonine protein kinases and phosphatases (Scheel 1998, Schenk and Snaar-Jagalska 1999), and MAP kinases (Jonak et al. 1999).

While these early elicitor-induced responses have been individually studied in numerous experimental systems, much less is known about the sequential order of these responses, their interdependence, and their role in the activation of distinct parts of the overall response of challenged plant cells (Scheel 1998). In addition, it has become increasingly clear that different elicitors may inaugurate independent signal transduction pathways that employ distinct sets of second messengers (Chandra et al. 1996, Scheel 1998).

We have previously reported that treatment of cultured parsley cells with elicitor preparations derived from different *Phytophthora* species results in transcriptional activation of defense-related genes as well as production and secretion of furanocoumarin phytoalexins (Kombrink and Hahlbrock 1986, Hahlbrock and Scheel 1996). Here we show that proteinaceous cell wall constituent(s) from the parsley pathogen, *Phytophthora parasitica*, activate a qualitatively similar spectrum of defense responses as the well-characterized oligopeptide elicitor Pep-25 derived from *Phytophthora sojae* (Nürnberger et al. 1994, Hahlbrock et al. 1995, Scheel 1998). Pronounced elicitor-specific differences in response kinetics and intensity suggest activation of a signaling cascade through separate perception systems.

Materials and Methods

Plant cell culture, oomycete growth, and treatment of cells with elicitors and inhibitors—Cell suspension cultures of *Petroselinum crispum* were propagated in the dark as previously described (Kombrink and Hahlbrock 1986). Protoplast preparation 5 d after subculturing, treatment with elicitor for 24 h, and quantification and thin-layer chromatographic analysis of furanocoumarins were performed according to Dangl et al. (1987). Cell viability was determined by double staining with fluorescein diacetate and propidium iodide (Jabs et al. 1997). Establishment of aequorin-transgenic parsley cell lines, reconstitution of active aequorin with coelenterazine and monitoring of bioluminescence was performed as described (Blume et al. 2000). *Phytophthora parasitica* strain 1828 was obtained from the German Collection of Microorganisms (Braunschweig, Germany). *P. parasitica* and *P. sojae* (race 1) were grown on agar plates or in liquid medium (Kombrink and Hahlbrock 1986). Pp and Ps-elicitor were prepared from hyphal cell walls (Ayers et al. 1976). The oligopeptide elicitor, Pep-25, was synthesized using Fmoc-chemistry (Nennstiel et al. 1998). A-9-C, DPI and IDP were applied as stock solutions dissolved in dimethyl sulfoxide (final solvent concentration, 0.1% v/v).

Proteinase treatment of elicitor—Aliquots of Pp-elicitor containing 1 mg ml^{-1} freeze-dried material were treated with 1 mg proteinase E or trypsin in 10 mM potassium phosphate, pH 6.5, 150 mM sodium chloride for 1 h at 26°C. The Pp-elicitor without added proteinase or with autoclaved proteinase was treated in the same way. After treatment samples were autoclaved to inactivate the proteinases. The samples were then added to parsley protoplasts at concentrations equivalent to 100 $\mu g\ ml^{-1}$ starting elicitor material.

RNA gel blot analysis—Total RNA from parsley cells was prepared as described (Dangl et al. 1987) and denatured with formaldehyde. RNA samples (15 μg) were electrophoresed in agarose using 3-(*N*-morpholino)propanesulfonic acid-EDTA buffer and transferred to nylon filters. UV crosslinking of RNA to the filter, prehybridization and hybridization were carried out according to Kawalleck et al. (1993). Digoxigenin-labeled cDNA probes were prepared by random prime labelling as described in the supplier's instruction (Boehringer Mannheim, Germany). RNA/DNA hybrids were visualized using a Storm 860 phospho imager (Molecular Dynamics, Sunnyvale, California).

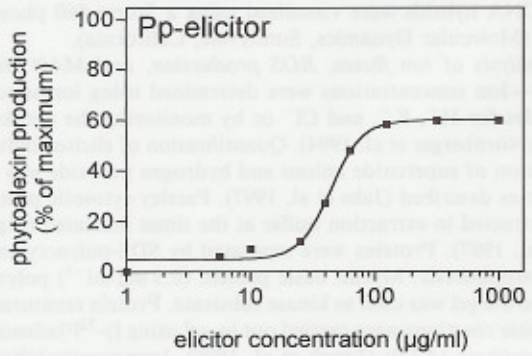
Analysis of ion fluxes, ROS production, and MAP kinase activity—Ion concentrations were determined using ion-selective electrodes for H^+, K^+, and Cl^- or by monitoring the uptake of $^{45}Ca^{2+}$ (Nürnberger et al. 1994). Quantification of elicitor-induced production of superoxide anions and hydrogen peroxide was carried out as described (Jabs et al. 1997). Parsley cytosolic proteins were extracted in extraction buffer at the times indicated (Ligterink et al. 1997). Proteins were separated by SDS-polyacrylamide gel electrophoresis. Myelin basic protein (0.5 mg ml^{-1}) polymerized into the gel was used as kinase substrate. Protein renaturation and kinase reactions were carried out in-gel using [γ-^{32}P]adenosine 5′-triphosphate (ATP) (Jonak et al. 1996). Immunoprecipitation of MAP kinase from parsley cell extracts was performed as described (Ligterink et al. 1997) with a polyclonal antibody raised against a synthetic peptide representing the C-terminal 10 amino acids of alfalfa MMK4 MAP kinase (Jonak et al. 1996).

Results

Defense-related gene activation and phytoalexin production—Treatment of cultured parsley cells or protoplasts with heat-released water-soluble cell wall fragments from the oomycete, *Phytophthora parasitica*, (Pp-elicitor) resulted in production and secretion of furanocoumarin phytoalexins. The total measurable amount of furanocoumarins was strongly dependent on the amount of elicitor added. The elicitor concentration necessary for half-maximal or maximum stimulation of phytoalexin formation in parsley protoplasts was 60 $\mu g\ ml^{-1}$ or 100 $\mu g\ ml^{-1}$, respectively. Corresponding concentrations of a *P. sojae*-derived cell wall elicitor (Ps-elicitor) were 2.2 or 10 $\mu g\ ml^{-1}$, respectively (data not shown). However, total amounts of phytoalexins produced in response to Pp-elicitor reproducibly represented approximately 60% of those produced

in response to treatment with Ps-elicitor or Pep-25, respectively (Fig. 1). Production of furanocoumarins increased linearly between 10 and 30 h after addition of elicitor. Parsley protoplasts remained viable for more than 50 h after the onset of elicitor treatment.

To examine whether Pp-elicitor activates the same defense-related genes as does the *P. sojae*-derived oligopeptide elicitor, Pep-25, total RNA prepared from elicitor-treated parsley cells was hybridized with digoxigenin-labelled cDNAs encoding phenylalanine ammonia lyase, 4-coumarate:coenzyme A ligase as well as the product of *eli12*, an elicitor-responsive gene of unknown function (Somssich et al. 1989). Fig. 2A shows that both elicitors indistinguishably induced expression of these genes. Messenger RNA levels of a constitutively expressed polyubiquitin gene from parsley (Kawalleck et al. 1993) were unaffected by elicitor treatment. Thin-layer chromatographic analysis of furanocoumarins secreted by parsley cells 24 h after addition of elicitor revealed that both elicitors stimulated synthesis of virtually the same pattern of phytoalexins (Fig. 2B). Major furanocoumarins produced in elicited parsley cells are bergapten, psoralen, isopimpinellin, xanthotoxin, umbelliferone, and marmesin.



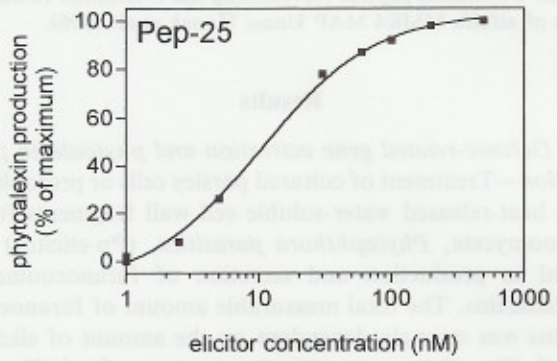


Fig. 1 Dose-response relationship of elicitor-induced phytoalexin production. Increasing concentrations of Pp-elicitor or Pep-25, respectively, were used to stimulate phytoalexin production in parsley protoplasts. Fluorimetric quantification of phytoalexins produced was performed 24 h after onset of treatment. Maximum phytoalexin production (100%) refers to that amount of which production was stimulated by 500 nM Pep-25. Each data point represents the average of triplicates.

Proteinase treatment of the Pp-elicitor—The Pp-elicitor consists of 80 µg protein and 330 µg anthrone-reactive carbohydrate per 1 mg freeze-dried material. Proteolytic digestion of the Pp-elicitor by proteinase E or trypsin resulted in a 96% or 88% reduction in elicitor activity, respectively, indicating that proteinaceous components are the elicitor-active structures within the cell wall of this oomycete (Table 1). Proteinase activity was completely inactivated by autoclaving the elicitor samples after treatment, ruling out adverse effects of the enzyme activity on parsley cell wall or plasma membrane proteins.

Activation of ion fluxes—Changes in permeability of the plasma membrane to H^+, Ca^{2+}, K^+ and Cl^- are among the earliest events detectable after treatment of parsley cells with both, Pp-elicitor and Pep-25 (Fig. 3). Stimulation of H^+, K^+ and Cl^- fluxes took place within 2–5 min after application of either elicitor (Fig. 3A–C). Net changes in ion concentrations were observed up to 30 min after addition of elicitor. At elicitor concentrations required to maximally activate ion fluxes and phytoalexin formation in parsley cells Pp-elicitor proved less efficient than Pep-25 with respect to activation of ion fluxes [35% (H^+), 65% (K^+), 70% (Cl^-) at 30 min after addition of elicitor]. Ca^{2+} uptake into parsley cells was stimulated within 2–5 min by either elicitor and lasted for about 15 min (Fig. 3D). Again, although phytoalexin response-saturating concentrations of either elicitor were used, Pp-elicitor stimulated only 30% of the increase in cell-associated Ca^{2+} when compared to Pep-25.

Table 1 Proteinaceous constituents of the Pp-elicitor determine its elicitor activity in parsley protoplasts

Sample	Phytoalexin accumulation (% of maximum)
Pp-elicitor only	100
Buffer only	0
Proteinase E only	1
Proteinase E + Pp-elicitor	4
Autoclaved Proteinase E + Pp-elicitor	95
Trypsin only	0
Trypsin + Pp-elicitor	12
Autoclaved Trypsin + Pp-elicitor	97

Furanocoumarin phytoalexin production in elicitor-treated parsley protoplasts was determined 24 h after application of 100 µg ml^{-1} Pp-elicitor. For protease digestions, 1 mg of Pp-elicitor was treated with 1 mg of proteinase E or trypsin in PBS buffer (10 mM potassium phosphate, pH 6.5, 150 mM sodium chloride). Samples were subsequently autoclaved to inactivate proteinases.

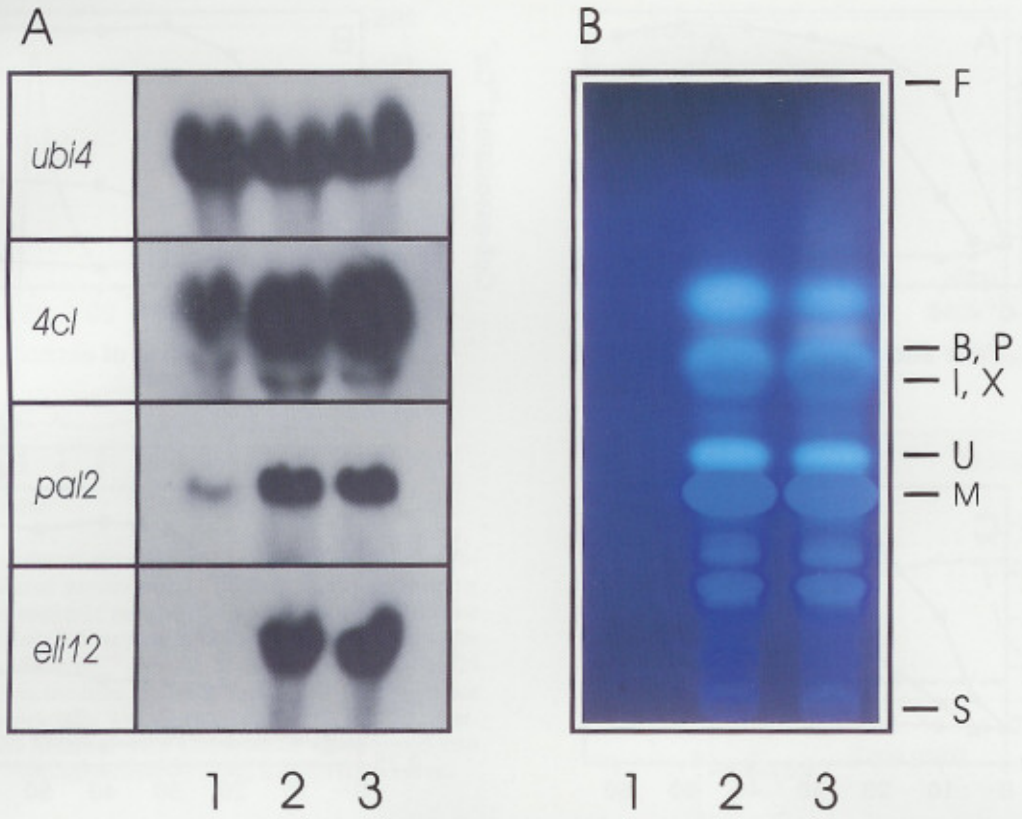


Fig. 2 Elicitor-induced accumulation of defense-related gene products and phytoalexin production. Accumulation of defense-related gene products (A) and furanocoumarin production (B) was analyzed in cultured parsley cells treated with water as control (lanes 1), 100 μg ml^{-1} Pp-elicitor (lanes 2), or 100 nM Pep-25 (lanes 3), respectively. Total RNA isolated from parsley cells treated for 3 h with elicitor or water was separated electrophoretically, transferred to nylon membranes, and subsequently hybridized to digoxigenin-labeled cDNAs complementary to mRNAs encoding polyubiquitin (*ubi4*), 4-coumarate:coenzyme A ligase (*4cl*), phenylalanine ammonia lyase 2 (*pal2*), and elicitor-responsive *eli12*. DNA/RNA hybrids were detected by fluoro imaging. Furanocoumarin phytoalexins were extracted from the culture medium of parsley cells treated for 24 h with either water or elicitor and analyzed by thin-layer chromatography. Co-chromatography of standard compounds is indicated (B, bergapten; P, psoralen; I, isopimpinellin; X, xanthotoxin; U, umbelliferone; M, marmesine; S, start; F, solvent front).

Elevation of cytoplasmic free calcium—In parsley cell lines stably expressing aequorin an elicitor-induced increase in cytosolic Ca^{2+} could be monitored (Fig. 4). However, the Ca^{2+} signature mediated by either elicitor varied significantly. Pp-elicitor-induced elevation of cytosolic Ca^{2+} levels started within 40–60 s upon elicitation and increased slowly from basal levels of 40 nM Ca^{2+} up to sustained levels of approximately 175 nM Ca^{2+} within 15 min. In contrast, Pep-25-induced increase in cytosolic Ca^{2+} peaked after 2–3 min at approximately 800 nM Ca^{2+} and subsequently declined to a sustained level of approximately 250–300 nM Ca^{2+}.

To investigate whether extracellular Ca^{2+} is required for elicitor-stimulated phytoalexin formation, thoroughly washed parsley cells were treated with either elicitor in the absence or presence of extracellular Ca^{2+}. As shown in Fig. 5A elicitor-induced phytoalexin production was significantly reduced in the absence of extracellular Ca^{2+} (by 73% or 94% in response to Pp-elicitor or Pep-25, respectively). Substitution of extracellular Ca^{2+} by 1 mM Mg^{2+} did not restore responsiveness of parsley cells, thus indicating that extracellular Ca^{2+} rather than divalent cations in general are required for activation of this plant defense in response to elicitor. ROS production also proved to be sensitive to extracellular Ca^{2+}-depletion (data not shown). Consistently, chelation of extracellular Ca^{2+} by 4 mM BAPTA administered at different times upon elicitation immediately abolished the Pp-elicitor-induced elevation of the cytoplasmic Ca^{2+} concentration (Fig. 5B) and ROS production, suggesting elicitation of pathogen defense reactions to be strictly dependent on Ca^{2+} influx via plasma membrane channels.

Anion channel activity—Anthracene-9-carboxylate (A-9-C, 100 μM), an anion channel blocker previously shown to inhibit ion fluxes and phytoalexin production in parsley cells treated with Ps-elicitor (Jabs et al. 1997),

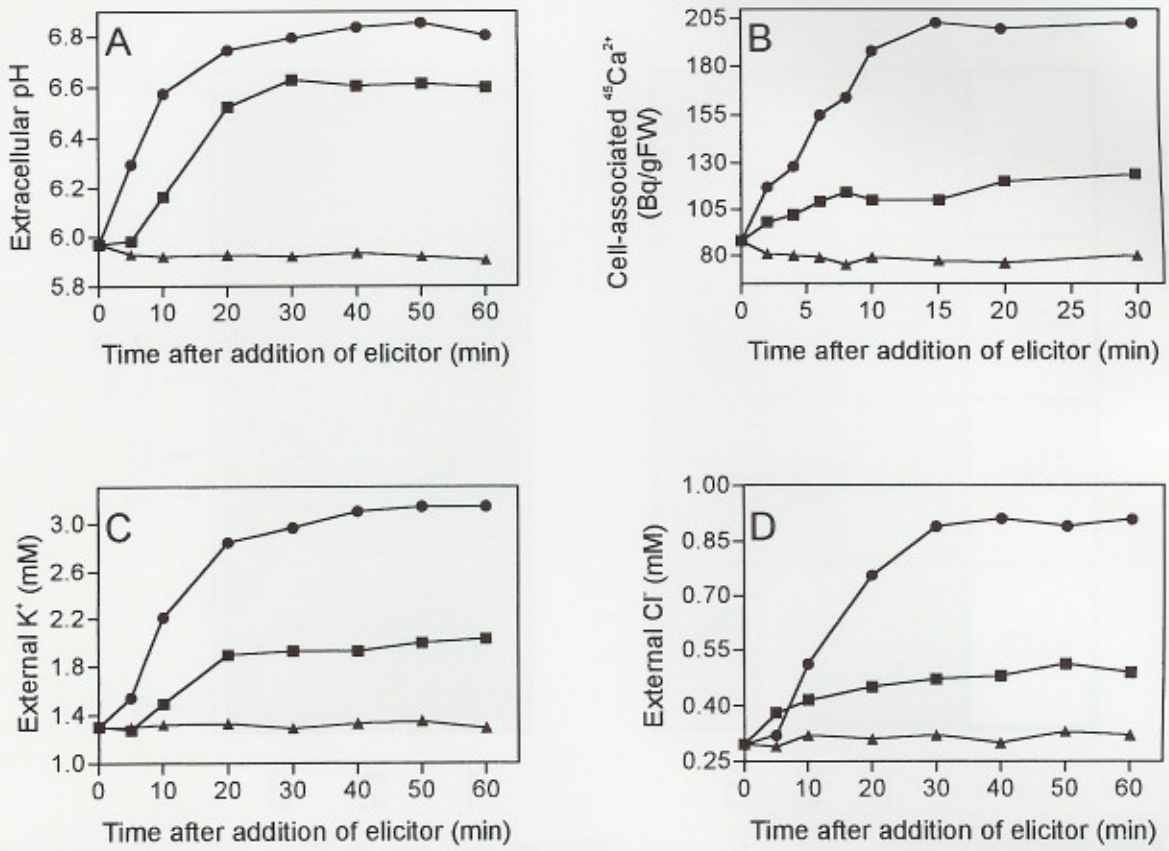


Fig. 3 Time courses of elicitor-stimulated plasma membrane ion fluxes in cultured parsley cells. (A) Extracellular alkalinization, (B) K^+ efflux, (C) Cl^- efflux, and (D) Ca^{2+} influx were determined in parsley cells treated with water as control (▲), 100 μg ml^{-1} Pp-elicitor (■), or 100 nM Pep-25 (●), respectively. Each data point represents the average of triplicates.

efficiently prevented phytoalexin production in parsley cells treated with either Pep-25 or Pp-elicitor, respectively. A-9-C also completely inhibited extracellular alkalinization and Ca^{2+} uptake induced by both, Pp-elicitor and Pep-25 (not shown). Similarly, Ca^{2+} channel inhibitors $La(NO_3)_3$ and $GdCl_3$ blocked elicitor-induced phytoalexin production at concentrations which did not affect viability of parsley cells. IC_{50} values obtained for these compounds were 100 μM and 110 μM $La(NO_3)_3$, and 60 μM and 65 μM $GdCl_3$ in parsley cells treated with either Pp-elicitor or Pep-25, respectively.

Production of ROS—Production of reactive oxygen species is frequently observed in pathogen-infected plants as well as in elicitor-treated plant cell suspensions or protoplasts (Lamb and Dixon 1997). Addition of Pp-elicitor or Pep-25 to parsley cells resulted in rapid, but transient generation of hydrogen peroxide (Fig. 6A). Increasing levels of extracellular hydrogen peroxide could be detected as early as 4 min upon addition of either elicitor, which peaked at about 15–20 min and subsequently declined to lower levels. As observed for other cellular responses as well, the Pp-elicitor stimulated only part of the ROS (approximately 40%) as compared to Pep-25 (Fig. 6C/D). Maximum concentrations of extracellular hydrogen peroxide were 8 and 20 μM in parsley cells treated with Pp-elicitor or Pep-25, respectively. As shown in Fig. 6A use of Pp-elicitor treated with trypsin prior to addition to parsley cells abolished generation of extracellular hydrogen peroxide.

Superoxide anions generated by a plasma membrane NADPH-dependent oxidase are assumed short-lived biosynthetic precursors for hydrogen peroxide production in elicited plant cells (Lamb and Dixon 1997). Inhibition of hydrogen peroxide formation by the superoxide dismutase inhibitor, sodium diethyldithiocarbamate (DDC), in elicited parsley cells should thus result in accumulation of superoxide anions. In the presence of DDC, superoxide anions accumulated in parsley cells treated with either elicitor with similar timing and to the same extent as hydrogen peroxide (not shown). In addition, diphenylene iodonium (DPI), a suicide substrate inhibitor of mammalian NADPH oxidase (Babior 1992), strongly inhibited the elicitor-in-

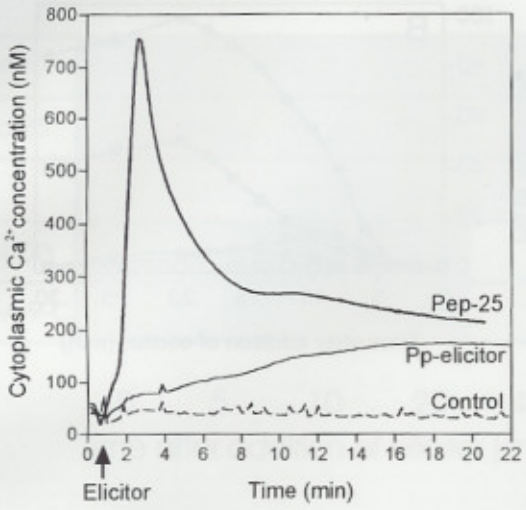


Fig. 4 Elicitor-induced elevation of the cytoplasmic Ca^{2+} concentration in cultured parsley cells. Cultured parsley cells stably transformed with aequorin were treated with 5 μM coelenterazine for 6 h to reconstitute holoaequorin. Cells were treated with water as control (····), 100 μg ml^{-1} Pp-elicitor (—), or 100 nM Pep-25 (—), respectively at the time indicated (arrow). Bioluminescence of transgenic parsley cells was determined with a luminometer. Relative light units obtained were subsequently transformed into cytoplasmic Ca^{2+} concentrations by using a calibration curve.

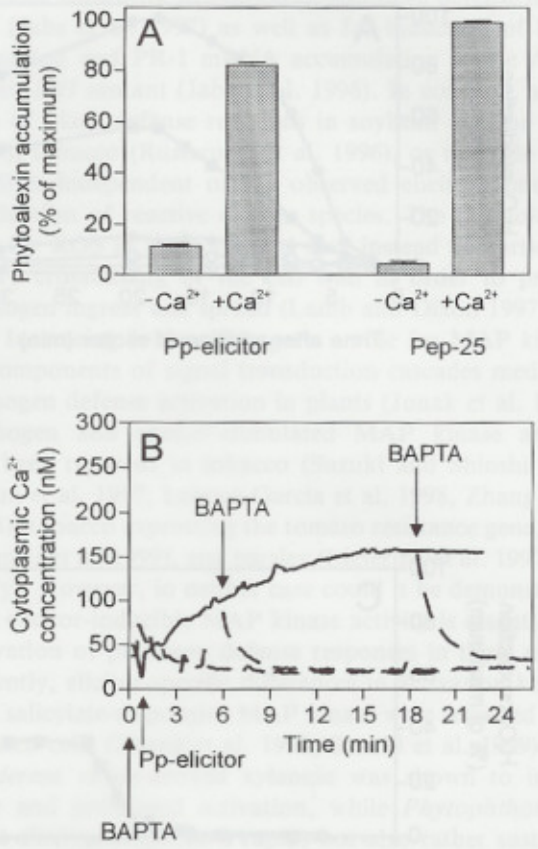


Fig. 5 Extracellular calcium requirement for elicitor-induced phytoalexin production (A) and elevation of cytoplasmic calcium concentration (B). Parsley cells were harvested by filtration, washed extensively with Ca^{2+}-free medium, and adjusted to a density of 60 mg of cell fresh weight ml^{-1} in either Ca^{2+}-containing (+Ca^{2+}) or Ca^{2+}-free (−Ca^{2+}; Ca^{2+} replaced by Mg^{2+}) culture medium 30 min before addition of 100 μg ml^{-1} Pp-elicitor or 100 nM Pep-25, respectively. Phytoalexin production was quantified 24 h after onset of treatment. Cultured parsley cells stably transformed with aequorin were treated as described in legend to Figure 3. Chelation of extracellular Ca^{2+} was initiated by addition of 4 mM BAPTA at the times indicated (arrows). Cytoplasmic Ca^{2+} concentration in transgenic parsley cells treated with 100 μg ml^{-1} Pp-elicitor (—) or Pp-elicitor and BAPTA (····), respectively.

duced oxidative burst (Fig. 6B) and phytoalexin production [IC_{50}=1.2 μM and 2.0 μM, respectively (Pp-elicitor); IC_{50}=1.2 μM and 2.1 μM, respectively (Pep-25)]. Diphenyl iodonium (IDP), a less potent inhibitor of mammalian NADPH oxidase (Babior 1992), inhibited elicitor-induced oxidative burst and phytoalexin production significantly less efficient than DPI [IC_{50}=250 μM and 280 μM, respectively, (Pp-elicitor); IC_{50}=220 μM and 250 μM, respectively (Pep-25)]. Neither DPI nor IDP treatment affected cell viability and elicitor-induced extracellular alkalinization and Ca^{2+} influx (data not shown). However, inhibitors of elicitor-induced ion fluxes, such as A-9-C, $La(NO_3)_3$ and $GdCl_3$, blocked Pp-elicitor or Pep-25-stimulated oxidative burst at comparable concentrations as were required for inhibition of proton and Ca^{2+} influx as well as phytoalexin production (see above). Taken together, these data suggest that reactive oxygen intermediates generated in response to elicitor treatment are involved in transmitting the elicitor signal, but themselves require elicitor-activated ion fluxes for their generation and physiological function.

Activation of a MAP kinase—A protein kinase that phosphorylated myelin basic protein (MBP) was activated within 5 min after treatment with Pep-25 and within 10 min after treatment with Pp-elicitor (Fig. 7A). In either case kinase activation was very transient and declined to nearly background levels within 40 min upon elicitation. To test whether the elicitor-responsive kinase belongs to the class of MAP kinases an antiserum raised against an alfalfa MAP kinase was employed for immunoprecipitation assays. Proteins precipitated from cell extracts of elicited as well as non-elicited parsley cells were subsequently examined for MBP-phosphorylating activity. As shown in Fig. 7B a MAP kinase activity was detectable exclusively in extracts from elicitor-treated parsley cells. Inhibition of the elicitor-induced MAP kinase activation by A-9-C but not by DPI (Fig. 7A) strongly suggests that this enzyme acts downstream of the elicitor-responsive ion channels but independently or upstream of the oxidative burst.

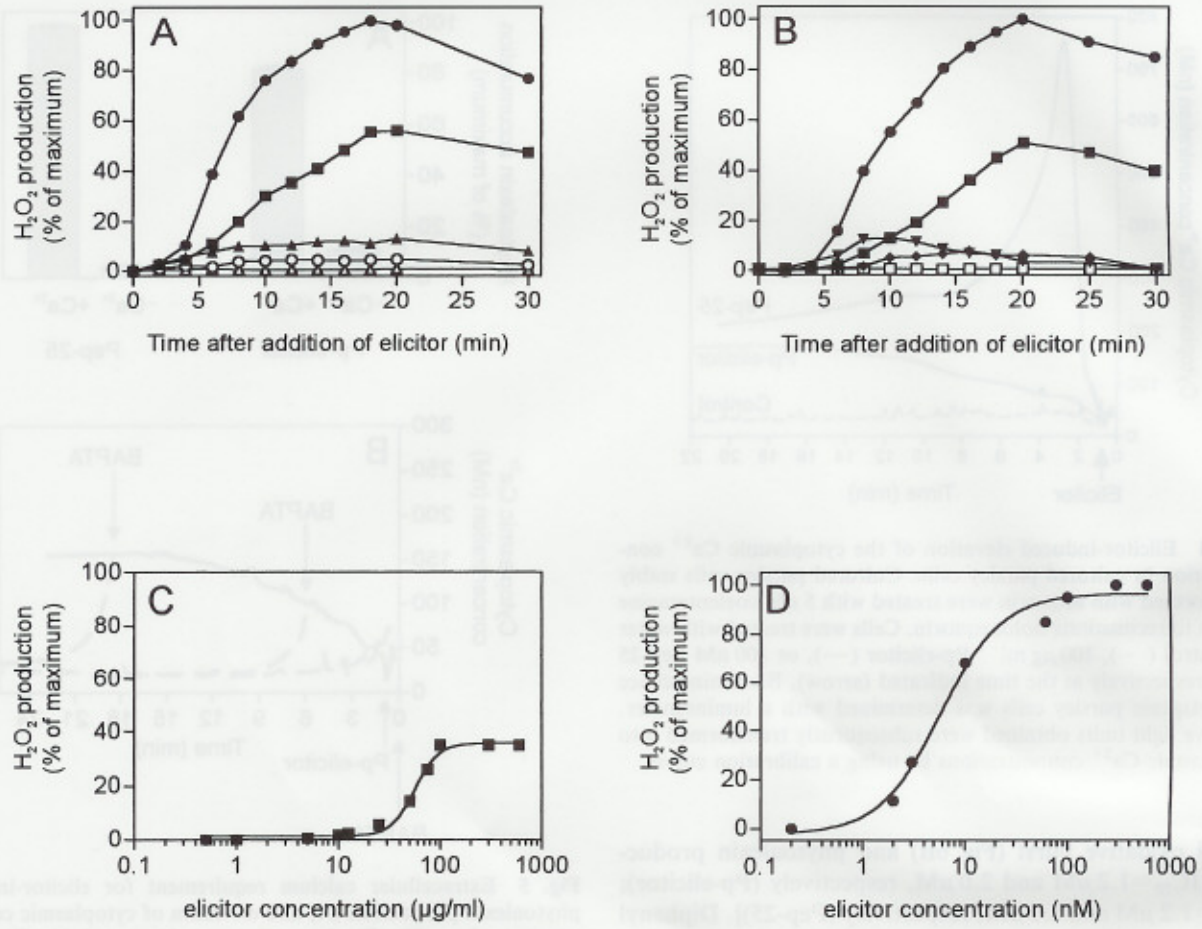


Fig. 6 Production of extracellular hydrogen peroxide (oxidative burst) by elicitor-treated parsley cells. Time courses of hydrogen peroxide accumulation in the medium of parsley cells (A) and the effect of DPI on the elicitor-induced oxidative burst (B) were analyzed. Suspension-cultured parsley cells were treated with water (△), 100 µg ml^{-1} Pp-elicitor (■), 100 nM Pep-25 (●), 100 µg ml^{-1} trypsinized Pp-elicitor (▲), or 100 µg ml^{-1} autoclaved trypsin (○), respectively. DPI (10 µM) was added to cultured parsley cells 30 min prior to addition of water (□), 100 µg ml^{-1} Pp-elicitor (◆), or 100 nM Pep-25 (▼), respectively. A dose-response relationship of elicitor-induced ROS production was performed using increasing concentrations of Pp-elicitor (C) or Pep-25 (D), respectively. Maximum phytoalexin production (100%) refers to that amount of which production was stimulated by 500 nM Pep-25. Each data point represents the average of triplicates.

Discussion

Here we report that proteinaceous constituents of the cell wall of *P. parasitica* stimulate production of the same furanocoumarins as does a 25-mer fragment of a *P. sojae*-derived 42-kDa cell wall glycoprotein (Nürnberger et al. 1994). Moreover, Pp-elicitor induced qualitatively the same early cellular responses as did Pep-25, such as elevation of cytoplasmic [Ca^{2+}] (Blume et al. 2000), Ca^{2+} and H^+ influxes, effluxes of K^+ and Cl^- ions, production of ROS (Nürnberger et al. 1994), post-transcriptional activation of a MAP-kinase pathway (Ligterink et al. 1997), and transcriptional activation of defense-related genes (Nürnberger et al. 1994). These early induced responses are assumed to contribute to signal transmission during activation of the plant's defensive arsenal.

Using pharmacological effectors a sequential order of early Pp-elicitor-induced responses could be established. Our studies revealed that extracellular Ca^{2+}, Ca^{2+} channel activity, and ROS are essential for elicitor-induced transcript accumulation of defense-related genes and phytoalexin production. Extracellular Ca^{2+} was further shown to be required for activation of the oxidative burst. In addition, PP-elicitor stimulated Ca^{2+}-dependent MAP kinase activity in parsley cells. Suppression of the elicitor-induced oxidative burst by DPI did not affect MAP kinase activation, indicating that the MAP kinase pathway acts either upstream or independently of the oxidative burst. These studies support observations in many systems which ascribe a pivotal role to Ca^{2+} for activation of downstream

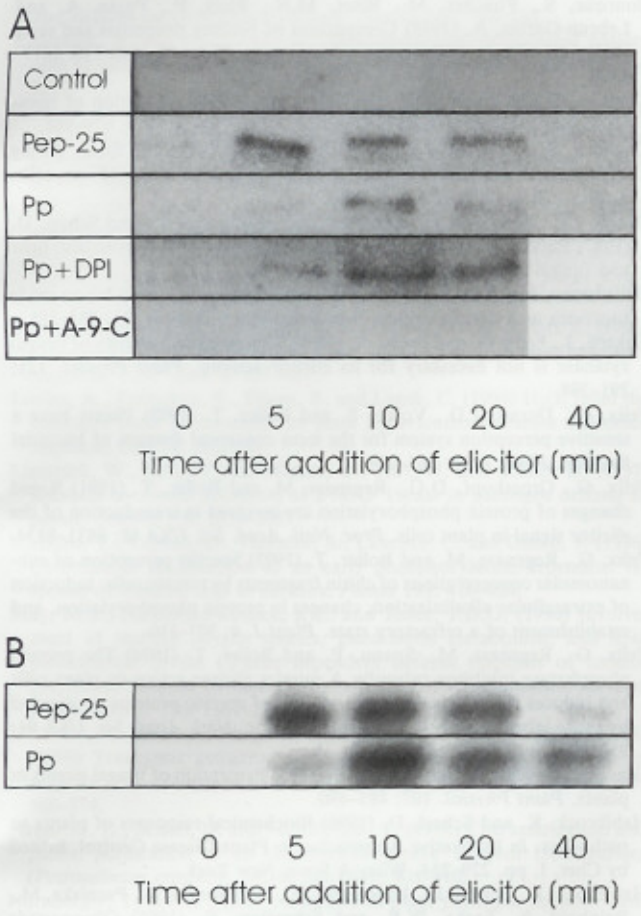


Fig. 7 Activation of MAP kinase activity in elicitor-treated parsley cells. Cultured parsley cells were treated with water as control, 100 nM Pep-25, 100 μg ml^{-1} Pp-elicitor, or 100 μg ml^{-1} Pp-elicitor in the presence of DPI or the ion channel inhibitor, A-9-C, respectively. Inhibitors were applied 30 min prior to addition of elicitor. (A) In-gel protein kinase assay. Cell extracts were prepared at the times indicated. Gelelectrophoretic separation, renaturation of protein kinase activity and protein kinase activity assays with myelin basic protein (MBP) as substrate were performed as described in Materials and Methods. (B) Immunoprecipitation of an elicitor-responsive MAP kinase. At the times indicated extracts were prepared from parsley cells treated with water as control, 100 nM Pep-25, or 100 μg ml^{-1} Pp-elicitor, respectively. Immunoprecipitation of MAP kinase activity, phosphorylation of MBP with [γ^{32}P]ATP, and gelelectrophoretic separation of phosphorylated MBP were performed as described in Materials and Methods. Phosphorylated MBP was visualized by phospho imaging.

responses (Yang et al. 1997, Scheel 1998). Series of gain-and-loss-of-function experiments have almost invariably revealed that Ca^{2+} influx is essential for activation of plant pathogen defense (Bach et al. 1993, Nürnberger et al. 1994, Tavernier et al. 1995, Levine et al. 1996, Kuchitsu et al. 1997, Mithöfer et al. 1999). Requirement of reactive oxygen species for activation of defense-associated responses has been shown for activation of pathogen defense in parsley (Jabs et al. 1997) as well as for induction of lesion formation and PR-1 mRNA accumulation in the Arabidopsis *lsd1* mutant (Jabs et al. 1996). In contrast, activation of plant defense reactions in soybean (Levine et al. 1994), tobacco (Rusterucci et al. 1996), or rice (He et al. 1998) is independent of the observed elicitor-stimulated production of reactive oxygen species. The physiological role for ROS in these systems may instead comprise oxidative crosslinking of the cell wall in order to prevent pathogen ingress and spread (Lamb and Dixon 1997).

Increasing evidence suggests a role for MAP kinases as components of signal transduction cascades mediating pathogen defense activation in plants (Jonak et al. 1999). Pathogen and elicitor-stimulated MAP kinase activity has been reported in tobacco (Suzuki and Shinshi 1995, Adam et al. 1997, Lebrun-Garcia et al. 1998, Zhang et al. 1998), tobacco expressing the tomato resistance gene, *Cf-9* (Romeis et al. 1999), and parsley (Ligterink et al. 1997, this study). However, in neither case could it be demonstrated that elicitor-inducible MAP kinase activity is essential for activation of pathogen defense responses in these plants. Recently, elicitor-specific differences in activation kinetics of a salicylate-responsive MAP kinase were reported from tobacco cells (Zhang et al. 1998, Suzuki et al. 1999). *Trichoderma viride*-derived xylanase was shown to induce slow and prolonged activation, while *Phytophthora*-derived elicitins triggered a rapid, but also rather sustained activation of the enzyme. Interestingly, all of these elicitors stimulated hypersensitive cell death in tobacco cell suspensions. In addition to suggesting a central role of MAP kinase activity during initiation of pathogen defense, this also exemplifies that distinct receptors mediate generation of stimulus-specific, temporally defined enzyme activity signatures. These may, subsequently, facilitate elicitor-specific activation of a complex plant defense response, which comprises common elements, such as HR, but also likely differs in activation of other defense responses, such as production of ROS, ethylene or defense gene activation. As Suzuki et al. (1999) anticipate it will be challenging to ascribe different phases of such activity profiles to activation of signal-specific reactions in overall defense responses of plant cells.

Pronounced differences in response amplitude or intensity, and to lesser extent in response kinetics, could be observed between the two elicitor preparations tested for activation of plant defense responses in parsley. The total amount of phytoalexins produced upon treatment with Pp-elicitor never exceeded two-third of that induced by Pep-25, even when applied at concentrations tenfold higher than those required to stimulate maximum phytoalexin production. The most immediate response of parsley cells to elicitor treatment is an increase in the cytoplasmic Ca^{2+} content, which was proven to originate from the cell

exterior (Blume et al. 2000). Concomitantly, elicitor treatment led to enhanced Ca^{2+} influx into parsley cells. Elevation of cytoplasmic Ca^{2+} levels in response to Pp-elicitor was 4 fold, while 6–7 fold in response to Pep-25. In addition, striking differences in the Ca^{2+} signature induced by either elicitor point towards the existence of different pathways for its activation. Elicitor-specific differences regarding the extent and intensity of elicitor-induced proton influx, efflux of K^+ and Cl^-, and production of ROS were consistently detected as well. Moreover, although less pronounced than differences between the elicitors with regard to response intensities elicitor-specific differences in induction kinetics of ion fluxes, oxidative burst and MAP kinase activation were evident. Activation of early responses of parsley cells by Pp-elicitor was reproducibly delayed as compared to activation by Pep-25.

These quantitative differences observed between both elicitors suggest that different extracellular, pathogen-derived proteinaceous signals target distinct ligand binding sites at the plant cell, which subsequently integrate intracellularly generated signals into a conserved signal transduction cascade. However, the possibility of two discrete ligand binding sites at the same receptor cannot be ruled out yet. Generally, this scenario is reminiscent of the responses of suspension-cultured tomato cells to treatment with elicitors of plant defense responses, such as fungus-derived glycopeptides (Felix et al. 1991), chitin fragments (Felix et al. 1993), ergosterol (Granado et al. 1995), bacterial xylanase (Enkerli et al. 1999), or flagellin (Felix et al. 1999). For some of these elicitors it was found that they are recognized by different plasma membrane receptors (Felix et al. 1999).

We gratefully acknowledge excellent technical assistance by Helga Nixdorf and Jutta Elster. This work was supported by the Deutsche Forschungsgemeinschaft (Nu 70/2-1), the European Community (ERB-BIO4-CT96-0101) and Kleinwanzlebener Saatzucht AG.

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(Received January 14, 2000; Accepted March 17, 2000)

The Plant Cell, Vol. 12, 1425–1440, August 2000, www.plantcell.org © 2000 American Society of Plant Physiologists

Receptor-Mediated Increase in Cytoplasmic Free Calcium Required for Activation of Pathogen Defense in Parsley

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Transient influx of Ca^{2+} constitutes an early element of signaling cascades triggering pathogen defense responses in plant cells. Treatment with the *Phytophthora sojae*–derived oligopeptide elicitor, Pep-13, of parsley cells stably expressing apoaequorin revealed a rapid increase in cytoplasmic free calcium ($[Ca^{2+}]_{cyt}$), which peaked at ~1 μM and subsequently declined to sustained values of 300 nM. Activation of this biphasic $[Ca^{2+}]_{cyt}$ signature was achieved by elicitor concentrations sufficient to stimulate Ca^{2+} influx across the plasma membrane, oxidative burst, and phytoalexin production. Sustained concentrations of $[Ca^{2+}]_{cyt}$ but not the rapidly induced $[Ca^{2+}]_{cyt}$ transient peak are required for activation of defense-associated responses. Modulation by pharmacological effectors of Ca^{2+} influx across the plasma membrane or of Ca^{2+} release from internal stores suggests that the elicitor-induced sustained increase of $[Ca^{2+}]_{cyt}$ predominantly results from the influx of extracellular Ca^{2+}. Identical structural features of Pep-13 were found to be essential for receptor binding, increases in $[Ca^{2+}]_{cyt}$, and activation of defense-associated responses. Thus, a receptor-mediated increase in $[Ca^{2+}]_{cyt}$ is causally involved in signaling the activation of pathogen defense in parsley.

INTRODUCTION

Cytoplasmic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) serves as a second messenger in plant processes as diverse as root nodule formation, phytochrome phototransduction, stomatal closure, geotropism, circadian rhythm, pollen tube growth, and stress adaptation (Rudd and Franklin-Tong, 1999). Stimulus-specific and spatially and temporally defined Ca^{2+} signatures of characteristic magnitude, frequency, and duration are assumed to maintain signal specificity of transduction cascades (Thuleau et al., 1998; Trewavas, 1999). Subsequently, the binding of $[Ca^{2+}]_{cyt}$ to calmodulin, Ca^{2+}-dependent protein kinases, Ca^{2+}-dependent protein phosphatases, Ca^{2+}-gated ion channels, or Ca^{2+}-activated phospholipases facilitates downstream signal transduction directed toward activation of a signal-specific cellular response (Blumwald et al., 1998).

Expression of the *Aequorea aequorea* apoaequorin gene in the cytoplasm of plant cells provides a means for accurate, noninvasive quantification of changes in $[Ca^{2+}]_{cyt}$ (Knight et al., 1991). When reconstituted with coelenterazine, holoaequorin acts as a bioluminescent indicator of $[Ca^{2+}]_{cyt}$. Since the pioneering work of Knight et al. (1991), aequorin technology has been widely applied in plants to report changes in $[Ca^{2+}]_{cyt}$ in response to abiotic stimuli, such as touch, wind, cold, heat, and drought (Knight et al., 1991, 1992, 1996, 1997; Haley et al., 1995; Gong et al., 1998; Plieth et al., 1999); blue light (Lewis et al., 1997); circadian rhythm (Johnson et al., 1995); ozone (Clayton et al., 1999); anoxia (Sedbrook et al., 1996); oxidative stress (Price et al., 1994); and hypoosmotic shock (Chandra and Low, 1997; Takahashi et al., 1997; Cessna et al., 1998).

Numerous recent studies have provided evidence that Ca^{2+} plays a pivotal role in activating the plant's surveillance system against attempted microbial invasion (Yang et al., 1997; Scheel, 1998). Activation of plant defense is believed to be receptor mediated through recognition of pathogen-derived elicitors (Yang et al., 1997; Scheel, 1998; Nürnberger, 1999). In contrast to elicitors from phytopathogenic bacteria, elicitors of fungal or oomycete origin appear to be recognized by high-affinity receptors residing in the plasma membrane of plant cells. Although several such plasma membrane binding sites have been characterized kinetically and structurally, our knowledge of the molecular mode of fungal pathogen perception in plants remains fragmentary: only one elicitor receptor has been isolated thus far, a soybean 70-kD plasma membrane protein that binds *Phytophthora sojae*–derived β-glucans (Umemoto et al., 1997).

Receptor–ligand interaction initiates an intracellular signal transduction cascade that mediates activation of the defense against the pathogen. Cellular components shown to be modulated by elicitor treatment include GTP binding proteins (Bischoff et al., 1999); plasma membrane ion channels

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(Thuleau et al., 1998); reactive oxygen intermediates (Lamb and Dixon, 1997); nitric oxide (Delledonne et al., 1998); lipid-derived metabolites (Chandra et al., 1997); and alterations in the phosphorylation status of various proteins catalyzed by serine/threonine protein kinases, protein phosphatases, and post-translationally activated mitogen-activated protein kinase (MAP kinase) cascades (Sopory and Munshi, 1998). Remarkably, many of these elements thought to be implicated in elicitor signaling have been identified in various plants, suggesting evolutionary conservation of signaling modules in plant pathogen defense (Yang et al., 1997; Scheel, 1998).

Plasma membrane ion channels are rapidly activated by pathogen infection or elicitor treatment of plant cells. In particular, extracellular alkalinization, Ca^{2+} influx, and effluxes of K^+ and Cl^- lead to depolarization of the plasma membrane (Scheel, 1998). Extracellular Ca^{2+} appears to be crucial to induction of plant defense against pathogens (Yang et al., 1997; Scheel, 1998). Elicitor-responsive Ca^{2+}-permeable ion channels residing in the plasma membrane of plant cells may mediate elicitor-induced Ca^{2+} influx and subsequently lead to increased amounts of $[Ca^{2+}]_{cyt}$ (Gelli et al., 1997; Zimmermann et al., 1997).

Rapidly induced transient increase of $[Ca^{2+}]_{cyt}$ was previously monitored in apoaequorin-transformed tobacco cells that had been treated with crude elicitors derived from yeast or *Gliocladium deliquescens* (Knight et al., 1991). In contrast, harpin from *Erwinia amylovora*, which induces an oxidative burst, defense-related gene expression, and hypersensitive cell death in tobacco, did not affect $[Ca^{2+}]_{cyt}$ in this plant (Chandra et al., 1997). Only recently were defined fungal chitin fragments or oomycete-derived glucan fragments shown to increase $[Ca^{2+}]_{cyt}$ in soybean cells (Mithöfer et al., 1999). Crucial questions, however, as to whether changes in $[Ca^{2+}]_{cyt}$ are receptor mediated and thus involved in signal activation of defense responses to pathogens remain to be resolved.

The nonhost resistance response of parsley (*Petroselinum crispum*) leaves to infection with zoospores of the phytopathogenic oomycete *P. sojae* was found to be closely mimicked in parsley cell cultures treated with cell wall–derived elicitors (Hahlbrock et al., 1995). An oligopeptide fragment (Pep-13) of a 42-kD *P. sojae* cell wall glycoprotein stimulates transcriptional activation of defense-related genes and phytoalexin production (Nürnberger et al., 1994). The binding of Pep-13 to a 100-kD parsley plasma membrane receptor (Nürnberger et al., 1995; Nennstiel et al., 1998) rapidly stimulates Ca^{2+} influx, effluxes of K^+ and Cl^-, extracellular alkalinization, production of reactive oxygen species (ROS), and post-translational activation of a MAP kinase (Nürnberger et al., 1994; Jabs et al., 1997; Ligterink et al., 1997). Extracellular Ca^{2+} was found to be indispensable for activation of all these plant responses (Nürnberger et al., 1994; Ligterink et al., 1997). Use of peptides structurally related to Pep-13 revealed a functional link between elicitor perception and activation of Ca^{2+} influx as a requirement for subsequent production of superoxide anions. ROS themselves are both necessary and sufficient to trigger pathogen defense responses in parsley (Nürnberger et al., 1994; Jabs et al., 1997). Patch-clamp analyses demonstrated the receptor-mediated activation of a Pep-13–responsive Ca^{2+}-permeable plasma membrane ion channel (Zimmermann et al., 1997). We now show that parsley cells stably expressing the apoaequorin gene respond to elicitor treatment with a characteristic biphasic $[Ca^{2+}]_{cyt}$ signature consisting of a rapidly induced $[Ca^{2+}]_{cyt}$ transient peak followed by sustained concentrations of $[Ca^{2+}]_{cyt}$. Intriguingly, although both phases of the Pep-13–induced $[Ca^{2+}]_{cyt}$ response in parsley cells are due to Pep-13 receptor activation, only sustained increases in $[Ca^{2+}]_{cyt}$ constitute an element of the signaling cascade triggering pathogen defense in parsley.

RESULTS

Establishment of Parsley Cell Lines Stably Expressing Cytoplasmic Apoaequorin

Particle bombardment technology was used to introduce a DNA cassette encoding apoaequorin into suspension-cultured parsley cells (Finer et al., 1992; Frohnmeyer et al., 1999). When holoaequorin was reconstituted with coelenterazine, 27 of 37 hygromycin-resistent calli exhibited Ca^{2+}-dependent bioluminescence. Cell suspensions were established from those calli showing the greatest amounts of reconstituted bioluminescent aequorin (Table 1).

DNA gel blot analysis confirmed integration of one to six copies of the apoaequorin-encoding DNA into the parsley genome. Maintenance of the Ca^{2+}-dependent bioluminescence in parsley cell lines cultivated for $>$12 months in the absence of selecting antibiotic proved that the integration of the transgene was stable. Phenotypic appearance and growth behavior of transgenic parsley cell lines were indistinguishable from those of nontransformed cell lines. Most importantly, when treated with the *P. sojae*–derived oligopeptide elicitor Pep-13, apoaequorin-transgenic parsley cell lines produced ROS and furanocoumarin phytoalexins in quantities comparable to those produced by nontransformed cell lines (Table 1). Nevertheless, to avoid misinterpretation of data in consideration of adverse effects of transgene integration on cellular responsiveness, all bioluminometric experiments were routinely performed in triplicate with at least two transgenic cell lines.

Because of the double logarithmic relationship between aequorin bioluminescence and $[Ca^{2+}]$ (Blinks et al., 1978), relative changes in light emission do not precisely reflect the extent of any change in actual $[Ca^{2+}]$. Moreover, because aequorin is consumed during the experiment and because the amount of reconstituted aequorin varies between individual experiments, meaningful analysis of recorded relative light units (RLUs) required conversion into $[Ca^{2+}]$ by using a

Table 1. Characterization of Transgenic Parsley Cell Lines Expressing Apoaequorin

Line	Number of Transgene Insertions[a]	Bioluminescence of Reconstituted Aequorin[b] (RLU/sec/mg FW)	Oxidative Burst[c] (Percentage of Untransformed Cells)	Phytoalexin Production[d] (Percentage of Untransformed Cells)
1/10	1	1.87×10^5	146×2	68×12
1/14	4–6	1.98×10^5	127×12	54×29
1/17	1	2.06×10^5	ND[e]	40×17
1/18	1	1.41×10^5	125×10	61×28
1/19	3	1.27×10^5	72×27	57×32
2/2	2	2.66×10^5	95×32	112×9
2/5	ND[e]	0.33×10^5	219×20	69×17
2/12	2	6.25×10^5	114×7	78×26

[a] Integration of the aequorin construct was investigated by genomic DNA gel blot analysis.
[b] Determined by complete discharge of aequorin with excess Ca^{2+}.
[c] Oxidative burst measurements were performed in triplicate.
[d] Phytoalexin production was quantified whenever cell lines were used for bioluminescence experiments.
[e] Not determined.
FW, fresh weight; RLU, relative light units.

calibration curve established in vitro. All relative changes in bioluminescence monitored were therefore transformed into absolute values for $[Ca^{2+}]_{cyt}$.

Increased $[Ca^{2+}]_{cyt}$ in Elicitor-Treated Parsley Cells

In transgenic parsley lines reconstituted aequorin reported a basal $[Ca^{2+}]_{cyt}$ level of 40 to 110 nM. Treating the parsley cells with the oligopeptide elicitor Pep-13 produced characteristic changes in $[Ca^{2+}]_{cyt}$ (Figure 1), which were similar in all transgenic cell lines listed in Table 1. After a delay of 30 to 40 sec, $[Ca^{2+}]_{cyt}$ increased rapidly, peaked at $\sim$1 μM after 2 min, and subsequently decreased to a slowly declining plateau of $\sim$300 nM during the next 10 to 40 min (Figure 1A). This biphasic response differed greatly from the immediate, transient, small peak detectable after addition of water or organic solvents. In contrast to the peak maximum (phase 1), which varied between 600 and 1100 nM among individual experiments performed with independent transgenic lines, the magnitude of the plateau value (phase 2) and the kinetics of the total response were consistently found to be identical across all lines.

The extent of the $[Ca^{2+}]_{cyt}$ response depended on the concentration of elicitor used, becoming saturated at Pep-13 concentrations $>$5 nM (Figure 1B). Decreasing the elicitor concentration preferentially reduced the transient $[Ca^{2+}]_{cyt}$ peak, whereas sustained increases of $[Ca^{2+}]_{cyt}$ could be elicited at Pep-13 concentrations as low as 0.25 nM. Lowering the Pep-13 concentration further also diminished the activation of the second phase of the $[Ca^{2+}]_{cyt}$ response. Importantly, we found a close quantitative correlation between the elicitor concentrations required to efficiently stimulate increased $[Ca^{2+}]_{cyt}$ and phytoalexin formation (Figure 1B). Maximum phytoalexin production was observed only at Pep-13 concentrations that elicited both phases of the $[Ca^{2+}]_{cyt}$ response. However, large amounts of phytoalexins (80% of maximum) were also produced in response to Pep-13 concentrations that stimulated only phase 2 of the $[Ca^{2+}]_{cyt}$ response. The EC_{50} value for Pep-13 to induce the sustained $[Ca^{2+}]_{cyt}$ plateau (0.2 nM) closely corresponded to the elicitor concentrations required to half-maximally elicit Ca^{2+} influx, K^+ and Cl^- efflux, medium alkalinization, oxidative burst, and phytoalexin formation (Table 2; Nürnberger et al., 1994).

In contrast to luminometric analyses of large cell populations (5×10^3 cells per experiment), in vivo imaging of aequorin activity using photon-counting video equipment permitted analysis of small cell clusters (10 to 20 cells). These experiments were performed to investigate whether the elicitor-induced $[Ca^{2+}]_{cyt}$ signature might be the result of cumulative light emission from cell populations responding asynchronously to elicitor with differing lag phases, and whether the $[Ca^{2+}]_{cyt}$ plateau might be brought about by alternately oscillating $[Ca^{2+}]_{cyt}$ spikes. As shown in Figure 2, the parsley cell clusters responded rather synchronously to elicitor treatment. Maximum light emission was observed between 60 and 160 sec after addition of elicitor (Figure 2A), corresponding precisely to the time of the large $[Ca^{2+}]_{cyt}$ spike seen in luminometric assays (Figure 1A). Subsequently, light emission declined (Figure 2B) but remained constantly higher than that observed in untreated cells (not shown) or in cells immediately after the administration of elicitor (first image, Figure 2A). This is reminiscent of the plateau phase monitored in luminometric measurements (Figure 1A). In all experiments performed, the majority of cell clusters responded to elicitor treatment (Figures 2C and 2D). Because the light emission of elicitor-treated parsley cells was synchronous and continuous rather than asynchronous or oscillating, the $[Ca^{2+}]_{cyt}$ signature observed in luminometric

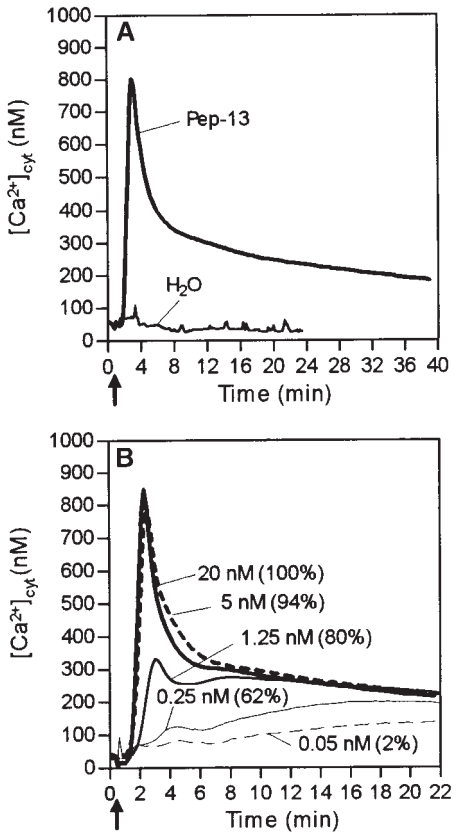

Figure 1. Elicitor-Induced Changes in $[Ca^{2+}]_{cyt}$ in Apoaequorin-Transformed Parsley Cells.

Bioluminescence of reconstituted aequorin was monitored in parsley cells treated with water or Pep-13 at the time (arrow) indicated.
(A) Addition of water or 100 nM Pep-13, respectively.
(B) Addition of Pep-13 at the concentrations indicated. Numbers in parentheses represent amounts of phytoalexins produced by parsley cells treated with the given Pep-13 concentrations relative to those produced by cells treated with 100 nM Pep-13.

assays (Figure 1) is unlikely to result from cell populations responding substantially differently from or later than the majority of cells.

Elicitor Specificity of the $[Ca^{2+}]_{cyt}$ Response

The 42-kD *P. sojae* cell wall glycoprotein harboring Pep-13 was tested for its ability to elicit the $[Ca^{2+}]_{cyt}$ response in parsley cells. Somewhat unexpectedly, this glycoprotein elicitor did not trigger the large $[Ca^{2+}]_{cyt}$ transient peak in the way Pep-13 did. Instead, a lag phase of ~4 min preceded the increase in $[Ca^{2+}]_{cyt}$, which reached a sustained value (phase 2) of 300 nM, the same as that of the Pep-13–treated cells, 12 to 15 min after the glycoprotein was added (Figure 3A). Because this elicitor stimulated phytoalexin production in parsley cells as efficiently as Pep-13 did, the characteristic transient $[Ca^{2+}]_{cyt}$ spike triggered by Pep-13 appears to be dispensable for activation of pathogen defense–associated responses in parsley.

The impact on parsley $[Ca^{2+}]_{cyt}$ of a series of plant defense elicitors was investigated. Interestingly, elicitors that induced phytoalexin production in parsley cells stimulated only phase 2 of the $[Ca^{2+}]_{cyt}$ response (Figure 3A). At concentrations sufficient to activate phytoalexin production in parsley cells, harpin, the product of the *HRPZ* gene from *Pseudomonas syringae* pv *phaseolicola*, yielded increased $[Ca^{2+}]_{cyt}$ with kinetics and magnitude (~250 nM) comparable to those of the *P. sojae* glycoprotein elicitor. Furthermore, the polyene antibiotic amphotericin B, previously shown to trigger sustained ion fluxes across the plasma membrane as well as phytoalexin production in parsley cells in the absence of elicitor (Jabs et al., 1997), increased the $[Ca^{2+}]_{cyt}$ with kinetics and magnitude (~220 nM) very similar to those shown by harpin and the *P. sojae* glycoprotein elicitor.

In contrast, elicitors incapable of inducing phytoalexin production in parsley cells were either ineffective with respect to increasing $[Ca^{2+}]_{cyt}$ (*Phytophthora megasperma* β-elicitin, β-megaspermin) or induced only a rapid, transient increase in $[Ca^{2+}]_{cyt}$ even at concentrations 10-fold greater than those reported from other plant systems to be sufficient to trigger pathogen defense reactions (Figure 3B). The $[Ca^{2+}]_{cyt}$ signature induced by fungal *N*-acetylchitoheptaose was reminiscent of phase 1 of the Pep-13–induced $[Ca^{2+}]_{cyt}$ response. Using less-polymerized chitooligosaccharides resulted in concomitant reduction (chitopentaose, not shown) or complete loss (chitotriose, not shown) of their $[Ca^{2+}]_{cyt}$-increasing activity. A 15-mer peptide derived from a conserved domain of bacterial flagellin, which was shown to elicit plant defense-associated responses in various dicot plant cells (Felix et al., 1999), increased $[Ca^{2+}]_{cyt}$ in parsley cells transiently up to 350 nM.

Among other abiotic stimuli, cold shock reportedly triggers an increase in $[Ca^{2+}]_{cyt}$ in apoaequorin-transgenic tobacco plants and cultured cells (Knight et al., 1991, 1996; Chandra and Low, 1997). As shown in Figure 3C, addition of ice-cold medium to transgenic parsley cells precipitously induced a $[Ca^{2+}]_{cyt}$ spike, which reached a maximum after 20 sec at concentrations >3 μM, subsequently declining to basal values within 150 sec. However, this cold shock could neither increase the $[Ca^{2+}]_{cyt}$ to sustained values nor stimulate phytoalexin production in parsley cells.

Requirement of Extracellular Ca^{2+} for Elicitor-Induced Increase of $[Ca^{2+}]_{cyt}$

Previous experiments proved that extracellular Ca^{2+} is essential for activation of the multifaceted defense response in elicitor-treated parsley cells (Nürnberger et al., 1994; Jabs et al., 1997; Ligterink et al., 1997). In addition, Pep-13 was

Table 2. Correlation between Elicitor-Specific Responses in Parsley Challenged with Pep-13 and Structural Analogs

	Activity Index (Pep-X/Pep-13)[a]				
		Elicitor Activity			
Peptide Sequence	Competitor Activity	Ca^{2+} Influx	Δ pCa_{cyt} (Aequorin)	H_2O_2 Formation	Phytoalexin Formation
Pep-13 VWNQPVRGFKVYE	1	1	1 (0.2 nM)[b]	1 (0.19 nM)	1 (0.4 nM)
Pep-13/A12 VWNQPVRGFKV**A**E[c]	1.3	1.7	1.6	1.3	1.6
Pep-13/A2 V**A**NQPVRGFKVYE	1,500	390	280	70	230
Pep-10 NQPVRGFKVY	7,000	—	15,500	3,400	1,100

[a] The activity index (Pep-X/Pep-13) represents the quotient of the half-maximal concentration of the respective Pep-13 structural derivative (Pep-X) required to stimulate the particular plant response and the half-maximal effector concentration of Pep-13. The difference in pCa_{cyt} between the unstimulated basal level and the sustained level obtained 10 min after addition of peptides was taken to compare the calcium response elicited by Pep-13 and structural analogs in aequorin-transformed parsley cells. To enable better comparison, we include previously determined indices for other elicitor-specific responses (Nürnberger et al., 1994).

[b] Numbers in parentheses represent the absolute EC_{50} values derived from dose–response curves using aequorin-transformed as well as untransformed parsley cells.

[c] Bold letters represent alanine substitution sites within Pep-13.

shown to initiate influx of $^{45}Ca^{2+}$ into parsley cells and to activate a plasma membrane Ca^{2+}-permeable ion channel (Nürnberger et al., 1994; Zimmermann et al., 1997). Thus, an elicitor-induced increase in $[Ca^{2+}]_{cyt}$ may be a direct consequence of Ca^{2+} influx through plasma membrane ion channels. Alternatively, generation of inositol trisphosphate (IP_3) or cyclic ADP-ribose and activation of their corresponding receptors could mobilize Ca^{2+} from internal stores, such as the vacuole. As shown in Figure 4A, chelation of extracellular Ca^{2+} by the chelating agent 1,2-bis(aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) abolished both phases of the Pep-13–induced increase in $[Ca^{2+}]_{cyt}$. Similarly, EGTA inhibited the $[Ca^{2+}]_{cyt}$ response in elicitor-treated parsley cells (not shown). Addition of BAPTA to parsley cells after onset of elicitor treatment immediately abrogated both the transient $[Ca^{2+}]_{cyt}$ peak and the plateau phase of the $[Ca^{2+}]_{cyt}$ response. In contrast, cold shock, a stimulus known to trigger Ca^{2+} release from vacuolar stores in addition to influx of external Ca^{2+} (Knight et al., 1996), evoked a $[Ca^{2+}]_{cyt}$ transient peak in parsley cells in the presence of BAPTA (Figure 4A). Addition of excess Ca^{2+} to parsley cells preincubated with BAPTA fully restored the $[Ca^{2+}]_{cyt}$ response (Figure 4B) and the ability of elicitor-treated parsley cells to produce ROS and phytoalexins (not shown). Thus, irreversible damage of parsley plasma membranes caused by BAPTA appears unlikely. Moreover, the Pep-13–induced $[Ca^{2+}]_{cyt}$ response of parsley cells treated simultaneously with BAPTA and excess $CaCl_2$ was indistinguishable from that of parsley cells treated with Pep-13 alone. Modulation of the extracellular free Ca^{2+} concentration by BAPTA resulted in a concomitant decrease in the ability of elicited parsley cells to mount a $[Ca^{2+}]_{cyt}$ response and an oxidative burst (Figure 4C). This suggests that induction of both cellular responses is similarly dependent on external Ca^{2+}. Strikingly, as long as the extracellular free $[Ca^{2+}]$ exceeded the $[Ca^{2+}]_{cyt}$, parsley cells remained responsive to Pep-13.

Lanthanides (Gd^{3+}, La^{3+}) are frequently used to inhibit Ca^{2+} importation across the plant plasma membrane. When added to parsley cells either before or together with Pep-13, 1 mM Gd^{3+} abolished the Pep-13–induced $[Ca^{2+}]_{cyt}$ signature (Figure 4D) and reduced phytoalexin production in elicitor-treated parsley cells by 91%. Use of 1 mM La^{3+} gave similar results (not shown). However, lanthanides are reported both to enter cells at millimolar external concentrations (Shimizu et al., 1997) and to affect plasma membrane Ca^{2+}-ATPase (Quiquampoix et al., 1990) and K^+ channels (Lewis and Spalding, 1998). Thus, our findings lend further support to the prime role of extracellular Ca^{2+} for activating plant defense in parsley but do not provide evidence for specific plasma membrane Ca^{2+} channels to mediate an elicitor-induced Ca^{2+} influx.

In a series of previous experiments, verapamil, nifedipine, and flunarizine, nonpermeable inhibitors of Ca^{2+} channels, failed to inhibit elicitor-induced alkalinization of the extracellular medium, Ca^{2+} influx, K^+ and Cl^- effluxes, oxidative burst, and phytoalexin production in parsley cells (Jabs et al., 1997). These compounds were now found to be also incapable of blocking the elicitor-induced increase in $[Ca^{2+}]_{cyt}$ after 15 min of preincubation with inhibitor (not shown).

Ruthenium Red (RR), a membrane-permeable Ca^{2+} channel blocker that predominantly inhibits Ca^{2+} release from intracellular compartments, was used to elucidate the contribution of internal Ca^{2+} stores to the elicitor-induced $[Ca^{2+}]_{cyt}$ signature response. At 100 μM, RR markedly inhibited

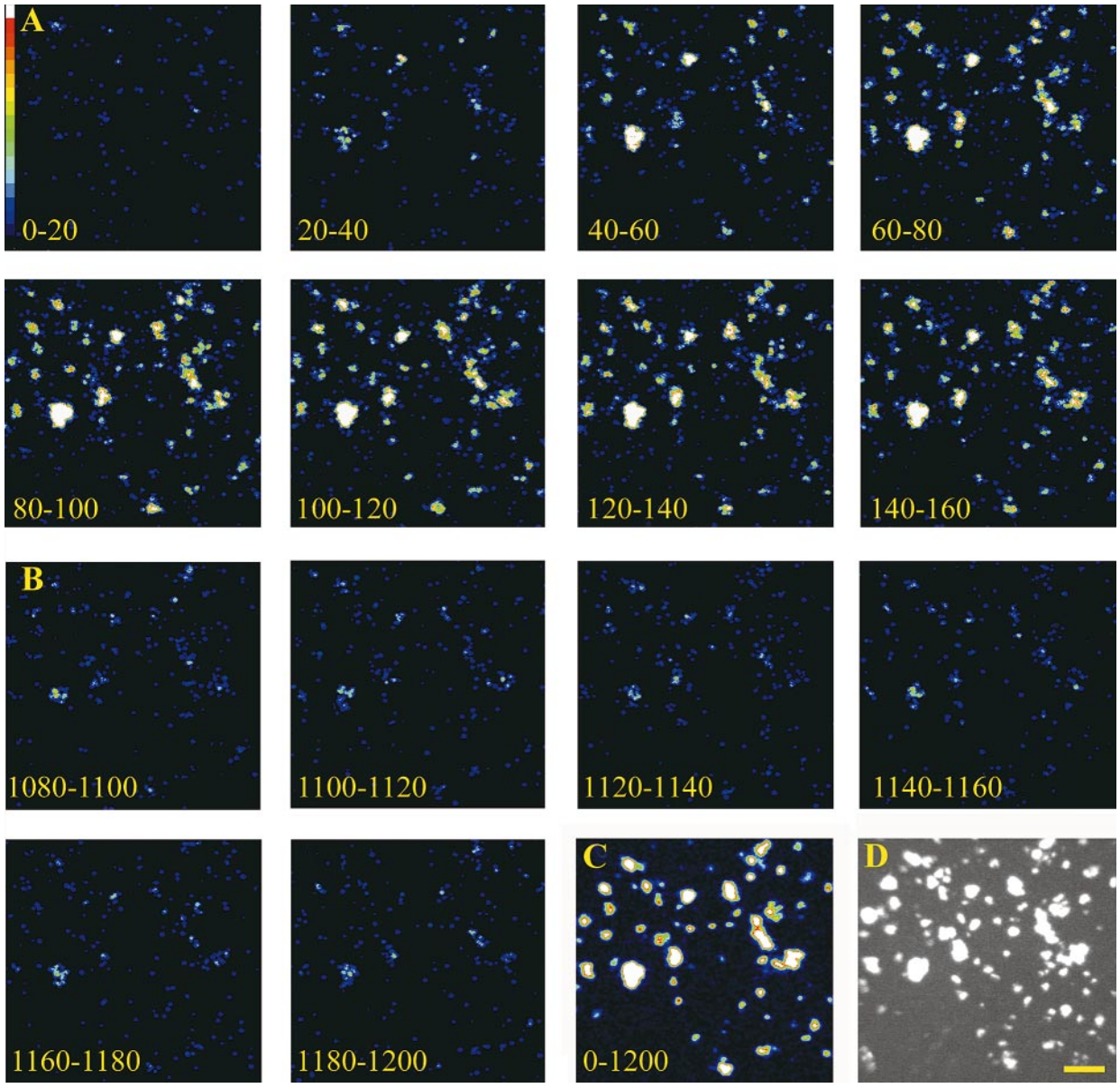

Figure 2. In Vivo Imaging of Pep-13–Induced Changes in $[Ca^{2+}]_{cyt}$ in Parsley Cells.

Bioluminescence of reconstituted aequorin in elicitor-treated parsley cells was monitored with a photon-counting video system. Individual images show light emission of parsley cells integrated for the times indicated. Pep-13 (100 nM) was added 30 sec before the start of measurements (i.e., before time zero).

(A) Image series (0 to 160 sec) corresponding to the peak phase observed in luminometric analyses (Figure 1).

(B) Image series (1080 to 1200 sec) corresponding to the plateau phase observed in luminometric analyses (Figure 1).

(C) Integration of light emission over the time course of the experiment (0 to 1200 sec).

(D) Bright-field image of parsley cells used in this experiment. Bar = 1.2 mm.

phase 1 but left phase 2 virtually unaffected (Figure 5A). Importantly, in the presence of RR, elicitor-treated parsley cells maintained the ability to produce phytoalexins, thus providing further evidence that phase 2 but not phase 1 of the $[Ca^{2+}]_{cyt}$ response is essential for activating the defense-associated reactions. This also suggests that Pep-13–induced sustained $[Ca^{2+}]_{cyt}$ increases may not be due to release from internal RR-sensitive stores.

Like RR, neomycin, an inhibitor of IP_3-releasing phospholipase C (Gabev et al., 1989), partially blocked phase 1 but not phase 2 of the Pep-13–induced $[Ca^{2+}]_{cyt}$ response (Figure 5B). Ca^{2+} release through IP_3-gated Ca^{2+} channels is therefore also unlikely to contribute to the elicitor-induced sustained increases of $[Ca^{2+}]_{cyt}$ associated with activation of pathogen defense. As shown in Figure 5B, another phospholipase C antagonist, U-73122 (Smallridge et al., 1992), did not inhibit the elicitor-induced $[Ca^{2+}]_{cyt}$ response. Both neomycin and RR strongly inhibited a mastoparan-induced $[Ca^{2+}]_{cyt}$ response (not shown), which is assumed to reflect release from internal stores. We therefore conclude that this experiment probably probed the actual intracellular pathways for Ca^{2+} release. Similarly, neomycin blocked a cold shock–induced $[Ca^{2+}]_{cyt}$ response, which is believed to result partially from the release of intracellular Ca^{2+}.

In summary, our findings strongly suggest that sustained increases of $[Ca^{2+}]_{cyt}$ in elicitor-treated parsley cells are predominantly the result of continuous Ca^{2+} influx through plasma membrane Ca^{2+} channels.

$[Ca^{2+}]_{cyt}$ Constitutes an Early Element of the Signaling Cascade Triggering Pathogen Defense in Parsley

A variety of pharmacological effectors of signal transduction pathways in eukaryotic cells were used to dissect the Pep-13–activated sequence of cellular responses in parsley. Major emphasis was put on elucidating the role of the elicitor-induced increase in $[Ca^{2+}]_{cyt}$ during activation of defense-associated responses, such as production of ROS and phytoalexins.

Heterotrimeric GTP binding proteins (G-proteins) are assumed to link cell surface receptors with intracellular effectors (Bischoff et al., 1999). Administration of the G-protein activator mastoparan to parsley cells yielded concentration-dependent large, rapid, and transient $[Ca^{2+}]_{cyt}$ responses (Figure 6). Mas-7, a mastoparan analog with fivefold more biological activity than mastoparan (Higashijima et al., 1990), evoked a substantially larger $[Ca^{2+}]_{cyt}$ transient peak than did mastoparan, whereas the inactive mastoparan analog Mas-17 stimulated only a small increase in $[Ca^{2+}]_{cyt}$. Our findings suggest that G-protein–activated signaling pathways may function in parsley. However, the $[Ca^{2+}]_{cyt}$ signature response stimulated by mastoparan greatly differed from that induced by the elicitors Pep-13, *P. sojae* glycoprotein, and harpin (Figures 1A and 3A). Consistently, moreover, unlike these elicitors, mastoparan was incapable of activating ROS and phytoalexin production and did not interfere with Pep-13–induced pathogen defense responses.

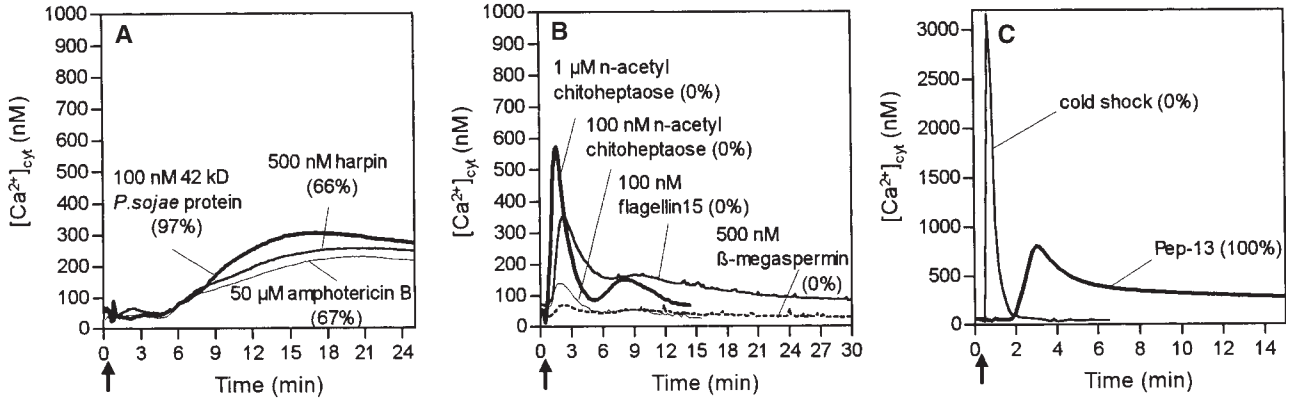

Figure 3. Effect of Abiotic and Biotic Stimuli on $[Ca^{2+}]_{cyt}$ Concentrations in Parsley Cells.

Bioluminescence of reconstituted aequorin was monitored in parsley cells treated with various stimuli at the times indicated (arrows). Numbers in parentheses represent amounts of phytoalexins produced by parsley cells treated as indicated relative to those produced by cells treated with 100 nM Pep-13.

(A) Addition of elicitors stimulating phytoalexin production in parsley cells. The *P. sojae*–derived 42-kD cell wall glycoprotein, recombinant *P. syringae* pv *phaseolicola*–derived harpin, and the polyene antibiotic amphotericin B were added at concentrations sufficient to stimulate elicitor-specific maximum phytoalexin production in parsley cells.

(B) Addition of elicitors of plant defense-associated responses incapable of stimulating phytoalexin production in parsley cells. Synthetic *N*-acetylchitoheptaose, a synthetic 15-mer fragment of bacterial flagellin, and purified β-megaspermin from *P. megasperma* were added at concentrations at least 10-fold greater than those reported to induce defense-associated responses in various plants.

(C) Cold shock was applied by adding an equal volume of ice-cold culture medium to parsley cells.

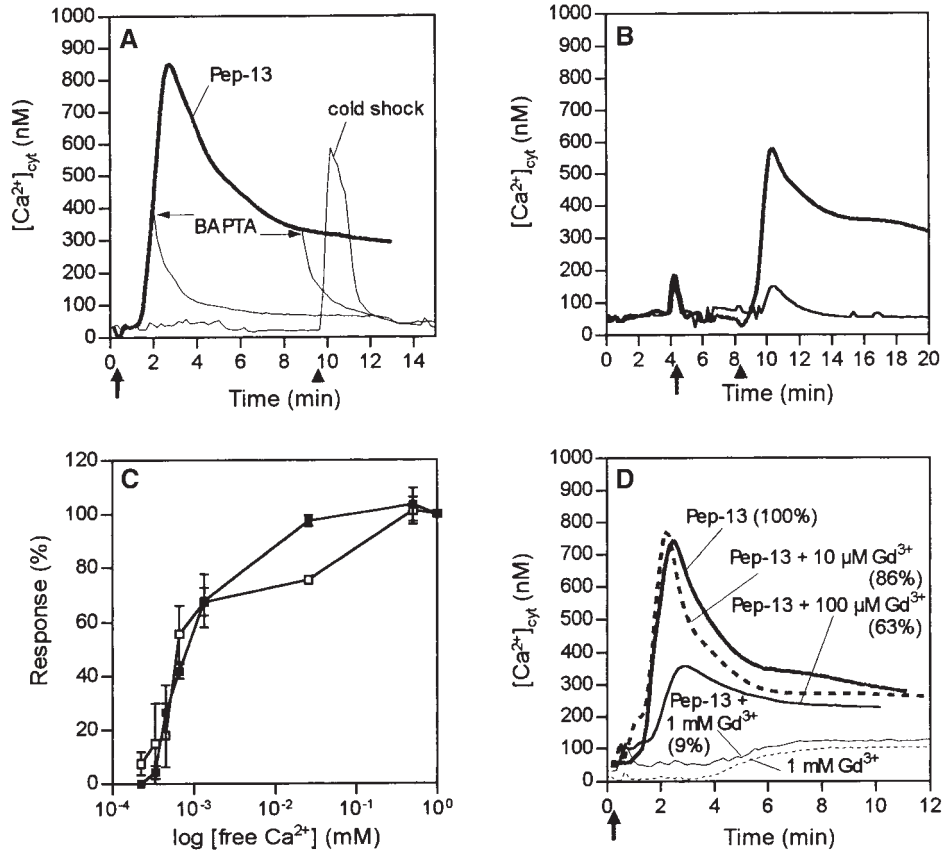

Figure 4. Inhibition of Pep-13–Induced Cellular Responses in Parsley Cells by Modulation of Ca^{2+} Influx across the Plasma Membrane.

(A) Parsley cells incubated in culture medium (containing 1 mM $CaCl_2$) were challenged with 100 nM Pep-13 at the time point indicated (thick arrow), and changes in $[Ca^{2+}]_{cyt}$ were recorded. The heavy line represents recordings obtained from parsley cells treated with Pep-13 only. The thin line represents recordings obtained from parsley cells treated first with the Ca^{2+} chelator BAPTA (4 mM) 5 min before the addition of Pep-13 and then with an equal volume of ice-cold culture medium (cold shock) as indicated (arrowhead). The thin lines descending from the Pep-13 line represent recordings obtained from parsley cells treated with 4 mM BAPTA at the times designated by thin arrows.

(B) Recording of $[Ca^{2+}]_{cyt}$ concentrations in parsley cells treated with BAPTA (4 mM) 8 min before the addition of 10 mM $CaCl_2$ (arrow). The cells were subsequently treated with 100 nM Pep-13 (heavy line) or water (thin line) at the time indicated (arrowhead).

(C) Dependence of the Pep-13–induced increase in $[Ca^{2+}]_{cyt}$ (filled squares) and oxidative burst (open squares) on extracellular $[Ca^{2+}]$. To progressively decrease the amount of extracellular free $[Ca^{2+}]$, parsley cells were incubated with increasing amounts of BAPTA (0 to 4 mM). Extracellular free $[Ca^{2+}]$ was calculated by using MaxChelator software (version 2.5 for Windows; http://www.stanford.edu/~cpatton/) (Bers et al., 1994). Data points represent means of four independent experiments. Error bars indicate SD.

(D) Changes in $[Ca^{2+}]_{cyt}$ were recorded from parsley cells treated with the Ca^{2+} channel inhibitor $GdCl_3$ at the concentrations indicated 5 min before the addition of water (dotted line) or 100 nM Pep-13 (as indicated) at the time noted (arrow). The solid line represents recordings obtained from parsley cells treated with Pep-13 only. Numbers in parentheses indicate the amounts of phytoalexins produced by parsley cells treated as indicated relative to those produced by cells treated with 100 nM Pep-13.

The G-protein activator, cholera toxin, failed to induce any Pep-13–specific response, including the $[Ca^{2+}]_{cyt}$ response (not shown). Moreover, cAMP, an element of a G-protein–activated signaling cascade, or cGMP, a product of receptor guanylyl cyclase activity, which is known to activate cyclic nucleotide–gated ion channels (both compounds tested as membrane-permeable dibutyryl derivatives), did not activate a $[Ca^{2+}]_{cyt}$ response. In summary, although involvement of G-proteins in pathogen defense signaling has been reported for tomato cells treated with a *Cladosporium fulvum*–derived elicitor (Blumwald et al., 1998, and references therein), our experiments do not support a role of G-protein–activated signaling pathways in transmitting the elicitor signal in parsley.

At concentrations previously reported to block chloride efflux, oxidative burst, MAP kinase activation, and phytoalexin production in parsley cells (Jabs et al., 1997; Ligterink

et al., 1997) (Figure 7A), use of the chloride channel inhibitors anthracene-9-carboxylate (A-9-C) and 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB) inhibited phase 1 but not phase 2 of the Pep-13–induced $[Ca^{2+}]_{cyt}$ response. The elicitor-induced Ca^{2+} influx giving rise to sustained increases in $[Ca^{2+}]_{cyt}$ may therefore act upstream of elicitor-induced Cl^- channels, which themselves have been shown to be essential for activation of pathogen defense by Pep-13 (Jabs et al., 1997).

The serine/threonine protein kinase inhibitors K-252a (Figure 7B) and staurosporine (not shown) completely blocked both phases of the Pep-13–induced $[Ca^{2+}]_{cyt}$ response and ROS production in parsley cells. In contrast, the tyrosine kinase inhibitors genistein and lavendustin did not affect either response (not shown), suggesting that the Pep-13 receptor is unlikely to belong to the tyrosine kinase family of plasma membrane receptors. Inhibitors of protein phosphatase 1 (calyculin A, tautomycin) and 2A (okadaic acid, cantharidin) blocked neither the $[Ca^{2+}]_{cyt}$ response nor ROS production in elicited parsley cells (not shown) at concentrations (1 μM) reported to abolish protein phosphatase activity in plant cells (Sopory and Munshi, 1998).

Receptor-Mediated Activation of the $[Ca^{2+}]_{cyt}$ Response in Elicitor-Treated Parsley Cells

Activation of elicitor-induced reactions in parsley cells is mediated through binding of Pep-13 to a 100-kD plasma membrane receptor protein (Nürnberger et al., 1994, 1995; Ligterink et al., 1997; Zimmermann et al. 1997; Nennstiel et al., 1998). To elucidate a possible functional link between Pep-13 perception, activation of Ca^{2+} influx, oxidative burst, phytoalexin production, and the Pep-13–induced increase in $[Ca^{2+}]_{cyt}$, we investigated a series of structural derivatives of Pep-13. As summarized in Figure 8A and Table 2, the $[Ca^{2+}]_{cyt}$ response of elicited parsley cells was activated by those Pep-13 derivatives that efficiently competed for binding of [^{125}I]Pep-13 to its receptor; they also strongly induced all other responses examined. Replacing Y12 (tyrosine at position 12) with alanine (Pep-13/A12) did not affect the ability of this peptide to bind to the Pep-13 receptor and to stimulate all responses in parsley cells. In contrast, replacing W2 with alanine (Pep-13/A2) rendered this derivative largely inactive with respect to increasing $[Ca^{2+}]_{cyt}$, corresponding to observed losses of competitor activity and the inability to trigger Ca^{2+} uptake and formation of ROS and phytoalexins. Similarly, deletion of one C-terminal and two N-terminal amino acid residues from Pep-13 (Pep-10) abolished the ability of this derivative to bind to the Pep-13 receptor and to induce increases in $[Ca^{2+}]_{cyt}$ and phytoalexin production. Taken together, our data provide evidence that the Pep-13–stimulated increase in $[Ca^{2+}]_{cyt}$ is a receptor-mediated process.

Treating apoaequorin-expressing cultured tobacco W38 cells with Pep-13 (0.1 to 1 μM) did not result in increased $[Ca^{2+}]_{cyt}$ (not shown). This is in agreement with the absence of a functional Pep-13 binding site in tobacco membranes (not shown) and corroborates previous observations that Pep-13 recognition and subsequent activation of defense responses is specific to parsley (Nürnberger et al., 1995). We further conclude from these experiments that integration of Pep-13 into plant cell membranes in an ionophore-like manner is unlikely.

Desensitization of plant cells to consecutive treatments with the same stimulus is commonly observed (Boller, 1995). To analyze a refractory state in elicitor-treated parsley cells,

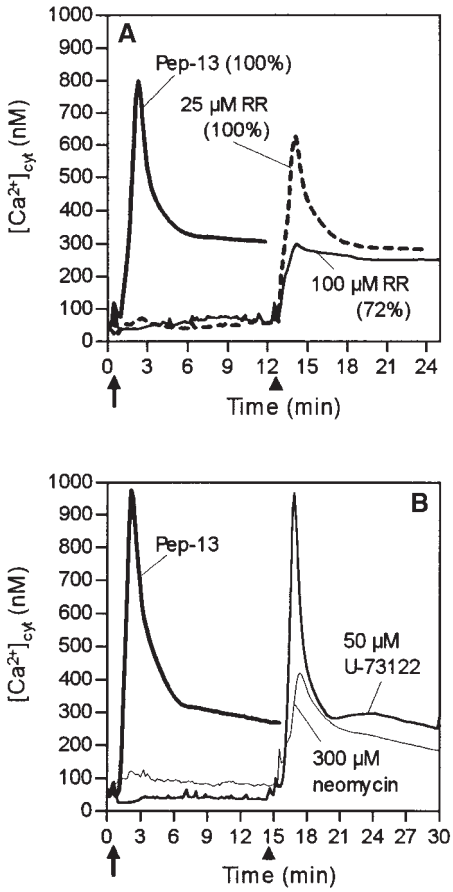

Figure 5. Inhibitors of Ca^{2+} Release or IP_3 Supply Affect the Pep-13–Induced Transient Increase but Not the Sustained Increase in $[Ca^{2+}]_{cyt}$.

Changes in bioluminescence were recorded from parsley cells treated with different inhibitors at times indicated (arrows) before the addition of 100 nM Pep-13. Arrowheads mark the times at which 100 nM Pep-13 was added to parsley cells. Numbers in parentheses indicated amounts of phytoalexins produced by parsley cells treated as indicated relative to those produced by cells treated with 100 nM Pep-13.
(A) Addition of the Ca^{2+} channel inhibitor RR (25 or 100 μM).
(B) Addition of phospholipase C inhibitors neomycin (300 μM) or U-73122 (50 μM).

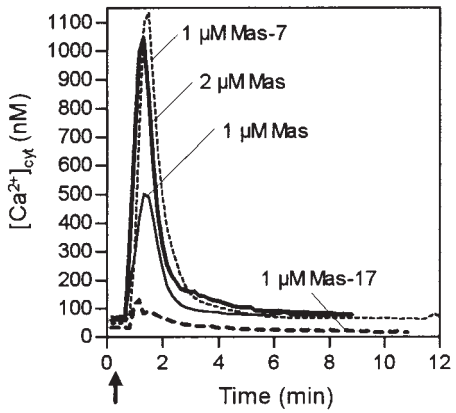

Figure 6. Mastoparan Evokes Changes in $[Ca^{2+}]_{cyt}$ in Parsley Cells.

Bioluminescence recordings were obtained from parsley cells treated with the G-protein activator mastoparan (Mas) or with mastoparan structural derivatives (Mas-7, Mas-17) at the time (arrow) and concentrations designated.

Pep-13 was administered at the start of bioluminescence recordings and after the decline of the $[Ca^{2+}]_{cyt}$ transient form. As shown in Figure 8B, no additional $[Ca^{2+}]_{cyt}$ response was evoked in cells that had been pretreated with Pep-13. Thus, addition of elicitor rendered the system refractory to repeated stimulation by the same signal for at least 40 min. Consistently, addition of the Pep-13–harboring *P. sojae* 42-kD glycoprotein elicitor to parsley cells pretreated with Pep-13 did not induce an additional increase in $[Ca^{2+}]_{cyt}$. Desensitization was stimulus specific, however, because subsequent addition of mastoparan to Pep-13–treated parsley cells activated a second $[Ca^{2+}]_{cyt}$ spike (Figure 8B). Loss of elicitor responsiveness is therefore assumed to indicate desensitization of the Pep-13 perception system.

DISCUSSION

Stimulus-dependent changes in $[Ca^{2+}]_{cyt}$ control a diverse range of cellular functions, including gene regulation and enzyme activation (Clapham, 1995; Parekh and Penner, 1997). Pulses, repetitive oscillations, and sustained plateaus constitute elements of $[Ca^{2+}]_{cyt}$ signatures (Dolmetsch et al., 1997, 1998; Li et al., 1998), all of which have been detected in plant cells (Thuleau et al., 1998; Rudd and Franklin-Tong, 1999; Trewavas, 1999). In parsley cell lines stably expressing the apoaequorin gene, we have monitored a biphasic $[Ca^{2+}]_{cyt}$ signature in response to elicitor treatment. We provide evidence that receptor-mediated increases in $[Ca^{2+}]_{cyt}$ to sustained higher values are required for activating pathogen defense responses in parsley. Hence, $[Ca^{2+}]_{cyt}$ is assumed to act as an early second messenger in transcriptional activation of an array of defense-related genes and subsequent phytoalexin production.

The Elicitor-Induced Increase in Cytoplasmic Free $[Ca^{2+}]$ in Parsley Cells Is Receptor Mediated

Three lines of experimental evidence indicate that elicitor-induced increase of $[Ca^{2+}]_{cyt}$ is a receptor-mediated process

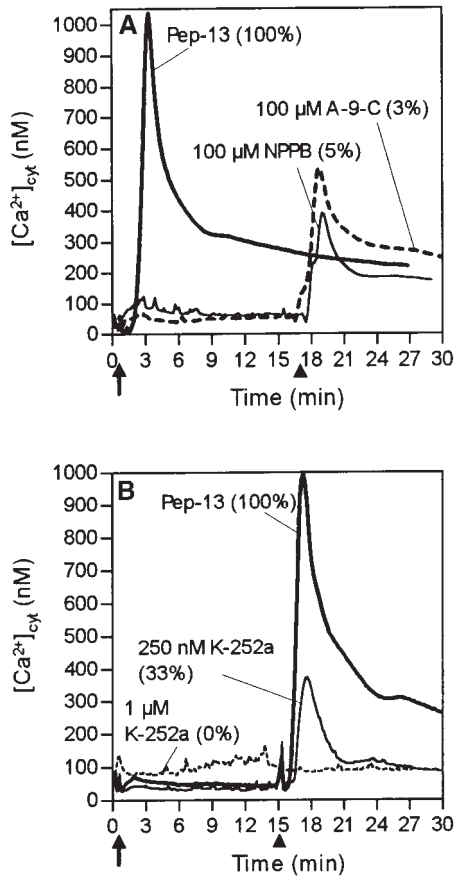

Figure 7. Effect of Chloride Channel and Protein Kinase Antagonists on Elicitor-Induced Changes in $[Ca^{2+}]_{cyt}$.

(A) and **(B)** Changes in bioluminescence were recorded from parsley cells treated with 100 nM Pep-13 (solid line) and either the chloride channel inhibitors A-9-C or NPPB **(A)** or protein kinase inhibitor K-252a **(B)** at the time (arrows) and concentrations designated. All compounds were applied from stock solutions made up in DMSO (final solvent concentration 0.1%). Arrowheads mark the time of addition of 100 nM Pep-13 to parsley cells pretreated with either inhibitor. Numbers in parentheses indicate the amount of phytoalexins **(A)** or hydrogen peroxide **(B)** produced by parsley cells treated as indicated relative to that produced by cells treated with 100 nM Pep-13.

(Table 2, and Figures 2 and 8). (1) Replacement of W2 by alanine or deletion of terminal residues markedly reduced the ability of these Pep-13 derivatives to trigger the $[Ca^{2+}]_{cyt}$ response. These residues were previously reported to be essential for elicitor as well as competitor activity of Pep-13 (Nürnberger et al., 1994, 1995; Zimmermann et al., 1997). Likewise, exchange of alanine for Y12 did not affect the ability of Pep-13 to bind to its receptor and to induce defense responses and left intact the ability of this derivative to trigger the $[Ca^{2+}]_{cyt}$ response. (2) Similar elicitor concentrations were sufficient to saturate the elicitor receptor and to trigger increases of $[Ca^{2+}]_{cyt}$ as well as other plant responses tested (Table 2). (3) Establishment of a refractory state with respect to repeated stimulation by the same signal was observed in parsley cells treated with Pep-13 (Figure 8B). Such a stimulus-specific desensitization of cellular responses is indicative of receptor-mediated activation and has also been reported for cultured tomato cells treated with fungal sterols or chitooligosaccharides (Boller, 1995).

A biphasic $[Ca^{2+}]_{cyt}$ signature of two transient peaks was observed in apoaequorin-transformed soybean cells after treatment with a *P. sojae*–derived β-glucan fraction or a synthetic hepta-β-glucan (Mithöfer et al., 1999). Although these experiments suggest a functional link between the elicitor-induced $[Ca^{2+}]_{cyt}$ response and phytoalexin production, as is reported here, the investigators could not demonstrate a receptor-mediated transient increase of $[Ca^{2+}]_{cyt}$ as a requirement for activation of pathogen defense. Moreover, hepta-β-glucan concentrations previously reported to saturate the elicitor receptor (Cosio et al., 1990) were insufficient to trigger the $[Ca^{2+}]_{cyt}$ response and phytoalexin production. However, the Mithöfer et al. study does not transform luminescence units into calibrated $[Ca^{2+}]_{cyt}$, which probably affects the proposed $[Ca^{2+}]_{cyt}$ signature. Thus, evidence for receptor-mediated activation of a defined $[Ca^{2+}]_{cyt}$ response signaling activation of pathogen defense was not given.

When treated with *Rhizobium meliloti* nodulation factors, alfalfa root hair cells mount a characteristic $[Ca^{2+}]_{cyt}$ signature (Ehrhardt et al., 1996). Image analysis of injected fluorescent Ca^{2+} indicators reveals oscillating $[Ca^{2+}]_{cyt}$ spikes with a mean oscillation period of 60 sec. Felle et al. (1999) reported a consistent, rapid decrease in extracellular Ca^{2+} and concomitant increase in $[Ca^{2+}]_{cyt}$ in alfalfa root cells treated with *R. meliloti*–derived Nod factors. This is remarkably similar to early responses of elicitor-treated parsley cells. Because the structural features of Nod factors required to cause nodulation in alfalfa were also essential for stimulating $[Ca^{2+}]_{cyt}$ spiking, Ehrhardt et al. (1996) concluded that there was a functional link between both responses. In addition, these findings also suggest a plasma membrane receptor–mediated activation of either response. However, although high- and low-affinity Nod factor binding sites were shown to reside in *Medicago truncatula* root cell plasma membranes (Gressent et al., 1999), genuine Nod factor receptors mediating establishment of symbiosis remain to be uncovered.

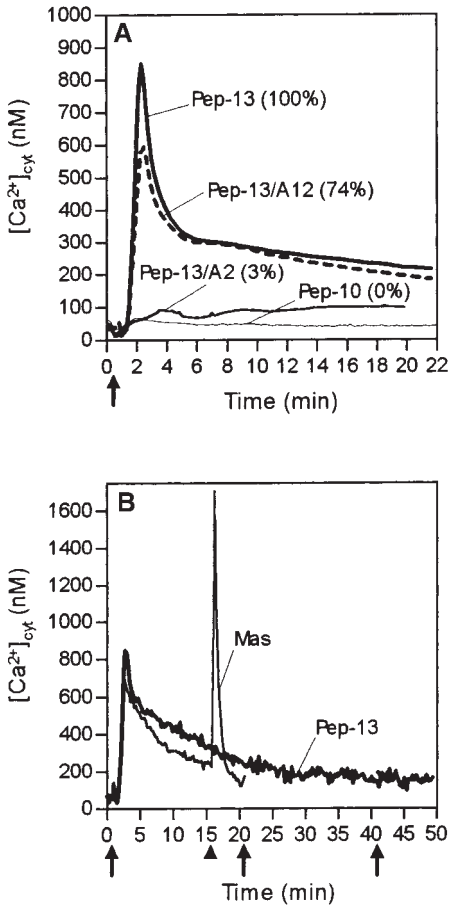

Figure 8. Elicitor-Induced Changes of $[Ca^{2+}]_{cyt}$ Are Receptor Mediated and Are Refractory to Consecutive Stimulation by Elicitor.

(A) Changes in bioluminescence recorded from parsley cells treated at the time indicated (arrow) with either 20 nM Pep-13 or 20 nM of the given Pep-13 structural derivatives. Numbers in parentheses indicate amounts of phytoalexins produced by parsley cells treated with each Pep-13 analog relative to those produced by cells treated with 100 nM Pep-13.

(B) Changes in bioluminescence recorded from parsley cells treated repeatedly with 100 nM Pep-13 at the times indicated (arrows). The arrowhead marks the time of addition of 1 μM mastoparan (Mas) to parsley cells pretreated with Pep-13. Measurements were performed in a microplate luminometer with continuous shaking of parsley cells.

Elicitor-Induced Sustained Increase in $[Ca^{2+}]_{cyt}$ Is Causally Involved in Activation of Pathogen Defense Responses in Parsley

Increases in $[Ca^{2+}]_{cyt}$ in parsley cells could be observed as early as 40 sec after addition of Pep-13. A large $[Ca^{2+}]_{cyt}$ transient peak of ~1 μM declined rapidly, reaching a plateau phase at ~250 to 350 nM $[Ca^{2+}]_{cyt}$ (Figure 1A). In vivo

imaging of aequorin activity revealed steady light emission from small parsley cell clusters during the course of the experiment (Figure 2), suggesting that there is no repeated spiking of $[Ca^{2+}]_{cyt}$ in individual cells. In particular, sustained values for $[Ca^{2+}]_{cyt}$ seem to be maintained continuously in elicitor-treated parsley cells. However, because the minimum interval for light signal integration was 5 sec, asynchronously responding cells of high-frequency $[Ca^{2+}]_{cyt}$ oscillations with mean period times of less than this are possible. On the other hand, studies on stimulus-induced $[Ca^{2+}]_{cyt}$ oscillations in plant cells noted mean period times between 15 sec and several minutes (Campbell et al., 1996; Ehrhardt et al., 1996; Calder et al., 1997; Staxen et al., 1999), which the technology applied would have been sufficient to detect.

Experimental evidence indicates that Pep-13–induced sustained increases in $[Ca^{2+}]_{cyt}$ and activation of pathogen defense in parsley are functionally inseparable. (1) Use of elicitors of pathogen defense in parsley (*P. sojae*–derived glycoprotein elicitor harboring Pep-13, harpin, amphotericin B) revealed that only the second phase of the $[Ca^{2+}]_{cyt}$ response, the prolonged increase in $[Ca^{2+}]_{cyt}$, was required for phytoalexin production (Figure 3A). Stimuli triggering only the rapid $[Ca^{2+}]_{cyt}$ transient peaks—chitooligosaccharides, a flagellin fragment, mastoparan, nystatin, cold shock—failed to induce sustained increases in $[Ca^{2+}]_{cyt}$ and phytoalexin production (Figures 3B and 3C). (2) Pep-13 concentrations that were insufficient to trigger the $[Ca^{2+}]_{cyt}$ transient response were sufficient to stimulate both the plateau phase and 80% of the phytoalexins produced at greater Pep-13 concentrations (Figure 1B). (3) The Ca^{2+} channel inhibitors Gd^{3+} and RR abolished the Pep-13–induced $[Ca^{2+}]_{cyt}$ spike at 100 μM but only slightly affected the $[Ca^{2+}]_{cyt}$ plateau value and phytoalexin production (Figures 4D and 5A). (4) Reduction of extracellular $[Ca^{2+}]$ by increasing concentrations of BAPTA resulted in gradual inhibition of the ability of Pep-13–treated parsley cells both to mount sustained increases in $[Ca^{2+}]_{cyt}$ and to produce ROS and phytoalexins. Similarly, removal of Ca^{2+} from the culture medium by several washes of cells with Ca^{2+}-free medium markedly reduced the elicitor-induced $[Ca^{2+}]_{cyt}$ response (not shown), the oxidative burst, the transcriptional activation of defense-related genes, and phytoalexin production in parsley cells (Nürnberger et al., 1994; Jabs et al., 1997). (5) Elicitor treatment of parsley cells in the presence of the Ca^{2+} channel blockers Gd^{3+} (Figure 4D) and La^{3+} or the protein kinase inhibitors (Figure 7B) inhibited the increase in $[Ca^{2+}]_{cyt}$ and production of ROS and phytoalexins. (6) Complementary gain-of-function experiments using amphotericin B revealed that artificially increasing $[Ca^{2+}]_{cyt}$ to sustained values triggered both the $[Ca^{2+}]_{cyt}$ response and the production of ROS and phytoalexins in the absence of elicitor (Figure 3A; Jabs et al., 1997). (7) Identical structural properties of Pep-13 were found to be required for efficient stimulation of the $[Ca^{2+}]_{cyt}$ plateau, of the oxidative burst, and of phytoalexin production (Table 2). (8) Initial changes in $[Ca^{2+}]_{cyt}$ clearly preceded ROS production and phytoalexin formation in elicitor-treated parsley cells (Figure 1A; Nürnberger et al., 1994).

Taken together, these findings indicate that the sustained increase of $[Ca^{2+}]_{cyt}$ rather than a single transient increase in $[Ca^{2+}]_{cyt}$ may encode signal specificity toward activation of pathogen defense. The physiological implication of the large $[Ca^{2+}]_{cyt}$ transient triggered by Pep-13, however, remains elusive.

A similar $[Ca^{2+}]_{cyt}$ response has been implicated in plant adaptation to abiotic stress. Ozone fumigation of Arabidopsis plants elicited a biphasic $[Ca^{2+}]_{cyt}$ response with a prolonged second phase required for transcriptional activation of a glutathione *S*-transferase gene (Clayton et al., 1999). Similarly, microinjection of cADP ribose into *Commelina communis* guard cells induced a sustained increase in $[Ca^{2+}]_{cyt}$ and resulted in stomatal closure (Leckie et al., 1998). A biphasic $[Ca^{2+}]_{cyt}$ response involving an initial transient peak followed by a second, prolonged, slow increase was also detected in Arabidopsis roots during cold acclimation (Plieth et al., 1999).

The importance of defined $[Ca^{2+}]_{cyt}$ signals for initiating different response pathways is intriguingly exemplified by activation of the inflammatory response in human B lymphocytes. The proinflammatory transcriptional regulators NF-κB and c-Jun N-terminal kinase (JNK) are activated by a $[Ca^{2+}]_{cyt}$ spike, whereas another essential transcriptional regulator, nuclear factor-activated T cell (NFAT), is activated by a low, sustained $[Ca^{2+}]_{cyt}$ plateau (Dolmetsch et al., 1997). Moreover, the $[Ca^{2+}]_{cyt}$ oscillation frequency was shown to modulate gene expression in these cell lines (Dolmetsch et al., 1998; Li et al., 1998). Apparently, downstream effectors decode complex $[Ca^{2+}]_{cyt}$ signatures, which probably constitute a mechanism by which the multifunctional second messenger $[Ca^{2+}]_{cyt}$ maintains specificity of signaling cascades. This view is supported by De Koninck and Schulman (1998), who reported that Ca^{2+}/calmodulin–dependent protein kinase II can decode the frequency of Ca^{2+} spikes into distinct amounts of its activity in vitro.

Elicitor-Induced Increase in $[Ca^{2+}]_{cyt}$ and Activation of Pathogen Defense Responses Requires Extracellular Ca^{2+}

Use of pharmacological effectors provided evidence that elicitor-induced sustained concentrations of $[Ca^{2+}]_{cyt}$ may predominantly reflect the influx of extracellular Ca^{2+}. Chelation of extracellular Ca^{2+} by membrane-impermeable BAPTA (Figures 4A and 4C) or EGTA completely abolished the increase in $[Ca^{2+}]_{cyt}$ and the activation of downstream responses in parsley cells, such as the oxidative burst (Figure 4C). In addition, only under experimental conditions in which an inwardly directed $[Ca^{2+}]$ gradient was maintained were parsley cells sensitive to elicitor treatment. Because BAPTA abrogated the $[Ca^{2+}]_{cyt}$ response also when added after the elicitor, a continuous Ca^{2+} influx appears to be required for

maintaining increased $[Ca^{2+}]_{cyt}$. To ensure a plateau value for $[Ca^{2+}]_{cyt}$, extrusion or sequestration of Ca^{2+} from the cytoplasm may therefore accompany elicitor-induced Ca^{2+} entry into the cytoplasm. Lanthanide inhibitors of plasma membrane Ca^{2+} channels blocked the elicitor-induced increase in $[Ca^{2+}]_{cyt}$ and the plant defense responses as well (Figure 4). The same inhibitors had previously also been found to block activation by Pep-13 of a parsley plasma membrane Ca^{2+}-permeable ion channel (Zimmermann et al., 1997). Likewise, treatment of parsley cells with the ionophore amphotericin B resulted in a Ca^{2+} influx, an increase in $[Ca^{2+}]_{cyt}$, the transcriptional activation of defense-related genes, and production of ROS and phytoalexins in the absence of elicitor (Jabs et al., 1997; Figure 3A). Furthermore, the attempted modulation of phospholipase C activity by neomycin, with the subsequent Ca^{2+} release from internal stores, and the inhibition by RR of RR-sensitive intracellular Ca^{2+} release channels failed to inhibit elicitor-induced sustained increases in $[Ca^{2+}]_{cyt}$ or activation of plant defense responses (Figure 5).

Increases in $[Ca^{2+}]_{cyt}$ in Nod factor–treated alfalfa root hair cells or in elicitor-treated soybean cells were also found to result mainly from a stimulus-induced Ca^{2+} influx through the plasma membrane (Felle et al., 1999; Mithöfer et al., 1999). In contrast, the increases of $[Ca^{2+}]_{cyt}$ monitored in plant cells in response to anoxia, cold shock, drought, or salinity have been ascribed to both a Ca^{2+} release from internal stores and an influx of external Ca^{2+} (Subbaiah et al., 1994; Haley et al., 1995; McAinsh et al., 1995; Knight et al., 1996, 1997). Thus, just as in animal cells, two major pathways for Ca^{2+} release into the cytoplasm may be operative in plant cells (Clapham, 1995; Parekh and Penner, 1997; Thuleau et al., 1998; Rudd and Franklin-Tong, 1999): Ca^{2+} influx directly through plasma membrane Ca^{2+} channels, and Ca^{2+} entry by second messenger–induced depletion of intracellular stores followed by store replenishment by way of Ca^{2+} release–activated Ca^{2+} currents across the plasma membrane.

Numerous Ca^{2+} channels have been identified in plant cells, but their physiological roles and probable cooperation in response to various stimuli are little understood. Challenging future tasks will be to understand how different Ca^{2+} channels contribute to signal-specific $[Ca^{2+}]_{cyt}$ signatures and how (spatio)temporal alterations in $[Ca^{2+}]_{cyt}$ can activate downstream effectors, such as Ca^{2+}- or Ca^{2+}/calmodulin–dependent protein kinases (Blumwald et al., 1998; Thuleau et al., 1998; Rudd and Franklin-Tong, 1999; Trewavas, 1999).

METHODS

Materials

The peptides Pep-13, Pep-13/A2, mastoparan, mastoparan-17, and flagellin-15 were synthesized by use of fluorenyl methoxycarbonyl chemistry as described by Nennstiel et al. (1998). Pep-13/A12 and Pep-10 were purchased from Kem-En-Tec A/S (Copenhagen, Denmark), and Mas-7 was from Calbiochem (Bad Soden, Germany). The *Phytophthora sojae* 42-kD cell wall glycoprotein elicitor was purified according to Nürnberger et al. (1994). Purified recombinant *Pseudomonas syringae* pv *phaseolicola* harpin was from Justin Lee (IPB, Halle, Germany). The *Phytophthora megasperma* elicitin β-megaspermin was provided by Serge Kauffmann (INRA, Strasbourg, France), *N*-acetylchitoheptaose was from Naoto Shibuya (University of Tsukuba, Tsukaba, Japan), and *N*-acetylchitopentaose was from Jürg Felix (FMI, Basel, Switzerland). Inhibitors were purchased from Alexis (Grünberg, Germany), anthracene-9-carboxylate (A-9-C) and *N*-acetylchitotriose from Sigma (Deisenhofen, Germany), amphotericin B from Calbiochem, and EGTA, $LaCl_3$, and $GdCl_3$ as well as high-purity chemicals for in vitro $[Ca^{2+}]$ calibration from Fluka (Deisenhofen, Germany). 1,2-Bis(aminophenoxy)ethane-*N*,*N*,*N′*,*N′*-tetraacetic acid (BAPTA), coelenterazine, and calcium calibration buffers were supplied by Molecular Probes (Leiden, The Netherlands).

Plant Cell Culture and Elicitor Treatment

Dark-grown cell suspension cultures of *Petroselinum crispum* were used for elicitor treatment and reconstitution of aequorin 5 days after inoculation. Culture maintenance and quantification of reactive oxygen species (ROS) and furanocoumarin phytoalexin production were performed according to Nürnberger et al. (1994). Cell viability was determined by double-staining with fluorescein diacetate and propidium iodide (Jabs et al., 1997) 30 min or 24 hr after treatment, respectively. Tobacco W38 cell suspensions stably transformed with apoaequorin were obtained from Phil Low (Purdue University, West Lafayette, IN) and maintained as described (Chandra et al., 1997).

Aequorin Constructs and Parsley Cell Transformation

Three-day-old parsley cells were transformed as described (Frohnmeyer et al., 1999) by particle bombardment with a plasmid carrying apoaequorin cDNA inserted between a double cauliflower mosaic virus 35S promoter, a tobacco mosaic virus Ω-element, and a 35S terminator (p*AEQ*/*HYG*). This plasmid was derived from pGN*AEQ*/*NEO2* (Mithöfer et al., 1999) by replacing the cassette for the selectable marker *NPTII* with a hygromycin-resistance gene (*HPT*) excised from plasmid pGL2 (Mithöfer et al., 1999). Plasmids pGN*AEQ*/*NEO2* and pGL2 were kindly provided by Gunther Neuhaus (University of Freiburg, Germany).

Aequorin Reconstitution and Bioluminescence Measurement

In vivo reconstitution of aequorin was initiated by adding 3 μL of coelenterazine (5 mM stock solution in methanol) to 3 mL of 5-day-old aequorin-transgenic parsley cells. Cells were adjusted to 60 mg (fresh weight) mL^{-1} culture medium supplemented with 1 mM $CaCl_2$. Subsequently, cells were incubated in the dark under continuous shaking (for 6 hr or overnight, at 26°C and 120 rpm).

Bioluminescence counts from 100-μL cell culture aliquots were recorded at 10-sec intervals (recorded as average relative light units (RLUs)/sec) with a digital luminometer (Lumat LB9501; Berthold, Bad Wildbad, Germany). At the end of each experiment, the remaining aequorin was discharged by adding two volumes of 37.5 mM $CaCl_2$/15% ethanol (v/v). The amount of aequorin consumed at each time

point of the experiment never exceeded 5% of the total remaining aequorin. Alternatively, bioluminescence assays were performed with a microplate luminometer (Luminoskan Ascent, version 2.4; Labsystems, Frankfurt/Main, Germany) under continuous shaking of cell suspensions (300-μL aliquots in 48-well plates, measured every 13 sec). No differences in reproducibility were observed between the two bioluminescence monitoring devices used.

In vivo calcium imaging of aequorin activity in parsley cells was performed with a C2400-40H Intensified (CCD) Charge-Coupled Device Camera device (Hamamatsu Photonics GmbH Deutschland, Herrsching, Germany) equipped with a Nikon lens system ($f = 35$ mm, 40-mm-long extension tube; Nikon Deutschland, Düsseldorf, Germany) and the operating software HiPic 5.1. Parsley cells (5×10^3 in 100 μL) were placed on a microscope slide, treated with elicitor, and subjected to photon counting in the dark. Bioluminescence was recorded in slice mode in dynamic photon-counting format.

Calibration of Calcium Measurements

To transform bioluminescence counts into absolute [Ca^{2+}] values, we established a calibration curve from in vitro measurements of protein extracts made from p*AEQ*/*HYG*–expressing parsley cells and a series of buffers with various [Ca^{2+}]. Transgenic parsley cells were washed with calcium-free buffer, frozen in liquid nitrogen, and homogenized in reconstitution buffer: 20 mM Tris/HCl, pH 7.4, 150 mM KCl, 5 mM EDTA, 0.1 mM EGTA, 50 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (5 min, 4°C, 12,100*g*) the supernatant was utilized for in vitro reconstitution of aequorin (3 hr, 4°C) in the presence of 0.1% gelatin and 2.5 μM coelenterazine.

Light production was measured in buffers (10 mM Hepes, pH 7.2, 100 mM KCl, 1 mM $MgSO_4$) with known [Ca^{2+}] (Molecular Probes calcium calibration buffer kit). [Ca^{2+}] values were subsequently calculated as rate constants from the total aequorin activity assessed at saturating concentrations of Ca^{2+} according to Cobbold and Lee (1991). Rate constants represent bioluminescence counts monitored per second divided by the total remaining bioluminescence counts.

All reagents tested were applied to lysates containing recombinant aequorin to examine the direct effects of the reagents on aequorin luminescence. None of these substances interfered with aequorin luminescence.

Extracellular free [Ca^{2+}] in the presence of the chelator BAPTA was calculated using MaxChelator software (version 2.5 for Windows; http://www.stanford.edu/~cpatton/) (Bers et al., 1994).

ACKNOWLEDGMENTS

We are indebted to Gunther Neuhaus for critical reading of the manuscript, and to him and Claudia Köhler for assisting in particle bombardment experiments as well as for kindly providing plasmids pGN*AEQ*/*NEO2* and pGL2. We thank Hanns Frohnmeyer and Linda Loyall for helpful discussions on parsley cell transformation, and Hans-Hubert Felle, Birgit Klüsener, Peter Buschmann, and Richard Nuccitelli for assistance in calculating free ion concentrations. Delivery of elicitors by Justin Lee, Serge Kauffmann, Naoto Shibuya, and Jürg Felix and of apoaequorin-transgenic tobacco cells by Phil Low is gratefully acknowledged. We thank Helga Nixdorf and Jutta Elster for peptide syntheses and Annette Romanski for performing Pep-13 binding assays on tobacco membranes. This work was supported by the CAST project of the European Community Biotech program (Grant No. BIO4-CT96-0101) and the Fonds der Chemischen Industrie.

Received March 1, 2000; accepted May 22, 2000.

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$HrpZ_{Psph}$ from the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* binds to lipid bilayers and forms an ion-conducting pore *in vitro*

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Edited by Eugene W. Nester, University of Washington, Seattle, WA, and approved November 9, 2000 (received for review June 8, 2000)

The *hrp* gene clusters of plant pathogenic bacteria control pathogenicity on their host plants and ability to elicit the hypersensitive reaction in resistant plants. Some *hrp* gene products constitute elements of the type III secretion system, by which effector proteins are exported and delivered into plant cells. Here, we show that the *hrpZ* gene product from the bean halo-blight pathogen, *Pseudomonas syringae* pv. *phaseolicola* ($HrpZ_{Psph}$), is secreted in an *hrp*-dependent manner in *P. syringae* pv. *phaseolicola* and exported by the type III secretion system in the mammalian pathogen *Yersinia enterocolitica*. $HrpZ_{Psph}$ was found to associate stably with liposomes and synthetic bilayer membranes. Under symmetric ionic conditions, addition of 2 nM of purified recombinant $HrpZ_{Psph}$ to the cis compartment of planar lipid bilayers provoked an ion current with a large unitary conductivity of 207 pS. $HrpZ_{Psph}$-related proteins from *P. syringae* pv. *tomato* or *syringae* triggered ion currents similar to those stimulated by $HrpZ_{Psph}$. The $HrpZ_{Psph}$-mediated ion-conducting pore was permeable for cations but did not mediate fluxes of Cl^-. Such pore-forming activity may allow nutrient release and/or delivery of virulence factors during bacterial colonization of host plants.

Pathogenic bacteria use highly specialized mechanisms to proliferate in their hosts. Recent work has revealed the importance of a type III protein-secretion system essential for pathogenicity of Gram-negative bacteria (1, 2). Type III secretion systems were reported initially from the mammalian pathogen *Yersinia enterocolitica* but have been identified also in various phytopathogenic bacteria (2). Specific effector proteins are believed to be injected into the cytosol of eukaryotic host cells by using this secretion apparatus (2). In contrast to the proteins that form the secretion apparatus, the export substrates often are not conserved among pathogenic bacteria (2, 3).

The *Yersinia* Yop (*Yersinia* outer proteins) virulon serves as a paradigm for the type III secretion system (1). A total of 25 *ysc* (Yop secretion) genes encode proteins implicated in protein translocation across the bacterial inner and outer membranes (1, 2, 4). Among 12 secreted Yops are three (YopB, D, and LcrV) that promote translocation of effector proteins across the mammalian-host cell-plasma membrane (1). YopB and YopD are hydrophobic proteins that interact with each other (1, 5). Pore-forming activity ascribed to both proteins (1, 4, 5) has been verified recently by experiments showing that YopB and YopD form an ion-conducting pore after insertion into planar lipid bilayers (6).

Phytopathogenic bacteria harbor a gene cluster (*hrp* for hypersensitive reaction and pathogenicity) that is essential for pathogenicity in susceptible plants and the ability to elicit the hypersensitive reaction in nonhosts or resistant cultivars of host plants (2, 7). *hrp* genes are expressed *in planta* or in media that mimic plant apoplastic conditions (8). Sequence analyses have uncovered a shared subset of *hrp* genes that were redefined as *hrc* (for *hrp* and conserved) (9) and that encode proteins homologous to *Yersinia ysc* gene products (2, 10–12). This observation suggests evolutionary conservation of molecular mechanisms of pathogenicity used by both mammalian and phytopathogenic bacteria (2). A number of proteins secreted by phytopathogenic bacteria in an hrp-dependent manner have been identified (2), but their molecular modes of action are understood poorly.

Harpins constitute a group of secreted effector proteins traveling along the type III pathway in plant pathogenic bacteria (2, 13). Genes encoding such proteins have been identified in *Erwinia amylovora*, *Erwinia chrysanthemi* (*hrpN*) (14), *Pseudomonas syringae* pvs. *syringae*, *tomato,* or *glycinea* (*hrpZ*) (7, 15), and *Ralstonia solanacearum* (*popA*) (16). Although not homologous in primary sequence, harpins from different bacterial genera trigger disease resistance-associated responses, such as hypersensitive cell death, and thus activate the plant's surveillance system (2, 13). However, the contribution of harpins to bacterial pathogenicity remains enigmatic. Mutations in *hrpN* significantly reduced pathogenicity of *E. amylovora* (14, 17), whereas mutations in *hrpZ* left the virulence of *P. syringae* pv. *tomato* apparently unaffected (18). The presence in *P. syringae* pathovars of genes functionally redundant to *hrpZ*, such as *hrpW*, may account for the unaltered pathogenicity of *hrpZ* mutants (18). Another open question concerns the site of action of harpins during infection. Immunolocalization studies revealed Ca^{2+}-dependent association of *P. syringae* pv. *syringae* harpin with plant cell walls (19). However, whether this interaction identifies a physiological target implicated in colonization of the host plant has yet to be determined.

Here, we report on the analysis of the structure and function of *hrpZ* in the bean halo-blight pathogen *P. syringae* pv. *phaseolicola* (*Psph*). Structural properties of $HrpZ_{Psph}$ resemble those of proteins assumed to interact with membranes such as YopB

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: POPC, 1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine; POPE, 1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF 268940).

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.011265298. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.011265298

from *Y. enterocolitica*. Similar to YopB (6), $HrpZ_{Psph}$ and related proteins from *P. syringae* pvs. *tomato* or *syringae* are integrated into bilayer membranes to form an ion-conducting pore *in vitro*. We propose that pore formation by harpins may either mediate nutrient release from host cells or assist delivery of virulence factors into the plant cell cytoplasm by facilitating movement of proteins through the plasma membrane.

Materials and Methods

Bacterial Strains, Plasmids, and Bacterial Growth. Wild-type and mutant strains of *Psph* race-6 and *pilM*-deficient *Psph* strain HB10Y were grown as described (20). Protein secretion was initiated in minimal-growth medium (21). The nonpolar *hrpA* and *hrpZ* mutants were constructed as follows: *hrpA* was deleted from bases 46 to 256 of the coding sequence in pPPY438 by a double digest with *Csp*45I and *Hpa*I and religated after end filling; *hrpZ* was deleted from bases 586 to 876 in pPPY438 by digests with *Mun*I and *Ppu*MI and religated. The truncated coding regions were cloned into the suicide vector pOK and introduced into *Psph* as described (22).

Y. enterocolitica strains were grown as described (5). MRS40(pABL 403) is a derivative of wild-type strain MRS40 lacking *yopH*, *yopO*, *yopP*, *yopE*, and *yopM* (23). Strain MRS40(pAB409) is a *yopB*-deficient derivative of MRS40(pABL403) (23). KNG22703(pSW2276) is a *yscN*-deficient mutant of wild-type strain KNG22703(pYV227) (24). For complementation of MRS40(pAB409) with *yopB*, pCNR27-encoding YopB was used (5). For expression of *hrpZ* in *Y. enterocolitica*, an *Xba*I/*Pst*I fragment from vector pT7-7-*hrpZ* containing the *hrpZ* ORF (25) was subcloned into pBluescript SK(+) (pSK-hrpZ), facilitating expression from the basal *lac* promoter. *Y. enterocolitica* transformation by electroporation, induction of the Yop virulon, and analysis of Yop proteins in culture supernatants were performed as described (26).

Plant Infiltration and Sheep Erythrocyte Hemolysis Assays. Pathogenicity tests of *Psph* strains on bean (*Phaseolus vulgaris* L.) were performed as described (27). Hemolytic activity was assayed as described (5).

Preparation of Recombinant HrpZ. A 1.38-kb *Nde*I/*Hin*dIII fragment from plasmid pT7-7-*hrpZ* was introduced into the modified pET vector, pJC40, encoding an N-terminal His_{10} tag (28). Expression in BL21 (DE3) pLysS *Escherichia coli* cells was initiated, and expressed proteins were isolated on Ni-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany). Stringent washes with buffer containing 60 mM of imidazole before elution were crucial for obtaining pure protein. Removal of the His_{10} tag was achieved by Factor Xa (Denzyme, Aarhus, Denmark) treatment for 12 h at 25°C in 50 mM Tris·HCl, pH 8.0 and 1 mM $CaCl_2$ containing 1/100 (wt/wt) protease. $HrpZ_{Psph}$ was separated by 40% $(NH_4)_2SO_4$ fractionation and desalted by ultrafiltration with a 30-kDa cut-off concentrator (Amicon). The purity of recombinant $HrpZ_{Psph}$ was checked by SDS/PAGE and silver staining. To express fragments of $HrpZ_{Psph}$, the corresponding PCR-generated products were cloned into vector pJC40 as *Nde*I/*Bam*HI or *Nde*I/*Eco*RI fragments, respectively. Recombinant proteins were purified as described above except that ultrafiltration was performed with 10- or 3.5-kDa cut-off filters, respectively. A PCR-generated *Hin*dIII/*Bam*HI fragment derived from *P. syringae* pv. *tomato* DC3000 DNA was subcloned into vector pJC40 for production of recombinant $HrpZ_{Pst}$. Recombinant $HrpZ_{Pss}$ was produced as described (7) with the vector pSYH10.

Protein Biochemistry. Phospholipid-binding assays were carried out as described (29). To determine β-glucuronidase activity, plasmid pDGUS (30) carrying the *E. coli uidA* gene was introduced into *Psph* strains by triparental mating as described (27). GUS activity was quantified as described (30).

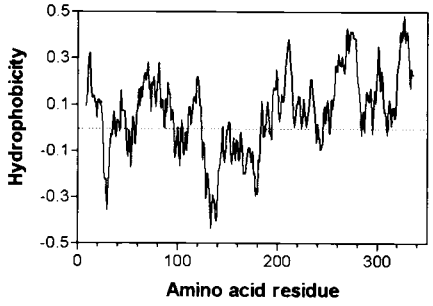

Fig. 1. Hydrophobicity plot of $HrpZ_{Psph}$ based on the Eisenberg algorithm (35).

Lipid-Binding and Planar Lipid Bilayer Experiments. Association of secreted proteins with liposomes and isolation of proteoliposomes was performed as described (6) except that liposomes were added to *Psph* grown in minimal medium. Lipid-bound proteins were precipitated in 80% (vol/vol) acetone before SDS/PAGE immunoblotting by using the anti-$HrpZ_{Psph}$ antiserum. In control experiments, PBS (pH 7.1) without liposomes was added to the bacteria to ensure that *hrp* gene induction was unaffected. Protein binding to silica beads coated with single lipid bilayers (TRANSIL, NIMBUS Biotechnology, Leipzig, Germany) was performed as described (31). Assays involved incubation of 5 μl of TRANSIL beads and 1 μM of purified recombinant protein for 1 h (room temperature; 10 mM Tris·HCl, pH 7.4, 150 mM NaCl, protein:lipid ratio 1:500/1,000). After centrifugation (5 min, 1,500 × *g*), the beads were washed three times with binding buffer. Unbound proteins and wash fractions were pooled and precipitated by 5% (vol/vol) ice-cold trichloroacetic acid. Bound proteins were eluted with sample buffer for SDS/PAGE and silver staining. Planar lipid bilayer experiments were performed as described (32). The trans compartment of the cuvette is defined to be at ground potential. The sign of the membrane voltage refers to the cis compartment, and a positive current (upward deflections) corresponds to a cation transfer from the cis to the trans compartment. $HrpZ_{Psph}$ was added to the aqueous solution of the cis compartment.

Results

$HrpZ_{Psph}$ Is Produced and Secreted in an hrp-Dependent, Type III-Specific Manner. The clone pPPY430 contains the *hrp* gene cluster from *Psph* race 6 except for part of *hrpRS* (33). Comparison of the region of PPY430 containing *hrpZ* (subclone pPPY438; GenBank accession no. AF 268940) with the *hrpZ* operon described for other pathovars of *P. syringae* (15) revealed *Psph* genes homologous to *hrpA*, *hrpZ, hrpB, hrcJ, hrpD*, and *hrpE.* In contrast to other pathovars of *P. syringae* (15, 34), Northern blot analysis of RNA prepared from *Psph* grown in minimal medium revealed separate *hrpA* and *hrpZ* + *hrpB*-specific transcripts (not shown). The deletion of basepairs 46–356 of *hrpZ* abolished accumulation of the gene product in bacteria grown under *hrp*-inducing conditions, but no alteration in pathogenicity on bean was associated with the *hrpZ* mutation (not shown). $hrpZ_{Psph}$ encodes a 345-aa protein (35.2 kDa), which shows 53 and 77% protein sequence similarity to the respective orthologs from *P. syringae* pv. *tomato* and *syringae.* Hydropathy plots (Eisenberg algorithm; ref. 35) revealed the amphipathic nature of the encoded protein, which comprises a hydrophilic central region flanked by two hydrophobic terminal domains (Fig. 1).

Expression of *hrpZ* from other *P. syringae* pvs. is *hrp* gene dependent (15). By using an antiserum raised against recombi-

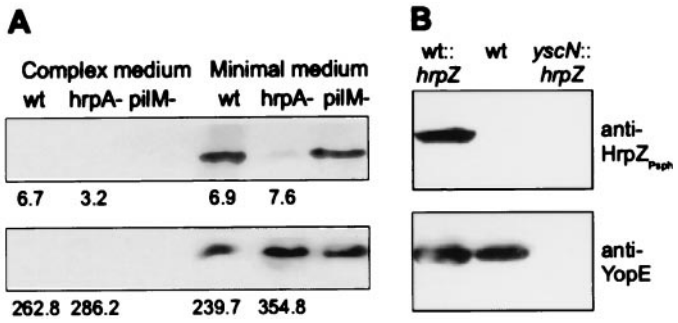

Fig. 2. Analysis of $HrpZ_{Psph}$ secretion by *Psph* and by *Y. enterocolitica*. (*A*) *hrp*-dependent secretion of $HrpZ_{Psph}$. *Psph* race-6 wild-type (wt), race-6 *hrpA*− mutant (hrpA−), and a type IV pili mutant of strain HB10Y (pilM−) were grown in complex media or *hrp*-inducing minimal media (8). Bacteria were pelleted by centrifugation, and secreted proteins were precipitated from the culture supernatant by 5% (vol/vol) trichloroacetic acid. Proteins prepared from the supernatant (*Upper*) and pellet (*Lower*) were analyzed by SDS/PAGE and immunoblotting by using an antiserum raised against recombinant $HrpZ_{Psph}$ (1:5,000 dilution). Numbers below individual lanes represent β-glucuronidase activity (nmol 4-methylumbelliferone released per minute per bacterium × 10^{10}), which was determined in *uidA*-transformed *Psph* strains grown in complex and minimal growth medium (30). (*B*) Type III-dependent secretion of $HrpZ_{Psph}$. *Y. enterocolitica* wild-type strain KNG22703(pYV227) (wt), this strain carrying pBluescript SK(+)-*hrpZ* (wt::*hrpZ*), and *Y. enterocolitica* type III secretion-deficient *yscN*- mutant KNG22703(pYV2276) transformed with plasmid pBluescript SK(+)-*hrpZ* (*yscN*::*hrpZ*) were grown under permissive conditions. Analysis of secreted proteins was performed by using antisera raised against $HrpZ_{Psph}$ or YopE, respectively.

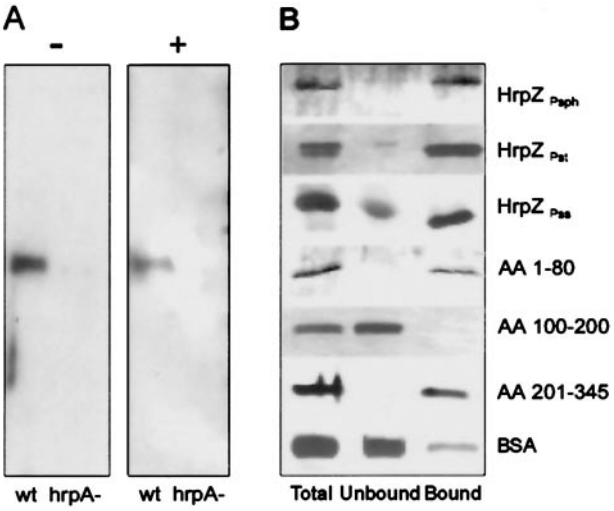

Fig. 3. $HrpZ_{Psph}$ interacts with lipid membranes. (*A*) *Psph* race-6 wild-type (wt) and race-6 mutant hrpA− were grown in *hrp*-inducing minimal media (8) in the absence (−) or presence (+) of liposomes. Proteins prepared from the supernatant or proteoliposomes were separated and analyzed by SDS/PAGE immunoblotting with an anti-$HrpZ_{Psph}$ antiserum. (*B*) TRANSIL beads coated with 1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine (POPC)/1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (POPE) (80:20) were incubated for 1 h with 1 μM of the proteins indicated (Total). After separation of lipid-bound (Bound) from unbound material, proteins were analyzed by SDS/PAGE and silver staining. $HrpZ_{Pst}$, HrpZ from *P. syringae* pv. *tomato* DC3000; $HrpZ_{Pss}$, HrpZ from *P. syringae* pv. *syringae*; AA 1–80, AA 100–200, AA 201–345, $HrpZ_{Psph}$ fragment encompassing the N-terminal 80 amino acid (AA) residues, amino acids 100–200, or the C-terminal amino acids 201–345, respectively.

nant $HrpZ_{Psph}$, we detected this protein in minimal, but not in complex, media (Fig. 2*A*). This was because of secretion rather than cell lysis, because no leakage of a constitutively expressed cytoplasmic marker protein β-glucuronidase was observed (Fig. 2*A*). The *hrpA* gene of *P. syringae* pv. *tomato*, which encodes the major constituent of the Hrp pilus (21), was shown recently to be required for full expression and secretion of putative virulence proteins such as HrpW and AvrPto (36). The *Psph hrpA* mutant, which was nonpathogenic on bean (not shown), accumulated $HrpZ_{Psph}$ intracellularly to the same levels as in wild-type bacteria, but secretion was reduced strongly (Fig. 2*A*). By contrast, a mutation in *pilM*, which is required for type IV pilus production (20), did not affect the secretion of $HrpZ_{Psph}$. An *hrcJ* mutant of *Psph* also did not secrete $HrpZ_{Psph}$ (not shown). Thus, $HrpZ_{Psph}$ is produced and secreted in an *hrp*-dependent and type III-specific manner.

The apparent evolutionary conservation of type III secretion systems prompted us to investigate whether *Psph* export substrates can be secreted by the mammalian pathogen *Y. enterocolitica*. Therefore, we introduced *hrpZ* under control of the basal *lac* promoter into strain KNG22703(pYV227). After induction of the *yop* virulon (5), the transformants produced and secreted $HrpZ_{Psph}$ (Fig. 2*B*). However, secretion of $HrpZ_{Psph}$ was not observed in its secretion-deficient *yscN* mutant KNG22703(pSW2276) (Fig. 2*B*). Thus, a putative phytopathogenic virulence factor, $HrpZ_{Psph}$, is exported from a mammalian pathogen, *Y. enterocolitica*, in a type III secretion-dependent manner.

The PROPSEARCH algorithm (37) allows the identification of proteins that may be dissimilar in primary sequence but share common structural properties. Comparison of $HrpZ_{Psph}$ predicted similarity to several proteins known to interact with membranes and to form protein complexes, among them *Yersinia* YopB (1, 5, 6) (reliability score 41%; ref 37). To test for a possible functional similarity between the two proteins, we attempted complementation by $hrpZ_{Psph}$ of *yopB*-deficient *Y. enterocolitica* strain MRS40(pABL409) lacking virulence factors *yopH*, *yopO*, *yopP*, *yopE*, and *yopM* in addition to *yopB*. Deletion of these genes was designed to eliminate adverse effects of other secreted virulence factors in the sheep erythrocyte hemolysis assay (1, 4). The mutant strain secreted $HrpZ_{Psph}$ (not shown) but failed to cause hemolysis ($OD_{570} < 0.01$). By contrast, the same strain expressing *yopB* [MRS40(pABL403)], or the *yopB*-deficient strain complemented by plasmid-borne *yopB*, possessed significant hemolytic activity ($OD_{570} = 0.161$). Despite structural similarities, $HrpZ_{Psph}$ is unable, therefore, to replace YopB in *Y. enterocolitica*.

Association of $HrpZ_{Psph}$ with Lipid Membranes. Hemolysis of sheep erythrocytes is an extreme measure of membrane-disintegrating activity. To pursue analysis of the predicted membrane-interacting activity of $HrpZ_{Psph}$ further, we tested whether the protein would associate with membrane-constituting lipids. In contrast to control experiments performed with human annexin (29), $HrpZ_{Psph}$ did not show Ca^{2+}-dependent binding to phospholipids (not shown). However, the phospholipid mixtures used were of amorphous structure rather than ordered membranes. To study the interaction of secreted native $HrpZ_{Psph}$ with lipid membranes, liposomes prepared from asolectin (6) were added to *Psph* grown in minimal medium. After 12 h of incubation, bacteria were pelleted, and the proteoliposomes were concentrated and purified by sucrose-density gradient centrifugation. Purified proteoliposomes were washed to eliminate sucrose and electrostatically bound proteins and were analyzed for $HrpZ_{Psph}$. Fig. 3*A* shows that $HrpZ_{Psph}$ was found in the culture supernatant in the absence of liposomes and in the purified proteoliposomes. When a *Psph hrpA* mutant was treated identically, neither secretion nor membrane association of $HrpZ_{Psph}$ was observed.

To verify membrane association of $HrpZ_{Psph}$ further, we used silica beads coated with a single phospholipid bilayer (TRAN-

Table 1. Binding* of $HrpZ_{Psph}$ to TRANSIL beads coated with single lipid membrane bilayers

Membrane composition	Bound protein (% of total)
1,2-diacyl-*sn*-glycero-3-phosphocholine	55 ± 5
POPC/POPE (80:20)	80 ± 5
1,2-di(*trans*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine/1,2-ditetradecanoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (98:2)	95 ± 5

*Binding of $HrpZ_{Psph}$ to TRANSIL beads coated with single lipid membrane bilayers was performed as described in *Materials and Methods*. Total protein applied, lipid-bound, and unbound protein were separated by SDS/PAGE, and Coomassie-stained proteins were quantified by light densitometry.

SIL), which allow measurement of protein binding to membranes (31). Because the lipid molecules are not covalently linked to the support and are separated by an ultrathin layer of water molecules, this material has characteristics reminiscent of the lipid bilayer of biological membranes (38). When purified recombinant $HrpZ_{Psph}$ was added to TRANSIL beads coated with POPC/POPE (80:20), approximately 80% of the protein applied was found to bind (Fig. 3*B*, Table 1). Interestingly, a mixture of phosphocholine alone esterified with various fatty acids yielded only 55% binding (Table 1). However, supplementing phosphocholine-coated beads carrying a neutral net charge with 2% of negatively charged 1,2-ditetradecanoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] increased binding to 95%.

Importantly, purified recombinant HrpZ from *P. syringae* pv. *tomato* or *syringae* bound to POPC/POPE (80:20) beads to a similar extent as $HrpZ_{Psph}$ (Fig. 3*B*). To identify regions within $HrpZ_{Psph}$ potentially involved in binding to membranes, we tested fragments of the protein. In contrast to the hydrophilic central core of the protein, both the hydrophobic N-terminal 80-mer fragment and the hydrophobic C-terminal 144-mer fragment bound to POPC/POPE (80:20) TRANSIL beads (Fig. 3*B*). Importantly, BSA, a major lipid and fatty acid carrier protein of the circulatory system (39), bound only very little to the TRANSIL beads. Thus, binding of HrpZ of different origin to lipid membranes is apparently specific. In addition, binding of 1 μM of different HrpZ suggests high affinity of the proteins to membranes.

$HrpZ_{Psph}$ Has Ion Pore-Forming Activity. The predicted structural similarity between YopB and $HrpZ_{Psph}$ as well as its association with membranes prompted an investigation of the formation of ion-conducting pores. We used the planar lipid bilayer technique, a protein-free membrane system widely used for isolation and characterization of membrane-active compounds (32, 40). His-tagged $HrpZ_{Psph}$ was engineered for purification to apparent homogeneity by Ni-nitrilotriacetic acid agarose ligand affinity chromatography. Removal of contaminating proteins was crucial to these assays, in particular the abundant *E. coli* porins (41). When control *E. coli* proteins prepared by an identical procedure as His-tagged $HrpZ_{Psph}$ were applied to bilayer membranes consisting of POPC/POPE (80:20), only marginal ion fluctuations were detectable (Fig. 4*A*). By contrast, addition of purified $HrpZ_{Psph}$ to the cis compartment of lipid bilayers induced distinct current fluctuations (Fig. 4*B*). Events induced by $HrpZ_{Psph}$ occurred without apparent lag phase and comprised rapid successions of open and closed states as well as prolonged phases of either state. $HrpZ_{Psph}$ concentrations required to induce the consistent channel-like ion fluxes were 2 nM. Raising this concentration to 22 nM resulted in increased fluctuation frequency, which eventually caused bilayer instabilities. Control experiments showed that BSA did not cause pore formation, demonstrating specificity of the observed event (data not shown; ref. 32). However, addition of HrpZ protein encoded by the *hrpZ* genes of *P. syringae* pv. *tomato* ($HrpZ_{Pst}$) or *syringae* ($HrpZ_{Pss}$), respectively, evoked ion currents of very similar unitary conductance to those stimulated by $HrpZ_{Psph}$ (Fig. 4 *C* and *D*).

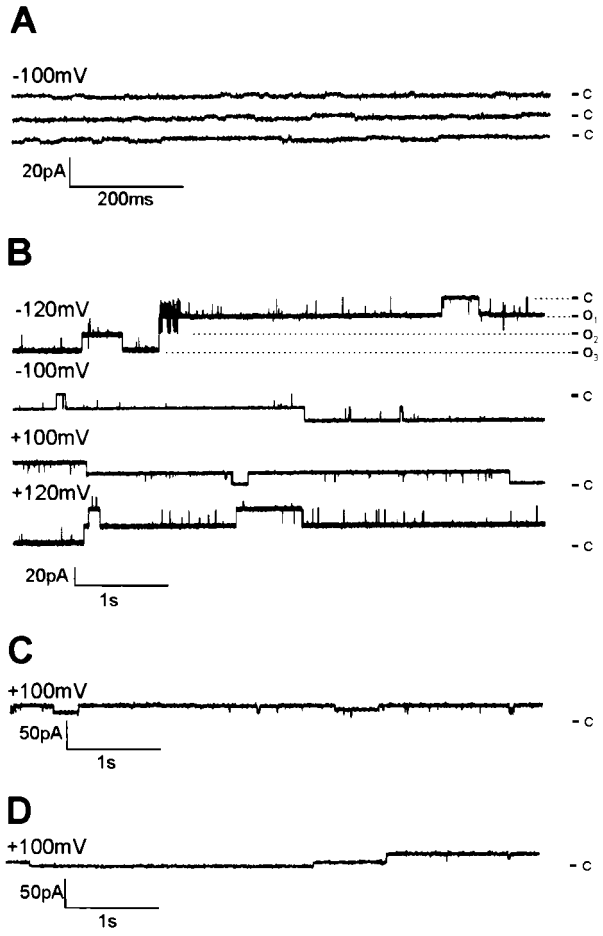

Fig. 4. $HrpZ_{Psph}$ and related $HrpZ_{Pst}$ and $HrpZ_{Pss}$ trigger ion-current fluctuations in planar lipid bilayers. (*A*) Protein from *E. coli* contaminants was prepared as described in *Materials and Methods*; purified recombinant $HrpZ_{Psph}$ (*B*), $HrpZ_{Pst}$ (*C*), or $HrpZ_{Pss}$ (*D*) was added to the cis-aqueous solution of the bilayer cuvette, and the induced current traces were recorded. In *B*, $HrpZ_{Psph}$-induced traces were recorded at different membrane potentials, as indicated. Electrolyte solutions (cis/trans) contained 100 mM KCl and 10 mM Hepes, pH 7.0. Dashed lines indicate different open states. c, closed, o_1-o_3, open states. Note different y axes in *A–D*.

Ion current fluctuation patterns induced by $HrpZ_{Psph}$ did not alter significantly in response to changes of the membrane potential applied (from −120 mV to +120 mV; Fig. 4*B*). Experiments in which the membrane potential was switched from negative to positive voltage revealed bidirectional ion currents mediated by the $HrpZ_{Psph}$ pore (Fig. 5*A*). Thus, the ion-conducting $HrpZ_{Psph}$ pore does not constitute an ion rectifier. A current–voltage relationship of $HrpZ_{Pshph}$-induced ion fluxes was established (Fig. 5*B*), facilitating determination of the pore unitary conductance as Λ_{K+} = 207 pS. Such a large ion-transport capacity is consistent with a proposed virulence function of $HrpZ_{Psph}$ as a mediator of nutrient release from host plant cells. Ion channel-like pores mediate currents along the electrochemical gradient of a particular ion. Because cytoplasmic K^+ and Cl^- concentrations of plant cells are much higher than apoplastic concentrations of these ions, both may be potential substrates for such an ion-conducting structure. Therefore, we tested the ion specificity of the pore formed by $HrpZ_{Psph}$

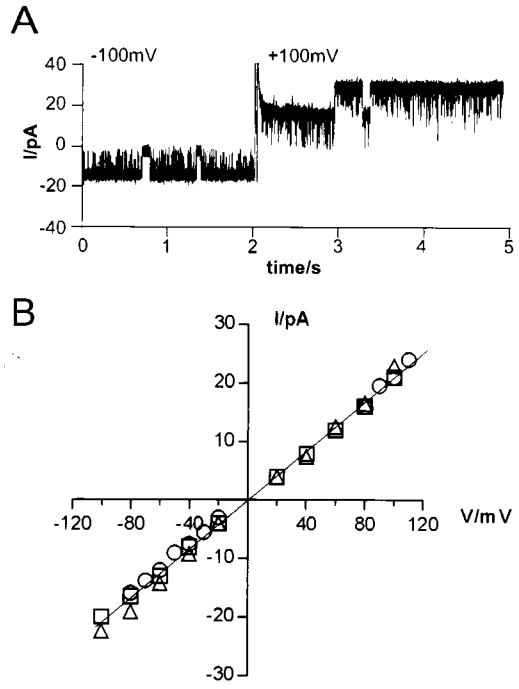

Fig. 5. Electrophysiological properties of $HrpZ_{Psph}$-induced currents. (*A*) $HrpZ_{Psph}$-induced currents do not exhibit rectifying properties. After addition of $HrpZ_{Psph}$ to the cis compartment of the bilayer cuvette, ion currents were recorded at a membrane potential of −100 mV and after immediately switching the membrane potential to +100 mV after 2 sec of total recording. (*B*) Linear current–voltage relationship of $HrpZ_{Psph}$-induced ion fluxes. Current–voltage relationships of $HrpZ_{Psph}$-induced nonselective cation currents were established in three different electrolyte solutions: 100 mM KCl and 10 mM Hepes, pH 7.0 (○); 100 mM NaCl and 10 mM Hepes, pH 7.0 (□); and 90 mM K^+-gluconate, 10 mM KCl, and 10 mM Hepes, pH 7.0 (△). The means of three recordings are given.

and found that cations K^+, Na^+ (Fig. 5*B*), and Ca^{2+} (not shown) permeated in a similar manner. However, when most of the KCl was substituted by K^+-gluconate, for which the anion is considered ion-channel impermeable, no apparent change in $HrpZ_{Psph}$-induced ion conductivity was observed. Thus, monovalent anions such as Cl^- may not pass the pore formed by $HrpZ_{Psph}$.

Discussion

The *Psph* harpin resembles the *hrpZ* gene products identified in *P. syringae* pvs. *glycinea*, *syringae*, or *tomato* (15). $HrpZ_{Psph}$ secretion depended on functional *hrp* genes and on a functional bacterial type III secretion system (Fig. 2). As shown for AvrPto from *P. syringae* pv. *tomato* and AvrB from *P. syringae* pv. *glycinea* (3), export of $HrpZ_{Psph}$ by the type III secretion system was achieved in the mammalian pathogen, *Y. enterocolitica*. However, whereas *avr* gene products are effector proteins assumed to be injected directly into host-plant cells (42, 43), our studies show that bacterial proteins that probably target the plant cell surface, such as $HrpZ_{Psph}$, are also export substrates of the type III pathway in *Y. enterocolitica*.

Like previous studies of *hrpZ* of other pathovars (18, 44), our research failed to provide unambiguous genetic evidence for a function of $HrpZ_{Psph}$ in bacterial pathogenicity. This fact may be explained by the presence of other proteins functionally redundant to $HrpZ_{Psph}$, such as HrpW, of which a crosshybridizing gene was found also in *Psph* (18). Although *hrpZ* is not essential for bacterial pathogenicity, maintenance of the gene in the *hrp* regulon, evolutionary conservation of this and structurally related genes among numerous plant pathogens, as well as the absence of the gene product in saprophytic *Pseudomonas fluorescens* (7), strongly suggest that there is a significant but as-yet-undefined role for the encoded proteins in colonization of host plants.

Both native and recombinant $HrpZ_{Psph}$ associated with lipid bilayer membranes (Fig. 3). Deletion experiments further showed that lipid binding is determined mainly by both termini of the protein, which harbor the most hydrophobic regions of this amphipathic molecule (Figs. 1 and 3*B*). For many membrane-interacting proteins, binding is mediated by ubiquitous membrane phosphoglycerolipids such as phosphatidic acid or phosphatidylserine [negatively charged lipids or neutral phosphatidylethanolamine (ref. 45 and references therein)]. Interestingly, association of $HrpZ_{Psph}$ to membranes was increased strongly in the presence of negatively charged phosphoglycerolipids and phosphatidylethanolamine (Table 1), both of which are constituents of plant plasma membranes (46).

Furthermore, addition of $HrpZ_{Psph}$ to protein-free lipid bilayers yielded a stable cation conductance, which is indicative of spontaneous self-organization of the protein into ion-conducting protein structures reminiscent of true ion channels (Fig. 4). This finding identified membranes as a physiological target for HrpZ proteins in addition to the plant cell wall (19). Using protein-free lipid bilayers rather than plant protoplasts to assess the membrane-integrating activity of $HrpZ_{Psph}$ allowed distinction between the abilities of proteins to form ion pores themselves or to trigger receptor-mediated activation of ion fluxes. In tobacco, $HrpZ_{Pss}$ from *P. syringae* pv. *syringae* activates a K^+/H^+ exchange (19), but it is unknown whether this activation is mediated by specific receptors, by direct interaction of $HrpZ_{Pss}$ with ion channels, or by direct insertion of $HrpZ_{Pss}$ into membranes. The predicted structural similarity of $HrpZ_{Psph}$ to *Y. enterocolitica* YopB led us to test whether $HrpZ_{Psph}$ could functionally replace YopB, but expression of $hrpZ_{Psph}$ in a *yopB*-deficient *Y. enterocolitica* strain failed to complement mutated *yopB*. Nevertheless, the electrophysiological properties of the ion-conducting pores formed by $HrpZ_{Psph}$ were reminiscent of those formed by YopB, i.e., distinct levels of open states, a large unitary conductance, and poor ion selectivity (6). Interestingly, current fluctuations mediated by YopB alone differed significantly from those monitored after insertion of YopB and YopD into lipid bilayers (6). In addition, genetic and biochemical evidence (1) indicated that both Yop proteins may interact to form a functional pore structure. The failure of $HrpZ_{Psph}$ in *Yersinia* complementation assays may therefore reflect its inability to interact with YopD. It is tempting to speculate that $HrpZ_{Psph}$, although able to form ion-conducting pores itself, may also interact with other bacterial proteins to establish a pore in the plasma membrane of host plant cells.

A direct consequence of $HrpZ_{Psph}$ pore formation may be a rapid hyperpolarization of the plant plasma membrane resulting predominantly from K^+ efflux. Interestingly, the $HrpZ_{Psph}$ channel seems impermeable to another major charge carrier of the plant cytoplasm, Cl^-, which may be because of the charge composition within the ion-conducting pore structure. Large unitary conductance and limited ion selectivity are also features reminiscent of bacterial homotrimeric porins (47). However, further investigations are required to elucidate the subunit structure of the $HrpZ_{Psph}$ pore and, thus, possible similarities to bacterial porins.

The electrophysiological properties of the $HrpZ_{Psph}$ pore are similar also to those of ion channels formed by toxins from plant pathogens, syringomycins, and syringopeptins from *P. syringae* pv. *syringae* and beticolins from the fungus *Cercospora beticola* (48, 49). This similarity raises the question whether $HrpZ_{Psph}$ may act simply as a toxin during infection of host plants. However, in contrast to syringomycins, harpins seem not to be toxic directly to plant cells. Hypersensitive cell death observed

after infiltration of $HrpZ_{Pss}$ into tobacco leaves was prevented completely by inhibitors of RNA and protein biosynthesis or ion channel antagonists and is thus caused by plant metabolic activities associated with programmed cell death (7, 18). More likely, pore formation benefits the bacterium by releasing cellular metabolites into the apoplast, thus providing an environment more advantageous to bacterial growth. Interestingly, a rapid efflux of sucrose was shown previously to accompany K^+ efflux in both susceptible and resistant responses of *Phaseolus vulgaris* plants to *P. syringae* pv. *syringae* infection (50).

Besides compromising the integrity of the plant plasma membrane to enable nourishment of the attacking pathogen, another role of $HrpZ_{Psph}$ during infection can be envisaged. $HrpZ_{Psph}$, like YopB, may constitute an element of the protein secretion/translocation apparatus, and the membrane-integrating activity of $HrpZ_{Psph}$ may serve to facilitate delivery of virulence factors into host plant cells via the type III secretion/translocation machinery. This view is supported by studies that revealed $HrpZ_{Pss}$ to be essential for delivery of putative virulence factors from saprophytic *P. fluorescens* or *E. coli* expressing the *P. syringae* pv. *syringae hrp* regulon (42, 51). Because *Psph hrpZ* mutants were not impaired in pathogenicity, other functional pore-forming proteins may exist. Future studies may entail isolation and identification of such proteins by use of liposomes and lipid-coated TRANSIL beads. Furthermore, it will be crucial to determine the size of the $HrpZ_{Psph}$ pore to identify substrates likely to be translocated into or out of plant cells through such a channel.

We thank Ulla Bonas (Martin-Luther-Universität, Halle, Germany), Dierk Scheel (Institut für Pflanzenbiochemie, Halle, Germany), and Dirk Nennstiel (Martin-Luther-Universität) for critical reading of the manuscript, Ulla Bonas for providing plasmid pDGUS, and Elina Roine and Martin Romantschuk (University of Helsinki, Finland) for the kind gift of *Psph pilM*-strain and *P. syringae* pv. *tomato* DC3000. We are grateful to Véronique Cabiaux (Université Libre de Bruxelles, Belgium) and Alan Collmer (Cornell University, Ithaca, New York) for providing freshly prepared liposomes and plasmid pSYH10, respectively. This work was supported by grants from the European Community (BIO-CT97–2244) (to J.L., G.T., C.S., A.T.), and by the U.K. Biotechnology and Biological Sciences Research Council (to J.W.M.).

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The Plant Cell, Vol. 13, 1079–1093, May 2001, www.plantcell.org © 2001 American Society of Plant Physiologists

A Harpin Binding Site in Tobacco Plasma Membranes Mediates Activation of the Pathogenesis-Related Gene *HIN1* Independent of Extracellular Calcium but Dependent on Mitogen-Activated Protein Kinase Activity

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Harpin from the bean halo-blight pathogen *Pseudomonas syringae* pv *phaseolicola* (harpin$_{Psph}$) elicits the hypersensitive response and the accumulation of pathogenesis-related gene transcripts in the nonhost plant tobacco. Here, we report the characterization of a nonproteinaceous binding site for harpin$_{Psph}$ in tobacco plasma membranes, which is assumed to mediate the activation of plant defense responses in a receptor-like manner. Binding of ^{125}I-harpin$_{Psph}$ to tobacco microsomal membranes (dissociation constant = 425 nM) and protoplasts (dissociation constant = 380 nM) was specific, reversible, and saturable. A close correlation was found between the abilities of harpin$_{Psph}$ fragments to elicit the transcript accumulation of the pathogenesis-related tobacco gene *HIN1* and to compete for binding of ^{125}I-harpin$_{Psph}$ to its binding site. Another elicitor of the hypersensitive response and *HIN1* induction in tobacco, the *Phytophthora megasperma*–derived β-elicitin β-megaspermin, failed to bind to the putative harpin$_{Psph}$ receptor. In contrast to activation by β-megaspermin, harpin$_{Psph}$-induced activation of the 48-kD salicylic acid–responsive mitogen-activated protein kinase (MAPK) and *HIN1* transcript accumulation were independent of extracellular calcium. Moreover, use of the MAPK kinase inhibitor U0126 revealed that MAPK activity was essential for pathogenesis-related gene expression in harpin$_{Psph}$-treated tobacco cells. Thus, a receptor-mediated MAPK-dependent signaling pathway may mediate the activation of plant defense responses induced by harpin$_{Psph}$.

INTRODUCTION

Phytopathogenic bacteria harbor a gene cluster (*HRP*, for hypersensitive reaction and pathogenicity) that controls pathogenicity in susceptible plants and the ability to elicit the hypersensitive reaction (HR) in nonhost plants or resistant cultivars of host plants (Lindgren et al., 1986; Galan and Collmer, 1999). Some *HRP* genes encode elements of a bacterial type III secretion system, by which effector proteins are exported and delivered into the cytosol of host plant cells (Galan and Collmer, 1999; Kjemtrup et al., 2000). Some of these effector proteins were found to interact with plant intracellular proteins and to activate the plant defense system.

Harpins constitute another group of effector proteins exported by the type III pathway of plant pathogenic *Erwinia*, *Pseudomonas*, and *Ralstonia* spp (Galan and Collmer, 1999). Although identified several years ago, the roles of these proteins during colonization of host plants and their site of action have remained unclear. However, when infiltrated into nonhost plants, harpins trigger disease resistance–associated responses, such as HR, transcript accumulation of pathogenesis-related (*PR*) genes, and systemic acquired resistance (Baker et al., 1993; He et al., 1993; Gopalan et al., 1996; Strobel et al., 1996; Dong et al., 1999; Galan and Collmer, 1999). Physiological target sites for harpin action, therefore, were suggested to reside at the plant cell surface. Immunolocalization studies revealed a Ca^{2+}-dependent association of *Pseudomonas syringae* pv *syringae* harpin with tobacco cell walls (Hoyos et al., 1996), but harpin-induced K^+/H^+ exchange at the plant plasma membrane and subsequent plasma membrane depolarization (Hoyos et al., 1996; Pike et al., 1998) raised questions regarding the concept of a cell wall binding site mediating such responses. Alternatively, bacterial elicitors may be recognized by the plant just like elicitors derived from phytopathogenic fungi and oomycetes, which bind to plasma membrane proteins (Nürnberger et al., 1995; Mithöfer et al.,

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1996; Umemoto et al., 1997; Bourque et al., 1999). An example of this phenomenon is provided by the recent identification of a 115-kD tomato microsomal membrane protein that binds the bacterial flagellin-derived elicitor flg22 (Meindl et al., 2000). On the other hand, harpins may interact with membranes directly and trigger plant defense responses in an ionophore-like manner, because harpins from various phytopathogenic *P. s. syringae* pathovars were found recently to associate stably with synthetic bilayer membranes and to evoke cation currents of large unitary conductance (Lee et al., 2001).

Elicitor binding to cell surface binding sites initiates an intracellular signaling cascade that results in the activation of plant-specific defense responses (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999; Nürnberger, 1999). Changes in cytoplasmic free calcium concentration ($[Ca^{2+}]_{cyt}$) are implicated in elicitor-induced signal transduction chains in various plants (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999). Previous work had demonstrated the importance of extracellular Ca^{2+} for the activation of pathogen defense responses (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999). More recently, receptor-mediated influx of extracellular Ca^{2+} in elicitor-treated plant cells was shown to cause characteristic $[Ca^{2+}]_{cyt}$ signatures as a prerequisite for the activation of pathogen defense (Mithöfer et al., 1999; Blume et al., 2000). In addition, mitogen-activated protein kinase (MAPK) cascades constitute another common element of intracellular signal transduction chains in eukaryotic cells (Herskowitz, 1995; Hirt, 2000). In plants, MAPK activation has been implicated in the adaptation to various environmental stimuli, including pathogen infection or treatment with pathogen-derived elicitors (Hirt, 2000). Tobacco mosaic virus infection of tobacco plants (Zhang and Klessig, 1998a) or treatment of tobacco cells with elicitins (Lebrun-Garcia et al., 1998; Zhang et al., 1998) and *Trichoderma viride*–derived xylanase (Suzuki et al., 1999) stimulated transient activation of a 48-kD salicylic acid–inducible MAPK (SIPK) (Zhang and Klessig, 1997, 2000). When the *Cladosporium fulvum*–derived race-specific elicitor AVR9 was infiltrated into tobacco plants expressing the tomato resistance gene *Cf-9*, both SIPK and another tobacco mosaic virus and elicitin-responsive tobacco MAPK, WIPK (for wounding-induced protein kinase) (Zhang and Klessig, 1998b), were activated (Romeis et al., 1999). Moreover, elicitor-responsive parsley SIPK and WIPK and Arabidopsis SIPK orthologous enzymes have been reported (Ligterink et al., 1997; Nühse et al., 2000; Scheel et al., 2000). In addition to specific $[Ca^{2+}]_{cyt}$ signatures, differentially induced MAPK isoenzymes with characteristic activity profiles are assumed to encode signal specificity during the activation of pathogen defense in plants (Hirt, 2000).

P. s. syringae–derived harpins and elicitins from various *Phytophthora* species trigger the HR, *PR* gene expression, and systemic acquired resistance in the nonhost plant tobacco (Ricci et al., 1989; He et al., 1993; Baillieul et al., 1995; Gopalan et al., 1996). Using harpin from *P. s. phaseolicola* ($harpin_{Psph}$) and the *Phytophthora megasperma* β-elicitin β-megaspermin, we have attempted a comparative analysis of signal perception and transduction mechanisms induced by bacterial and oomycete elicitors. We provide evidence that tobacco plasma membranes harbor a specific binding site for $harpin_{Psph}$ that does not bind β-megaspermin. This binding site is likely to mediate $harpin_{Psph}$-induced expression of the *PR* gene *HIN1* (Gopalan et al., 1996). In contrast to the β-elicitin–induced activation of *HIN1*, $harpin_{Psph}$-induced *HIN1* expression was independent of extracellular Ca^{2+}. We further show that MAPK activity, which includes SIPK activity, is required for $harpin_{Psph}$-induced *HIN1* expression.

RESULTS

Tobacco Cell Responses to Treatment with $Harpin_{Psph}$

When infiltrated into tobacco leaves, purified recombinant $harpin_{Psph}$ elicited an HR (Figure 1A). At concentrations of 1 μM, symptoms became visible 8 hr after infiltration. In addition, treatment with $harpin_{Psph}$ of cultured tobacco cells resulted in transcript accumulation of the *PR* genes *PR1*, *PR2*, acidic chitinase (*PR3*), and chitinase/lysozyme (Heitz et al., 1994) (Figure 1B). Transcripts derived from genes considered HR marker genes (*HSR203*, *HSR201*, *HSR515*, and *HIN1*) (Gopalan et al., 1996; Pontier et al., 1999) also accumulated in $harpin_{Psph}$-treated tobacco cells (Figure 1B).

$Harpin_{Psph}$-induced *HIN1* transcript accumulation was observed as early as 30 min after elicitation and persisted for a minimum of 5 hr (Figure 1C). Therefore, we chose *HIN1* as an exemplary gene to analyze elicitor-induced *PR* gene expression by single tube multiplex reverse transcription–polymerase chain reaction (RT-PCR). A constitutively expressed gene encoding the translation elongation factor *EF1α* served as an internal standard in these assays. *HIN1* transcript accumulation in tobacco cell cultures did not differ quantitatively from that observed in $harpin_{Psph}$-infiltrated tobacco leaves (Figure 1D), thus validating the use of cell suspensions for studies of $harpin_{Psph}$ perception and signal transduction.

The concentration of $harpin_{Psph}$ required to trigger half-maximum expression (EC_{50}) of *HIN1* and *HSR203* in tobacco cells was 120 and 82 nM, respectively (Table 1). This is in good agreement with concentrations of the elicitor required to stimulate a rapidly induced K^+/H^+ exchange and Cl^- efflux (Table 1). Similar ion fluxes have been associated with pathogen resistance responses in many plant systems (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999) and are assumed to be involved in signaling *PR* gene expression and HR. Such a correlation of EC_{50} values strongly suggests that $harpin_{Psph}$-induced cellular responses are activated upon recognition of the elicitor at a signal-specific binding site.

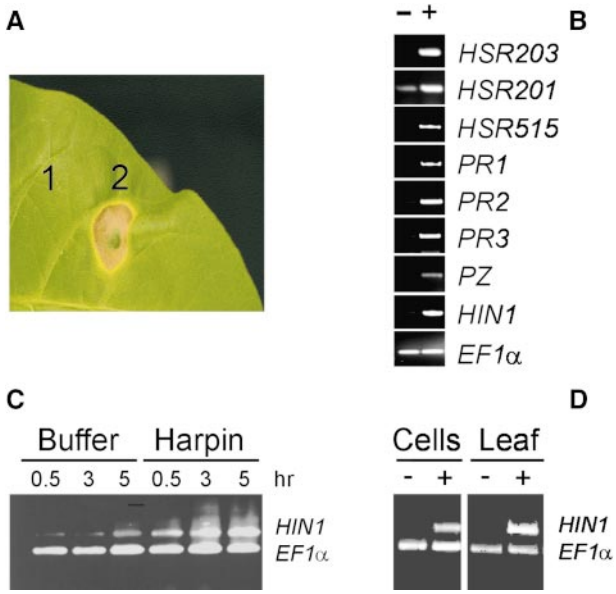

Figure 1. Harpin$_{Psph}$-Induced Hypersensitive Cell Death and *PR* Gene Expression in Tobacco.

Harpin$_{Psph}$ (1 μM) was infiltrated into tobacco leaves or added to suspension cultured tobacco cells.

(A) Tobacco leaf treated with 5 mM Mes buffer, pH 5.5 (1), or harpin$_{Psph}$ (2) 2 days after infiltration.

(B) Total RNA prepared from tobacco cells treated for 3 hr with buffer (−) or harpin$_{Psph}$ (+) was used as a template in RT-PCR assays with DNA primers derived from the tobacco genes indicated (see text). The tobacco gene encoding *EF1α* served as an internal control. *PZ*, chitinase/lysozyme.

(C) Kinetics of *HIN1* expression in tobacco cells treated with buffer or harpin$_{Psph}$. Total RNA from tobacco cells was prepared at the times after infiltration indicated and used in RT-PCR to simultaneously amplify *HIN1* and *EF1α* transcripts.

(D) *HIN1* expression in tobacco cells or leaves treated for 3 hr with buffer (−) or harpin$_{Psph}$ (+). RT-PCR analysis was performed as described in **(C)**.

Specific Binding of Harpin$_{Psph}$ to Tobacco Plasma Membranes

To characterize harpin$_{Psph}$ binding sites on tobacco membranes, the protein was radioiodinated (^{125}I) at the meta position of the phenoxyl ring of a tyrosine residue. Because native harpin$_{Psph}$ lacked tyrosine, PCR was used to attach this amino acid to the C terminus. Expression products were nonradioactively iodinated and separated by reverse phase HPLC. Matrix-assisted laser-desorption ionization time of flight mass spectrometry analysis of the reaction products confirmed complete iodination of harpin$_{Psph}$ and revealed that harpin$_{Psph}$ was not post-translationally modified during heterologous expression. Because iodination did not affect HR- or *HIN1*-inducing activities of harpin$_{Psph}$ in tobacco leaves (data not shown), ^{125}I-harpin$_{Psph}$ (specific radioactivity, 2200 Ci/mmol) was prepared and used as ligand in binding assays.

Binding of ^{125}I-harpin$_{Psph}$ to tobacco microsomal membranes was investigated by filtration to separate free from bound label, which ensured that any loss of ligand caused by rapid dissociation of the receptor–ligand complex was negligible. Specific binding of ^{125}I-harpin$_{Psph}$ to tobacco microsomes was not affected significantly by ionic strength (up to 1 M NaCl) and was greatest at pH 7.0 (84% at pH 6.0, 51% at pH 8.0, and 33% at pH 10.0). Therefore, binding assays were performed in neutral buffer containing 100 mM NaCl. The stability of the radioligand under binding assay conditions was confirmed by SDS-PAGE/autoradiography of aliquots taken from the binding mixture after various times of incubation (data not shown). In all experiments, specific binding constituted no more than 5% of the initially added ligand, ensuring that ligand depletion did not obscure binding assays.

Kinetic analysis of ^{125}I-harpin$_{Psph}$ binding demonstrated that ligand association with tobacco microsomal membranes was initially faster than dissociation (Figure 2A). Half-maximal binding was achieved within 15 sec after addition of the ligand, and equilibrium between association and dissociation was reached after 60 sec. Addition of a 100-fold molar excess of unlabeled harpin$_{Psph}$ 45 min after the addition of the radioligand to tobacco membranes resulted in the rapid dissociation of bound label (Figure 2B). Thus, binding of ^{125}I-harpin$_{Psph}$ was reversible.

Saturation analyses with increasing concentrations of ^{125}I-harpin$_{Psph}$ (50 to 700 nM) were performed. Specific binding increased exponentially at radioligand concentrations up to 250 nM. At higher concentrations, specific binding began to plateau (Figure 2C), suggesting that saturation of microsomal binding sites was approached. Nonspecific binding showed a linear increase with increasing ligand concentrations (Figure 2C). Unfortunately, experiments with higher radioligand/competitor concentrations were impeded by the tendency of harpin$_{Psph}$ to precipitate. Thus, the competitor

Table 1. EC_{50} Values[a] of Harpin$_{Psph}$-Induced Responses in Tobacco Cells

Response	EC_{50} Value (nM)
Medium alkalinization	100
K^+ efflux	50
Cl^- efflux	120
Expression of *HIN1*[b]	120
Expression of *HSR203*[b]	82

[a] Concentrations of harpin$_{Psph}$ required to stimulate 50% of the particular plant response as derived from dose–response curves.

[b] Transcript accumulation was quantified by phosphorimaging RNA gel blots hybridized with α-^{32}P-dATP–labeled *HIN1* and *HSR203* cDNA, respectively. Hybridization of filters with α-^{32}P-dATP–labeled rDNA was performed to normalize RNA loading.

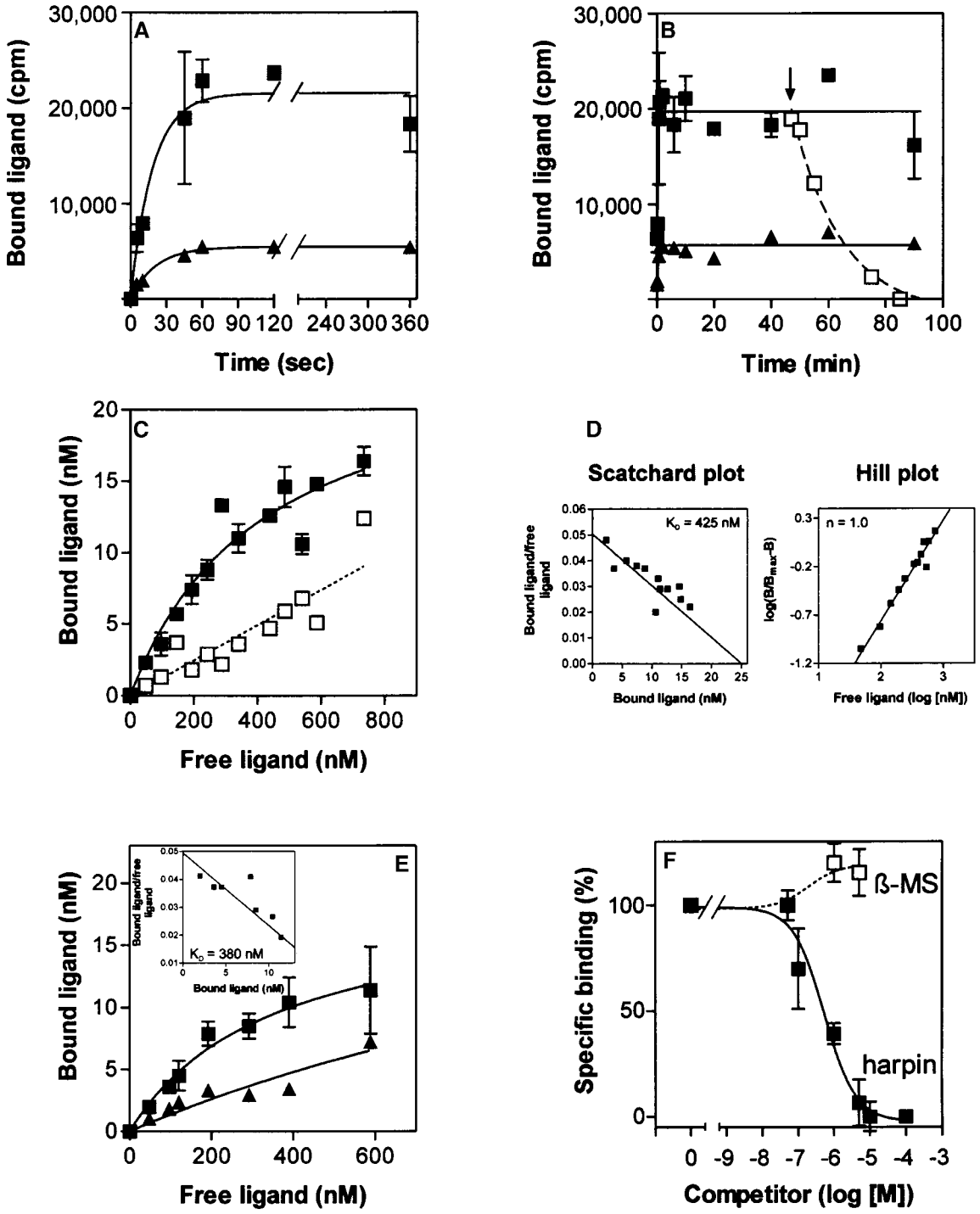

Figure 2. Binding of ^{125}I-Harpin$_{Psph}$ to Tobacco Membranes.

Data points represent the average of triplicate experiments. Data points representing nonspecific binding are the average of duplicate experiments. **(A)** Time course of binding of 50 nM ^{125}I-harpin$_{Psph}$ (1:10 dilution with unlabeled harpin$_{Psph}$) to tobacco microsomal membranes added at time 0. Membrane-bound radioligand was determined at the times indicated. Squares show the amount of radioligand that was bound specifically by the ^{125}I-harpin$_{Psph}$ binding site. Nonspecific binding is indicated by triangles. Specific binding was obtained by subtracting nonspecific binding from total binding. Nonspecific binding was determined in the presence of 5 μM unlabeled harpin$_{Psph}$.

concentrations required to determine the degree of nonspecific binding at higher ^{125}I-harpin$_{Psph}$ levels could not be used. Linearization of the data in a Scatchard plot (Figure 2D) indicated the existence of a ^{125}I-harpin$_{Psph}$ binding site in tobacco microsomal membranes with a dissociation constant (K_D) of 425 nM and an apparent binding site concentration of 6.7 pmol/mg of protein. Hill plot analysis of the binding data yielded a Hill coefficient of 1 (Figure 2D), indicating that there is no cooperativity in the binding of ^{125}I-harpin$_{Psph}$ to tobacco membranes.

Binding of ^{125}I-harpin$_{Psph}$ to tobacco protoplasts revealed a saturable binding site with an apparent K_D of 380 nM and a binding site concentration of 1.5 pmol/10^6 protoplasts (Figure 2E). Consistent with the experiments performed with microsomal membranes, ^{125}I-harpin$_{Psph}$ binding to protoplasts did not show any cooperativity (data not shown). Because binding experiments were performed under conditions believed to prevent radioligand endocytosis (15 min at 0°C; Hulme and Birdsall, 1990), our results indicated that the harpin$_{Psph}$ binding site is localized predominantly in the plasma membrane.

Competition experiments with increasing concentrations of unlabeled harpin$_{Psph}$ yielded a concentration resulting in 50% inhibition of specific binding of 550 nM (Figure 2F). This is consistent with the K_D value determined in ligand saturation analyses (Figures 2C and 2D). When added at a 50-fold molar excess over ^{125}I-harpin$_{Psph}$, unlabeled harpin$_{Psph}$ reduced specific binding of the radioligand by 92%. In contrast, β-megaspermin, from the phytopathogenic oomycete *Phytophthora megasperma* (Baillieul et al., 1995), did not compete for binding of ^{125}I-harpin$_{Psph}$ when applied at the same concentration (Figure 2F). Therefore, the harpin$_{Psph}$ binding site and the recently characterized elicitin receptor (Bourque et al., 1998, 1999) are unlikely to be identical.

Treatment of tobacco microsomal membranes with proteinase E before binding assays did not abolish the binding of ^{125}I-harpin$_{Psph}$ (data not shown). In addition, use of the radioligand in chemical cross-linking assays with the homobifunctional reagent, 3,3′-dithiobis[sulfosuccinimidyl propionate] (DTSSP), failed to identify one or more microsomal membrane proteins as possible constituents of the ^{125}I-harpin$_{Psph}$ binding site (data not shown). Thus, the ^{125}I-harpin$_{Psph}$ binding site may not be a protein.

Harpin$_{Psph}$ Fragments Are Sufficient for *HIN1* Activation and Competition of ^{125}I-Harpin$_{Psph}$ Binding

To identify the minimum portion of harpin$_{Psph}$ required to elicit *HIN1* expression, cDNAs encoding harpin$_{Psph}$ or portions of the polypeptide were expressed as His$_{10}$-tag fusion proteins in *Escherichia coli* (Figure 3A). The expression products were purified to apparent homogeneity on nickel–nitrilotriacetic acid agarose and subsequently assessed for their ability to induce *HIN1* transcript accumulation in tobacco cells. Deletion of 100 or 200 N-terminal amino acid residues of a total of 345 amino acids of harpin$_{Psph}$ did not adversely affect *HIN1*-inducing activity (Figure 3A, fragments II and III). Fragment V, corresponding to amino acids 100 to 300, also was elicitor active. Further C-terminal deletion of this fragment resulted in the complete loss of elicitor activity (Figure 3A, fragment VI). In addition, an N-terminal 80–amino acid peptide was found to be devoid of *HIN1*-inducing activity (Figure 3A, fragment VII). Fragment III represented the smallest elicitor-active polypeptide that could be expressed in *E. coli*.

Together, these data suggest that the elicitor activity of harpin$_{Psph}$ resided in a C-terminal fragment corresponding to amino acids 200 to 300 (Figure 3A, fragment VIII). Because attempts to produce this minimal fragment in *E. coli* proved unsuccessful, we chemically synthesized overlapping peptides covering amino acids 174 to 345 (amino acids 174 to 218, 200 to 224, 219 to 263, 225 to 257, 236 to 280, 264 to 300, and 301 to 345). However, neither alone nor in combination did these peptides exhibit *HIN1*-inducing activity

Figure 2. (continued).

(B) Time course of displacement of ^{125}I-harpin$_{Psph}$. ^{125}I-harpin$_{Psph}$ (50 nM; 1:10 dilution with unlabeled harpin$_{Psph}$) was incubated with tobacco microsomal membranes for the times indicated. To initiate the displacement of ^{125}I-harpin$_{Psph}$, a 100-fold molar excess of unlabeled harpin$_{Psph}$ (open squares) was added 45 min (arrow) after the radioligand. Specific binding (closed squares) and nonspecific binding (triangles) are indicated.

(C) Saturability of ^{125}I-harpin$_{Psph}$ binding to tobacco microsomal membranes. Specific binding (closed squares) and nonspecific binding (open squares) were determined in the presence of a 100-fold molar excess of unlabeled harpin$_{Psph}$.

(D) Scatchard plot and Hill plot of the binding data shown in **(C)**. The binding constant (K_D) and the Hill coefficient (n) were determined according to Hulme and Birdsall (1990).

(E) Saturability of ^{125}I-harpin$_{Psph}$ binding to tobacco protoplasts. Graphs for specific binding (squares) and nonspecific binding (triangles) are shown. The inset shows a Scatchard plot of the binding data. The dissociation constant (K_D) was determined according to Hulme and Birdsall (1990).

(F) Competitive inhibition of ^{125}I-harpin$_{Psph}$ (100 nM) binding to tobacco microsomal membranes by increasing concentrations of harpin$_{Psph}$ or β-megaspermin (β-MS). One hundred percent specific binding corresponded to the binding detected in the absence of competitor (1,600,000 cpm), whereas 0% specific binding corresponded to the binding detected in the presence of a 100-fold molar excess of competitor (10 μM) (nonspecific binding, 374,000 cpm).

Error bars indicate standard error.

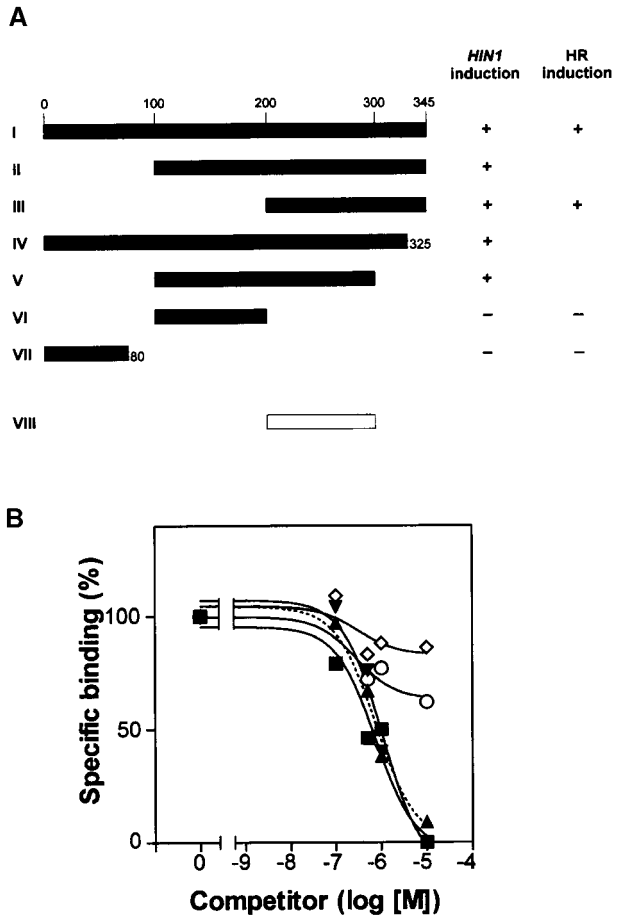

Figure 3. Structure/Activity Relationship of $Harpin_{Psph}$ and $Harpin_{Psph}$ Deletion Derivatives Expressed in *E. coli*.

(A) cDNAs encoding $harpin_{Psph}$ (fragment I) and deletion derivatives (fragments II to VII) fused to a His_{10}-encoding tag were expressed in *E. coli*. Expression products were purified to apparent homogeneity on nickel–nitrilotriacetic acid agarose and tested for their ability to induce *HIN1* expression or the HR in tobacco cells at a concentration of 1 μM. Total RNA was prepared from tobacco cells treated with $harpin_{Psph}$ for 3 hr, and *HIN1* expression was monitored by RT-PCR as described in the legend to Figure 1C. The white bar (fragment VIII) denotes the overlapping region of two active derivatives (fragments III and V) and defines the smallest fragment deduced to harbor elicitor activity. +, full activity relative to that of the full-length expression product; −, no detectable activity.

(B) Competitive inhibition of ^{125}I-$harpin_{Psph}$ (100 nM) binding to tobacco microsomal membranes by recombinant deletion derivatives shown in **(A)** and $harpin_{Pss}$ from *P. s. syringae*. Increasing concentrations of fragment I (closed squares), fragment III (closed triangles), $harpin_{Pss}$ (closed inverted triangles), fragment VI (open circles), or fragment VII (open diamonds) were used as competitors of binding of 100 nM ^{125}I-$harpin_{Psph}$. The graphs show the amount of specific binding as described in the legend to Figure 2A. One hundred percent specific binding corresponded to the binding detected in the absence of competitor (1,850,000 cpm), whereas 0% specific binding corresponded to the binding detected in the presence of a 100-fold molar excess of competitor (10 μM) (nonspecific binding, 525,000 cpm). Each data point represents the average of duplicate experiments.

(data not shown). In radioligand competition assays, the elicitor-active fragment III proved to be as active as the canonical $harpin_{Psph}$ in blocking the binding of ^{125}I-$harpin_{Psph}$, whereas the two elicitor-inactive fragments (VI and VII) failed to do so (Figure 3B). Consistently, neither alone nor in combination did the synthetic peptides encompassing amino acids 174 to 345 compete for binding of ^{125}I-$harpin_{Psph}$ as effectively as canonical $harpin_{Psph}$. Hence, a qualitative and quantitative correlation was found between the abilities of $harpin_{Psph}$ fragments to bind to the receptor and to elicit *HIN1* expression. Residual binding activity of 35 to 50% of the radioligand was detected when one of three synthetic peptides (amino acids 219 to 263, 236 to 280, or 301 to 345) was used as a competitor at a 100-fold molar excess over ^{125}I-$harpin_{Psph}$ (data not shown).

Recombinant harpins from *P. s. syringae* ($harpin_{Pss}$; Figure 3B) and *P. s. tomato* ($harpin_{Pst}$; data not shown), which are structurally related to $harpin_{Psph}$ (77 and 53% identical at the amino acid level; GenBank accession number AF268940), exhibited competitor activity similar to that of $harpin_{Psph}$, indicating that they targeted the same binding site in tobacco. $Harpin_{Pss}$ and $harpin_{Pst}$ also were found to induce *HIN1* expression in tobacco (Gopalan et al., 1996; our unpublished data). Fragment VIII of $harpin_{Psph}$ (Figure 3A) exhibited 60% identity to both $harpin_{Pss}$ and $harpin_{Pst}$, which is not very different from the overall identity observed between the three proteins. Thus, a secondary structure motif, rather than a highly conserved peptide fragment, is likely to represent the recognition determinant for the stimulation of *HIN1* expression.

$Harpin_{Psph}$ Activates a Salicylic Acid–Inducible MAPK in Tobacco Cells Independent of Extracellular Calcium

Recent studies have provided evidence that rapidly induced influxes of extracellular Ca^{2+} and subsequent changes in the $[Ca^{2+}]_{cyt}$ contribute to the activation of defense-associated responses in various plants (Xu and Heath, 1998; Mithöfer et al., 1999; Blume et al., 2000). However, decreasing the extracellular free $[Ca^{2+}]$ to 20 nM using the membrane-impermeable chelator 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA) did not affect $harpin_{Psph}$-induced *HIN1* transcript accumulation (Figure 4). The Ca^{2+} influx inhibitors La^{3+} and Gd^{3+} also failed to block $harpin_{Psph}$-induced *HIN1* activation (Figure 4). In contrast, extracellular Ca^{2+} and Ca^{2+} influx proved indispensable for β-megaspermin–induced *HIN1* activation, because BAPTA and lanthanides inhibited this response in elicited tobacco cells (Figure 4).

A myelin basic protein (MBP)-phosphorylating protein ki-

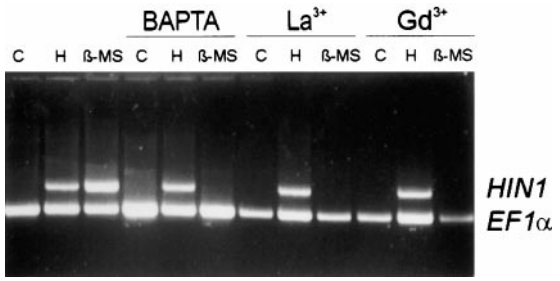

Figure 4. Harpin$_{Psph}$-Induced *HIN1* Expression Is Independent of Extracellular Calcium.

Effects of Ca^{2+} chelator and Ca^{2+} channel inhibitors on elicitor-induced *HIN1* expression in tobacco cells. Tobacco cells were treated for 1.5 hr with buffer (C), 1 μM harpin$_{Psph}$ (H), or 50 nM β-megaspermin (β-MS) in the absence or presence of BAPTA (8 mM), $LaCl_3$ (0.25 mM; La^{3+}), or $GdCl_3$ (0.25 mM; Gd^{3+}). $MgSO_4$ (20 mM) was included in the buffer together with BAPTA to prevent membrane destabilization due to Ca^{2+} depletion. Total RNA prepared from elicited tobacco cells was analyzed by RT-PCR as described in the legend to Figure 1C.

nase of 48 kD was activated within 5 min after treatment of tobacco cells with harpin$_{Psph}$ (Figure 5A). Protein kinase activation was transient and decreased to nearly background levels within 60 min after elicitation. In immunoblot analyses performed with a MAPK-specific antibody that recognized the dually phosphorylated threonine-glutamic acid-tyrosine tripeptide motif $_pTE_pY$, a 48-kD protein was detected in protein extracts from harpin$_{Psph}$-treated cells (Figure 5B). Thus, harpin$_{Psph}$ activated a tobacco MAPK that was likely SIPK (Zhang and Klessig, 1997). To verify this, we used a monospecific antiserum raised against a unique N-terminal peptide of SIPK (Zhang et al., 1998) for immunoprecipitation and subsequent in vitro protein kinase assay with MBP as a substrate. As shown in Figure 5C, the antiserum precipitated a MBP kinase activity from extracts of harpin$_{Psph}$-induced cells (lane 2) that was not precipitated from buffer-treated cells (lane 1). Most importantly, immunoprecipitation of this kinase could be inhibited with an excess of the SIPK-specific peptide used for antibody production (lane 3). Thus, harpin$_{Psph}$ stimulated SIPK activity in tobacco cells.

The activation of *HIN1* expression by harpin$_{Psph}$, but not by β-megaspermin, was independent of extracellular Ca^{2+} (Figure 4). Because the activation of SIPK by elicitins was reported recently for the *Phytophthora parasitica*–derived α-elicitin parasiticein and the *Phytophthora cryptogea* β-elicitin cryptogein (Zhang et al., 1998), we wondered if extracellular Ca^{2+} was required for SIPK activation in response to harpin$_{Psph}$ or β-megaspermin. Protein extracts prepared from tobacco cells treated with elicitor either in the absence or presence of BAPTA were fractionated by SDS-PAGE, blotted, and analyzed with the anti-$_pTE_pY$ antiserum. Harpin$_{Psph}$-mediated activation of the 48-kD MAPK (SIPK) was not affected by BAPTA treatment (Figure 6), indicating that MAPK activation was independent of extracellular Ca^{2+}. In contrast, β-megaspermin–induced activation of this enzyme was sensitive to BAPTA treatment and thus dependent on extracellular Ca^{2+}.

Harpin$_{Psph}$-Induced MAPK Activation Is Required for *PR* Gene Expression in Tobacco

MAPK cascades are assumed to constitute an element of elicitor-induced signal transduction cascades in various plant systems (Hirt, 2000). However, the direct involvement of activated MAPK in triggering plant defense responses has yet to be demonstrated. To causally link harpin$_{Psph}$-induced MAPK activity and *HIN1* expression in tobacco, we used the inhibitor of MAPK kinases (MAPKK) U0126 (Favata et al., 1998). This inhibitor is believed to be more specific and more active against MAPKK than inhibitor PD98059 (Favata et al., 1998), which was reported recently to block the MAPK pathway in Arabidopsis and tobacco (Desikan et al., 1999; Romeis et al., 1999). Although PD98059 blocks MAPKK activity by binding directly to the inactive (nonphosphorylated) form of the enzyme, U0126 does not affect MAPKK activation by phosphorylation but inhibits the activated (phosphorylated) MAPKK at the catalytic site (Favata et al., 1998). When tobacco cells were pretreated with U0126, harpin$_{Psph}$-mediated activation of the 48-kD MBP kinase was strongly reduced (at 50 μM) or compromised (at 100 μM) (Figure 7A, top section). SIPK activation was reduced by 50% in the presence of 5 μM U0126 (not shown). Similar U0126 concentrations (25 μM) have been found to efficiently block activation of the human MAPK, ERK2, by the MAPKK MEK1 in vitro (Favata et al., 1998; Goueli et al., 1999). Incubation of tobacco cells with U0126 for 4 hr had no apparent effect on the viability of the cells, which was determined in a combined viability/lethality assay using fluorescein diacetate and propidium iodide (Blume et al., 2000). In addition, the harpin$_{Psph}$-induced oxidative burst remained unaffected in tobacco cells treated with 100 μM U0126, suggesting that MAPK activation and reactive oxygen species production are not linked functionally (not shown).

The level of harpin$_{Psph}$-induced *HIN1* transcript accumulation in U0126-treated tobacco cells was diminished compared with that in DMSO-treated control cells (Figure 7A, bottom section). To quantify the effect of the inhibitor on harpin$_{Psph}$-induced *HIN1* activation, RT-PCR products (Figure 7A) were blotted for hybridization with α-^{32}P-dATP–labeled cDNA and relative *HIN1* expression levels were determined by phosphorimaging. This was necessary because the inhibitor itself slightly induced the transcript accumulation of *HIN1* and other *PR* genes in nonelicited tobacco cells (Figure 7A). Pooled data from three independently performed experiments revealed that 100 μM U0126 reduced *HIN1* expression by 78% compared with control levels (Figure 7B). Interestingly, albeit to a variable extent, transcript accumulation of PR genes such as *PR1*, *PR2*, and acidic chitinase

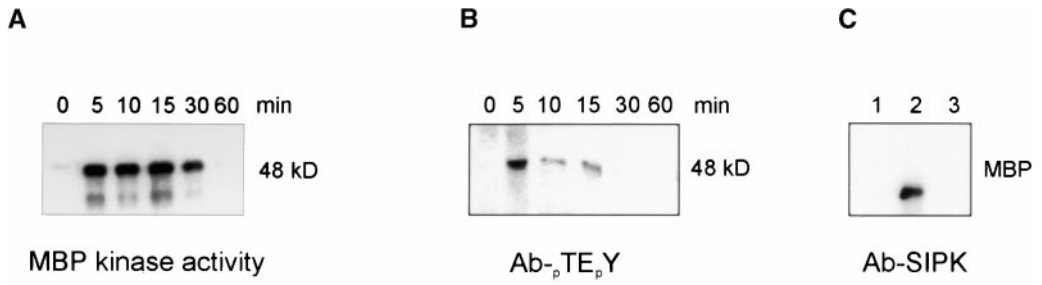

Figure 5. Activation of the SIPK in Tobacco Cells Treated with $Harpin_{Psph}$.

Tobacco cells treated with 1 μM $harpin_{Psph}$ were harvested at the times after infiltration indicated and used to prepare protein extracts.
(A) Kinase activity determined by an in-gel kinase assay using MBP as the substrate.
(B) Protein extracts analyzed by immunoblotting using an antiserum (Ab) recognizing the $_pTE_pY$ motif of activated MAPK.
(C) Protein extracts were prepared from tobacco cells treated with buffer (lane 1) or $harpin_{Psph}$ (lanes 2 and 3) for 5 min. For immunoprecipitation, the tobacco SIPK-specific antibody (Ab-p48N) (Zhang et al., 1998) was added alone (lanes 1 and 2) or together with the competitor peptide used for antibody production (lane 3). Kinase activity of immunoprecipitated material was assayed using MBP as the substrate. The phosphorylated MBP was visualized by autoradiography.

(*PR3*) or of the HR marker genes *HSR201*, *HSR515*, and *HSR203* also was affected by the inhibitor (Figure 7A). A similar reduction in *HSR203* expression was found when RNA gel blots were probed with *HSR203* cDNA (Figure 7A). Thus, a $harpin_{Psph}$-activated MAPK cascade appears to be involved in signaling *PR* gene expression in tobacco.

DISCUSSION

A Harpin Binding Site Involved in *PR* Gene Expression in Tobacco

We have shown that tobacco plasma membranes harbor a binding site for *P. s. syringae*–derived harpins (Figures 2 and 3). Binding of ^{125}I-$harpin_{Psph}$ was inhibited by excess of unlabeled $harpin_{Psph}$ and binding was saturable (Figure 2), as was expected of an authentic receptor (Hulme and Birdsall, 1990). Ligand saturation analyses performed with tobacco protoplasts revealed a dissociation constant of the ligand/binding site interaction of 380 nM, which is in good agreement with the EC_{50} value obtained for a number of $harpin_{Psph}$-induced responses of tobacco cells (Figure 2D, Table 1). Moreover, use of a series of deletion derivatives of $harpin_{Psph}$ showed a close quantitative and qualitative correlation between the abilities of ligands to inhibit binding of the radioligand and to induce *HIN1* expression (Figure 3). Thus, the $harpin_{Psph}$ binding site detected in the plasma membrane is likely to mediate the activation of defense responses in tobacco.

A comparably high degree of correlation between elicitor and displacement activities of ligand derivatives was found for other elicitors and their binding sites as well (Cheong and Hahn, 1991; Nürnberger et al., 1994; Bourque et al., 1998; Kooman-Gersmann et al., 1998; Meindl et al., 2000). Unfortunately, the preparation of tobacco protoplasts caused *PR* gene expression in the absence of $harpin_{Psph}$ (data not shown); hence, it was impossible to determine if protoplasts would respond to $harpin_{Psph}$ treatment in a manner similar to intact cells. This would have been most desirable, because Hoyos et al. (1996) reported binding of $harpin_{Pss}$ exclusively to the uppermost layer of tobacco cell walls but not to tobacco protoplasts; this binding was detected by confocal laser microscopy using an anti-$harpin_{Pss}$ antibody and a fluorochrome-tagged anti-IgG antibody. This association was Ca^{2+} dependent and detectable only at $harpin_{Pss}$ concentrations of ~5 μM, which exceeded by far the concentrations required for stimulation of $harpin_{Psph}$-induced plant cell responses (Table 1).

Important questions regarding whether binding was saturable and reversible were not addressed in that study. Therefore, we performed radioligand saturation assays with intact tobacco cells. Scatchard plot analysis of the data yielded a scattered distribution of data points (data not shown), which prevented meaningful analysis but led us to conclude that ^{125}I-$harpin_{Psph}$ may bind to tobacco cells at multiple sites with very different ligand affinities. Although it cannot be excluded that harpins may interact with the plant cell wall in addition to the plasma membrane, it is difficult to reconcile a cell wall binding site with a $harpin_{Pss}$-induced K^+/H^+ exchange response and plasma membrane depolarization (Hoyos et al., 1996; Pike et al., 1998). Signal perception remote from the plasma membrane and initiation of an intracellular signaling cascade would require the involvement of extracellular matrix receptor-like molecules, as in mammalian cells (Turner and Burridge, 1991). Although such proteins appear to exist in plants, their function has yet to be elucidated (Kohorn, 2000). Thus, it is not clear if the study by Hoyos et al. (1996) identified a physiological target implicated in the activation of plant defense responses or if this

association was due to an ionic interaction with cell wall pectic polysaccharides.

Using harpin$_{Psph}$ fragments, we identified regions within the protein that were capable of binding to the binding site to some extent but were unable to elicit *HIN1* expression in tobacco cells. When added at a 100-fold molar excess over ^{125}I-harpin$_{Psph}$, two elicitor-inactive peptide fragments (covering amino acids 219 to 280) showed residual competitor activity, suggesting that different regions of the elicitor are implicated in elicitor perception and generation of an intracellular signaling cascade. This is similar to the address-message concept according to which the yeast invertase-derived glycopeptide elicitor gp8 or the bacterial elicitor flg22 activates defense responses in tomato cells (Basse et al., 1992; Meindl et al., 2000). Similar to our findings, biologically inactive fragments of these elicitors partially retained the ability to interact with their binding sites. A somewhat surprising observation in our studies was that a peptide fragment (amino acids 301 to 345) that was apparently dispensable for the elicitor activity of harpin$_{Psph}$ showed partial competitor activity. However, it is possible that this region contributed only marginally to the binding of ^{125}I-harpin$_{Psph}$ to tobacco membranes and that deletion of this fragment had so little impact on its elicitor activity that we did not detect it in *HIN1* expression assays.

Recent studies that complement our analyses of harpin$_{Psph}$-induced signal perception and transduction in tobacco cells attempted to elucidate the role of harpin$_{Psph}$ in bacterial pathogenicity. Like structurally related YopB from the mammalian pathogen *Yersinia enterocolitica* (Tardy et al., 1999), harpin$_{Psph}$ and homologous proteins from *P. s. tomato* or *P. s. syringae* were found to integrate into protein-free bilayer membranes and to form an ion-conducting pore in vitro (Lee et al., 2001). Binding of membrane-interacting proteins is most often mediated by specific membrane phosphoglycerolipids, such as negatively charged phosphatidic acid or neutral phosphatidylethanolamine (Thevissen et al., 2000a, and references therein). Interestingly, the association of harpin$_{Psph}$ to lipids was strongly enhanced in the presence of negatively charged phosphoglycerolipids and phosphatidylethanolamine (Lee et al., 2001), both of which are constituents of plant plasma membranes (Staehelin and Newcomb, 2000). Direct interaction of harpin$_{Psph}$ with lipids seems to be consistent with a protease-insensitive binding site detected in tobacco membranes. In addition, activation of plant defense responses by ionophore-like compounds has been reported in many plant systems (Yang et al., 1997; Scheel, 1998). Because binding of protein ligands to specific lipids may resemble very closely the molecular interactions between proteinaceous ligands and receptors, it appears likely that the harpin$_{Psph}$ binding site detected in tobacco plasma membranes is not a protein.

Precedent for this may be provided by high affinity binding sites for plant defensins, which were detected on fungal plasma membranes (Thevissen et al., 2000b). Defensins trigger ion fluxes similar to those observed in harpin$_{Psph}$-treated tobacco cells. Recently, Thevissen et al. (2000a) reported that defensin binding to fungal plasma membranes was strongly dependent on mannose-(inositol-phosphate)$_2$-ceramide, the major sphingolipid in membranes of *Saccharomyces cerevisiae*. Their data support a model in which membrane patches containing sphingolipids act as specific binding sites for defensins or, alternatively, are required to anchor membrane- or cell wall–associated proteins, which themselves interact with defensins (Thevissen et al., 2000a). Similarly, the protein antibiotic nisin Z from *Lactococcus lactis* binds with high affinity to the membrane-anchored cell wall precursor lipid II of Gram-positive bacteria (Breukink et al., 1999). Like harpin$_{Psph}$ (Lee et al., 2001), nisin Z is an amphipathic, highly charged protein. Remarkably, nisin Z exerted its biological function through high affinity binding to lipid II and subsequent formation of an ion-conducting pore (Breukink et al., 1999).

At present it is unknown if activation by harpin$_{Psph}$ of pathogen defense responses in tobacco is mediated by a specific (non)proteinaceous receptor, by direct insertion of the protein into membranes, or by receptor-mediated membrane insertion (Figure 8). It is also conceivable that membrane insertion and receptor-mediated recognition of harpin$_{Psph}$ occur independently in tobacco membranes, and that either or both pathways could lead to activation of defense responses in tobacco. The latter case seems to be supported by our observations that harpin$_{Psph}$-induced ion pore formation was detectable at concentrations as low as 2 nM (Lee et al., 2001), whereas binding of harpin$_{Psph}$ (Figures 2D and 2E) and transcriptional activation of *PR* genes (Table 1) required significantly higher elicitor concentrations. On the other hand, pore formation sufficient to trigger plant defense responses may require a threshold concentration of harpin$_{Psph}$ higher than 2 nM. However, specific receptors would explain why plant species respond differently to harpin$_{Psph}$

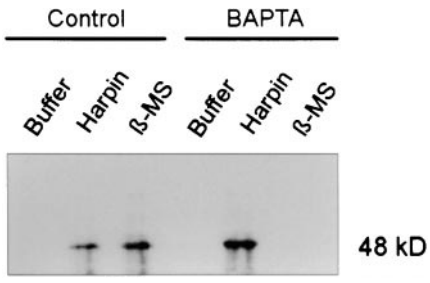

Figure 6. Harpin$_{Psph}$-Induced SIPK Activation Is Independent of Extracellular Calcium.

Protein extracts prepared from tobacco cells treated for 5 min with buffer, 1 μM harpin$_{Psph}$, or 50 nM β-megaspermin (β-MS) in the absence (Control) or presence of the Ca^{2+} chelator BAPTA were analyzed for MAPK phosphorylation by immunoblotting using the anti-$_pTE_pY$-antiserum.

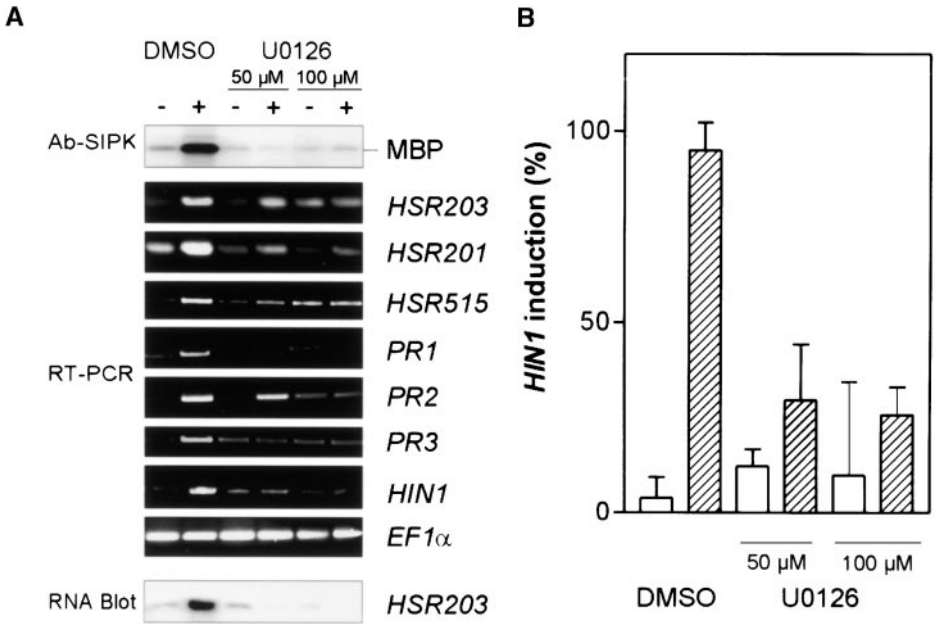

Figure 7. The MAPKK-Specific Inhibitor U0126 Compromises Both Harpin$_{Psph}$-Induced SIPK Activation and *PR* Gene Expression in Tobacco.

(A) Tobacco cells were treated for 3 hr with buffer (−) or 1 μM harpin$_{Psph}$ (+) in the absence (DMSO) or presence of 50 or 100 μM U0126, a MAPKK-specific inhibitor (Favata et al., 1998). The highest concentration of DMSO used was 0.2%. Protein extracts from elicited tobacco cells were analyzed for SIPK activity as described in the legend to Figure 5C. The expression of *PR* genes was analyzed by RT-PCR with total RNA and specific DNA primers for the indicated genes or, alternatively, by RNA gel blot analysis using total RNA and α-^{32}P-dATP–labeled *HSR203* cDNA.
(B) Quantification of the inhibitory effect of U0126 on harpin$_{Psph}$-induced *HIN1* expression. RT-PCR products obtained from three independent elicitation experiments as described in **(A)** were separated electrophoretically, blotted onto nylon membranes, and probed with α-^{32}P-dATP–labeled *HIN1* cDNA. The signal intensity was quantified by phosphorimaging and normalized to 100% for the maximum induction in each experiment. Buffer treatment (blank bars); harpin$_{Psph}$ treatment (shaded bars). Error bars indicate ±SE.

treatment, whereas insertion into membranes may reflect the role of harpin$_{Psph}$ during bacterial infection of host plants.

MAPK Activity but Not Extracellular Calcium Is Required for Harpin$_{Psph}$-Induced *HIN1* Expression

Extracellular Ca^{2+} is important for the induction of pathogen defense in various plants (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999). However, we found that BAPTA or lanthanide inhibitors of Ca^{2+} influx did not affect harpin$_{Psph}$-induced *HIN1* expression but abrogated β-elicitin–induced *HIN1* expression (Figure 4). Thus, structurally diverse elicitors, which target different receptors (Figure 2F), trigger *PR* gene expression dependent on or independent of extracellular Ca^{2+} in the same plant. Moreover, because harpin$_{Pss}$-induced HR has been shown to depend on extracellular Ca^{2+} (He et al., 1993), several emerging signaling cascades appear to be used for the activation of a complex plant defense response triggered by a single elicitor.

We have provided evidence that harpin$_{Psph}$ stimulated SIPK rapidly and transiently (Figure 5). Similar activation kinetics have been reported for a 49-kD MBP-phosphorylating protein kinase that was induced in tobacco leaves upon treatment with harpin$_{Ea}$ from *Erwinia amylovora* (Adam et al., 1997) and that is likely SIPK (Zhang and Klessig, 2000). In contrast, activation of SIPK by β-elicitins (Lebrun-Garcia et al., 1998; Zhang et al., 1998; Zhang and Klessig, 2000; our unpublished data) or the β-elicitin parasiticein (Zhang et al., 1998) was much more prolonged. Similarly prolonged activation of SIPK activity has been reported in tobacco cells treated with *Trichoderma viride*–derived xylanase (Suzuki et al., 1999) or in *Cf-9*–transformed tobacco cells treated with the race-specific *C. fulvum* elicitor AVR9 (Romeis et al., 1999). Moreover, elicitins but not harpin$_{Psph}$ induced prolonged activation of the 44-kD MAPK, WIPK, and another yet undefined tobacco MAPK of 40 kD (Zhang et al., 1998; Zhang and Klessig, 2000). These findings demonstrate that not only elicitor- or pathogen-induced MAPK activity but signal-specific MAPK activity profiles and isoenzyme patterns may be key features of the signal transduction cascades involved in the activation of plant pathogen defense. In addition, MAPK activity is controlled both post-translationally and transcriptionally, which is considered another regulatory

mechanism through which the specificity of signal transduction cascades is maintained (Hirt, 2000).

In those cases investigated, MAPK activation by elicitor was dependent on extracellular Ca^{2+} (Ligterink et al., 1997; Lebrun-Garcia et al., 1998; Romeis et al., 1999; Suzuki et al., 1999; Fellbrich et al., 2000). Particularly, β-elicitin–induced SIPK activity (Lebrun-Garcia et al., 1998; Figure 6) and *HIN1* expression (Figure 4) were dependent on extracellular Ca^{2+}. In contrast, SIPK activation by $harpin_{Psph}$ was independent of extracellular Ca^{2+} and Ca^{2+} influx (Figures 4 and 6). Recently, Ca^{2+}-independent activation of SIPK was observed in tobacco cells undergoing hyperosmotic stress (Hoyos and Zhang, 2000), thus exemplifying the central role of this enzyme in stress adaptation as well as its implication in differentially regulated signaling chains.

$Harpin_{Psph}$-induced Ca^{2+}-independent SIPK activation and *HIN1* expression prompted us to investigate a possible causal link between the two responses. Specific inhibition of MAPKK activity by U0126 (Favata et al., 1998) not only abolished $harpin_{Psph}$-induced SIPK activation but also suppressed the expression of numerous *PR* genes in tobacco cells (Figure 7). This is novel in that it suggests the functional involvement of MAPK (SIPK) activity in plant defense activation. Romeis et al. (1999) showed inhibition of elicitor-induced tobacco SIPK activity by another MAPKK inhibitor, PD98059, which, however, did not block the elicitation of defense-related responses, such as production of reactive oxygen species. Furthermore, PD98059 inhibited the activation of two MAPKs of 39 and 44 kD and of *PR* gene expression in $harpin_{Pss}$-treated Arabidopsis cells (Desikan et al., 1999), but because the Arabidopsis SIPK ortholog AtMPK6 is a 49-kD protein (Nühse et al., 2000), it is not certain if either one of the PD98059-sensitive MAPKs represented AtMPK6.

Taken together, $harpin_{Psph}$ and β-elicitin-induced *HIN1* expression in tobacco cells was shown to be mediated through different receptors (Figure 2F). Subsequently, Ca^{2+}-independent and Ca^{2+}-dependent signaling cascades are initiated, which merge upstream of MAPK activity and give rise to expression of *PR* genes, such as *HIN1*.

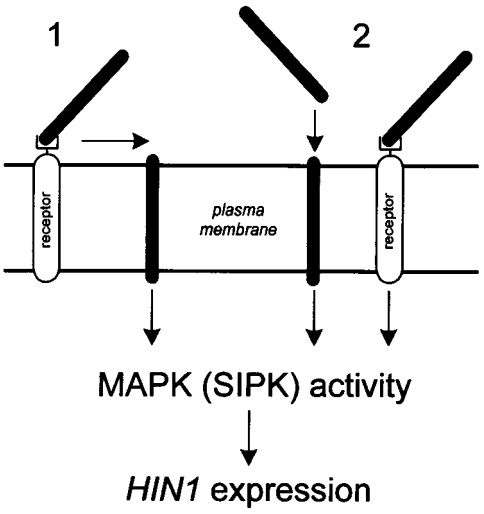

Figure 8. Hypothetical Model for $Harpin_{Psph}$-Induced Signal Transduction in Tobacco.

Activation by $harpin_{Psph}$ of pathogen defense responses in tobacco may be mediated by receptor-mediated membrane insertion (1), by direct insertion of $harpin_{Psph}$ into membranes, or by a specific (non)proteinaceous receptor (2). Alternatively, membrane insertion and receptor-mediated recognition of $harpin_{Psph}$ may occur independently, with either or both pathways activating MAPK-dependent *HIN1* expression in tobacco.

METHODS

Plant Growth, Maintenance of Plant Cell Cultures, and Elicitor Application

Tobacco (*Nicotiana tabacum* cv Samsun NN) plants were grown in a greenhouse at 22°C with a 14-hr-light/10-hr-dark cycle. Six- to 8-week-old plants were used for infiltration experiments. Test substances were infiltrated into the apoplast using a 1-mL disposable plastic syringe. Tobacco BY2 cell lines were maintained as described (Nagata et al., 1992). For subculturing, 2 mL of cells was transferred weekly into 50 mL of fresh medium. Experiments with cultured cells were performed 3 days after subculture. To maintain uniform conditions for all experiments, cultured cells were filtered and resuspended in incubation buffer (0.5 mM Mes, 4% [v/v] B5 salts, and 3% sucrose, pH 5.7) at a density of 5 g/100 mL. Diluted cells (5 mL) were transferred into Petri dishes and equilibrated for a minimum of 1 hr at 22°C with shaking (125 rpm) before the addition of effectors. Inhibitors were added 30 min before elicitor treatment. Harpin from *Pseudomonas syringae* pv *phaseolicola* ($harpin_{Psph}$) and β-megaspermin were added at final concentrations of 1 μM and 50 nM, respectively. Cells were collected by filtration 3 hr after elicitor treatment or at the indicated times and stored in liquid nitrogen. For experiments with Ca^{2+} channel blockers or 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid, the elicitation time was reduced to 1.5 hr. Cell viability was determined routinely by fluorescein diacetate/propidium iodide staining (Blume et al., 2000). Tobacco BY2 protoplasts were prepared using the protocol described previously for the preparation of parsley protoplasts (Dangl et al., 1987).

Elicitor Preparation

For the production of recombinant harpins, a DNA fragment encoding $harpin_{Psph}$ (GenBank accession number AF268940) or a polymerase chain reaction (PCR) fragment of harpin from *P. s. tomato* amplified from genomic DNA (Preston et al., 1995) was placed under the control of a T7 promoter (pT7-7 or pJC40) (Clos and Brandau, 1994; Lee et al., 2001). For the expression of harpin from *P. s. syringae,* the plasmid pSYH10 was used (He et al., 1993). DNA constructs were transformed into BL21 (DE3) pLysS *Escherichia coli* cells, and protein expression in bacteria grown to midlogarithmic phase was induced with 1 mM isopropyl-β-D-thiogalactoside for 5 hr. Subsequently, cells were harvested and lysed by sonication in extraction

buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 8.0). The sonicated material was boiled (100°C, 10 min) and centrifuged to remove denatured proteins, and heat-stable proteins were precipitated by ammonium sulfate fractionation (45% saturation). The precipitated protein was resuspended in 5 mM Mes buffer, pH 5.5, and desalted using PD-10 columns (Amersham Pharmacia, Freiburg, Germany). This procedure allowed purification to more than 95% homogeneity according to SDS-PAGE/silver staining and reverse phase HPLC analysis (Nürnberger et al., 1994).

To verify the integrity of the recombinant protein, the N terminus was sequenced using a G1050 Protein Sequencer (Hewlett-Packard, Palo Alto, CA). For the expression of truncated forms of harpin$_{Psph}$, DNAs encoding the fragments shown in Figure 3A were amplified by PCR from plasmid pT7-7/hrpZ (Lee et al., 2001). Primers were designed from the harpin$_{Psph}$-encoding DNA sequence and modified to construct appropriate restriction sites. NdeI–BamHI fragments (except fragment IV, which was encoded by a NdeI–EcoRI DNA fragment) were introduced into the modified pET vector pJC40 encoding an N-terminal His$_{10}$ tag (Clos and Brandau, 1994). The clone encoding harpin$_{Psph}$ fragment VII (Figure 3A) was obtained by removal of an internal XhoI DNA fragment. Expression in BL21 (DE3) pLysS *E. coli* cells was initiated by 1 mM isopropyl-β-D-thiogalactoside, and expressed proteins were isolated on nickel–nitrilotriacetic acid agarose using the Qiagen (Hilden, Germany) protocol. Harpin$_{Psph}$ fragments were dialyzed against 5 mM Mes, pH 5.5. The homogeneity of purified recombinant harpin$_{Psph}$ was tested by SDS-PAGE/silver staining. β-Megaspermin was prepared as described (Baillieul et al., 1995). Protein concentrations were determined with the bicinchoninic acid assay kit (Pierce, Sankt Augustin, Germany) using BSA as the standard. Solid phase peptide synthesis with N-α-9-fluorophenylmethoxycarbonyl (Fmoc)–protected amino acids was performed with an Economy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany) according to the manufacturer's instructions. Synthesized peptides were purified to homogeneity as described (Nürnberger et al., 1994).

RNA Extraction and Analyses

RNA was extracted by the hot phenol/chloroform/LiCl precipitation method (Sambrook et al., 1989). RNA prepared from 250 mg of frozen tobacco cells was resuspended in 50 μL of water. Routinely, 1 to 2 μL of RNA solution was used in reverse transcription (RT)-PCR analyses. RT was initiated in the presence of oligo(dT) primers (42°C, 30 min), and after inactivation of the reverse transcriptase (95°C, 5 min), the appropriate primers were added for PCR cycling (25 cycles of 15 sec at 95°C, 1 min at 55°C, and 1 min at 72°C). Initially, reverse transcriptase and Taq polymerase were added separately, but subsequently, a single tube reaction was used (Ready-To-Go RT-PCR beads; Amersham Pharmacia). Each rehydrated bead was used for one reaction of 50 μL. Amplification of a constitutively expressed gene (translation elongation factor 1α [*EF1α*]) served as an internal control in RT-PCR assays. For analysis of *HIN1* expression, multiplex PCR was used to amplify simultaneously transcripts of *HIN1* and *EF1α*. The RT-PCR products were analyzed by agarose gel electrophoresis. For quantification, RT-PCR products were transferred to nylon membranes and hybridized to α-^{32}P-dATP–labeled *HIN1* cDNA (Sambrook et al., 1989). Signal intensity was determined by phosphorimaging.

For amplification of tobacco genes by RT-PCR, the following primers were used: *HSR203* (forward, 5′-TGTACTACACTGTCTACACGC-3′; reverse, 5′-GATAAAAGCTATGTCCCACTCC-3′); *HSR201* (forward, 5′-CATCACGAATACGATGAAGTACG-3′; reverse, 5′-CAGGCAAACAAATTGGAACC-3′); *HSR515* (forward, 5′-AACTCTCCCTTAAGTACGGAC-3′; reverse, 5′-CAATAGTCCATACACTCACGA-3′); *PR1* (forward, 5′-GATGCCCATAACACAGCTCG-3′; reverse, 5′-TTTACAGATCCAGTTCTTCAGAGG-3′); *PR2* (forward, 5′-CTGCCCTTGTACTTGTTGGG-3′; reverse, 5′-TCCAGGTTTCTTTGGAGTTCC-3′); *PR3* (forward, 5′-GGTTCTATTGTAACGAGTGAC-3′; reverse, 5′-TTCTATGTAACGAAGCCTAGC-3′); chitinase/lysozyme (forward, 5′-TCTCATGTTTCCTTCTCCGG-3′; reverse, 5′-CAAAGTAACCTAGCAATCCTCTACC-3′); *HIN1* (forward, 5′-GAACGGAGCCTATTATGGCCCTTCC-3′; reverse, 5′-CATGTATATCAATGAACACTAAACGCCGG-3′); and *EF1α* (forward, 5′-TCACATCAACATTGTGGTCATTGGC-3′; reverse, 5′-TTGATCTGGTCAAGAGCCTCAAG-3′). RNA gel blotting with total RNA from tobacco cells and α-^{32}P-dATP–labeled DNA was performed as described (Sambrook et al., 1989).

Iodination of Harpin$_{Psph}$, Microsomal Membrane Preparation, and Radioligand Binding Assays

A tyrosine residue was added to the C terminus of harpin$_{Psph}$ for radioiodination. PCR with corresponding primers was performed to change the TGA stop codon by the deletion of one nucleotide into a tyrosine-encoding TAC codon, which was followed by a TGA stop codon created by the frameshift. The introduced mutation and the integrity of the coding sequence were verified by DNA sequencing. Nonradioactive iodination of harpin was performed by the addition of IODO-BEADS (Pierce) and NaI according to the supplier's instructions. Labeling with Na^{125}I to a specific radioactivity of 2200 Ci/mmol was performed by Biotrend Chemikalien (Köln, Germany). Tobacco BY2 cells (6 days old) were used for the preparation of microsomes as described (Nürnberger et al., 1994). The microsomal pellet recovered from 150 g of frozen tobacco cells was resuspended in 5 mL of 20 mM sodium phosphate and 100 mM NaCl, pH 7.0. Microsomal protein (200 μg) was resuspended in a total volume of 200 μL of buffer and kept on ice during the course of the experiment. If not indicated otherwise, binding was initiated by the addition of 100 nM ^{125}I-harpin$_{Psph}$. The binding reaction was terminated by adding 5 mL of ice-cold buffer, and membranes were harvested by filtration on Whatman GF/B glass filters (Maidstone, UK) preblocked with 5% BSA in binding buffer (Nürnberger et al., 1994). Subsequently, filters were subjected to γ-counting in a Wizard counter (Amersham Pharmacia). Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled harpin$_{Psph}$. Binding of ^{125}I-harpin$_{Psph}$ to 2.5 × 10^6 tobacco protoplasts resuspended in 250 μL of Murashige and Skoog (1962) medium containing 0.4 M sucrose was performed as described above except that protoplasts were rinsed three times with 5 mL of ice-cold 0.24 M $CaCl_2$. Appropriate dilutions of ^{125}I-harpin$_{Psph}$ with unlabeled harpin$_{Psph}$ were used to reduce the costs and use of radioactivity.

Ion Flux Measurements and MAPK Activity Assays

Changes in ion concentrations (H^+, K^+, and Cl^-) of elicited tobacco cells were determined as described (Nürnberger et al., 1994). Extracellular free Ca^{2+} concentration was calculated using MaxChelator software (version 2.5 for Windows; http://www.stanford.edu/~cpatton/) (Bers et al., 1994). Preparation of protein extracts from tobacco cells, in-gel protein kinase assays with myelin basic protein (MBP) embed-

ded in 10% polyacrylamide gels, and immune complex kinase activity assays using the antiserum Ab-p48N were performed as described (Zhang et al., 1998). For immunoblot analyses with the antiACTIVE mitogen-activated protein kinase (MAPK) antibody (Promega, Mannheim, Germany), tobacco protein extracts (30 μg of protein) were separated on 10% SDS-polyacrylamide gels, proteins were transferred to nitrocellulose by semidry blotting, and membranes were incubated with the antiserum according to the supplier's instructions. A secondary goat anti-rabbit IgG antibody coupled to alkaline phosphatase was used to visualize immunoreactive proteins.

ACKNOWLEDGMENTS

We thank Serge Kauffmann (Centre National de la Recherche Scientifique, Strasbourg, France) for the kind gift of purified β-megaspermin and Alan Collmer (Cornell University, Ithaca, NY) for providing plasmid pSYH10. Angela Schierhorn (Martin-Luther-University, Halle-Wittenberg, Germany) is gratefully acknowledged for matrix-assisted laser-desorption ionization time of flight analyses of iodinated $harpin_{Psph}$. We thank Beatrix Blume, Dierk Scheel, and Dirk Nennstiel for critical reading of the manuscript and Jason J. Rudd for sharing with us his expertise on MAPK assays. This work was funded by grants from the European Community Biotechnology program (Grant No. BIO-CT97-2244) and the United States Department of Agriculture (Grant No. 9862200).

Received January 5, 2001; accepted March 6, 2001.

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Signal transmission in the plant immune response

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Genetic and biochemical dissection of signaling pathways regulating plant pathogen defense has revealed remarkable similarities with the innate immune system of mammals and *Drosophila*. Numerous plant proteins resembling eukaryotic receptors have been implicated in the perception of pathogen-derived signal molecules. Receptor-mediated changes in levels of free calcium in the cytoplasm and production of reactive oxygen species and nitric oxide constitute early events generally observed in plant–pathogen interactions. Positive and negative regulation of plant pathogen defense responses has been attributed to mitogen-activated protein kinase cascades. In addition, salicylic acid, jasmonic acid and ethylene are components of signaling networks that provide the molecular basis for specificity of plant defense responses. This article reviews recent advances in our understanding of early signaling events involved in the establishment of plant disease resistance.

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Innate immunity is an ancient form of defense against microbial infection that is shared by plants, insects and vertebrates[1–3]. The discrimination of many potential pathogens from self is a formidable task for the innate immune system. This challenge is met by the evolution of a family of receptors that recognize conserved surface components of microbial pathogens called pathogen-associated molecular patterns. Immune modulators in mammals include the LIPOPOLYSACCHARIDE (LPS) fraction of Gram-negative bacteria (see Glossary), bacterial PEPTIDOGLYCANS and lipopeptides or fungal cell-wall constituents, such as chitin, glucan, mannans or proteins[1]. TOLL-LIKE RECEPTORS (TLR, a class of mammalian plasma membrane proteins with similarity to the Toll family of receptors from *Drosophila*) consisting of multiple LEUCINE-RICH REPEATS (LRR) are part of multicomponent complexes that bring about ligand-specific signal perception and initiation of an intracellular signaling cascade (Fig. 1). Such TLR-activated pathways in mammals and *Drosophila* show remarkable evolutionary conservation[1,2]. Perception complex-associated kinases (IRAK or Pelle) initiate MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascades that activate transcription factors, such as mammalian NF-κB or Dif/Relish from *Drosophila*. In addition, mammalian TLRs have the ability to activate NO-dependent as well as NO-independent antimicrobial effector pathways at the site of infection[4]. Finally, the expression of immune response genes stimulates the mammalian inflammatory response or the production of antimicrobial peptides and, thus protects against microbial invasion (Fig. 1)[5].

Research over the past years has provided compelling evidence that plants have evolved similar molecular modules to recognize potential pathogens and to mount appropriate defensive measures against attempted microbial infection[3,6]. In this review, we cover some of the findings that support the intriguing view of an evolutionarily conserved molecular basis of innate immunity in different kingdoms.

Signals and signal delivery

Plant disease resistance occurs at the species level (non-host resistance) as well as at the subspecies or varietal level (race or cultivar-specific resistance)[7]. Regardless of the type of resistance employed, the activation of a plant's surveillance system requires sensitive perception of pathogen-derived molecular patterns or motifs. Activation of non-host resistance can be triggered by numerous pathogen-derived surface structures that are similar to the modulators of the innate immune response in mammals or *Drosophila*. For example, lipid A and the O-antigen side chain of the lipopolysaccharide (LPS) fraction from Gram-negative bacteria stimulate a subset of defense-associated plant responses, and complex LPS strongly enhances the plant's response to subsequent bacterial infection[8]. Carbohydrate or proteinaceous constituents of fungal or oomycete cell walls are also well known modulators of plant defense responses[9]. Likewise, plant cell wall carbohydrate fragments that are likely to be released by phytopathogen-derived hydrolytic enzymes trigger plant defense. This signal diversity might even expand to pathogen-associated patterns that have stimulatory effects on the innate immune system of animal cells, but have not been tested in plants yet, such as bacterial DNA (Ref. 10).

Race or cultivar-specific resistance is determined by complementary pairs of dominant pathogen-encoded AVIRULENCE (*Avr*) genes and dominant plant resistance (*R*) genes[3,6,7,11]. AVR proteins are considered to be virulence factors during the colonization of host plants, but in the case of resistant host-plant cultivars, betray the pathogen to the plant's surveillance system[3,6,12]. Gram-negative phytopathogenic bacteria use an evolutionarily conserved type-III secretion system to export and deliver effector proteins, including AVR proteins, into the cytosol of host plant cells. Bacterial pilus structures unique to phytopathogenic bacteria might ease the passage of effector proteins across the plant cell wall[12]. Immunocytochemical analyses have now shown that type-III effector proteins of *Erwinia amylovora* (HrpN) and *Pseudomonas syringae* pv.

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Glossary of terms commonly used to describe innate immunity

Avirulence (AVR)
Inability of an infecting pathogen race carrying an avirulence gene to colonize a resistant host plant cultivar expressing a complementary resistance gene.
Calcium-dependent protein kinase (CDPK)
A class of calcium-dependent protein kinases assumed to be the plant homologs of human protein kinase C.
Interleukin 1-receptor-associated kinase (IRAK)
A cytoplasmic human protein kinase implicated in downstream signaling linked to the TIR domain of TLR by the adaptor protein MyD88.
Leucine-rich repeat (LRR)
A structural domain of animal, insect and plant proteins assumed to mediate protein–protein interactions.
Lipopolysaccharide (LPS)
A major cell wall constituent of Gram-negative bacteria.
Mitogen-activated protein kinase (MAPK)
Signal transducing eukaryotic enzymes involved in various facets of cellular regulation.
Nitric oxide (NO)
A highly reactive second messenger implicated in a multitude of signaling pathways in animals, insects and plants.
Pathogenesis-related gene (*PR* gene)
A class of plant genes encoding proteins with antimicrobial activity. Expression of *PR* genes is triggered upon pathogen infection or elicitor treatment.
Peptidoglycan (PGN)
A major cell wall constituent of Gram-positive bacteria.
Reactive oxygen species (ROS)
A heterogenous class of small molecules (i.e. superoxide anions and hydrogen peroxide) with direct antimicrobial potential, but also implicated in plant signal transduction cascades.
Resistance gene (*R* gene)
Encodes a protein that conditions cultivar-specific resistance of a host plant to pathogen races carrying the complementary avirulence gene.
Toll-interleukin1 receptor domain (TIR)
A cytoplasmic domain of *Drosophila* Toll and human interleukin1 receptors implicated in downstream signaling.
Toll-like receptor (TLR)
A class of animal membrane receptors with similarity to the *Drosophila* Toll receptor family.

tomato (HrpW, AvrPto) are associated with these pili, suggesting that these structures guide the transport of effector proteins outside the bacterial cell[13]. Although direct evidence for their translocation across the plant plasma membrane is still lacking, bacterial AVR proteins confer cultivar-specific resistance when produced *in planta*[3,6,12]. For some AVR proteins (*P. syringae* AvrRPM1, AvrB, AvrPto), targeting to the plasma membrane subsequent to injection into the plant cytosol has been shown[14,15]. Consensus myristoylation sites within these AVR proteins provide substrates for this eukaryote-specific post-translational modification, which subsequently enables favorable subcellular compartmentation of the injected effector molecules. The make up of AvrD from *P. syringae* pv. *glycinea* is a remarkable exception to *Avr* gene-encoded effector proteins[16]. AvrD does not directly act as an effector protein in host plant cells, but probably encodes an enzyme involved in the production of an acyl glucoside elicitor, syringolide. Upon transfer across the plant plasma membrane, syringolides trigger a resistance response in soybean plants carrying the *Rpg4* gene, but not in susceptible cultivars lacking a functional *Rpg4*.

Phytopathogenic fungi secrete several Avr proteins, which activate cultivar-specific resistance responses in plant cultivars expressing the matching *R* gene. The causal agent of tomato leaf mould, *Cladosporium fulvum*, produces a 28-mer polypeptide, AVR9, which triggers hypersensitive cell death in tomato plants carrying the *Cf-9* gene[17]. Potato virus X-based expression of the AVR9-encoding cDNA or infiltration of AVR9 into Cf-9 tomato cultivars results in HR-associated resistance, suggesting that recognition of the AVR protein occurs at the tomato plasma membrane[17,18]. By contrast, *in vitro*, AvrPita from the rice blast fungus, *Magnaporthe grisea*, interacts with the matching *R* gene product, Pi-ta, a predicted cytoplasmic rice protein[19]. This suggests direct introduction of a fungal effector protein into the plant cell cytoplasm by a secretion–translocation system functionally similar to that used by phytopathogenic bacteria.

Signal perception – a matter of complexity

Recent findings have significantly improved our understanding of signal perception mechanisms at the plasma membrane of both non-host and host plants. For example, a 75-kDa plasma membrane-bound protein found in various *Fabaceae* is a binding site for a *Phytophthora sojae*-derived hepta-β-glucan elicitor of phytoalexin production[20,21]. Heterologous expression of the encoding soybean gene in tomato confers the high-affinity binding of the elicitor. Because this putative receptor protein does not contain recognizable functional domains for signal transmission across the plasma membrane or for intracellular signal generation, it might be recruited into a multi-component perception complex[21]. Consistent with this, several proteins closely associated with the heptaglucan binding site have been detected in photoaffinity labeling experiments.

A 22-amino acid fragment of flagellin (flg22), the major constituent of bacterial flagellae, induced plant defense-associated responses in a variety of plants including *Arabidopsis*[22]. A genetic screen based on the ability of flg22 to inhibit growth of *Arabidopsis* ecotype Landsberg *erecta* led to the isolation of two independent, recessive mutants affected at the locus, *fls2* (for flagellin-sensing)[23]. *FLS2* encodes a 129-kDa receptor-like kinase made of an extracellular LRR domain, a membrane-spanning domain and a protein kinase domain. Interestingly, the mutants, which carried a point mutation either in the LRR-domain or in the kinase domain, were both impaired in their ability to bind flg22, suggesting that both parts of the protein are important for ligand perception and formation of a functional receptor complex[24]. Biochemical analyses must now prove or disprove whether flg22 and FLS2 interact physically, and thus reveal whether FLS2 constitutes the flg22 receptor or, alternatively, is part of the signal perception mechanism downstream of the elicitor binding site.

The prevalent biochemical interpretation of the gene-for-gene relationship predicts *Avr* gene products to be ligands, which are recognized by *R* gene-encoded plant receptors. More than 30 *R* genes conferring

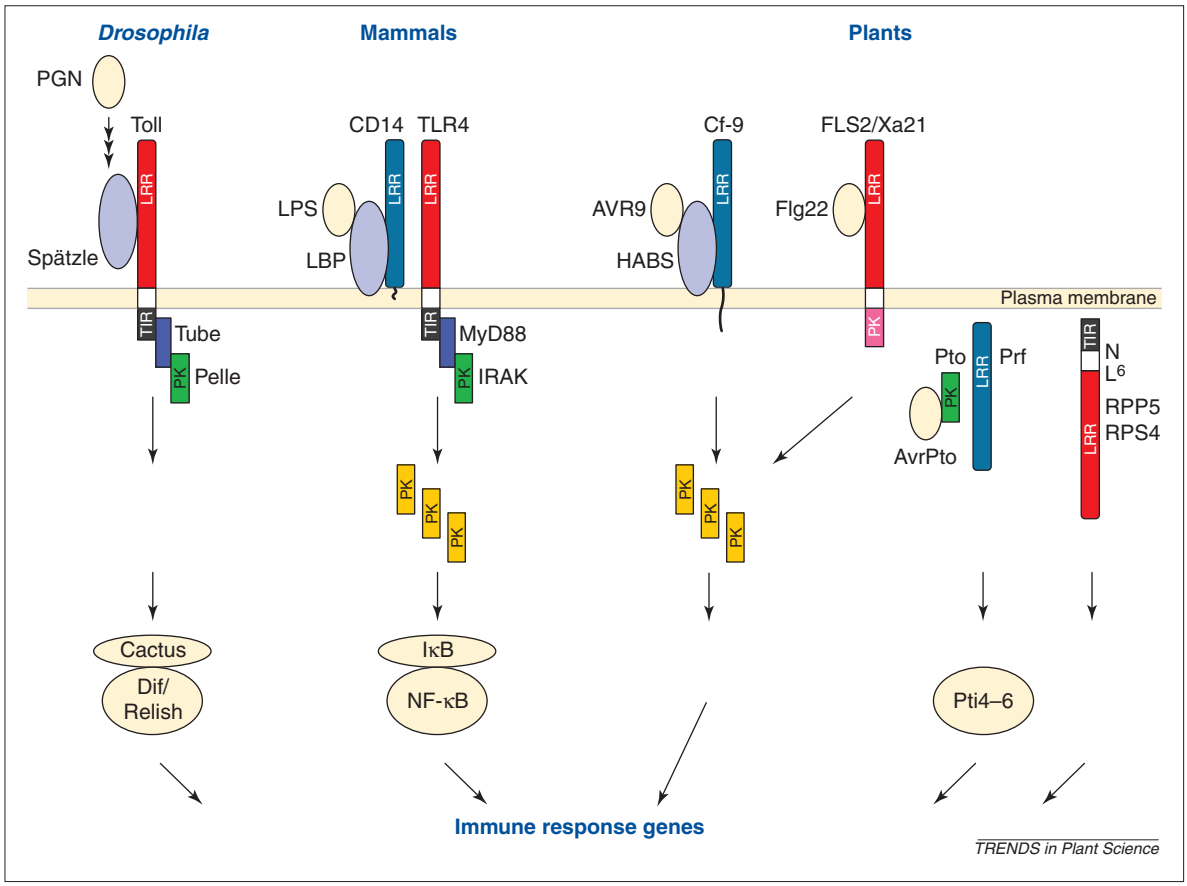

Fig. 1. Signaling cascades in the innate immune response of insects, mammals and plants. Pathogen recognition by the innate immune system relies on interactions between pathogen-derived molecules and corresponding host receptors. Sugars and proteins on microbial surfaces, but also various molecules that infecting pathogens produce, trigger innate immunity[1–3]. In *Drosophila*, peptidoglycans (PGN) from Gram-positive bacteria initiate a proteolytic cascade, upon which Spätzle, a proteinaceous ligand for Toll is generated. Adaptor proteins MyD88 (myeloid differentiation factor) or Tube link the cytoplasmic TOLL-INTERLEUKIN 1 RECEPTOR (TIR) domain of TLR4 or Toll, respectively, to protein kinases IRAK (interleukin 1 receptor protein kinase) or Pelle. Subsequently, a series of protein kinases including mitogen-activated protein kinases (MAPKs) mediate activation of transcription factors, NF-κB or Dif/Relish, through the inactivation of repressor proteins, IκB or Cactus, and expression of immune response genes. The lipopolysaccharide (LPS) envelope of Gram-negative bacteria stimulates innate immunity in mammals. Upon recognition by LBP, an LPS-binding protein, a complex with leucine-rich repeat (LRR-proteins) CD14 and TLR4 (which contains a cytoplasmic TIR domain) is formed. In plants, various LRR-type proteins with similarity to CD14, TLR4 or Toll appear to be involved in pathogen defense activation[3,6]. AVR9, which is structurally similar to Spätzle[6], is recognized by a high-affinity binding site in tomato[31]. This complex interacts directly or indirectly with Cf-9 and activates various MAPKs and CDPK (Refs 47,61). *Arabidopsis* FLS2 and rice Xa21 probably transduce the pathogen signal through their cytoplasmic protein kinase domain. Flg22 might directly bind to FLS2 and activate MAPK AtMPK6 (Ref. 60). Gram-negative phytopathogenic bacteria employ a type-III secretion system to deliver effector proteins, such as AvrPto, into the plant cell cytoplasm[12]. Pto-mediated resistance requires Prf, a cytoplasmic LRR-type protein[3]. AvrPto binds directly to Pto, a tomato protein kinase with homology to IRAK and Pelle. Phosphorylation by Pto of transcription factor Pti4 enhances its ability to bind to GCC elements present in the promoter region of various *PR* genes[3,36]. Several cytoplasmic proteins carrying LRR and TIR domains have been implicated in plant resistance against viral (tobacco N), bacterial (*Arabidopsis* RPS4), oomycete (*Arabidopsis* RPP5), and fungal (flax L^6) pathogens. Modified from Refs 3,6.

resistance to viruses, bacteria, fungi, nematodes or aphids have been isolated from monocot and dicot plants[6]. The predominant structural motifs found in R proteins are LRR and coiled coils, both of which suggest a role in protein–protein interaction. Thus, *R* proteins might function in ligand perception. However, only for the cytoplasmic rice protein Pi-ta has an *R* gene-encoded LRR-protein been shown to interact directly with the matching Avr protein[19]. Instead, several studies have revealed that LRR proteins apparently constitute components of larger signal perception complexes. LRR-type R proteins from viruses (HRT)[25] and fungi (Cf-9)[26] have been identified that might form complexes with Avr proteins or plant proteins binding Avr proteins. These plant binding proteins appear to interact physically with Avr proteins. Moreover, these proteins have been detected in both resistant and susceptible cultivars of host plants. These findings have led to the 'guard hypothesis'[27], which predicts that Avr proteins act as virulence factors that contact their cognate pathogenicity targets in host plants or even non-host plants, but can only function as avirulence factors if the complementary resistance protein is recruited into a functional signal perception complex. The following examples illustrate this:

- The capsid protein (CP) of turnip crinkle mosaic virus (TCV) serves as an avirulence determinant in *Arabidopsis* ecotypes carrying the *HRT* RESISTANCE GENE[25]. TCV-interacting protein (TIP), which binds to the TCV CP, is a transcriptional activator present

in both susceptible and resistant ecotypes. CP–TIP interaction is crucial to *HRT*-mediated resistance[28]. It has been suggested[28] that the viral CP produced in infected plant cells functions as a virulence factor by binding to TIP and, thus, interfering with its biological function. Resistant plants expressing the HRT protein might guard TIP by detecting a change in TIP caused by the TIP–CP interaction, which then results in resistance.

- The tomato *Cf-9* resistance gene encodes a membrane-anchored glycoprotein with an extracellular LRR and a small cytoplasmic domain without apparent function in downstream signaling[26,29]. Recent studies on the topology of the Cf-9 protein showed that it is present in the plasma membrane[29] as well as in the endoplasmic reticulum[30]. Cf-9 mediates tomato cultivar-specific recognition of AVR9, a race-specific elicitor from *C. fulvum*. However, both susceptible and resistant cultivars of tomato, as well as other solanaceous plants, harbor a high-affinity binding site for AVR9. Hence, the Cf-9 protein is unlikely to be the AVR9 receptor[31]. Consistently, comprehensive biochemical analyses failed to show a physical interaction between the two proteins[32].
- *P. syringae* pv. *glycinea*-derived syringolides trigger resistance in soybean cultivars expressing the *Rpg4* gene. However, syringolides bind to a cytoplasmic 34-kDa protein found in both resistant and susceptible soybean cultivars, suggesting again that the elicitor receptor and the *R* gene product are not identical[16]. Co-purification of two additional proteins together with the syringolide binding protein is indicative of the existence of a larger signal perception complex, of which Rpg4 might be a constituent[16].
- Similarly, complex formation of the *Arabidopsis* LRR-type resistance protein, RPS2, with *P. syringae*-derived AvrRpt2 and an additional 75-kDa plant protein, supports the idea of multicomponent complexes in pathogen sensing[33].

Taken together, plant receptors for pathogen-associated molecular motifs often appear to be present in many plant species, including non-host plant species. Hence, *R* gene-encoded specificity of host resistance is brought about by structurally conserved R proteins that assemble into larger complexes in conjunction with ligand–receptor complexes. Notably, the soybean heptaglucan receptor and the *Arabidopsis* FLS2 protein also fit into this model, suggesting that pathogen perception systems are conserved independently of the type of plant resistance exhibited. As in mammals and *Drosophila*, LRR proteins also constitute the predominant structural basis for pathogen perception in plants (Fig. 1). Furthermore, plant R proteins with a TIR domain that is characteristic of mammalian TLR have been found (tobacco N, flax L6, *Arabidopsis* RPP5, RPS4)[6]. Downstream signaling through cytoplasmic protein kinase domains of LRR-type plant R proteins (rice Xa21)[34] or plant cytoplasmic protein kinases with homology to IRAK/Pelle (tomato Pto)[35] is assumed to facilitate defense gene expression. For example, phosphorylation of the transcription factor, Pti4, by the serine/threonine kinase, Pto, strongly enhanced its capacity to bind to GCC boxes present in the promoters of many PATHOGENESIS-RELATED (*PR*) genes[36]. Activation of MAPK cascades through TLR receptors is another characteristic feature of the signaling pathway, bringing about mammalian innate immunity. Hence, plant MAPKs recently implicated in plant defense signaling are probably regulated in a similar way (see below).

Downstream signaling

Signal transduction cascades link recognition and defense responses using second messengers that are conserved among most eukaryotes. In plants, no principal differences in signaling mechanisms have been observed between host and non-host recognition or perception of race-cultivar-specific and general elicitors, respectively[37]. However, individual recognition events appear to dictate specific signaling routes that use a distinct set of secondary messengers and activate a characteristic portion of the complex defense machinery[11].

Transient changes in the ion permeability of the plasma membrane appear to be a common early element in defense signaling[38–41]. *R* gene- and receptor-mediated regulation of plasma membrane-located ion channels appear to stimulate ion fluxes across the plasma membrane (Ca^{2+} and H^+ influx, K^+ and Cl^- efflux) and thereby activate defense reactions[38–41]. Two K^+ channels were found to be differentially regulated in an *R* gene- and protein kinase-dependent manner in tobacco guard cells expressing the tomato *Cf-9* gene[41]. Treatment of these cells with AVR9 resulted in activation of an outward-rectifying K^+ channel and simultaneous inactivation of an inward-rectifying K^+ channel, explaining the frequently observed net K^+ efflux. Elicitor-responsive Ca^{2+} channels of the plasma membrane can mediate Ca^{2+} entry[38,42] resulting in transiently increased cytosolic Ca^{2+} levels[43–46]. The sensitivity of elicitor-stimulated increases in cytosolic Ca^{2+} concentration to protein kinase inhibitors suggests that protein phosphorylation is involved in receptor-mediated regulation of Ca^{2+} channels[43]. Pharmacological analyses have shown that an influx of extracellular Ca^{2+} is needed to increase the level of cytosolic Ca^{2+} and elicitor stimulation of downstream reactions, although this does not rule out the possibility that Ca^{2+} could be released from internal stores[43–46]. Amplitude and duration of *R* gene- or elicitor-stimulated Ca^{2+} transients vary, but prolonged, modest elevation of cytosolic Ca^{2+} levels rather than spikes of large intensity are often found to be essential for the activation of defense reactions[43,44,46].

One of the earliest elicitor-responsive downstream targets of cytosolic Ca^{2+} was identified as a CALCIUM-DEPENDENT PROTEIN KINASE (CDPK) in transgenic tobacco expressing the tomato *Cf-9* gene[47]. Treatment of these cells with AVR9 resulted in rapid phosphorylation and activation of this CDPK in a Ca^{2+}-dependent manner. The target of plant CDPKs, which might be analogous to animal protein kinase C, has not been identified.

Calmodulin is a universal Ca^{2+}-binding signal mediator. Ca^{2+}-dependent rapid accumulation of two specific calmodulin isoforms (SCaM-4, SCaM-5) and the encoding transcripts is induced upon pathogen infection and elicitor treatment in soybean[48]. In contrast to other calmodulin isoforms, SCaM-4 and 5 were barely detectable in healthy plants. Constitutive expression of either isoform in tobacco resulted in spontaneous lesion formation, constitutive *PR* gene expression and enhanced resistance against virulent oomycete, bacterial and viral pathogens. Surprisingly, in contrast to salicylate-dependent activation of these pathogen-inducible reactions in wild-type tobacco, their constitutive activation in transgenic tobacco plants did not require salicylate.

The oxidative burst, an important early component of pathogen defense and defense signaling in many plants, is activated by elicitor by transient increases of cytosolic Ca^{2+} levels[43]. Extracellular production of REACTIVE OXYGEN SPECIES (ROS) during the oxidative burst appears to be mechanistically similar to the respiratory burst of human phagocytes, which is catalyzed by an NADPH oxidase[49]. The catalytic subunit, gp91, resides silently in the plasma membrane associated with another subunit, p22. The active human enzyme complex is formed by Rac- and phosphorylation-mediated association of the two membrane-bound subunits with the cytosolic subunits p47 and p67. Interestingly, although *Arabidopsis* harbors eight genes homologous to human gp91, none of the other subunits of the human enzyme appear to be encoded in the genome[50]. To date, all plant gp91 homologs that have been isolated carry an N-terminal extension comprising an EF-hand motif indicative of Ca^{2+} regulation. Expression of a parsley (*Petroselinum crispum*) gp91 homolog in yeast revealed Ca^{2+}- and NADPH-dependent O_2^--generating enzyme activity in the microsomal cell fraction (T. Nürnberger and D. Scheel, unpublished).

Three genes (*OsRac1-3*) encoding proteins with 60% amino acid sequence identity to human Rac proteins were identified in rice[51]. Transgenic rice plants or cell cultures expressing a constitutively active derivative of OsRac1 produced increased ROS and phytoalexin levels, developed symptoms of programmed cell death and displayed increased resistance against virulent fungal and bacterial rice pathogens[51,52]. By contrast, the dominant-negative OsRac1 derivative suppressed elicitor-stimulated ROS production and pathogen-induced cell death in transgenic rice[52].

In addition to ROS production, NITRIC OXIDE (NO) was found to be generated in tobacco, soybean and *Arabidopsis* upon infection with avirulent bacteria or viruses, as well as upon elicitor treatment[53–56]. Together, ROS and NO appear to be essential second messengers for the activation of defense-related genes and programmed cell death[53–55]. Although NO synthase inhibitors efficiently block infection- or elicitor-stimulated NO production, cell death and defense gene activation[53,54], neither the enzyme itself nor a gene with obvious homologies to mammalian NO synthase-encoding genes have been detected in plants[50]. As in mammals, downstream signaling of NO appears to employ cyclic GMP and cyclic ADP ribose, which might stimulate another transient increase in cytosolic Ca^{2+} levels[54]. The *Arabidopsis* cyclic nucleotide-gated ion channel, AtCNGC2, might be regulated through this pathway. AtCNGC2 harbors a functional calmodulin-binding domain within its C-terminal region and was shown to mediate fluxes of Ca^{2+}, K^+ and other cations[57,58]. AtCNGC2 is the protein that is affected by the *dnd1* mutation[57]. The *dnd1* mutant fails to develop programmed cell death upon infection with avirulent pathogens, but resistance itself is not affected. Thus, activation of this ion channel by Ca^{2+} and/or cyclic nucleotides might be involved in signaling cell death programs in pathogen defense and plant development[57,58]. Interestingly, *dnd1* plants exhibit elevated levels of salicylate and systemic resistance.

Research over the past years has revealed that plant MAPKs are activated by a large variety of abiotic and biotic stimuli. At least two MAPK cascades appear to be early positive regulators in plant defense signaling[59–62]. The tobacco MAPKs, SIPK and WIPK, are activated upon infection and elicitor treatment[61,62]. Elicitor treatment of parsley and alfalfa results in rapid activation of the two MAPKs orthologous to SIPK and WIPK (Refs 59,63). Interestingly, in *Arabidopsis*, only the SIPK ortholog, AtMPK6, is activated (Fig. 2)[60]. Elicitor-induced MAPK activation is dependent on Ca^{2+} influx, but is itself not required for elicitor-induced ROS production[61,64,65]. In elicitor-treated parsley cells, the WIPK orthologous MAPK translocates to the nucleus, where it might be involved in the regulation of defense gene expression[64].

A causal link between SIPK and WIPK activation and defense gene activation, as well as programmed cell death, was suggested recently by a gain-of-function approach[65]. The MAPK kinase (MAPKK), NtMEK2, was found to activate specifically both SIPK and WIPK, and thereby induce the activation of two but not a third defense-related gene, and initiate programmed cell death. Interestingly, salicylate was not required for NtMEK2-mediated initiation of the cell death program. Furthermore, activation of SIPK and WIPK by a constitutively active NtMEK2 derivative was not accompanied by the generation of H_2O_2 (Ref. 65), which, in concert with NO, signals

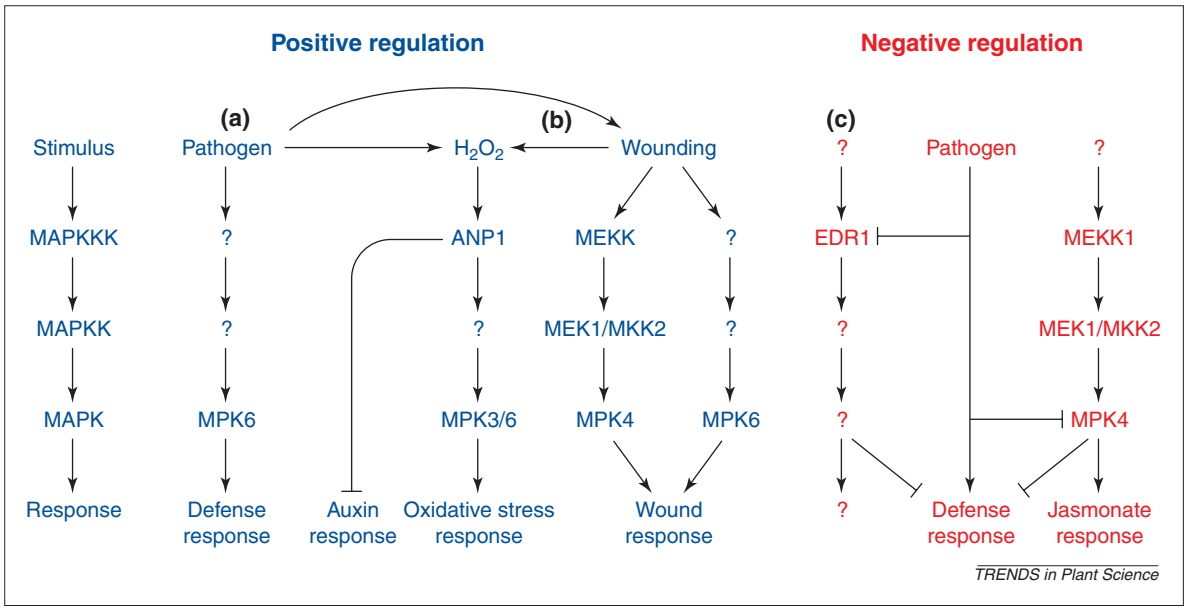

Fig. 2. Hypothetical model of mitogen-activated protein kinase (MAPK) cascade involvement in pathogen defense signaling in *Arabidopsis thaliana*. Pathogen attack activates distinct MAPK pathways either (a) directly or (b) indirectly via H_2O_2 produced by the oxidative burst, or upon wounding (positive regulation). (c) In addition, plant defense also appears to be under the negative control of various MAPK cascades (negative regulation).

defense gene activation and programmed cell death in tobacco and *Arabidopsis*[53–55]. Therefore, alternative salicylate- and ROS-independent HR signal transduction pathways appear to exist. However, this conclusion would benefit greatly from confirmation by a complementary loss-of-function experiment. H_2O_2, the most long-lived and, therefore, major ROS of the oxidative burst, initiates MAPK cascades[65,66]. In tobacco, SIPK was activated by H_2O_2 (Ref. 65), whereas in *Arabidopsis*, AtMPK3 and AtMPK6, the orthologs of WIPK and SIPK, were activated via the MAPKK kinase (MAPKKK), ANP1 (Fig. 2)[66]. Apparently, pathogen-induced MAPK-independent production of H_2O_2 can activate pathogen-induced MAPK cascades and might thereby increase the effectiveness of MAPK-regulated defense reactions.

Recently, MAPK cascades that are different from those activated upon pathogen infection or elicitor treatment, were shown to negatively regulate plant defense (Fig. 2). Transposon inactivation of the gene encoding *Arabidopsis* AtMPK4 resulted in dwarfed mutants that exhibited elevated levels of salicylate, constitutive *PR* gene expression and increased resistance to virulent pathogens[67]. This resistance required salicylate, but was independent of NPR1 (also known as NIM1). A second study showed that a mutation in a putative *Arabidopsis* MAPKKK resulted in a mutant (*edr1*, enhanced disease resistance) with increased resistance to powdery mildew and *Pseudomonas syringae* without any phenotypic alterations[68]. Because *edr1*-mediated resistance was salicylate and NPR1/NIM1-dependent and did not lead to enhanced levels of salicylate and constitutive *PR* gene expression, EDR1 is unlikely to control the AtMPK4 MAPK cascade. EDR1 is homologous to members of the Raf-like MAPKKK family, such as the negative regulator of the ethylene response, CTR1 (Ref. 69). Interestingly, the *Arabidopsis* MAPKs, AtMPK3 and ATMPK6, are regulated by the MAPKKK, ANP1, which does not belong to the EDR1/CTR1 family of MAPKKK (Ref. 66).

Intriguingly, negative and positive regulation of different signaling pathways can be brought about by one particular MAPK cascade (Fig. 2). Activation of oxidative stress responses upon expression of a constitutively active derivative of the MAPKKK, ANP1, resulted in concomitant inhibition of the auxin response pathway[66]. Likewise, inactivation of AtMPK4 not only leads to activation of salicylate-mediated defense responses, but also inhibits salicylate-independent jasmonate-responsive gene expression, suggesting positive regulation by AtMPK4 of the salicylate-independent jasmonate-response pathway[67]. AtMPK4 is indeed rapidly activated upon wounding, which initiates a jasmonate-mediated signaling pathway[70]. Apparently, MAPK cascades constitute central elements in complex signaling networks that regulate a plant's response to a multitude of stimuli. This complexity, together with the 20 MAPKs found in the *Arabidopsis* genome[50], provides an enormous future challenge for plant biologists with an interest in MAPK function.

Conclusions and perspectives

The functional conservation of components of the innate immune system in mammals, insects and plants suggests an early evolutionary origin of eukaryotic pathogen defense systems[1–3]. Structurally similar receptor complexes recognize pathogen-derived signals and thereby initiate complex signaling networks that trigger transient

changes in defense gene expression. Similar second messengers, such as Ca^{2+}, ROS and NO are employed in the innate immune response of higher eukaryotes. MAPK cascades constitute another common element of defense signaling chains in various organisms. In plants, early changes in the activity of these messengers regulate at least two alternative resistance mechanisms mediated by the plant hormones, salicylate or jasmonate and ethylene, respectively[3,11]. Such signal transduction networks are characterized by multiple points of convergence and divergence that enable signal integration at different levels, and provide the molecular basis for appropriate protective measures. Intriguingly, similarities between innate immunity in different kingdoms even extend to the products of immune response genes. Antimicrobial peptides produced by mammals, insects and plants contribute to resistance against microbial invasion[5]. Recent reports show the potential for the production of resistant crop plants of (modified) antimicrobial peptides with a proven role in plant and non-plant innate immunity[71,72]. In addition, detailed knowledge of signaling cascades regulating innate immunity should help develop sophisticated strategies for durable crop protection. A candidate element for such an approach is, for example, mutant EDR1 protein from *Arabidopsis*[68]. Inactivation of a MAPK pathway generated a phenotypically normal plant with increased disease resistance.

Acknowledgements
We apologize to all our colleagues whose work could not be reviewed here because of space limitations. We thank Sabine Rosahl for critical reading of the manuscript. Work in our laboratories is supported by the Fonds der Chemischen Industrie, Deutsche Forschungsgemeinschaft, and the European Community.

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PCI complexes: pretty complex interactions in diverse signaling pathways

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Three protein complexes (the proteasome regulatory lid, the COP9 signalosome and eukaryotic translation initiation factor 3) contain protein subunits with a well defined protein domain, the PCI domain. At least two (the COP9 signalosome and the lid) appear to share a common evolutionary origin. Recent advances in our understanding of the structure and function of the three complexes point to intriguing and unanticipated connections between the cellular functions performed by these three protein assemblies, especially between translation initiation and proteolytic protein degradation.

The PCI complexes are a recently discovered family of multisubunit protein complexes that regulate development and signal transduction. There are three known PCI complexes: the regulatory lid of the 26S proteasome ('P'), the COP9 signalosome (CSN) ('C') and the translation initiation factor eIF3 ('I'). All three are structurally related and are highly conserved among higher eukaryotes. The situation in *Saccharomyces cerevisiae* is less clear, because there is an obvious proteasome regulatory lid and a smaller but obvious eIF3, but no recognizable CSN. All three complexes are ~500 kDa. Subunits of all three complexes contain one of two domains, the PCI domain or the Mpr1–Pad1 N-terminal (MPN) domain[1–4]. The primary sequence of the PCI domain is not well conserved, which hinders classic alignment and phylogenetic analysis. Instead, the PCI domain is characterized by a conserved secondary structure that is a largely α-helical fold.

http://plants.trends.com 1360-1385/01/$ – see front matter PII: S1360-1385(01)02015-5

A Novel Protein Elicitor (PaNie) from *Pythium aphanidermatum* Induces Multiple Defense Responses in Carrot, Arabidopsis, and Tobacco[1]

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A novel protein elicitor ($PaNie_{234}$) from *Pythium aphanidermatum* (Edson) Fitzp. was purified, microsequenced, and the corresponding cDNA was cloned. The deduced amino acid sequence contains a putative eukaryotic secretion signal with a proteinase cleavage site. The heterologously expressed elicitor protein without the secretion signal of 21 amino acids ($PaNie_{213}$) triggered programmed cell death and de novo formation of 4-hydroxybenzoic acid in cultured cells of carrot (*Daucus carota*). Programmed cell death was determined using the tetrazolium assay and DNA laddering. Infiltration of $PaNie_{213}$ into the intercellular space of leaves of Arabidopsis (Columbia-0, wild type) resulted in necroses and deposition of callose on the cell walls of spongy parenchyma cells surrounding the necrotic mesophyll cells. Necroses were also formed in tobacco (*Nicotiana tabacum* cv Wisconsin W38, wild type) and tomato (*Lycopersicon esculentum* Mill.) but not in maize (*Zea mays*), oat (*Avena sativa*), and *Tradescantia zebrina* (Bosse), indicating that monocotyledonous plants are unable to perceive the signal. The reactions observed after treatment with the purified $PaNie_{213}$ were identical to responses measured after treatment with a crude elicitor preparation from the culture medium of *P. aphanidermatum,* described previously. The availability of the pure protein offers the possibility to isolate the corresponding receptor and its connection to downstream signaling-inducing defense reactions.

Plants are able to defend themselves successfully with a complex set of preformed structures and inducible reactions. The inducible reactions require the perception of either plant-derived (endogenous) or pathogen-derived (exogenous) signal molecules. These so-called elicitors are of diverse chemical nature and include proteins, peptides, glycoproteins, lipids, and oligosaccharides (Nürnberger, 1999). Elicitors trigger plant defense responses that are part of the basic or non-host resistance of plants (Nürnberger, 1999). Defense is often associated with localized hypersensitive cell death (Mittler et al., 1997) and the de novo formation of low-M_r antimicrobial compounds called phytoalexins (Hammond-Kosack and Jones, 1996). The reinforcement of cell wall constituents is also part of the defense response (Bruce and West, 1989). The structural and cultivar specificity of elicitors and their ability to trigger plant defense responses at very low concentrations strongly suggest the existence of receptors at the plasma membrane and a downstream signal transduction cascade (Ebel and Cosio, 1994). Despite many years of work, there are very few cases described in which the causal link between elicitors from oomycetes and putative receptors or high-affinity binding sites has unequivocally been linked to induced defense responses (Nennstiel et al., 1998). To study the elicitor-receptor interaction and the downstream signaling, pure elicitor molecules are necessary prerequisites.

Protein elicitors have been found in bacterial pathogens as well as in oomycetes and ascomycetes (Ebel and Cosio, 1994). Gram-negative phytopathogenic bacteria like *Erwinia amylovora* and *Pseudomonas syringae* secrete proteins, which induce a hypersensitive response, the so-called harpins (Wei et al., 1992; He et al., 1993). Bacterial flagellin and the corresponding receptor-like kinase were found more recently to induce defense responses (Felix et al., 1999; Gómez-Gómez and Boller, 2000).

In the order of *Peronosporales*, protein elicitors with a relative molecular mass of 10 kD and necrosis-inducing activity were identified and designated the elicitins (Ponchet et al., 1999). The race-specific Avr9-peptide from *Cladosporium fulvum* is responsible for the induction of active defense responses in tomato (*Lycopersicon esculentum* Mill.) cell cultures (May et al., 1996).

The cell wall of *Phytophthora sojae* contains a 42-kD glycoprotein that induces the activation of defense-related genes in parsley (Nürnberger et al., 1994). The

[1] This work was supported by the Deutsche Forschungsgemeinschaft (grant no. Se 229/19-1) and by the Land Baden-Württemberg (grant to W.K.).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010350.

active part of this glycoprotein is an internal peptide of 13 amino acids (Pep-13; Hahlbrock et al., 1995). From *Phytophthora parasitica*, an elicitor-active protein (Pp-elicitor) has been described recently that activates defense responses in parsley suspension cultures similar to those induced by the Pep-25 elicitor, containing the sequence of Pep-13 (Felbrich et al., 2000).

A 24-kD (Nep1) necrosis- and ethylene-inducing protein has been purified from culture filtrates of *Fusarium oxysporum* f. sp. *erythroxyli* (Bailey, 1995). When applied to weed species as a foliar spray it causes necrosis (Jennings et al., 2000).

Pythium aphanidermatum is a cosmopolitan pathogen with a wide host range causing economic losses on several important crops. *P. aphanidermatum* infects preferably juvenile tissues like seedling stems. Suspension-cultured hyphae of *P. aphanidermatum* release a variety of elicitor-active molecules into the culture medium. Among these elicitors are carbohydrates as well as proteins (Schnitzler 1992). In carrot (*Daucus carota*) cell cultures, and protoplasts derived from the cultured cells, these elicitors induce the de novo formation of 4-hydroxybenzoic acid (4-HBA). In intact cells, this compound is transferred to the cell wall and covalently linked to cell wall constituents, whereas in protoplasts lacking cell walls, the compound is secreted in a conjugated form into the culture medium (Schnitzler and Seitz, 1989). The carrot system described here reflects a non-host interaction or a basic resistance. The formation of active Phe ammonia-lyase (PAL) is necessary for 4-HBA synthesis and the elicitor induces the synthesis on a transcriptional level (Koch et al., 1998). The *P. aphanidermatum*-derived elicitor also induces programmed cell death in carrot cell cultures (Koch et al., 1998). Using an assay for loss of carrot cell viability, a 25-kD protein with elicitor activity was identified and partially purified (Koch et al., 1998). Using this crude elicitor preparation and specific inhibitors, several components of the signal transduction pathway have been identified. It was shown that an increase of cytoplasmic calcium concentration is essential for cell death induction and 4-HBA synthesis (Bach et al., 1993; Koch et al., 1998) and it was also demonstrated by these inhibitor experiments that G proteins are involved in signal transduction leading to programmed cell death, but not to 4-HBA accumulation (Koch et al., 1998).

To rule out effects of contaminating proteins and carbohydrates in the crude preparation, a pure elicitor protein is necessary. In the present paper, we describe the isolation of a cDNA encoding the elicitor protein ($PaNie_{234}$) from *P. aphanidermatum*. The protein contains 234 amino acids and has a putative eukaryotic secretion signal harboring a proteinase cleavage site. The mature elicitor protein without the secretion signal ($PaNie_{213}$) consists of 213 amino acids. $PaNie_{213}$ has been heterologously expressed in *Escherichia coli* and can be detected by a rabbit antiserum raised against the elicitor protein. Using affinity chromatography, the His-tagged $PaNie_{213}$ was purified and assayed for its elicitor activity in suspension-cultured carrot cells and by infiltration into leaves of dicotyledones Arabidopsis, tobacco (*Nicotiana tabacum*), tomato, and monocotyledons maize (*Zea mays*), oat (*Avena sativa*), and *Tradescantia zebrina*.

With the purified $PaNie_{213}$ in hand, we were able to show that a single pure elicitor protein is sufficient to trigger multiple defense pathways.

RESULTS

To study elicitor-receptor interactions and the link to downstream defense reactions, a pure elicitor protein is necessary. Therefore, heterologous expression and purification of the elicitor from *P. aphanidermatum* was initiated.

Heterologous Expression of the His-Tagged Protein Elicitor from *P. aphanidermatum* ($PaNie_{213}$)

The elicitor protein was purified from the culture medium of *P. aphanidermatum* with preparative SDS-PAGE as the final step. Because it was blocked at its N terminus, the protein was proteolytically digested and the released oligopeptides were microsequenced. On the basis of these sequences, degenerated primers were used to screen a cDNA library from *P. aphanidermatum*. The amino acid sequence is illustrated in Figure 1. By analyzing the sequence according to Nielsen et al. (1997), we can predict a putative eukaryotic secretion signal that is not present in $PaNie_{213}$ and also reveal a putative proteinase cleavage site. This deduced amino acid sequence has no predicted transmembrane domain (Frishman and Argos, 1996). We ruled out the existence of a glycosyl residue using the DIG Glycan Double Labeling Kit (Boehringer Mannheim, Mann-

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                    ▼
MVRFVSALLL AAAGVLASTN AAVINHDAVP VWPQPEPADA TQALAVRFKP   50
CCCHHHHHHH HHCCCCCCCE EEEEECCCCC CCCCCCCCHH HHHHHHHCC

QLDVVNGCQP YPAVDPQGNT SGGLKPSGSQ AAACRDMSKA QVYSRSGTYN  100
CCEEEECCCC CCCCCCCCCC CCCCCCCCCH HHHHHHCCCC CCCCCCCCCC

GYYAIMYSWY MPKDSPSTGI GHRHDWENVV VWLDNAASAN IVALSASAHS  150
EEEEEEEECC CCCCCCCCCC CCCCCCEEE EEECCCCHHH HHHHHCCCCC

GYKKSFPADK SYLDGITAKI SYKSTWPLDH ELGFTTSAGK QQPLIQWEQM  200
CCCCCCCCCC CCCCCEEEE EEECCCCCCC CCCCCCCCCC CCCCHHHHHH

TQAARDALES TDFGNANVPF KSNFQDKLVK AFFQ                  234
HHHHHHHHHH CCCCCCCCCC CCCHHHHHHH HCCC
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Figure 1. Amino acid sequence of $PaNie_{234}$ in the one letter code. A predicted secondary structure is given in the lower line (Frishman and Argos, 1996). H, Alpha helix; C, random coiled; E, extended strands; the arrowhead points to a proteinase cleavage site at the end of the eukaryotic secretory signal sequence (Nielsen et al., 1997). Underlined, Microsequenced oligopeptides; shadowed box, amino acid sequence used for degenerated primer (pep2rev) design.

heim, Germany; data not shown). In Table I, the sequence of $PaNie_{234}$ was compared with sequences in the National Center for Biotechnology Information database. Similar proteins with 70% to 84% similarity were found with *F. oxysporum* cv *erythroxyli* (accession no. AAC97382), *P. sojae* (accession no. AAK01636), *P. parasitica* (accession no. AAK19753), *P. infestans* (accession no. AAK25828), and the eubacterium *B. halodurans* (accession no. BAB04114). This may represent a novel family of elicitor proteins.

Appropriate oligopeptides were synthesized as antigens to raise an antiserum against the protein elicitor. In Figure 2, the heterologous expression and purification of $PaNie_{213}$ is followed by SDS-PAGE and western blotting. The antiserum detects a single band after the final purification step.

The heterologous expression of C-terminal His-tagged $PaNie_{213}$ in *E. coli* and purification using nickel-nitrilotriacetic acid agarose (Ni-NTA) resulted in a 25-kD protein that is the functional and mature part of the total $PaNie_{234}$ secreted by the oomycete *P. aphanidermatum* into the culture medium. Purification leads to a single protein band with a molecular mass of 25 kD.

Genomic Organization

DNA gel blotting was performed to provide information on genomic organization (Fig. 3). Genomic DNA of *P. aphanidermatum* was digested with various combinations of restriction enzymes and probed with a cDNA clone containing the total ORF from $PaNie_{213}$. The restriction enzymes used for digestions are not blocked by methylation sites on the DNA. Two strong bands were always present in all four lanes regardless of the enzyme combinations used. This is an indication that a multicopy gene is present, provided that no intron with a cleavage site exists. The absence of introns was demonstrated by comparing the product length after PCR on genomic DNA and cDNA. The resulting two molecules had identical lengths (data not shown).

Table I. *Comparison of deduced amino acid sequences of $PaNie_{234}$ with other necrosis-inducing elicitor proteins*

Elicitor Proteins	Similarity in % to $PaNie_{234}$
P. aphanidermatum (25-kD protein elicitor)	100.0
Phytophthora infestans (necrosis-inducing protein NPP1)	84.2
P. parasitica (necrosis-inducing protein NPP1)	83.8
P. sojae (necrosis-inducing peptide)	83.1
Bacillus halodurans[a] (necrosis- and ethylene-inducing protein)	78.2
F. oxysporum f. sp. *erythroxyli* (necrosis- and ethylene-inducing peptide)	70.4

[a] Translated open reading frame (ORF) only; no activity assays were performed.

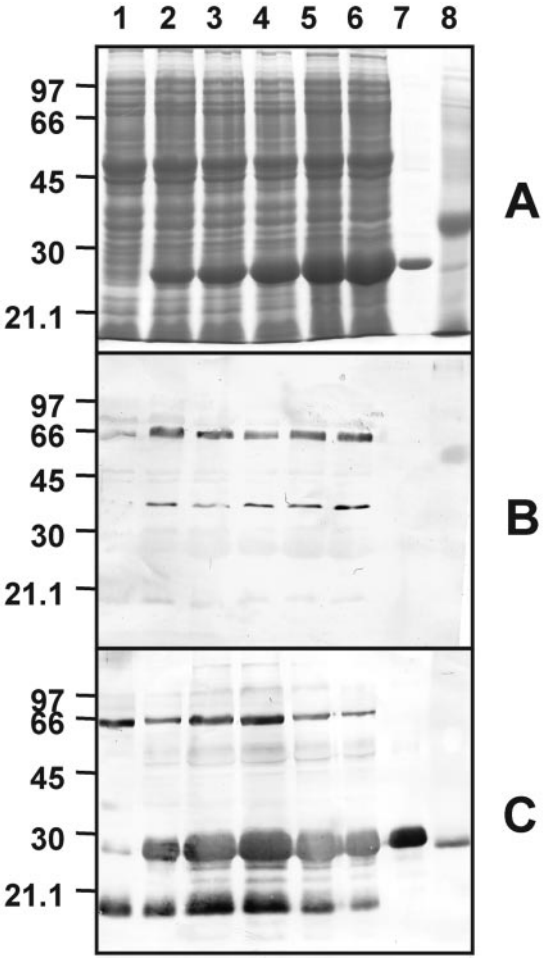

Figure 2. SDS-PAGE of fractions from purification of heterologously expressed C-terminal His-tagged $PaNie_{213}$ in *E. coli* in comparison to the crude elicitor. A, Coomassie Blue-stained SDS-PAGE. Lane 1, Control; lanes 2 through 6, 1 to 5 h after induction with isopropyl-thio-β-galactoside; lane 7, purified $PaNie_{213}$; lane 8, crude elicitor. B, Hybridization with pre-immuneserum (1:500); C, hybridization with $PaNie_{213}$ antiserum (1:20,000).

Defense-Related Responses of Carrot Cell Cultures

To show that $PaNie_{213}$, a single pure elicitor protein, is sufficient to trigger multiple defense reactions, carrot cell cultures were treated with $PaNie_{213}$. As can be seen in Figure 4A, the viability decreased rapidly within the first 30 min after elicitor application. This time course is nearly identical with that observed with a $(NH_4)_2SO_4$-precipitated crude elicitor described previously (Koch et al., 1998). $PaNie_{213}$ was active at very low protein concentrations, inducing marked viability losses (IC_{50} = 50 nM) and the accumulation of large amounts of 4-HBA (data not shown). However, to obtain maximum effects in the experiments depicted in Figure 4, final concentrations of 500 nM were applied.

The accumulation of 4-HBA was determined (Fig. 4B). As already demonstrated for the crude elicitor preparation (Schnitzler and Seitz, 1989; Koch et al.,

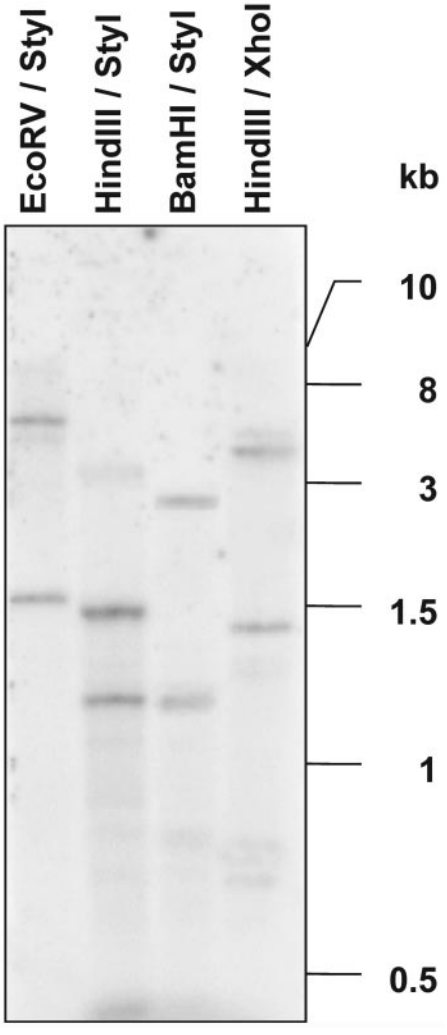

Figure 3. Southern-blot analysis of genomic DNA from *P. aphanidermatum*. Each of 30 μg of DNA was digested with the enzyme combination indicated and then separated on a 0.8% (w/v) agarose gel. The blot was probed with ^{32}P-labeled PaNie$_{213}$ ORF cDNA. The blots were washed with 0.1% (w/v) SDS and 0.2× SSC at 65°C.

1998), the pure PaNie$_{213}$ also enhanced the accumulation of 4-HBA. Sixteen hours after the application of PaNie$_{213}$ to carrot cells, 4-HBA was accumulated rapidly. As already shown previously, this process is preceded by an increase of PAL-mRNA (Koch et al., 1998). As previously shown, the 4-HBA could only be released from the carrot cell wall by alkaline hydrolysis indicating a covalent linkage of this compound to wall constituents (Schnitzler and Seitz, 1989). These results demonstrate that the pure elicitor and the crude preparation induce the same responses.

PaNie$_{213}$-Induced Chromatin Fragmentation

The fragmentation of nDNA is one of the best established criterion for confirming an elicitor-dependent programmed cell death during the hypersensitive response (Peitsch et al., 1993; Ryerson and Heath, 1996). Therefore, we isolated protoplasts from cultured carrot cells and treated them with PaNie$_{213}$. These protoplasts responded to the pure elicitor protein PaNie$_{213}$ in the same way that they did to the crude preparation, as described previously. In Figure 5, DNA laddering after treatment with PaNie$_{213}$ is shown. The fragmentation of chromatin to multiples of 180 bp is already visible 48 h after the onset of elicitation and continues during the next 48 h. As shown by Koch et al. (1998), this active chromatin fragmentation is dependent on the import of external Ca^{2+}, as was shown in a previous communication. G-proteins are involved in this active process as is shown by the fact that mastoparan and Mas-7 incubation mimic the elicitor effect (Koch et al., 1998).

PaNie$_{213}$ Treatment of Intact Plants

To study the effects of the pure elicitor protein on intact plant organs of genetically well defined sys-

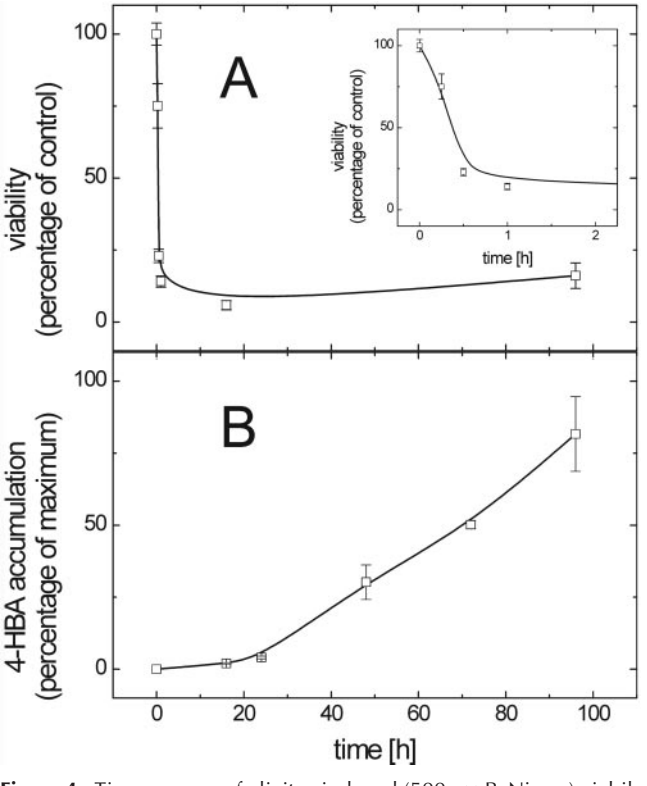

Figure 4. Time courses of elicitor-induced (500 nM PaNie$_{213}$) viability changes and accumulation of 4-HBA in suspension-cultured carrot cells. A, The viability was monitored using the tetrazolium-assay and expressed as percentage of control (untreated cells). The inset shows the data for the first 2 h with an enlarged abscissa. B, Accumulation of cell wall-bound 4-HBA. Phenolic acids were released by saponification with 1 M NaOH from crude cell wall preparations and separated by HPLC (ODS Hypersil with a linear gradient of water: acetic acid (95:5; v/v) and methanol ranging from 10% to 50% (v/v) methanol over 30 min. 4-HBA was detected at 260 nm. The 4-HBA concentration is expressed as percentage of the highest accumulation of 4-HBA (100% = 10.6 μg mg^{-1} cell wall carbohydrates). Each data point represents the average of triplicates. Error bars represent SD.

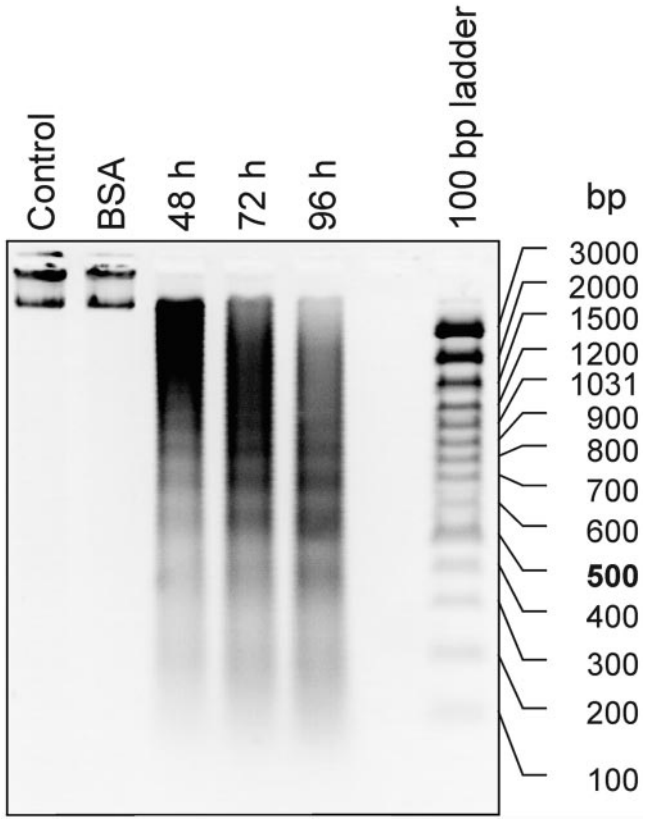

Figure 5. Elicitor–induced fragmentation of nDNA in protoplasts derived from suspension-cultured cell cultures of carrot. The DNA was extracted from protoplast at the indicated times and equal amounts were separated on a 1.2% (w/v) agarose gel and stained with ethidium bromide. The concentration of $PaNie_{213}$ was 100 nM. In the controls, water or equal bovine serum albumin (BSA) concentrations were applied.

tems, we treated leaves of Arabidopsis (Columbia-0 [Col-0], wild type [WT]), tobacco (W38; WT), and maize. In the first series (Fig. 6A), 5 μL of a $PaNie_{213}$ solution (10 μM) was infiltrated into the intercellular space of Arabidopsis leaves through stomatal pores. This treatment resulted in the formation of clearly defined necrotic areas at the infiltration site (see also Fig. 7). The necrotic area is bordered by a ring of callose deposition on mesophyll cell walls (Fig. 6A). At lower elicitor concentrations (1 μM), no necrotic effects were observed; in contrast to the reaction seen at higher concentrations, callose is present only in a diffuse pattern (Fig. 6B). Equimolar protein concentrations of bovine serum albumin had no effect (Fig. 6C). The same is true for incubation with the corresponding buffer (data not shown).

In an additional series of experiments, the reactions of different plant species to $PaNie_{213}$ were compared. Infiltration of a 10-μM elicitor solution into Arabidopsis leaves resulted in strong necrotic effects (Fig. 7), as did infiltration into tobacco, which responded more rapidly, already forming necrotic lesions after 6 h (data not shown). Tomato showed also necrotic lesions. Infiltration of maize failed to lead to a reaction even 72 h after the onset of elicitation (Fig. 7). Other monocotyledons like *Avena sativa* and *T. zebrina* (Bosse) also show no response to elicitation.

DISCUSSION

Here, we report a novel protein elicitor from the culture medium of the pathogenic oomycete *P. aphanidermatum* that triggers an array of defense responses in carrot cell cultures and in intact plants of Arabidopsis and tobacco.

The protein was purified by preparative SDS-PAGE (Koch et al., 1998). Sequence comparison of proteins in the National Center for Biotechnology Information database yielded similar sequences for various phytopathogenic fungi and the eubacterium *B. halodurans* (only the sequence was published but no physiological function of the deduced protein was described; see Table I) indicating a conserved gene family. Such a gene must have an indispensable function in the pathogen; otherwise, it would have been eliminated during evolution. Analysis of the genomic organization of *P. aphanidermatum* using Southern blot presents strong evidence for the existence of at least two copies of the PaNie gene.

To find the smallest peptide with elicitor activity, N-terminal truncated cDNAs missing 63 or 106 amino acids were expressed in *E. coli*. Both products did not show any elicitor activity in carrot cell cultures with regard to 4-HBA accumulation and loss of viability (data not shown). Because all attempts to produce C-terminal truncated peptides proved to be unsuccessful and therefore no physiological assays concerning elicitor activity of these truncated peptides could be performed. These experiments revealed that the entire $PaNie_{213}$ is necessary for elicitor activity, suggesting that the intact secondary structure must be preserved for its activity. This is in contrast to the Pep-13 from *P. sojae* in which the elicitor active peptide was only 13 amino acides (Hahlbrock et al., 1995).

Rapid loss of viability and the induction of 4-HBA accumulation clearly demonstrates that a single protein elicitor ($PaNie_{213}$) is sufficient to trigger both defense responses, namely programmed cell death and phytoalexin synthesis in suspension-cultured carrot cells.

This apparent contradiction between viability loss and the concomitant induction of de novo synthesis of 4-HBA suggests that the remaining viable cells have greatly elevated 4-HBA biosynthetic activity. In an earlier paper, we demonstrated that the transcription of PAL-mRNA proceeded even after cell death was initiated (Koch et al., 1998). Dying cells presumably can still be active 4-HBA producers. It has been shown for *Lactuca sativa* after infection with *Bremia lactucae* that dying cells are still able to synthesize

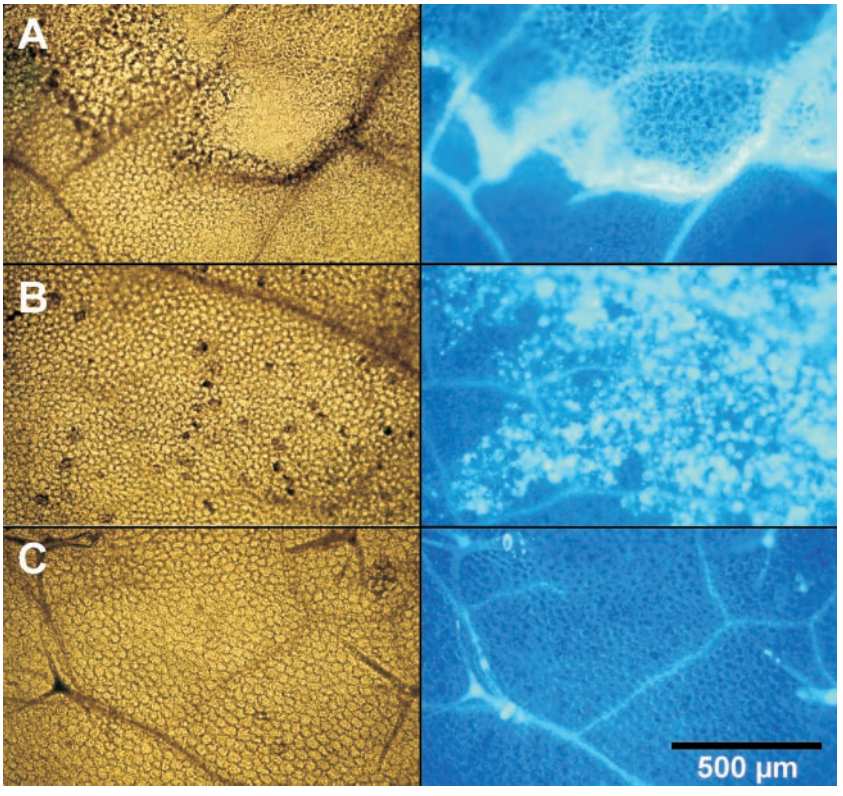

Figure 6. Elicitor-induced callose deposition in leaves of Arabidopsis (Col-0, WT). The 3-week-old leaves were infiltrated using a 1-mL syringe without a needle through stomatal pores with solutions of $PaNie_{213}$ (5 μL of a 10-μM or 1-μM solution of $PaNie_{213}$). As a control, equal concentration of BSA was applied. After an incubation period of 24 h, the chlorophyll was removed and the bleached leaves were stained with aniline blue and photographed in bright field (left) and under UV light (right). A, Ten micromolar $PaNie_{213}$; B, 1 μM $PaNie_{213}$; C, BSA control.

defense-related compounds (Bennet et al., 1996). A second reasonable explanation would be that the tetrazolium assay as an indicator for initiated programmed cell death measures only the impairment of mitochondrial activity (Berridge and Tan, 1993).

A typical feature of programmed cell death is the digestion of the chromatin to nucleosomal fragments with multiples of 180 bp (Ryerson and Heath, 1996). Again, with the purified elicitor $PaNie_{213}$ we obtained a DNA laddering that was also observed with the crude elicitor preparation (Koch et al., 1998).

In addition to the elicitor-triggered defense response of the carrot cell culture, responses of intact plants to this novel elicitor protein were examined. As previously shown, carrot leaves respond to injections of the crude elicitor preparation by senescence at the leaf tips and by the accumulation of 4-HBA and other wall-bound phenols (Koch et al., 1998).

To broaden our understanding of the elicitor action $PaNie_{213}$ was applied to genetically well-defined systems like Arabidopsis and tobacco. Infiltration of Arabidopsis leaves resulted in necrotic lesions that are surrounded by a ring of cells with callose deposits at their cell walls. At the border of the necrosis, a higher concentration of brownish material was present that could be due to a reinforcement of these cell walls with wall-bound phenols. The callose deposits are thought to form a barrier between necrotic and healthy tissue (Vleeshouwers et al., 2000). It has been shown that 1,3-β-glucan synthase is merely activated by calcium and no de novo synthesis is necessary (for review, see Kauss, 1987).

These callose deposits appeared at a threshold concentration of 8 to 10 μM. At lower elicitor concentrations, the callose was distributed in a diffuse manner (see Fig. 6B). Similar to carrot cells, Arabidopsis also seems to react with multiple responses. Preliminary infiltration experiments with elicited Arabidopsis leaves showed a dose-dependent increase in camalexin accumulation measured according to Tsuji et al. (1992) and Thomma et al. (1999) by fluorescence and UV light detection after thin-layer chromatography and HPLC separation (M. Malcherowitz, H.U. Seitz, unpublished data).

Tobacco leaves infiltrated with $PaNie_{213}$ also showed necroses. However, maize and other monocotyledons (see "Materials and Methods") did not respond to this treatment by forming necroses. This is an indication that dicotyledonous and monocotyledonous plants respond differently to protein elicitors, demonstrated here for $PaNie_{213}$. This has raised the question whether monocotyledonous plants are unable to perceive the elicitor signal, at least as expressed by rapid cell death. Jennings et al. (2000) reported similar behavior after spraying various weed plants with a protein isolated from culture filtrates of *F. oxysporum*.

In summary, we present strong evidence here that a single pure elicitor protein is sufficient to trigger multiple defense reactions in the cell culture system

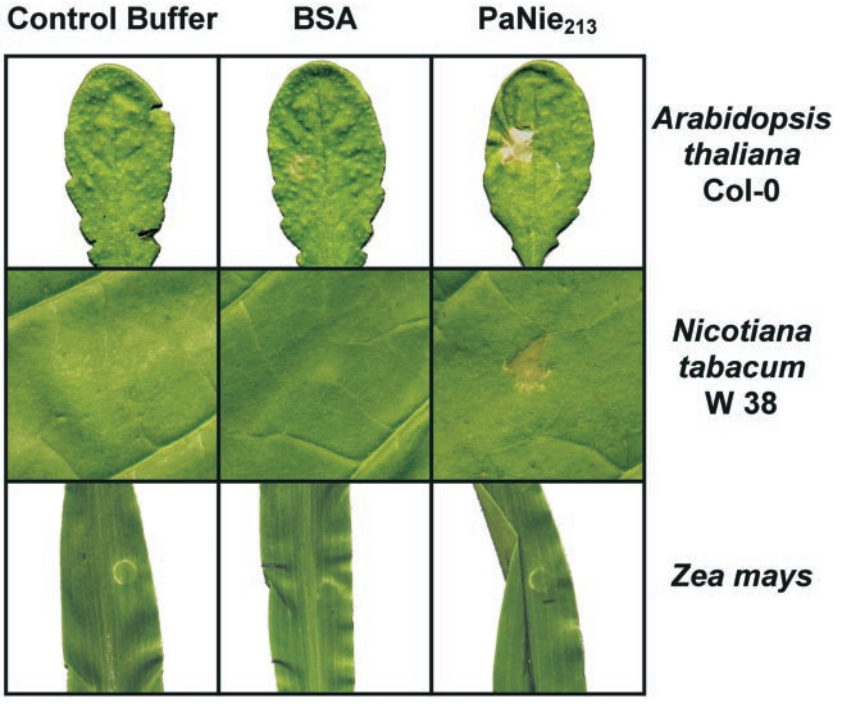

Figure 7. Comparison of effects of $PaNie_{213}$ (10 μM) infiltrated (5 μL) into leaves of Arabidopsis (Col-0; 3-week-old plants), tobacco (W38, WT, 4-month-old plants), and maize (1-week-old plants). Buffer and BSA were used as controls. For infiltration, see Figure 5. The photographs were taken 48 h after elicitation.

of carrot, Arabidopsis, and tobacco leaves. This study provides the basis for a better understanding of the recognition process and the causal connection with downstream signaling toward different defense reac-tions. The Arabidopsis system offers the possibility of taking a genetic approach to isolate a receptor protein and link it to the downstream signal pathway and the de novo synthesis of defense compounds.

MATERIALS AND METHODS

Culture Conditions for Cell Cultures of Carrot (*Daucus carota*) and for *Pythium aphanidermatum* (Edson) Fitzp.

Cell suspension cultures of carrot were cultivated as previously described (Noé et al., 1980). *P. aphanidermatum* was propagated in liquid media as previously described (Schnitzler and Seitz, 1989).

Treatment of Carrot Protoplasts with $PaNie_{213}$ and Isolation of Genomic DNA (DNA Laddering)

The protoplasts were isolated from carrot cell cultures with a protocol described previously (Koch et al., 1998).

Purified protoplasts were counted in a Fuchs-Rosenthal hematocytometer. The suspension was brought to a cell titer of 2×10^5 protoplasts mL^{-1}. The samples were incubated in aliquots of 10 mL in petri dishes at 26°C. The elicitor was applied directly after protoplast isolation.

After the incubation with $PaNie_{213}$, the protoplasts were collected at 100*g* for 5 min and the supernatant was discarded. Lysis buffer (500 μL containing 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA, 2% [w/v] SDS, and 0.1% [v/v] 2-mercaptoethanol) was added and the mixture was incubated for 10 min at 65°C. After extraction in phenol:chloroform:isoamyl-alcohol (25:24:1, v/v), the aqueous phase was precipitated with ethanol (0.1 volume 3 M sodium acetate and 2.5 volumes of ethanol). The DNA was dissolved in Tris-EDTA buffer to a final concentration of 0.5 $\mu g \times \mu L^{-1}$. Equal amounts of DNA were separated on 1.2% (w/v) agarose gels by electrophoresis and stained with ethidium bromide.

Screening of cDNA and Microsequencing

Microsequencing of a purified protein from *P. aphanidermatum* with elicitor activity (Koch et al., 1998) yielded sequences of oligopeptides (peptide1, N′-AVINXDAVPVX PQPEPADXT-C′; and peptide2, N′- LGFTTSAGKQQPL IQWEQMTQAARD-C′) that were used to design a degenerated primer (degenerated primer derived from peptide2; pep2rev 5′-ATHCARTGGGARCARARGAC-3′). PCR (primer: pep2rev and T7 5′-GTAATACGACTCACTATA GGGC-3′) with cDNA from *P. aphanidermatum* as a template produced a 260-bp fragment which contained parts of the oligopeptide coding region. A second PCR on cDNA from *P. aphanidermatum* with pep2rev and a forward primer binds in front of the poly A tail (5′-GTCGACAGCACTTTACTGG-3′) led to a fragment that was used as an [α-^{32}P]dCTP probe to screen a cDNA library of *P. aphanidermatum*. The cDNA library was established with the ZAP-cDNA Synthesis Kit (Stratagene, Heidelberg). A clone lacking the 5′-end was completed with 5′-Race (5′-Race System for Rapid Amplification of cDNA Ends, Version 2.0; GibcoBRL Life Technologies, Karlsruhe, Germany).

Heterologous Expression and Purification of the His-Tagged $PaNie_{213}$

The pQE60 expression vector (Qiagen, Hilden, Germany) containing the ORF coding for $PaNie_{213}$ was used for the heterologous expression of the elicitor protein with a C-terminal His tag in *Escherichia coli* (strain M15). An artificial translation initiation site was inserted using PCR-based mutagenesis, starting after the putative eukaryotic secretory signal sequence. This protein, coding for a protein of 213 amino acids, was designated $PaNie_{213}$. For the PCR, we used the reverse primer 5′-GAGACCATGGCC GTGATCAACCATG-3′ and the forward primer 5′-CTC TGGATCCCTGGAAAAACGCCTTCACGAG-3′.

The following PCR conditions were chosen: 5 min at 94°C, cycling denaturation for 20 s at 94°C, annealing for 20 s at 56°C, and elongation for 90 s at 70°C using *Pyrococcus furiosus* DNA polymerase (Stratagene, La Jolla, CA).

Preparation and purification of $PaNie_{213}$ under denaturing conditions was performed using the batch purification protocol for QIAexpressionist Ni-NTA technology (Qiagen). Transformed *E. coli* cells from a 1-L batch were induced with isopropylthio-β-galactoside (1 mM) for 4 h and then disrupted by ultrasonication (Micro Tip Sonifier B-12, Branson, Danbury, CT) in 20 mL of buffer B (Qiagen; 8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris-HCl, pH 8.0). The lysate was added to 10 mL of Ni-NTA and incubated for 5 h at 4°C with gentle agitation. The matrix was washed three times stepwise with buffer C (urea; 0.1 M NaH_2PO_4, and 0.01 M Tris-HCl, pH 6.3) containing 20 mM imidazole with decreasing concentrations of urea (first step, 2 M; second step, 0.5 M; and third step, 0.1 M urea). Elution occurred following a final wash step (50 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 8.0, 300 mM NaCl, and 300 mM imidazole) with 20 mL of 6 M guanidine-HCl and 0.2 M acetic acid. The elicitor protein $PaNie_{213}$ was dialyzed against water. This protein was used for the elicitation of cell cultures and for infiltration into leaves.

Isolation of Genomic DNA of *P. aphanidermatum* and Southern-Blot Analysis

Genomic DNA was isolated according to Dellaporta et al. (1983) from mycelium (10 g fresh weight) frozen in liquid nitrogen and ground with mortar and pestle. DNA samples (30 μg) were digested with *Eco*R V and *Nde*I, *Hin*dIII and *Sty*I, *Bam*HI and *Sty*I, *Hin*dIII and *Xho*I. The products were fractionated on 0.8% (w/v) agarose gel and then transferred onto a Hybond-N(+) membrane (Amersham Pharmacia Biotech, Freiburg, Germany). The $PaNie_{213}$ ORF cDNA was labeled with [α-^{32}P]dCTP and used as a probe. Hybridization was performed at 55°C in 0.33 M NaH_2PO_4/Na_2HPO_4, pH 7.0, 1 mM EDTA, and 7% (w/v) SDS for 16 h. The membranes were washed for at least 20 min with 0.1% (w/v) SDS, 0.2× SSC (20× SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.5) at 65°C and then subjected to autoradiography.

Preparation of the Antiserum and Immunoblotting

A synthetic oligopeptide ($PaNIE_{201-214}$) was used for immunisation of a rabbit. The pre-immunesera and antisera were provided by BioTrend (Köln, Germany) and used for immunoblotting in a dilution of 1:20,000.

Determination of 4-HBA

The 4-HBA content of carrot cell walls was determined as previously described (Schnitzler and Seitz, 1989). Vanillic acid was used as an internal standard for the quantification of the 4-HBA concentration.

Tetrazolium Assay

The loss of viability of suspended carrot cells was measured at the indicated times after elicitor application. Relative viability was calculated as $A_{555\text{-}655} * mg^{-1}$ fresh weight using the tetrazolium assay according to Koch et al. (1998).

In Situ Infiltration of Leaves with $PaNie_{213}$

Plants were grown under constant greenhouse conditions (60% relative humidity, long day: 16 h at 22°C and 8 h at 18°C). Leaves of 3-week-old Arabidopsis plants (Col-0), 4-month-old tobacco (*Nicotiana tabacum*) plants (W38), tomato (*Lycopersicon esculentum* Mill.) plants, 1-week-old maize (*Zea mays*) plants, *Tradescantia zebrina* (Bosse) plants, and oat (*Avena sativa*) plants were infiltrated in situ with constant volumes (5 μL) of $PaNie_{213}$ solutions. BSA and the corresponding buffer were used as controls. The solutions were injected into the intercellular space through the stomata pore using a 1-mL syringe without hypodermic needle. Leaves were harvested 24 h after infiltration to visualize callose deposition and after 48 h to monitor necrotic effects.

Analysis of Callose Deposition

To visualize callose deposition, seedlings were treated and stained as described by Gómez-Gómez et al. (1999) according to Currier and Strugger (1956). The tissue was fixed overnight in 1% (v/v) glutaraldehyde, 5 mM citric acid, 90 mM Na_2HPO_4 (pH 7.4). The chlorophyll was removed and the specimens were dehydrated in ethanol. The transparent leaves were transferred to 50% (v/v) ethanol and afterward equilibrated in 67 mM K_2HPO_4 (pH 12.0) and then stained for 1 h at room temperature in 0.1% (w/v) aniline blue dissolved in 67 mM K_2HPO_4 (pH 12.0). The stained leaves were transferred to a microscopic slide in 70% (v/v) glycerol and 30% (v/v) staining solution and examined under UV epifluorescence (Zeiss, Axioplan, Oberkochen, Germany). The callose deposits were visible as pale-blue fluorescence.

Received April 12, 2001; returned for revision June 18, 2001; accepted July 20, 2001.

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MPMI Vol. 15, No. 6, 2002, pp. 608–616. Publication no. M-2002-0429-02R. © 2002 The American Phytopathological Society

NHL25 and *NHL3*, Two *NDR1/HIN1-Like* Genes in *Arabidopsis thaliana* with Potential Role(s) in Plant Defense

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Submitted 17 December 2001. Accepted 5 March 2002.

The *Arabidopsis* genome contains 28 genes with sequence homology to the *Arabidopsis NDR1* gene and the tobacco *HIN1* gene. Expression analysis of eight of these genes identified two (*NHL25* and *NHL3* for *NDR1/HIN1*-like) that show pathogen-dependent mRNA accumulation. Transcripts did not accumulate during infection with virulent *Pseudomonas syringae* pv. *tomato* DC3000 but did accumulate specifically when the bacteria carried any of the four avirulence genes *avrRpm1*, *avrRpt2*, *avrB*, or *avrRps4*. Furthermore, expression of *avrRpt2* in plants containing the corresponding resistance gene, *RPS2*, was sufficient to induce transcript accumulation. However, during infection with an avirulent oomycete, *Peronospora parasitica* isolate Cala-2, only *NHL25* expression was reproducibly induced. Salicylic acid (SA) treatment can induce expression of *NHL25* and *NHL3*. Studies performed on *nahG* plants showed that, during interaction with avirulent bacteria, only the expression of *NHL25* but not that of *NHL3* was affected. This suggests involvement of separate SA-dependent and SA-independent pathways, respectively, in the transcriptional activation of these genes. Bacteria-induced gene expression was not abolished in ethylene- (*etr1-3* and *ein2-1*) and jasmonate- (*coi1-1*) insensitive mutants or in mutants impaired in disease resistance (*ndr1-1* and *pad4-1*). Interestingly, *NHL3* transcripts accumulated after infiltration with the avirulent *hrcC* mutant of *Pseudomonas syringae* pv. *tomato* DC3000 and nonhost bacteria but not with the virulent *Pseudomonas syringae* pv. *tomato* DC3000, suggesting that virulent bacteria may suppress *NHL3* expression during pathogenesis. Hence, the expression patterns and sequence homology to *NDR1* and *HIN1* suggest one or more potential roles for these genes in plant resistance.

Keywords: gene-for-gene, wounding.

Plants are constantly exposed to potential pathogens and can resist most attacks by activating defense mechanisms. The first crucial step to mounting defense reactions is the recognition of the pathogen (Dangl and Jones 2001). This relies on sophisticated sensing mechanisms for signal molecules that could be pathogen-derived or generated during the infection process (Bent 1996). Defense reactions initiated are usually multicomponent and complex but often occur as a rapid localized cell death at the site of infection (referred to as hypersensitive response [HR]) to contain the pathogen, as well as a systemic acquired resistance (SAR) throughout the plant (Grant and Mansfield 1999).

The "gene-for-gene" hypothesis, demonstrated to control race- and cultivar-specific plant-pathogen interaction, relies on the presence or the absence of an avirulence (*avr*) gene in the pathogen and a corresponding resistance (*R*) gene in the plant (Flor 1971). A majority of *R* genes isolated encode proteins with putative nucleotide binding sites (NBS) and contain leucine-rich repeats (LRR). These NBS-LRR resistance proteins harbor in their amino terminus either a leucine zipper (LZ) or a so-called TIR domain that has homology to the Drosophila Toll and human interleukin-1 receptors (Dangl and Jones 2001).

Genetic analysis in Arabidopsis has been instrumental in unraveling the complex signal transduction in disease resistance, and simplified signaling models have been proposed (Glazebrook 2001). Various signaling pathways can lead to expression of defense-related genes after pathogen attack, and mutant analyses suggest these pathways are required for successful resistance. One pathway is salicylate-dependent and requires genes like *EDS1*, *PAD 4*, and *NPR1*. Two other pathways are dependent on jasmonate and ethylene: one pathway does not require *NPR1* and is defined by mutants like *coi1*, *ein2*, and *etr1*, and one functions during interaction with nonpathogenic rhizobacteria and requires *NPR1*. These pathways are not strictly independent of each other, and a complex network may be activated in response to a particular pathogen (Feys and Parker 2000).

Three different networks of *R* gene-mediated signaling have also been proposed through the analysis of Arabidopsis mutants (Feys and Parker 2000). *R* genes known to require *EDS1* and *PAD4* for mediating resistance belong to the TIR-NBS-LRR class, while *R* genes known to require *NDR1* and *PBS2* belong to the LZ-NBS-LRR class (Aarts et al. 1998; Glazebrook et al. 1997; Warren et al. 1999). There are, however, exceptions from this general classification indicating existence of one or more other signaling networks. The *RPP8* gene does not require *EDS1* or *NDR1*, while *EDS1* and *NDR1* in combination appear to mediate full *RPP7* function (McDowell et al. 2000). Like *RPP8*, *RPP13* encodes an LZ-NBS-LRR protein, and *RPP13*-mediated resistance functions independently of *NDR1*, *PBS2*, *EDS1*, and *PAD4* (Bittner-Eddy and Beynon 2001). Another

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exception is *RPW8*, a broad-spectrum *R* gene with low similarity to the NBS-LRR genes that requires salicylate and *EDS1* (Xiao et al. 2001).

The EDS1 and PAD4 proteins share sequence similarity with lipases, although enzyme activity has not been shown (Falk et al. 1999; Jirage et al. 1999). The sequence of NDR1 does not provide any clue to possible biochemical function, but it is predicted to be a membrane protein (Century et al. 1997). Furthermore, NDR1 has limited sequence similarity to the tobacco HIN1 protein, and its transcript also accumulates upon pathogen attack (Century et al. 1997). The *HIN1* gene is activated by the bacterial elicitor harpin and bacteria with a functional *hrp* gene cluster (Gopalan et al. 1996). A partial sequence of the tobacco gene, *NG2*, identified as an inducer of HR-like cell death (Karrer et al. 1998) also shares sequence similarity with *HIN1*.

The *Arabidopsis* genome contains 28 genes that display similarities to *NDR1* and *HIN1* (The Arabidopsis Genome Initiative 2000). These have been tentatively designated as *NHL1-28* (*NDR1/HIN1*-like) and grouped into several subclasses on the basis of sequence similarities (Dörmann et al. 2000). Like NDR1, one to two putative transmembrane domains are predicted for these NHL proteins although, in some cases, these domains also coincide with predicted cleavage sites of signal peptides. However, similarities to NDR1/HIN1 are restricted to short amino acid stretches. This suggests that the NHLs might represent protein families sharing common structural motifs but not necessarily similar biochemical properties or signaling roles. We postulate that those members having a role in pathogen response may share similar expression patterns with *NDR1* and *HIN1*. Therefore, we have screened some of these *NHL* genes for their capacity to respond to bacterial infection. Two out of eight genes studied showed transcript accumulation specifically during an incompatible interaction, and detailed expression studies suggested that they potentially define two new response pathways to pathogen infection.

RESULTS

Differential expression of a subset of the *NHL* genes.

To screen for pathogen-responsive members of the *NHL* gene family, we infiltrated *Arabidopsis* Columbia leaves with phytopathogenic bacteria and analyzed gene expression by RNA gel blot and reverse transcription-polymerase chain reaction (RT-PCR). Only a subset of the *NHL* sequences were available in the public databases when this project was initiated; thus, only eight of these genes (*NHL3*, *NHL9*, *NHL19*, *NHL23*, *NHL24*, *NHL25*, *NHL26*, and *NHL27*) (Dörmann et al. 2000) have been investigated. The use of RT-PCR allowed us to distinguish between related cross-hybridizing sequences as well as increasing the detection of low expression levels. In one case, RT-PCR and DNA sequencing allowed the detection of an unpredicted 80-bp intron in *NHL25* (accession number

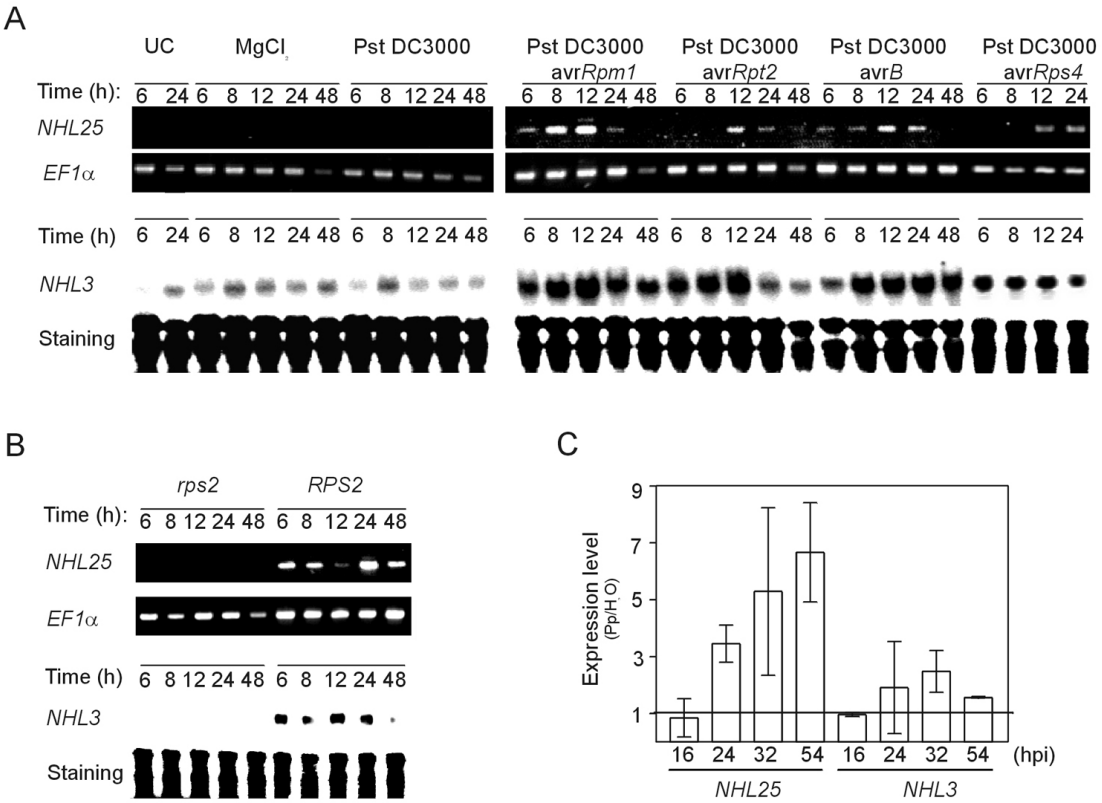

Fig. 1. Pathogen-induced mRNA accumulation of two *NHL* genes. **A,** *Pseudomonas syringae* pv. *tomato* DC3000 infiltration. Six-week-old plants were infiltrated with magnesium chloride ($MgCl_2$), *P. syringae pv. tomato* DC3000 (*Pst* DC3000), and *Pst* DC3000 carrying the indicated avirulence genes. Leaf tissues (including two untreated controls [UC]) were collected at the indicated time points and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (*NHL25*) or RNA gel blots (*NHL3*). Equal loading of RNA was checked by amplifying a constitutively expressed gene (translation elongation factor 1 alpha, *EF1*α) or by methylene-blue staining of the ribosomal bands, respectively. Similar results were obtained in two other independent experiments. **B,** Transgenic plants expressing *avrRpt2* under the control of an estradiol-inducible promoter were sprayed with 10 μM estradiol to express *avrRpt2* in plants containing a mutated (*rps2*) or a functional *RPS2* gene (*RPS2*). RNA was extracted and analyzed by RT-PCR or RNA gel blot as described above. **C,** *Peronospora parasitica* infection. Arabidopsis plants were sprayed with spores of *P. parasitica* (*Pp*) isolate Cala-2 resuspended in water. Leaf tissues were collected at the indicated time points and were analyzed by RT-PCR. The expression levels of *NHL25* and *NHL3* are shown as the ratio of transcript levels in *Pp*-inoculated to water-treated tissues (normalized to the 16 h time point). Bars represent the means and standard errors of three independent experiments.

NM123055). In all other cases, the sizes of the RT-PCR amplified bands corresponded well to the predicted values, confirming a lack of introns in the other seven genes studied.

Increased transcript levels of two genes (*NHL3* and *NHL25*, accession numbers NM120715 and NM123055) were detected upon infiltration with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) strains that carried an avirulence gene (*avrRpm1*, *avrRpt2*, *avrB*, or *avrRps4*), all of which initiate incompatible interactions (Fig. 1A). Depending on the type of avirulence gene involved, transcript accumulation began about 6 to 12 h after infiltration and decreased after 24 to 48 h (Fig. 1A). While expression of both genes is induced, the overall levels are quite different. *NHL25* transcripts accumulate to much lower levels than *NHL3* transcripts and are detectable only with RT-PCR. Reprobing the blots (data not shown) revealed very similar expression patterns of *NDR1* (Century et al. 1997). However, gene expression was not induced by infiltration with a 10 μM solution of the *HrpZ*-encoded harpin over a period of 24 h (data not shown). Thus, the expression pattern of these two genes resembles that of *NDR1* but not of *HIN1*. The virulent *Pst* DC3000 strain did not lead to increased expression of either of the two genes within the time period tested (Fig. 1A). Interestingly, the timing of *NHL25* and *NHL3* transcript accumulation preceded HR development and the expression of *PR1* transcripts, which were first detected 12 to 24 h postinfection (not shown).

Since increased expression of both *NHL25* and *NHL3* appears to be confined to incompatible interactions, it is likely that this relies on the recognition of the avirulence gene products. Indeed, transgenic plants containing the avirulence gene *avrRpt2* under the control of an estradiol-inducible promoter (Tornero et al. 2002) expressed both *NHL25* and *NHL3* when treated with estradiol (Fig. 1B). This induced expression is dependent on a functional *RPS2* gene for recognition of *avrRpt2* gene product, as is evident from the lack of *NHL25* and *NHL3* expression in *rps2* mutant plants after estradiol treatment. Hence, expression of an avirulence gene in plant cells containing the corresponding *R* gene is sufficient to activate one or more signal cascades leading to increased *NHL25* and *NHL3* expression.

In order to verify if the enhanced gene expression was restricted to bacterial pathogens, we analyzed the incompatible interaction between *Arabidopsis thaliana* ecotype Col-0 and the oomycete pathogen *Peronospora parasitica* isolate Cala-2. Interestingly, RT-PCR analysis revealed a clear accumulation of *NHL25* mRNA, whereas no significant changes in *NHL3* transcript levels were detected (Fig. 1C).

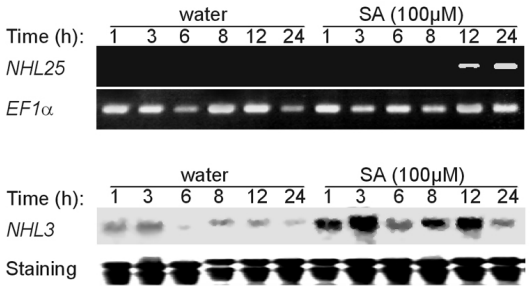

Fig. 2. Salicylic acid-mediated mRNA accumulation of *NHL25* and *NHL3*. Plants were sprayed with 100 μM salicylic acid (SA) and analyzed by reverse transcription-polymerase chain reaction (*NHL25*) or RNA gel blots (*NHL3*). Equal loading of RNA was checked by amplifying a constitutively expressed gene (*EF1α*) or by methylene-blue staining of the ribosomal bands, respectively. Similar results were obtained in two other independent experiments.

Salicylic acid accumulation is required for bacteria-mediated expression of *NHL25* but not of *NHL3*.

Since salicylic acid (SA) has been shown to play a central role in the activation of defense genes and SAR (Glazebrook 2001), we tested the effect of SA on *NHL25* and *NHL3* expression. Levels of *NHL3* transcripts increased rapidly in a biphasic manner, while *NHL25* transcripts started to accumulate only 12 h after SA treatment (Fig. 2). Therefore, SA is sufficient for inducing *NHL25* and *NHL3* expression. To investigate if SA is also necessary for pathogen-induced expression of *NHL25* and *NHL3,* SA-deficient *Arabidopsis* (Col-0) plants expressing the salicylate hydroxylase gene (*nahG*) (Delaney et al. 1995) were infiltrated with *Pst* DC3000 (*avrRpm1*). Expression of *NHL25* in these plants was reduced compared with wild-type plants, whereas expression of *NHL3* was not affected (Fig. 3A). Taken together, the results demonstrate that while SA treatment is sufficient to induce transcript accumulation of both genes, it is not necessary for the enhancement of *NHL3* expression during incompatible interactions with bacterial pathogens.

Ethylene and jasmonate are not required for bacteria-induced expression of *NHL25* and *NHL3*.

Jasmonate and ethylene are, like SA, potential signaling molecules involved in the regulation of many defense-related genes (Reymond and Farmer 1998). To investigate the role of ethylene, the bacteria-induced expression of *NHL25* and *NHL3* was tested in ethylene response mutants. Mutant *etr1* (Bleecker et al. 1988) is altered in its ability to perceive and react to ethylene due to a dominant mutation in the *ETR1* gene that encodes an ethylene receptor (Chang et al. 1993). This mutation does not significantly affect the *NHL25* and *NHL3* transcript levels after avirulent bacterial treatment (data not shown). To eliminate the possibility that the 'leaky' phenotype of *etr1* (Chang and Shockey 1999) may mask any requirement for ethylene, we analyzed the effect of another ethylene response mutant, *ein2* (Guzman and Ecker 1990). The enhanced expression of *NHL25* and *NHL3* during the incompatible interaction is, as in *etr1*, not affected by *ein2* (Fig. 3A). Hence, sensitivity to ethylene is not essential for induction of *NHL25* and *NHL3* expression after infiltration with avirulent bacterial pathogens.

Exogenous application of methyl jasmonate failed to induce transcript accumulation of *NHL25* or *NHL3,* although transcripts of other jasmonate-responsive genes, such as *Atjrg21* (Bau 2001), accumulated (data not shown). To exclude a possible role of endogenous jasmonate, we studied *NHL25* and *NHL3* expression levels after pathogen challenge in the *coi1-1* mutant. The *coi1-1* mutant is insensitive to methyl jasmonate and impaired in the jasmonate (JA) signaling pathway (Feys et al. 1994). As shown in Figure 3A, this mutation does not significantly affect *NHL25* and *NHL3* transcript levels after infection with avirulent bacteria. Thus, jasmonate is not an essential signal molecule in mediating *NHL25* and *NHL3* mRNA accumulation.

***NHL25* and *NHL3* expression during bacterial incompatible interaction is independent of *NDR1*, *EDS1*, *and PAD4*.**

The *ndr1-1* mutant is susceptible to *Pst* DC3000 carrying any one of the four avirulence genes *avrB*, *avrRpm1*, *avrRpt2*, or *avrPphB* (Century et al. 1995), and defines a specific *R* gene-dependent signaling pathway (Aarts et al. 1998). Since NHL25 and NHL3 display some sequence homology with NDR1 and respond similarly to infection with avirulent bacteria, the dependence of *NHL25* and *NHL3* transcript accumulation on *NDR1* was investigated. Neither *NHL25* expression nor

NHL3 expression were found to be affected by the *ndr1-1* mutation during the *avrRpm1-RPM1* interaction (Fig. 3A).

EDS1 and *PAD4* are required for resistance mediated by TIR domain-containing *R* genes (Aarts et al. 1998). The increased expression of *NHL25* and *NHL3* was not abolished after infiltration of the *pad4-1* mutant (Glazebrook et al. 1996) with *Pst* DC3000 (*avrRps4*), which stimulates the TIR *RPS4*-mediated resistance pathway (Gassman et al. 1999) (Fig. 3B). In agreement with the finding that EDS1 and PAD4 interact in plant cells (Feys et al. 2001), the *eds1-1* mutation (Parker et al. 1996) also did not block the induction of *NHL25* and *NHL3* expression after infiltration with *Pst* DC3000 (*avrRps4*) (data not shown). However, the induction of *NHL25* transcript accumulation appears to be slightly delayed in the *pad4-1* mutant (Fig. 3B).

Virulent *Pst* DC3000 may suppress the bacteria-induced *NHL3* expression.

Inoculation with virulent bacteria did not lead to increased expression of *NHL25* and *NHL3* (Fig. 1A). From these data, it seems unlikely that the expression of these genes is mediated by general elicitors such as surface components of the bacterium. Accordingly, *NHL25* and *NHL3* expression was not altered after infiltration of *Arabidopsis* leaves with the flagellin peptide elicitor flg15 (Felix et al. 1999) (data not shown). However, it remains possible that the virulent bacterial strains produce suppressor molecules that block *NHL25* and *NHL3* expression. Thus, we analyzed *NHL25* and *NHL3* expression after challenge with a *Pst* DC3000 *hrcC* Type III-secretion deficient mutant that is incapable of delivery of effector proteins (Roine et al. 1997) as well as with the nonhost bacteria *P. syringae* pv. *phaseolicola* Race 6 and its corresponding *hrpA* pilus mutant (Lee et al. 2001). *NHL25* expression was not significantly induced by these bacteria (Fig. 4). Interestingly, elevated levels of *NHL3* mRNAs were observed after treatment with the *Pst* DC3000 *hrcC* mutant strain, *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *phaseolicola hrpA* mutant (Fig. 4). Hence, *NHL3* expression can be induced by an unknown elicitor that may be common to at least the two tested phytopathogenic bacteria. More importantly, this activity appears to be suppressed by virulent *Pst* DC3000.

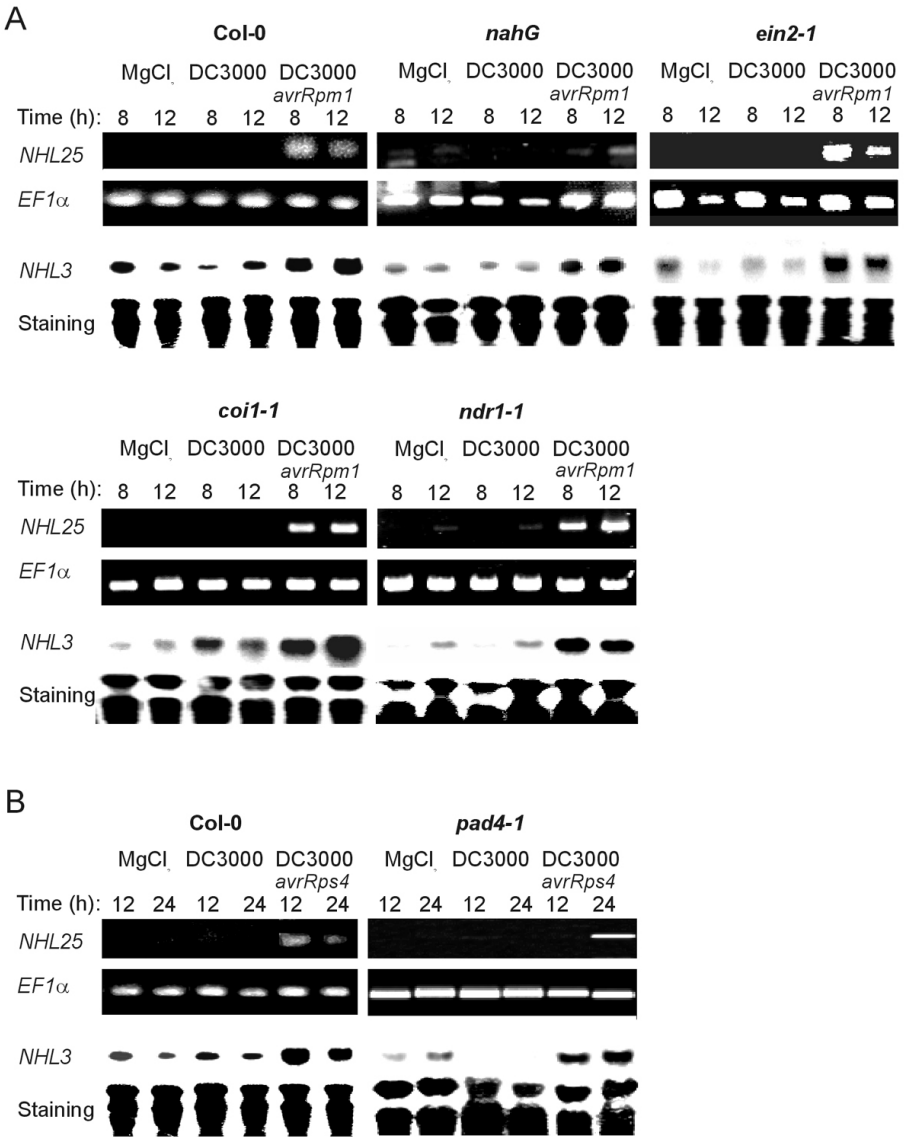

Fig. 3. Pathogen-induced expression of *NHL25* and *NHL3* in **A,** salicylic acid (SA)-deficient (*nahG*), ethylene-insensitive (*ein2-1*), jasmonate-insensitive (*coi1-1*), *ndr1-1* mutants and **B,** *pad4-1* mutants. Plants were treated and analyzed by reverse transcription-polymerase chain reaction (*NHL25*) or RNA gel blots (*NHL3*). Equal loading of RNA was checked by amplifying a constitutively expressed gene (*EF1α*) or by methylene-blue staining of the ribosomal bands, respectively. Similar results were obtained in two other independent experiments. Note that four more polymerase chain reaction cycles were necessary to visualize the low level of *NHL25* expression in *nahG* plants (top middle panel) compared with wild-type Columbia (Col-0) plants.

***NHL3* transcripts accumulate rapidly in local and systemic tissues after wounding.**

In some experiments, *NHL3* transcript levels also increased transiently in leaves infiltrated with a solution of 10 mM $MgCl_2$ (data not shown). This rapid but transient induction may be due to a wounding effect. Indeed, after wounding of leaves with forceps, *NHL3* transcripts rapidly accumulated locally in wounded leaves but also systemically in the unwounded leaves (Fig. 5). Dissection of the components of both signaling cascades leading to expression of *NHL3* may provide insights into how wound and pathogen signaling overlap. We therefore investigated the possible involvement of some of the signals ascribed to pathogen attack and wounding, such as SA, jasmonate, and ethylene.

The wound-induced expression pattern was unaltered in SA-deficient *nahG* plants (Fig. 5). *Arabidopsis* exhibits a transient increase in ethylene production after wounding (Rojo et al. 1999), but no effect of the *etr1* mutation or of the *ein2* mutation was detectable on the *NHL3* expression pattern in response to wounding (Fig. 5). Similarly, the wounding response of *NHL3* was also not significantly affected in the jasmonate-insensitive *coi1-1* mutant (Fig. 5). Hence, none of the hormone signals studied were found to be essential for wound-mediated expression of *NHL3*.

DISCUSSION

On the basis of sequence homology, *NDR1/HIN1*-like (*NHL*) genes have been identified in the Arabidopsis genome (Dörmann et al. 2000). The encoded proteins are thought to be possible mediators of pathogen defense (Dörmann et al. 2000). It is, however, not known if all of them are required for *R* function or are involved in defense gene activation. Several genes encoding important defense signal components including *NDR1* (Century et al. 1997) or *EDS1* and *PAD4* (Falk et al. 1999; Jirage et al. 1999) are also pathogen-responsive, probably as part of a signal feedback amplification loop. Pathogen-responsive members of the *NHLs* are thus likely to be involved in the defense response. We have identified two members of this *NHL* family (*NHL25* and *NHL3*) that show transcript accumulation after pathogen attack. Transcripts for six other genes from this gene family that were studied did not accumulate after inoculation with virulent or avirulent bacteria. As yet, we cannot rule out the possibility that these six genes might also play significant roles in the plant's response to other pathogens. We focused on the characterization of two *NHL* genes that are clearly responsive to the tested pathogens.

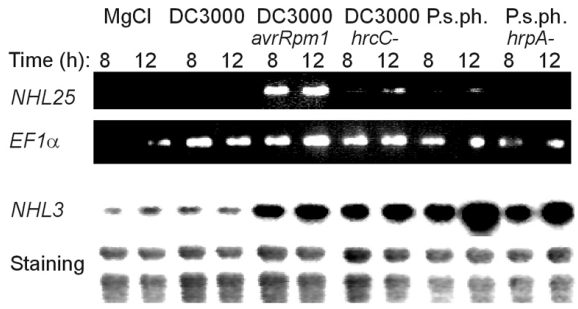

Fig. 4. *Pseudomonas syringae*-induced mRNA accumulation of *NHL25* and *NHL3*. Plants were treated with the indicated bacterial strains and analyzed by reverse transcription-polymerase chain reaction (*NHL25*) or RNA gel blots (*NHL3*). Equal loading of RNA was checked by amplifying a constitutively expressed gene (*EF1*α) or by methylene-blue staining of the ribosomal bands, respectively. Similar results were obtained in two parallel experiments. P. s. ph. = *Pseudomonas syringae* pv. *phaseolica*.

Figure 6 shows a model summarizing the findings of this work. We found that interaction with bacteria expressing four different avirulence genes and also the expression of the avirulence gene *avrRpt2* in planta led to the increased expression of *NHL25* and *NHL3* (Fig. 1). Our studies involved genetic interactions of *R* genes comprising both the TIR (*RPS4*, *RPP2*) and the LZ (*RPM1*, *RPS2*) types with their matching *avr* genes (Fig. 1). The results showed that the bacteria-induced expression of *NHL25* and *NHL3* can be mediated through either structural class of R protein. Recognition of the avirulence gene product by the *R* gene product, whether direct or indirect, is sufficient to evoke one or more signal events leading to *NHL25* and *NHL3* expression. The increased expression of both genes correlated with the subsequent appearance of HR. In this respect, it is interesting that the tobacco *NG2* gene (Karrer et al. 1998), which has some sequence homology to the *NDR1* and *HIN1* gene family, has been isolated through a functional screen for HR induction in tobacco. Furthermore, transgenic plants expressing *NHL2* ectopically led to 'light-dependent speck-like' disease symptoms and elevated levels of *PR1* expression (Dörmann et al. 2000). Hence, it is possible that expression of genes such as *NHL25* and *NHL3* may be involved in *PR* gene expression or HR development, or both.

Interestingly, only *NHL25* reproducibly showed mRNA accumulation after infection with *P. parasitica* isolate Cala2 (Figs. 1C and 6). Another difference between the two genes is the differential requirement of SA for enhanced gene expression after avirulent bacterial challenge (Fig. 3). Thus, after recognition of the potential pathogen, signal events diverged into SA-dependent and SA-independent pathways for *NHL25* and *NHL3*, respectively (Fig. 6).

Depending on the *avr* and *R* genes involved, the transcript accumulation of *NHL25* occurs as early as 6 h after pathogen challenge (Fig. 1A) and requires SA (Fig. 3). Thus, it is surprising that *NHL25* transcripts accumulate relatively late after SA treatment, which is not an uptake problem since expression of *NHL3* is already induced (Fig. 2). This suggests that SA is not the intermediate signal between infection and induction of *NHL25* expression. More likely, one or more unknown factors generated in incompatible plant-pathogen interactions acts in synergy with SA to induce *NHL25* expression (Factor X in Fig. 6). This factor X alone would be insufficient to induce *NHL25* expression in the *nahG* plants, and SA alone would only lead to late transcript accumulation in the absence of this factor (Fig. 6).

SA treatment leads to a biphasic accumulation of *NHL3* transcripts (Fig. 2). This is reminiscent of the biphasic pattern recently described for the SA-induced physical interaction between NPR1/NIM1 and TGA2 transcription factor within the plant nucleus (Subramaniam et al. 2001); these proteins are known to mediate response to SA (Despres et al. 2000). Interestingly, the *NHL3* promoter contains two inverted TGACG sequence elements. TGACG motifs were found to be required for the binding of TGA-bZIP transcription factors (Schindler et al. 1992) and were shown to be important within the *PR1* promoter for response to treatment with the SAR-inducing chemical, INA (Lebel et al. 1998). It is thus tempting to speculate that the inverted TGACG sequences within the *NHL3* promoter may be involved in the response to SA treatment.

Strikingly, inoculation with nonhost bacteria *P. syringae* pv. *phaseolicola* and its Type III pilus (*hrpA*) mutant led to enhanced *NHL3* (but not *NHL25*) expression (Fig. 4). This indicates that delivery of avr or effector proteins into the plant cell via the Type III secretion system (TTSS) is not necessary for the nonhost bacteria-induced expression of *NHL3*. Furthermore, this suggests the existence in *P. syrin-*

gae pv. *phaseolicola* of an elicitor that does not require the TTSS to induce *NHL3* mRNA accumulation. The nature of this elicitor is unknown, but it is unlikely to be the flg15 peptide (Felix et al. 1999), since this peptide did not trigger *NHL3* expression (data not shown). Such an elicitor could also be responsible for the *NHL3* induction obtained with the *Pst* DC3000 *hrcC* mutant (Fig. 4). The absence of *NHL3* transcript accumulation after treatment with the virulent strain *Pst* DC3000 suggests that the bacteria secrete via the TTSS one or more effector proteins that interfere with *NHL3* mRNA accumulation (Fig. 6, effector Y). By contrast, although this suppressor is still present during incompatible interactions, coinjected Avr proteins such as AvrRpm1, AvrRpt2, AvrB, or AvrRps4 may overcome this suppressor effect (Figs. 1 and 4). It has been shown that animal and plant bacterial pathogens are able to suppress host defense mechanisms by means of effector proteins secreted by the TTSS (Brown et al. 1995; Jackson et al. 1999; Orth et al. 2000; Tsiamis et al. 2000). Hence, the possibility that virulent *Pst* DC3000 may suppress *NHL3* transcript accumulation and the fact that avirulent as well as nonhost bacteria induced *NHL3* expression suggest a potential role for *NHL3* in the *Arabidopsis* general resistance against bacteria. This is reminiscent of the suppression of defense gene expression and phytoalexin accumulation in bean by virulent *P. syringae* pv. *phaseolicola* (Jakobek et al. 1993; Jakobek and Lindgren 1993).

NHL3 showed a rapid wound-inducible expression in local and systemic leaves (Figs. 5 and 6). It is conceivable that pathogens may take advantage of wound sites for entry into plants and, hence, the rapid activation of wound and pathogen-responsive genes such as *NHL3* may serve as an early attempt to elevate the defense status against possible infections. The tomato *TWI1* gene, encoding a putative glycosyl transferase (O'Donnell et al. 1998), shows an expression pattern strikingly similar to *NHL3.* This gene responded rapidly to wounding, pathogen-derived factors, and also SA. The time frame of local and systemic gene activation after wounding and the fact that this wound response was independent of ethylene and SA are also similar to that of *NHL3*. Further studies are needed to clarify the nature of the systemic signal that activates *NHL3* after wounding. However, we can rule out the role of SA, JA, or ethylene. It remains to be determined if electrical, hydraulic, or cytosolic acidification events (Bowles 1998; Herde et al. 1999) are wound signals for activation of *NHL3*.

With the aid of Arabidopsis mutants, we attempted to dissect the signal pathways involved in regulating expression of *NHL25* and *NHL3*. Neither gene required ethylene and jasmonate for increased expression (Fig. 3). They therefore belong to a different set of pathogen-responsive genes than e.g. plant defensins that require these hormones for induced expression (Penninckx et al. 1998). Enhanced *NHL25* expression depends on the recognition of Avr proteins by the matching plant R proteins (Fig. 1). Interestingly, this expression, mediated through either TIR-NBS-LRR or LZ-NBS-LRR *R* genes, was not abolished in the *ndr1-1* mutant and only slightly delayed in the *pad4-1* mutant (Fig. 3). Thus, the signal events leading to the induction of *NHL25* belong to either a pathway upstream of or parallel to these mediated by *NDR1* or *PAD4* and *EDS1*. In the case of *NHL3*, the induced expression during incompatible plant-bacteria interaction clearly defines a novel pathway, as no such pathway independent of SA, ethylene, and jasmonate has been described.

Although *NHL25* and *NHL3* share sequence homology and similar expression patterns after inoculation with avirulent bacteria, they are differentially regulated by virulent and nonhost bacteria and by wounding (Figs. 1A, 4, 5, and 6). We propose that *NHL25* may be used as a specific marker gene for incompatible interactions with pathogens and possibly for HR development. *NHL3* expression, on the other hand, can be triggered by multiple biotic and abiotic stresses in addition to regulation by avirulent bacteria in a gene-for-gene manner.

Most pathogen-related genes studied to date show transcript accumulation in response to both avirulent and virulent pathogens although, in the latter case, a delayed reaction of lower intensity has frequently been observed (Kombrink and Somssich 1997). Genes that are exclusively induced during interaction with avirulent pathogens are likely to be implicated in mediating resistance. Here, we describe one gene (*NHL25*) that fits this description for gene-for-gene interactions. *NHL3* is also such a defense-related gene but, in addition, is induced after inoculation with nonhost bacteria and appears to be suppressed by virulent bacteria in a TTSS-dependent manner (Fig. 4). This suggests the importance of the gene product in counteracting susceptibility to bacteria. Unfortunately, we failed to isolate any "knock-out" lines for

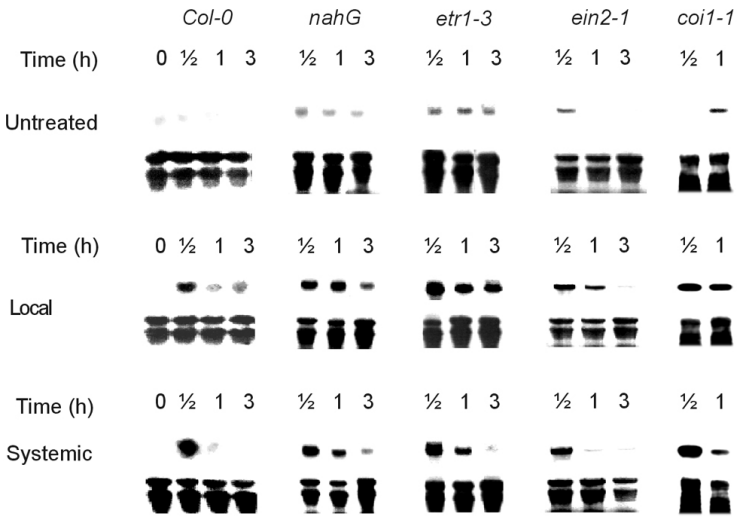

Fig. 5. Wound-induced expression of *NHL3* in *Arabidopsis* and mutant plants. Plants were wounded with forceps and analyzed by RNA blots. Unwounded plants were also included as untreated controls (top panel). Internal controls for RNA levels were checked by methylene-blue staining of the ribosomal bands.

NHL25 and *NHL3*, even after screening three different T-DNA or transposon insertion libraries.

In conclusion, the exclusive expression patterns and their homology to the resistance-mediating gene, *NDR1*, are suggestive that these two genes may be involved in resistance.

MATERIALS AND METHODS

Plant and growth conditions.

All experiments were performed with *Arabidopsis thaliana* ecotype Columbia (Col-0). The Col-0 *etr1-3* and the Col-0 *ein2-1* plants were obtained from the Ohio State University *Arabidopsis* Biological Resource Center (Columbus, OH, U.S.A.). Plants were grown in a phytochamber (Heraeus Voetsch, Balingen, Germany) at 22°C, either under short-day conditions (8 h light and 16 h darkness) for infection experiments or under long-day conditions (16 h light and 8 h darkness) for seed set in a potting mixture consisting of soil and sand (2:1).

Bacterial strains and plasmids.

The bacterial pathogen *P. syringae* pv. *tomato* DC3000, its corresponding avirulent strains expressing the avirulence genes *avrRpt2*, *avrRpm1*, *avrB*, and *avrRps4* and *Pst* DC3000 *hrcC* mutant have been described previously (Debener et al. 1991; Hinsch and Staskawicz 1996; Staskawicz et al. 1987; Whalen et al. 1991; Yuan and He 1996). Generation of the *hrpA* mutant of *P. syringae* pv. *phaseolicola* Race 6 was described in Lee and associates (2001). Bacteria were grown at 28°C in King's B medium (King et al. 1954) containing 50 µg of rifampicin per ml and the appropriate antibiotics required for plasmid maintenance. The *avrRpt2*, *avrRpm1*, *avrB*, and *avrRps4* genes were expressed in *P. syringae* strains on plasmids pV288, pK48, pVB01 (Kunkel et al. 1993), and pVSP61 (Hinsch and Staskawicz 1996), respectively.

Bacterial and oomycete inoculation.

Plants were infected by infiltration with bacterial suspensions of 10^8 CFU per ml in 10 mM $MgCl_2$ (optical density at 600 nm of 0.2) as described previously (Kiedrowski et al. 1992). *Peronospora* Cala2 infections were performed as described by Aarts and associates 1998. Leaf material was harvested at the indicated time points, frozen in liquid N_2, and stored at –70°C. For each time point, four leaves per plant from three individual plants were pooled.

Treatment with salicylic acid and methyl jasmonate.

For hormone treatments, leaves were sprayed with a 100 µM solution of salicylic acid (SERVA, Heidelberg, Germany) or methyl jasmonate (ZEON Corporation, Tokyo). Methyl jasmonate treatment was also performed by floating excised leaves at 25°C under constant light conditions (120 µmol/m^2/s). For harpin treatment, leaves were infiltrated with 10 µM harpin (gene product of HrpZ) diluted in 5 mM MES pH5.5 (Lee et al. 2001).

RNA extraction and analysis.

Plant material was ground in liquid N_2, and RNA was isolated using TRIZOL-reagent (Gibco Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. RNA was quantified by UV-spectroscopy and 10 µg or 0.2 µg were used for RNA blot or RT-PCR analyses, respectively. RT-PCR was performed using the Ready-to-Go RT-PCR system (Amersham Pharmacia Biotech, Freiburg, Germany). First-strand cDNA synthesis was primed using oligo-dT primer for 30 min at 42°C. The reaction was divided into two tubes. One reaction was used as internal control by amplifying a fragment of translation elongation factor 1-alpha, *EF-1α* (Curie et al. 1993). The second reaction was used for amplifying the gene of interest by adding the corresponding primer combinations. PCR was initiated at 95°C (4 min), followed by 25 cycles of 10 s denaturation at 94°C, 10 s annealing at a suitable T_m for the primer pairs, and 40 s synthesis at 72°C. A final step of synthesis at 72°C for 10 min was used to complete the reaction. Preliminary experiments were used to verify that PCR conditions were not saturated.

For quantification, the PCR-amplified products were separated on agarose gels, transferred onto nylon-membranes (HybondN+, Amersham Pharmacia Biotech) and hybridized to ^{32}P-labeled DNA probes. DNA probes used in RNA and DNA

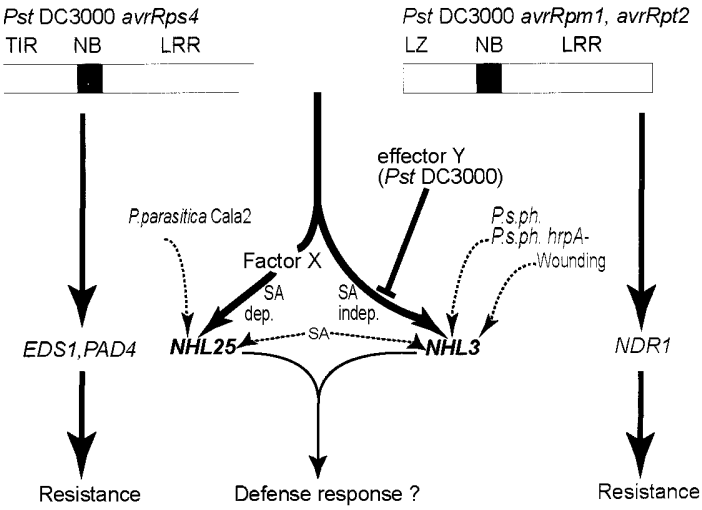

Fig. 6. Model summarizing the deduced pathways of treatments that induce *NHL25* and *NHL3* expression. Avirulent bacteria induce mRNA accumulation of *NHL25* and *NHL3* in a gene-for-gene-dependent manner but via two different signaling pathways (SA-dependent and SA-independent pathways). Although salicylic acid (SA) is sufficient in inducing *NHL25* and *NHL3* expression and is required for *NHL25* expression, it is probably not the intermediate signal; an unknown factor (X) has to be proposed. Transcript accumulation of *NHL25* is further induced by *Peronospora parasitica* isolate Cala2, while *NHL3* is responsive to nonhost bacteria and wounding. Virulent *Pst* DC3000 probably suppresses the *NHL3* transcript accumulation during compatible interactions via an unknown effector Y in a *Hrp*-dependent manner. The pathogen responsiveness, homology to *NDR1*, and suppression of *NHL3* induction by virulent *Pst* DC3000 suggest potential roles of these two *NHL* genes in defense response.

analyses were synthesized using the Megaprime kit (Amersham Pharmacia Biotech) and α^{32}P-dATP. Hybridization was performed in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 2 mM EDTA [pH 7.7]), 5× Denhardt's reagent (Denhardt 1966), 0.1% sodium dodecyl sulfate (SDS), 100 µg of denatured salmon sperm DNA per ml, and 50% formamide at 42°C overnight, and the blots were washed twice at 60°C in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS for 20 min and in 0.1× SSC and 0.1% SDS for 10 min. Radioactivity was visualized and quantified using a PhosphorImager (STORM, Molecular Dynamics, Amersham Pharmacia Biotech). For quantitative analysis of RNA gel blots, a 26S-rDNA probe was used to normalize differences in loading.

Primers used for amplifications are as follows: 5′-TCACATCAACATTGTGGTCATTGGC-3′ and 5′-TTGATCTGGTCAAGAGCCTCAAG-3′ for *EF-1α*, 5′-CCAAGACACAGCAAGCAGCACC-3′ and 5′-CCCGAGTTTGATCCGAACCG-3′ for *NHL25* (NM_123055); 5′ATGGCGGACTTAAACGGTGCG-3′ and 5′-TCAAAAGTCAACGTCACACTTGGTCGG-3′ for *NHL3* (NM_120715), 5′-CAGCCACTCATCATGCAACCG-3′ and 5′-TCCAACGAACTCGACCGTCG-3′ for *NHL23* (NM_120716), 5′-GCCCAAGCTAGAATTGATGCCG-3′ and 5′-GTTAGGCTGCGACAAAGAGACCG-3′ for NHL24 (NM_122192), 5′-ACGTCTCCGGCAATCCATCG-3′ and 5′-GCTGATCGTCAAGATACAAGGCG-3′ for *NHL27* (accession number AB005237, nucleotide 9667-10146), 5′-GGCGAACGGATTAAACGGCG-3′ and 5′-TCAACGTGGCACTTGGTGGG-3′ for *NHL9* (NM_129098), 5′-CATTGCGCCAAGAAAGGAGGG-3′ and 5′-TATGCACCCGAGACCCATGTCC-3′ for *NHL26* (NM_124752), 5′-AAGCAGAGCACGCAGCCAAAGC-3′ and 5′-CGTCCAGTCTTAATCGCACCAGC-3′ for *NHL19* (NM_116371).

ACKNOWLEDGMENTS

The Col-0 *nahG* and *coi1-1* lines were kindly provided by Syngenta (Research Triangle Park, NC, U.S.A.) and J. Turner, respectively. We also thank J. Boch (Genetic Institute, University of Halle, Germany) for providing the *Pseudomonas* strains carrying avirulence genes.

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Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns

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Recent findings have highlighted remarkable similarities in the innate pathogen defense systems of plants, animals and insects. Pathogen-associated molecular patterns (PAMP) that are similar to those activating innate immune responses in animals have been shown to mediate the activation of plant defense. Moreover, recognition complexes that are structurally related to animal PAMP receptors are now being discovered in plants, suggesting a common evolutionary origin of pathogen defense systems in higher eukaryotes.

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Current Opinion in Plant Biology 2002, **5**:

1369-5266/02/$ – see front matter

DOI 10.1016/S1369-5266(02)00265-0

Abbreviations

AVR	avirulence gene
CHRK1	chitinase-related receptor-like kinase1
FLS2	*Flagellin-sensing2*
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
PAMP	pathogen-associated molecular pattern
PEN1	*Penetration1*
R	resistance gene
TIR	*Drosophila* Toll and human interleukin-1 receptor
TLR	Toll-like receptor

Introduction

The ability to discriminate between self and non-self is a key feature of all living organisms, and forms the basis for the activation of innate defense mechanisms against attempted microbial infection. Generally, pathogen recognition and the subsequent activation of disease resistance responses in plants occurs either at the non-cultivar-specific level (i.e. non-host or species resistance, non-cultivar-specific host resistance) or at the cultivar level (i.e. cultivar-specific host resistance) [1–5]. Cultivar-specific resistance, which is expressed only by particular plant cultivars against some races of a pathogen species, conforms to the gene-for-gene-hypothesis and is genetically determined by complementary pairs of pathogen-encoded avirulence (*AVR*) genes and plant resistance (*R*) genes. The absence of either gene or the failure of either gene to produce a functional product results in disease. Most AVR proteins are considered to be virulence factors that are required for the colonization of host plants. When AVR proteins are recognized by resistant host plant cultivars, they act as 'specific elicitors' of plant defense and hence betray the pathogen to the plant's surveillance system [2,3,6].

Although often overlooked, the immunity of an entire plant species (i.e. non-host or species resistance) towards most phytopathogenic microorganisms is the predominant form of plant disease resistance [1]. Infrequent changes in the host range of phytopathogens are indicative of the stability of species immunity. This type of resistance is determined by several intermingled layers of defense, which include both constitutive barriers and inducible reactions [1,4]. A large variety of microbe-associated products, which are referred to as 'general elicitors', triggers defense responses in many plant species in a non-cultivar-specific manner [7,8]. Until recently, however, it was not clear why plants should possess recognition capacities for such 'antigenic' signals. In this review, we address the striking similarities that have been revealed recently between the molecular basis of innate immunity in plants and that known for insects and animals. These findings provide an intriguing explanation for the recognition of general elicitors by plants, and support the idea of a common, early evolutionary origin of eukaryotic non-self recognition systems [2,3,5,9–11,12••,13].

General elicitors as pathogen-associated molecular patterns

Research carried out over the past decade has demonstrated that plants have evolved recognition capacities for numerous microbial surface-derived compounds, which induce plant defense responses in both host and non-host plants [7,8,14]. These elicitors include (glyco)proteins, peptides, carbohydrates, and lipids, all of which can trigger plant defense responses comparable to those observed upon *R*-gene-mediated pathogen recognition in resistant host plant cultivars.

A better understanding of why plants may recognize 'antigenic' epitopes on microbial surfaces is provided by a set of definitions that formally describes the components of the mammalian innate immune system [9]. In this model, pathogen surface-derived molecules are referred to as pathogen-associated molecular patterns (PAMPs). These bind to pattern recognition receptors and, thereby, trigger the expression of immune response genes and the production of antimicrobial compounds [9,10,15]. In recent years, innate immunity has been studied thoroughly in humans, mice and insects, and it has been revealed that its molecular basis shows remarkable evolutionary conservation across kingdom borders [9–11,12••]. PAMPs

that trigger innate immune responses in various vertebrate and invertebrate organisms include the lipopolysaccharide (LPS) fraction of Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, eubacterial flagellin, unmethylated bacterial DNA fragments, as well as glucans, chitins, mannans and proteins that are derived from the fungal cell wall [10,11,12••]. Intriguingly, many of these molecules have long been known to act as general elicitors of defense responses in a multitude of plant species [1,5,7]. For example, various structural elements of LPS from Gram-negative bacteria are potent inducers of plant defense reactions [14,16,17]. Furthermore, flg*22* — a highly conserved amino-terminal fragment of flagellin, the main building block of eubacterial flagellae — triggers defense-associated reactions in plant species as diverse as *Arabidopsis* and tomato [18]. These findings are important as they strongly suggest that plants have acquired and maintained the ability to recognize microbe-associated patterns (both LPS and flagellin decorate Gram-negative bacteria).

It appears that plants also recognize patterns similar to those reported to activate innate defense mechanisms in mammals and *Drosophila*. However, the minimum structural requirements for elicitor activity in plants and animals may differ. Non-self recognition capacities already vary considerably between monocot and dicot plants, as illustrated by the apparent insensitivity of rice cells to the bacterial flagellin fragment, flg*22* [18]. Nevertheless, rice cells appear to possess the ability to recognize bacterial flagellins, but the structural properties of the defense-eliciting 'epitope' likely differ from those of flg*22* [19]. Similarly, a hepta-β-glucoside derived from the cell wall of *Phytophthora* that is an elicitor of phytoalexin production in soybean does not trigger defense responses in rice or tobacco [20•,21•,*22*]. Conversely, a tetraglucosyl glucitol (a β-glucan that is structurally different from hepta-β-glucoside) derived from the cell wall of the fungus *Pyricularia oryzae* triggered plant defense in rice but not in soybean [21•].

Unifying features of PAMPs are their highly conserved structures, their functional importance for and their presence in various microorganisms, and their apparent absence in potential host organisms. Do general elicitors of non-cultivar-specific plant defense responses also display such characteristics? Our studies have revealed that Pep-13 [23], a surface-exposed peptide sequence that is present within a 42-kDa cell wall glycoprotein, can serve as a recognition determinant for the activation of plant defense in parsley and potato during interactions with *Phytophthora* species. More recently, the 42-kDa glycoprotein elicitor was shown to possess calcium-dependent transglutaminase activity (F Brunner, S Rosahl, D Scheel, T Nürnberger, unpublished data). Pep-13 sequences were highly conserved among the ten *Phytophthora* species analyzed, but were virtually absent from plant protein sequences. In addition, mutational analysis of the Pep-13 sequence, which itself is not part of the catalytic center of the transglutaminase, identified amino acid residues that are indispensable for both transglutaminase activity and the activation of plant defense responses. This suggests that plants recognize PAMPs that have characteristics identical to those that trigger innate defense in humans and *Drosophila*. The activation of plant defense upon recognition of pathogen-associated structures that are not subject to frequent mutation is likely to provide a fitness penalty to the pathogen [4]. Like Pep-13, fungal chitin, oomycete glucans and bacterial flagellin all represent microbe-specific structures that are expected to be indispensable for the microbial host. Thus, these compounds should also be considered to be PAMPs.

Plant cells encounter a variety of pathogen-associated signals when interacting with microorganisms *in vivo*, and the plant's ability to recognize complex PAMPs is likely to determine its efficiency in inducing innate defense mechanisms. For example, the cell walls of many phytopathogenic fungi harbor chitins, *N*-mannosylated glycopeptides and ergosterol, all of which trigger plant defense responses [7,24]. Various phytopathogenic Gram-negative bacteria harbor LPS and flagellin, which stimulate plant defenses, and produce harpins upon contact with plants [14,18,25,26•]. Moreover, phytopathogenic oomycetes of the genera *Phytophthora* and *Pythium* possess heptaglucan structures, elicitins and other cell wall proteins [4,8,20•,27,28•], all of which elicit plant defense.

Although not all plant species recognize and respond to all of these signals, plant cells have systems that recognize multiple signals derived from individual microbial species. For example, tobacco and *Arabidopsis* cells recognize harpins and flagellin that are derived from *Pseudomonas syringae* [18,29,30,31•], whereas tomato cells perceive fungal chitin fragments, glycopeptides and ergosterol [7]. Complex pattern recognition by plants is yet another phenomenon that is reminiscent of the activation of innate defense responses in animals. For example, innate immune responses in humans are activated by a number of signals that are derived from Gram-negative bacteria including LPS, flagellin and unmethylated CpG dinucleotide motifs that are characteristic of bacterial DNA [11,12••]. There is an ongoing debate among immunologists as to whether the recognition of multiple signals from one pathogen mediates more sensitive perception or if redundant recognition systems act as independent back-up systems in the same or different tissues [12••].

PAMP recognition in animals and plants

PAMPs are sensed by pattern recognition receptors that distinguish self from conserved microbial structures that are shared by different pathogens [9–11,12••]. *Drosophila* Toll and mammalian Toll-like receptors (TLRs) have pattern recognition capabilities. They recognize PAMPs through an extracellular leucine-rich repeat (LRR) domain and transduce the PAMP signal through a cytoplasmic TIR domain (*Drosophila* Toll and human interleukin-1 receptor).

For example, the mammalian innate immune response to Gram-negative bacteria is triggered through TLR4 (which binds LPS), TLR5 (which binds flagellin), and TLR9 (which binds bacterial CpG dinucleotide motifs) [12••]. As shown recently, the repertoire for pattern recognition (i.e. the number of recognized PAMPs) can be significantly enhanced through cooperation between different TLRs [12••]. TLRs are often found in molecular complexes that involve soluble ligand binding sites and various accessory membrane-attached or transmembrane proteins. LPS, for example, is bound by a soluble LPS-binding protein (LBP) before recruitment into a complex that comprises soluble MD-2, membrane-attached CD14 and the transmembrane protein TLR4 [12••]. Likewise, the recognition of peptidoglycans that are derived from Gram-positive bacteria by *Drosophila* Toll involves a circulating peptidoglycan recognition protein [32]. Interestingly, multi-component complexes also appear to be involved in pathogen perception by plants.

Binding proteins for general elicitors of plant defense have been kinetically and biochemically characterized, but the isolation and cloning of the encoding genes has proven notoriously difficult [7,8]. However, purification of a 75-kDa plasma membrane protein from soybean and expression of the encoding gene allowed tomato to recognize hepta-β-glucan fragments, which bind to and elicit phytoalexin production in various *Fabaceae* species [20•,27]. The absence of recognizable functional domains for transmembrane signaling within the heptaglucan binding protein, and the detection of multiple labeled proteins in photoaffinity experiments, suggest that this protein may form part of a multi-component recognition complex [20•]. Similarly, chemical cross-linking experiments conducted with Pep-13 and parsley membranes identified two protein species (of 100 and 145-kDa) as putative binding proteins. As the 100-kDa protein bound Pep-13 in the absence of the 145-kDa protein, however, their functional interrelationship remains to be elucidated [33].

The elicitin receptor represents another example in which the formation of a complex is implicated in PAMP perception by plants. Elicitins, which constitute a molecular pattern that is associated with various *Phytophthora* and *Pythium* species [4], trigger plant defense in tobacco upon binding to a receptor complex comprising two *N*-glycoproteins of 162 and 50 kDa [34]. High-affinity binding sites for elicitins have also been reported in *Arabidopsis* and *Acer pseudoplatanus* cells. Elicitins possess the ability to bind sterols, suggesting that the function of these proteins during plant infection is to provide the oomycete with essential lipids [35]. Recently, sterol–elicitin complexes were shown to bind more efficiently to the elicitin receptor than elicitins alone, and it was proposed that sterol loading by elicitins may precede the binding of the sterol–elicitin complex to the plant receptor [36••]. Apparently, the elicitin receptor 'guards' against pathogens that use elicitins to manipulate plant sterol homeostasis. Thus, the 'guard' hypothesis [3,6,37] that originally described AVR–R protein interactions (see below) may also explain the pathogen recognition processes that mediate the activation of non-cultivar-specific plant defense.

The perception of fungal chitin is widespread among plant species [7,24,38]. A chitinase-related receptor-like kinase, CHRK1, that exhibits autophosphorylation activity but no chitinase activity has been identified in tobacco plasma membranes [39]. However, binding of chitin fragments to CHRK1 has yet to be shown. As CHRK1-encoding transcripts accumulated strongly upon infection, it is conceivable that CHRK1 might function as a surface receptor for fungus-derived chitin fragments.

Unarguably, the most valuable recent contribution to our understanding of PAMP recognition in plants originates from the Boller lab. This group has provided ample evidence that similarities between innate immune systems in plants, animals, and insects extend beyond the nature of the PAMPs recognized, and may also be seen in the corresponding perception complexes. Boller and colleagues used the amino-terminal fragment of eubacterial flagellin, flg22 [18], to screen an ethylmethane sulfonate (EMS)-mutagenized population of *Arabidopsis thaliana* ecotype La-er for flagellin-insensitive plants [40••]. This screen provided two independent mutations, which mapped to a single gene (*Flagellin-sensing2* [*FLS2*]) that encodes a putative transmembrane receptor-kinase with an extracellular LRR domain. Strikingly, this protein shares significant structural homology with *Drosophila* Toll and human TLRs [2,5,12••]. Thus, a PAMP that activates human innate immunity through TLR5 [12••] appears to activate plant innate immune responses through a related perception system. A close correlation between the flagellin sensitivity of different *Arabidopsis* ecotypes or *FLS2* mutants and the presence of flagellin-binding sites in the membranes of the plants strongly suggests that FLS2 is part of the flagellin-perception complex [31•,41••]. It remains to be seen, however, whether FLS2 does indeed constitute the primary target site for flg22. Using an *Arabidopsis* protoplast transient expression system, Sheen and colleagues [42••] identified a complete mitogen-activated protein kinase (MAPK) cascade and WRKY transcription factors that act downstream of FLS2, and described a role for MAPK in activating the early transcription of defense genes. MAPK activity is also implicated in the activation of innate immune responses in other plants [26•,43••,44] and can be added to the growing list of parallels in the molecular organization of innate immunity in the plant and animal kingdoms.

PAMP-mediated species resistance versus AVR protein-mediated cultivar-specific resistance in plants

It is tempting to speculate that the activation of innate immune responses in plants is a consequence of PAMP recognition events, and that the 'antigenic' potential of multiple microbe-associated general elicitors (i.e. PAMPs),

in conjunction with plant pattern recognition receptors, provides the basis for durable non-host resistance (species immunity) in plants. However, it should be stated very clearly that this view is based upon correlative data rather than upon causal (i.e. genetic) evidence. For example, the crucial question of whether general elicitors induce plant defenses during natural encounters between plants and would-be pathogens has yet to be answered. The extent to which recognition of PAMPs contributes to plant immunity may also vary between plant species. In parsley, receptor-mediated recognition of Pep-13 triggers defense reactions that contribute to, or are sufficient for, resistance against *Phytophthora* infection [23] (T Nürnberger, F Brunner, unpublished data). However, in the potato–*Phytophthora infestans* interaction that causes disease, pathogen recognition through the Pep-13 motif is insufficient to provide resistance. This is reminiscent of the stimulation of the innate immune system of humans or *Drosophila* by bacterial LPS or flagellin, which does not always sufficiently protect the host from infection by bacteria that display either one or both PAMPs. However, in both mammals and *Drosophila*, PAMP-mediated activation of innate immune responses does contribute to successful defense against microbial invasion [32,45]. It is now important to determine the contribution of PAMP-mediated recognition systems to non-cultivar-specific pathogen defense in plants.

We should remember that the stability of species resistance is likely the result of multiple, intertwined layers of resistance that are activated upon recognition of multiple microbe-associated signals [1,4]. Therefore, the impairment of individual recognition events may not substantially change the interaction between non-host plants and microbial invaders. Nevertheless, mutant screens in *Arabidopsis* have revealed loci that specify non-host resistance against *P. syringae* pv. *phaseolicola* (namely *NON-HOST1* [*NHO1*]; [46•]) and powdery mildew (namely *Penetration1* [*PEN1*] [H Thordal-Christensen, personal communication]; and *PEN2* [P Schulze-Lefert, personal communication]). Both, PEN1 and PEN2, appear to be directly or indirectly involved in the control of cell-wall architecture (P Schulze-Lefert, personal communication). This nicely corroborates the idea that constitutive barriers, such as the plant cell wall, are of central importance for species resistance. Recently, Mellersh and Heath [47•] demonstrated that intact plant plasma membrane–cell wall adhesions and H_2O_2-mediated activation of local defense responses (possibly through the involvement of phenolic compounds) [48••] were required to block fungal penetration of non-host plants.

What is the proposed relationship between species resistance and cultivar-specific host resistance in plants? We assume that during evolution plant species resistance was overcome by phytopathogens through the acquisition of virulence factors, which enabled them to either evade or suppress plant defense mechanisms. Such newly evolved pathogen race-specific virulence factors have driven the co-evolution of plant resistance genes and, thus, the development of pathogen race-/plant cultivar-specific disease resistance that is phylogenetically more recent [1,3,4]. The simple biochemical interpretation of the gene-for-gene-hypothesis implies a receptor–ligand-like interaction between plant *R* gene products and the corresponding pathogen-derived *AVR* gene products. However, the isolation and functional characterization of numerous plant *R* genes that confer resistance to a variety of phytopathogenic viruses, bacteria, oomycetes, fungi, nematodes, and insects suggests that the situation in many plant–pathogen interactions is likely to be more complex than suggested by this simple interpretation [2,3,6,37].

The predominant structural motifs found in R proteins are LRRs and coiled coils, both of which have a role in protein–protein interaction. Direct interaction between AVR proteins and R proteins has indeed been demonstrated (reviewed in [2,3,5,6]). However, several studies have provided evidence that LRR-type R proteins constitute components of larger signal perception complexes, and do not necessarily bind directly to their matching AVR proteins [2,3,5,6,49••,50]. These findings led to the 'guard hypothesis', which predicts that AVR proteins act as virulence factors that contact their cognate pathogenicity targets in host plants or even non-host plants, but function as elicitors of cultivar-specific plant resistance only when the complementary R protein is recruited into a functional signal perception complex [2,3,6,37]. Thus, the role of the R protein is to monitor (i.e. 'guard' against) the AVR-mediated perturbance of cellular functions. In this respect, it is intriguing that FLS2 shares similarity not only with Toll/TLR but also with a number of R proteins that are implicated in race-/cultivar-specific host resistance. Examples include the LRR receptor kinase Xa21, which confers the resistance of rice to particular *Xanthomonas oryzae* pv. *oryzae* strains [51], and other LRR-type R proteins [2,3,6]. Taken together with the presence of TIR domains (which are also found in Toll and TLR) in various R proteins [2,3,5,6], these observations lend further support to the theory of a common evolutionary origin of eukaryotic defense systems.

Conclusions

There is increasing evidence to show that plants employ pathogen perception and defense pathways that closely resemble those present in animals and insects. Recognition of pathogen-associated molecular patterns (some of which possess defense-inducing activity in species from all three kingdoms), formation of pattern recognition complexes involving TLR LRR proteins, MAPK-mediated activation of immune response genes and subsequent production of antimicrobial products all occur across kingdom borders. Comparative genome analyses indicate that various facets of development evolved independently in the lineages leading to plants and animals [13]. However, proteins similar to Toll and TLRs are implicated in both the animal and plant

responses to pathogens. Thus, it is reasonable to assume that the last common ancestor of plants and animals used some relative of Toll/TLR for pathogen recognition, and that this system has evolved extensively to provide resistance in both kingdoms [13].

The isolation and characterization of FLS2 from *Arabidopsis* has provided a valuable insight into the molecular mechanisms of immunity in plants. However, only the identification of many more elements that are implicated in the activation of this defensive system will enable us to prove or disprove the intriguing hypothesis of a common evolutionary origin of innate immunity in animals and plants. Moreover, efforts must be intensified to causally link the recognition of general elicitors to species resistance in plants. Forward genetics approaches in the model plant *Arabidopsis thaliana*, and advanced biochemical and cell biological techniques developed for different experimental systems, should help us to meet these goals.

Acknowledgements

We thank our colleagues Sabine Rosahl, Dierk Scheel and Jason J Rudd for critical reading of the manuscript. FB received support from Kleinwanzlebener Saatzucht AG, Einbeck, Germany.

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The Plant Journal (2002) **32**, 375–390

NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and *Arabidopsis*

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Summary

Activation of non-cultivar-specific plant defense against attempted microbial infection is mediated through the recognition of pathogen-derived elicitors. Previously, we have identified a peptide fragment (Pep-13) within a 42-kDa cell wall transglutaminase from various *Phytophthora* species that triggers a multifacetted defense response in parsley cells. Many of these oomycete species have now been shown to possess another cell wall protein (24 kDa), that evoked the same pattern of responses in parsley as Pep-13. Unlike Pep-13, necrosis-inducing *Phytophthora* protein 1 (NPP1) purified from *P. parasitica* also induced hypersensitive cell death-like lesions in parsley. NPP1 structural homologs were found in oomycetes, fungi, and bacteria, but not in plants. Structure–activity relationship studies revealed the intact protein as well as two cysteine residues to be essential for elicitor activity. NPP1-mediated activation of pathogen defense in parsley does not employ the Pep-13 receptor. However, early induced cellular responses implicated in elicitor signal transmission (increased levels of cytoplasmic calcium, production of reactive oxygen species, MAP kinase activation) were stimulated by either elicitor, suggesting the existence of converging signaling pathways in parsley. Infiltration of NPP1 into leaves of *Arabidopsis thaliana* Col-0 plants resulted in transcript accumulation of pathogenesis-related (*PR*) genes, production of ROS and ethylene, callose apposition, and HR-like cell death. NPP1-mediated induction of the *PR1* gene is salicylic acid-dependent, and, unlike the *P. syringae* pv. *tomato* DC3000(*avrRpm1*)-induced *PR1* gene expression, requires both functional NDR1 and PAD4. In summary, *Arabidopsis* plants infiltrated with NPP1 constitute an experimental system that is amenable to forward genetic approaches aiming at the dissection of signaling pathways implicated in the activation of non-cultivar-specific plant defense.

Keywords: cell death, elicitor, oomycete, *Phytophthora*, signal transduction.

Introduction

The ability to discriminate between self and non-self is a key feature of all living organisms, and forms the basis for the activation of innate defense mechanisms against microbial infection. In 1997, a set of definitions was provided to formalize the description of the components of the mammalian innate immune system (Medzhitov and Janeway, 1997). These authors referred to pathogen surface-derived molecules as pathogen-associated molecular patterns (PAMPs) which bind to pattern recognition receptors, and thereby trigger the transcriptional activation of immune-response genes and subsequent production of antimicrobial compounds (Boman, 1998). Innate immune responses have been thoroughly studied in human, mice, and insects and it was shown that their molecular basis shows remarkable evolutionary conservation across kingdom borders (Aderem and Ulevitch, 2000; Imler and Hoffmann, 2001; Khush and Lemaitre, 2000; Medzhitov and Janeway, 1997; Underhill and Ozinsky, 2002). Unifying features of immune modulators are their highly conserved structures, their functional importance for the microorganism, their presence in various microorganisms, and their apparent absence in potential host organisms. PAMPs that have been

shown to trigger innate immune responses in various vertebrate and non-vertebrate organisms include the lipopolysaccharide (LPS) fraction of Gram-negative bacteria, peptidoglycans derived from Gram-positive bacteria, eubacterial flagellin, bacterial DNA as well as fungal cell wall-derived glucans, chitins, mannans, and proteins (Aderem and Ulevitch, 2000; Hemmi *et al.*, 2000; Imler and Hoffmann, 2001; Thoma-Uszynski *et al.*, 2001; Underhill and Ozinsky, 2002). Intriguingly, many of these molecules have long been recognized to be triggers of non-cultivar-specific defense responses in a multitude of plants (Boller, 1995; Dow *et al.*, 2000; Ebel and Scheel, 1997; Heath, 2000; Nürnberger, 1999). This suggests that plants, like numerous other eukaryotic organisms, have evolved recognition systems for common pathogen-associated surface structures, which initiate intracellular signaling cascades and, ultimately, the activation of protective measures.

Genetic and biochemical dissection of signaling pathways regulating plant pathogen defense has revealed further remarkable similarities with innate defense mechanisms of humans and *Drosophila* (Cohn *et al.*, 2001; Dangl and Jones, 2001; Nürnberger and Scheel, 2001; Takken and Joosten, 2000). For example, work of the Boller group has provided evidence that such similarities extend beyond the nature of the PAMPs recognized, and are found for the corresponding perception systems as well. They showed that a conserved *N*-terminal fragment within eubacterial flagellin, flg22, serves as elicitor of non-cultivar-specific defense responses in a number of plants including *Arabidopsis* (Felix *et al.*, 1999). Moreover, FLS2, a membrane receptor kinase with an extracellular leucine-rich repeat domain was implicated in flagellin perception in *Arabidopsis* (Bauer *et al.*, 2001; Gomez-Gomez and Boller, 2000). Strikingly, this protein shares significant structural homology with *Drosophila* Toll and mammalian Toll-like receptors (TLR), which form a class of 10 plasma membrane proteins with similarity to the Toll receptor family (Underhill and Ozinsky, 2002). Both Toll and TLR receptors are pattern recognition receptors that have key roles in pathogen perception and initiating innate defense mechanisms (Aderem and Ulevitch, 2000; Imler and Hoffmann, 2001; Khush and Lemaitre, 2000; Medzhitov and Janeway, 1997; Underhill and Ozinsky, 2002).

Although it has been useful to study defense pathways triggered by isolated microbial compounds, it must be kept in mind that eukaryotic cells face a multitude of these molecules during attempted microbial infection, and activated defense responses are likely to result from complex recognition events. For example, the mammalian innate immune response to Gram-negative bacteria is triggered through the TLR-mediated recognition of LPS (TLR2/4), flagellin (TLR5) as well as bacterial DNA fragments (TLR9) (Hayashi *et al.*, 2001; Hemmi *et al.*, 2000; Underhill and Ozinsky, 2002; Werts *et al.*, 2001). Similarly, plant cells have recognition systems for a variety of signals derived from individual microbial species. Tobacco and *Arabidopsis* cells recognize *P. syringae*-derived harpins and flagellin (Bauer *et al.*, 2001; Desikan *et al.*, 1999; Felix *et al.*, 1999; He *et al.*, 1993), while tomato cells were shown to perceive fungal chitin fragments, glycopeptides, or ergosterol (Basse *et al.*, 1993; Baureithel *et al.*, 1994; Granado *et al.*, 1995). All of these molecules trigger plant defense responses in a non-cultivar-specific manner. In many cases, defense activation is mediated through high affinity binding sites in the plasma membrane of these plants.

In our longstanding attempt to explore the 'antigenic' potential of *Phytophthora*-derived surface structures to trigger non-cultivar-specific defense responses in parsley, we have previously identified various proteinaceous cell wall fractions to contain active elicitors (Fellbrich *et al.*, 2000; Parker *et al.*, 1991). A 13 amino acid fragment (Pep-13) derived from a surface-exposed loop structure of a 42-kDa *P. sojae* transglutaminase was shown to serve as a recognition determinant for the activation of plant defense responses in parsley through binding to a 100-kDa plasma membrane receptor (Brunner *et al.*, 2002a; Nennstiel *et al.*, 1998; Nürnberger *et al.*, 1994; Nürnberger *et al.*, 1995). Pep-13 was almost invariably found to be associated with transglutaminases from various *Phytophthora* species (Brunner *et al.*, 2002a). The evolutionary conservation of Pep-13 together with its proven indispensability for both elicitor and enzyme activity of the intact protein characterizes it as a genus-specific PAMP mediating pathogen recognition similar to that described for many species from various kingdoms (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 1997; Underhill and Ozinsky, 2002). Receptor binding of Pep-13 evokes an elicitor-specific cytoplasmic calcium signature, production of reactive oxygen species as well as post-translational activation of MAP kinases, all of which are important elements for the transmission of the elicitor signal (Blume *et al.*, 2000; Jabs *et al.*, 1997; Ligterink *et al.*, 1997; Zimmermann *et al.*, 1997). Now, we have isolated a protein elicitor from *P. parasitica*, NPP1, which is found also in the devastating plant pathogens *P. infestans* and *P. sojae*. Using both Pep-13 and NPP1 as triggers of plant defense in parsley enabled us to study the molecular architecture of converging signaling cascades in one plant system. In addition, the ability of NPP1 to activate a multi-facetted defense response in *Arabidopsis* provides the basis for genetic dissection of signal perception and transduction systems employed by *Phytophthora*-derived signals.

Results

Purification of a 24-kDa protein elicitor from P. parasitica *and isolation of the encoding gene*

We have shown previously that parsley cells and protoplasts respond to proteinaceous constituents of hyphal cell

walls from *P. parasitica* with the production of ROS and furanocoumarin phytoalexins (Fellbrich *et al.*, 2000). Activation of these responses differed quantitatively, but not qualitatively from those evoked by the *P. sojae*-derived oligopeptide elicitor, Pep-13 (Fellbrich *et al.*, 2000; Nürnberger *et al.*, 1994). To isolate a protein elicitor structurally different from Pep-13, we chose the culture filtrate of axenically grown *P. parasitica* as starting material. Elicitor-active protein within various protein fractions was monitored by its ability to induce phytoalexin production in parsley protoplasts. As shown in Figure 1(a), a combination of anion exchange chromatography steps yielded a homogenous elicitor preparation containing a 24-kDa protein.

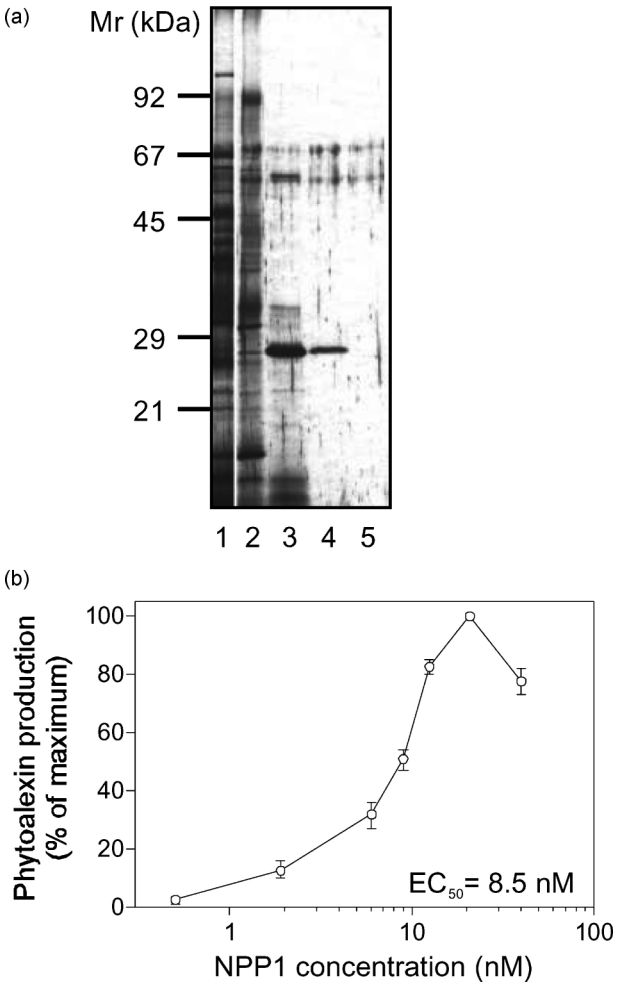

Figure 1. Isolation and phytoalexin production-inducing activity of a *P. parasitica* protein elicitor.
(a) SDS-PAGE of 20 μg of *P. parasitica* culture filtrate protein (1), 10 μg protein eluted from a DE-52 anion exchange chromatographic matrix (2), 0.8 μg protein eluted from HQ/M anion exchange FPLC (3), 0.3 μg protein re-chromatographed by HQ/M anion exchange FPLC (4), and 20 μl SDS-PAGE sample loading buffer (5). Proteins were visualized by silver staining.
(b) Dose–response relationship graph for the elicitor-induced phytoalexin production in parsley protoplasts. Data points represent average values from three independent experiments.

Approximately, 10 μg of pure elicitor protein was obtained from 5 l of culture filtrate. In size exclusion chromatography experiments elicitor activity co-eluted with the molecular size standard protein myoglobin (17 kDa), suggesting that the elicitor protein is a monomer (not shown). Addition of increasing amounts of this protein to parsley protoplasts revealed a concentration of 20 nM to be sufficient to trigger maximum phytoalexin production (Figure 1b). The elicitor concentration required for the stimulation of half-maximal phytoalexin production (EC_{50}) was 8.5 nM as deduced from a sigmoidal dose–response relationship graph (for comparison, $EC_{50,Pep\text{-}13} = 31$ nM; Nürnberger *et al.*, 1994).

Protein micro-sequencing of the *N*-terminus and of internal peptides (Figure 2a) allowed isolation of the encoding gene by means of PCR and rapid amplification of cDNA ends (RACE). In a first step, a 534-bp product was obtained by PCR performed with primers deduced from peptide sequences and *P. parasitica* genomic DNA as template. Subsequently, RACE experiments performed with primers derived from the first PCR product and *P. parasitica* mycelial poly(A)+ RNA, provided sequence information on the complete 3′ and 5′ termini, respectively (Figure 2a). A cDNA encoding the entire open reading frame of 238 codons was obtained by RT–PCR using appropriate primers derived from the RACE products (GenBank accession No. AAK19753). The analysis of protein sequences deduced from the full-length cDNA revealed that all peptide sequences obtained by protein micro-sequencing (representing 102 of 237 aa) were present, strongly suggesting that the isolated cDNA encodes the purified protein elicitor. The *N*-terminus of the mature elicitor protein corresponded to codon 20 of the ORF, which is consistent with a 19 amino acid leader peptide upstream of the mature *N*-terminus. This sequence exhibited the hallmarks of a eukaryotic signal peptide, such as a hydrophobic core region and two small uncharged amino acids at positions -3 and -1, reminiscent of a signal peptidase cleavage site (von Heijne and Abrahmsen, 1989). One putative *N*-glycosylation site was found at codon 67 (Figure 2a). However, an enzyme immunoassay employed for the detection of sugars in glycoconjugates did not reveal a carbohydrate moiety within the purified elicitor protein (not shown). In addition, the deduced molecular mass of the mature protein (23 463 Da) is in good agreement with the molecular mass of the purified protein as determined by MALDI-TOF mass spectrometry (23 498 Da). The mature protein has a predicted pI of 5.03, which is in agreement with the value of 5.1 as determined by isolelectric focusing (not shown).

Expressed sequence tag (EST) sequences found in the *Phytophthora* Genome Initiative Database (Waugh *et al.*, 2000) were used to isolate orthologous genes from *P. infestans* (AAK25828) and *P. sojae* (AAK01636; Qutob *et al.*, 2002), respectively, by RACE/RT–PCR. Sequence alignment revealed the encoded proteins to be highly similar to the

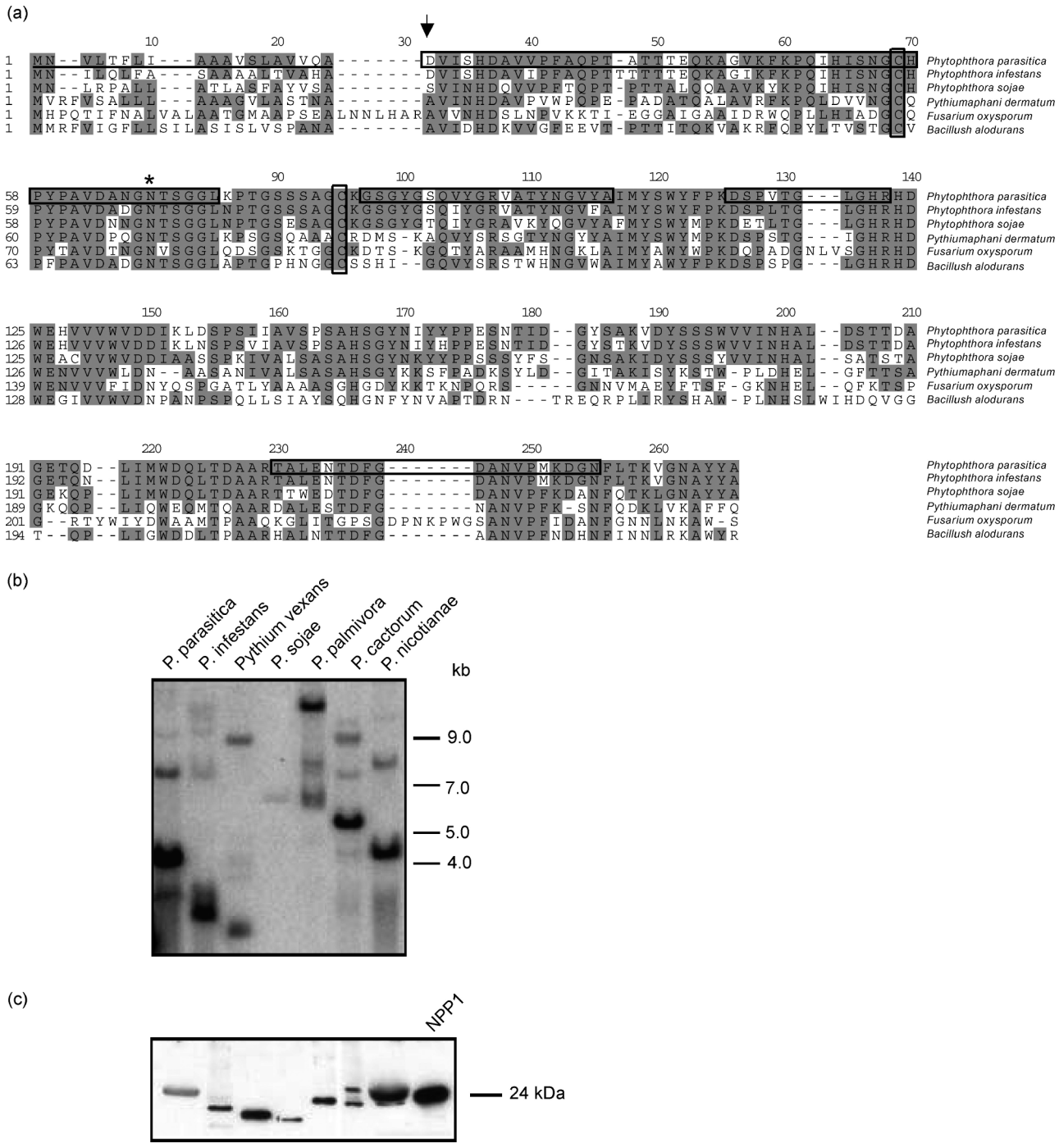

Figure 2. Alignment of the amino acid sequence of the *P. parasitica* elicitor with related protein sequences, and analysis of the encoding genes and gene products in various oomycete species.

(a) Amino acid sequences of the *P. parasitica* protein elicitor and related proteins from *P. infestans* and *P. sojae*, respectively, were deduced from the corresponding cDNAs and aligned to related sequences found in public databases. The *N*-terminal leader peptide is underlined. Horizontally boxed sequences denote peptides which were released from purified protein by tryptic digestion and of which sequence was determined by Edman degradation. Vertical boxes mark cysteine residues conserved between all sequences aligned. The asterisk denotes a potential *N*-glycosylation site. Shaded amino acid residues are those which are conserved in at least two of the sequences aligned.

(b) DNA blot analysis of the protein elicitor-encoding gene. Genomic DNA was isolated from the oomycete species indicated and analyzed as described in Experimental procedures.

(c) Immunoblot analysis of oomycete culture filtrate protein. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, incubated with a polyclonal antiserum raised against a peptide comprising amino acids 54–83 of the *P. parasitica* protein, followed by an incubation with goat antirabbit IgG horseradish peroxidase conjugate, and visualized by chemiluminescence. NPP1, purified mature protein elicitor *from P. parasitica*.

P. parasitica sequence (91 and 76% identity, respectively). In addition, related proteins from another oomycete, *Pythium aphanidermatum* (AAD53944, 51% identity; Veit *et al.*, 2001), from the fungus *Fusarium oxysporum* pv. *erythroxyli* (AAC97382, 37% identity; Nelson *et al.*, 1998), and the bacterial species, *Bacillus halodurans* (BAB04114, 46% identity; Takami *et al.*, 2000) could be retrieved from databases. Interestingly, no sequence similarities to proteins from higher eukaryotic organisms including plants were detected, suggesting these proteins to be a feature of microbial organisms. Algorithms for the prediction of secondary structures did not reveal any characteristic domains within these proteins. However, two cysteine residues (C56 and C82) were found to be fully conserved in all sequences.

DNA blot analysis performed with *P. parasitica* genomic DNA at high hybridization stringency detected one major signal (not shown). However, when genomic DNA from various *Phytophthora* species as well as from *P. vexans* was probed at medium stringency, up to four signals appeared, thus indicating the existence of a small family of related genes in all species tested (Figure 2b). RNA blot analyses performed with *P. parasitica* poly(A)+ RNA detected an mRNA of approximately 0.75 kb, which is in agreement with an ORF of 238 codons (not shown). A peptide antiserum raised against an *N*-terminal motif representing the most conserved region among all orthologous proteins (AA 54–83 of the *P. parasitica* protein) recognized related protein(s) in the culture filtrate of all oomycete species tested (Figure 2c).

NPP1 induces necrotic cell death and ethylene biosynthesis in parsley

Sequence homology of the *P. parasitica* protein elicitor to previously identified plant necrosis and ethylene production-inducing proteins NEP1 from *F. oxysporum* (Bailey, 1995; Nelson *et al.*, 1998) and PaNie from *P. aphanidermatum* (Veit *et al.*, 2001), prompted us to investigate a similar activity of NPP1 in parsley. As shown in Figure 3(a), parsley protoplasts treated with 20 nM NPP1 died within 24 h. The EC_{50} value for this response was determined to be 8 nM, which is very close to that obtained for the elicitor-induced phytoalexin production (Figure 1b). A kinetic analysis of the elicitor-induced cell death (20 nM) showed a significant reduction (24%) of the viability of parsley protoplasts already after 6 h. After 12 h, the viability was reduced by 58% and after 24 h, only 12% of the protoplasts remained viable (not shown, Figure 3a, upper panel). Moreover, when 2.5 μM protein was infiltrated into parsley leaves, necrotic lesions occurred within 4 h (Figure 3a, lower panel). Consequently, we termed the elicitor protein NPP1 (for necrosis-inducing *Phytophthora* protein). Similar to *F. oxysporum* NEP1, infiltration of NPP1 into leaves of tobacco (Figure 4b) or *Arabidopsis* (Figure 6a), but not into leaves of the monocotyledonous plants maize or barley (even when applied at 10 μM, not shown), evoked lesion formation. The NPP1 threshold concentration for lesion formation was 500 nM. Furthermore, treatment of parsley cells with NPP1 stimulated ethylene biosynthesis fivefold within 5 h relative to cells treated with water (Figure 3b). The EC_{50} value determined for the NPP1-induced ethylene production was 3.5 nM (not shown).

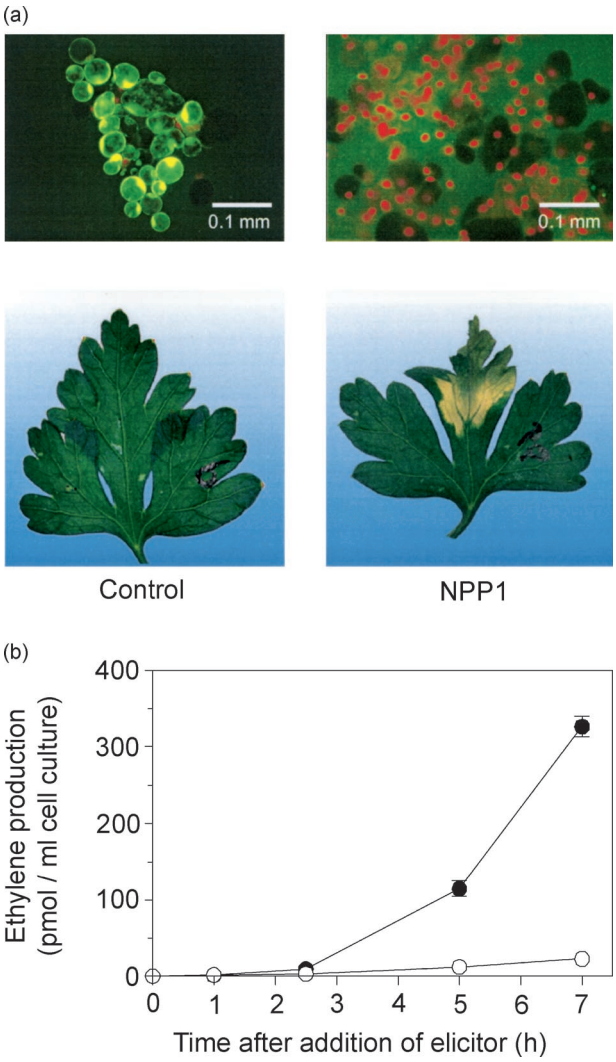

Figure 3. NPP1-induced cell death and ethylene biosynthesis in parsley.
(a) Viability of parsley protoplasts treated with 20 nM NPP1 or water (control) was determined 24 h upon elicitation (upper panel). NPP1 (2.5 μM) or water (control) infiltrated into parsley leaves for 48 h (lower panel). Viability of parsley protoplasts (5×10^5 ml^{-1}) was determined by double-staining with 50 μg ml^{-1} fluorescein diacetate and 10 μg ml^{-1} propidium iodide 24 h after treatment (Jabs *et al.*, 1997).
(b) Kinetics of NPP1 (67 nM)-induced ethylene biosynthesis in cultured parsley cells. Recombinant NPP1 was produced as GST fusion protein in *E. coli* and purified as described in Experimental procedures. Control treatments were performed with 67 nM GST dissolved in water. Data points represent average values from two independent experiments.

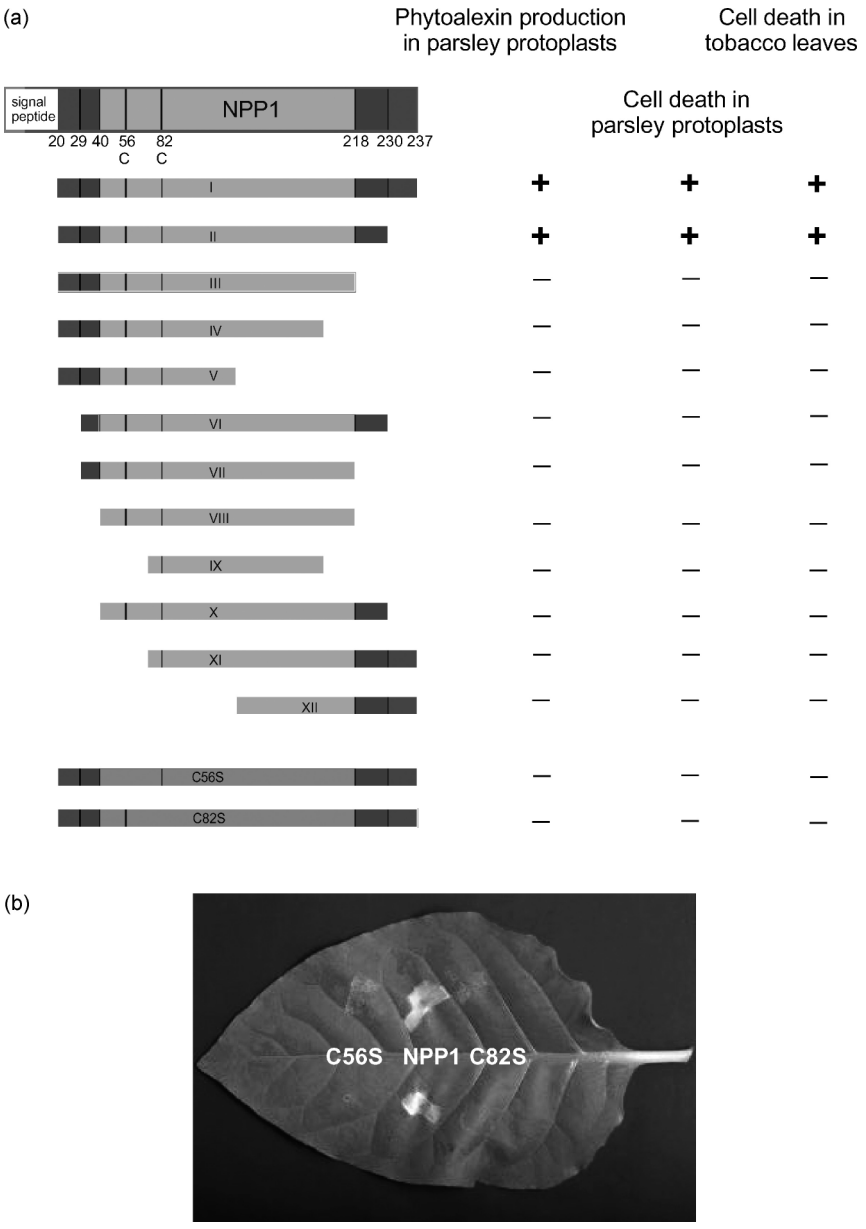

Figure 4. Structural and functional comparison of NPP1 with various deletion derivatives expressed in *E. coli*. cDNAs encoding intact NPP1, NPP1 deletion products, and NPP1 derivatives carrying either C56S or C82S mutations were expressed in *E. coli* as described in Experimental procedures. Recombinant proteins were assessed for their ability to trigger phytoalexin production and cell death in parsley protoplasts at a concentration of 100 nM (a) as well as for their ability to form necrotic lesions in tobacco leaves at a concentration of 2 μM (a, b).

Structural requirements for NPP1 elicitor activity

We have previously shown that an oligopeptide of 13 amino acids (Pep-13) identified within a 42-kDa transglutaminase from *P. sojae* was necessary and sufficient to stimulate a complex defense response in parsley including the biosynthesis of ethylene and phytoalexins (Brunner *et al.*, 2002a; Nürnberger *et al.*, 1994). In order to identify a minimal elicitor motif within NPP1, we synthesized a series of peptides spanning overlapping sequences of the entire mature protein (peptides corresponding to amino acids 20–53, 39–68, 54–83, 69–99, 84–113, 100–129, 114–143, 130–159, 144–173, 160–189,174–203, 190–219, and 204–237). However, neither alone nor in various combinations were these peptides capable of stimulating phytoalexin biosynthesis in parsley protoplasts (not shown).

Recombinant mature NPP1 produced in *E. coli* proved as active as native, purified NPP1 in eliciting phytoalexin production and cell death in parsley protoplasts as well as necrotic lesion formation in tobacco leaves. Likewise, recombinant mature NPP1 from *P. infestans* or *P. sojae* produced in *E. coli* was shown to trigger these responses with the same efficiency as did the *P. parasitica* NPP1 (not shown). Various recombinant deletion derivatives of mature *P. parasitica* NPP1 were produced and assessed for elicitor activity. However, all but one expression

product, carrying an eight amino acid *C*-terminal deletion, lacked elicitor activity (Figure 4a). This finding, together with our failure to identify an elicitor-active minimal peptide motif within NPP1, suggested that the tertiary structure of the entire protein may be a prerequisite for elicitor activity. This view is further supported by the finding that heating the protein for 15 min at 65°C reduced the elicitor activity by 92% (not shown). To assess the importance for elicitor activity of the two cysteine residues that were invariably found in all proteins homologous to NPP1, we employed site-directed mutagenesis to replace either C56 or C82 by serine. As shown in Figure 4, both recombinant mutant proteins were impaired in their abilities to trigger phytoalexin production and cell death in parsley protoplasts and necrotic lesion formation in tobacco leaves.

NPP1 and Pep-13 initiate converging signaling cascades in parsley via separate perception systems

Treatment of cultured parsley cells or protoplasts with either NPP1 or Pep-13 resulted in production and secretion of furanocoumarin phytoalexins (Nürnberger *et al.*, 1994) (Figures 1b and 5f). Thin-layer chromatographic analysis of furanocoumarins secreted by parsley cells 24 h after addition of either elicitor revealed no significant differences in the phytoalexin patterns (Figure 5f). Similarly, as shown in Figure 5(e), both elicitors indistinguishably induced accumulation of transcripts encoding phenylalanine ammonia-lyase, 4-coumarate:coenzyme A ligase as well as ELI12, function of which is unknown. Activation of nearly identical response patterns by both elicitors (the only exception being NPP1-induced cell death in parsley) prompted us to investigate if both signals employed the same or different perception systems in parsley membranes. Therefore, an increasing molar excess of NPP1 was used as competitor in binding assays using [^{125}I]Pep-13 as radioligand. However, at none of the NPP1 concentrations tested binding of [^{125}I]Pep-13 to its receptor was reduced, strongly suggesting the existence of separate perception sites in parsley membranes for either elicitor (Figure 5a).

Changes in the cytoplasmic calcium concentration, calcium-dependent production of reactive oxygen species, and post-translational activation of MAP kinase activity have been identified as necessary elements of Pep-13-induced signaling pathways, mediating phytoalexin production in parsley cells (Blume *et al.*, 2000; Jabs *et al.*, 1997; Ligterink *et al.*, 1997). Since Pep-13 and NPP1 were shown to engage different perception systems for the activation of plant defense responses, we now studied which of these processes were also activated by NPP1. In parsley protoplasts prepared from cell lines stably expressing the calcium indicator apoaequorin, an NPP1-induced increase in cytoplasmic calcium could be monitored. However, the calcium signature mediated by NPP1 varied significantly from that evoked by Pep-13 (Figure 5b). A prolonged lag phase of approximately 300 sec was followed by a slow increase from basal levels of 70 nM Ca^{2+} up to sustained levels of approximately 150 nM. In contrast, in Pep-13-treated parsley cells cytoplasmic [Ca^{2+}] rose sharply after 150 sec and reached plateau levels of approximately 480 nM.

No drastic differences were found in the ability of either elicitor to trigger superoxide anion production in parsley cells (Figure 5c). In the presence of the superoxide dismutase inhibitor, sodium diethyl dithiocarbamate, both elicitors initiated superoxide anion production within 10 min, which proceeded for approximately 1 h. As previously shown for the Pep-13-induced phytoalexin production (Jabs *et al.*, 1997), the NPP1-induced phytoalexin production was inhibited by 10 μM diphenylene iodonium (DPI) (not shown), suggesting that NADPH oxidase activity was required for this plant response. Interestingly, the NPP1-induced death of parsley cells was not affected by DPI (not shown).

An antiserum raised against a Pep-13 responsive parsley MAP kinase (PcMPK6), which is orthologous to the salicylic acid responsive MAPK, SIPK (Kroj, 1999), was employed to precipitate MAP kinase activity from parsley cells treated with either Pep-13 or NPP1, respectively. Proteins precipitated from both cell extracts were examined for phosphorylation of the MAP kinase substrate, myelin basic protein (MBP). As shown in Figure 5(d), post-translationally activated MAP kinase activity could be detected in cells treated with either elicitor. As observed for the elicitor-induced increase in cytoplasmic [Ca^{2+}], the Pep-13-mediated activation of PcMPK6 was faster and more pronounced than that triggered by NPP1. However, despite the obvious quantitative differences in the ability of both elicitors to trigger these early cellular reactions, the qualitative pattern of the responses evoked by both Pep-13 and NPP1, was similar.

NPP1 triggers a complex defense response in Arabidopsis

When infiltrated into leaves of *A. thaliana* accession Col-0, recombinant mature NPP1 (2.5 μM) evoked necrotic lesion formation, while the structural derivative C56S failed to produce a similar phenotype (Figure 6a). Symptoms became visible within 3–4 h and developed into brownish, dry leaf sections after 24–48 h. The lesion phenotype on *Arabidopsis* accessions Ler, Ws-0, Blanes-4, Mt-0, C24, Nossen, Umkirch-4, and Halle was indistinguishable from that on Col-0 (not shown). Addition of NPP1 to *Arabidopsis* leaf pieces resulted in the production of ethylene within 2 h, which was comparable to that stimulated by the bacterial elicitor, flg22 (Felix *et al.*, 1999) (Figure 6b). In addition, NPP1, but not its derivative C56S, triggered production of reactive oxygen species within 3 h upon infiltration into

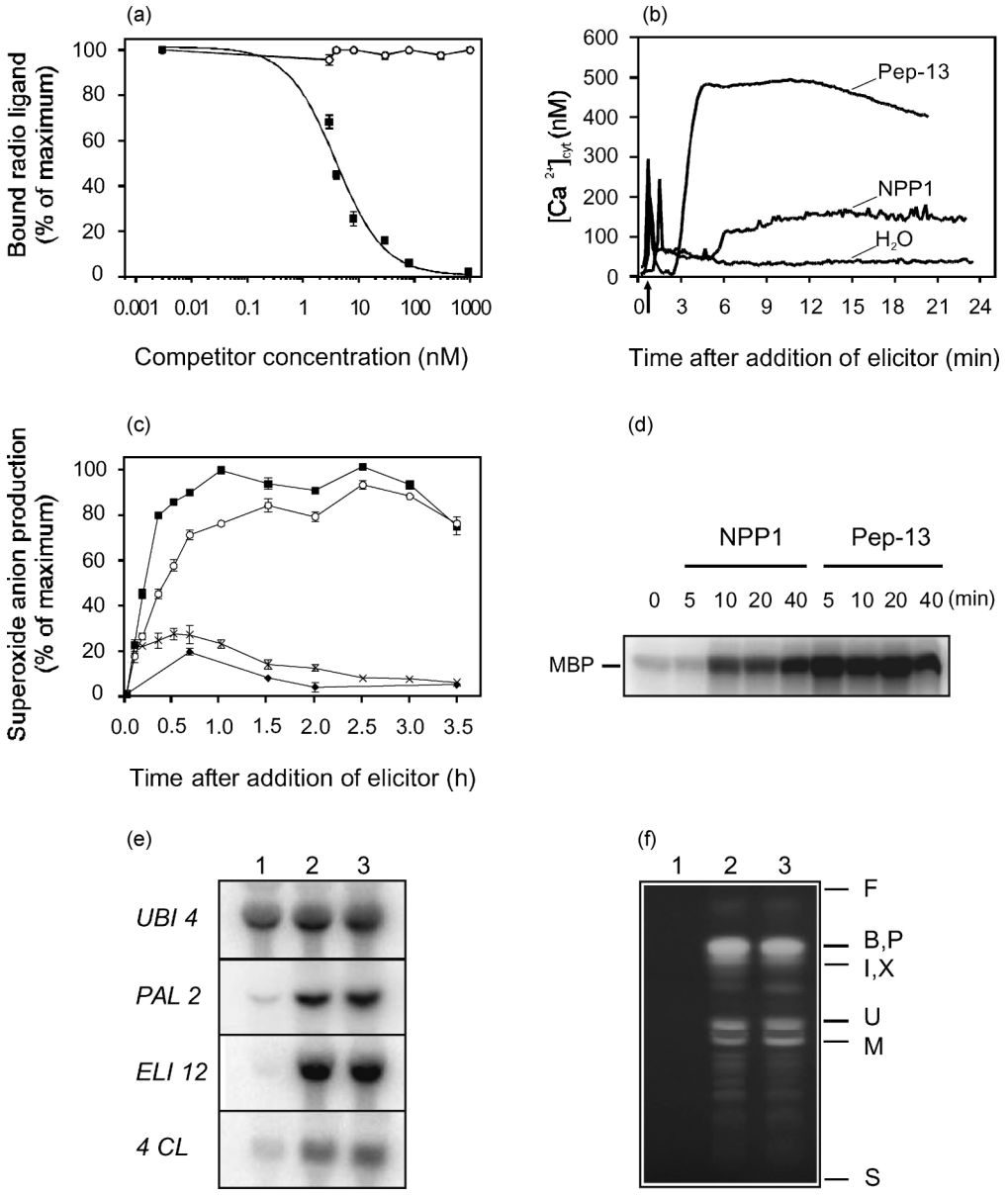

Figure 5. NPP1 and Pep-13 trigger converging signaling cascades in parsley.
(a) Competition of binding of [^{125}I]Pep-13 to parsley microsomal membranes by increasing concentrations of Pep-13 (closed symbols) or NPP1 (open symbols). Data points represent average values from three independent experiments.
(b) Elicitor-induced changes in cytoplasmic [Ca^{2+}] in apoaequorin-transformed parsley protoplasts treated with water, 100 nM Pep-13, or 20 nM NPP1, respectively. Very rapid, transient increases in chemiluminescence were observed indistinguishably upon administration of any compound, and are thus considered to be non-specific. One out of three experiments performed is shown.
(c) Elicitor-induced production of superoxide anions in parsley cells treated with 100 nM Pep-13 (■), 20 nM recombinant NPP1 (○), water (x) or 20 nM GST (◆), respectively. Experiments were performed in duplicate in the presence of 0.5 mM diethyl dithiocarbamate as superoxide dismutase inhibitor.
(d) Activation of MAP kinase activity in parsley cells treated with 100 nM Pep-13 or 20 nM NPP1, respectively. Cells were harvested at the times indicated and subjected to protein extraction. Immunoprecipitation of the tobacco SIPK ortholog from parsley and quantification of MAPK activity (phosphorylation of myelin basic protein, MBP) was as described in Experimental procedures.
(e) Elicitor-induced transcript accumulation in parsley cells treated for 3 h with water (1), 20 nM NPP1 (2) or 100 nM Pep-13 (3), respectively. [α-^{32}P]dATP-labeled cDNAs encoding parsley polyubiquitin (*UBI4*), phenylalanine ammonia-lyase (*PAL2*), the product of the elicitor-responsive gene, *ELI12*, and *4CL* were hybridized to total RNA prepared from elicitor-treated parsley cells.
(f) Thin-layer chromatographic analysis of furanocoumarin phytoalexins prepared from parsley cells treated for 24 h with water (1), 20 nM NPP1 (2), or 100 nM Pep-13 (3), respectively. Furanocoumarins were visualized under UV light. B, bergapten; I, isopimpinellin; M, marmesin; P, psoralen; U, umbelliferone; X, xanthotoxin; F, front; S, start.

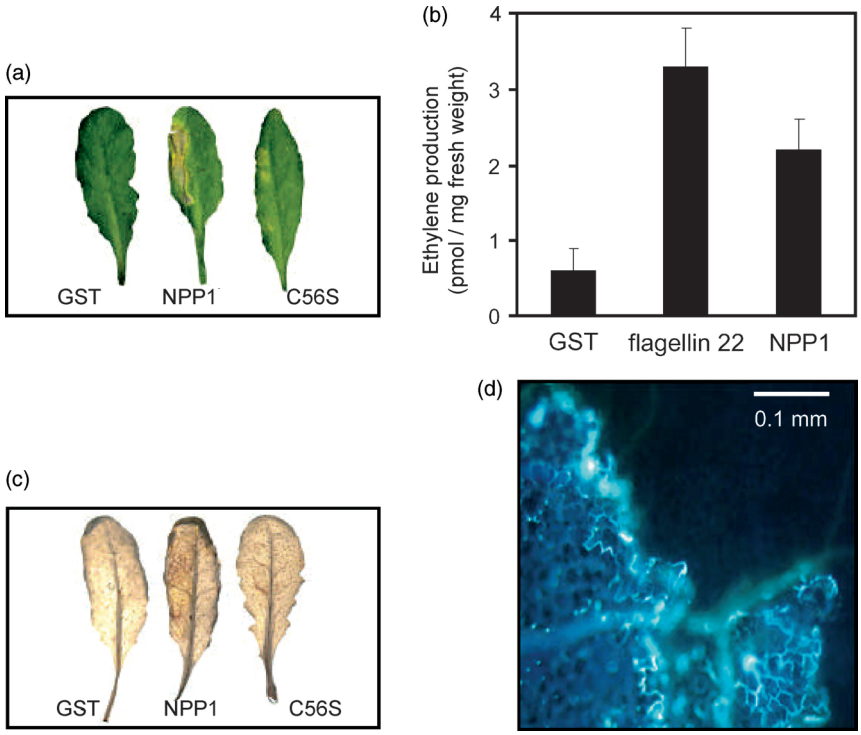

Figure 6. NPP1 induces a complex defense response in *A. thaliana* Col-0. Infiltrations were performed with 2.5 μM of each recombinant NPP1, mutant protein C56S, or GST as control. necrotic lesion formation 48 h upon elicitation (a), ethylene production in leaf pieces 2 h upon elicitation (b), production of reactive oxygen species 3 h upon elicitation (c), and callose apposition 24 h upon elicitation (d). Representative results from experiments performed in triplicate are shown. In (b), ethylene production stimulated by bacterial flagellin (flg22) was used as a positive control.

Arabidopsis leaves, thus preceding the onset of lesion formation (Figure 6c). Moreover, cell wall reinforcement due to the apposition of callose at the interface between necrotic and healthy leaf tissue could be detected 24 h after application of NPP1 (Figure 6d). As shown in Figure 7(a), NPP1 triggered the rapid accumulation of transcripts encoding the pathogenesis-related protein 1 (PR1) as well as of a recently identified *NDR1/HIN1*-like *Arabidopsis* gene, *NHL25* (Varet *et al.*, 2002). Interestingly, NPP1 carrying a mutation in C56 lacked the ability to trigger transcript accumulation of either gene (Figure 7a).

Several loci have been genetically defined to contribute to cultivar-specific activation of defense responses, such as *PR1* expression in *A. thaliana* Col-0 infiltrated with avirulent pathogens (Feys and Parker, 2000). Here, we assessed the effect of mutations in such loci on the NPP1-mediated *PR1* expression, and compared it to that evoked upon infiltration of avirulent *P. syringae* pv. *tomato* DC3000 (*avrRpm1*) (Figure 7b). Both NPP1 and bacteria-induced *PR1* expression were found to be dependent on salicylic acid, as no transcript accumulation could be detected in salicylate-deficient *nahG* plants. In contrast, mutants insensitive to the plant hormones ethylene (*ein2-1*) or jasmonate (*coi1-1*) were not impaired in their ability to trigger *PR1* expression in response to both NPP1 or *P. syringae* pv. *tomato* DC3000 (*avrRpm1*). NDR1, but not PAD4, is assumed to contribute to the activation of plant resistance responses specified by LZ-NBS-LRR-type resistance genes, such as *RPM1*, conferring resistance to *P. syringae* pv. *tomato* DC3000 (*avrRpm1*) (Feys and Parker, 2000). Vice versa, PAD4, but not NDR1, was found to be implicated exclusively in the activation of defense pathways specified by TIR-NBS-LRR resistance genes, such as *RPP2*, conferring resistance to *Peronospora parasitica*, race Cala2 (Aarts *et al.*, 1998; Feys *et al.*, 2001). Intriguingly, unlike *P. syringae* pv. *tomato* DC3000 (*avrRpm1*)-mediated *PR1* expression, which showed the expected requirement for NDR1, but not for PAD4, NPP1-mediated *PR1* expression was found to be dependent on both functional NDR1 and PAD4 (Figure 7b).

Discussion

Recognition of molecular patterns common to microbial organisms is a key event in the activation of innate defense systems in vertebrates and insects (Aderem and Ulevitch, 2000; Imler and Hoffmann, 2001; Medzhitov and Janeway, 1997; Underhill and Ozinsky, 2002). Research over the past decade has provided compelling evidence that plants have evolved recognition capacities for similarly complex arrays of microbial surfaces (Boller, 1995; Dow *et al.*, 2000;

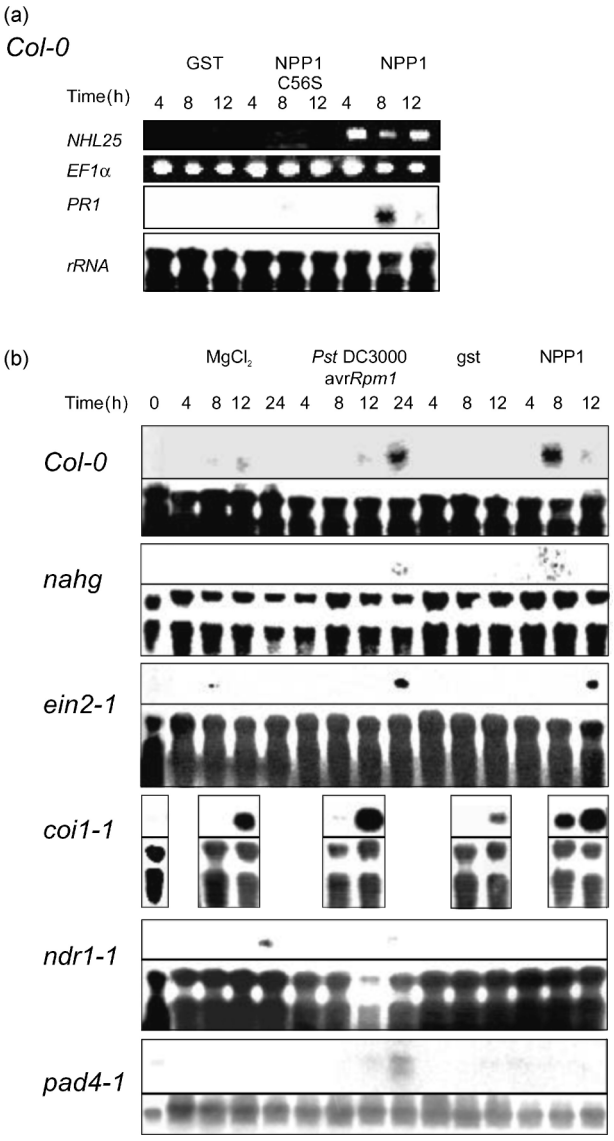

Figure 7. NPP1-mediated *PR* gene expression in *Arabidopsis* wild-type and mutant plants. Representative results from experiments performed in triplicate are shown.

(a) *A. thaliana* Col-0 plants were infiltrated with recombinant NPP1 or mutant NPP1 carrying the amino acid substitution C56S (2.5 μM each) for the times indicated. Control inoculations were performed with 2.5 μM GST. Three leaves per plant from three individual plants were harvested at the indicated time points, pooled, and subjected to RNA preparation. Expression of the *NDR1/HIN1*-like gene, *NHL25*, was analyzed by RT–PCR using total RNA and gene-specific primers. *EF1α* expression was used as control in RT–PCR assays. *PR1* expression was monitored by RNA gel blot analysis using total RNA and [α-^{32}P]dATP-labeled *PR1* cDNA as described in Experimental procedures. Equal loading of RNA was checked by methylene blue staining after blotting.

(b) *A. thaliana* Col-0, Col-0 expressing the *nahG* gene (*nahG*), *and* Col-0 mutant alleles *ein2-1*, *coi1-1*, *ndr1-1*, or *pad4-1* were infiltrated with 2.5 μM NPP1 or avirulent *P. syringae* pv. *tomato* DC3000(*avrRpm1*) (1×10^8 cfu ml^{-1}), respectively, for the times indicated. Control inoculations were performed with 2.5 μM GST (for NPP1 infiltrations) or 10 mM $MgCl_2$ (for bacterial infiltration). Monitoring of *PR1* expression was as in (a) with the exception that RNA loading in the *pad4-1* line was checked by RNA blot analysis using [α-^{32}P]dATP-labeled *18S rRNA* cDNA.

Nürnberger, 1999). For example, the cell walls of many phytopathogenic fungi harbor chitins, *N*-mannosylated glycopeptides, and ergosterol, all of which have been reported to trigger plant defense responses (Basse *et al.*, 1993; Baureithel *et al.*, 1994; Granado *et al.*, 1995; Ito *et al.*, 1997). Similarly, various phytopathogenic Gram-negative bacteria harbor plant defense-stimulating lipopolysaccharides and flagellin fragments and produce harpins upon contact with plants (Dow *et al.*, 2000; Felix *et al.*, 1999; He, 1996; Lee *et al.*, 2001). Moreover, phytopathogenic oomycetes of the genus *Phytophthora* have been shown to contain defense-eliciting heptaglucan structures, elicitins, and other cell wall (glyco)proteins (Mithöfer *et al.*, 2000; Nürnberger *et al.*, 1994; Ricci *et al.*, 1989; Umemoto *et al.*, 1997). Common to all of these signals is that they evoke plant defense responses in a non-cultivar-specific manner. However, recognition of such signals was often reported to be restricted to only a few plant species. Here, we report isolation and characterization of a protein elicitor from *P. parasitica* (NPP1), which belongs to a protein family widespread among microorganisms. NPP1 and related proteins were found to evoke a complex defense response including cell death in an exceptionally large number of different plant species. Homologous proteins with the same activity profile have been found in other species of the genus *Phytophthora*, such as *P. infestans* or *P. sojae* (our findings; Qutob *et al.*, 2002). Plants responding to NPP1 and homologous proteins are as diverse as parsley, tobacco, *Arabidopsis*, or soybean (our findings; Qutob *et al.*, 2002). Similarly, NPP1-related proteins have been reported from another oomycete, *P. aphanidermatum* and the true fungus, *F. oxysporum* pv. *erythroxyli* (Bailey, 1995; Veit *et al.*, 2001). The activity spectrum of the latter protein was shown to comprise as many as 20 out of 24 tested dicotyledonous plants, but no monocotyledonous plants (Bailey, 1995). This is consistent with our finding that monocotyledonous plants (maize, barley) did not show any cell death symptoms after infiltration of NPP1, suggesting that non-self recognition capacities between monocotyledonous and dicotyledonous plants vary considerably. This view is also supported by the apparent insensitivity of rice cells to the bacterial flagellin fragment, flg22, which triggered plant defense in a variety of dicotyledonous plants (Felix *et al.*, 1999). However, rice cells appear to have the ability to recognize bacterial flagellin, but the structural properties of the defense-eliciting 'epitope' is likely to differ from flg22 (Che *et al.*, 2000).

The phylogenetically wide distribution of NPP1 homologous proteins in oomycete, fungal as well as bacterial species (structural homologs are present in *B. halodurans*, *Streptomyces coelicolor* and *Vibrio* spp. (our findings; Qutob *et al.*, 2002), but apparently not in higher eukaryotes including plants, suggests that these proteins are a common feature of microbial organisms and are, thus, likely of

central importance to their life style. Such functions could range from involvement in the organization of the microbial cell wall to direct action as microbial toxin. Identification of the intrinsic function of these proteins, their role during the life cycle of oomycetes, and/or its possible implication in oomycete pathogenicity will be most appropriately addressed by inactivation of the encoding gene. Isolation of the *NPP1* gene and establishment of gene silencing technologies for diploid oomycetes (van West *et al.*, 1999) now provide the tools for this experiment.

The presence in a broad spectrum of microbial organisms, the absence in potential host organisms, and the ability to evoke defense responses in those hosts are characteristics of pathogen-associated molecular patterns, which trigger innate defense systems in vertebrates or *Drosophila* (Aderem and Ulevitch, 2000; Underhill and Ozinsky, 2002). NPP1 and related proteins may therefore be considered an element of a *Phytophthora*-associated molecular pattern which, upon recognition, triggers innate defense mechanisms in dicotyledonous plants. At present, however, it cannot be ruled out completely that NPP1 may trigger plant defense responses rather indirectly. For example, due to elusive intrinsic enzyme activity or due to toxic activity, NPP1 may facilitate the release of an endogenous elicitor from the plant plasma membrane (NPP1 induces defense responses in parsley protoplasts), which itself may be the trigger of defense responses in numerous dicotyledonous plants. Instability to heat of NPP1 elicitor activity and our failure to identify a minimal motif within NPP1 to be sufficient for elicitor activity may support this view. On the other hand, NPP1 does not show any similarity to known enzymes or toxins, and release of plant plasma membrane-derived elicitors by microbial enzymes is thus far unprecedented in the literature. Moreover, since a tertiary structure motif within NPP1 appears to be responsible for elicitor activity, it may not be surprising that (i) such a structure may be susceptible to heat denaturation and (ii) that we failed to identify a 'minimum' elicitor motif within the intact NPP1 protein. In general, identification of NPP1 broadens our understanding of the plant defense-inducing ('antigenic') potential of *Phytophthora* species, and adds to the known elements β-heptaglucans (recognized in Fabaceae; Mithöfer *et al.*, 2000), elicitins (tobacco, radish; Ricci *et al.*, 1989), and Pep-13 as part of a *Phytophthora* transglutaminase (parsley, potato; Brunner *et al.*, 2002a; Nürnberger *et al.*, 1994).

As both NPP1 and Pep-13 have been shown to induce overlapping patterns of defense responses in parsley (this paper; Nürnberger *et al.*, 1994), this system offers possibilities to study the molecular architecture of converging signaling pathways and possible synergisms between them in one plant. Both molecules are present in the cell walls of the same *Phytophthora* species and both have the potential to trigger plant defense reactions upon contact with parsley plants. We have shown that perception of either signal requires distinct binding sites. However, early elicitor-induced responses (increase in cytoplasmic [Ca^{2+}], ROS production, MAP kinase activation) likely to be implicated in elicitor signal transmission are activated by both triggers, although quantitative differences have been observed (which are also indicative of the existence of different perception systems). This suggests that signaling cascades initiated by structurally different molecules and mediated through specific perception modules converge rapidly, and employ components commonly implicated in the activation of non-cultivar-specific as well as cultivar-specific plant defense (Boller, 1995; Cohn *et al.*, 2001; Dangl and Jones, 2001; Nürnberger and Scheel, 2001). Such similarities have indeed not only been found among signaling cascades triggered by non-race-specific elicitors, such as flagellin, elicitins, Pep-13, or NPP1, but are also reported from *Cf-9*-transformed tobacco cells treated with AVR9 (Romeis *et al.*, 1999; Takken and Joosten 2000). In our system, it will now be interesting to study if simultaneous recognition of both NPP1 and Pep-13 will have co-operative or synergistic effects on the activation of defense responses, which may result from more sensitive pathogen perception by the plant. Taken together, recognition by plants of complex structures characteristic of whole genera of potential pathogens and subsequent activation of defense responses is very likely to occur in many plant–pathogen interactions. Such a scenario is reminiscent of the activation of innate defense responses in mammals, which was, for example, shown to be activated by Gram-negative bacteria-derived LPS, flagellin, and unmethylated CpG dinucleotides (Hayashi *et al.*, 2001; Hemmi *et al.*, 2000; Underhill and Ozinsky, 2002; Werts *et al.*, 2001).

In comparison to the activation of plant cultivar-specific resistance, relatively little is known about the signaling pathways mediating non-cultivar-specific plant resistance (Cohn *et al.*, 2001; Dangl and Jones, 2001; Feys and Parker, 2000). Since the latter work has benefitted substantially from forward genetic screens in *Arabidopsis*, an enormous gain of information can be expected from experiments aiming at delineating signaling cascades implicated in the activation of non-cultivar-specific plant defense. Isolation of *Phytophthora*-derived NPP1 and characterization of its defense-inducing potential in the model plant, *Arabidopsis*, now provides suitable tools for such an approach. Towards this end, identification of the *FLS2* locus and biochemical characterization of the FLS2 protein as the flg22 receptor can serve as paradigm (Bauer *et al.*, 2001; Felix *et al.*, 1999; Gomez-Gomez and Boller, 2000). It is assumed that such screens will unveil loci undetected in previous approaches, which aimed at analyzing cultivar-specific defense pathways or pathways implicated exclusively in the activation of non-cultivar-specific defense responses. This is illustrated by our analysis of *Arabidopsis*

mutants impaired in the activation of cultivar-specific defense responses, which revealed that NPP1-mediated *PR1* gene expression was salicylic acid dependent and required NDR1 and PAD4 (Figure 7). This is remarkable, as it has often, but not exclusively been reported (activation of the *EDS5* gene in Col-0 plants infiltrated with avirulent *P. syringae* pv. *tomato* DC3000 (*AvrRpt2*); Nawrath *et al.*, 2002) that activation of cultivar-specific defense reactions (including *PR1* expression) in response to avirulent pathogens required either PAD4 or NDR1, but not both proteins (Aarts *et al.*, 1998; Dangl and Jones, 2001; Feys *et al.*, 2001). Cultivar-specific resistance specified by *TIR-NBS-LRR* resistance genes was usually found to require PAD4, while activation of defense pathways governed by LZ-NBS-LRR-type resistance genes was dependent on NDR1 (Aarts *et al.*, 1998; Dangl and Jones, 2001; Feys *et al.*, 2001). Thus, signaling pathways for plant defense responses in addition to those already defined are likely to exist. Therefore, forward genetic approaches towards the identification of additional loci implicated in the activation of plant defense responses will help unravel similarities and differences in the molecular basis of cultivar-specific and-non-cultivar-specific plant defense.

Experimental procedures

Materials

Synthetic elicitors Pep-13, flg22, and NPP1 fragments were synthesized as described (Lee *et al.*, 2001). An antiserum raised in rabbits against an *N*-terminal fragment of NPP1 comprising amino acid residues 54–83 was obtained from Eurogentec (Herstal, Belgium) 66 days after immunization.

Oomycete and plant cultivation

P. parasitica strain 1828 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Other oomycete isolates were obtained from INRA (Unité Santé Végétale et Environnement, Antibes, France). *P. cactorum*, *P. infestans*, *P. nicotianae*, *P. palmivora* and *P. vexans* were grown in liquid Henniger medium (Henniger, 1959), while *P. parasitica* and *P. sojae* were grown in liquid asparagine medium (Keen, 1975). Dark-grown parsley cell cultures were maintained and parsley protoplasts were prepared as described (Nürnberger *et al.*, 1994). *Arabidopsis* Col-0, Col-0 *nahG*, and Col-0 mutant alleles *ein2-1*, *coi1-1*, *ndr1-1*, *pad4-1* were grown in a phytochamber (Heraeus Voetsch, Balingen, Germany) at 22°C under short-day conditions (8/16 h light/dark) in a 1 : 1 soil/sand mix. Parsley (*Petroselinum crispum* L. cv. Hamburger Schnitt), tobacco (*Nicotiana tabacum* L. cv. Samsun NN), maize (*Zea mays*), and barley (*Hordeum vulgare* cv. Salome) were grown on 1 : 1 soil/sand mix in the greenhouse at 23–25°C (16-h light period).

Elicitation of plant responses in parsley

Binding of [^{125}I]Pep-13 to parsley microsomal membranes, accumulation of superoxide anions in elicitor-treated parsley cells, elicitor-induced changes in the cytoplasmic Ca^{2+} concentration in apoaequorin-expressing parsley cells, and parsley MAP kinase activity were quantified as described (Blume *et al.*, 2000; Jabs *et al.*, 1997; Kroj, 1999; Nürnberger *et al.*, 1994). RNA blot analysis of transcripts encoding parsley polyubiquitin (*UBI4*), phenylalanine ammonia-lyase (*PAL2*), elicitor-induced protein ELI12 (*ELI12*), and 4-coumarate:CoA-ligase (*4CL*) was carried out as described (Fellbrich *et al.*, 2000). Elicitation and quantification of phytoalexin production in parsley cells and protoplasts was as described (Blume *et al.*, 2000; Nürnberger *et al.*, 1994) with the modification that parsley cells were incubated in B5-medium (Gamborg *et al.*, 1968) supplemented with 13.9 mg ml^{-1} $FeSO_4 \cdot 7H_2O$ and 18.6 mg ml^{-1} Na_2-EDTA. Thin-layer chromatographic analysis of parsley furanocoumarins was performed as described (Nürnberger *et al.*, 1994). Viability of parsley protoplasts (5×10^5 ml^{-1}) was determined by double-staining with 50 μg ml^{-1} fluorescein diacetate and 10 μg ml^{-1} propidium iodide 24 h after treatment (Jabs *et al.*, 1997). Ethylene production in cultured parsley cells was determined as described (Chappell *et al.*, 1984).

Elicitation of plant defense responses in Arabidopsis, tobacco, maize, and barley

NPP1 dissolved in water was infiltrated abaxial into leaf tissue by using needleless 1 ml plastic syringes (Roth, Karlsruhe, Germany). Routinely, infiltrations were performed on 5-week-old *Arabidopsis*, 4-week-old tobacco, and 4-week-old maize and barley plants. Leaves were harvested 48 h after infiltration to monitor lesion formation. Measurement of ethylene production in *A. thaliana* was performed on 2–3 mm slices (approximately 20 mg fresh weight per assay) floated overnight on water. The leaf slices were transferred to 6-ml glass tubes containing 0.5 ml H_2O or NPP1 dissolved in H_2O. After vacuum infiltration (3 min), vials were closed with rubber septa and placed horizontally on an orbital shaker (100 r.p.m.). Ethylene accumulating in the aerial space was measured by gas chromatography after 2 h of incubation. Determination of callose apposition and H_2O_2 production in *Arabidopsis* leaves was performed as described (Gomez-Gomez *et al.*, 1999; Thordal-Christensen *et al.*, 1997). Infiltration into *Arabidopsis* leaves of *P. syringae* pv. *tomato* strain DC3000 (*avrRpm1*) was carried out with bacterial suspensions of 10^8 cfu ml^{-1} in 10 mM $MgCl_2$ ($OD_{600} = 0.2$) (Varet *et al.*, 2002). Leaf material was harvested at the time points indicated and frozen in liquid N_2. Plant RNA was prepared from ground material using Trizol (Invitrogen Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. Total RNA was used for RNA blot (20 μg) or reverse transcription-polymerase chain reaction (RT–PCR) analyses (0.2 μg), respectively. RT–PCR was performed using the Ready-to-Go® RT–PCR system (AP Biotech, Freiburg, Germany). First-strand cDNA synthesis was primed using oligo-dT primer for 30 min at 42°C. Amplification of constitutively expressed elongation factor 1α (EF1α) served as an internal control in RT–PCR assays (Lee *et al.*, 2001). PCR cycling was 1 cycle 95°C, 4 min; 25 cycles of 10 sec at 94°C, 10 sec at 60°C, 40 sec at 72°C; 1 cycle of 10 min at 72°C). Primers were 5′-TCACATCAACATTGTGGTCATTGGC-3′ (forward) and 5′-TTGATCTGGTCAAGAGCCTCAAG-3′ (reverse) for EF1α, 5′-CCAAGACACAGCAAGCAGCACC-3′ (forward) and 5′-CCCGAGTTTGATCCGAACCG-3′ (reverse) for *NHL25*. For RNA blot analyses, *PR1*-encoding DNA was labeled with [α-^{32}P]-dATP using the Megaprime kit (AP Biotech, Freiburg, Germany). Hybridization was performed in 5× SSPE, 5× Denhardt's reagent, 0.1% SDS, 100 μg ml^{-1} denatured salmon sperm DNA, 50% formamide at 42°C for 16 h. Subsequently, filters were washed twice for 20 min at 60°C in 0.5× SSC/0.1% SDS, and for 10 min in 0.1×

SSC/0.1% SDS. Signal quantification was performed by phosphoimaging (STORM II, AP Biotech, Freiburg, Germany).

Purification of NPP1

P. parasitica was grown in liquid culture for 5 weeks before the culture filtrate (5 l) was harvested, cleared by filtration through a 40-μm nylon mesh, and concentrated by lyophilization. Freeze-dried material was dissolved in 50 mM Tris–HCl, pH 8.0 and subjected to anion exchange chromatography on DE-52 cellulose (C16/40, AP Biotech, Freiburg, Germany) equilibrated in the same buffer. After protein loading the matrix was washed with 5 column volumes of 50 mM Tris–HCl, pH 8.0. Bound proteins were eluted with a linear gradient of 0–500 mM KCl in 50 mM Tris–HCl, pH 8.0 for 360 min at a flow rate of 1 ml min^{-1}. Eluted material was fractionated in 5 ml portions, and absorbance was monitored at 280 nm. Elicitor-active fractions were de-salted by dialysis against 20 mM Bis–Tris, pH 6.0, and applied onto a POROS HQ/M anion exchange column (Perkin Elmer Biosystems, Weiterstadt, Germany) equilibrated with 20 mM Bis–Tris, pH 6.0 by using a Hewlett Packard 1090 HPLC device (Waldbronn, Germany). Bound proteins were eluted with a linear gradient of 20 mM Bis–Tris, pH 6.0/20 mM Bis–Tris, pH 6.0, 500 mM KCl for 30 min at a flow rate of 1 ml min^{-1}. Absorbance was monitored continuously at 280 nm. Eluted fractions (0.5 ml) were collected automatically (fraction collector 201, Gilson, Middleton, USA) and, upon dialysis against 20 mM Bis–Tris, pH 6.3, subjected to re-chromatography on POROS HQ/M using the same salt gradient buffered at pH 6.3.

Isolation and characterization of the NPP1 cDNA

Genomic DNA prepared from frozen mycelium of *P. parasitica* (Brunner *et al.*, 2002b) as template and the following primers (forward, 5′-AAGTTCAAGCCICAAATICATAT-3′; reverse, 5′-TTCATIGGIACATTIGCATCICCGAAGTC-3′) were used in a PCR reaction, which yielded a 534-bp product, from which RACE primers (5′-RACE, 5′-AGTGGATGCGAACGGCAATACCAG; 3′-RACE, 5′-TTGATCCCACATGATCAGATCCTG-3′) for completion of the terminal sequences were deduced. RACE was performed with the SMART RACE cDNA amplification kit (Clontech, Palo Alto, USA) and poly(A)+RNA according to the supplier's instructions. Total RNA prepared from frozen mycelium of *P. parasitica* (Brunner *et al.*, 2002b) was used for isolation of poly(A)+ RNA using the PolyA-Tract mRNA isolation system (Promega, Madison, USA). PCR fragments were cloned into pGEM-T (Promega, Madison, USA) and sequenced. The primers (forward, 5′-CCCCGAATTCATGA-ACGTCCTTACGTTC-3′ encoding an *Eco*RI restriction site; reverse, 5′-GAAACTCGAGCTAAGCGTAGTAAGCGTT-3′ encoding an *Xho*I restriction site) derived from RACE fragments and *P. parasitica* genomic DNA (Brunner *et al.*, 2002b) as template were used to obtain NPP1 full-length cDNA by PCR. PCR was performed with 10 ng genomic DNA, 0.5 μM primers, 20 nM deoxynucleotides, 15 mM $MgCl_2$, 5 U Taq-polymerase (Invitrogen Life Technologies) as follows: 1 cycle, 1 min at 94°C; 25 cycles, 20 sec at 94°C, 30 sec at 68°C, 1 min at 72°C; 1 cycle, 7 min at 72°C. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and introduced directly into pGEM-T. Full-length cDNAs encoding NPP1 from *P. infestans* and *P. sojae*, respectively, were isolated by the same strategy with the exception that primers for the first PCR were deduced from EST sequences found in the *Phytophthora* Genome Initiative Database (Waugh *et al.*, 2000). For DNA blot analysis, genomic DNA (2 μg) from various oomycete mycelia was prepared, digested with *Sac*I, separated on 0.8% agarose, blotted onto nylon membranes (Hybond-N, AP Biotech, Freiburg, Germany), and hybridized to the 534 bp NPP1 fragment labeled with [α-^{32}P]dATP as described (Brunner *et al.*, 2002b). RNA blot analysis of poly(A)+ RNA prepared from *P. parasitica* was carried out as in (Brunner *et al.*, 2002b).

Heterologous expression of NPP1 and its structural derivatives

A cDNA encoding mature NPP1 was obtained by PCR as described for the production of NPP1 full-length cDNA using NPP1 cDNA as template and the following primers: NPP1 forward, 5′-CGTCGAATTCGACGTGATCTCGCACGAT-3′ encoding an *Eco*RI restriction site; NPP1 reverse, 5′-GAAACTCGAGCTAAGCGTAG-TAAGCGTT-3′ encoding an *Xho*I restriction site. The purified PCR fragment was cleaved with *Eco*RI/*Xho*I, introduced into expression vector pGEX-5X-1 (AP Biotech, Freiburg, Germany) and transformed into *E. coli* strain BL21. PCR fragments encoding mature NPP1 from *P. infestans* and *P. sojae*, respectively, were generated using the appropriate cDNAs as template, forward primers 5′-CGCGGAATTCGATGTGATTTCACACGAT-3′ (*P. infestans*) or 5′-CGTGGAATTCAGCGTTATCAACCACGAC-3′ (*P. sojae*), respectively, and the reverse primer used for amplification of *P. parasitica* mature NPP1. Replacement by serine (TCC) of cysteine 56 (TGT) (C56S) or cysteine 82 (TGC) (C82S) within native *P. parasitica* NPP1 was achieved by PCR. Both codons are preceded by a glycine residue-encoding codon (GGC), which upon mutation into another glycine-encoding codon (GGA), created a *Bam*HI restriction site in the following primers: C56S forward, 5′-GGATCC-CATCCGTATCCTGCAGTG-3′; C56S reverse, 5′-GGATCCGTTCGA-GATGTGGATCTG-3′; C82S forward, 5′-GGATCCAAGGGCTCCG-GATACGGC-3′; C82S reverse, 5′-GGATCCCGCACTAGAGGAAC-CGGT-3′. Four primer combinations (NPP1 forward/C56S reverse, C56S forward/NPP1 reverse, NPP1 forward/C82S reverse, C82S forward/NPP1 reverse) and template cDNA encoding mature NPP1 were used for PCR amplification of the *N*-terminal and *C*-terminal halves of C56S and C82S NPP1 derivatives, respectively. PCR conditions were as described before. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and introduced directly into pGEM-T for DNA sequencing. DNA fragments encoding either C56S or C82S constructs were released from the vector by treatment with *Bam*HI, *Eco*RI, and *Xho*I, and introduced into pGEX-5X-1 before transformation of *E. coli* strain BL21.

Transformed BL21 cultures grown overnight and subsequently transferred into LB-medium containing 100 μg ml^{-1} ampicillin, were grown at 37°C to an OD_{600} of 0.6, supplemented with 0.4 mM isopropyl-D-thiogalactoside and cultivated for 4 h at 37°C under constant agitation (180 r.p.m.). Bacteria were resuspended in 10 mM NaH_2PO_4, 1.8 mM K_2HPO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.0, containing 10 mM DTT, 0.001% PMSF and treated with lysozyme (100 μg ml^{-1}) for 15 min on ice. One per cent lauryl sarcosine was added to the extracts prior to sonication (three times 20 sec at 4°C). Supernatants were cleared by centrifugation (5000 *g*, 20 min, 4°C), supplemented with 2% Triton X-100 and 0.2% glutathione (GSH) agarose (Sigma, Deisenhofen, Germany), and agitated gently for 12 h at 4°C. GSH agarose was pelleted (2000 *g*, 2 min, 4°C), washed three times with PBS containing 1 M NaCl followed by five washes with PBS alone. Bound proteins were eluted for 10 min in two volumes of 50 mM Tris–HCl, pH 8.0, containing 10 mM glutathione. NPP1 was released from glutathione-S-transferase by factor Xa (AP Biotech, Freiburg, Germany) treatment (10 U mg^{-1} protein) for 16 h at 25°C. Recombinant NPP1 was dialyzed against water, aliquoted and stored at −80°C. For expression of NPP1 deletion products, cDNAs spanning the respective

protein portions were constructed by PCR as described above with cDNA-derived primers encoding *Eco*RI (forward) or *Xho*I (reverse) restriction sites, respectively.

Since NPP1 preparations contained contaminations of GST, control inoculations with GST dissolved in H_2O were carried out in all experiments performed with recombinant NPP1 or its structural derivatives.

Protein biochemistry

For Western blot analyses of oomycete culture filtrates, proteins (50 μg) were separated on 12.5% SDS-PAGE and, subsequently, transferred electrophoretically (100 V, 1 h, 25 mM Tris–HCl, pH 8.3; 0.192 M glycine, 20% methanol) to nitrocellulose membranes (Porablot NCL, Macherey & Nagel, Düren, Germany). Membranes were incubated with a polyclonal antiserum raised against a peptide comprising amino acids 54–83 of the *P. parasitica* protein (1 : 5000 dilution) followed by an incubation with goat antirabbit IgG horseradish peroxidase conjugate (1 : 3000 dilution). Immunodetection was performed using the ECL Plus chemiluminescence detection kit (AP Biotech, Freiburg, Germany). Glycosylation of NPP1 was investigated using a Glycan Detection Kit (Roche Diagnostics, Mannheim, Germany).

To release internal NPP1 peptide fragments, purified protein (50 pmol) was dissolved in 100 μl 100 mM Tris–HCl, pH 8.5, containing 200 ng trypsin, sequencing grade (Roche Diagnostics) and was incubated for 24 h at 37°C. Peptides were separated by reverse phase HPLC as described (Nürnberger *et al.*, 1994). Purified peptide fragments or intact NPP1 were dissolved in 2% trifluoroacetic acid and subjected to Edman degradation using a Hewlett-Packard G1050 device (Waldbronn, Germany).

Acknowledgements

We gratefully acknowledge Helga Nixdorf, Jutta Elster, and Christel Rülke for technical assistance as well as Fred Rosche's (probiodrug Halle) assistance in MALDI-TOF analyses. We thank H. Keller and P. Venard (INRA, Unité Santé Végétale et Environnement, Antibes) for providing oomycete isolates, and Jeff Dangl (UNC, Chapel Hill, USA) for providing *P. syringae* pv. *tomato* DC3000(*avrRpm1*). Many thanks go to Jason Rudd for language editing. Arabidopsis Col-0 *nahG* seeds were obtained from Bob Dietrich (Syngenta, Research Triangle Park, USA). *Arabidopsis* Col-0 mutants *coi1-1*, *ndr1-1*, and *pad4-1* were a kind gift of Paul Schulze-Lefert (MPIZ Köln, Germany). The Col-0 *ein2-1* mutant was obtained from the Ohio State University *Arabidopsis* Biological Resource Center (Columbus, USA). We are grateful to Dierk Scheel and Thomas Boller for critical reading of the manuscript. This work was supported by the European Community (Grant No. BIO4-CT96-0101), the Kleinwanzlebener Saatzucht AG, Einbeck, Germany, and the Alexander-von-Humboldt Foundation.

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The EMBO Journal Vol. 21 No. 24 pp. 6681–6688, 2002

Pep-13, a plant defense-inducing pathogen-associated pattern from *Phytophthora* transglutaminases

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Innate immunity, an ancient form of defense against microbial infection, is well described for animals and is also suggested to be important for plants. Discrimination from self is achieved through receptors that recognize pathogen-associated molecular patterns (PAMPs) not found in the host. PAMPs are evolutionarily conserved structures which are functionally important and, thus, not subject to frequent mutation. Here we report that the previously described peptide elicitor of defense responses in parsley, Pep-13, constitutes a surface-exposed fragment within a novel calcium-dependent cell wall transglutaminase (TGase) from *Phytophthora sojae*. TGase transcripts and TGase activity are detectable in all *Phytophthora* species analyzed, among which are some of the most destructive plant pathogens. Mutational analysis within Pep-13 identified the same amino acids indispensable for both TGase and defense-eliciting activity. Pep-13, conserved among *Phytophthora* TGases, activates defense in parsley and potato, suggesting its function as a genus-specific recognition determinant for the activation of plant defense in host and non-host plants. In summary, plants may recognize PAMPs with characteristics resembling those known to trigger innate immune responses in animals.

Keywords: elicitor/innate immunity/pathogen-associated molecular pattern/*Phytophthora*/transglutaminase

Introduction

The innate immune response is a well-studied phenomenon in human, mice and insects, and its molecular basis shows remarkable evolutionary conservation across kingdom borders (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000). Activation of inflammatory responses or production of antimicrobial compounds relies on the recognition through Toll-like receptors (TLRs) of pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000; Imler and Hoffmann, 2001; Underhill and Ozinsky, 2002). Common features of such immune modulators are their highly conserved structures, their functional importance for the microorganism and their presence in a broad range of microbial species. Recognized PAMPs that trigger innate immune responses include bacterial lipopolysaccharide (LPS), lipoproteins and flagellin, in addition to fungal cell wall-derived carbohydrates and proteins (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000; Imler and Hoffmann, 2001; Underhill and Ozinsky, 2002). Plants also possess non-self recognition systems (receptors) for numerous microbe-derived molecules which mediate activation of plant defense responses in a non-cultivar-specific manner and have been described as 'general elicitors' (Heath, 2000; Cohn *et al*., 2001; Dangl and Jones, 2001). These include β-heptaglucan structures from oomycete cell walls, fungal cell wall chitin fragments and an N-terminal fragment of bacterial flagellin, flg22 (Felix *et al*., 1999; Nürnberger and Scheel, 2001). In particular, flg22, which was found in several but not all bacterial flagellins, triggered defense responses in a range of different plants (Felix *et al*., 1999). However, in none of the cases to date has any such motif been shown to be indispensable for the host microbe and, hence, to be physiologically equivalent to the PAMPs described for humans and *Drosophila*.

TGases (*R*-glutaminyl-peptide:amine-γ-glutamyltransferase, EC 2.3.2.13), which catalyze an acyl transfer reaction between peptide-bound glutamine residues and primary amines including the ε-amino group of peptide-bound lysine residues, form intra- or intermolecular isopeptide bonds resulting in irreversible protein cross-linking (Folk, 1980; Aeschlimann and Paulsson, 1994). TGase activity has been implicated in a variety of physiological activities in animals, including neuronal growth and regeneration, bone development, angiogenesis, wound healing, cellular differentiation and apoptosis (Liu *et al*., 2002). However, no physiological function has been elucidated for either bacterial, fungal or plant TGases.

We have previously identified a peptide fragment (Pep-13), within an abundant cell wall glycoprotein (GP42) from the phytopathogenic oomycete *Phytophthora sojae*, that was necessary and sufficient for receptor-mediated defense gene expression and synthesis of antimicrobial phytoalexins in parsley (Nürnberger *et al*., 1994; Hahlbrock *et al*., 1995). Now, we provide evidence that GP42 is a *P.sojae* cell wall-associated Ca^{2+}-dependent TGase, which is the first such enzyme reported from an oomycete species. TGases with a highly conserved Pep-13 motif were found in all *Phytophthora* species analyzed. Mutational analysis within the Pep-13 motif revealed that the same amino acid residues that were shown to be important for plant defense-eliciting activity in parsley (Nürnberger *et al*., 1994) and potato were also essential for

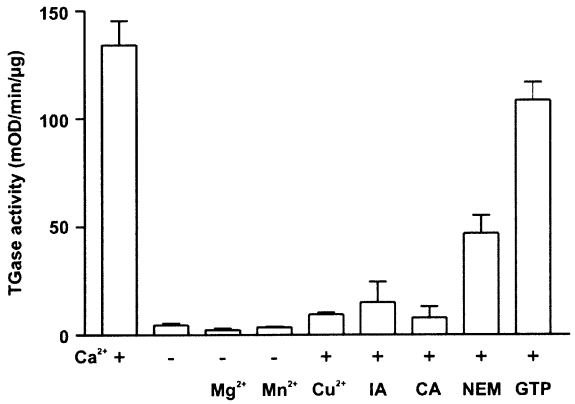

Fig. 1. TGase activity of the *P.sojae* GP42 elicitor protein. Recombinant *P.sojae* GP42 was expressed in *A.oryzae* and assayed for TGase activity in the presence (+) or absence (–) of 5 mM Ca^{2+}. The specificity for Ca^{2+} was determined by replacement with 5 mM Mg^{2+} or 5 mM Mn^{2+}, respectively. TGase inhibitors, including 1 mM Cu^{2+}, 10 µM iodoacetamide (IA), 10 mM cystamine (CA) or 10 mM *N*-ethylmaleimide (NEM), inhibited this Ca^{2+}-dependent activity. No significant inhibition was observed in the presence of 5 mM GTP. Bars represent the mean values ± SD of three independent experiments.

TGase activity. Our data support the intriguing view that plants may have evolved receptors that recognize genus-specific, 'epitope-like' motifs present within, and essential for, the function of pathogen-derived molecules. Thus, Pep-13 exhibits characteristics reminiscent of PAMPs modulating innate immune responses in vertebrate and invertebrate organisms (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000; Underhill and Ozinsky, 2002). Implications of PAMP-mediated pathogen recognition for the activation of defense responses, in both host plants (potato) and non-host plants (parsley), will be discussed.

Results

The P.sojae GP42 is a TGase

We have previously reported that an oligopeptide fragment (Pep-13) identified within a 42 kDa glycoprotein elicitor from *P.sojae* (GP42) is necessary and sufficient to trigger a multifaceted defense response in parsley (Nürnberger *et al*., 1994; Hahlbrock *et al*., 1995; Jabs *et al*., 1997; Ligterink *et al*., 1997; Zimmermann *et al*., 1997; Blume *et al*., 2000). Since the GP42 amino acid sequence exhibited significant homology to a recently purified *Phytophthora cactorum* TGase (our unpublished data), we tested whether the *P.sojae* GP42 possessed TGase activity. Therefore, a solid-phase microtiter plate assay based on the incorporation of 5-(biotinamido) pentylamine into *N*,*N*-dimethylcasein was employed. TGase activity was shown to be associated with both recombinant (Figure 1) and purified GP42 (not shown). The activity of both purified and recombinant *P.sojae* TGase was strictly dependent on Ca^{2+} [$K_{M(pentylamine)}$ = 0.249 mM at 5 mM Ca^{2+}], which could not be substituted by 5 mM Mg^{2+} or Mn^{2+}. The TGase inhibitors Cu^{2+}, iodoacetamide, cystamine and *N*-ethylmaleimide blocked this Ca^{2+}-dependent activity efficiently. GTP, an inhibitor of human tissue TGase (Melino and Piacentini, 1998), did not significantly affect TGase activity. In addition, as reported for guinea pig liver TGase (Folk, 1980), the oomycete enzyme catalyzed Ca^{2+}-dependent auto-oligomerization (data not shown). In summary, the *Phytophthora* TGase shares biochemical characteristics of mammalian Ca^{2+}-dependent TGases (Folk, 1980; Aeschlimann and Paulsson, 1994; Melino and Piacentini, 1998). The lack of sequence homology to any TGases present in databases (Sacks *et al*., 1995) suggests, however, that GP42 belongs to a novel class of these enzymes.

The Pep-13 motif is highly conserved among Phytophthora TGases

Non-self recognition through phytopathogen-derived PAMPs requires that the motifs selected as recognition determinants are not present within the recipient organism, but are widely found among various microbial species (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000; Imler and Hoffmann, 2001; Underhill and Ozinsky, 2002). Database analysis of all plant sequences, including the fully sequenced *Arabidopsis thaliana* genome (The Arabidopsis Genome Initiative, 2000), against the complete TGase sequence (Sacks *et al*., 1995), or against the Pep-13 sequence (NCBI Blast Search for short nearly exact matches), suggested that plants possess neither orthologs of the *Phytophthora* TGase nor proteins containing peptide motifs with Pep-13 elicitor activity (not shown). In contrast, genomic DNA blot analysis demonstrated that several species of the oomycete genus *Phytophthora*, but not of the related genus *Pythium*, possess a gene family encoding GP42 TGase-related proteins (Sacks *et al*., 1995). A RT–PCR analysis performed on poly(A)$^+$ RNA from 10 *Phytophthora* species revealed the presence of GP42 TGase homologs in all species tested (Figure 2A). However, no GP42 TGase-related transcripts were obtained from *Pythium vexans*, *Phytophthora undulata*, the latter being taxonomically more closely related to *Pythium* species (Erwin and Ribeiro, 1996), or from the obligate biotroph *Peronospora parasitica* (not shown). Consistently, a peptide antiserum raised against the Pep-13 motif of *P.sojae* GP42 recognized a protein of ~42 kDa in the culture filtrate of all *Phytophthora* species tested that was not detected in *P.undulata* or *P.vexans* (Figure 2A). A zymogram of TGase activity associated with these 42 kDa proteins confirmed that homologs of GP42 possessing TGase activity were present in all *Phytophthora* species (Figure 2A). Interestingly, enzyme activity prepared from *Phytophthora infestans* was reproducibly found to be associated with an 85 kDa protein, which cross-reacted with the anti-Pep-13 antiserum and most likely represents a TGase dimer (not shown).

Analysis of the partial TGase sequences at the deduced amino acid level revealed >60% identity between all sequences (Figure 2B). Remarkably, the sequence comprising Pep-13, the peptide fragment essential for activation of defense responses, was highly conserved among all species analyzed (Figure 2B). The only exception involved a tyrosine residue (corresponding to Y241 in the *P.sojae* protein), which in two species was replaced by another aromatic amino acid, phenylalanine. A synthetic peptide containing this amino acid substitution

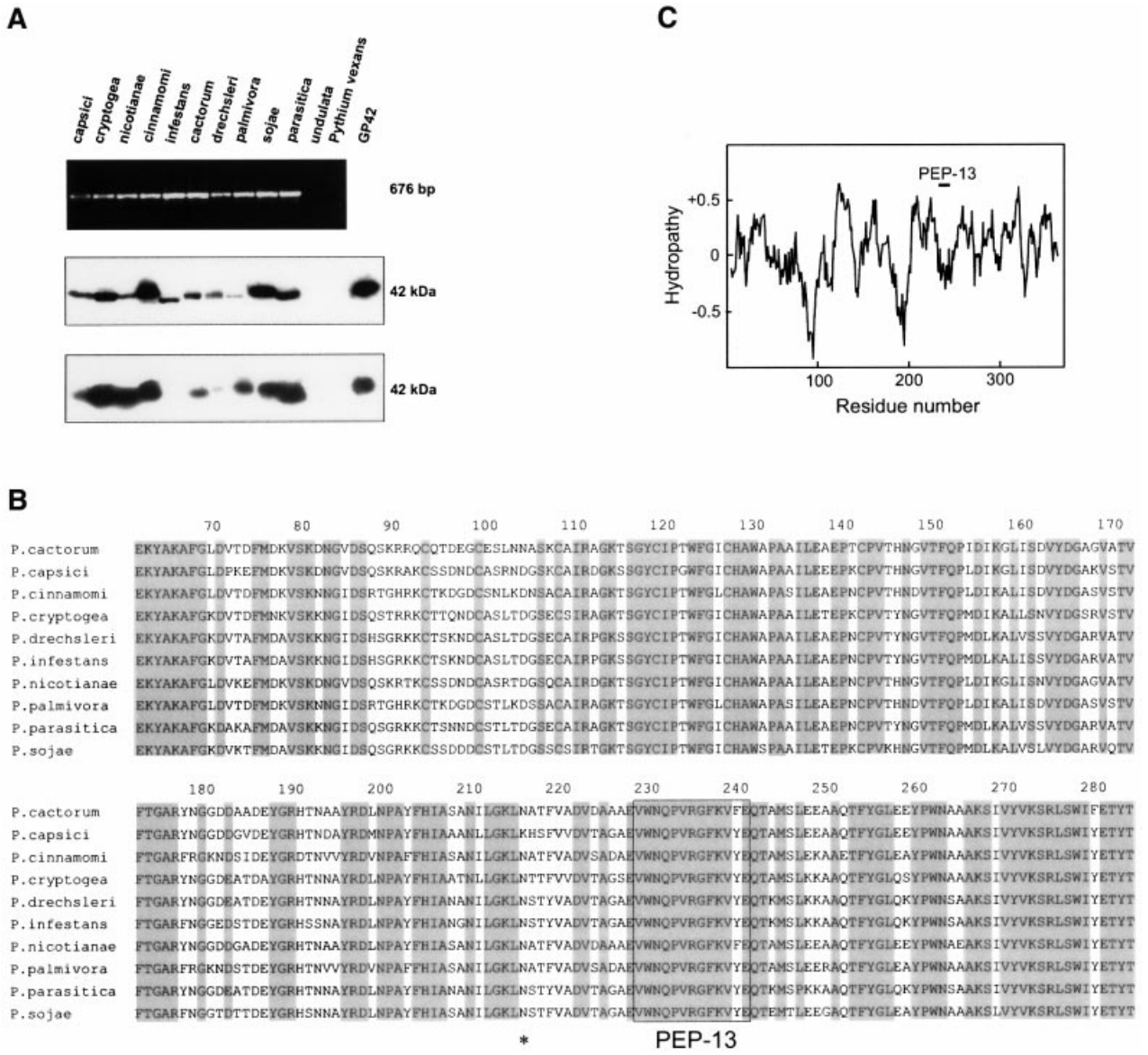

Fig. 2. GP42 TGase homologs containing the surface-exposed Pep-13 motif are highly conserved among the genus *Phytophthora*. (**A**) RT–PCR demonstrated the presence of GP42 TGase-related transcripts in *Phytophthora* species (upper panel). Immunoblot analysis of culture filtrates (50 µg protein/lane) using anti-Pep13 antibodies revealed that each species possessed a GP42-like protein containing the Pep-13 motif (middle panel). In-gel TGase assays demonstrated that the GP42-related proteins possessed TGase activity (lower panel). Purified *P.sojae* GP42 (100 ng) was used as a positive control for both immunodetection and TGase activity (lane GP42). (**B**) The alignment of the deduced amino acid sequences of the RT–PCR products highlights the conservation of the Pep-13 motif (boxed). The asterisk marks the position of the sole *N*-glycosylation site of the *P.sojae* GP42. (**C**) The hydropathy plot based on the Eisenberg algorithm (Eisenberg *et al*., 1984) predicts the Pep-13 motif to reside in a hydrophilic region of the protein.

was found to bind to the Pep-13 receptor and activate defense responses in parsley in a manner indistinguishable from Pep-13 (data not shown). Hydrophobicity analysis predicted Pep-13 to be present within a hydrophilic region of the enzyme (Figure 2C). In addition, secondary structure prediction analysis (Rost, 1996) suggested that Pep-13 resides in a surface-exposed loop structure containing the sole *N*-glycosylation site (Parker *et al*., 1991; Sacks *et al*., 1995). Thus, the strong sequence conservation and surface exposure of the Pep-13 motif are consistent with its role as a recognition determinant for the activation of plant defense responses during the interaction with *Phytophthora* species.

W231 and P234 are important for both elicitor activity and TGase activity

The strict conservation of the Pep-13 motif within the *Phytophthora* TGases prompted us to investigate whether this sequence was important for enzyme activity. We had previously shown that Pep-13 was sufficient for the activation of plant defense responses by intact GP42 (Nürnberger *et al*., 1994). In addition, replacement within Pep-13 of each individual amino acid revealed W231 and P234 to be important for elicitor activity (Nürnberger *et al*., 1994). Moreover, conservative mutations W231F (phenylalanine) and P234Hyp (hydroxyproline) retained the ability of the Pep-13 mutants to trigger phytoalexin

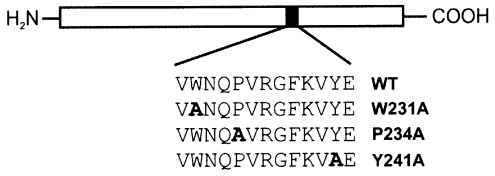

Fig. 3. Schematic representation indicating the position of mutations introduced into the Pep-13 sequence of the *P.sojae* GP42 TGase. WT represents the wild-type GP42 sequence. W231A, P234A and Y241A correspond to single amino acid exchanges of Trp231, Pro234 and Tyr241 with alanine, respectively. All proteins were heterologously expressed in *A.oryzae* and prepared from culture filtrate as described in Materials and methods.

Table I. Mutational analysis of GP42 reveals amino acid residues indispensable for both TGase and elicitor activities[a]

Protein	TGase activity (mOD/min/μg)	Elicitor activity [EC_{50} (nM)]
WT TGase	104.0 ± 22.8	0.5
W231A	1.5 ± 1.3	–[b]
P234A	6.5 ± 2.6	11.2
Y241A	98.9 ± 2.2	1.1

[a]Elicitor activity of TGase is expressed as the EC_{50} value, which corresponds to the protein concentration required to half-maximally stimulate phytoalexin production in parsley protoplasts (Parker *et al.*, 1991).
[b]No detectable activity (tested up to 50 nM).

production in parsley, whereas non-conservative mutations W231T (threonine) and P234I (isoleucine) abolished the elicitor activity of these mutant peptides (not shown). Thus, non-conservative mutations introduced into the codons encoding either W231 or P234 would be likely to impair TGase-mediated recognition of *P.sojae* by the plant. To test whether such mutations would affect the TGase activity, *P.sojae* wild-type GP42 TGase and mutant proteins containing single alanine exchanges within the Pep-13 sequence (Figure 3) were expressed and purified from *Aspergillus oryzae*. In control experiments, neither TGase activity nor TGase protein was detected in culture supernatants of *A.oryzae* transformed with an empty expression vector (not shown). Both elicitor and TGase activities of the mutant proteins were determined and compared with the activity of the wild-type protein. Significantly, mutations that compromised the ability of the protein to elicit defense responses in parsley protoplasts also markedly reduced TGase activity (Table I). Substitution of Trp231 by alanine (W231A) resulted in a 98% reduction in TGase activity. This substitution also abolished the elicitor activity of the protein and thus we were unable to determine an EC_{50} value. Replacement of Pro234 by alanine (P234A) resulted in a reduction in TGase activity to ~6% of wild-type activity and a concurrent 20-fold higher EC_{50} value for elicitor activity. In contrast, substitution of Tyr241 by alanine (Y241A) had only a modest effect on both TGase and elicitor activities. These data demonstrate that amino acid residues important for the TGase activity of the GP42 protein are identical to those necessary to elicit defense reactions in parsley protoplasts. Thus, it appears that the evolutionary stability of this functionally indispensable epitope may have favored its selection as a PAMP that is recognized by the plant in order to detect and respond to attack by *Phytophthora* species.

Pep-13-mediated defense responses in potato

The interaction between potato and *P.infestans*, the causal agent of late blight disease, can result in devastating crop losses, as illustrated by the Irish potato famine of the 19th century (Govers, 2001; Kamoun, 2001). Both infection of potato plants and treatment of potato cells with *P.infestans* culture filtrate triggered defense gene expression and the synthesis of antimicrobial compounds (Rohwer *et al.*, 1987; Göbel *et al.*, 2001). We therefore tested whether potato has the ability to recognize and respond to the conserved Pep-13 motif. Treatment of potato cells with Pep-13 led to the accumulation of defense-related transcripts encoding lipoxygenase, 4-coumarate:CoA ligase and pathogenesis-related protein 1 (Figure 4). Likewise, increased transcript levels of the same genes were detected in intact potato leaves upon infiltration of Pep-13 (not shown). Dose–response experiments revealed that 10 nM Pep-13 was sufficient to induce accumulation of defense gene transcripts in potato cells (Figure 4). We next tested whether activation of defense in potato through recognition of Pep-13 resembled that described for parsley, using the Pep-13 mutant derivatives. No defense-related transcript accumulation was detectable in potato cells treated with Pep-13 containing the W231A mutation (Figure 4), even at higher concentrations (1 μM), demonstrating that this mutation abolishes the ability of Pep-13 to elicit defense responses in potato. This compares favorably with our failure to measure phytoalexin production in parsley protoplasts treated with GP42 TGase containing the W231A mutation. However, treatment of potato cells with Pep-13 containing the P234A mutation induced defense gene activation (Figure 4) only at concentrations significantly higher than wild-type Pep-13 (⩾100 nM). This is in agreement with a significantly higher EC_{50} value for initiation of defense responses in parsley following treatment with the corresponding GP42 TGase mutant. Thus, the ability of Pep-13 to elicit defense responses in potato correlates quantitatively and qualitatively with the elicitor activity described for parsley. These data suggest that this recognition capacity for *Phytophthora* species is a more widespread feature of plants, and that the Pep-13 motif could function as a PAMP for the activation of innate defense reactions during these interactions.

Discussion

Here we describe for the first time a microbial TGase that is Ca^{2+} dependent. Microbial TGases known to date are of bacterial or plasmodial origin, but these enzymes are very sequence divergent from the animal and *Phytophthora* TGases, and their enzyme activities were found to be independent of Ca^{2+} (Kanaji *et al.*, 1993; Adini *et al.*, 2001). Ca^{2+}-dependent TGases have been isolated from a variety of animal species (Aeschlimann and Paulsson, 1994; Noguchi *et al.*, 2001; Ahvazi *et al.*, 2002), but not from fungi (including yeast) or plants. Interestingly, *Phytophthora* TGases also lack sequence similarity to all of the known Ca^{2+}-dependent TGases, but share with these enzymes (in those cases where tested) a similar co-factor

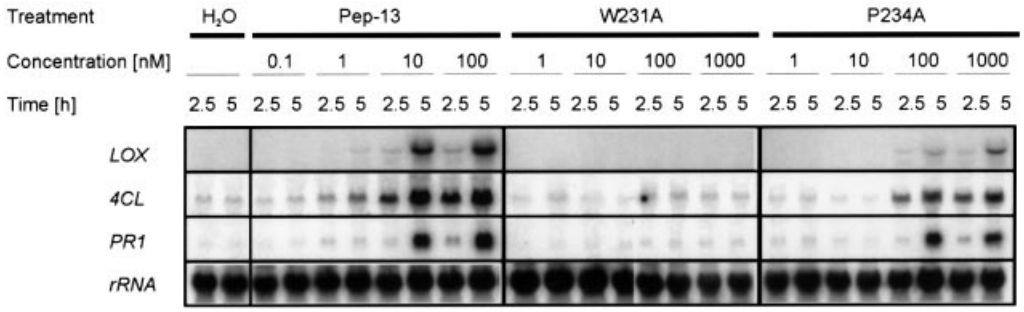

Fig. 4. Functionally indispensable residues selected as recognition determinants for the activation of defense in parsley also serve as defense-inducing determinants in potato. RNA isolated from potato cells 2.5 and 5 h following treatment with Pep-13 (0.1–100 nM), or with Pep-13 mutant derivatives W231A or P234A (1–1000 nM), was analyzed for the accumulation of defense-related transcripts. The RNA blots were probed with radiolabeled cDNA fragments encoding lipoxygenase (*LOX*), 4-coumarate:CoA ligase (*4CL*), pathogenesis-related protein 1 (*PR1*) and 25S rRNA (*rRNA*) as loading control.

requirement and inhibitor sensitivity. Limited, but significant, sequence similarity between *Phytophthora* and mammalian TGases, as well as cysteine proteases, was observed in the regions adjacent to the catalytic site cysteine residue of these enzymes (our unpublished data). Thus, it is intriguing to speculate whether the apparent sequence dissimilarity between the TGases is the result of divergent evolution or, alternatively, whether similarities in the active site domain are indicative of convergent evolution.

Seminal reviews (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000; Imler and Hoffmann, 2001; Underhill and Ozinsky, 2002) have highlighted striking similarities between the molecular organization of innate immunity in vertebrates and insects. The authors referred to pathogen-derived signals as PAMPs, which enable potential host cells to discriminate between potential microbial pathogens and self, no matter whether these microbes are pathogenic or not. Receptor-mediated PAMP recognition results in the production of antimicrobial compounds. PAMPs are not only shared by particular pathogen races, but are broader signatures of a given class of microorganisms. They constitute evolutionarily conserved structures that are unique to microorganisms, have important roles in microbial physiology and may therefore not be subject to frequent mutation. Typical PAMPs include LPS of Gram-negative bacteria, bacterial flagellin, and fungal cell wall-derived carbohydrates or proteins, some of which were also shown to trigger plant defense in a non-cultivar-specific manner (Boller, 1995; Nürnberger and Brunner, 2002). The concept of PAMP-mediated non-self recognition has found renewed interest among plant biologists, as it may provide an explanation for why plants may recognize and respond to non-race-specific elicitors of plant defense.

In this study, we attempted to show whether Pep-13 exhibits characteristics of PAMPs. Such studies are part of our general attempt to elucidate why microbe-specific surface structures do induce plant defense reactions, and if such perception systems resemble those described in vertebrates and insects. We show that Pep-13 is a surface-exposed motif present within a cell wall TGase, which is apparently unique to *Phytophthora* species and is not found in potential host plants. In addition, mutational analyses within the Pep-13 motif revealed amino acid residues to be important for both TGase activity and elicitor activity of the parent protein. Thus, mutations within this conserved and functionally important region may not allow the microbe to evade Pep-13-mediated recognition by plants. The genus-wide presence of the protein (including the highly conserved Pep-13 region) is indicative of an important function of this protein for the life cycle of *Phytophthora*. Unfortunately, our attempts to inactivate *P.infestans* TGase gene expression failed. Among the 14 stable transformant cell lines produced, none was found to exhibit significantly reduced TGase levels (F.Brunner, I.Vijn, F.Glovers and T.Nürnberger, unpublished data). However, as gene silencing in *Phytophthora* (and oomycetes in general) is not yet a routine application, we were unable to produce significantly larger numbers of transgenic lines. Nevertheless, it is reasonable to assume that the genus-wide presence of the TGase (including the highly conserved Pep-13 region) is supportive of an essential function for this protein. Taken together, the elicitor Pep-13 shows hallmarks of PAMPs known to evoke innate immune responses in vertebrates and insects, and may thus serve a similar role in the interaction between plants and phytopathogenic *Phytophthora.*

Elicitors, such as Pep-13, may act (often as one of many) as non-self recognition determinants for the activation of plant defense responses in a non-cultivar-specific manner, but may not necessarily mediate resistance. This (in addition to the broad distribution among pathogen races) clearly distinguishes PAMPs from avirulence gene products conferring plant cultivar-specific pathogen recognition and disease resistance (Nürnberger *et al*., 1994; Cohn *et al*., 2001; Dangl and Jones, 2001). PAMP-based alert systems seem to function with different efficiencies in both host and non-host plants. In the case of the non-host plant, parsley, receptor-mediated recognition of this PAMP may trigger defense reactions that contribute to, or are sufficient for, resistance against *Phytophthora* infection (Nürnberger *et al*., 1994). However, in the potato–*P.infestans* disease-causing interaction, pathogen recognition through the Pep-13 motif is clearly insufficient to provide resistance. It is assumed that during evolution plant species resistance was overcome by phytopathogens through the acquisition of virulence factors, which enabled them to either evade or (partially) suppress host plant

defense mechanisms. Such newly evolved pathogen race-specific virulence factors have driven the co-evolution of plant resistance genes and thus the development of phylogenetically more recent pathogen race/plant cultivar-specific disease resistance (Heath, 2000; Dangl and Jones, 2001; Kamoun, 2001). Importantly, susceptibility of host cells in spite of PAMP-mediated pathogen recognition (probably through repression of host defense by the pathogen) is found in animals as well. Stimulation of the innate immune system in human or *Drosophila* by, for example, bacterial LPS or flagellin, may not in all cases sufficiently protect the host from infection by Gram-negative bacteria displaying either one or both PAMPs. Nevertheless, PAMP-mediated activation of innate immune responses was shown to contribute to successful defense against microbial invasion in both *Drosophila* (Lemaitre *et al*., 1996) and mammals (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000; Underhill and Ozinsky, 2002). Importantly, two natural mutations (*lps*) that render mice insensitive to Gram-negative bacterial LPS, yet highly susceptible to Gram-negative infection, were shown to be defective in the TLR4 receptor (Poltorak *et al*., 1998; Hoshino *et al*., 1999), a homolog of which was first cloned from human and shown to be essential for activation of adaptive immune responses as well (Medzhitov *et al*., 1997). In total, 10 vertebrate TLR receptors sensing different microbial molecules have been identified, covering the whole array of pathogens with which a potential host must cope. Gene knockout work confirmed that various TLR–/– mice showed decreased resistance to a variety of Gram-positive as well as Gram-negative bacteria (Underhill and Ozinsky, 2002). Intriguingly, the repertoire for the recognition of TLR stimuli can be significantly enlarged through hetero-oligomerization of different TLRs (Ozinsky *et al*., 2000).

It will now be important to determine whether and to what extent PAMP-mediated pathogen recognition may contribute to the activation of innate immune responses in both host and non-host plants. For example, plant varieties susceptible under certain test conditions may not necessarily be susceptible under less favorable infection conditions determined by humidity, temperature or reduced inoculum densities (Tyler, 2002). Moreover, PAMP-mediated alert systems for the activation of plant defense in non-host plants may contribute to non-host resistance, which is the predominant form of plant disease resistance (Heath, 2000; Kamoun, 2001; Nürnberger and Brunner, 2002). Instrumental to the assessment of individual PAMP recognition events for overall disease resistance will be the genetic inactivation of PAMP-encoding pathogen genes (such as the TGase gene in *Phytophthora* species) as well as those encoding PAMP receptors in plants. Once technically feasible, inactivation of PAMP-encoding genes in *Phytophthora* will enable assessment of the effects of such mutations on overall fitness of the pathogen as well as on its virulence. Likewise, we have previously devised a strategy to isolate the Pep-13 receptor from parsley (Nennstiel *et al*., 1998), which upon completion will allow us to isolate the encoding receptor genes from parsley and potato. Subsequently, upon inactivation of this gene in potato, it will be interesting to assess whether such a mutation would result in an enhanced susceptibility phenotype. However, it should also be taken into consideration that another, yet unexplored role of PAMP-mediated 'non-self' recognition might be to establish/maintain a beneficial homeostasis between plants and commensal or potentially mutualistic symbionts.

The finding that an elicitor of non-cultivar-specific plant defense exhibits characteristics of PAMPs known to trigger innate immune responses in animals adds to the growing list of parallels in the molecular organization of innate immunity in various kingdoms. The intriguing view of an evolutionary conservation of innate defense mechanisms across kingdom borders (Cohn *et al*., 2001; Dangl and Jones, 2001; Nürnberger and Brunner, 2002) is further supported by structural similarities found between the flagellin receptor in human (TLR5) (Hayashi *et al*., 2001) and the *Arabidopsis* flg22 receptor FLS2 (Gomez-Gomez and Boller, 2000), as well as by the identification of MAP kinase cascades implicated in the activation of innate immune responses in both plants and animals (Asai *et al*., 2002; Dong *et al*., 2002).

Materials and methods

Cultivation of oomycetes and plant cell cultures

Oomycetes were grown on vegetable juice agar (Rohwer *et al*., 1987). Liquid cultures were harvested after 3–4 weeks of growth and filtered through a 200 μm nylon mesh. The filtrate was cleared by centrifugation at 4100 *g* for 20 min. The culture filtrate material, stored as freeze-dried preparations, was dissolved in water and concentrated in Centriprep or Centricon YM10 filters (Millipore).

Dark-grown, 5-day-old suspension-cultured potato cells (cv. Désirée) or parsley cells were used for elicitor treatment (Göbel *et al*., 2001) or protoplast preparation (Parker *et al*., 1991). Quantification of furanocoumarin phytoalexin production in parsley protoplasts was performed as described previously (Parker *et al*., 1991). Elicited potato cells were harvested by filtration and stored at –80°C.

Protein biochemistry

Proteins were separated on 12.5% SDS–polyacrylamide gels and blotted according to standard protocols. Both primary (anti-Pep-13) and secondary (goat anti-rabbit IgG–horseradish peroxidase conjugate; Bio-Rad) antibodies were used at 1:5000 dilution. Immunodetection was performed using the ECL Plus detection system (Amersham Pharmacia Biotech). For in-gel determination of TGase activity, a PVDF membrane was first incubated overnight in a 10–20 mg/ml *N*,*N*-dimethylcasein solution in 0.1 M Tris–HCl pH 8.5. The membrane was blocked with non-fat dry milk (1% in 0.1 M Tris–HCl pH 8.5) for 1 h, followed by two washes in 0.1 M Tris–HCl pH 8.5 and two washes with 0.1 M sodium acetate pH 5.2.

In-gel denaturation and renaturation of TGases were performed as described previously (Usami *et al*., 1995). After two washes in 50 mM Tris–HCl pH 8.5 and 0.1 M sodium acetate pH 5.2 for 5 min, the gel was overlaid upon the *N*,*N*-dimethylcasein-coated PVDF membrane and maintained immersed in TGase buffer [0.1 M sodium acetate pH 5.2, 0.5 mM 5-(biotinamido) pentylamine, 10 mM DTT, 5 mM $CaCl_2$] for 12 h at 20°C. The membrane was washed twice with 0.2 M EDTA pH 8.0 and twice with PBS buffer prior to 1 h incubation with avidin–horseradish peroxidase (Bio-Rad), diluted 1:5000 in PBS buffer, 1% non-fat dry milk, 0.05% Tween-20. After four additional washes with PBS buffer, TGase activity was detected by chemiluminescence.

The TGase solid-phase microtiter plate assays were carried out as described previously (Slaughter *et al*., 1992) with the following modifications: 100 ng/ml recombinant wild-type or mutant GP42 was used in each reaction. The reaction was performed in 0.1 M sodium acetate pH 5.2 and the streptavidin–alkaline phosphatase (1000 U/ml; Roche) was diluted 1:1000. A kinetic measurement of absorbance at 405 nm was determined at 15 min intervals for a period of 1–4 h using an MRX microplate reader (Dynatech Laboratories).

Recombinant protein expression in A.oryzae

The *P.sojae* GP42 encoding cDNA (U10471) was introduced into the pBluescript II KS(-) vector (Stratagene) and subjected to site-directed

mutagenesis using the GeneEditor *in vitro* site-directed mutagenesis system (Promega). Trp231, Pro234 and Tyr241 were each substituted by alanine. The mutant cDNA constructs were sequenced and confirmed to contain only the expected mutations. The constructs were subcloned into the pHD464 expression vector (Dalbøge and Heldt-Hansen, 1994) and transformation of *A.oryzae* JaL228 was carried out as described previously (Fuglsang *et al*., 2000). Transformants were grown for 3 days at 30°C before harvesting the culture supernatant. Aliquots of this material were desalted by gel filtration on PD-10 columns (Amersham Pharmacia Biotech) and stored at –80°C until use in assays for TGase and elicitor activity.

RNA blot and RT–PCR analysis

Twenty micrograms of total RNA isolated from elicitor-treated potato cells were subjected to RNA blot analysis as described previously (Göbel *et al*., 2001). As probes, the following potato cDNA fragments were used: a 0.4 kb PCR fragment from *LOX* (*POTLX-3*; Kolomiets *et al*., 2000), a 2.0 kb *Eco*RI fragment from *4CL* and a 0.3 kb *Eco*RI fragment from *PR1* (Göbel *et al*., 2001). For standardization, blots were probed with a 1.3 kb *Bam*HI fragment from potato 25S rRNA.

Oomycete poly(A)$^+$ RNA was purified from 100 µg of total RNA (Dunsmuir *et al*., 1989) using oligo(dT) cellulose (Amersham Pharmacia Biotech). First-strand cDNA was synthesized from 50 ng of poly(A)$^+$ RNA using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). PCR amplification was performed using degenerate primers 5′-gatgaattcGA(A/G)AA(A/G)TA(C/T)GC(N)AA(A/G)GC(N)TT(C/T)GG-3′ (sense) and 5′-cccgggtcgaCGT(A/G)TA(N)GT(C/T)TC(A/G)TA(A/G/T)ATCCA-3′ (antisense) encoding, respectively, amino acids (one-letter code) EKYAKAF and WIYETYT in the sequence of the *P.sojae* GP42. The following PCR conditions were used: 30–32 cycles (1 min, 94°C; 1 min, 54°C; 1.5 min, 72°C). Subsequently, the PCR fragments were cloned into the pGEM-T vector (Promega) and sequenced.

Acknowledgements

We thank H.Keller and P.Venard (INRA, Unité Santé Végétale et Environnement, Antibes) for providing oomycete isolates, W.Wirtz for cloning of the TGase wild-type construct and M.-A.Allerslev for skillful technical assistance. We are grateful to Jane Parker (MPIZ Cologne) for kindly providing *P.parasitica*-infected *Arabidopsis* leaves. F.B. received support from KWS Einbeck, Germany. J.J.R., J.L., D.S. and T.N. were funded by the EC, DFG and Fonds der Chemischen Industrie. B.Blume and A.Ozinsky are gratefully acknowledged for critical reading of the manuscript.

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Received August 2, 2002; revised October 18, 2002; accepted October 22, 2002

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 4, Issue of January 24, pp. 2256–2264, 2003

Printed in U.S.A.

Mitogen-activated Protein Kinases Play an Essential Role in Oxidative Burst-independent Expression of Pathogenesis-related Genes in Parsley*

Received for publication, August 11, 2002, and in revised form, November 6, 2002
Published, JBC Papers in Press, November 7, 2002, DOI 10.1074/jbc.M208200200

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Plants are continuously exposed to attack by potential phytopathogens. Disease prevention requires pathogen recognition and the induction of a multifaceted defense response. We are studying the non-host disease resistance response of parsley to the oomycete, *Phytophthora sojae* using a cell culture-based system. Receptor-mediated recognition of *P. sojae* may be achieved through a thirteen amino acid peptide sequence (Pep-13) present within an abundant cell wall transglutaminase. Following recognition of this elicitor molecule, parsley cells mount a defense response, which includes the generation of reactive oxygen species (ROS) and transcriptional activation of genes encoding pathogenesis-related (PR) proteins or enzymes involved in the synthesis of antimicrobial phytoalexins. Treatment of parsley cells with the NADPH oxidase inhibitor, diphenylene iodonium (DPI), blocked both Pep-13-induced phytoalexin production and the accumulation of transcripts encoding enzymes involved in their synthesis. In contrast, DPI treatment had no effect upon Pep-13-induced *PR* gene expression, suggesting the existence of an oxidative burst-independent mechanism for the transcriptional activation of *PR* genes. The use of specific antibodies enabled the identification of three parsley mitogen-activated protein kinases (MAPKs) that are activated within the signal transduction pathway(s) triggered following recognition of Pep-13. Other environmental challenges failed to activate these kinases in parsley cells, suggesting that their activation plays a key role in defense signal transduction. Moreover, by making use of a protoplast co-transfection system overexpressing wild-type and loss-of-function MAPK mutants, we show an essential role for post-translational phosphorylation and activation of MAPKs for oxidative burst-independent *PR* promoter activation.

In most circumstances plants are able to defend themselves against pathogen attack. This is primarily facilitated through recognition mechanisms, which plants use to sense the presence of the pathogen (1–3), and through triggering intrinsic defense mechanisms that either kill the pathogen or limit its spread to the site of immediate infection (4, 5). Parsley (*Petroselinum crispum*) exhibits a non-host resistance response to attack by the oomycetes, *Phytophthora infestans* and *Phytophthora sojae* (6, 7). Defense reactions are triggered through the recognition of an abundant cell wall transglutaminase present and conserved in all but one tested member of *Phytophthora* (8). This protein was previously characterized as a 42-kDa glycoprotein purified from *P. sojae* that was able to trigger phytoalexin accumulation when added to cultured parsley cells (9, 10). Within this protein resides a conserved peptide sequence of 13 amino acids (Pep-13) that is necessary and sufficient for its elicitor activity (11). The ability of Pep-13 to trigger defense responses in parsley requires its interaction with a 100-kDa receptor protein present in the plasma membrane of parsley cells (12, 13), since all mutations made within the Pep-13 sequence that prevented binding to the receptor also inhibited the elicitation of defense reactions (11, 14–17). The defense response itself is multifaceted and involves the generation of reactive oxygen species (ROS)[1] (15), the synthesis of antimicrobial furanocoumarin phytoalexins (10), and the expression of defense-related genes including pathogenesis-related (*PR*) genes (18). Pep-13-induced defense gene activation is temporally regulated (18). Transcripts of immediate early genes, including the *WRKY1*, *-3*, *-4*, and *–5* transcription factor genes, accumulate rapidly after elicitation apparently without the requirement of *de novo* protein synthesis (19). With a slight delay, transient activation of another group of early genes is observed, among these are the *PR1* and *PR2* genes (18, 20, 21). Many *PR*-type defense-related genes appear to be regulated by WRKY transcription factors (22, 23), which have been analyzed in particular for the parsley *PR1* promoter (21, 24). Transcripts encoding enzymes implicated in phenylpropanoid metabolism and the synthesis of the furanocoumarin phytoalexins, including phenylalanine ammonia-lyase (PAL), 4-coumarate:CoA ligase (4CL), and *S*-adenosyl-L-methionine:bergaptol *O*-methyltransferase (BMT) accumulate even later (20). Treatment of

* This work was supported by Deutsche Forschungsgemeinschaft Grant Sche 235/3-1-235/3-4), European Commission Grant HPRN-CT-2000-00093, CRISP, and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y12875, AY173415, AY173414, and AY17343.

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[1] The abbreviations used are: ROS, reactive oxygen species; PR, pathogenesis-related; MAPK, mitogen-activated protein kinase; GST, glutathione *S*-transferase; GUS, β-glucuronidase; LUC, luciferase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MBP, myelin basic protein; DPI, diphenylene iodonium; Me_2SO, dimethyl sulfoxide; BMT, *S*-adenosyl-L-methionine:bergaptol *O*-methyltransferase; ERM kinase, elicitor-responsive MAPK; 2,4-D,2,4-dichlorophenoxy acetic acid.

parsley cells with diphenylene iodonium chloride (DPI) blocks both the induction of the oxidative burst and phytoalexin biosynthesis by elicited parsley cells (15). Moreover, it has been shown that the generation of O_2^- via the oxidative burst is necessary and sufficient to drive phytoalexin biosynthesis by the cells (15). Calcium influx through Pep-13-responsive ion channels of the plasma membrane (15, 17) followed by elevation of cytosolic calcium levels (14) were found to be located upstream of the oxidative burst and the activation of a mitogen-activated protein kinase (MAPK) (14, 16). The DPI insensitivity of this Pep-13-induced MAPK activation positions this kinase between calcium influx and oxidative burst or indicates bifurcation of the signaling pathway into DPI-sensitive and -insensitive branches (16).

Pharmacological and ^{32}P-labeling studies have long since indicated the importance of protein phosphorylation and protein kinase activities in bringing about pathogen defense responses both in parsley and other systems (25, 26). Among the many implicated protein kinases, the activation of MAPKs has been shown to be a consistent and common response of plant cells following infection and exposure to microbial elicitors (27, 28). Based upon analysis of the fully sequenced *Arabidopsis thaliana* genome, plants appear to contain more putative MAPKs than any other known organism, including humans (29). *Arabidopsis* possesses at least 20 MAPK-encoding genes that fall into a minimum of four subgroups (30, 31). In all systems whereby MAPK activity has been studied with respect to elicitor responses, activation of members of the AtMPK6 subgroup has been described (1, 27). This includes the responses of tobacco SIPK to general elicitors, such as Harpin and elicitins, TMV infection, and race-specific elicitation (32–36); alfalfa SIMK to chitin, ergosterol, and β-glucans (37); *A. thaliana* AtMPK6 to bacterial elicitors including the flg22 peptide from flagellin (38) and Harpin (39). It was recently demonstrated for *A. thaliana* that MAPKs can also act as negative regulators of defense responses, as shown for AtMPK4 mutants (40); however, this would appear to be contradictory to the activation of this kinase described in response to Harpin (39). Members of a second closely related class of MAPKs, initially characterized in tobacco as being activated following wounding (WIPK) (41, 42), and having homology to AtMPK3, have also been implicated in pathogen defense signaling (34, 43, 44). Our previous studies demonstrated the activation of such a homologue, described as ERM kinase, following treatment of parsley cells with the Pep-13 elicitor (16).

Evidence indicating the importance of MAPK activation for the elicitation of defense reactions has recently emerged from gain-of-function experiments whereby MAPKs themselves, or constitutively active forms of their upstream activators, MAPK kinases (MAPKKs), were transiently overexpressed in tobacco and *Arabidopsis* leaves (45–47). This resulted in a hypersensitive response-type phenotype in leaves in addition to activation of genes implicated in the biosynthesis of defense-related antimicrobial compounds. These observations have recently been supported by the identification of a complete MAPK cascade from *A. thaliana* that is triggered through recognition of flg22 (48). This resulted not only in the accumulation of transcripts of a group of defense-related genes, but also in increased resistance to attack by both fungal and bacterial pathogens (48). In addition to these functions in defense, AtMPK6 homologues have been shown to be activated in response to various abiotic stresses including osmotic stresses, ozone exposure, oxidative stress, cold stress, drought, and treatment with salicylic acid (32, 49–58). It has therefore been suggested that members of this class of MAPKs may function as points of cross-talk between various stress signaling pathways in plants (3, 27, 30).

In this article we demonstrate the existence of parallel pathways that operate to induce the transcriptional activation of particular sets of defense-related genes in parsley. One pathway is triggered downstream of the oxidative burst and controls genes implicated in phytoalexin biosynthesis. The second pathway is independent of the oxidative burst, but is dependent on MAPK activity. The MAPKs involved are activated in parsley cells through receptor-mediated recognition of the Pep-13 elicitor and other elicitors of defense reactions, but appear largely insensitive to abiotic stresses, suggesting that their activation is primarily associated with pathogen defense. Furthermore, by utilizing a protoplast transient transfection system employing loss-of-function MAPK mutants, we demonstrate a requirement of MAPK activity for the elicitor-mediated oxidative burst-independent activation of *PR* genes, which represent classical markers for pathogen defense responses in plants.

EXPERIMENTAL PROCEDURES

Elicitor Preparations—The Pep-13 elicitor was chemically synthesized as previously described (13). *Pseudomonas syringae* HrpZ was expressed and purified as a recombinant protein from *Escherichia coli* (59). Synthetic *N*-acetyl chitoheptaose was provided by Naota Shibuya (University of Tsukuba, Tsukuba, Japan).

Cell Culture Handling, Treatment, and Protoplast Isolation—Cultured parsley cells were maintained in modified Gamborg's B5-Medium containing 1 mg/liter of 2,4-D as previously described (60). Protoplasts were isolated 5 days following transfer of the culture to fresh medium according to previously described methods (61). Cells were treated by addition of the stimulus to cells previously washed and allowed to equilibrate for 30 min in fresh medium. All treatments were performed by direct application from appropriate stock solutions, or in the case of hypo-osmotic treatment, following dilution in four volumes of medium lacking the osmoticum (sucrose-free). Following appropriate time points cells were collected by vacuum filtration, quickly frozen in liquid N_2, and stored at −80 °C until use.

RNA Isolation and Reverse Transcription (RT)-PCR Analysis—Total RNA was extracted from parsley cells at different times after elicitor treatment by using the TRIzol reagent (Invitrogen) according to the manufacturer's guidelines. For RT-PCR analysis cDNA was synthesized from 2 μg of total RNA by using reverse transcriptase and oligo(dT) or 18Sr primers. The cDNA was amplified by PCR with the following gene-specific primers: PR2f (5′-AGGGCTTTCTTCTTGACAT-3′), PR2r (5′-CTTCGATTGACTTTATTATTCTTA-3′), BMTf (5′-CAAAGCTGGCCCTGGTAACTATT-3′), BMTr (5′-GGCGTCTCCTTTTGGCACAC-3′), WRKY1f (5′-AATCATAACCATCCAAAGC-3′), WRKY1r (5′-CATATTTCAAACAAGGTACACT-3′), PAL2f (5′-TG AAATTGCGATGGCTAG-3′) PAL2r (5′-TTTAAGTAGCAAGAGCCTT-3′), 18Sf (5′-GATGGTAGGATAGTGGCCTA-3′), and 18Sr (5′-TGGTTCAGACTAGGACGATA-3′). PCR was performed in a 50-μl reaction volume containing 1× *Taq*PCR buffer (Promega, Madison, WI), 0.25 mM dNTPs, 0.5 units of *Taq*, and 0.5 μM concentrations of each primer. The PCR cycle consisted of 2 min at 94 °C, 18 cycles of 30 s at 94 °C, 45 s at 50 °C, 80 s at 72 °C, and one final extension step of 7 min at 72 °C. The products were analyzed by agarose gel electrophoresis.

Acquisition and Analysis of MAPK cDNAs—A λ-ZAP™ II (Stratagene, Heidelberg, Germany) phage cDNA library was prepared from mRNA of elicited and un-elicited cultured parsley cells according to the manufacturer's guidelines and screened with radioactively labeled probes corresponding to the open reading frames of the MAPK-encoding genes *MMK1* (62, 63) and *MMK4* from alfalfa (55). Each probe was used to screen 6×10^5 plaques and resulted in the acquisition of 8 cDNA clones encoding 4 different full-length open reading frames. Sequence analysis of the cDNAs and their encoded proteins were performed using the DNASIS 2.1 software (Hitachi, Tokyo, Japan).

Site-directed Mutagenesis—Single point mutations were introduced into MAPK sequences present within vector pGEM-T (Promega, Mannheim, Germany) by PCR-based site-directed mutagenesis using the GeneEditor system (Promega, Mannheim, Germany) and the following 5′-phosphorylated oligonucleotides: PcMPK6Y214F, 5′-GATTTTATGACAGAATTTGTTGTTACAAGATGG; PcMPK6D348N, 5′-CTGCACGACATCAGTAACGAGCCTGTATGTG; PcMPK4Y200F, 5′-GATTTTATGACAGAATTTGTTGTTACTCGCTGG. The manufacturer's guidelines were followed, and the resulting mutants were verified by sequencing.

Generation and Bacterial Expression of Glutathione S-Transferase Fusion Proteins—MAPK-encoding open reading frames were cloned as *Bam*H1/*Xho*I PCR fragments into vector pGEX 2T-2 (Amersham Biosciences) for expression in *E. coli* (strain BL-21) as fusion proteins containing an N-terminal GST moiety. Recombinant proteins were subsequently purified using glutathione-Sepharose 4B according to the manufacturer's guidelines.

Protoplast Transfection and Co-transfection—For MAPK activity measurements wild-type or mutated open reading frames were cloned as *Nco*I/*Bam*H1 fragments into vector pRT100 (64) behind an introduced c-Myc-encoding sequence and the 35S-cauliflower mosaic virus promoter. 30 μg of each construct were then used to transfect 2×10^6 protoplasts (~200 μl). Protoplasts and DNA were mixed before the addition of 600 μl of 25% (w/v) polyethylene glycol (PEG) 6000, pH 9.0, containing 100 mM $Ca(NO_3)_2$ and 45 mM mannitol. Following a 20-min incubation, the protoplasts were collected by centrifugation after the addition of 7 ml of 0.275 M $Ca(NO_3)_2$, pH 6.0, then resuspended in 4 ml of B5-sucrose solution (0.28 M sucrose, 1 mg/ml 2,4-D, 3.2 mg/ml B5 medium (solid)) and divided into two Petri dishes. Following 24 h of incubation, the dishes were treated with either 100 nM Pep-13 or water for 15 min. The protoplasts were then collected by centrifugation following the addition of 25 ml of 0.24 M $CaCl_2$ and quickly frozen in liquid N_2. Co-transfection experiments were performed as already described with the following modifications: 20 μg of MAPK constructs were transfected in combination with 5 μg of PR2-promoter-GUS (β-glucuronidase) construct (24) and 5 μg of the normalization plasmid, pRTLUC (65). Following an 8-h incubation in B5-sucrose medium, the protoplasts were treated either with water or 100 nM Pep-13 and incubated for a further 14 h. Protoplasts were then collected and stored as described.

Protein Extraction—Proteins were extracted by grinding frozen cells in extraction buffer (25 mM Tris-HCl, pH 7.8, 75 mM NaCl, 15 mM EGTA, 15 mM glycerophosphate, 15 mM 4-nitrophenylphosphate, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1% (v/v) Tween 20) followed by centrifugation ($23{,}000 \times g$) for 10 min at 4 °C. Protoplasts were extracted in the same buffer by vortexing for 30 s. For studies involving luciferase (LUC) and GUS measurements protoplasts were extracted in K_2HPO_4/KH_2PO_4, pH 7.5, containing 1 mM dithiothreitol.

GUS and LUC Determinations—For LUC activities, 10 μl of protoplast extracts were mixed with 90 μl of LUC substrate (20 mM Tricine, pH 7.8, 2.5 mM $MgSO_4$, 1 mM $(MgCO_3)_4/Mg(OH)_2{\cdot}5H_2O$, 0.1 mM EDTA, 30 mM dithiothreitol, 300 μM coenzyme A, 500 μM ATP, 500 μM luciferin) and measured for 5 s in a luminometer (Luminoscan Ascent plate reader, Labsystems, Frankfurt, Germany). For GUS activities, 10 μl of protoplast extract was mixed with 40 μl of substrate (50 mM Na_2HPO_4/NaH_2PO_4, pH 7, 10 mM mercaptoethanol, 2 mM 4-methylumbelliferyl β-D-glucopyranoside, 0.1 mM EDTA, 0.1% (v/v) Triton X-100) and incubated at 37 °C for 1 h. Following addition of 200 μl of 0.4 M $Na(CO_3)_2$ fluorescence was measured at 360 nm excitation/440 nm emission using the Cytofluor II apparatus (Biosearch, Bedford, MA).

In-gel Protein Kinase Assays—Cell extracts containing 20 μg of protein per lane were separated on 10% PAGE gels containing 0.1 mg/ml myelin basic protein (MBP) (Sigma). All subsequent denaturation, renaturation, kinase activity, and washing steps were performed as previously described (66). Protein kinase activity was visualized by phosphorimaging (Molecular Dynamics, Krefeld, Germany).

Antibody Production—Peptides were synthesized corresponding to amino acid sequences 2–15 in PcMPK6 (DGSTQPSDTVMSDAC); 1–11 in PcMPK3b (MANPGDGQYDC); and 360–374 in PcMPK4 (CEQHALTEEQMRE). The peptides were then coupled to keyhole limpet hemocyanin and used to raise antiserum following immunization of rabbits (Eurogentec, Seraing, Belgium).

Western Blotting—SDS-PAGE gels were semidry-blotted onto nitrocellulose membrane (Porablot-NCL, Machery-Nagel, Düren, Germany). Membranes were blocked at 4 °C overnight in either TBS (20 mM Tris-HCl, 150 mM NaCl), 0.1% (v/v) Tween 20 (TBST) containing 5% (w/v) skimmed milk powder or 5% (w/v) bovine serum albumin. Primary antibody solutions were prepared in blocking solution at the following dilutions: 1:10,000 anti-PcMPK6, 3, or 4; 1:500 monoclonal anti-c-Myc (Sigma); 1:15,000 anti-ACTIVE™ MAPK (Promega, Mannheim, Germany). Secondary antibodies coupled to either horseradish peroxidase or alkaline phosphatase were also prepared in blocking solution. All washes were performed in TBST. Blots were developed using either enhanced chemiluminescence (Amersham Biosciences) or nitro blue terazolium/5-bromo-4-chloro-3-indolyl phosphate precipitate formation.

Immunoprecipitation/Protein Kinase Assays—Cell or protoplast extracts containing 100 μg of protein were immunoprecipitated for 1 h at 4 °C with either MAPK-specific or c-Myc antibodies coupled to protein A- or protein G-Sepharose (Amersham Biosciences). Subsequent washing and *in vitro* MBP phosphorylation reactions were as described previously (16). Reactions were stopped by the addition of SDS sample buffer and boiling. The proteins were then separated by SDS-PAGE, and MBP phosphorylation was determined by phosphorimaging.

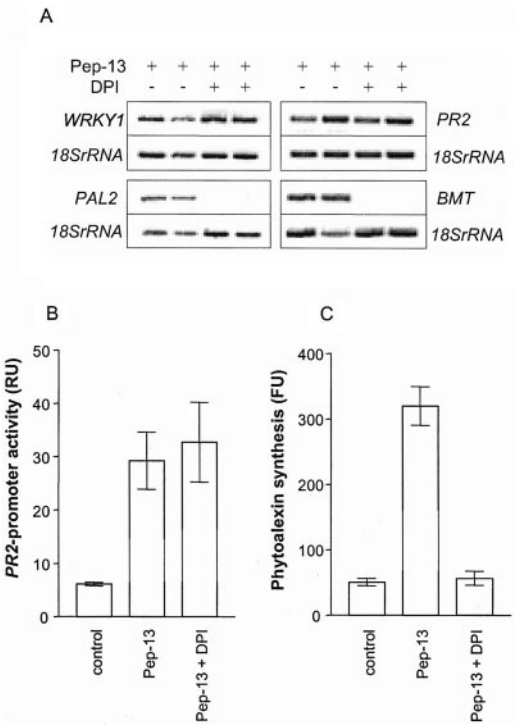

FIG. 1. **Differential activation of parsley defense-related genes through oxidative burst-dependent and -independent pathways in response to the Pep-13 elicitor.** *A*, RT-PCR analysis of transcript accumulation demonstrates the existence of parallel independent pathways leading to defense gene expression. Parsley cells were pretreated for 30 min with either 10 μM DPI (+) or an equivalent volume of Me_2SO (−) prior to addition of 100 nM Pep-13 (+). Cells were then harvested at the following time points: *WRKY1*, 1 h post-elicitation; *PR2*, 4 h; *PAL2*, 8 h; and *BMT*, 24 h. RNA was isolated and used for RT-PCR analysis in order to determine defense gene transcript levels. The transcript level of 18 S rRNA was also determined for each time point for normalization purposes, and each treatment is shown in duplicate. *B*, promoter activity studies confirm oxidative burst-independent transcriptional activation of the *PR2* gene. Parsley protoplasts were transfected with a *PR2* promoter fused to the gene encoding GUS in addition to an 35S-promoter-driven LUC construct (*35S-LUC*). Protoplasts were then treated with 10 μM DPI or a corresponding volume of Me_2SO 30 min prior to addition of 100 nM Pep-13. Following a further 14-h incubation, the protoplasts were harvested, extracts were generated, and GUS and LUC activity determinations were performed. The data are expressed as GUS/LUC activities for each treatment ($n = 4$). *C*, fluorescence of the culture medium was also measured prior to protoplast harvesting to confirm the inhibitory effect of 10 μM DPI upon phytoalexin production.

RESULTS

Differential Activation of Defense Genes through Oxidative Burst-dependent and -independent Pathways—We sought to identify genes whose transcriptional activation occurred independently of the oxidative burst signaling pathway, by performing RT-PCR analysis of defense transcript accumulation in Pep-13- and DPI-treated parsley cells. Prior to RNA isolation, treated cells were tested to ensure that 10 μM DPI had effectively blocked Pep-13-induced phytoalexin production, measured 24 h after elicitation (not shown). Transcripts were examined belonging to each of the "immediate early," "early," and "late" responses in addition to an 18 S rRNA control, and typical results were seen as illustrated by the duplicate treatments shown in Fig. 1*A*. The *WRKY1* transcription factor and *PR2* genes are characteristic immediate early and early elici-

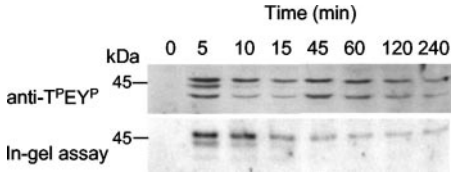

FIG. 2. **The Pep-13 elicitor activates at least three independent MAPKs in cultured parsley cells.** Cells treated with 100 nM Pep-13 were harvested after various time periods and cell extracts were prepared. Proteins (20 μg/lane) were then separated by SDS-PAGE, blotted, and probed with antibodies cross-reacting with activated MAPKs (*anti-T^{P}EY^P*, *upper panel*), or separated on SDS-PAGE gels containing 0.1 mg/ml MBP to test in-gel kinase activities (*lower panel*). Both techniques revealed the activation of at least three MAPKs (M_r ~46, 44, and 42) in Pep-13-treated cells.

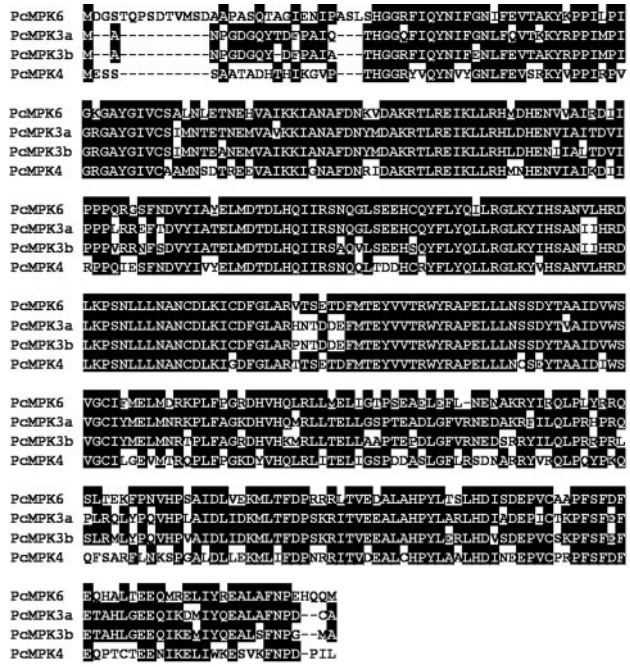

FIG. 3. **Sequence alignments of the encoded proteins of four parsley MAPK cDNA clones.** Four MAPK encoding cDNA clones were isolated from a library generated from a mixture of Pep-13-treated and untreated parsley cells. Based upon the homology to *A. thaliana* MAPKs the parsley MAPK are referred to as PcMPK6, 3a, 3b, and 4. Alignments between the encoded amino acid sequences are shown, and fully conserved residues are indicated in *black boxes*.

tor-responsive genes, respectively (24, 67). As illustrated in Fig. 1*A*, the transcriptional activation of each gene, measured at 1 and 4 h post-elicitation, respectively, was unaffected by DPI treatment. This was in contrast to genes characteristic of the late response, including *PAL2* and *BMT* genes (20), whose activation was inhibited by DPI treatment at all time points tested (Fig. 1 displays the 8-h *PAL2* and 24-h *BMT*). In addition to the genes shown in Fig. 1*A*, other genes were examined that were either sensitive, such as the *S*-adenosyl-L-methionine: caffeoyl-CoA *O*-methyltransferase gene, or insensitive to DPI, such as the *PR1-3* gene. In principle, those genes encoding enzymes of phenylpropanoid metabolism were most strongly affected (data not shown). The only unexpected variation to this theme was 4CL whose transcript accumulation reproducibly showed no inhibition by DPI under the conditions tested. However, as illustrated by Fig. 1*A*, which clearly and consistently showed inhibition by DPI of *PAL* gene expression, transcript accumulation of the *PR2* gene was not affected, suggesting that this gene is regulated by an oxidative burst-independent pathway. To further test this hypothesis we performed additional experiments aimed at studying *PR2* promoter activity in Pep-13- and DPI-treated transfected protoplasts. Parsley protoplasts were co-transfected with a plasmid containing a *PR2* promoter-*GUS* construct (24, 67) in addition to a 35S-promoter-*LUC* construct for normalization. Twenty-four hours after elicitation, the protoplasts were first tested for phytoalexin synthesis prior to their harvesting and the determination of GUS and LUC activities in extracts. Fig. 1*C* shows that 10 μM DPI effectively blocked phytoalexin synthesis by the transfected protoplasts, which is in agreement with the responses seen in cells. However, this treatment had no effect upon the elicitor responsiveness of the *PR2* promoter (Fig. 1*B*), whose activation was indistinguishable to that seen in solvent-treated cells in response to Pep-13. These data support the hypothesis that there exist parallel signaling pathways leading to defense gene expression in parsley cells, one being mediated through the oxidative burst, while the other appears independent of this response and results in the activation of *PR2* and *WRKY1* genes.

Treatment of Cultured Parsley Cells with the Pep-13 Elicitor Induces the Activation of At Least Three MAPKs—We reported previously that in parsley cells a MAPK is activated in a receptor-mediated manner following treatment with the Pep-13 elicitor peptide (16). This activation was shown to be DPI-insensitive, suggesting that these activities are located upstream or independent of the oxidative burst and may be involved in the oxidative burst-insensitive pathway leading to PR gene expression. By using a modified MBP in-gel assay we found that in fact three MBP kinases were rapidly activated in response to Pep-13 treatment (Fig. 2, *lower panel*). The largest kinase had an apparent molecular weight of 46 kDa and showed a sustained activation lasting for up to 240 min, while two other proteins (44 and 42 kDa in size) showed a more transient activation profile. As the MBP in-gel kinase assay is a sensitive detection method for activated MAPKs, and as the size of the detected kinases are in agreement with those of this class of protein (31, 68), we hypothesized that all the elicitor-responsive MBP kinases are indeed MAPKs. To verify this we used an antiserum that recognizes the dually phosphorylated T^{P}EY^P motif, that is present in the activation loop of most MAPKs from mammals and yeast (68), and also from plants (31). The phosphorylation of this motif is mediated by dual specificity upstream MAPKKs, and leads to the activation of the kinase activity of the MAPKs (30, 68). In Western blot experiments with protein extracts from elicitor-treated cells, this anti-T^{P}EY^P antiserum detected three bands of sizes identical to those seen in the in-gel kinase assay (Fig. 2, *upper panel*). In contrast to this, no signals were detected in protein extracts from non-treated cells, confirming that the elicitor-responsive MBP kinases are MAPKs. The activation characteristics of the 46- and 44-kDa kinases matched the pattern seen in the in-gel assays. In contrast to this, the 42-kDa MAPK gave a relatively stronger signal in the Western blot experiments and was detectable up to 240 min after initiation of elicitor treatment.

Cloning of Parsley MAPK cDNA Clones—In order to identify the MAPKs detected in the Western blotting and in-gel kinase assays, and to address the question of their function in elicitor signal transduction, we initiated efforts to clone a variety of different MAPK-encoding cDNAs. Screening of a parsley cDNA library generated from a mixture of elicited and un-elicited cells with a DNA probe derived from the alfalfa SIMK/MMK1 cDNA (52, 55) was performed. This resulted in the identification of five independent cDNAs, out of which four contained complete open reading frames. Comparison of the deduced amino acid sequences of the encoded kinases (Fig. 3) indicated that they fall into three characteristic subgroups. One cDNA encoded a 46-kDa MAPK with strongest homology to a subclass

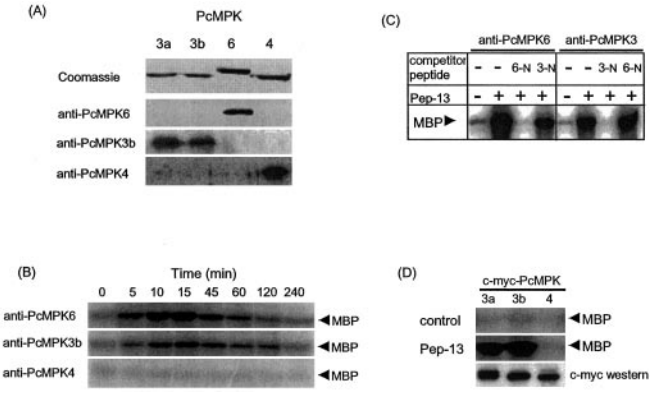

FIG. 4. **Use of MAPK-specific antisera and immunoprecipitation/protein kinase assays identify PcMPK6, 3a, and 3b as Pep-13-responsive.** *A*, specificity of peptide antibodies raised against peptide sequences contained within PcMPK6, 3b, and 4. Antisera cross-reactivity was tested by Western blotting against each of the recombinant MAPKs (100 ng/lane). *B*, immune complex-protein kinase assays. Cultured parsley cells were elicited with 100 nM Pep-13, and cell extracts containing 200 μg of protein were immunoprecipitated with the indicated antiserum coupled to protein A-Sepharose. The immune complexes were then tested for kinase activity by measuring incorporation of ^{32}P into MBP visualized following separation by SDS-PAGE. *C*, specificity of antisera in immune kinase assays. MAPKs were immunoprecipitated from extracts (200 μg of protein) of Pep-13-elicited parsley cells with PcMPK6 and PcMPK3b sera in the presence or absence of competitor peptides (20 μg/ml) corresponding to the N termini of PcMPK6 (6-N) and PcMPK3a and 3b (3-N). Kinase activity of the immune complexes was again determined using MBP as substrate. *D*, Pep-13 activates both PcMPK3a and PcMPK3b. PcMPK3a and 3b possessing an N-terminal c-Myc epitope tag were transiently expressed in parsley protoplasts through the activity of the 35S-promoter. Protoplasts were elicited with Pep-13 for 15 min 24 h after transfection. Proteins (100 μg) were extracted and immunoprecipitated with an antibody to the c-Myc tag. The kinase activity of the immune complexes was then determined using MBP as substrate.

of enzymes containing AtMPK6 from *A. thaliana*, and thus we refer to this sequence as PcMPK6. Additionally, two parsley cDNAs showed high sequence homology to one another (89% identity) and encode proteins of indistinguishable molecular weight (~44 kDa). These proteins exhibit closest homology to a subgroup of plant MAPKs containing *Arabidopsis* AtMPK3 and we thus refer to them as PcMPK3a (formerly described as ERMK, Ref. 16) and PcMPK3b. The final cDNA encodes a MAPK of 44 kDa with closest homology to AtMPK4 and is therefore named PcMPK4.

Use of Specific Antisera Reveal Pep-13-induced Activation of PcMPK6 and 3a/b—We next wished to determine, whether any of the parsley MAPK cDNAs we had cloned encoded one of the elicitor-responsive enzymes seen in the in-gel assay and Western blotting experiments. For this purpose antibodies discriminating between the different MAPK subgroups were produced by immunizing rabbits with synthetic peptides corresponding to the extreme N-terminal amino acid sequences of PcMPK6 and 3b and the extreme C terminus of PcMPK4, respectively. The specificity of the obtained antisera was tested in Western blot experiments with recombinant MAPKs produced as GST fusion proteins in *E. coli* (Fig. 4*A*). The antiserum generated against the peptide sequence of PcMPK6 only detected MPK6, while anti-PcMPK4 peptide antiserum only cross-reacted with MPK4. As predicted from the amino acid sequence conservation, the antiserum generated against the N-terminal peptide of PcMPK3b detected both PcMPK3b and 3a recombinant kinases with equal affinity but did not recognize either of the other tested MAPKs (Fig. 4*A*).

The antisera were then used in coupled immunoprecipitation/*in vitro* MBP kinase assays. Immunoprecipitations performed with anti-PcMPK6 and 3b sera precipitated MBP kinase activity from extracts of Pep-13-treated cells (Fig. 4*B*). These activities increased rapidly, within 5 min of elicitor treatment, and persisted at high levels for up to 240 min thereafter. To further test the specificity of the antisera in the immunoprecipitation experiments we performed competition studies using the peptides to which the antisera were generated. Fig. 4*C* demonstrates that the addition of a large excess of the peptide corresponding to the N terminus of PcMPK6 (6-N) prevented the immunoprecipitation of the activated PcMPK6 from elicited extracts. In contrast, addition of the peptide corresponding to the N terminus of the PcMPK3 proteins did not affect the immunoprecipitation of PcMPK6 by this antibody. Fig. 4*C* also shows the same pattern for the immunoprecipitation of the activated PcMPK3(s), whose immunoprecipitation was only blocked by addition of the N-terminal peptide of PcMPK3b (3-N). In contrast, the antiserum specific for PcMPK4 failed to immunoprecipitate an activated protein kinase from Pep-13-treated cells. These observations demonstrate that PcMPK6 and PcMPK3a and/or 3b are activated following Pep-13 treatment while PcMPK4 is not.

Transient Protoplast Transformation Confirms Pep-13-induced Activation of both PcMPK 3a and 3b—As described above and shown in Fig. 4*A*, the antiserum that immunoprecipitates activated PcMPK3 is unable to discriminate between the PcMPK3a and 3b homologues. Peptides that diverge in the highly homologous MPK3a and MPK3b proteins were found to be unsuitable for antibody production. We therefore decided to test, whether both these kinases were activated during the elicitor response by employing a protoplast transient expression system. N-terminal c-Myc-tagged PcMPK3a, 3b or 4 were overexpressed through the activity of the 35S-promoter in parsley protoplasts. The protoplasts were then treated with Pep-13, and immunoprecipitations were performed on cell extracts using c-Myc antibodies. The kinase activities of the immunoprecipitated epitope-tagged MAPKs were then determined by MBP phosphorylation. Equal expression of the constructs was verified by Western blotting with the c-Myc antiserum. As shown in Fig. 4*D*, both c-Myc-PcMPK3a and c-Myc-PcMPK3b were activated following Pep-13 treatment, suggesting that both kinases are activated in the parsley elicitor response, and make up together one of the activated MAPKs seen in the initial in-gel and Western blot experiments. In contrast to this, and in agreement with the immunoprecipitation experiments performed with the kinase-specific antibodies, c-Myc-PcMPK4 was not activated following treatment with Pep-13 (Fig. 4*B*).

Responses of PcMPK6 and PcMPK3 to Biotic and Abiotic Stress Stimuli—Studies performed in other plant systems have demonstrated that MAPK activation occurs as a common feature of many plant stress responses (30). In order to determine whether any of the parsley MAPKs plays a more general role in plant stress adaptation, we tested whether a selection of commonly studied stress treatments would induce activation of PcMPK6 and PcMPK3a/b. A range of treatments was applied to parsley cell cultures based upon conditions shown to activate MAPK signaling in cell cultures or protoplasts of alfalfa, tobacco, and *Arabidopsis* (32, 49–58). Immunoprecipitation/MBP phosphorylation assays were then performed and kinase activities were expressed against that seen in response to treatment with 100 nM Pep-13, which reproducibly gave the strongest kinase activation. The results of these investigations are presented in Fig. 5. No significant activation of either the PcMPK6 or PcMPK3a/b kinases were observed following treatments of parsley cells with 1 μM *N*-acetyl chitoheptaose (chitin), 250 μM salicylic acid, 250 mM NaCl, 500 nM sorbitol, or 4 volumes of hypotonic buffer (hypo-osmotic). These treatments did also not stimulate phytoalexin synthesis in parsley cells (not shown).

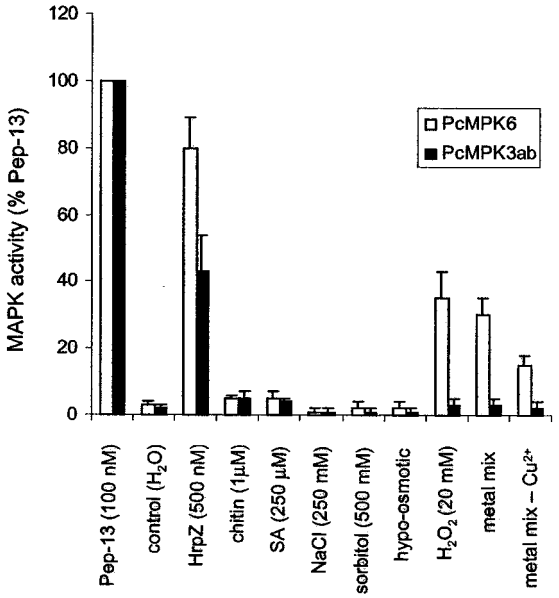

FIG. 5. **The responses of PcMPK6 and 3a/b to a range of biotic and abiotic stresses.** The responses of the PcMPK6 and PcMPK3a/b in parsley cells treated with various abiotic and biotic stress stimuli were determined by immunoprecipitation/protein kinase assays using MBP as substrate. All treatments were applied for 15 min. The metal mix contained 100 μM $CdCl_2$, 250 μM $ZnCl_2$, and 250 μM $CuCl_2$. Proteins (100 μg) were extracted and immunoprecipitated with either PcMPK6- or PcMPK3a/b-specific antisera. Kinase activities in response to each treatment were determined in triplicate by phosphorimage analysis and plotted against the maximum measurable response seen following treatment of cells with 100 nM Pep-13. The kinase activity of PcMPK6 is represented by the *white bars* and for PcMPK3a and 3b by the *black bars*.

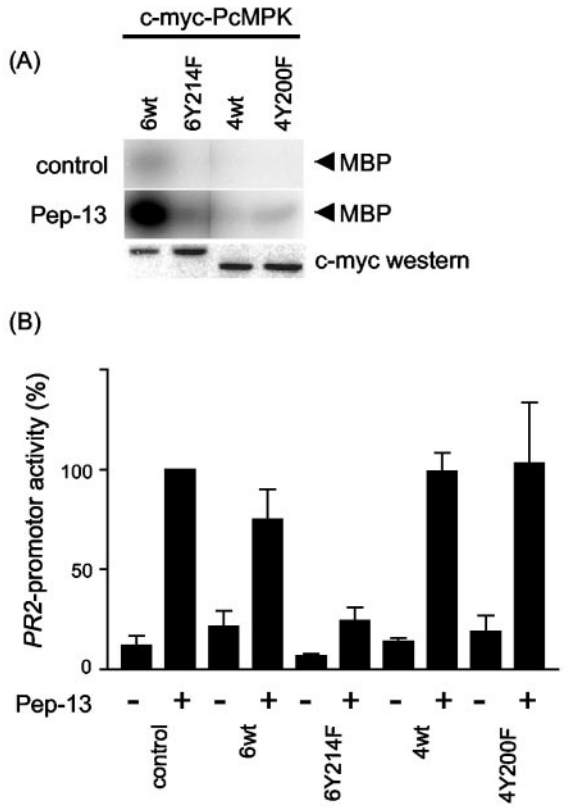

FIG. 6. **PcMPK6 activation through phosphorylation of Tyr-214 is required for Pep-13-induced *PR2* promoter activity.** *A*, activity of MAPK mutants in transfected protoplasts treated with 100 nM Pep-13. PcMPK6 and PcMPK4 wild-type and mutant constructs containing single point mutations in the activation loop motif, TEY (6Y214F, 4Y200F), were generated and transfected into protoplasts as c-Myc-tagged versions. Following 24 h expression under the control of the 35S-promoter the protoplasts were treated with either water (control) or 100 nM Pep-13 for 15 min. Proteins (100 μg) were extracted and MAPKs immunoprecipitated with an anti-c-Myc antibody. Kinase activities present in the immune complexes were then determined by MBP phosphorylation (*upper* and *middle panels*). Correct and equal expression of all constructs was tested by Western blotting of 10 μg of protein with c-Myc antibody (*lower panel*). *B*, transient expression of a dominant negative form of PcMPK6 (6Y214F) blocks the elicitor responsiveness of the parsley *PR2* promoter. Parsley protoplasts were co-transfected with a *PR2* promoter construct fused to *GUS*, together with the MAPK constructs shown in *A* or empty vector (*control*), and a 35S-promoter-*LUC* construct for normalization. Eight hours after transfection the protoplasts were treated with either water (−) or 100 nM Pep-13 (+). Following another 14-h incubation, the protoplasts were harvested, and total extracts were prepared and assayed for GUS and LUC activities. The influence of each co-transfected MAPK construct on the *PR2* promoter activity was determined in triplicate and plotted against the effect of co-transfection with the empty vector (*control*).

Treatment of cells with 500 nM recombinant HrpZ from *P. syringae* pv. *phaseolicola* activated both PcMPK6 (~80% of Pep-13 response) and the PcMPK3 kinases (~45% of Pep-13 response). HrpZ also acted as an elicitor of parsley cells and induced phytoalexin synthesis with an EC_{50} in the nanomolar range.[2] The concentration of 500 nM HrpZ used here gave maximal responses with respect to phytoalexin synthesis by parsley cells (not shown). Only two treatments were able to separate the activation of the different elicitor-responsive MAPKs. Treatment with 20 mM H_2O_2 induced the activation of PcMPK6 (~35% of Pep-13 response), but did not activate the PcMPK3 kinases. PcMPK6 was found to be activated by H_2O_2 concentrations of between 2 and 20 mM in a dose-dependent manner, whereas concentrations up to 1 mM had no effect (not shown). PcMPK6 was also activated in the absence of PcMPK3a/b activity following addition of a combination of heavy metals. This suggests that under some circumstances the elicitor-responsive MAPKs can be activated independently of one another, possibly during oxidative or heavy metal stress signaling. All activity measurements were performed in triplicate 15 min following the application of the treatment. All treatments were also analyzed after 30 min (data not shown) and yielded identical results to those shown for 15 min (Fig. 5). Neither H_2O_2 nor heavy metals stimulated phytoalexin accumulation 24 h after elicitation (not shown).

PcMPK6 Activation through Phosphorylation of Tyrosine 214 Is Required for PR Gene Promoter Activity Following Pep-13 Treatment of Parsley Protoplasts—Previous work had suggested that activated MAPKs might play roles in the control of elicitor-responsive gene expression in parsley (16). In order to directly test this, we performed transient expression experiments using dominant negative MAPK mutants to address, through a loss-of-function approach, the involvement of MAPKs in the activation of Pep-13-induced defense gene activation. Single point mutations predicted to influence kinase activity were introduced into the elicitor-responsive PcMPK6 and the un-responsive PcMPK4. The conserved tyrosine residue present within the TEY activation loop motif was mutated to phenylalanine in both PcMPK6 (6Y214F) and PcMPK4 (4Y200F). These mutations were predicted to render the protein kinases incapable of activation by upstream MAPKK-type activities (69). Both constructs contained an N-terminal c-Myc tag that enabled the determination of expression levels via Western blotting in addition to kinase activities through immunoprecipitation/MBP kinase assays on protoplast extracts. As shown in Fig. 6*A*, wild-type c-Myc-PcMPK6 was activated following treatment of transfected protoplasts with Pep-13.

[2] J. Lee and T. Nürnberger, personal communication.

This supported the previous data that demonstrated activation of this MAPK in parsley cells. However, we were unable to detect any activation of the PcMPK6 Y214F mutant in Pep-13-treated transfected protoplasts. Given that the expression levels were equal to those of the wild-type construct (Fig. 6*A*, lower *panel*), it appears that the Y214F mutation renders PcMPK6 incapable of activation through upstream MAPKK activities. For this reason the PcMPK6Y214F construct provided us with an ideal dominant negative form of the MAPK for further analysis of its influence on defense gene expression. As expected, and also shown in Fig. 6*A*, the PcMPK4 wild-type and 4Y200F kinases were again seen to be un-responsive to Pep-13 elicitor treatment.

The parsley *PR2* gene promoter has been studied in much detail (67, 70), and we have already demonstrated that its activation, like MAPK activation, occurs independently of the Pep-13-triggered oxidative burst response (Fig. 1, *A* and *B*). Therefore, we selected this promoter for use in co-transfection assays, to determine whether overexpression of an inactive MAPK impairs *PR2* promoter activation. Plasmids of the *PR2-GUS* construct (24) were co-transfected along with the constructs shown in Fig. 6*A* and an 35S-promoter-*LUC* construct for normalization purposes. Following 8 h of incubation, the protoplasts were treated with 100 nM Pep-13 and then left for a further 14 h. Protoplasts were then harvested, and GUS and LUC activity determinations were performed upon protein extracts. A typical data set for these co-transfection experiments is shown in Fig. 6*B*. Transfection with the PcMPK6 wild-type construct led to little or no reduction (~20%) in Pep-13-induced promoter activity compared with the co-transfections performed with the empty vector controls. However, co-transfection of the dominant negative form (6Y214F) of PcMPK6 led to a dramatic reduction in Pep-13-induced *PR2* promoter activity (~80% inhibition). In addition to this, the basal (non-elicited) levels of activity were also reduced, suggesting that the PcMPK6Y214F construct has a strong negative effect on both, activity and Pep-13 responsiveness of this promoter. Importantly, Fig. 6*B* also shows that co-transfection with either wild-type or Y200F forms of PcMPK4 had no effect on the Pep-13 responsiveness of the promoter. This agrees well with the fact that PcMPK4 is not activated in response to the Pep-13 elicitor and is therefore unlikely to trigger downstream events of the defense response.

DISCUSSION

Receptor-mediated perception of plant pathogens results in the activation of intracellular signaling pathways that function in triggering downstream defense reactions (3, 4). Defense reactions themselves are characterized by large-scale transcriptional activation of genes, whose products are believed to be actively involved in resisting pathogen attack (20, 71). Our studies have demonstrated that particular signaling pathways are responsible for the transcriptional activation of distinct subsets of defense genes. It is clear that both oxidative burst-dependent and -independent pathways play roles in this response. Previous studies, and those presented here, have demonstrated that the generation of O_2^-, most likely through the activity of an NADPH oxidase homologue(s), is necessary and sufficient to drive the synthesis of antimicrobial phytoalexins in parsley cells (15). The use in these studies of the NADPH oxidase inhibitor, DPI, to block Pep-13-induced phytoalexin biosynthesis, correlated with its ability to inhibit the transcript accumulation of genes encoding enzymes involved in this process. The transcriptional activation of these genes belongs to the late reactions of elicited parsley cells (18, 20). In contrast, transcript accumulation of genes involved in the immediate early and early reactions (21, 23) was unaffected by this treatment suggesting that a separate, albeit parallel, oxidative burst-independent pathway controls the transcriptional activation of such genes.

Changes in protein phosphorylation have long been known to occur as a consequence of treatment of plant cells with microbial elicitors (25, 26). Among the many protein kinases believed to be involved in these events, members of the MAPK family are becoming increasingly recognized as playing important roles in defense signaling (27, 28). In the present study we have shown that in parsley cells at least four different MAPKs are activated in a receptor-dependent manner by the *Phytophthora*-derived elicitor peptide, Pep-13. Three of these MAPKs could be identified by molecular cloning, immunoprecipitation, and transient transformation assays, and they were found to be homologous to MAPKs implicated in defense signaling in other plant species (3, 27, 31, 48). The initial in-gel and Western blotting experiments also suggest that at least one elicitor-responsive MAPK remains to be identified. Based upon the activation profile seen for each of the kinases with these methods, and compared with the activities determined through specific immunoprecipitation/kinase assays, this remaining kinase would appear to be activated more transiently than the PcMPK6 and PcMPK3 kinases. Given the lack of any cross-reacting antisera we have as yet been unable to identify this additional activity.

The MAPKs we have identified as Pep-13-responsive have homology to those seen to be implicated in elicitor signaling in other systems, *i.e.* homologues of the AtMPK6 and AtMPK3 MAPKs from *Arabidopsis* (27). In addition, we isolated a parsley homologue of AtMPK4, a MAPK shown to be a negative regulator of disease-resistance responses in *Arabidopsis* (40). This MAPK was not responsive to elicitors (Pep-13 or HrpZ) in parsley cells, and we cannot say whether it is functionally homologous to the *Arabidopsis* MAP kinase 4, which was previously described as being activated in response to Harpin treatments (39). We also isolated two parsley MAPKs belonging to the AtMPK3 class and have shown that both become activated following Pep-13 treatment. Whether these two kinases share a common function remains to be determined. One might suppose that they could have, despite their high degree of sequence identity, slight differences with respect to substrate specificities and interaction with activators and deactivators, or even that their expression profile *in planta* might differ. In *Arabidopsis* quite a number of such highly homologous MAPK pairs have been identified (29, 31), and it will be interesting in the future to learn to what extent their functions are redundant.

The other Pep-13-responsive MAPK was shown to be PcMPK6, a homologue of the AtMPK6, SIPK, and SIMK MAPKs from *Arabidopsis*, tobacco, and alfalfa, respectively, each of which has been shown to be activated following elicitation (32, 35, 39). As reflected in their nomenclature, many of these kinases have also been shown to become activated following abiotic stress treatments including salicylic acid (54), salt, or hyper-osmotic (52), hypo-osmotic, and oxidative stresses (51, 58). It was therefore surprising that no significant increases in PcMPK6 or PcMPK3 activities were observed when cultured parsley cells were placed under conditions described to activate MAPKs in other systems. The exceptions, from the conditions tested, were H_2O_2 and heavy metal treatments that activated PcMPK6 alone. This may reflect a role for this class of MAPKs in responses to oxidative stress, which has been suggested, with respect to treatment with millimolar concentrations of H_2O_2, by the activation of AtMPK6 in *Arabidopsis* (51, 58). It has also been shown that treatment of plants with micromolar concentrations of heavy metals, including copper, results in the

transcript accumulation of many oxidative stress-protective and -responsive genes (72). AtMPK6 class MAPKs may therefore operate as components of signal cascades initiated by these environmental stimuli. In this case the specificity of the outcome may be determined by the relative duration of the kinase activation (as in our hands, the oxidative stress PcMPK6 activation was more transient than that seen in response to elicitors, not shown) or in the contribution made by parallel signaling pathways. Perhaps significantly, none of the abiotic treatments described resulted in the activation of PcMPK3a or 3b. Even with respect to PcMPK6, the highest and most persistent levels of activity strictly correlated with treatments that induce phytoalexin synthesis in parsley cells, *i.e.* elicitors. *P. syringae* HrpZ and the NPP1 protein from *P. parasitica* effectively and strongly activated both PcMPK6 and PcMPK3, although to levels not quite that seen following Pep-13 treatment (not shown) (73).[3] However, these activities also remained significantly higher than the activity of PcMPK6 during the oxidative stress responses. This alone suggests that these MAPKs play important roles in plant defense signaling. The use of different elicitors also highlights the way in which different perception mechanisms can and do converge upon these kinases, as has also been reported in other systems (37). The identification of the sequential upstream components of these MAPK cascades, and the determination of the initial convergence points will be of significant interest in the future.

What functions do MAPKs have in plant defense responses? Recent gain-of-function experiments in tobacco and *Arabidopsis* leaves overexpressing constitutively active MAPKK or wild-type SIPK resulted in the formation of hypersensitive response-like necrotic lesions (45–47). In addition, accumulation of transcripts associated with defense responses was observed. This clearly shows that SIPK/AtMPK6 homologues or their upstream MAPKK activities when overexpressed can trigger defense-related reactions. The mechanism by which this is achieved remains, however, unclear and corresponding loss-of-function approaches were not presented. The recent complete functional identification of a MAPK cascade from *Arabidopsis* that is sufficient to provide increased resistance to pathogen attack has now confirmed the importance of MAPK signaling for plant defense (48). We chose to investigate the importance of MAPK activity for the induction of downstream defense responses that occurred independently of the oxidative burst, using a loss-of-function approach. Our studies have shown that *PR* gene expression (this study) and MAPK activation (16) in parsley cells occurred upstream or independently of the oxidative burst. It was therefore of interest to see whether one response was linked to the other. Overexpression of PcMPK6 in parsley protoplasts followed by Pep-13 treatment resulted in activation of the kinase in a manner indistinguishable to that observed in cells. However a Y-F activation loop mutant could not be activated in this system, confirming this tyrosine phosphorylation reaction as essential for kinase activation during the elicitor response that likely results from activation of an upstream MAPKK activity. Moreover, in co-transfection experiments, this Y-F mutant gave a strong inhibition of the elicitor responsiveness of the *PR2* promoter activity. As *PR* gene expression is regarded as a classical marker for plant defense, we can conclude that PcMPK6 plays an essential role in the induction of these defense reactions. It is unlikely that the kinase is solely responsible for this activity, since, as we demonstrated here with respect to oxidative stress, it can be activated by treatments that do not trigger typical defense reactions. We therefore believe PcMPK6 activation to be a necessary, but not sufficient component for *PR2* gene expression during defense. Interestingly, co-transfection experiments using a Y-F activation loop mutant of PcMPK3b also showed a degree of inhibition of the *PR2* promoter, although not to the levels shown for the PcMPK6 mutant construct (not shown). This may perhaps represent some redundancy in MAPK signaling pathways during defense, where in almost all cases studied to date, activity of the MPK3 class kinases is seen in addition to the MPK6 class. However, hypotheses of this type need to be addressed with the use of specific knockouts, which is seen as difficult in plants, or the identification of specific substrates. It is most likely that the overexpression of the PcMPK6Y214F construct blocks the correct activation of the endogenous wild-type activity and results in a reduced level of phosphorylation of a protein(s) that regulates *PR2* promoter activity. WRKY-type transcription factors were first identified in parsley as proteins that bind to response elements in these promoters (24), which are not present in promoters of Pep-13-responsive parsley genes encoding phytoalexin biosynthetic enzymes activated *via* the oxidative burst (74). WRKY transcription factors have since been implicated in disease resistance responses of *Arabidopsis*, occurring downstream of MAPK signaling (48). Future studies should address the link between MAPK and WRKY activities and will require the identification of MAPK substrates, which at present remain unknown. The identification of these unknown proteins represents a major future challenge for research in plant MAPK signaling and function in mediating plant defense.

Acknowledgments—We thank Dr. Heribert Hirt for providing the *MMK1* and *MMK4* cDNAs and Dr. Naoto Shibuya for a gift of *N*-acetyl chitoheptaose. Advice provided by Christoph Veβ and Dr. Stephan Clemens for heavy metal treatment of cultured parsley cells is gratefully acknowledged.

[3] T. Nürnberger, unpublished data.

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