Struktur, Erkennungsspezifität und Regulationsmechanismus des Resistenzproteins Bs4 aus Tomate

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Zusammenfassung

Das Resistenz (*R*)-Gen *Bs4* aus Tomate (*Lycopersicon esculentum*) vermittelt Resistenz gegen Stämme des Erregers der Bakteriellen Fleckenkrankheit *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), die das Avirulenz (Avr)-Protein AvrBs4 exprimieren. *Bs4* ist konstitutiv schwach exprimiert und kodiert für ein cytoplasmatisches Protein mit einer aminoterminalen Toll/Interleukin1-Rezeptor-ähnlichen (TIR)-Domäne, einer Nukleotidbindedomäne (NB) und carboxyterminalen Leucin-reichen Wiederholungen (LRR). *Bs4* induziert in mehreren Pflanzenarten bei gemeinsamer *Agrobacterium*-vermittelter transienter Expression mit kern- bzw. cytoplasmatisch lokalisierten AvrBs4-Derivaten eine hypersensitive Reaktion (HR), die unabhängig von Kernlokalisierungssequenzen in AvrBs4 ist. Für die HR sind die Signalkomponenten *EDS1, SGT1* und *Hsp90* notwendig. *Bs4* vermittelt auch die Erkennung von Hax3 und Hax4, zwei AvrBs3-ähnlichen Proteinen, sowohl bei *Xcv*-vermittelter Translokation, als auch bei niedriger Expression unter *Bs4*-Promotor-Kontrolle. Dagegen erfolgt AvrBs3-Erkennung nur bei Überexpression. Um zu testen, ob Bs4-Domänen intramolekulare Interaktionen eingehen können, wurden die Bs4-Domänen-Derivate *in planta* exprimiert. Durch Expression von TIR und NB-LRR *in trans* wurde eine AvrBs4-spezifische HR induziert. Außerdem interagierten TIR- und NB-LRR-Domäne bzw. TIR-NB- und LRR-Domäne miteinander, was auf intramolekulare Interaktionen in Bs4 hindeutet. Diese Domänen-Interaktionen sind in Gegenwart von AvrBs4 nicht mehr nachweisbar. Außerdem werden die Protein-Mengen von Bs4 und Bs4-Domänen, die allein keine HR auslösen, stark reduziert. Diese AvrBs4-induzierten Effekte weisen auf eine Zustandsänderung des Bs4-Proteins hin. Um die Rolle von Hsp90 bei der Bs4-vermittelten Resistenz zu analysieren, wurden Überexpressions- und Silencing-Experimente gekoppelt mit phänotypischen und biochemischen Analysen durchgeführt. Hsp90 interagiert mit Bs4 und *Hsp90* ist notwendig für detektierbare Bs4-Mengen. Sowohl Silencing als auch Überexpression von *Hsp90* hemmt die Bs4-HR und die HRs anderer NB-LRR-Proteine. *Hsp90*-Überexpression führt zu mehr Bs4 und bremst den AvrBs4-induzierten Abbau von Bs4. Dieser Abbau erfolgt nicht über Polyubiquitinierung, da deren Inhibierung durch Expression der Ubiquitin-Mutante *ubR48* den Bs4-Abbau nicht stoppt. Vielmehr sind die Bs4-Mengen dann bereits ohne AvrBs4 stark reduziert. Die Expression von *ubR48* beeinträchtigt auch die HRs anderer NB-LRR-Proteine.

Summary

The tomato (*Lycopersicon esculentum*) Bs4 disease resistance (*R*)-gene mediates resistance towards strains of *Xanthomonas campestris* pv. *vesicatoria (Xcv)*, the causal agent of bacterial spot disease, that express the avirulence (Avr) protein AvrBs4. Bs4 is constitutively expressed at low levels and encodes a cytoplasmic R-protein consisting of an amino terminal Toll/Interleukin1-like (TIR) domain, a central nucleotide binding site (NB) and carboxy terminal leucine rich repeats (LRR). Bs4 induces a hypersensitive response (HR) upon transient *Agrobacterium* mediated coexpression with nuclear or cytoplasmically localized AvrBs4 derivatives in tomato, *Nicotiana benthamiana* and other plant species independent of Avr encoded nuclear localisation sequences. This HR requires the signaling components *EDS1, SGT1* and *Hsp90.* Bs4 also mediates recognition of Hax3 and Hax4, two other AvrBs3-like proteins upon *Xcv* type III-dependent translocation as well as low-level transient expression driven by the *Bs4* promoter. By contrast, AvrBs3 requires to be overexpressed to trigger Bs4-HR.

Bs4 domains were expressed *in planta* to address whether they interact *in trans*, indicating intramolecular interactions. Indeed, Bs4 TIR and NB-LRR domain derivatives conferred AvrBs4-induced HR upon expression *in trans*. Furthermore the TIR interacts with the NB-LRR domain and the TIR-NB interacts with the LRR domain *in planta*, and these interactions are resolved in presence of AvrBs4. Protein levels of Bs4 and also Bs4 domains that are alone incapable of eliciting HR decline AvrBs4-dependently pointing towards a state transition of the Bs4 protein.

Hsp90 interacts with Bs4 and is required to maintain detectable Bs4 amounts. Both, silencing and overexpression of *Hsp90* inhibit the Bs4 HR and HRs of other NB-LRR proteins. Conversely, *Hsp90* overexpression results in increased Bs4 levels and blocks the Avr induced disappearance of Bs4. This does not involve polyubiquitinatiation, as *ubR48* expression that blocks polyubiqutination does not prevent disappearance. Instead, *ubR48* expression attenuates Bs4 levels independent of AvrBs4. Expression of *ubR48* does not only interfere with Bs4 but also with HRs triggered by other NB-LRR proteins

Inhalt

Zusammenfassung II
Summary III
Inhalt IV
Verzeichnis der Abbildungen und Tabellen VI
1. Einleitung 1
1.1. Pflanzliche Resistenzmechanismen 1
1.2. Pflanzen-Pathogen-Interaktionen 3
1.3. Das bakterielle Pathogen *Xanthomonas campestris* pv. *vesicatoria* 3
1.4. Die AvrBs3-Effektor-Familie 4
1.5. AvrBs3-ähnliche Proteine als Avirulenzdeterminanten 6
1.6. Resistenzgene gegen AvrBs3-ähnliche Proteine 8
1.7. Das Resistenzgen *Bs4* aus Tomate kodiert für ein TIR-NB-LRR-Protein 9
1.8. Modelle für den Funktionsmechanismus von NB-LRR-Proteinen 12
1.9. Ebenen der Regulation pflanzlicher Resistenz 13
1.10. Zielstellung 15
2. Ergebnisse 16
2.1. Übersicht der Publikationen und Manuskripte 16
2.2. Das Tomaten-Resistenzprotein Bs4 ist ein vorhergesagtes nicht-nukleäres TIR-NB-LRR Protein, das Abwehrreaktionen gegen stark verkürzte Derivate von AvrBs4 und überexprimiertem AvrBs3 vermittelt. 18
2.2.1 Zusammenfassung 18
2.2.2 Manuskript 1 19
2.2.3 Ergänzende Ergebnisse 34
2.2.3.1. Erkennung AvrBs3-ähnlicher Proteine in verschiedenen Pflanzenarten 34
2.2.3.2. Bs4-Funktionalität in heterologen Pflanzenarten 37
2.3. Das Expressionsniveau *avrBs3*-ähnlicher Gene beeinflusst die Spezifität der Tomaten-*Bs4*-, jedoch nicht der Paprika-*Bs3*-vermittelten Erkennung. 39
2.3.1 Zusammenfassung 39
2.3.2 Manuskript 2 40
2.3.3 Zusätzliche Ergebnisse 51
2.3.3.1. Bs4- und AvrBs4-Strukturvorhersagen über *in silico* 3D-Modellierung 51
2.3.3.2. 3D-Modell der TIR-Domäne von Bs4 51

2.3.3.3. Die NB-Domäne von Bs4 weist eine ARC3-ähnliche Region auf 52
2.3.3.4. 3D-Strukturmodell der LRR-Domäne von Bs4 54
2.3.3.5. 3D-Modell einer möglichen AvrBs4-Bs4-Interaktion 55
2.4. Die Erkennung des AvrBs4-Proteins aus *Xanthomonas campestris* pv. *vesicatoria* wird durch miteinander interagierende Bs4-TIR-NB- und LRR-Domänen vermittelt, durch Überexpression oder Silencing von *Hsp90* aufgehoben und geht außerdem mit dem Abbau von Bs4 einher 57
2.4.1 Zusammenfassung 57
2.4.2 Manuskript 3 58
3. Diskussion 100
3.1. Die Bs4-vermittelte Erkennung von AvrBs4 weicht vom generellen Erkennungsprinzip AvrBs3-ähnlicher Proteine ab 100
3.2. Die Fähigkeit zur Erkennung von verkürztem AvrBs4 ist auf die Gattung *Lycopersicon* beschränkt 102
3.3. Die Bs4-Erkennungsspezifität ist abhängig von der Avr-Menge 104
3.4. Bs4 und AvrBs4: indirekte oder direkte Interaktion? 106
3.5. Die Bs4-Menge wird posttranslational reguliert 108
3.6. AvrBs4 induziert die Auflösung von Bs4-Domänen-Interaktionen und führt zum Verschwinden von Bs4 109
3.7. Hsp90 reguliert die Bs4-Aktivität 111
3.8. Die Bs4-HR benötigt Polyubiquitinierung 112
3.9. Die Blockierung der Ubiquitinierung hat einen indirekten Effekt auf die Bs4-Funktion. 113
3.10. Das Bs4-AvrBs4-System birgt einige ungelöste Fragen und bietet vielfältige Möglichkeiten zur weiteren Charakterisierung 114
4. Literatur 116
Verzeichnis der verwendeten Abkürzungen 128
Lebenslauf 129
Veröffentlichungen 130

Verzeichnis der Abbildungen und Tabellen

Abbildungen sind fortlaufend nummeriert. Querverweise zu Abbildungen in Manuskripten sind durch Manuskript-Nummer und dortige Abbildungsnummer angegeben (z.B. M2-1, Manuskript 2, Abb. 1).

Abbildungen

Abb. 1. Modell der Anpassung von bakteriellen Pathogenen an pflanzliche Resistenzmechanismen. ... 2
Abb. 2. Phänotypen der *Xanthomonas* – Tomate – Interaktion ... 4
Abb. 3. Struktur AvrBs3-ähnlicher Proteine ... 5
Abb. 4. Effekte von AvrBs4 in resistenten Pflanzen ... 7
Abb. 5. Domänen-Struktur von NB-LRR-Proteinen. ... 10
Abb. 6. Direktes und indirektes Erkennungsprinzip. ... 12
Abb. 7. Schalter-Modell der NB-LRR-Aktivierung. ... 14
Abb. 8. Phänotypen nach transienter Expression von *avr*-Genen, *Bs4* und Reportergenen 38
Abb. 9. 3D-Modell der Bs4-TIR-Domäne ... 51
Abb. 10. 3D-Modell der Bs4-NB-Domäne.. ... 53
Abb. 11. 3D-Modell der Bs4-LRR-Domäne ... 54
Abb. 12. AvrBs4-Strukturvorhersage. ... 55
Abb. 13. Modell einer Bs4-AvrBs4-Assoziation.. ... 56
Abb. 14. Taxonomische Verbreitung der Erkennung AvrBs3-ähnlicher Proteine und der Bs4-Funktionalität innerhalb der getesteten Nachtschattengewächse.. ... 103
Abb. 15. Funktionsprinzip von ubR48. ... 112
Abb. 16. Dosis-Modell des ubR48-Einflusses auf die Bs4-HR. ... 113

Tabellen

Tabelle 1. Pflanzliche Resistenzgene, die die Erkennung AvrBs3-ähnlicher Proteine vermitteln. ... 8
Tabelle 2. Verbreitung der Avr-Aktivität von AvrBs3-ähnlichen Proteinen bzw. der *Bs4*-Funktionsfähigkeit ... 35

1. Einleitung

1.1. Pflanzliche Resistenzmechanismen

Pflanzen sind ständig Mikroorganismen ausgesetzt. Die Fähigkeit, Pathogene zu detektieren und sich davor zu schützen, ist deshalb ein lebensnotwendiger Mechanismus. Die Resistenz von Pflanzen gegen die meisten potentiell phytopathogenen Mikroorganismen, wird als Spezies- oder Nichtwirtsresistenz bezeichnet. Dieses, auch als Basisresistenz bezeichnete Prinzip, ist die vorherrschende Form der pflanzlichen Krankheitsresistenz und beschreibt allgemein die Unfähigkeit eines Mikroorganismus, Pflanzenarten zu besiedeln (Heath, 2000). Teil der Nichtwirtsresistenz sind membranständige Rezeptoren, die typische Pathogen-assoziierte Strukturen (*pathogen-associated molecular patterns*, PAMPs), wie z. B. Flagellin erkennen (Gómez-Gómez und Boller, 2000; Zipfel *et al.*, 2004). Dies führt zur Abwehr der Mehrzahl aller Mikroorganismen, bevor diese Zugang zum Wirtszellinneren erlangen (Abb. 1A).

Phytopathogene Bakterien können jedoch die PAMP-basierte Erkennung verhindern, z. B. durch Modifikation ihrer PAMPs (Gómez-Gómez *et al.*, 1999) oder durch Typ-III-Sekretion von Effektor-Proteinen ins Wirtscytoplasma (Nürnberger *et al.*, 2004). Typ-III-sekretierte Effektoren können mit den Mechanismen der Nichtwirtsresistenz interferieren und eine Abwehrreaktion einschränken (Abb. 1B, Abramovitch *et al.*, 2006; Janjusevic *et al.*, 2006). Einige Pflanzen-Kultivare besitzen wiederum Mechanismen, um Effektor-Proteine (auch bezeichnet als Avirulenz-Proteine, Avr) zu detektieren und eine Effektor-induzierte Resistenz auszulösen (Abb. 1C, Keen, 1990). Diese kultivarspezifische Erkennung von Pathogenen ist vom Effektor-kodierenden *Avr*-Gen und von einem korrespondierenden pflanzlichen Resistenz- (*R*-) Gen abhängig (Gen-für-Gen-Hypothese, Flor, 1942). Ein typisches Kennzeichen von Resistenzreaktionen ist die damit einhergehende hypersensitive Reaktion (HR, Greenberg 2005), ein schneller, lokaler Zelltod des infizierten Gewebes (Klement, 1963; Lam und Blumwald, 2002; van Doorn und Woltering, 2005). Weitere Merkmale sind die Anreicherung reaktiver Sauerstoff-Spezies (*oxidative burst*, Lam *et al.*, 2001) bzw. Stickstoff-Monoxid (Wendehenne *et al.*, 2004), Kallose-Ablagerungen, transkriptionelle Genaktivierung (Dixon und Harrison, 1990) und Phytoalexin-Akummulierung (Hammond-Kosack und Jones, 1996; Greenberg und Yao, 2004). Außerdem können Pflanzen eine systemische Resistenz ausbilden (*systemic acquired resistance*, Durrant und Dong, 2004).

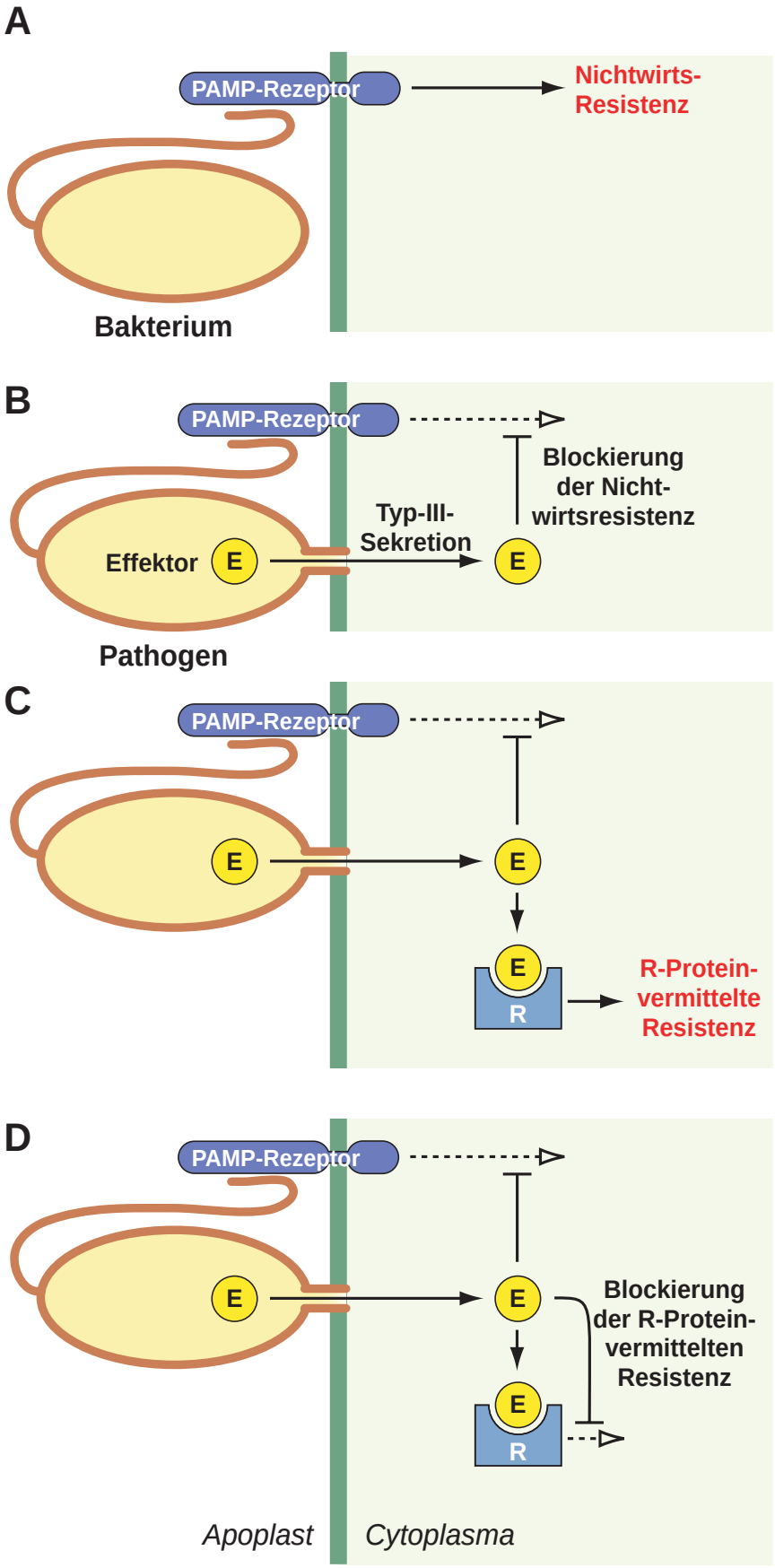

Abb. 1. Modell der Anpassung von bakteriellen Pathogenen an pflanzliche Resistenzmechanismen. **(A)** Pathogen-assoziierte molekulare Strukturen (PAMPs, z. B. Flagellin) induzieren die Nichtwirtsresistenz. **(B)** Bakterielle Effektor-Proteine unterbinden die Nichtwirtsresistenz. **(C)** R-Proteine vermitteln Erkennung einzelner Effektor-Proteine. **(D)** Andere Effektor-Proteine verhindern die Auslösung einer Resistenz (R)-Protein-vermittelten Resistenzreaktion.

Einige bakterielle Effektor-Proteine blockieren die Ausbildung der HR. Bakterien besitzen dadurch weitere spezielle Anpassungsmechanismen zur Überwindung der pflanzlichen Resistenz (Abb. 1D, siehe z.B Jamir *et al.*, 2004).

1.2. Pflanzen-Pathogen-Interaktionen

Die Erforschung der Interaktionen zwischen Pathogen und Pflanze begann mit ökonomisch relevanten Wirtspflanzen und deren Pathogenen. Die ersten Untersuchungen beschrieben die Resistenz von Weizen gegen Weizenrost (Biffen, 1905). Untersuchungen an Flachs und Flachsrost führten zur Formulierung der Gen-für-Gen-Hypothese (Flor, 1971). Aufgrund der Zugänglichkeit gegenüber molekularen Techniken wurden das erste *avr*-Gen aus Bakterien isoliert (Staskawicz *et al.*, 1984). Die spätere Isolierung von *avr*-Genen (*avrPto*, Ronald *et al.*, 1992) und korrespondierenden *R*-Genen (*Pto*, Martin *et al.*, 1993) bestätigte die Gen-für-Gen-Hypothese. Aktuell sind bereits für alle Pathogen-Klassen (Viren, Bakterien, Pilze, Oomyceten, Nematoden und Insekten) *R*-Gene isoliert wurden (Bent *et al.*, 1994; Jones *et al.*, 1994; Whitham *et al.*, 1994; Parker *et al.*, 1997; Milligan *et al.*, 1998; Vos *et al.*, 1998). Dagegen sind korrespondierende Avr-Proteine bisher nur aus Viren, Bakterien, Pilzen und Oomyceten bekannt.

Die Analyse von *R*-Genen zeigte, dass zwei Grundprinzipien der Pathogen-Perzeption durch R-Proteine existieren. Membranständige R-Proteine vermitteln die extrazelluläre Erkennung (z. B. Avr2 und Cf2, Rooney *et al.*, 2005). Die für die Mehrzahl der R-Proteine vorhergesagte intrazelluläre Lokalisation weist aber darauf hin, dass der Transfer von Avr-Proteinen in die Pflanzenzelle die Basis vieler Resistenzreaktionen ist (Bonas und Lahaye, 2002). Dieser Transfer kann bei phytopathogenen Bakterien z. B. über ein Typ-III-Sekretionssystem erfolgen (Hueck, 1998). Für pathogene Pilze und Oomyceten ist dagegen bisher nicht bekannt, wie die vom Pathogen meistens sekretierten Proteine ins Wirtszellinnere gelangen (Ellis *et al.*, 2006). Die HR-Induktion nach Expression von Avr-Proteinen in Zellen resistenter Pflanzen bestätigte jedoch die Hypothese der intrazellulären Erkennung (Van der Hoorn *et al.*, 2000; Dodds *et al.*, 2006).

1.3. Das bakterielle Pathogen *Xanthomonas campestris* pv. *vesicatoria*

Das 1921 nahezu gleichzeitig in Südafrika (Doidge, 1921) und Amerika (Gardner und Kendrick, 1921) aus Tomaten isolierte Bakterium *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) (Dye, 1978) ist der Erreger der bakteriellen Fleckenkrankheit von Paprika und

Tomate (Abb. 2A,B) und besonders ertragsschädigend in feuchten warmen Klimaten. *Xcv* dringt über Spaltöffnungen und Verwundungen ins Blatt ein und vermehrt sich im Apoplasten in anfälligen (suszeptiblen) Pflanzen. Im Gegensatz zu anderen *Xanthomonas*-Spezies (z.B. *X. campestris* pv. *campestris*) verbreitet sich *Xcv* nicht systemisch in der Pflanze, sondern bleibt auf die lokale Infektionsstelle begrenzt. Makroskopisch werden wässrige Läsionen (*watersoaked lesions*, WS, Abb. 2B,D) sichtbar, die später nekrotische Flecken auf Blättern und Früchten bilden (Abb. 2A,B). Dagegen zeigen resistente Kultivare keine sichtbaren Symptome. Bei Infiltration hoher *Xcv*-Titer kommt es jedoch zu einer HR des inokulierten Gewebeabschnitts innerhalb von zwei Tagen (Abb. 2C, Laborphänotyp).

Notwendig für die Ausprägung der Krankheitssymptome und auch für die Auslösung der HR ist ein durch die *Xanthomonas hrp*-Gene (hypersensitive Reaktion und Pathogenität) kodiertes Typ-III-Sekretionssystem, welches bakterielle Effektor-Proteine direkt ins Cytoplasma der Wirtszelle transloziert (Hueck, 1998; Büttner und Bonas, 2006)

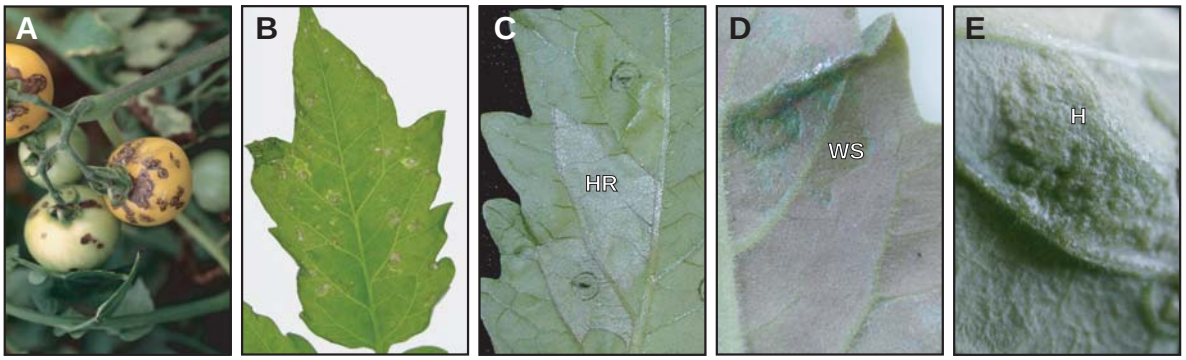

Abb. 2. Phänotypen der *Xanthomonas*–Tomate–Interaktion. Bakterielle Fleckenkrankheit auf Früchten **(A)** und Blättern **(B)**. Laborphänotyp auf resistenten **(C)** und suszeptiblen **(D)** Tomaten-Kultivaren 72 h p.i., Hypersensitive Reaktion nach Infiltration eines avirulenten Stammes (HR) und beginnende wässrige Läsionen (WS) nach Infiltration eines virulenten Stammes. Hypertrophie (H) nach Infiltration eines AvrBs3-exprimierenden *Xcv*-Stammes in Tomate **(E)**. Abb.2A reproduziert von http://plaza.ufl.edu/jbjones/joneslab

1.4. Die AvrBs3-Effektor-Familie

AvrBs3-ähnliche Proteine repräsentieren eine nach ihrem ersten Vertreter benannte Familie von bakteriellen Effektoren, die sich durch eine hohe Aminosäure (AS)-Sequenzidentität auszeichnen (Schornack *et al.*, 2006; Abb. 3A). Gene, die für Proteine dieses Typs kodieren, wurden bislang ausschliesslich in Xanthomonaden nachgewiesen, mit Ausnahme von *brg11* aus *Ralstonia solanacearum* (Salanoubat *et al.*, 2002; Cunnac *et al.*, 2004). Zwei bereits gut charakterisierte Vertreter aus *Xcv* sind *avrBs3* und *avrBs4* (vormals *avrBs3-2*)(Bonas *et al.*, 1989; Bonas *et al.*, 1993). Weitere Sequenz-Homologe

von *avrBs3* wurden aus *X. campestris pv. armoraciae* isoliert (*hax2* , *hax3* und *hax4*, Abb. 3B, Kay *et al.*, 2005).

Im C-Terminus tragen alle von *avrBs3*-homologen Genen abgeleitete Proteine typische Motive eukaryotischer Transkriptionsaktivatoren, nukleäre Lokalisierungssignale (NLS), sowie eine saure Aktivierungsdomäne (AD, (Abb. 3A, Yang und Gabriel, 1995; Lahaye und Bonas, 2001). NLS-Sequenzen dirigieren AvrBs3 nach Typ-III-Sekretion Importin α-vermittelt in den Zellkern der Pflanze (Szurek *et al.*, 2002). Über die AD induziert AvrBs3 die Transkription von Genen, welche zur Ausbildung von phänotypisch sichtbarer

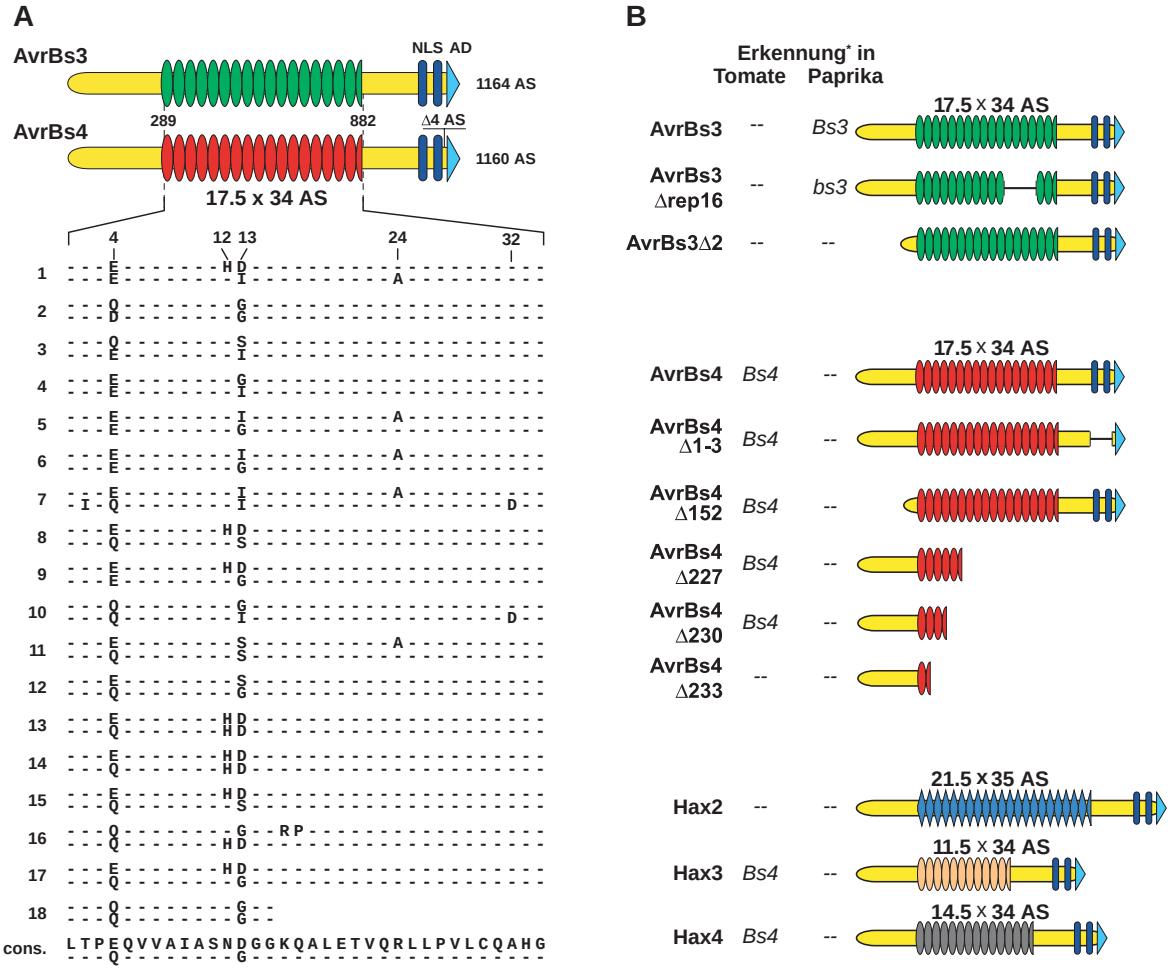

Abb. 3. Struktur AvrBs3-ähnlicher Proteine (A) Struktur- und Sequenzvergleich von AvrBs3 und AvrBs4. Beide Proteine sind zu 97% identisch in ihrer AS-Sequenz. Die zentrale Region besteht aus 17,5 nahezu identischen Repeats. Unterschiede befinden sich bis auf eine Deletion von 4 AS-Resten (Δ4 AS) ausschließlich in der Repeat-Region. AS-Unterschiede zwischen den nummerierten Repeats von AvrBs3 (jeweils obere Zeile) und AvrBs4 (jeweils untere Zeile) und innerhalb aller Repeats sind dargestellt. Die letzten beiden Zeilen enthalten die Konsensussequenz aller AvrBs3- bzw. AvrBs4-Einzelrepeats. -, identische AS-Reste, NLS, Kernlokalisierungssignale; AD, saure Aktivierungsdomäne (aus Schornack *et al.*, 2006, modifiziert). **(B)** Struktur relevanter AvrBs3-ähnliche Proteine (siehe Tabelle 1 für Referenzen) und von Deletionsderivaten. * Die *R*-Gen-Spezifität von *Xcv*-translozierten AvrBs3-ähnlichen Proteinen ist angegeben.

Symptome beitragen können (Szurek *et al.*, 2001; Marois *et al.*, 2002). Infiltriert man AvrBs3-exprimierende Xanthomonaden in Blätter suszeptibler Paprika-, bzw. Tomaten-Pflanzen, so reagiert das infiltrierte Gewebe mit einer Hypertrophie des abaxialen Mesophylls (Abb. 2, Marois *et al.*, 2002). Andere AvrBs3-ähnliche Proteine induzieren ebenfalls Symptome in anfälligen Pflanzen, die auf unterschiedliche Weise zur Virulenz beitragen (z.B. Hyperplasie, verstärkte Aggressivität, verstärktes bakterielles Wachstum, ein Überblick findet sich bei Gürlebeck *et al.*, 2006). Für AvrBs4 konnte verbessertes bakterielles Wachstum, verbesserte Transmission und Läsionsbildung bisher nur unter Feldbedingungen, jedoch nicht im Labor nachgewiesen werden (Wichmann und Bergelson, 2004). In der Regel sind für die beobachteten Symptome C-terminale NLS und AD notwendig (Marois *et al.*, 2002). Ob dies auch für AvrBs4 zutrifft, wurde bisher nicht geprüft.

Weiterhin kennzeichnend für alle AvrBs3-ähnlichen Proteine ist eine zentrale Region von 5,5 (Avrxa5, Liang *et al.*, 2004) bis 28,5 (PthXo3, Yang und White, 2004) direkten, nahezu sequenzidentischen Wiederholungen aus je 34, oder seltener 35 AS-Resten (Brg11, Cunnac *et al.*, 2004 und Hax2), von denen einzelne hypervariabel sind (Abb. 3A,B). Analysen von AvrBs3-ähnlichen Proteinen mit Unterschieden ausschließlich in der Repeat-Region belegen eine Abhängigkeit des Spektrums induzierter pflanzlicher Gene von diesem Proteinabschnitt (Marois *et al.*, 2002). Für AvrXa7 wurde außerdem eine nicht spezifische DNA-Affinität nachgewiesen (Yang *et al.*, 2000). Deshalb könnte diese Region eine Bindung des Avr-Proteins an DNA-Abschnitte vermitteln.

1.5. AvrBs3-ähnliche Proteine als Avirulenzdeterminanten

Xcv-Stämme, die AvrBs3-ähnliche Proteine exprimieren, induzieren in resistenten Pflanzenlinien eine *R*-Gen-abhängige HR. Notwendig dafür ist ein N-terminal lokalisiertes Typ-III-Sekretionssignal. Die Deletion dieser Region (z.B. AvrBs3Δ2 und AvrBs4Δ152, Abb. 3B) führt zum Verlust der Erkennung in resistenten Pflanzen, weil das Avr-Protein nicht mehr in die Wirtszelle transloziert wird (Szurek *et al.*, 2001; Szurek *et al.*, 2002). AvrBs3 wird in Paprikapflanzen (*Capsicum annuum*) erkannt, die das korrespondierende *Bs3*-Resistenzgen tragen (Pierre *et al.*, 2000). Das 97% identische AvrBs4 induziert dagegen eine *Bs4*-vermittelte HR in Tomate (Abb. 3B, Ballvora *et al.*, 2001). Bemerkenswert ist, dass bei *Xcv*-vermittelter Translokation keine Kreuzreaktion auftritt, d. h. *Xcv* (*avrBs3*) induziert keine *Bs4*-HR und *Xcv* (*avrBs4*) keine HR in *Bs3*-Paprika

(Schornack *et al.*, 2005). Da sich AvrBs3 und AvrBs4 bis auf vier AS-Reste nur im Bereich der Repeat-Region unterscheiden, muss dort die Spezifität der Avr-Aktivität liegen (Bonas *et al.*, 1993). Interessanterweise wird ein im Bereich der Repeat-Region deletiertes AvrBs3Δrep16-Derivat in *bs3*-, aber nicht in *Bs3*-Paprika erkannt (Abb. 3B, Herbers *et al.*, 1992). Die Repeat-Region von AvrBs3-ähnlichen Proteinen definiert also nicht nur die Ausbildung von Symptomen, sondern die Avr-Aktivität (Herbers *et al.*, 1992; Yang *et al.*, 1994; Zhu *et al.*, 1998; White *et al.*, 2000).

Die AvrBs3-Repeats vermitteln, ähnlich wie Transkriptionsregulatoren, eine Dimerisierung. Dies deutet auf eine transkriptionelle Regulation von Pflanzen-Genen hin, die in kompatible und inkompatible Interaktionen involviert sind (Gürlebeck *et al.*, 2005). In Übereinstimmung mit ihrer Avr-Aktivität durch Transkriptionsaktivierung im Zellkern wurde für Vertreter der AvrBs3-Familie eine NLS-Abhängigkeit der HR-Induktion gezeigt (Szurek *et al.*, 2001; Gu *et al.*, 2005).

Eine Ausnahme von dieser NLS-Abhängigkeit der HR-Induktion ist das AvrBs3-ähnliche Protein AvrBs4. Die funktionsbasierte Suche nach *Xanthomonas*-Genen, die für eine HR in Tomate notwendig sind, identifizierte *avrBsP*, das für ein Protein mit hoher Ähnlichkeit zur N-terminalen Hälfte von AvrBs3 kodiert (Canteros *et al.*, 1991). Allerdings war *avrBsP* kürzer als *avrBs3* und kodierte nicht für die C-terminalen NLS und AD-Motive. Später stellte sich heraus, dass *avrBsP* ein sequenzidentisches Fragment von *avrBs4* war. Da AvrBs4 auch ohne NLS und AD in Tomate erkannt wird (Bonas *et al.*, 1993), besitzt

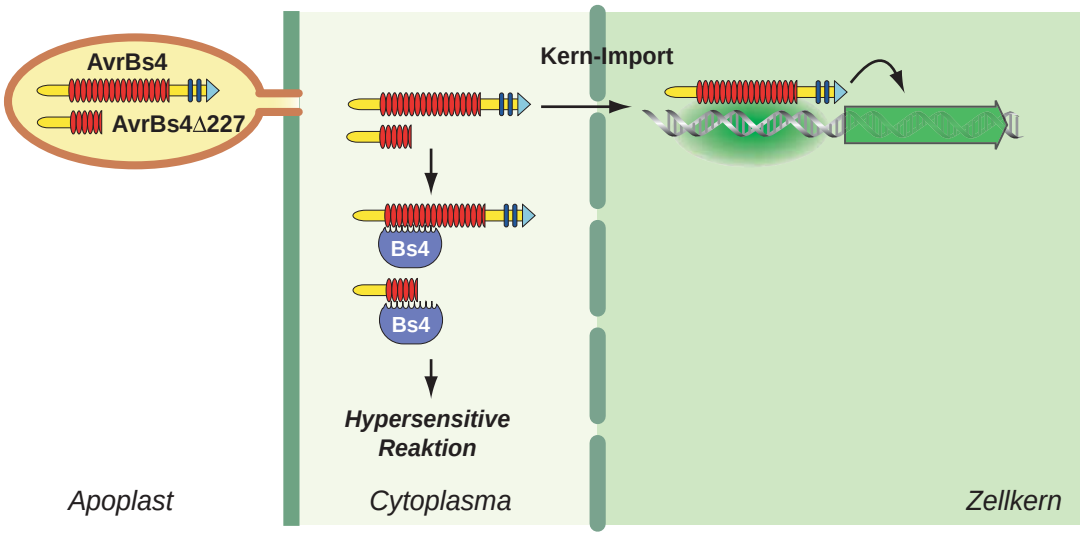

Abb. 4. Effekte von AvrBs4 in resistenten Pflanzen. (A) AvrBs4 wird nach Translokation durch das Typ-III-System ins Wirtscytoplasma wahrscheinlich Importin α-vermittelt in den Zellkern transportiert, wo es in Analogie zu anderen AvrBs3-ähnlichen Proteinen die Wirtsgen-Transkription beeinflusst. AvrBs4 und AvrBs4-Derivate ohne NLS und AD (AvrBs4Δ227) induzieren wahrscheinlich im Cytoplasma eine *Bs4*-vermittelte HR.

Tomate eine Erkennung im Cytoplasma, die von der anderer AvrBs3-ähnlichen Proteine abweicht (Abb. 4, Bonas *et al.*, 1993; Ballvora *et al.*, 2001). Die Identifizierung des zugrunde liegenden *R*-Gens führte zur Isolierung von *Bs4*.

1.6. Resistenzgene gegen AvrBs3-ähnliche Proteine

Trotz der Kenntnis von mehr als 40 *avrBs3*-ähnlichen Genen aus *Xanthomonas* und *Ralstonia*, ist nur für elf ein korrespondierender *R*-Gen (-Lokus) in Pflanzen bekannt. Insgesamt sind bisher vier *R*-Gene (*Bs4*, *xa5*, *xa13* und *Xa27*) isoliert und charakterisiert worden (Tabelle 1). Obwohl alle die Erkennung eines spezifischen AvrBs3-ähnlichen Proteins vermitteln, kodieren sie für Proteine ohne Ähnlichkeit zueinander (Schornack *et al.*, 2006). *Xa27* aus Reis hat nur im Reis-Genom sequenzverwandte Gene und kodiert für ein Protein ohne Homologie zu bekannten Proteinen.

Tabelle 1. Pflanzliche Resistenzgene, die die Erkennung AvrBs3-ähnlicher Proteine vermitteln.

***R*-Gen**	**Referenzen[a]**	**Avr-Protein**	**Repeat-Anzahl**	**Patho-gen[b]**	**Referenz[c]**
Paprika					
Bs3	(Pierre *et al.*, 2000)	AvrBs3	17,5	*Xcv*	(Bonas *et al.*, 1989)
Tomate					
Bs4	(Ballvora *et al.*, 2001), (Schornack *et al.*, 2004)*	AvrBs4	17,5	*Xcv*	(Bonas *et al.*, 1993)
Bs4		Hax3	11,5	*Xca*	(Kay *et al.*, 2005)
Bs4		Hax4	14,5	*Xca*	(Kay *et al.*, 2005)
Reis					
Xa3	(Ezuka *et al.*, 1975{Ogawa, 1986 #5400; Yoshimura *et al.*, 1992)	AvrXa3	8,5	*Xoo*	(Li *et al.*, 2004; Lee *et al.*, 2005)
xa5	(Blair *et al.*, 2003; Iyer und McCouch, 2004)*, (Zhong *et al.*, 2003)	Avrxa5	5,5	*Xoo*	(Hopkins *et al.*, 1992; Bai *et al.*, 2000)
Xa7	(Sidhu *et al.*, 1978; Porter *et al.*, 2003)	AvrXa7	25,5	*Xoo*	(Hopkins *et al.*, 1992; Vera Cruz *et al.*, 2000)
Xa10	(Yoshimura *et al.*, 1983; Yoshimura *et al.*, 1995; Xinghua *et al.*, 1996)	AvrXa10	15,5	*Xoo*	(Hopkins *et al.*, 1992; Zhu *et al.*, 1998)
xa13 (Os8N3)	(Chu *et al.*, 2006)* ,(Yang *et al.*, 2006)*	PthXo1	23,5	*Xoo*	(Yang und White, 2004)
Xa27	(Gu *et al.*, 2005)*	AvrXa27	16,5	*Xoo*	(Gu *et al.*, 2005)
Baumwolle					
B1	(Gabriel *et al.*, 1986; De Feyter *et al.*, 1993)	Avrb6	13,5	*Xcm*	(De Feyter und Gabriel, 1991; De Feyter *et al.*, 1993)

[a]) Referenzen, die die genetische Kartierung und Isolierung (markiert durch *) von *R*-Genen beschreiben
[b]) *Xcv, X. campestris* pv. *vesicatoria; Xca, X. campestris* pv. *armoraciae; Xoo, X. oryzae* pv. *oryzae; Xcm, X. campestris* pv. *malvacearum*
[c]) Referenzen, die die Isolierung und Charakterisierung der korrespondierenden *avr*-Gene beschreiben.

Unterschiede zwischen resistentem und suszeptiblem Allel liegen besonders im Promotorbereich. Avrxa27 aus X. *oryzae* pv. *oryzae* (*Xoo*) induziert nur die Expression des *Xa27*–Allels aus resistenten Pflanzen (Gu *et al.*, 2005).

Bei der *xa13* (*Os8N3*)-vermittelten Resistenz erfolgt dagegen eine für das bakterielle Wachstum notwendige transkriptionelle Induktion des suszeptiblen *Xa13*-Allels durch PthXo1 (entspricht Avrxa13) aus *Xoo*. Sequenzunterschiede im *xa13*-Promoter verhindern eine *xa13*-Aktivierung in resistenten Pflanzen (Chu *et al.*, 2006; Yang *et al.*, 2006). Als Untereinheit des Präinitiationskomplexes ist das von *xa5* kodierte Protein an der Regulation der Transkription in Reis beteiligt. Obwohl der Resistenzmechanismus noch ungeklärt ist, wird vermutet, dass Avrxa5 aus *Xoo* durch Interaktion mit xa5 die Transkription krankheitsfördernder Gene beeinflusst (Iyer und McCouch, 2004).

1.7. Das Resistenzgen *Bs4* aus Tomate kodiert für ein TIR-NB-LRR-Protein

AvrBs4-exprimierende *Xcv*-Stämme induzieren eine Resistenzreaktion in nahezu allen daraufhin untersuchten *Lycopersicon*-Linien (Bonas *et al.*, 1993; Ballvora *et al.*, 2001). Die Identifizierung phänotypisch abweichender, suszeptibler Linien ist jedoch zum Aufbau einer Kreuzungspopulation für eine kartengestützte Klonierung notwendig. Nur *L. pennellii* LA2963 zeigte einen von der schnellen HR abweichenden Phänotyp. AvrBs4-exprimierende *Xcv*-Stämme induzierten zwar auch hier eine nekroseähnliche Reaktion, diese war aber zeitlich verzögert (Schornack *et al.*, 2005). Dieser differenzielle Phänotyp bildete die Basis für die Isolierung des in Anlehnung an andere *Xcv*-Resistenzgene *(Bs1, Bs2, Bs3*, Hibberd *et al.*, 1987) als *Bs4* bezeichneten *R*-Gens aus Tomate (Ballvora *et al.*, 2001). *Bs4* kodiert für ein NB-LRR-Protein mit 54%-Sequenzidentität zum N-Protein aus Tabak (Whitham *et al.*, 1994) bzw. 57% zu Y-1 aus Kartoffel (Vidal *et al.*, 2002). Beide homologen Proteine vermitteln aber keine Resistenz gegen bakterielle AvrBs3-ähnliche Proteine, sondern gegen Viren. DNA-Hybridisierungen identifizierten neben *Bs4* keine weiteren hoch homologen Sequenzen im Tomaten-Genom (Schornack *et al.*, 2004).

Die *Bs4*-Erkennungsspezifität wurde durch Infiltration von *Xcv*-Stämmen mit verschiedenen AvrBs4-Derivaten in Tomate getestet. Nur *Xcv* (*avrBs4*) bzw. dessen NLS/AD-Deletionsderivat (AvrBs4Δ227, Abb. 3B), aber nicht *avrBs3*-exprimierende Xanthomonaden lösten eine HR aus. AvrBs4-Deletionsstudien zeigten, dass ein Derivat mit 3,5 Repeats (AvrBs4Δ230, Abb. 3B) noch eine HR induziert. Eine weitere Verkürzung

auf 1,5 Einzelrepeats (AvrBs4Δ233, Abb. 3B) führt zum Verlust der *Bs4*-spezifischen Erkennung (Bonas *et al.*, 1993; Ballvora *et al.*, 2001; Schornack *et al.*, 2004). Die Funktionalität von *Bs4* konnte auch durch *Agrobacterium*-vermittelte Koexpression mit *avrBs4*-Derivaten in *Nicotiana benthamiana* gezeigt werden, da auch in dieser Pflanze eine *Bs4*-HR induziert wurde (Schornack *et al.*, 2004).

Das TIR-NB-LRR-Protein Bs4 gehört zur großen Familie intrazellulärer NB-LRR-Proteine mit zentraler Nukleotid-Bindestelle (NB) und C-terminalen Leucin-reichen Sequenzwiederholungen (*leucine-rich repeats*, LRR; Abb. 5).
Innerhalb dieser Klasse wird aufgrund der unterschiedlichen N-Termini zwischen TIR-NB-LRR-Proteinen (enthalten einen TOLL/Interleukin-1 Rezeptor-ähnlichen N-Terminus) und NB-LRR-Proteinen ohne konservierte N-terminale Domäne unterschieden, die oft einen Leucin-Zipper oder eine putative *coiled-coil* (CC) Struktur tragen. Beide NB-LRR-Klassen lassen sich zusätzlich anhand spezifischer AS-Reste in der NB-Domäne unterscheiden (Meyers *et al.*, 1999; Pan *et al.*, 2000; Young, 2000).
Die zwischen Tieren und Pflanzen konservierte **TIR-Domäne** interagiert beim Toll-Protein aus *Drosophila* und bei Toll-ähnlichen Rezeptoren der Säugetiere mit Proteinen der Signalkaskade. Aufgrund der Konservierung in TIR-NB-LRR-Proteinen wird auch in Pflanzen eine Rolle bei der Signalweiterleitung angenommen. Interessanterweise ist die Funktion nahezu aller TIR-NB-LRR Proteine abhängig von *EDS1* (*enhanced disease susceptibility*), einem ursprünglich aus *Arabidopsis* isolierten Lipase-ähnlichen Protein (Falk *et al.*, 1999). Dagegen zeigen CC-NB-LRR Proteine keine generelle *EDS1*-

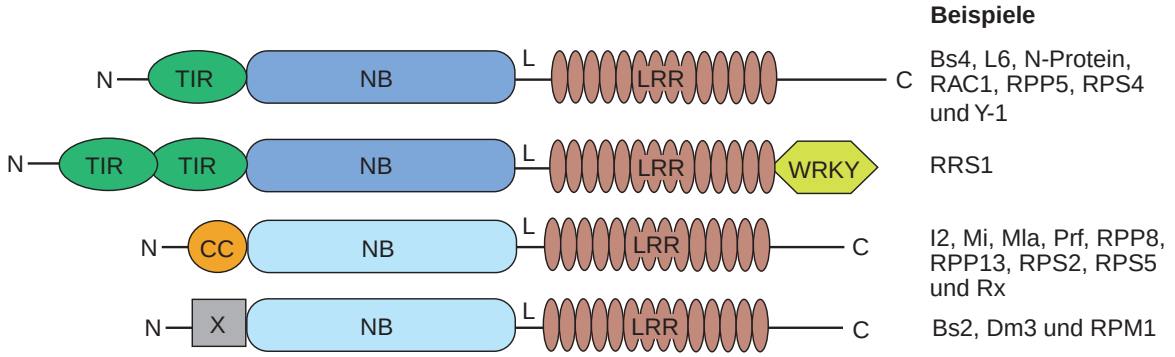

Abb. 5. Domänen-Struktur von NB-LRR-Proteinen. Beispiele für jede Strukturklasse sind rechts aufgeführt. Bs4, I2, Mi, und Prf aus Tomate; L6 aus Flachs; N aus Tabak; RAC1, RPP5, RPS4, RRS1, RPP8, RPP13, RPS2, RPS5 und RPM1 aus *Arabidopsis*; Y-1 und Rx aus Kartoffel; Mla aus Gerste; Dm3 aus Salat; Bs2 aus Paprika. N, Amino-Terminus; TIR, Toll/interleukin-1 Rezeptor-ähnliche Domäne; CC, *Coiled-coil*-Domäne; X, Domäne ohne eindeutiges CC-Motiv; NB, Nukleotid-Bindungsstelle; L, Linker; LRR, Leucin-reiche Wiederholungen (*leucine-rich repeats*); WRKY, Zink-Finger Transkriptionsfaktor-verwandte Domäne mit WRKY-AS-Motiv; C, Carboxyl-Terminus. (aus McHale *et al.*, 2006, modifiziert).

Abhängigkeit (Aarts *et al.*, 1998). Die TIR-Domäne könnte aber auch in die Erkennung des korrespondierenden Avr-Proteins involviert sein (Luck *et al.*, 2000). Das bei Bs4 und zahlreichen anderen TIR-NB-LRR-Proteinen vorhandene N-terminale Alanin-Polyserin-Motiv könnte eine Rolle bei der Protein-Stabilität spielen (Meyers *et al.*, 2003).

Die **NB-Domäne** enthält konservierte Motive: eine N-terminale AAA+ATPase (*extended ATPase with a variety of activities*, Neuwald et al., 1999) und eine sich anschließende APAF-, R-Protein- und CED4-homologe Region (ARC, Van der Biezen und Jones, 1998a). Mutationen im konservierten ATPase-Motiv P-Loop führen zum Funktionsverlust (Dinesh-Kumar *et al.*, 2000; Bendahmane *et al.*, 2002). Weiterhin weist die gesamte NB-Domäne Homologie zu STAND-ATPasen (*signal transduction ATPases with numerous domains*) auf, zu denen auch die tierischen NOD (*N-terminal oligomerisation domain*)-Proteine gehören (Leipe *et al.*, 2004; Albrecht und Takken, 2006; Takken *et al.*, 2006). STAND-ATPasen sind Nukleotidtriphosphat-hydrolysierende Schalter, welche Signaltransduktionsprozesse durch Konformationsänderungen steuern (Leipe *et al.*, 2004). Biochemische Analysen der NB-LRR-Proteine I-2 und Mi-1 aus Tomate belegten eine ATP-Bindung und ATPase-Aktiviät (Tameling *et al.*, 2002). Außerdem vermittelt die NB-Domäne zusammen mit der TIR-Domäne die von tierischen NOD-Proteinen bekannte Oligomerisierung des N-Proteins aus Tabak (Mestre und Baulcombe, 2006). Mutationen im Bereich der ARC-Region (z. B. im Methionin-Histidin-Aspartat-Motiv, MHD) führen zur Autoaktivierung einer HR in Abwesenheit des Elicitors (Bendahmane *et al.*, 2002; de la Fuente van Bentem *et al.*, 2005; Howles *et al.*, 2005). Weitere autoaktivierende Mutationen wurden auch im Bereich des LRR identifiziert, was auf ein Zusammenwirken beider Domänen bei der Regulation der Signaltransduktion hindeutet (Bendahmane *et al.*, 2002).

Der **LRR** ist eine Protein-Protein-Interaktionsdomäne in Prokaryonten und Eukaryonten (Kobe und Deisenhofer, 1995). Bei NB-LRR-Proteinen wird für diese Domäne die Interaktion mit dem Avr-Protein postuliert. Basierend auf der 3D-Struktur des Ribonuklease-Inhibitors des Schweins bildet der LRR eine hufeisenförmige 3D-Struktur aus, in deren konkaver Innenseite sich parallele β-Stränge befinden, die eine Interaktionsoberfläche darstellen könnten (Kobe und Deisenhofer, 1995). Im Bereich der β-Stränge wurden Lösungsmittel-orientierte AS-Reste identifiziert, die im intergenischen Sequenzvergleich hypervariabel sind und deshalb die Erkennungsspezifität des R-Proteins definieren könnten (Parniske *et al.*, 1997; Meyers *et al.*, 1998). Eine direkte Interaktion

zwischen der LRR-Domäne und einem Avr-Protein wurde bisher jedoch nur einmal gezeigt (Jia *et al.*, 2000)

Während CC-NB-LRR-Proteine einen eher kurzen AS-Abschnitt C-terminal vom LRR haben, weisen TIR-NB-LRR-Proteine größere C-terminale Nicht-LRR-Bereiche auf. Diese können durch ihre Ähnlichkeit zu Transkriptionsfaktoren (z.B. WRKY-Trankriptionsfaktor-Motiv in RRS1, Deslandes *et al.*, 2002) oder anhand von konservierten AS-Motiven vom LRR abgegrenzt werden (Dodds *et al.*, 2001).

1.8. Modelle für den Funktionsmechanismus von NB-LRR-Proteinen

Die Vermittlung einer Resistenzreaktion ist die einzige bislang beschriebene Funktion pflanzlicher NB-LRR-Proteine (Dangl und Jones, 2001). NB-LRR-kodierende Gene repräsentieren eine der umfangreichsten Genfamilien in sequenzierten Pflanzengenomen (McHale *et al.*, 2006). Aktuelle Modelle postulieren direkte oder indirekte Interaktionen des NB-LRR-Proteins mit dem korrespondierenden Avr-Protein (Abb. 6, Dangl und McDowell, 2006). Im Rezeptor-Liganden-Modell interagiert das Avr-Protein als Ligand direkt mit dem NB-LRR-Protein (Gabriel und Rolfe, 1990; Keen, 1990). Eine mögliche Interaktionsoberfläche dafür bietet der LRR, über dessen hypervariable AS-Reste die Interaktionsspezifität vermittelt werden könnte. Bislang wurde eine direkte Avr-Interaktion mit drei NB-LRR-Proteinen demonstriert (Jia *et al.*, 2000; Deslandes *et al.*, 2003; Dodds *et al.*, 2006). Der fehlende Nachweis einer direkten Interaktion anderer NB-LRR-Proteine mit ihren korrespondierenden Avr-Proteinen führte zur Beschreibung eines indirekten Interaktionsmodells. Das *guard*-Modell postuliert die Interaktion des Avr-Protein mit einem Zielprotein in der Pflanze (Pathogenitätsziel, Abb. 6B). NB-LRR-Proteine (Wächter, *guards*) erkennen Avr-bedingte Änderungen des Zielproteins und lösen eine Resistenzreaktion aus (Van der Biezen und Jones, 1998b; Dangl und Jones, 2001). *Arabidopsis* RPM1 detektiert z. B. die Phosphorylierung des RPM1-Interaktors 4 (RIN4) durch die *Pseudomonas*-Effektoren AvrB und AvrRpm1 (Mackey, 2003). Das *Arabidopsis*

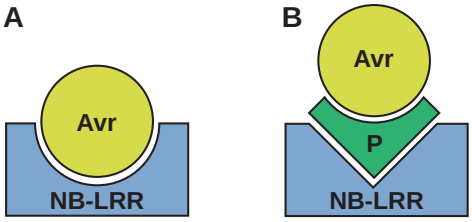

Abb. 6. Direktes und indirektes Erkennungsprinzip. **(A)** Im Rezeptor-Liganden-Modell interagiert das Avr-Protein (Avr) direkt mit dem NB-LRR-Protein und induziert dadurch eine Resistenzreaktion. **(B)** Im *guard*-Modell interagiert das Avr-Protein mit einem Pathogeniätsziel-Protein (P), welches wiederum mit dem NB-LRR-Protein interagiert. Veränderungen des Zielproteins sind der Auslöser für eine NB-LRR-vermittelte Resistenzreaktion.

R-Protein RPS2 dagegen „prüft“ das Vorhandensein von RIN4 und induziert eine Resistenzreaktion nach *Pseudomonas* AvrRpt2-Protease-vermitteltem Abbau von RIN4 (Axtell *et al.*, 2003; Axtell und Staskawicz, 2003; Mackey *et al.*, 2003; Kim *et al.*, 2005). Andere NB-LRR-Protein-Analysen unterstützen dieses Modell (Krüger *et al.*, 2002; Shao *et al.*, 2003; Rooney *et al.*, 2005).

1.9. Ebenen der Regulation pflanzlicher Resistenz

Proteine, die zur Induktion von Zelltod führen können, müssen strenger Regulation durch die Pflanze unterliegen, um eine unkontrollierte Aktivierung zu verhindern. In Pflanzen wurden mehrere Regulationsebenen der NB-LRR-Aktivität gefunden.

Zahlreiche NB-LRR-kodierenden *R*-Gene sind konstitutiv niedrig exprimiert (Mindrinos *et al.*, 1994; Grant *et al.*, 1995; Parker *et al.*, 1997; De Ilarduya und Kaloshian, 2001; Shen *et al.*, 2002; Goggin *et al.*, 2004; Paal *et al.*, 2004), andere *R*-Gene dagegen stark induziert (Yoshimura *et al.*, 1998; Levy *et al.*, 2004; Sobczak *et al.*, 2005). Neben der transkriptionellen Ebene erfolgt auch eine Regulation auf Proteinebene. So können NB-LRR-Proteine durch intramolekulare Interaktionen in inaktiver Konfiguration gehalten werden (Moffett *et al.*, 2002). Die Anwesenheit des Avr-Proteins aktiviert das NB-LRR-Protein, einhergehend mit einem Verlust der intramolekularen Interaktionen und ATP-Statusänderung (Schaltermodell der Regulation, Abb. 7A, Moffett *et al.*, 2002; Tameling *et al.*, 2002; Rathjen und Moffett, 2003). Außerdem resultieren Mutationen kritischer AS-Reste der intramolekularen Interaktionen oder der ATP-Bindung (z.B. MHD-Motiv der NB-Domäne, McHale *et al.*, 2006) in Autoaktivierung des NB-LRR-Proteins ohne Avr-Protein (Abb. 7B, Howles *et al.*, 2005). Eine Autoaktivierung wurde auch bei Überexpression einiger NB-LRR-Proteine beobachtet (Abb. 7C; Belkhadir *et al.*, 2004; Zhang *et al.*, 2004).

Eine zusätzliche Möglichkeit zur Regulierung der NB-LRR-Aktivität ist die Stabilitätskontrolle des NB-LRR-Proteins. So lässt sich das CC-NB-LRR-Protein RPM1 nach Avr-Induktion nicht mehr nachweisen (Boyes *et al.*, 1998; Kawasaki *et al.*, 2005). Identifizierte RPM1-Interaktoren und Signalkomponenten der NB-LRR-vermittelten Resistenz geben weitere Hinweise auf einen solchen Mechanismus. Zwei RPM1-Interaktoren sind Ubiquitin-Ligasen, die in die Protein-Degradierung involviert ist. Beide RPM1-Interaktoren sind notwendig für die RPM1-vermittelte HR (Kawasaki *et al.*, 2005).

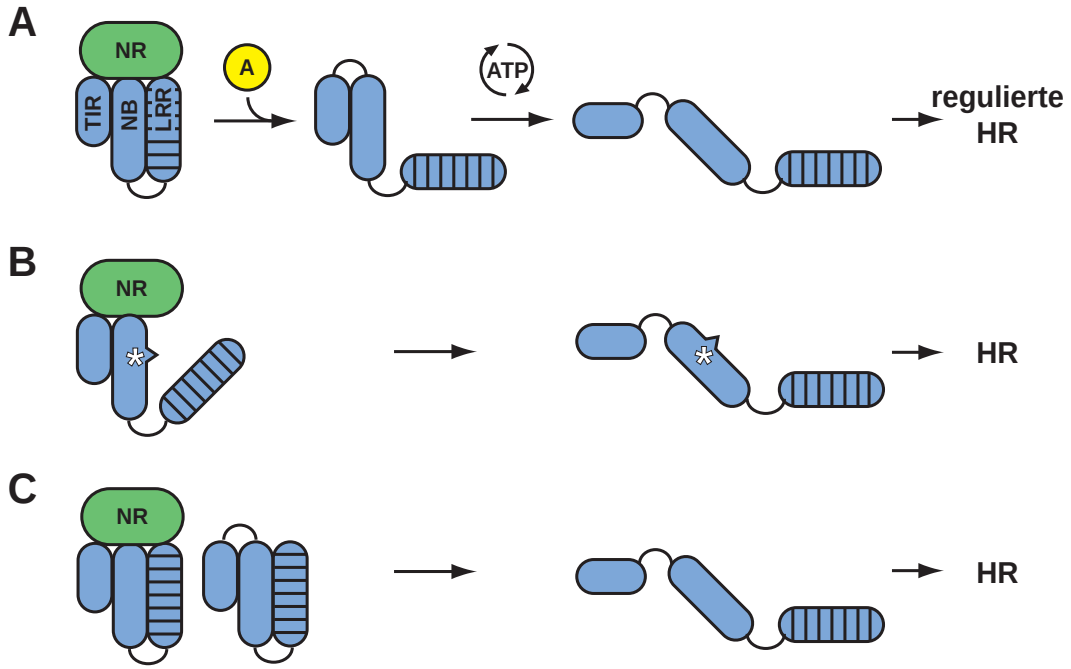

Abb. 7. Schalter-Modell der NB-LRR-Aktivierung. (A) Negative Regulatoren (NR) halten das NB-LRR-Protein im inaktiven Zustand. Das Avr-Protein (A) induziert die Auflösung intramolekularer Interaktionen zwischen NB- und LRR-Domäne. Die Auflösung von Interaktionen zwischen N-Terminus und NB-Domäne geht mit einer ATP-Statusänderung einher und resultiert in HR-Induktion. **(B)** Mutationen kritischer AS für intramolekulare Interaktionen oder ATP-Bindung (*) können zur Aktivierung führen. **(C)** Ebenso kann NB-LRR-Überexpression zur Austitrierung negativer Regulatoren führen und dadurch HR auslösen. (nach Rathjen und Moffett, 2003 und)

Außerdem ist die Funktion zahlreicher NB-LRR-Proteine abhängig von *Rar1* (*required for Mla12 resistance*), *SGT1* (*suppressor of G-two allele of SKP1/suppressor of kinetochore protein1*) und Komponenten des COP9–Signalosoms (*constitutive photomorphogenic*). Die Funktion dieser Proteine wird unter anderem im regulierten Abbau von NB-LRR-Proteinen gesehen (Liu *et al.*, 2002; Muskett und Parker, 2003; Shirasu und Schulze-Lefert, 2003; Schulze-Lefert, 2004).

Ein Gegenspieler der NB-LRR-Destabilisierung könnte das in Proteinfaltung und Proteinstabilisierung involvierte Hsp90 (*heat shock protein 90*) sein. Hsp90 interagiert mit allen daraufhin getesteten NB-LRR-Proteinen und reduzierte *Hsp90*-Mengen führen zum NB-LRR-Funktionsverlust (Schulze-Lefert, 2004). Destabilisierende und stabilisierende Proteine könnten demnach ein fein abgestimmtes Gleichgewicht generieren, dessen Störung zur Auslösung einer Resistenzreaktion führt.

1.10. Zielstellung

Mittelpunkt dieser Arbeit war die Charakterisierung des *Bs4*-Gens aus Tomate auf transkriptioneller, bzw. des Bs4-Proteins auf posttranslationeller Ebene. Es sollte die molekulare Basis der Bs4-Erkennungsspezifität gegenüber AvrBs4 und nahe verwandten Proteinen identifiziert werden.

Ein weiteres Ziel war die funktionelle Analyse der *Bs4*-vermittelten Resistenz in artfremden, taxonomisch verwandten und nicht verwandten Spezies mittels *Agrobacterium*-vermittelter Expression.

Die Notwendigkeit bekannter Signalkomponenten im *Bs4*-Kontext war zu prüfen und etwaige neue Komponenten zu identifizieren. Beteiligte Proteine sollten über genetische und biochemische Ansätze näher charakterisiert werden.

2. Ergebnisse

2.1. Übersicht der Publikationen und Manuskripte

Schornack, S., Ballvora, A., Gürlebeck, D., Peart, J., Baulcombe, D., Baker, B., Ganal, M., Bonas, U., and Lahaye, T. (2004). The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. Plant Journal **37**, 46-60.

Vorarbeiten: Die vorangegangene Diplom-Arbeit hatte ergänzende Arbeiten zur Isolierung von *Bs4* zum Thema. Folgende Daten sind deshalb nicht als Promotionsleistung zu berücksichtigen: die *Bs4*-abhängige Erkennung von AvrBs4-Deletionsderivaten (Schornack *et al.*, 2004, Abb. 1), Kartierung und Isolierung eines *Bs4*-Kandidatengens (Schornack *et al.*, 2004, Abb. 2,3), Identifizierung alternativer Transkripte (Schornack *et al.*, 2004, Abb. 5B) und Sequenzierung des *bs4*-Allels aus *L. pennellii* (Schornack *et al.*, 2004, Abb. 7).

Eigenanteil: Erkennungsspezifität von AvrBs3- und AvrBs4-Derivaten in *Xanthomonas* bzw. nach *Agrobacterium*-vermittelter *in planta*-Expression; Aufklärung der Bs4-Exon/Intron-Struktur; Analyse der intronfreien Bs4-Sequenz und des *L. pennellii bs4*-Allels; Silencing-Experimente; Generierung von Konstrukten für Hefe-Dihybrid-Analysen; Manuskriptentwurf

Schornack, S., Peter, K., Bonas, U., and Lahaye, T. (2005). Expression levels of *avrBs3*-like genes affect recognition specificity in tomato *Bs4*- but not in pepper *Bs3*-mediated perception. Molecular Plant-Microbe Interactions **18**, 1215-1225.

Eigenanteil: Manuskriptentwurf, Planung, Konstrukt-Erstellung, Durchführung und Auswertung aller Experimente, mit Ausnahme der Erstellung der Plasmide pBs4P3 und pBs4P6.

Schornack, S., Fick, A., Meyer, A., Peter, K. and Lahaye, T. (2006). Recognition of the *Xanthomonas campestris* pv. *vesicatoria* AvrBs4 protein is mediated by interacting Bs4 TIR-NB and LRR domains, abolished by overexpression or silencing of *Hsp90*, and coincides with disappearance of Bs4. *bei The Plant Cell eingereicht*

Eigenanteil: Manuskript, Planung, Konstrukt-Erstellung, Durchführung und Auswertung aller Experimente, mit Ausnahme der Erstellung eines AvrBs4-NLS-Deletionsderivats, der Bs4-Domänen-Expressionsanalyse im GAL4-System und einiger Tabak-N-Domänen-Konstrukte.

weitere Publikationen:

Von Roepenack-Lahaye, E., Newman, M.-A., Schornack, S., Hammond-Kosack, K.E., Lahaye, T., Jones, J.D.G., Daniels, M.J., and Dow, J.M. (2003). *p*-Coumaroylnoradrenaline, a novel plant metabolite implicated in tomato defense against pathogens. Journal of Biological Chemistry **278**, 43373-43383.

Eigenanteil: Durchführung und Auswertung der quantitativen PCR-Experimente zur THT-Expression

Übersichtsartikel:

Jordan, T., Schornack, S., and Lahaye, T. (2002). Alternative splicing of transcripts encoding Toll-like plant resistance proteins – what is the functional relevance to innate immunity? Trends in Plant Science **7**, 392-398.

Schornack, S., Meyer, A., Römer, P., Jordan, T., and Lahaye, T. (2006). Gene-for-gene-mediated recognition of nuclear-targeted AvrBs3-like bacterial effector proteins. Journal of Plant Physiology **163**, 256-272.

2.2. Das Tomaten-Resistenzprotein Bs4 ist ein vorhergesagtes nicht-nukleäres TIR-NB-LRR Protein, das Abwehrreaktionen gegen stark verkürzte Derivate von AvrBs4 und überexprimiertem AvrBs3 vermittelt.

2.2.1 Zusammenfassung

Das *R*-Gen *Bs4* aus Tomate (*Lycopersicon esculentum*) vermittelt die Erkennung von Stämmen des Pflanzen-Pathogens *Xanthomonas campestris* pv. *vesicatoria*, die das korrespondierende Avr-Protein AvrBs4 exprimieren. *Bs4* wurde über positionelle Klonierung isoliert und kodiert für ein NB-LRR-Protein, das Ähnlichkeit zu den Virusresistenz-Proteinen N aus Tabak (54%) und Y-1 (57%) aus Kartoffel hat. *Bs4* vermittelte in *Xcv*-Infektionsexperimenten die Erkennung von AvrBs4, aber nicht von AvrBs3, welches 97% Aminosäureidentität aufweist. Wird jedoch *avrBs3 Agrobacterium*-vermittelt und unter Kontrolle des *cauliflower mosaic virus*-35S-Promotors exprimiert, so induziert auch AvrBs3 eine *Bs4*-abhängige HR. Vermutlich übersteigen die *in planta* produzierten Avr-Mengen deutlich die von *Xanthomonas*-translozierten Avr-Mengen. Durch Transkript-Analysen wurde in einigen *Bs4*-Transkripten Intron-Retention nachgewiesen. Da aber ein *Bs4*-Konstrukt, aus dem die Introns entfernt wurden, immer noch AvrBs4-Erkennung vermittelt, sind die identifizierten Spleißvarianten für die Resistenz nicht notwendig. Das zu mehr als 98% identische *L. pennellii bs4*-Allel hat eine mit *Bs4* übereinstimmende Exon/Intron-Struktur, mit Ausnahme eines Spleißstellen-Polymorphismus in Intron 2, der verantwortlich für die Generierung eines verkürzten bs4-Proteins sein könnte. Hefe-Dihybrid-Experimente sollten die Gültigkeit des Rezeptor-Liganden-Modells für die Bs4-AvrBs4-Interaktion prüfen. Es konnte aber keine direkte Interaktion nachgewiesen werden. Die Signalweiterleitung der *Bs4*-vermittelten Abwehrreaktion wurde durch Virus-induziertes Gen-Silencing in *N. benthamiana* untersucht. Es konnte gezeigt werden, dass die *Bs4*-vermittelte Resistenz von den Signalkomponenten *EDS1* und *SGT1* abhängig ist. Durch transiente, *Agrobacterium*-vermittelte Koexpression von *avrBs4*- und *Bs4* konnte gezeigt werden, dass *Bs4* auch in verschiedenen *Nicotiana*-Arten und in Kartoffel (*Solanum tuberosum*) funktional ist.

2.2.2 Manuskript 1

The Plant Journal (2004) **37**, 46–60 doi: 10.1046/j.1365-313X.2003.01937.x

The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3

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Summary

The *Lycopersicon esculentum Bs4* resistance (*R*) gene specifies recognition of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) strains that express the cognate AvrBs4 avirulence protein. *Bs4* was isolated by positional cloning and is predicted to encode a nucleotide-binding leucine-rich repeat (NB-LRR) protein that is homologous to tobacco N and potato Y-1 resistance proteins. *Xcv* infection tests demonstrate that *Bs4* confers perception of AvrBs4 but not the 97% identical AvrBs3 protein. However, when delivered via *Agrobacterium* T-DNA transfer, both, *avrBs4* and *avrBs3* trigger a *Bs4*-dependent hypersensitive response, indicating that naturally occurring AvrBs3-homologues provide a unique experimental platform for molecular dissection of recognition specificity. Transcript studies revealed intron retention in *Bs4* transcripts. Yet, an intron-deprived *Bs4* derivative still mediates AvrBs4 detection, suggesting that the identified splice variants are not crucial to resistance. The *L. pennellii bs4* allele, which is >98% identical to *L. esculentum Bs4*, has a *Bs4*-like exon-intron structure with exception of a splice polymorphism in intron 2 that causes truncation of the predicted bs4 protein. To test if the receptor-ligand model is a valid molecular description of *Bs4*-mediated AvrBs4 perception, we conducted yeast two-hybrid studies. However, a direct interaction was not observed. Defense signaling of the *Bs4*-governed reaction was studied in *Nicotiana benthamiana* by virus-induced gene silencing and showed that *Bs4*-mediated resistance is *EDS1*- and *SGT1*-dependent.

Keywords: *Xanthomonas campestris* pv. *vesicatoria*, gene-for-gene interaction, type III effector, disease resistance gene, tomato, map-based cloning.

Introduction

Plants are a nutritious habitat for phytopathogenic microbes and thus have had to evolve countermeasures that minimize assimilate plundering. Cells of resistant plant genotypes challenged by pathogens frequently respond with a controlled suicide program, termed the hypersensitive response (HR; Klement and Goodman, 1967). Genetic analysis of plant–microbe interactions has shown that perception of microbial invaders is often determined by complementary pairs of plant resistance (*R*) and pathogen avirulence (*avr*) genes. The receptor-ligand model is one biochemical interpretation of gene-for-gene resistance and predicts that the Avr ligand binds directly to an R receptor that subsequently activates a defense reaction (Gabriel and Rolfe, 1990; Keen, 1990). Analysis of two gene-for-gene interactions have provided experimental support for this model (Jia *et al.*, 2000; Kim *et al.*, 2002; Scofield *et al.*, 1996;

Tang *et al.*, 1996). An alternative biochemical interpretation of gene-for-gene resistance known as the guard model (Van der Biezen and Jones, 1998b) postulates that the R protein (the 'guard') detects Avr-triggered changes of a plant virulence target (the 'guardee'). Identification and analysis of the *Arabidopsis* RIN4 protein provided experimental support for this model as RIN4 interacts physically with both the *Arabidopsis* RPM1 R protein and its cognate Avr proteins AvrRpm1 and AvrB (Mackey *et al.*, 2002).

The majority of *R* genes cloned to date encode putatively cytoplasmic proteins with nucleotide-binding leucine-rich repeat (NB-LRR) domains (Dangl and Jones, 2001; Ellis *et al.*, 2000b; Holub, 2001; Martin *et al.*, 2003; Young, 2000), and this structure–function relationship has inspired homology-based approaches aimed at identification of potential candidate *R* genes (Pflieger *et al.*, 2001). NB-LRR proteins differ structurally in their N-termini, comprising either a Toll/interleukin-1-receptor (TIR)-homologous region or a coiled-coil (CC) domain. Genetic dissection of defense signaling in *Arabidopsis* has shown that TIR-NB-LRR proteins signal through EDS1, whereas CC-NB-LRR proteins signal predominantly through NDR1 (reviewed in Feys and Parker, 2000; Glazebrook, 2001). By contrast, analysis of *Arabidopsis* Rar1 and SGT1 showed that these signaling components are engaged by both structural NB-LRR subtypes. Mutational studies of the *Nicotiana benthamiana* homologs of Rar1, EDS1 and SGT1, as well as the barley homologs of Rar1 and SGT1 indicate that these components are functionally conserved in *Arabidopsis*, *N. benthamiana* and barley (reviewed by Shirasu and Schulze-Lefert, 2003).

Genetic screens initially identified bacterial *avr* genes as mediators of gene-for-gene resistance (Staskawicz *et al.*, 1984). The ability of many avirulent bacteria to elicit HR also depends upon the hypersensitive response and pathogenicity (*hrp*) genes. As the name implies, these genes are also required for bacteria to cause disease on susceptible plants. *hrp* genes encode a type III secretion system that injects bacterial Avr proteins into the host cell (Alfano and Collmer, 1997). This would allow these proteins to interact with NB-LRR type R proteins, which have an intracellular location. The existence of this sophisticated injection machinery implies that the primary function of Avr proteins is in virulence rather than avirulence. Indeed increasing evidence suggests a dual role of Avr proteins as recognition determinants in resistant and virulence determinants in susceptible hosts (Gabriel, 1999a; Luderer and Joosten, 2001; Van't Slot and Knogge, 2002).

Xanthomonas AvrBs3, the founder of a large protein family, exemplifies a well-studied Avr protein that has a documented contribution to both virulence and avirulence (reviewed in Büttner and Bonas, 2002). AvrBs3-like proteins share 90–97% sequence identity to each other and contain in the center of their polypeptide chain nearly perfect copies of a 34-amino-acid (aa) repeat motif that determines recognition specificity (reviewed by Gabriel, 1999b; Lahaye and Bonas, 2001; Leach *et al.*, 2001; White *et al.*, 2000). Other structural hallmarks of AvrBs3-homologous proteins are nuclear localization signals (NLSs) and an acidic transcriptional activation domain (AAD). Functional studies have shown that NLS and AADs are essential to nuclear import (Szurek *et al.*, 2001) and for transcriptional activation of host genes (Marois *et al.*, 2002), respectively. Mutational studies of multiple AvrBs3 family members revealed that NLSs and AADs are not only crucial for virulence but also for their avirulence function (Van den Ackerveken *et al.*, 1996; Yang and Gabriel, 1995b; Yang *et al.*, 2000; Zhu *et al.*, 1998, 1999).

We study tomato and pepper bacterial spot disease, which is caused by the Gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). In particular, our interest is in the *R* gene-mediated perception of AvrBs3 and AvrBs4 – two members of the *Xanthomonas* AvrBs3 family that share 97% sequence identity (Bonas *et al.*, 1993). AvrBs3 and AvrBs4 are recognized specifically by the corresponding pepper *Bs3* and tomato *Bs4 R* genes, respectively (Ballvora *et al.*, 2001a). We have shown that perception mediated by pepper *Bs3* but not by tomato *Bs4* depends on functional NLSs in the corresponding Avr proteins, which suggests different recognition principles for the detection of almost identical avirulence proteins in pepper and tomato (Ballvora *et al.*, 2001a). The abundance and highly conserved structure of AvrBs3 family members make them a useful experimental system in which to study the specificity of *R* gene-mediated Avr protein perception. However, the molecular isolation of a corresponding *R* gene for any of the family members has not yet been reported.

Previously, we presented the genetic mapping and physical delimitation of the tomato *Bs4* locus (Ballvora *et al.*, 2001a,b). Here, we report the isolation and functional analysis of the tomato *Bs4* gene. *Bs4* encodes a TIR-NB-LRR protein with most similarity to the tobacco N (Whitham *et al.*, 1994) and potato Y-1 proteins (Vidal *et al.*, 2002). Transcript studies uncovered *Bs4* splice variants, which, however, appear to be not required for *Bs4*-mediated HR. Analysis of recognition specificity indicates that Bs4 has the ability to mediate not only detection of AvrBs4 but also other AvrBs3-like proteins. Yeast two-hybrid (Y2H) studies suggest that Bs4 does not directly interact with AvrBs4. Furthermore, we demonstrate that *Bs4* is functional in *Solanum tuberosum* and *Nicotiana* species and that its function is *EDS1*- and *SGT1*-dependent.

Results

Bs4 *mediates recognition of AvrBs4-deletion derivatives*

Analysis of F_2 segregants derived from a cross between *Lycopersicon esculentum* cultivar (cv.) Moneymaker (MM)

AvrBs4 derivatives		Infection phenotype[b]		
		Plant genotype		
Avr protein (vector designation)	protein structure[a]	*MM*[c]	*Bs4/—*	*bs4/bs4*
AvrBs4 (pLAT211)		+	+	–
AvrBs4 Δ215 (pLAT215)		+	+	–
AvrBs4 Δ218 (pLAT218)		+	+	–
AvrBs4 Δ221 (pLAT221)		+	+	–
AvrBs4 Δ227 (pLAT227)		+	+	–
AvrBs4 Δ230 (pLAT230)		+	+	–
AvrBs4 Δ233 (pLAT233)		–	–	–

Figure 1. The tomato *Bs4* gene mediates perception of AvrBs4 and deletion derivatives.
Inheritance of AvrBs4-induced HR was studied in F_2 segregants derived from a cross between *L. esculentum* cv. MM (*Bs4*) and *L. pennellii* LA2963 (*bs4*). Tomato plants were infiltrated with *Xcv* transconjugants delivering the depicted AvrBs4 derivative. Infection phenotypes were scored 48 h after inoculation. a, Boxed areas represent repeat units and white and black diamonds represent nuclear localization signals and the transcriptional activation domain, respectively; b, +/– indicates presence/absence of HR, respectively; and c, described previously by Bonas *et al.* (1993).

and *L. pennellii* LA2963 has shown that recognition of *Xcv* strains that express AvrBs4 is mediated by the *L. esculentum* cv. MM *Bs4* locus (Ballvora *et al.*, 2001a). Earlier studies have shown that not only full-length AvrBs4 but also AvrBs4-deletion derivatives trigger an HR on *L. esculentum* cv. MM (Figure 1; Bonas *et al.*, 1993). Yet, it remained unclear, if *Bs4* or other unlinked *R* loci govern recognition of these AvrBs4-deletion derivatives. We therefore analyzed six different C-terminal AvrBs4-deletion derivatives on a set of 20 F_2 progenies derived from a cross between *L. esculentum* cv. MM (*Bs4*) and *L. pennellii* LA2963 (*bs4*) (Figure 1). The allele configuration of these F_2 segregants at the *Bs4* locus was determined with molecular markers that flank *Bs4* on either side of the locus (see Methods for details). *Xcv* infection tests showed that C-terminal AvrBs4-deletion derivatives, which contain 3.5 or more repeat units, triggered an HR in *Bs4/–* segregants and the *L. esculentum* cv. MM but not in *bs4/bs4* segregants (Figure 1). On the contrary, an AvrBs4 derivative that contains 26 residues of the first repeat unit (AvrBs4 Δ233) did not induce an HR in any of the tested tomato genotypes. Notably, all tested *Bs4/–* segregants and *L. esculentum* cv. MM showed identical infection phenotypes with AvrBs4 and its deletion derivatives. These findings indicate that the previously determined avirulence activity of AvrBs4-deletion derivatives (Bonas *et al.*, 1993) was because of *Bs4*-dependent recognition.

Degenerate PCR and high-resolution mapping uncovers a Bs4 *candidate gene*

A PCR-based strategy with degenerate primers targeting the conserved TIR motif of TIR-NB-LRR-encoding *R* genes was applied to isolate *Bs4* candidate genes from tomato genomic DNA. The chromosomal positions of the cloned PCR products were assessed by RFLP mapping in a standard tomato mapping population (Tanksley *et al.*, 1992) and placed one of the isolated fragments (T26 RGC) in the vicinity of the *Bs4*-linked RFLP marker TG432 (Figure 2; Ballvora *et al.*, 2001b). T26 RGC was converted into a PCR-based RFLP marker and employed for the analysis of a mapping population that segregates for *Bs4* resistance. Linkage analysis of 1972 meiotic events revealed that T26 RGC fragments diagnostic for the *Bs4*-parental genotype *L. esculentum* cv. MM co-segregated with an HR phenotype that was visible 48 h after infiltration of *avrBs4*-expressing *Xcv*. Furthermore, analysis of two yeast artificial chromosome (YAC) clones, that were shown previously to span the

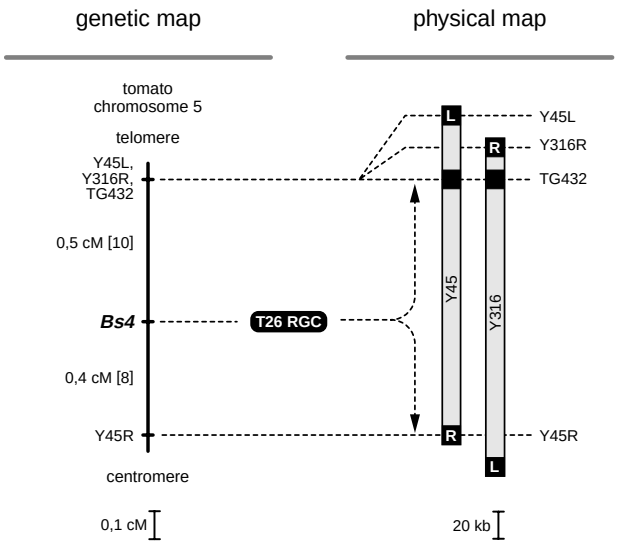

Figure 2. Integrated genetic/physical map of the *Bs4* locus.
Based on the analysis of 1972 meiotic events, T26 RGC shows complete linkage to the *Bs4* target locus. Physical mapping placed T26 RGC between TG432 and Y45R. Arrows indicate the area within which T26 RGC was physically delimited. Marker loci are shown as horizontal, dashed lines. Numbers in square brackets denote the number of recombinants identified. YAC inserts are displayed to scale as bars with their respective right (R) and left (L) ends. Note that spaces between markers in the YAC insert do not represent defined physical distances.

Bs4 locus (Ballvora *et al.*, 2001b), demonstrated that both YACs contain the T26 RGC marker locus. In summary, high-resolution genetic and physical mapping supported the notion that T26 RGC was part of a potential *Bs4* candidate gene.

A TIR-NB-LRR-encoding Bs4 *candidate gene mediates specific recognition of AvrBs4*

To determine if the YAC insert DNA that was used for physical delimitation of *Bs4* could be employed for complementation studies, we performed infection tests with *L. esculentum* cv. VFNT Cherry, the DNA source for YAC library construction (Bonnema *et al.*, 1996; Martin *et al.*, 1992). Inoculation tests showed that *avrBs4*- but not *avrBs3*-expressing *Xcv* triggers an HR, indicating that the *L. esculentum* cv. VFNT Cherry contains the *Bs4* gene.

In order to isolate T26 RGC-flanking sequences, we generated a cosmid library of YAC clone Y45, which was shown previously to span the *Bs4* locus (Ballvora *et al.*, 2001b). Sequence analysis of T26 RGC-containing cosmids revealed a putative TIR-NB-LRR-encoding candidate gene (termed *Bs4*^{c}). Amplification and sequence analysis of the *Bs4*^{c} homolog from *L. esculentum* cv. MM, the genotype that was used for high-resolution linkage mapping of *Bs4*, revealed that the nucleotide sequence of *Bs4*^{c} is identical in VFNT Cherry and MM. Southern analysis of *L. esculentum* cv. MM and YAC Y45 DNA indicated that *Bs4*^{c} is a single-copy gene (data not shown).

To determine whether *Bs4*^{c} mediates AvrBs4 recognition, the previously established *L. esculentum bs4* backcross line MM^{bs4}–BC4 (Ballvora *et al.*, 2001a) was transformed with a binary vector (pVTSB1) in which the *Bs4*^{c} genomic fragment is under transcriptional control of the CaMV 35S promoter. *Xcv* infection tests showed that 16 out of 22 transgenic tomato plants displayed an AvrBs4-dependent HR (Figure 3). Analysis of several independent T_1 and T_2 lines showed that inheritance of AvrBs4-responsiveness was strictly dependent on the presence of *Bs4*^{c}, indicating that *Bs4*^{c} is indeed the *Bs4* gene.

Recognition specificity of the *Bs4*^{c}-transgenic plants was tested with *Xcv* strains that deliver (i) AvrBs4, (ii) AvrBs4 Δ227 (C-terminal deletion derivative of AvrBs4), (iii) AvrBs3, or (iv) AvrBs1 (no sequence homology with AvrBs4; Ronald and Staskawicz, 1988), respectively (Figure 3). Infiltration of the *Bs4*^{c}-transgenic lines with xanthomonads that express *avrBs4* or its deletion derivative *avrBs4 Δ227* triggered a rapid HR. By contrast, *avrBs3*- and *avrBs1*-expressing *Xcv* did not induce an HR. Notably, all *avr* derivatives produced identical infection phenotypes in *Bs4*^{c}-transgenic lines and the *Bs4*-containing genotype *L. esculentum* cv. MM and did not trigger HR in the *bs4* genotype MM^{bs4}–BC4 (Figure 3). Taken together, our complementation studies led us to conclude that *Bs4*^{c} is indeed the tomato *Bs4* gene.

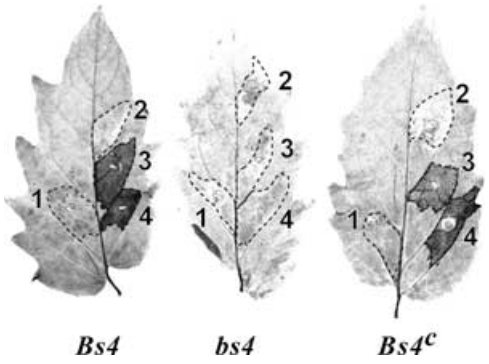

Figure 3. Functional analysis of the tomato *Bs4* candidate gene.
The tomato genotypes *L. esculentum* cv. MM (*Bs4*), the near isogenic line MM^{bs4}–BC4 (*bs4*) and a transgenic plant that contains the *Bs4* candidate (*Bs4*^{c}) gene were infiltrated with *Xcv* strains that deliver AvrBs1 (1), AvrBs3 (2), AvrBs4 Δ227 (3), and AvrBs4 (4), respectively. Leaves have been bleached by ethanol treatment for better visualization of the HR. Dashed lines indicate the infiltrated leaf areas. Photographs were taken 48 h post infection.

Agrobacterium tumefaciens*-mediated expression of* avrBs3 *triggers a* Bs4*-dependent HR*

Recognition specificity of the *L. esculentum* cv. MM *Bs4* allele was also studied by *A. tumefaciens*-mediated transient expression (agroinfiltration). In contrast to our *Xcv* infection tests we found that, when delivered by agroinfiltration, not only *avrBs4* and its deletion derivative *avrBs4 Δ227* but also *avrBs3* triggered an HR in *L. esculentum* cv. MM and the *Bs4* transgenic lines (Figure 4a). We did not observe an HR in the *bs4* genotype MM^{bs4}–BC4, indicating that agroinfiltrated *avrBs3* was detected in a *Bs4*-dependent manner. It is worth noting that the HR was indistinguishable in the *Bs4*-transgenic lines and *L. esculentum* cv. MM although the *Bs4* transgene is under control of the CaMV 35S promoter (Figure 4a).

Analysis of unchallenged tissue by real-time PCR revealed that the *Bs4* transcript levels in our 35S::*Bs4* transgenic tomato lines were approximately 100-fold higher when compared to *L. esculentum* cv. MM (*Bs4*) (data not shown). To examine whether *Bs4* overexpression influences the infection phenotypes, we cloned a genomic fragment containing the *Bs4* open-reading frame (ORF) and approximately 3.5-kbp upstream sequence into a promoterless binary vector (pVTSB3). Functionality of *Bs4* under transcriptional control of (i) its putative native promoter or (ii) the CaMV 35S promoter was compared by *Agrobacterium*-mediated co-expression with *avrBs4* (Figure 4b). In this assay, a *bs4* plant genotype was infiltrated with a mixture of two *Agrobacterium* cultures, one expressing the respective *avr* gene, and the other expressing *Bs4* under transcriptional control of either the CaMV 35S or the putative *Bs4* native promoter. Phenotypic inspection of the reactions mediated by the CaMV 35S and the native promoter constructs showed no

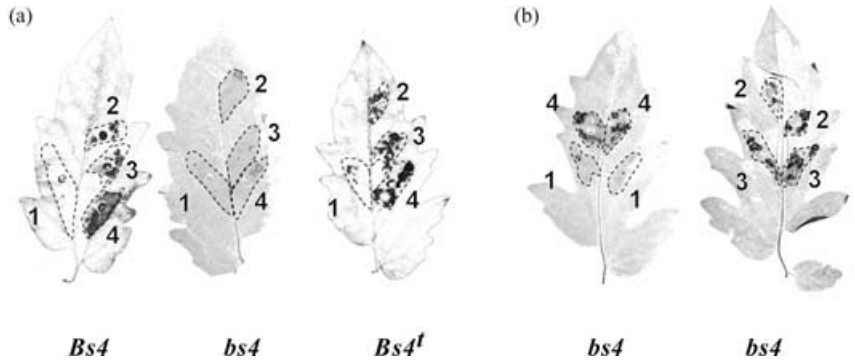

Figure 4. *Agrobacterium*-mediated delivery of *avrBs3* triggers a *Bs4*-dependent HR.
(a) Analysis of *Bs4* recognition specificity by *Agrobacterium*-mediated delivery of *avr* derivatives. *Agrobacterium* strains that mediate T-DNA-based delivery of *avrBs1* (1), *avrBs3* (2), *avrBs4 Δ227* (3), or *avrBs4* (4), respectively, were infiltrated into *L. esculentum* cv. MM (*Bs4*), the near isogenic line MM^{bs4}–BC4 (*bs4*) and *Bs4*-transgenic line (*Bs4*^{t}).
(b) *Agrobacterium*-mediated coexpression of the *Bs4* and *avr* derivatives. For transient co-expression, respective *avr*-containing *Agrobacterium* strains 1–4 (see a) were mixed with equal amounts of *Agrobacterium* strains that contain the *Bs4* candidate gene under transcriptional control of the CaMV 35S promoter (left side of the leaf) or its own promoter (right side of the leaf). Leaves have been bleached by ethanol treatment for better visualization of the HR. Dashed lines indicate the infiltrated leaf tissue. Photographs were taken 6 days post infection.

differences with respect to recognitional specificity, extent, timing, and intensity of the HR (Figure 4b).

The Bs4 *transcript undergoes alternative splicing*

Reverse transcriptase (RT)-PCR was used to determine the *Bs4* exon-intron structure (summarized in Figure 5), because attempts to isolate a *Bs4* full-length cDNA clone were unsuccessful. Based on rapid amplification of cDNA ends (RACE) analysis, the *Bs4* transcript contains 25 and 500 bp of 5′- and 3′-untranslated region (UTR), respectively. A comparison of the *Bs4* genomic sequence with the RACE products revealed the presence of a 90-bp intron in the 3′-UTR. Inspection of the *Bs4* ORF by means of intron finder algorithms suggested the presence of three introns in the *Bs4* ORF that could be confirmed by RT-PCR with primers flanking the predicted introns. In addition, we obtained *Bs4*-splice variants that still contained intron 2 or 3 but lacked intron 1. To determine the functional relevance of these splice variants, we removed introns 1–3 and used this intron-deprived *Bs4* derivative for agroinfiltration of the *bs4* genotype MM^{bs4}–BC4. We found, that the intron-deprived *Bs4* ORF mediates *avrBs4* recognition, indicating that the introns are not crucial to *Bs4* functionality. Likewise, we tested *Bs4* constructs lacking the 5′- or 3′-UTRs and found that UTRs are functionally dispensable.

The predicted Bs4 protein is highly related to the virus resistance proteins potato Y-1 and tobacco N

The *Bs4* transcript encodes a predicted protein of 1146 amino acids with a molecular weight of 131 kDa. Among

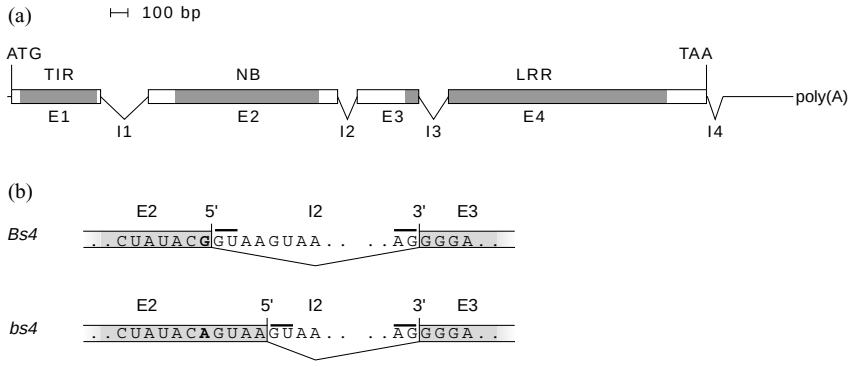

Figure 5. *Bs4* transcript structure.
(a) Schematic representation of the *Bs4* exon-intron structure. Putatively coding or intronic regions are depicted as boxes and angled lines, respectively. Exons (E) and introns (I) are numbered above and below the drawings. Shaded areas represent the TIR-, NB-, and LRR-encoding regions. Please note that the LRR region is encoded by sequence stretches in exon 3 (E3) and exon 4 (E4). The putative ATG start codon, TAA stop codon, and the poly(A) tail are indicated.
(b) Sequence comparison of *Bs4*- and *bs4*-derived cDNAs reveals a splice-site polymorphism at intron I2 that causes a frameshift in *bs4* transcripts. Boldface letters highlight a G/A polymorphism between the *Bs4* and *bs4* alleles. Thick horizontal lines highlight the 5′-splice donor and 3′-splice acceptor sites in *Bs4*/*bs4* alleles.

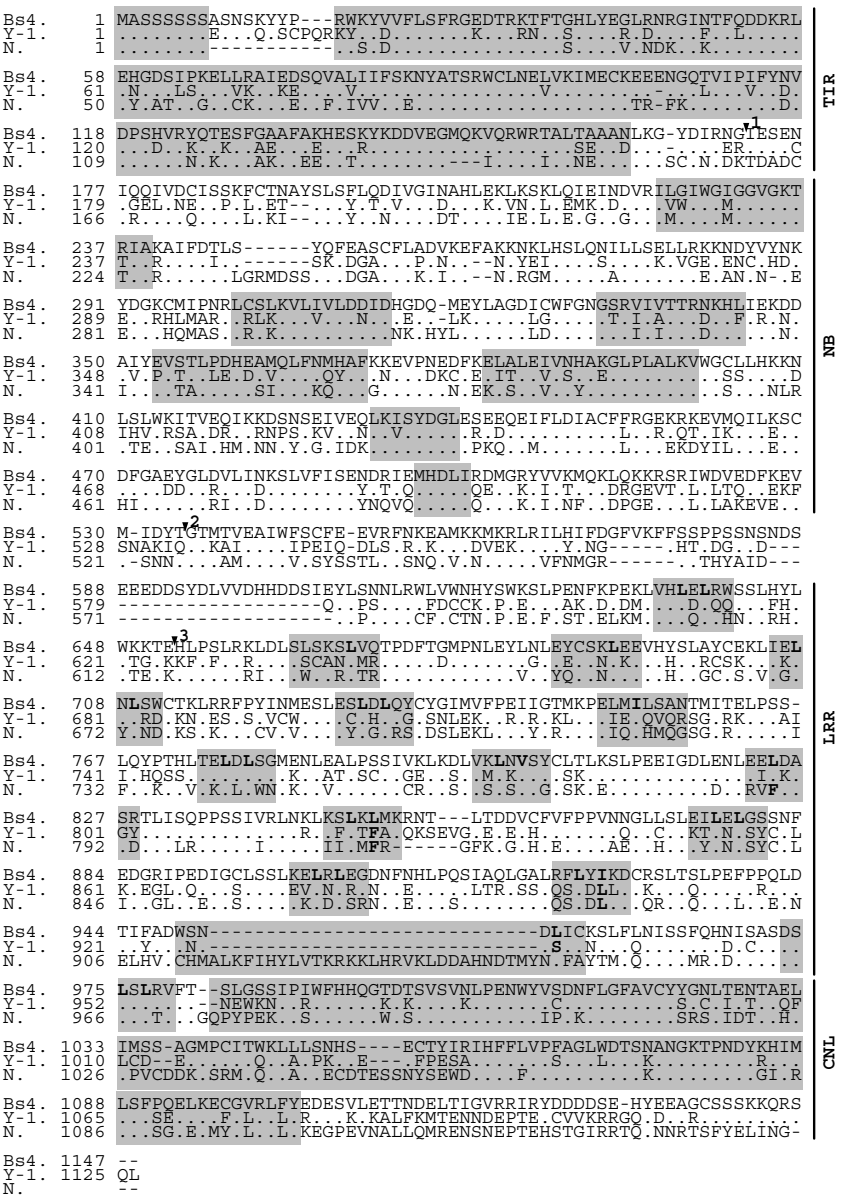

Figure 6. Bs4 is highly similar to the virus resistance proteins Y-1 and N.

Alignment of the deduced amino-acid sequences of tomato *Bs4*, potato *Y-1*, and tobacco *N*. TIR, NB, LRR, and C-terminal non-LRR (CNL) homology domains are indicated by gray boxes. Dots represent residues in Y-1 and N that are conserved with respect to Bs4. Sequence gaps inserted to maintain the alignment are indicated by dashes. Arrowheads mark the intron positions in *Bs4*. NB and CNL domains were defined according to Van der Biezen and Jones (1998a) and Dodds *et al.* (2001), respectively. Residues that form the structural backbone of the LRR units were defined according to Ellis *et al.* (2000a) and are shown in bold.

proteins with a known function, Bs4 most closely resembles the TIR-NB-LRR proteins potato Y-1 (57% identity, 71% similarity; Vidal *et al.*, 2002) and tobacco N (54% identity, 67% similarity; Whitham *et al.*, 1994) (Figure 6). The Bs4 protein displays at its far N-terminus (aa residues 1–8) a characteristic sequence motif (MASSSSSS), which is also present in potato Y-1 (Vidal *et al.*, 2002), tobacco N (Whitham *et al.*, 1994), *Arabidopsis* RPP4 (Van der Biezen *et al.*, 2002), *Arabidopsis* RPP5 (Parker *et al.*, 1997), and other putative TIR-NB-LRR proteins (Hehl *et al.*, 1999). Comparison of the Bs4 TIR (aa residues 17–162), NB (aa residues 224–500), and LRR (aa residues 626–987) regions with the corresponding domains of Y-1 and N shows that the TIR is the most conserved and the LRR the most divergent domain between these R proteins. The C-terminal region of Bs4 is composed of 15 repeat units with similarity to the cytoplasmic LRR consensus sequence (Jones and Jones, 1997). We found no apparent N-terminal signal sequences in Bs4, suggesting that it is a cytoplasmic protein. However, PROSITE motif search (http://www.expasy.ch/prosite) identified potential myristoylation sites in Bs4, which might mediate membrane anchoring of the protein.

bs4 *transcripts encode a truncated bs4 protein*

Sequence comparison of the *L. pennellii* LA2963 *bs4* allele with the *L. esculentum* cv. MM *Bs4* allele revealed in total 74 nucleotide polymorphisms (Figure 7). Analysis of

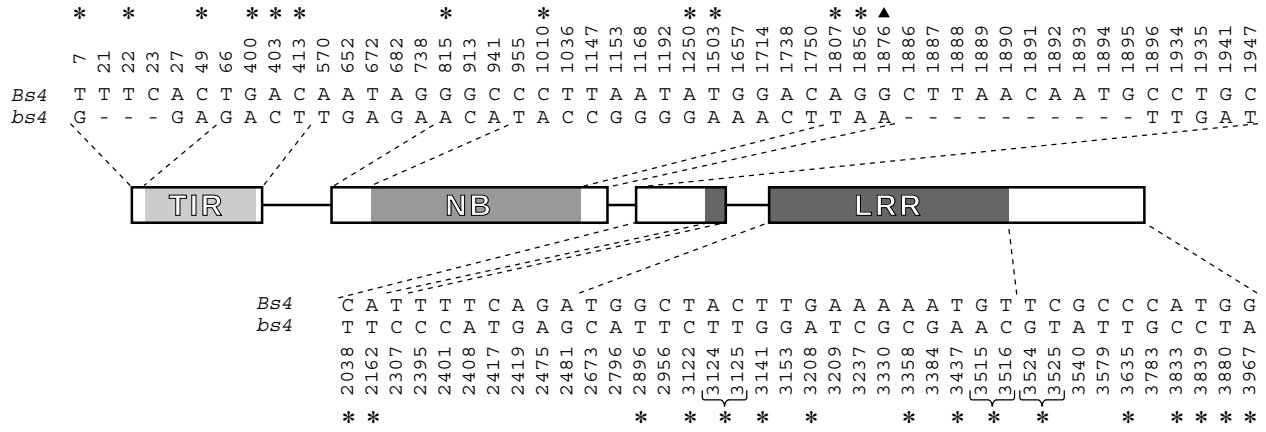

Figure 7. Sequence comparison between the *L. esculentum* cv. MM *Bs4* and *L. pennellii* LA2963 *bs4* alleles.
Exonic and intronic sequence are shown as boxes and lines, respectively. Dashes indicate deletions in *bs4* with respect to *Bs4*. Gray-boxed areas indicate TIR, NB and LRR regions. Asterisks mark nucleotide polymorphisms that cause amino-acid differences between the predicted Bs4 and bs4 proteins. Brackets indicate amino-acid differences between Bs4 and bs4 that are caused by two base pair differences within one codon. The triangle marks a G/A polymorphism that is located next to the *Bs4*-splice donor site of intron 2 and possibly causes a translational stop codon 21 bp downstream of this polymorphism. To compare *Bs4*- and *bs4*-encoded proteins downstream of intron 2, we assumed the presence of *bs4* transcripts with a *Bs4*-like exon-intron structure. Polymorphisms are numbered with respect to *Bs4*.

putative exons predicts that 28 out of 60 nucleotide polymorphisms would lead to aa differences between the *bs4*- and *Bs4*-encoded proteins. The predicted *bs4* exons do not contain an early stop codon that would unequivocally define *bs4* as a null allele. Transcript analysis showed that *bs4* and *Bs4* share an almost perfect conservation of exon–intron junctions with the exception of the 5′ splice site of intron 2 (Figure 7). This modified exon–intron boundary is predicted to cause a frame-shift and hence to encode a truncated protein that lacks the LRR region. This is confirmed by sequence analysis of 32 cloned RT-PCR fragments, which revealed no *bs4* transcripts that would encode an LRR region.

Bs4 and AvrBs4 do not show interaction in the Y2H system

To test whether AvrBs4 interacts physically with Bs4, we performed Y2H studies. An AvrBs4 bait construct (N-terminal fusion of AvrBs4 to the LexA DNA-binding domain) was found to activate both the *leu2* and the *lacZ* reporter genes in the absence of the prey construct. To overcome this autoactivation problem, we constructed an N-terminal deletion derivative of AvrBs4 (AvrBs4 Δ152) lacking aa residues 1–152. AvrBs4 Δ152 no longer activates the yeast reporter genes but still triggers a *Bs4*-dependent HR when the corresponding gene is delivered by *Agrobacterium*. This finding suggests that the N-terminus of AvrBs4 is not required for *Bs4*-dependent recognition. Next, an intron-deprived *Bs4* gene, that lacks introns 1–3, and that was shown to be functional (see above) was cloned into the prey vector. We also generated reciprocal bait and prey constructs (AvrBs4 Δ152, prey vector; Bs4, bait vector) and confirmed by Western blot analyses for all constructs expression of appropriate-sized proteins in yeast (data not shown). A repression assay (Brent and Ptashne, 1984) showed that the LexA-AvrBs4 Δ152 and LexA-Bs4 fusion proteins can bind to nuclear-localized operator sequences, indicating that both proteins are suitable for Y2H interaction studies. Pepper importin α1 (Caimpα1), previously identified as an interactor of AvrBs3 (Szurek *et al.*, 2001), also interacts with AvrBs4 and was used as a positive control for our Y2H studies. The *Drosophila* bicoid protein was used as a control for non-specific interactions.

To study interaction of Bs4 and AvrBs4, we tested AvrBs4 Δ152 bait and Bs4 prey constructs and the reciprocal combination (AvrBs4 Δ152 prey; Bs4 bait). The *lacZ* reporter gene activity measured in AvrBs4 Δ152–Bs4 co-transformants in no case exceeded the levels of the negative controls (bicoid in combination with AvrBs4 Δ152 or Bs4) and was about 10 times lower than that for the positive control (Figure 8). The same yeast co-transformants lacked leucine prototrophy, indicating that AvrBs4 Δ152 and Bs4 proteins do not interact in yeast. We also cloned different Bs4 regions (TIR, NB-LRR, and LRR) into the prey vector and tested these for interaction with the AvrBs4 Δ152 bait protein. In accordance with the data observed for the full-length Bs4 protein, co-transformation of the separated Bs4 regions with AvrBs4 Δ152 (Figure 8) did not activate the *leu2* or the *lacZ* reporter gene, suggesting that a simple receptor-ligand model does not hold true for the *avrBs4–Bs4* gene-for-gene interaction.

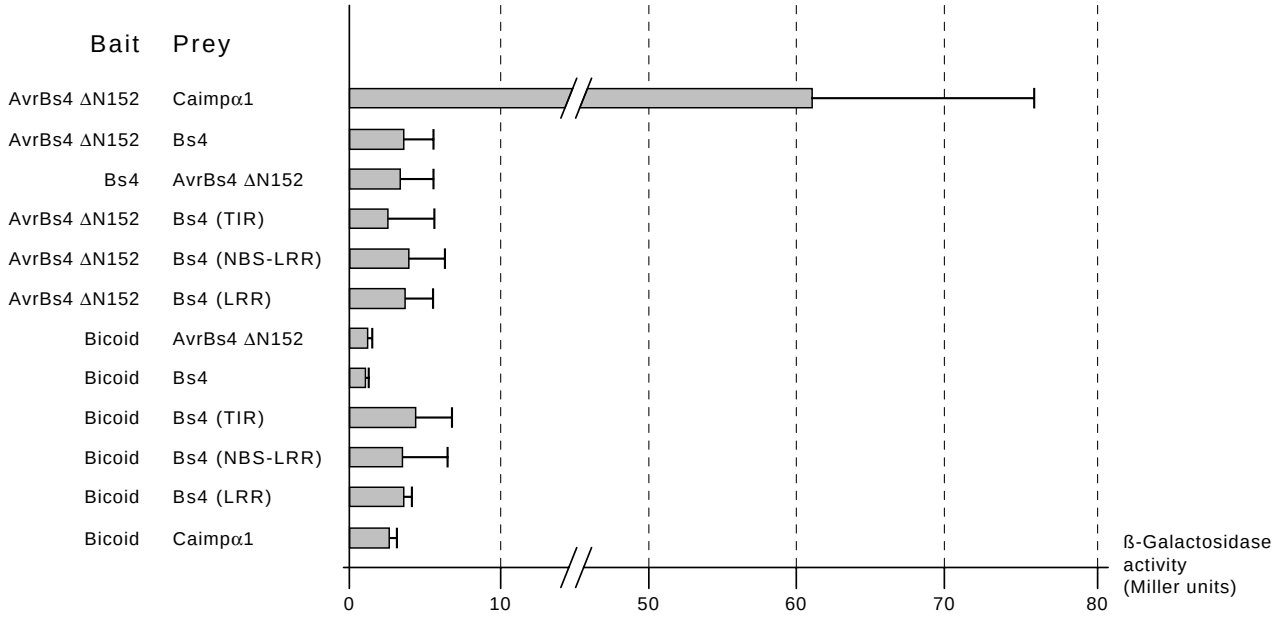

Figure 8. Y2H analysis of the Bs4–AvrBs4 interaction.
Reporter gene activation was determined by measuring β-galactosidase activity of yeast strains expressing the respective bait and prey proteins. The interaction of AvrBs4 Δ152 and Caimpα1 was used as positive control and *Drosophila* bicoid as negative control.

Tomato Bs4 *is functional in* S. tuberosum *and* Nicotiana *species*

To examine whether species other than tomato have all accessory proteins that are needed for a *Bs4*-mediated defense response, we performed agroinfiltration of the solanaceous plant species *N. tabacum*, *N. clevelandii*, *N. benthamiana*, *Capsicum annuum*, *C. frutescens*, *S. tuberosum*, and *Petunia hybrida*. Agroinfiltration of a binary vector carrying the β-glucuronidase (GUS) gene induced reporter activity in the infiltrated tissues, thereby indicating that all tested plant species are transformable by *Agrobacterium* (data not shown). Subsequently, we performed agroinfiltration of these different plant species using *Bs4* in combination with *avrBs4*, *avrBs4 Δ227*, *avrBs3*, and *avrBs1*. Co-expression of *Bs4* with *avrBs4*, *avrBs4 Δ227* and *avrBs3*, but not *avrBs1* triggered an HR in *N. tabacum*, *N. clevelandii*, *N. benthamiana*, and *S. tuberosum* (Figure 9; data not shown). No HR was observed in *P. hybrida*, *C. annuum*, and *C. frutescens*. We noted in *S. tuberosum* that agroinfiltration of *avrBs4*, but not *avrBs3*, induced a *Bs4*-independent HR, which is most likely because of an intrinsic *R* gene. However, as the *avrBs3*-triggered HR in *S. tuberosum* was only observed when co-expressed with *Bs4*, we conclude that *Bs4* is functional in *S. tuberosum*. In summary, our results indicate that *S. tuberosum* and *Nicotiana* species but not *Capsicum* or *Petunia* species contain the elements that are required for the *Bs4*-mediated HR.

Bs4-*mediated HR is* EDS1- *and* SGT1-*dependent*

As a first step towards elucidation of the signal components that mediate the *Bs4*-specified defense response we analysed known *R* gene pathway elements. To study the

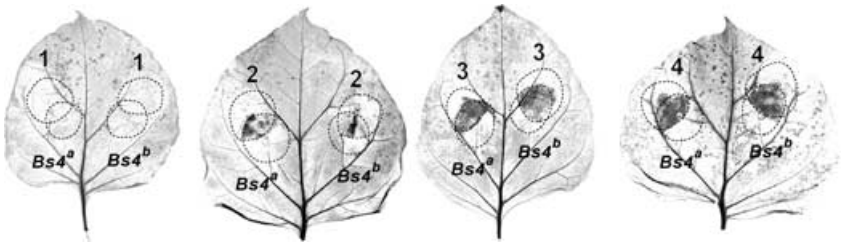

Figure 9. Tomato *Bs4* is functional in *N. benthamiana*.
Agrobacterium-based co-expression of *Bs4* and different *avr* genes. *Agrobacterium* strains containing *avrBs1* (1), *avrBs3* (2), *avrBs4 Δ227* (3), or *avrBs4* (4) were infiltrated into leaves of *N. benthamiana*. Subsequently, *Agrobacterium* strains that contain *Bs4* under transcriptional control of the CaMV 35S promoter (*Bs4*^{a}) or *Bs4* under transcriptional control of its own promoter (*Bs4*^{b}), were infiltrated. Leaves were bleached by ethanol treatment seven days after agroinfiltration for better visualization of the HR. Dashed lines indicate the infiltrated leaf tissue.

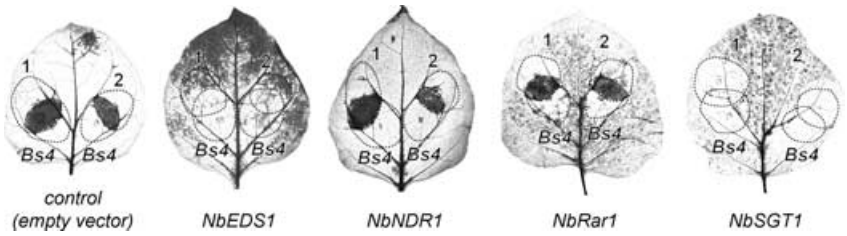

Figure 10. Virus-induced gene silencing of *NbEDS1* or *NbSGT1* suppresses *Bs4* function in *N. benthamiana*.
Plants were infected with TRV derivatives of *NbEDS1*, *NbNDR1*, *NbSGT1*, *NbRar1*, or an empty TRV vector. Twenty-one days later *Agrobacterium* strains that deliver *Bs4*, *avrBs3* (1), or *avrBs4* (2), respectively, were infiltrated. HR symptoms are diagnostic for functionality of the *Bs4* gene. Seven days after agroinfiltration, the leaves were bleached by ethanol treatment to visualize HR. Dashed lines indicate infiltrated leaf areas.

functional relevance of EDS1, NDR1, RAR1, and SGT1 in the *Bs4*-mediated HR, we employed virus-induced gene silencing (VIGS), a well-established tool for transient gene silencing in *N. benthamiana* (Baulcombe, 1999). As VIGS requires a high degree of sequence similarity between the recombinant virus and the target RNA (Mueller *et al.*, 1995), it was necessary to use the *N. benthamiana* orthologs of *EDS1* (*NbEDS1*), *NDR1* (*NbNDR1*), *Rar1* (*NbRar1*), and *SGT1* (*NbSGT1*). The *NbEDS1* and *NbSGT1* genes have been described before (Peart *et al.*, 2002a,b) and for isolation of *NbNDR1* and *NbRar1*, we employed a homology-based approach (see Experimental procedures for details). VIGS was initiated by inoculation of tobacco rattle virus (TRV) vector derivatives carrying a fragment of *NbEDS1*, *NbNDR1*, *NbRar1*, or *NbSGT1*, respectively. Approximately 21 days after the first inoculation, *Bs4* was agroinfiltrated in combination with *avrBs4* or *avrBs3*, respectively. Plants that were infiltrated with an empty virus vector showed an HR in the leaf area, in which *Bs4* was co-expressed with *avrBs4* or *avrBs3* (Figure 10). This indicates that the viral infection did not interfere with the *Bs4*-mediated HR. However, in *NbEDS1*- and *NbSGT1*-silenced plants co-expression of *Bs4* with *avrBs4* or *avrBs3* (Figure 10) triggered no HR, indicating that both genes are crucial to *Bs4*-governed HR execution. On the contrary, *Bs4*-mediated HR was not affected in *NbNDR1* and *NbRar1*-silenced plants.

Discussion

A putatively cytoplasmic R protein mediates recognition of a nuclear-targeted Avr protein

A positional candidate approach was used to isolate the tomato *Bs4* gene, which encodes a novel member of the TIR-NB-LRR class of R proteins. Tomato *Bs4* was shown to mediate recognition of the *Xanthomonas* AvrBs4 protein, which is a member of the well-characterized AvrBs3 family (Lahaye and Bonas, 2001). AvrBs3-like proteins contain NLS sequences that are indicative for nuclear targeting (Gabriel, 1997; Leach and White, 1996; White *et al.*, 2000), and recent immunocytochemical studies indeed demonstrated that AvrBs3 and AvrBs4 are localized to the plant nucleus (Szurek *et al.*, 2002; Szurek and Bonas, unpublished results). Molecular analysis of several gene-for-gene interactions suggests that R-protein localization is generally dictated by the Avr protein destination (reviewed by Bonas and Lahaye, 2002; Martin *et al.*, 2003) and thus *Bs4* would be predicted to encode a nuclear protein. However, tomato *Bs4* has no apparent sequence signatures that would indicate nuclear localization. This prediction is in agreement with the fact that *Bs4* mediates recognition of NLS-deprived AvrBs4-deletion derivatives (this study and Ballvora *et al.*, 2001a) and supports our previously proposed working model that *Bs4*-mediated perception of AvrBs4 occurs in the cytoplasm before AvrBs4 has reached its final destination, the nucleus (Ballvora *et al.*, 2001a). However, it needs to be considered that computer-based predictions are error-prone as exemplified by the RPM1 R protein that was found to be membrane bound although it was predicted to be cytoplasmic (Boyes *et al.*, 1998). Hence, localization studies are needed to clarify the subcellular localization of Bs4.

Agrobacterium*-mediated* avrBs3 *expression triggers a* Bs4*-dependent HR*

Xanthomonas infection tests demonstrated that *Bs4* mediates recognition of AvrBs4 and its C-terminal deletion derivatives, but not the 97% identical AvrBs3 protein (Figure 3). However, when the corresponding genes are delivered via *Agrobacterium*, both AvrBs4 and AvrBs3 but not AvrBs1 trigger a *Bs4*-dependent HR (Figure 4). This finding indicates that *Agrobacterium*-mediated delivery causes a partial loss of recognition specificity. The reasons for this are not fully understood.

One possibility is that increased AvrBs3 expression levels generate the observed loss of Bs4 recognition specificity as transgene expression in the binary vector is under control of the CaMV 35S promoter. Thus, AvrBs3 levels in the *Agrobacterium*-mediated expression system might exceed

the quantity that is delivered by the *Xanthomonas*-type III secretion system, possibly causing loss of Bs4 recognition specificity. However, in the converse experiment, the pepper *Bs3 R* gene that confers perception of AvrBs3 in an NLS-dependent fashion (Van den Ackerveken *et al.*, 1996) does not mediate recognition of agroinfiltrated AvrBs4 (S. Schornack and T. Lahaye, unpublished results). The seemingly different degrees of specificity in *Bs3*- and *Bs4*-mediated resistance in agroinfiltration assays might be related to the fact that *Bs3* confers only recognition of NLS-bearing AvrBs3 derivatives (Ballvora *et al.*, 2001a; Van den Ackerveken *et al.*, 1996). Previous studies have shown that AvrBs3 recruits the host's nuclear import machinery in order to reach the plant nucleus (Szurek *et al.*, 2001). Assuming that the host's nucleocytoplasmic shuttle system has only a limited transfer capacity, it might be possible that in agroinfiltration assays, only a small fraction of NLS-bearing AvrBs3-like proteins are actually transferred into the nuclear compartment where it can interact with Bs3. In contrast, the putatively cytoplasmic tomato Bs4 might be confronted with high levels of AvrBs3-like proteins. Hence, the apparently different degrees of recognition specificity of Bs3 and Bs4 may reflect their subcellular localization rather than intrinsic properties of the proteins.

A second possibility is that 'relaxed' recognition specificity is an intrinsic property of Bs4. This hypothesis is supported by the identification of two novel AvrBs3-like proteins that are distinct from AvrBs4, which are both recognized in a *Bs4*-dependent manner when delivered by *Xanthomonas* (S. Kay and U. Bonas, unpublished results). Considering that several *Xanthomonas* strains contain multiple AvrBs3-like proteins (Leach *et al.*, 2001; Van't Slot and Knogge, 2002) and given that genes encoding AvrBs3-like proteins can rapidly change their structure by intra- and inter-repeat recombination (Yang and Gabriel, 1995a), it seems economically sensible to employ one R protein with a 'relaxed' recognition specificity rather than expressing multiple, highly specific R proteins.

Intron-deprived Bs4 *derivatives mediate AvrBs4 detection*

Molecular analysis of transcripts encoding TIR-NB-LRR class R proteins has revealed many cases of alternative splicing (Anderson *et al.*, 1997; Ayliffe *et al.*, 1999; Dinesh-Kumar and Baker, 2000; Gassmann *et al.*, 1999; Lawrence *et al.*, 1995; Whitham *et al.*, 1994). Recent studies of tobacco *N* and *Arabidopsis RPS4*, both encoding TIR-NB-LRR proteins, showed that intron-deprived genes have no or only reduced activity, suggesting that alternative splicing is crucial to these defense-signaling pathways (reviewed by Jordan *et al.*, 2002).

The *Bs4* exon-intron structure is characteristic of an *R* gene transcript that encodes an TIR-NB-LRR protein (reviewed by Jordan *et al.*, 2002) and has three conserved introns that are located between the TIR-, NB-, and LRR-encoding regions. RT-PCR uncovered in several *Bs4* transcripts retention of introns 2 and 3, which has also been described for the *Arabidopsis RPS4* (Gassmann *et al.*, 1999) and the flax *L6* gene (Ayliffe *et al.*, 1999). Complementation analysis showed that intronless *RPS4* derivatives mediate no or only a reduced defense response in comparison to genomic *RPS4* constructs (Zhang and Gassmann, 2003). In contrast to the findings observed for *RPS4*, we did not observe obvious functional differences between genomic and intron-deprived constructs. Nonetheless, one cannot exclude the possibility that these *Bs4* splice variants confer subtle biological effects.

Sequence analysis of the bs4 *allele provides no unequivocal evidence for a complete-loss-of-function (null) allele*

Analysis of 74 DNA polymorphisms between the *L. pennellii* LA2963 *bs4* and *L. esculentum* cv. MM *Bs4* genomic sequences (Figure 6) failed to uncover mutations that would unequivocally classify *bs4* as a null allele. RT-PCR revealed that *bs4* and *Bs4* transcripts differ with respect to the 5′ splice site of intron 2 (Figure 4b). Usage of an alternative GT splice donor site in *bs4* transcripts generates a frame-shift and is predicted to encode truncated bs4 proteins that lack the LRR region. However, we analyzed only 32 cloned RT-PCR fragments and it might well be possible that *bs4*-derived transcripts that encode a functional, full-length TIR-NB-LRR protein remained undetected. Phenotypic inspection of *L. pennellii* LA2963 and segregants that harbor the corresponding *bs4* allele revealed a delayed HR in *bs4* genotypes that appears about 10 days after *Xanthomonas* infection (data not shown; *Bs4* mediates HR 48 h after infection). Notably, only *avrBs4*-expressing xanthomonads trigger this delayed HR, indicating that the *L. pennellii* LA2963 *bs4* allele still has residual function with respect to AvrBs4 perception. In summary, the lack of mutations that would clearly classify *L. pennellii bs4* as a null allele and the delayed AvrBs4-dependent HR in *bs4* genotypes supports the hypothesis that *bs4* is not a non-functional allele but rather an allele with reduced activity.

VIGS of NbEDS1 *and* NbSGT1 *suppresses* Bs4*-mediated HR execution*

Genetic dissection has shown that *EDS1* and *NDR1* define defense-signaling pathways that are differentially employed by TIR-NB-LRR and CC-NB-LRR proteins, respectively (reviewed by Feys and Parker, 2000; Glazebrook, 2001). In agreement with this postulate, we found that

functionality of the TIR-NB-LRR protein Bs4 was compromised by *EDS1*- but not by *NDR1*-silencing.

Unlike *EDS1* and *NDR1*, the pathway elements *Rar1* and *SGT1* were shown to be engaged by both TIR–NB-LRR and CC-NB-LRR subtypes (reviewed by Dodds and Schwechheimer, 2002). Noteworthy, activity of the tobacco N protein, which shares 54% sequence identity with Bs4, is impaired by VIGS of *Rar1* and *SGT1* (Liu *et al.*, 2002a,b). By contrast, our studies show that *Bs4*-mediated HR execution is only impaired by *SGT1*- but not by *Rar1*-silencing. This is somewhat surprising, given that Bs4 and N share extensive sequence homology. However, previous studies of the barley MLA1, MLA6, and MLA12 mildew R proteins, which share approximately 90% sequence identity (Halterman *et al.*, 2001; Shen *et al.*, 2003; Zhou *et al.*, 2001), have shown that, despite their pronounced sequence homology, only MLA6 and MLA12 require RAR1 and SGT1 for execution of a defense response (Halterman *et al.*, 2001; Shen *et al.*, 2003; Zhou *et al.*, 2001). Domain swaps between the *Rar1/SGT1*-independent *Mla1* and the *Rar1/SGT1*-dependent *Mla6* alleles generated *Rar1/SGT1*-independent chimeras with *Mla6* recognition specificity (Shen *et al.*, 2003), thereby demonstrating that recognitional specificity and *Rar1*/*SGT1* requirements are defined by distinct protein regions. Analysis of the barley *Mla* alleles demonstrates also that highly similar R proteins that recruit distinct signaling elements provide a functional tool for allocation of protein regions that specify dependency on certain downstream components. In analogy to the MLA variants, Bs4 and N represent highly similar R proteins, which however differ only with respect to their *Rar1* but not with respect to their *SGT1* dependency. Hence, domain swaps between Bs4 and N might allow allocation of domains that define *Rar1*-dependency.

However, there is a caveat to our VIGS-based *Rar1* and *NDR1* knockdown assays as gene-silencing does not generally facilitate complete elimination of the targeted transcripts. Thus, we cannot exclude that Bs4 acts in a *NDR1*- and *Rar1*-dependent manner and that residual amounts of the corresponding transcripts are sufficient for *Bs4*-mediated HR execution.

No direct interaction between Bs4 and AvrBs4?

Resistance proteins are thought to detect Avr proteins either directly by physical interaction or indirectly because of virulence-associated Avr actions (Bonas and Lahaye, 2002; Dangl and Jones, 2001; Martin *et al.*, 2003; Van der Biezen and Jones, 1998b). Thus far, no virulence function has been assigned to AvrBs4. However, as AvrBs4 has been maintained in nature, despite the fact that it exerts negative selective pressure in the interaction with most tomato hosts, it is likely to have a virulence function. Conceivably, AvrBs4 employs molecular virulence strategies similar to those of the 97% identical AvrBs3 protein, and hence it seems likely that AvrBs4-deletion derivatives that lack C-terminal AAD and NLS domains have no or only reduced virulence activity. We showed by analysis of deletion constructs that an AvrBs4-derivative (AvrBs4 Δ230), which contains only 3.5 out of 17.5 repeat units and lacks its AAD and NLS regions was still able to trigger a *Bs4*-dependent HR. This might be interpreted as an argument against the guard model as recognition in the conceptual framework of the guard model requires biologically active, disease-promoting Avr effectors. Taken together, genetic analysis of the *avrBs4–Bs4* gene-for-gene interaction favors a receptor-ligand rather than a guard model. However, AvrBs4 and Bs4 seem not to interact *in yeast*, despite the fact that Western analyses and repression assays showed that AvrBs4 and Bs4 derivatives are in principle suitable for Y2H studies. How can we explain these seemingly contradictory findings? Recent biochemical studies suggest that R proteins are part of multiprotein complexes (Bogdanove, 2002; Ellis *et al.*, 2002; Leister and Katagiri, 2000), and it may well be possible that R proteins fulfill their Avr-receptor function only in the context of this multicomponent recognition complex. Further clarification of the molecular principles that govern the AvrBs4-Bs4 interplay will therefore foreseeable require techniques that allow analysis of proteins in their natural environment.

Experimental procedures

avr *derivatives and* Xcv *inoculations*

Plants were grown and inoculated as described previously by Bonas *et al.*, (1989, 1993). Analysis of *avrBs4*-deletion derivatives in pLAFR6 (description of constructs by Bonas *et al.*, 1993) was conducted with respective *Xcv* transconjugants. All other *avr* genes were assayed in *Xcv* transconjugants that carry pDSK602 constructs of *avrBs1* (pDS100, Escolar *et al.*, 2001), *avrBs3* (pDS300F, Van den Ackerveken *et al.*, 1996), *avrBs4* (pDS200F, this study) and *avrBs4 Δ227* (pDS227, this study). *avrBs4* and *avrBs4 Δ227* were cloned into pDSK602 (Murillo *et al.*, 1994) as follows. An *Eco*RI/*Hin*dIII fragment from pUS200F, bearing *avrBs4* (Ballvora *et al.*, 2001a), was cloned into pDSK602, resulting in plasmid pDS200F. AvrBs4 Δ227 (pAT227) is a C-terminal *avrBs4*-deletion derivative that was generated by DNAse I digest of an *Eco*RI-linearized pAT200 (Bonas *et al.*, 1993) and released from pAT227 by *Bam*HI digest and replaced the corresponding *avrBs3* fragment in pDSK602-36 giving rise to pDS227. pDSK602-36 was generated by cloning a *Bam*HI–*Nde*I–*Bam*HI linker into the *Bam*HI site of pUXV1006 (Bonas *et al.*, 1989) and ligating the *Nde*I–*Hin*dIII fragment with the *avrBs3*-ORF into *Eco*RI/*Hin*dIII sites of pDSK602.

Plant material

Inoculation tests of *avrBs4*-deletion derivatives were performed on 10 *bs4/bs4* and 10 *Bs4/–* F_2 segregants of a cross between the *L. esculentum* cv. MM (*Bs4*) and *L. pennellii* LA 2963 (*bs4*) (Ballvora *et al.*, 2001b). The genotype of F_2 plants in the *Bs4* locus

was determined with the *Bs4*-flanking markers TG432 and P11M6 (Ballvora *et al.*, 2001a,b).

PCR with degenerate oligonucleotide primers targeting the TIR motif

Primer pairs based on the conserved TIR motif of NB-LRR-encoding *R* genes were used for amplification of *Bs4* candidate genes from VF36 tomato DNA. Their sequences were: RD5: GT(T/G)TT(T/C)TT(A/G)AGTTT(C/T)AG(A/G)GG; and RD10: GGATCCAC(A/C)(T/A)(C/T)ATA(G/A)AA(A/T)AT(A/C)GG. Amplification was carried out with the following program: 5 cycles of 94°C for 45 sec, 42°C for 45 sec, and 72°C for 90 sec; 30 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 90 sec. PCR products were mapped on a population of 47 F_2 plants from *L. esculentum* × *L. pennellii* (Tanksley *et al.*, 1992) using MAPMAKER (Lander *et al.*, 1987).

Genetic mapping of a Bs4 *candidate gene*

We developed two PCR-based RFLP markers within the *Bs4* candidate gene; RGA2 (primers: Bs4-A02: CTACCATCATCTCTTCAGTACCAACTC; and Bs4-B02: GAAATTGGAGGAACCGAGCTCCAG; *Msp*I-polymorphism); and RGA3 (primers: Bs4-A03: GGGTTGGAGTCCGAAGAGCAGG; and Bs4-B03: GACTAACCAACGCAAGTTATTGGACAGG; *Rsa*I-polymorphism). Inheritance of AvrBs4 recognition in tomato was studied using a cross between the *L. esculentum* cv. MM (resistant parent) and *L. pennellii* LA 2963 (susceptible parent; Ballvora *et al.*, 2001b).

Construction of binary vectors carrying Bs4

For construction of a cosmid library, YAC Y45 was partially digested with *Sau*3A, and cloned into the *Bam*HI site of the binary vector pCLD04541 (Jones *et al.*, 1992). The T26 RGC-bearing cosmid T2-2 was identified by colony hybridization and confirmed by PCR. A T2-2-derived genomic fragment containing *Bs4* and 1.2 kbp upstream of the ATG was transferred into the *Sma*I site of pCP60 (kindly provided by C. Coronado and P. Ratet; *Bs4* transgene is under control of CaMV 35S promoter) yielding pVTSB1. We removed the 35S* promoter from the binary vector pVB60 (Van den Ackerveken *et al.*, 1996) yielding pVB61. Next, we cloned *Bs4* and 3.5 kbp upstream sequence into the *Sal*I–*Not*I sites of pVB61 yielding pVTSB3.

Construction of binary vectors containing avrBs4 *derivatives*

The binary vector pVB60 (Van den Ackerveken *et al.*, 1996) was used for *Agrobacterium*-mediated transient expression of *avr* derivatives. We assayed *avrBs4* (pVS200F, Ballvora *et al.*, 2001a), *avrBs4 Δ227* (pVS227, this study), and *avrBs4 Δ152* (pVS256F, this study). pVS227 and pVS256F originate from pAT227 (avrBs4 Δ227 in pUC118; Bonas *et al.*, 1993) and pUS256 (this study, see below for description), respectively. *Eco*RI/*Hin*dIII inserts from pAT227 and pUS256 were cloned into pBluescript KS yielding pBS227F and pBS256F, respectively. Subsequently *Eco*RI/*Xho*I fragments from pBS227F and pBS256F were transferred into pVB60 creating pVS227F and pVS256F, respectively.

Complementation studies

The binary vector pVTSB1, containing *Bs4* under transcriptional control of the CaMV 35S promoter, was transferred into *A. tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) and transformed into the previously described *bs4/bs4 L. esculentum* genotype MM^{bs4}–BC4 (Ballvora *et al.*, 2001a). Transformation and plant regeneration was performed as described by Ling *et al.* (1998). Transgenic plants were confirmed by PCR with primers for the neomycin phosphotransferase (*npt*II) gene and primers that distinguished between the transgenic *Bs4* and endogenous *bs4* sequences.

RACE

Total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and enriched for poly(A) RNA using the Oligotex mRNA Mini kit (Qiagen). RACE PCR was carried out using the SMART RACE cDNA Amplification Kit (Clontech, Heidelberg, Germany). Amplicons were cloned into pCR2.1 (Invitrogen, Karlsruhe, Germany) and sequenced using vector-specific primers.

Construction of an intron-deprived Bs4*-derivative*

An intron-deprived *Bs4* derivative was generated by amplification of exonic regions. Restriction sites present at the end of Bs4 exons were used to fuse PCR-amplified exons. Briefly, an *Xba*I – *Not*I fragment from pVTSB1 containing the *Bs4* genomic sequence was subcloned into pBluescript SK II (Stratagene, La Jolla, CA, USA) to yield pBlue:Bs4. A *Xba*I–*Kpn*I fragment of pBlue:Bs4 that contained all introns to be removed was subcloned into pUC119 (Vieira and Messing, 1987) to yield pUCSB6. A *Bs4* clone with pBluescript backbone lacking *Eco*RV, *Eco*RI, and *Pst*I was generated by *Eco*RV–*Sma*I digest, religation, and *Xba*I–*Not*I introduction of *Bs4* from pBlue:Bs4 into this vector to yield pBSB3. Primers I1fwd-*Eco*RV, I2rev-*Bst*XI, and I3rev-*Bgl*II and the corresponding reverse primers derived from the genomic *Bs4* sequence were used to amplify exonic regions from pBlue:Bs4 and introduce exon–exon junctions instead of introns. These amplicons replaced corresponding restriction fragments in pUCSB6 by *Eco*RV–*Sac*II, *Sac*II–*Bst*XI, and *Bst*XI–*Bgl*II digest, respectively, to create pUCSB6 Δ123. A *Xba*I–*Pst*I fragment from pUCSB6 Δ123 was then cloned into pBSB3, thus replacing the corresponding genomic *Bs4* fragment and creating pBSB5. pBSB5 represents an intron-deprived *Bs4* fragment containing flanking genomic sequences. A *Xba*I–*Not*I fragment from pBSB5 was subsequently cloned into the binary vector pCP60 for *in planta* assays.

Bs4 *pathway dissection by VIGS*

Expressed sequence tag (EST) database searches using *Arabidopsis* NDR1 (AI776252.1) as input returned a closely related tomato EST (EST257352). Based on this EST, we designed primers in regions of highest homology to *Arabidopsis* NDR1 (JP NDR 2: TATAATCTCGTCGTAACGAACACCTTTGTC and JP NDR 1: ACTGCAGGCTTAACAGCTCTCTTTATCTGG) and performed RT-PCR on *N. benthamiana* cDNA. We observed a 198-bp PCR fragment that was cloned into pGEM-T Easy vector (Promega, Mannheim, Germany). The 5′ and 3′ ends of *NbNDR1* were determined by RACE, using the MARATHON cDNA amplification kit (Clontech). A 480-bp *NbNDR1* fragment used to produce TRV:NbNDR1 was generated by PCR on a plasmid containing *NbNDR1* cDNA, using primers JP NDR 4: (TACCTGGCTTTTACCAAGGTCATGAC) and JP NDR 5: (CCAACTACAGTCAGTTTGCACCGA). We designed primers for amplification of *NbRar1* based on the *N. tabacum Rar1* mRNA sequence (AF480487). A 585-bp *NbRar1* fragment used to produce TRV:NbRar1 was generated by RT-PCR on *N. benthamiana* cDNA,

using primers Rar1-f2 (TGCACTTATCACGAATCCGG) and Rar1-r2 (TGGGCTGGCGTTGTGCCATCCTG). Construction of TRV:EDS1 and TRV:SGT1 has been described before (Peart *et al.*, 2002a,b). Infection of plants with TRV derivatives was performed by agroinfiltration as described previously by Peart *et al.* (2002a).

Sequence analysis of the L. esculentum *cv. MM* Bs4 *and* L. pennellii *LA2963* bs4 *genes*

The *Bs4* sequence from *L. esculentum* cv. VFNT Cherry was obtained from cosmid T2-2. Corresponding sequences from *L. esculentum* cv. MM (*Bs4*), and *L. pennellii* LA2963 (*bs4*) were determined by sequencing of respective PCR products. Sequences were obtained using the BigDye Terminator Kit (PE Biosystems, Foster City, CA, USA) and analyzed using Sequencher 4.0 (Gene Codes Corp., Ann Arbor, MI, USA).

Y2H interaction studies

For Y2H studies, the yeast interaction trap was used (Gyuris *et al.*, 1993). Bait and prey plasmids were co-transformed into yeast strain EGY48 (Estojak *et al.*, 1995) containing the *lacZ*-reporter plasmid pSH18-34. Transformants grown on selective glucose medium were transferred on galactose medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to determine β-galactosidase (*LacZ*) activity, and on galactose plates lacking leucine to measure *Leu2* expression. Quantitative assays were performed on liquid cultures. Three individual yeast transformant colonies for each construct were inoculated into liquid glucose medium and incubated overnight (o.n.) at 30°C. The culture was diluted in galactose medium inducing the expression of the prey and grown until an OD_{600} of 0.5–1.0. β-galactosidase activity was assayed using *o*-Nitrophenyl β-D-galactopyranoside as described previously by Ausubel *et al.* (1996). For Y2H analysis, *avrBs4* and *Bs4* were cloned into bait (pYB) and prey (pYP) vectors. Full-length AvrBs4 autoactivated transcription in yeast and to overcome this problem, we cloned the N-terminal deletion derivative AvrBs4 Δ152 (lacking aa residues 1–152) into bait (pYB256) and prey (pYP256) vectors, respectively. The N-terminal deletion was generated by replacing the *Stu*I/*Hin*dIII fragment of pUS356 (contains N-terminal deletion of avrBs3, Szurek *et al.*, 2001) with the corresponding fragment of pAT200 (contains avrBs4, Bonas *et al.*, 1993), yielding pUS256 (*avrBs4 Δ152* in pUC118). The *Eco*RI/*Xho*I fragment from pUS256 was transferred into pEG202 and pJG4-5 (OriGene Technologies Inc., Rockville, MD, USA) yielding pYB256 and pYP256. *Bs4* domains were amplified from cDNA using primers annealing adjacent to TIR (Bs4-TIR-fwd, Bs4-TIR-rev), NB (Bs4-NB-fwd, Bs4-NB-rev) and LRR (Bs4-LRR-fwd, Bs4-LRR-rev) or combinations, all containing *Mun*I/*Sal*I sites that allowed cloning into *Eco*RI/*Xho*I of pEG202 and pJG4-5. The previously described Caimpα1 (Szurek *et al.*, 2001) was used as a positive control for Y2H studies. In all cases, expression and stability of fusion proteins was confirmed by immunoblotting using a monoclonal anti-LexA antibody (Clontech Laboratories) and monoclonal anti-HA antibody (3F10, Roche, Mannheim, Germany), respectively.

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2.2.3 Ergänzende Ergebnisse

2.2.3.1. Erkennung AvrBs3-ähnlicher Proteine in verschiedenen Pflanzenarten

Auf der Basis existierender Daten konnten bereits zwei Erkennungsprinzipien für AvrBs3-ähnliche Proteine identifiziert werden. Während der *Bs3*-Typ auf das Vorhandensein von NLS-Sequenzen im AvrBs3-ähnlichen Protein angewiesen ist, induzieren beim *Bs4*-Typ der Erkennung auch verkürzte AvrBs3-ähnliche Proteine ohne NLS eine HR (Ballvora *et al.*, 2001). Transiente *Agrobacterium*-vermittelte Expressionen zeigten bereits, dass die AvrBs4-Erkennung jedoch nicht notwendigerweise NLS-unabhängig erfolgt, da die Erkennung in Kartoffel von NLS-Sequenzen abhängig ist, also dem *Bs3*-Typ entspricht (siehe vorangegangenes Manuskript). Weitere Pflanzenarten wurden auf ihre Erkennung von AvrBs3, AvrBs4 und weiteren AvrBs3-ähnlichen Proteine analysiert, um sie hinsichtlich *Bs3*- oder *Bs4*-Erkennungstyp zu klassifizieren. Dazu wurden *Agrobacterium* Stämme (GV3101) mit verschiedenen T-DNA-Konstrukten bei einer optischen Dichte von 0,4 bei 600 nm in Infiltrationsmedium in die Blattunterseiten von verschiedenen Pflanzen infiltriert (zur Methodik siehe Schornack *et al.*, 2005).

AvrBs4 induzierte außer in Kartoffel und Tomate auch eine HR in *Capsicum pubescens* PI585270 (siehe auch Minsavage *et al.*, 1999), *Solanum suaveolens* und *Nicandra physalodes* (Tabelle 2). Im Gegensatz zur *Bs4*-vermittelten Erkennung in Tomate erfolgte in den genannten Spezies keine Erkennung des AvrBs4Δ227-Derivats, sondern eine dem *Bs3*-Typ entsprechende NLS-abhängige Erkennung. Die Expression von *avrBs3* zeigte, dass *Solanum nigrum* und *Nicandra physalodes* ebenso wie die Paprika-Linie ECW-30R in der Lage sind, AvrBs3 zu erkennen. In *S. nigrum* erfolgte keine Erkennung eines NLS-Deletionsderivats von AvrBs3, d. h. mit Ausnahme der *Bs4*-vermittelten Erkennung von überexprimiertem AvrBs3 in Tomate wird AvrBs3 *Bs3*-Typ spezifisch erkannt. Nur AvrBs3, aber keines der anderen AvrBs3-ähnlichen Proteine induzierte eine Hypertrophie in zahlreichen Arten, in denen keine HR ausgelöst wurde. AvrBs3Δrep16 ist ein AvrBs3-Derivat, dessen Erkennung in *bs3*-Paprika (cv. ECW), aber nicht in *Bs3*-Paprika (cv. ECW-30R) erfolgt (Abb. 3B; Herbers *et al.*, 1992). Interessanterweise zeigten, mit Ausnahme von *Bs3*-Paprika, alle getesteten Arten (*C. pubescens, N. benthamiana, S. nigrum, N. physalodes*) eine mehr oder weniger starke HR nach Expression von AvrBs3Δrep16.

Tabelle 2. Verbreitung der Avr-Aktivität von AvrBs3-ähnlichen Proteinen bzw. der Bs4-Funktionsfähigkeit

		AvrBs3-ähnliche Proteine[b]								Bs4-Funktion[c]	Kontrollen[d]		
Familie	**Spezies[a]**	**AvrBs4**	**AvrBs4 Δ227**	**AvrBs3**	**AvrBs3 Δrep16**	**AvrBs3 Δ1–3**	**Hax2**	**Hax3**	**Hax4**	**Bs4 + AvrBs4 Δ227**	**AvrBs1**	**Bax**	**GUS***
Solanaceae	*Capsicum pubescens* PI585270	++*	-	-	++	-				-	-		
	Nicotiana benthamiana	-	-	HH	+		-	-	-	++*	AA	++	++
	Nicotiana tabacum 'Petit Havana'	-	-	H			-	++	-	++*	A	++	++
	Nicotiana clevelandii	-	-	HH			-	++	-	++*	AA	+	+
	Petunia hybrida	-	-				-	-	-	-	-	-	+
	Solanum nigrum (A)	-	-	+++	+	-	-	-	-	-	A	-	++
	Solanum americanum PI 268152	-	-	H			-	++	-	-	-	-	
	Solanum atropurpureum PI 305320	-	-	-						-	-		
	Solanum tuberosum 'Desiree'	++*	-*	H						+*	A		+
	Solanum melongena Grif 14189	-	-	-							-		
	Solanum aviculare PI 280049	-	-	H						+	-		
	Solanum pseudocapsicum PI 368425	-	-	-			-	-	-		-	-	
	Solanum suaveolens PI 203339	++	-	HHH			-	-	-	++	-	-	
	Tubocapsicum anomalum PI 501532	-	-	-						-	-		
	Datura suaveolens (H)	-	-	-							-		+
	Nicandra physalodes (B)	++	-	+	++		-	-	-		-	++	+
	Mandragora sp. (H)	-	-	H			-	+	-	++	-	-	
	Physalis alkekengi (H)	-	-	H			-	-	-	-	-	-	
	Physalis peruviana (H)	-	-	H			-	++	-	-	-	-	
Chenopodiaceae	*Spinacia oleracea* (H)	-	-	-			-	-	-	++	-	-	
Fabaceae	*Lupinus* sp. (H)	-	-	-							-		+
	Pisum sativum (H)						-	-	-	-	-	-	
Asteraceae	*Lactuca sativa* (H)	-	-	-							-		+
Valerianaceae	*Valerianella* sp. (H)	-	-	H							-		
Brassicaceae	*Arabidopsis thaliana* Col-0	-	-	-						-	-		+
	Raphanus sativus sativus (H)	-	-	-							-		+

Außer AvrBs4 und AvrBs3 wurden die AvrBs3-ähnlichen Proteine Hax2, Hax3 und Hax4 getestet. Die aus *X. campestris* pv. *armoraciae* isolierten *hax3*- und *hax4*-Gene werden *Bs4*-vermittelt detektiert (Kay *et al.*, 2005). Bei den hier durchgeführten Analysen konnte nur eine Erkennung von Hax3 nachgewiesen werden. Dieses induzierte in mehreren Arten eine HR (*Nicotiana tabacum, N. clevelandii, S. americanum, Mandragora sp.* und *Physalis peruviana*). Da keine NLS-Deletionsderivate getestet wurden, kann keine Zuordnung zum *Bs4*- bzw. *Bs3*-Typ der Erkennung erfolgen. Da aber der *Bs4*-Typ der Erkennung bisher auf *Lycopersicon* beschränkt ist, ist es wahrscheinlich, dass die weit verbreitete Erkennung von Hax3 eher dem NLS-abhängigen *Bs3*-Typ folgt.

Um *Agrobacterium*-induzierte Effekte auszuschließen, wurde AvrBs1 exprimiert, welches eine HR in *C. annuum* cv. ECW-10R induziert und keine Sequenzhomologie zu AvrBs4 aufweist. In keiner der hier getesteten Spezies wurde eine AvrBs1-Erkennung nachgewiesen. AvrBs1 eignet sich damit als Negativkontrolle. In *Nicotiana*-Arten, *S. nigrum* und Kartoffel wurde aber eine AvrBs1-spezifische Aufwölbung des mit *Agrobacterium* infiltrierten Bereiches detektiert. Ursache dafür könnte eine bisher unbekannte Funktion von AvrBs1 sein. Bei *Petunia, S. atropurpureum, S. melongena, S. pseudocapsicum, Tubocapsicum anomalum, Datura suaveolens, Lupinus* sp., *Lactuca sativa, Arabidopsis, Raphanus sativus* und *Pisum sativum* wurde nach *Agrobacterium*-vermittelter Expression mit keinem *avr*-Gen eine Reaktion beobachtet. Um zu prüfen, ob die transiente, *Agrobacterium*-vermittelte Expression in diesen Pflanzen generell möglich ist, wurde in einigen Spezies 35S-*uidA* (Gen der β-Glucuronidase) exprimiert und mittels Nachweis des X-Glucuronid-Substratumsatzes (Blaufärbung) detektiert (siehe Manuskript 2). Dadurch konnte gezeigt werden, dass *Petunia, Datura, Lupinus, Lactuca, Arabidopsis* und *Raphanus* generell eine *Agrobacterium*–vermittelte Expression ermöglichen.

Tabelle 2 (vorhergehende Seite).

[a] wiss. Artnamen, Sortenname, Akzessionsnr. oder anderweitige Herkunft sind angegeben (A, Handaufsammlung des Autors; H, handelsübliches Saatgut; B, Botanischer Garten Halle.

[b] alle *avr*-Konstrukte wurden mittels 35S-Promotor exprimiert. AvrBs4Δ227 und AvrBs3Δ1-3 enthalten keine NLS-Sequenzen. Bei AvrBs3Δrep16 fehlen vier Repeats.

[c] Bs4 und AvrBs4Δ227 wurden überexprimiert. Die Bs4-Expression ergab in keiner Spezies eine Reaktion.

[d] AvrBs1 induzierte eine spezifische HR in *C. annuum* ECW 10R. Das proapoptotische Bax-Protein wurde als 35S-*Bax:GFP*-Konstrukt exprimiert. GUS (*uidA*)-Expression wurde mittels eines X-Glucuronid-Enzymassays nachgewiesen (siehe Manuskript 2).

* Diese Phänotypen stimmen mit publizierten Daten überein (Schornack, 2004; Ref. Minsavage)

-, keine Reaktion; A/AA, schwache/starke Aufwölbung des infiltrierten Gewebes; +/++/+++, schwache/starke/sehr starke Reaktion (HR/GUS-abhängige Färbung); H/HH/HHH, schwache/starke/sehr starke Hypertrophie; ? leichte unspezifische Reaktion; kein Eintrag, Konstrukt wurde nicht getestet.

Außerdem erfolgte die Expression des proapoptotischen Bax-Proteins der Maus, dessen Zelltod-auslösende Wirkung in *N. benthamiana* und *Arabidopsis* bereits gezeigt wurde (Jamir, 2004). Interessanterweise induzierte 35S-*Bax:GFP* außer in *Nicotiana*-Arten nur in *N. physalodes* eine Zelltodreaktion. Demnach handelt es sich nicht um eine universell einsetzbare Zelltod-Kontrolle.

Insgesamt ergaben die transienten Expressionsexperimente mit AvrBs3-ähnlichen Proteinen, dass deren Erkennung nicht auf eine bestimmte Pflanzenspezies beschränkt ist. Interessanterweise ist aber nur *N. physalodes* in der Lage, gleichzeitig mehrere in *Xanthomonas* vorkommende AvrBs3-ähnliche Proteine zu detektieren. Eine besonders auffällige Ausnahme ist die durch *Bs4* vermittelte AvrBs4-, Hax3- und Hax4-Erkennung in Tomate, die aber NLS-unabhängig erfolgt.

2.2.3.2. Bs4-Funktionalität in heterologen Pflanzenarten

Agrobacterium-Infiltrationsexperimente belegen eine Funktionsfähigkeit von *Bs4* in mehreren Solanaceae-Arten (siehe vorangegangenes Manuskript). In den hier getesteten Arten wurde *Bs4* stets zusammen mit einem NLS-freien *avrBs4*-Derivat exprimiert, da dieses im Gegensatz zu AvrBs4 außer in Tomate in keiner Spezies eine HR induzierte. Neben den bereits getesteten *Nicotiana*-Arten und *S. tuberosum* wurde eine spezifische Bs4-HR in *S. aviculare, S. suaveolens, Mandragora* sp. und *Spinacia oleracea* induziert. Damit ist Bs4 nicht nur in Solanaceen, sondern auch in einem nicht verwandten Gänsefussgewächs (Chenopodiaceae) funktionsfähig. Interessanterweise ist die Funktionalität von Bs4 nicht in allen Solanaceen gegeben (*Capsicum*, einige *Solanum*-Arten, *Petunia*, *Tubocapsicum*). *N. benthamiana* und *S. aviculare* induzieren eine HR bei Expression von Bs4 und AvrBs4Δ227, sind aber nicht in der Lage eines der getesteten AvrBs3-ähnlichen Proteine unabhängig von *Bs4* zu detektieren. Es gibt also keine Korrelation zwischen der Fähigkeit zur Erkennung AvrBs3-ähnlicher Proteine und Bs4-Funktionalität.

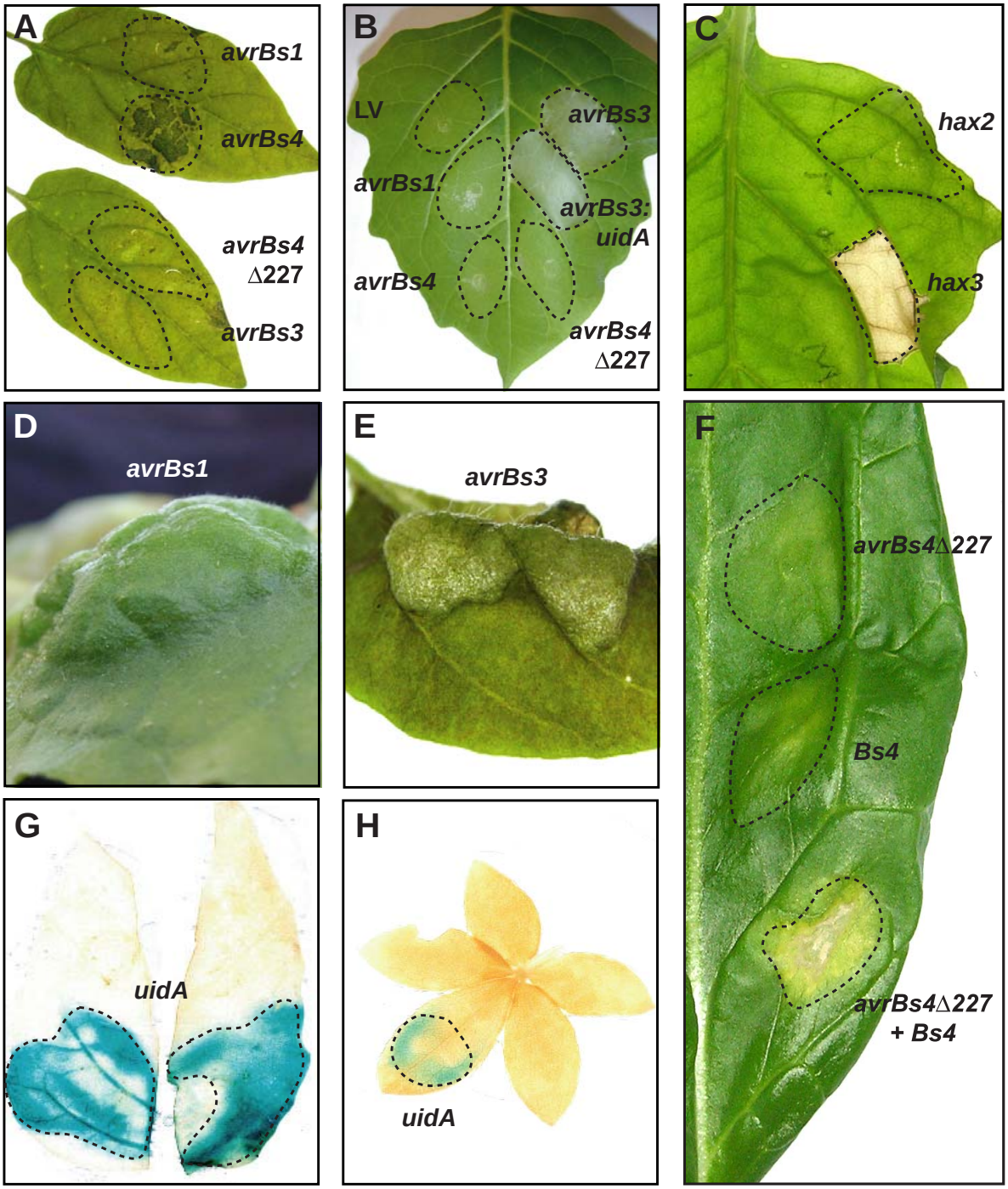

Abb. 8. Phänotypen nach transienter Expression von *avr*-Genen, *Bs4* und Reportergenen.
Dargestellt sind Blattabschnitte, in denen lokal mittels *Agrobacterium* GV3101-vermittelter Expression die angegebenen Gene exprimiert wurden (siehe auch Tabelle 2). HR-Phänotypen wurden 3 d.p.i. fotografiert. Die Visualisierung von Hypertrophie und GUS-Expression erfolgte 10 d.p.i. **(A)** In *Solanum suaveolens* erfolgt NLS-abhängige AvrBs4-Erkennung. **(B)** Dagegen wird in *S. nigrum* AvrBs3 (auch als Fusion mit GUS) und in *S. americanum* Hax3 **(C)** detektiert. **(D)** Die *avrBs1*-Expression führt zur Aufwölbung des *N. benthamiana*-Gewebes. **(E)** AvrBs3 induziert eine sehr starke Hypertrophie in *S. suaveolens*. **(F)** Die bei gemeinsamer *avrBs4*Δ227+*Bs4*-Expression detektierbare HR belegt die Bs4-Funktionalität in Spinat. *Nicandra physalodes* **(G)** und *Lupinus* sp. **(H)** zeigen unterschiedliche GUS-Enzymaktivität (mittels Blaufärbung durch Substratumsatz visualisiert) nach transienter *uidA*-Expression.

2.3. Das Expressionsniveau *avrBs3*-ähnlicher Gene beeinflusst die Spezifität der Tomaten-*Bs4*-, jedoch nicht der Paprika-*Bs3*-vermittelten Erkennung.

2.3.1 Zusammenfassung

Dieses Manuskript beschreibt die Charakterisierung des *Bs4*-Promotors und seine Verwendung zur konstitutiv niedrigen Expression von bakteriellen Typ-III-Effektor-Proteinen in der Pflanze. Quantitative RT-PCR-Analysen zeigten, dass *Bs4* konstitutiv niedrig exprimiert wird und die *Bs4*-Transkriptmengen nicht signifikant durch AvrBs4-exprimierende Xanthomonaden beeinflusst werden. Die stufenweise Deletion der *Bs4*-Promotorregion, gekoppelt mit funktioneller Analyse, führte zur Identifizierung eines 302 bp-Fragments stromaufwärts des *Bs4*-Startcodons, das ausreichend für eine *Bs4*-vermittelte HR war. Unter Verwendung von *Bs4*-Promotorfragmenten verschiedener Länge wurden GATEWAY Expressionskonstrukte generiert, welche in Paprika, Tomate, *N. benthamiana*, *Nicandra physalodes*, *Physalis* sp. und *Lactuca sativa* einsetzbar sind.

Vorangegangene Experimente zeigten bereits, dass die 35S-Promotor-vermittelte konstitutive Überexpression des zu AvrBs4 97% identischen AvrBs3-Proteins eine *Bs4*-abhängige HR induziert. Dagegen wird AvrBs3 bei Translokation durch *Xanthomonas* in die Pflanze nicht *Bs4*-abhängig erkannt. Diese Arbeit belegt, dass zwar *avrBs4*, jedoch nicht mehr *avrBs3* bei Expression unter Kontrolle des schwachen *Bs4*-Promotors eine *Bs4*-HR induziert. Im Gegensatz zu *Bs4* aus Tomate bleibt die *Bs3*-Erkennungsspezifität in Paprika bei 35S-Promotor gesteuerter Avr-Expression unverändert. *Bs3* vermittelt die Erkennung von *avrBs3*-exprimierenden, aber nicht *avrBs4*-exprimierenden *Xcv* und *avrBs4* induziert selbst bei 35S-Promotor-basierter Expression in der Pflanze keine *Bs3*-HR. Wichtig ist, dass die *Bs4*-Promotor-basierte Expression von *hax3*, *hax4, avrBs3* und *avrBs4* ein vergleichbares Reaktionsmuster hervorruft, wie die Infiltration der entsprechenden *avr*-exprimierenden *X. campestris* pv. *vesicatoria*-Stämme. Dies deutet darauf hin, dass die unter *Bs4*-Promotor-Kontrolle exprimierten Proteinmengen den über das bakterielle Typ III-System translozierten Mengen ähnlich sind.

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2.3.2 Manuskript 2

Expression Levels of *avrBs3*-Like Genes Affect Recognition Specificity in Tomato *Bs4*- But Not in Pepper *Bs3*-Mediated Perception

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The tomato *Bs4* disease resistance gene mediates recognition of *avrBs4*-expressing strains of the bacterial spot pathogen *Xanthomonas campestris* pv. *vesicatoria* to give a hypersensitive response (HR). Here, we present the characterization of the *Bs4* promoter and its application for low-level expression of bacterial type III effector proteins in planta. Real-time polymerase chain reaction showed that *Bs4* is constitutively expressed at low levels and that transcript abundance does not change significantly upon infection with *avrBs4*-containing xanthomonads. A 302-bp promoter fragment was found to be sufficient to promote *Bs4* gene function. Previous studies have shown that high, constitutive in planta expression of *avrBs3* (AvrBs3 and AvrBs4 proteins are 96.6% identical) via the *Cauliflower mosaic virus* 35S (35S) promoter triggers a *Bs4*-dependent HR whereas *X. campestris* pv. *vesicatoria*-mediated delivery of AvrBs3 into the plant cytoplasm does not. Here, we demonstrate that, when expressed under control of the weak *Bs4* promoter, *avrBs3* does not trigger a *Bs4*-dependent HR whereas *avrBs4* does. In contrast, the pepper *Bs3* gene, which mediates recognition of AvrBs3- but not AvrBs4-delivering xanthomonads, retains its recognition specificity even if *avrBs4* was expressed in planta from the strong 35S promoter. Importantly, *Bs4* promoter-driven expression of *hax3*, *hax4* (two recently isolated *avrBs3*-like genes), *avrBs3,* and *avrBs4* resulted in identical reactions as observed upon infection with *X. campestris* pv. *vesicatoria* strains that express the respective *avr* gene, suggesting that the protein levels expressed under control of the *Bs4* promoter are similar to those that are translocated by the bacterial type III secretion system.

Additional keywords: Agrobacterium, agroinfiltration, *Nicotiana benthamiana,* TIR-NB-LRR.

The ability to sense and respond to pathogens is crucial for survival of all living creatures. Like mammals and insects, plants are capable of detecting conserved pathogen-associated molecular patterns (PAMPs) such as flagellin and lipopolysaccharides (Boller 2005; Nürnberger and Lipka 2005; Zipfel and Felix 2005). Superimposed onto this basal line of defense is the so-called varietal (cultivar-specific) resistance, which mediates protection of individual cultivars within an otherwise susceptible plant species (Jones and Takemoto 2004). Varietal resistance is triggered when plants express a plant resistance (*R*) gene and pathogens express a complementary pathogen avirulence (*avr*) gene (Flor 1971). This gene-for-gene-governed resistance often is associated with a spatially confined plant cell suicide, termed hypersensitive response (HR), which is concomitant with halt of pathogen spread (Lam 2004; van Doorn and Woltering 2005).

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Isolation of more than 40 *R* genes revealed that most encode intracellular proteins with nucleotide-binding (NB) and leucine-rich repeat (LRR) domains (Hammond-Kosack and Parker 2003; Martin et al. 2003). The NB region is preceded by either a coiled-coil (CC) domain or a toll and interleukin 1 receptor (TIR) domain that is homologous with the effector domains of Drosophila Toll and human Interleukin-1 receptors. The biochemical basis of gene-for-gene resistance is still poorly understood, and it remains to be seen whether R proteins mediate recognition predominantly by direct or indirect recognition of *avr*-encoded proteins. Irrespective of the molecular basis, R proteins mediate pathogen detection and, therefore, should be present before microbial attack. This view is supported by multiple studies that reported a low-level constitutive expression of a given *R* gene in the absence of the corresponding pathogen elicitor (De Ilarduya and Kaloshian 2001; Goggin et al. 2004; Grant et al. 1995; Huang et al. 2005; Mindrinos et al. 1994; Paal et al. 2004; Parker et al. 1997; Shen et al. 2002). Yet, some *R* genes are transcriptionally induced upon pathogen challenge (Levy et al. 2004; Sobczak et al. 2005; Thurau et al. 2003; Yoshimura et al. 1998), indicating that modification of *R* gene expression levels is crucial for their function in defense.

The term *avr* gene is operational: a given pathogen strain expressing an *avr* gene is avirulent on plants that contain the corresponding *R* gene (Staskawicz et al. 1984). However, this definition is somewhat one-sided because *avr* genes often are required for maximal virulence of a given pathogen in plants that lack the matching *R* gene (Chang et al. 2004; Gabriel 1999a; Ponciano et al. 2003; Van't Slot and Knogge 2002; White et al. 2000). Both the virulence and the avirulence functions of bacterial *avr* genes generally depend on type III secretion systems (TTSS) that translocate the bacterial effector proteins into plant cells (Büttner and Bonas 2002).

One of the most well-studied Avr proteins is AvrBs3 from the bacterial spot pathogen *Xanthomonas campestris* pv. *vesicatoria* (also referred to as *X. euvesicatoria* or *X. axonopodis* pv. *vesicatoria*) (Jones et al. 2004; Vauterin et al. 1995). AvrBs3 is the founder of a large Avr family (Gabriel 1999b; Lahaye and Bonas 2001; Ponciano et al. 2003; White et al. 2000), and homologs have been identified in many species and pathovars of *Xanthomonas* (Vivian and Gibbon 1997) and *Ralstonia solanacearum* (Cunnac et al. 2004). AvrBs3-like proteins from *Xanthomonas* share 90 to 97% sequence identity and contain 5.5 to 25.5 nearly

perfect copies of a 34-amino-acid tandem repeat motif that determines recognition specificity (Ballvora et al. 2001; Gu et al. 2005; Herbers et al. 1992; Yang et al. 2000; Zhu et al. 1998). AvrBs3-like proteins also contain nuclear localization signals (NLSs) and an acidic transcriptional activation domain (AAD), motifs that usually are restricted to eukaryotic proteins (Lahaye and Bonas 2001). Functional studies have shown that NLS and AADs are essential to nuclear import (Szurek et al. 2001) and transcriptional activation of host genes (Marois et al. 2002) and that mutations in these motifs usually abolish recognition of AvrBs3-homologs by the corresponding *R* gene (Lahaye and Bonas 2001).

We are studying the tomato (*Lycopersicon esculentum*) *Bs4* and pepper (*Capsicum annuum*) *Bs3* disease resistance genes that mediate specific recognition of xanthomonads containing the 96.6% identical AvrBs3 and AvrBs4 proteins, respectively (Ballvora et al. 2001). Analysis of *Bs4*-mediated resistance revealed that *X. campestris* pv. *vesicatoria*-delivered AvrBs3 did not trigger a *Bs4*-dependent HR, even if *avrBs3* was expressed under transcriptional control of the strong, constitutive p*lac* promoter (Ballvora et al. 2001). However, AvrBs3 triggers a *Bs4*-dependent HR when the corresponding gene was expressed in planta under control of the strong *Cauliflower mosaic virus* 35S (35S) promoter (Schornack et al. 2004). It is unclear if quantitative or qualitative differences (e.g., post-translational modifications) between *X. campestris* pv. *vesicatoria*-delivered and in planta-produced AvrBs3 protein account for the observed differences in *Bs4*-mediated recognition. Recent studies revealed that tomato *Bs4* may be promiscuous because it mediates not only recognition of *avrBs4*-containing *X. campestris* pv. *vesicatoria* strains but also of xanthomonads that express *hax3* or *hax4*, two recently isolated *avrBs3*-homologs that encode proteins with 96.1 and 96.2% identity to AvrBs4, respectively (Kay et al. 2005). In contrast, pepper *Bs3* mediates specifically recognition of AvrBs3 but not of the AvrBs3-like AvrBs4, Hax3, and Hax4 proteins, regardless of whether these Avr proteins are *X. campestris* pv. *vesicatoria* delivered or if the corresponding genes are expressed in planta under control of the strong 35S promoter (Ballvora et al. 2001; Kay et al. 2005). Thus, the pepper *Bs3* gene shows a more pronounced recognition specificity than tomato *Bs4*. Tomato *Bs4* was cloned recently and encodes a TIR- NB-LRR class R protein (Schornack et al. 2004). Here, we describe the characterization of the *Bs4* promoter and its use for in planta expression of *Xanthomonas* type III effector proteins. Our data suggest that *Bs4* promoter-driven in planta expression of Avr proteins resembles the protein amount translocated by the *X. campestris* pv. *vesicatoria* TTSS better than the regularly used 35S-driven expression.

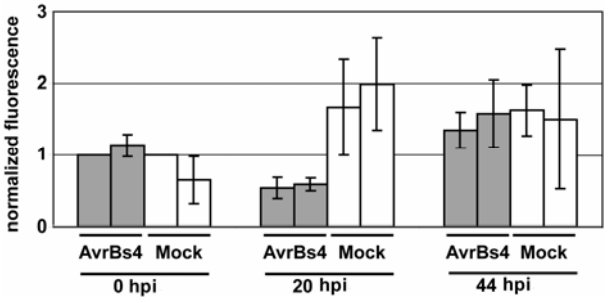

Fig. 1. *Bs4* transcript levels in leaf tissue of *Lycopersicon esculentum* cv. Moneymaker. Leaves were infiltrated with *Xanthomonas campestris* pv. *vesicatoria* strain 75-3 expressing *avrBs4* (gray columns) or infiltration-medium (white columns). Infiltrations were conducted twice (biological replicates) and are depicted by separate columns. *Bs4* transcripts were quantified by quantitative reverse-transcription polymerase chain reaction. Samples were harvested at 0, 20, and 44 h postinfiltration (hpi). Transcript levels are shown as *n*-fold increase in normalized fluorescence relative to the transcript levels measured at 0 hpi. Error bars represent standard deviations calculated using six technical replicates.

RESULTS

Bs4 transcript levels do not increase upon infection with *avrBs4*-expressing xanthomonads.

To determine whether tomato *Bs4* is transcriptionally regulated upon delivery of the matching AvrBs4 protein, we inoculated tomato *Bs4* plants (*L. esculentum* cv. Moneymaker [MM]) with *avrBs4*-expressing *X. campestris* pv. *vesicatoria* or infiltration medium (mock control). Leaf tissue samples were taken at 0, 20, and 44 h postinfiltration (hpi), which is prior to HR formation (48 to 72 hpi). RNA from challenge-inoculated leaf tissue of two independent infiltration experiments was reverse transcribed and subsequently analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using primers that anneal to the *Bs4* region encoding the NB-domain. qRT-PCR measurements were normalized to the expression of elongation factor 1α (EF1α) (Pokalsky et al. 1989). We observed a slight but significant transient decrease of *Bs4* transcript levels at 20 hpi upon delivery of AvrBs4 when compared with the mock control (Fig. 1). However, overall the *Bs4* transcript levels showed only minor changes upon inoculation with the avirulent strain, suggesting that *Bs4* promoter activity is constitutive rather than inducible.

Bs4 mediates identical recognition specificity in tomato and *Nicotiana benthamiana.*

Previously, we studied the *Bs4*-mediated HR by transient and stable transformation of tomato and showed that the recognition specificity, timing, and intensity of the reactions are indistinguishable between *Bs4* driven by its own promoter ($p3516_{Bs4}$::*Bs4*, previously termed pVTSB3) (Schornack et al. 2004) or by the strong 35S promoter (p35S::*Bs4*). We demonstrated that agroinfiltration of p35S::*Bs4* mediates HR in *N. benthamiana* when coexpressed with p35S::*avrBs4* (Schornack et al. 2004). To clarify whether the heterologous *N. benthamiana* expression system is a viable means to study tomato Bs4, we then investigated whether $p3516_{Bs4}$::*Bs4* and p35S::*Bs4* mediate identical reaction patterns in tomato and *N. benthamiana.* For this, we used *Agrobacterium*-mediated delivery of $p3516_{Bs4}$::*Bs4* or p35S::*Bs4* in combination with different 35S-driven *avr* constructs. Agroinfiltration assays showed that both $p3516_{Bs4}$::*Bs4* and p35S::*Bs4* mediated HR upon coinfiltration of p35S::*avrBs4*, p35S::*avrBs4*Δ227 (C-terminal *avrBs4*-deletion construct) (Schornack et al. 2004), or p35S::*avrBs3* but not upon coinfiltration of p35S::*avrBs1* (*avrBs1* and *avrBs4* are not sequence related) (Fig. 2). *Agrobacterium*-mediated delivery of the p35S::*avrBs1* T-DNA triggered an HR in *Bs1*- but not *bs1*-pepper genotypes, thus confirming functionality of the construct. The 35S and *Bs4* promoter-driven expression of the *Bs4* gene in *N. benthamiana* resulted in identical reactions with respect to both timing and intensity of HRs. Importantly, all *avr-R* combinations tested in *N. benthamiana* resulted in reactions qualitatively identical to those in tomato.

In summary, our findings demonstrate that (i) the tomato *Bs4* promoter is functional in the heterologous plant host *N. benthamiana* and (ii) that p35S::*Bs4* and $p3516_{Bs4}$::*Bs4* constructs mediate identical recognition specificity in tomato and *N. benthamiana*.

A 302-bp fragment comprises the *Bs4* minimal promoter.

Previously, we reported that the *Bs4* coding region and 3,516 bp of 5′ sequence ($p3516_{Bs4}$::*Bs4*) are capable of mediat-

ing AvrBs4 recognition (Schornack et al. 2004). Homology searches identified a hypothetical gene approximately 2.1 kbp upstream of the *Bs4* translational start codon which is predicted to encode a member of the mTERF mitochondrial transcription factor family (Pfam profile PF02536) (Fig. 3A). Thus, the *Bs4* promoter is most likely confined to the 2.1 kbp between *mTERF* and *Bs4*. To define a minimal *Bs4* promoter, we generated progressive 5′ deletions of p3516$_{Bs4}$::*Bs4* and functionally tested the corresponding T-DNA constructs by agro-coinfiltration with p35S::*avrBs4* (Fig. 4). p3516$_{Bs4}$::*Bs4* deletion derivatives containing 2,123, 604, or 302 bp (p2123$_{Bs4}$::*Bs4*, p604$_{Bs4}$::*Bs4*, or p302$_{Bs4}$::*Bs4*, respectively) of *Bs4*-upstream sequence mediated an *avrBs4*-dependent HR. By contrast, 74 or 0 bp (p74$_{Bs4}$::*Bs4* or p0::*Bs4*, respectively) of *Bs4*-upstream sequence in front of *Bs4* did not mediate AvrBs4 recognition. None of the *Bs4* constructs mediated HR when coexpressed with *avrBs1* (Fig. 4), which demonstrates the specificity of the observed reactions. The HRs mediated by p2123$_{Bs4}$::*Bs4*, p604$_{Bs4}$::*Bs4,* or p302$_{Bs4}$::*Bs4* were phenotypically indistinguishable from p3516$_{Bs4}$::*Bs4*-induced responses (Fig. 4). We also studied functionality of all transcriptional fusions to *Bs4* upon co-expression with p35S::*avrBs3* and found identical phenotypes, as observed with p35S::*avrBs4* (data not shown). In summary, we conclude that a 302-bp fragment of *Bs4* 5′ sequence comprises a minimal promoter that is sufficient to promote functionality of a downstream *Bs4* open reading frame (ORF).

To quantify the level of *Bs4* promoter activity, we generated transcriptional fusions to a *gfp:uidA* (green fluorescent protein translationally fused to β-glucuronidase [GUS]) reporter gene and functionally tested the 2,123-bp fragment and its truncations by agroinfiltration into *N. benthamiana*. GUS assays revealed a direct correlation between fragment length and promoter activity (Fig. 5). Furthermore, fragments that promoted AvrBs4 recognition when fused to the *Bs4* ORF (p2123$_{Bs4}$, p604$_{Bs4}$, and p302$_{Bs4}$) consistently generated higher GUS values than p74$_{Bs4}$ (Fig. 5), which did not promote Bs4 function. Although p74$_{Bs4}$ did not promote Bs4 function, the corresponding GUS activities were significantly higher than with the promoterless reporter construct, suggesting that a certain threshold level is crucial to *Bs4*-mediated HR elicitation.

The wild potato *Solanum demissum* contains two genomic regions with sequence homology to the *Bs4* promoter.

To identify *cis*-regulatory elements in the *Bs4* promoter, we extended our study to include the promoters of sequence-related R proteins. However, no promoter data is available for proteins with known function and which closely resemble Bs4, namely potato Y-1 (Vidal et al. 2002) and tobacco N (Whitham et al. 1994). Therefore, we screened the GenBank database for sequences with high sequence identity to the *Bs4* upstream region. BlastN search using the noncoding region as query resulted in identification of two sequences from *S. demissum* (BACs PGEC979H09 and PGEC132D05). Both sequences are located upstream of a putative disease resistance gene and were designated here as *S. demissum-Bs4*-homolog number 1 (*SdBs4H1*) and *SdBs4H2* (GenBank accession numbers AC151803 and AC154033, respectively). The linear order of sequence elements at the tomato *Bs4* locus, consisting of the *mTERF*, the *Bs4* promoter, and the *Bs4* ORF, is conserved in the *S. demissum* BAC sequences (Fig. 3B; Table 1). Sequence identity in the noncoding region was lower compared with the flanking ORFs (Fig. 3B; Table 1). Within the noncoding area, the *S. demissum* sequences showed greater sequence identity to each other than to *L. esculentum* cv. MM. However, the *SdBs4H2* 5′ upstream sequence harbors a 200-bp insertion with respect to *SdBs4H1* promoter and *L. esculentum* cv. MM at a position approximately 300 bp upstream of the Bs4 start codon (Fig. 3B; data not shown). A 302-bp fragment defines the minimal promoter that is sufficient for *Bs4*-mediated AvrBs4 recognition (Fig. 4); therefore, we focused our analysis on this 300-bp genomic stretch. Mapping of *cis*-regulatory elements revealed a putative TATA box at sequence position –35 in the tomato *Bs4* upstream sequence which was not present in the *SdBs4H1* and *SdBs4H2* upstream sequences. CAAT boxes in the minimal 300-bp *Bs4* promoter fragment were predicted only downstream but not upstream of the TATA box (Fig. 3C).

We also performed comparative sequence analysis of the *L. esculentum* cv. MM *Bs4* and the *L. pennellii bs4* 300-bp upstream area. However, these comparisons were uninformative due to extensive (>98%) sequence identity (S. Schornack, K. Peter, and T. Lahaye, *unpublished results*). Furthermore, we searched in *Arabidopsis thaliana* adjacent *mTERF*- and *Bs4*-homologous sequences. However, inspection of *mTERF*-encoding genes did not uncover nearby TIR-NB-LRR-encoding genes in the *Arabidopsis* genome (data not shown).

The fact that *SdBs4H1* and *SdBs4H2* share approximately 86% sequence identity with *Bs4* raises the question of whether these homologs mediate a function similar to that of tomato *Bs4*. However, agroinfiltration of a p35S::*avrBs3* or p35S::*avrBs4* T-DNA does not trigger an HR in *S. demissum* (data not shown), indicating that the *S. demissum Bs4* homologs mediate neither AvrBs4 nor AvrBs3 recognition.

***Bs4* recognition specificity depends upon *avr* expression level.**

Previous studies showed that *Bs4* HR is induced by agroinfiltration of a p35S::*avrBs3* T-DNA but not by *X. campestris* pv. *vesicatoria*-delivered AvrBs3 (Schornack et al. 2004). However, it remained unclear whether quantitative or qualitative alterations in AvrBs3 accounted for the differences in *Bs4*-dependent recognition of AvrBs3. We used transcriptional fu-

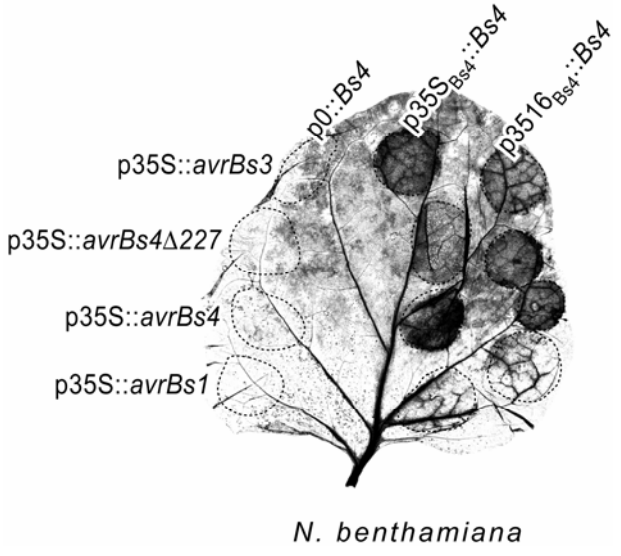

Fig. 2. Functional analysis of the *Bs4* gene under control of different promoters. *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana* was used to study the functionality of different promoter-*Bs4* fusions. The following promoters were transcriptionally fused to the *Bs4* gene: the native *Bs4* (p3516$_{Bs4}$::*Bs4*), the strong 35S (p35S::*Bs4*), and no promoter (p0::*Bs4*). *Agrobacterium* strains carrying one of these *Bs4* constructs were mixed prior to infiltration with equal amounts of a second *Agrobacterium* strain delivering p35S::*AvrBs1,* p35S::*AvrBs4,* p35S::*AvrBs4*Δ227, or p35S::*AvrBs3*, respectively. Dashed lines indicate the infiltrated leaf areas. The leaf was bleached by ethanol treatment for better visualization of the hypersensitive response at 8 days postinfiltration.

sions of *avrBs3* and *avrBs4* to the weak *Bs4* or the strong 35S promoter to study the interdependence of Avr expression levels and recognition specificity. We generated GATEWAY-based binary vectors harboring $p604_{Bs4}$ (pBs4P6) or $p302_{Bs4}$ promoter fragments (pBs4P3) to drive low-level in planta expression of *avrBs3* and *avrBs4*. *Bs4* promoter-driven *avrBs3* and *avrBs4* constructs ($p604_{Bs4}$::*avrBs3* and $p604_{Bs4}$::*avrBs4*) and the corresponding 35S-driven *avr* genes (p35S::*avrBs3* and p35S::*avrBs4*) were agroinfiltrated in *N. benthamiana* and the expression levels of the corresponding proteins were quanti-

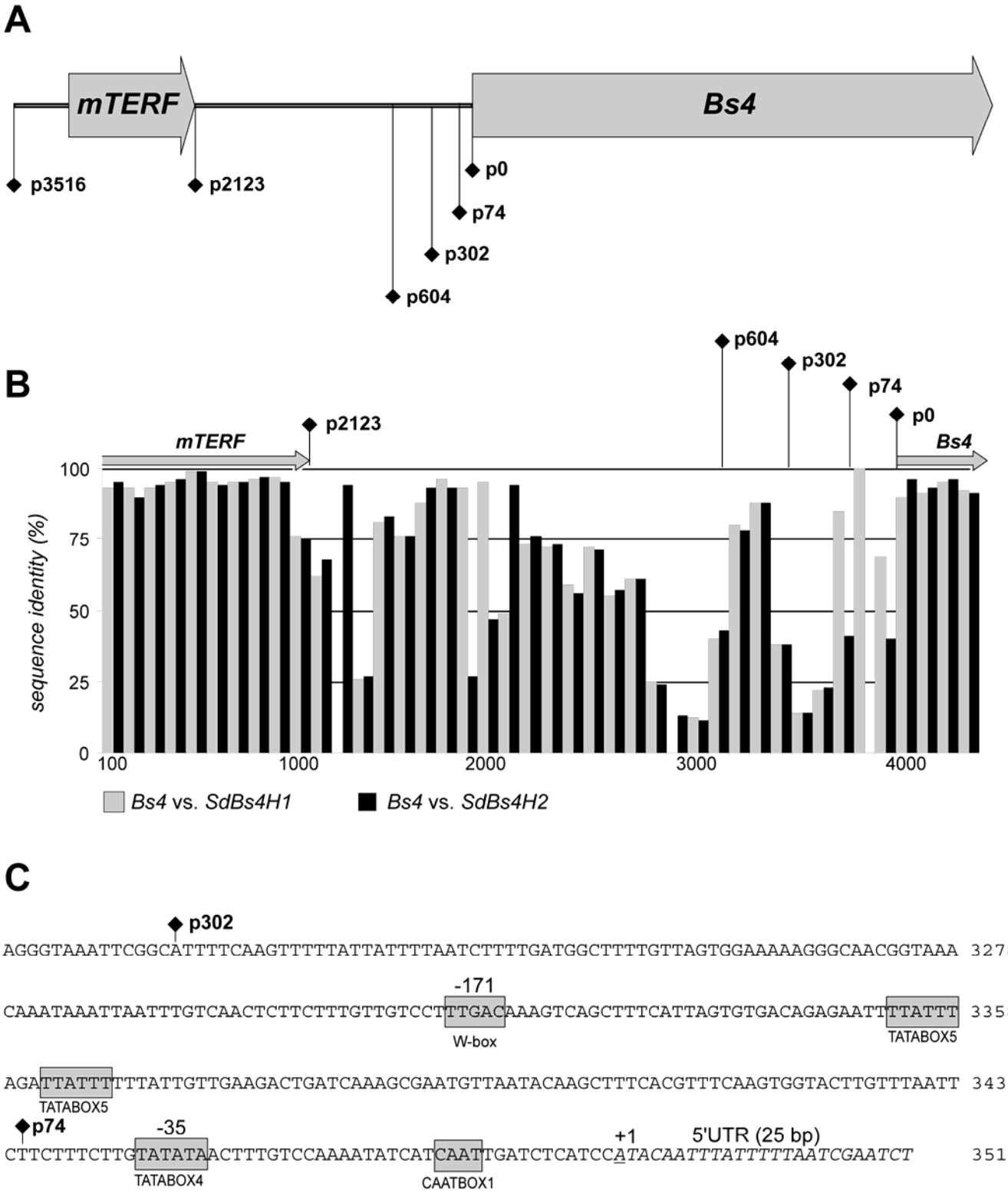

Fig. 3. Analysis of the B*s4* promoter region. **A,** Structure of the *Bs4* upstream region. Black diamonds indicate the 5′ end of different transcriptional fusions to the *Bs4* gene that were functionally tested. The *mTERF* and *Bs4* open reading frames (ORFs) are shown as arrows. **B,** Sliding window sequence comparison of *Lycopersicon esculentum* cv. Moneymaker and corresponding *Solanum demissum* sequences. Window size was 100 bp. Note that this graph covers only parts of the *Bs4* ORF. **C,** Promoter motifs prediction and designation according to the PLACE (Plant *cis*-acting regulatory DNA elements database (Higo et al. 1999). Start of 5′ untranslated region (UTR), putative TATA- (TATABOX4 and TATABOX5), CAAT- (CAATBOX1), and W-boxes (W-box) are indicated.

fied with an AvrBs3-specific antibody (Knoop et al. 1991) that was shown previously to cross-react with AvrBs4 (Ballvora et al. 2001; Bonas et al. 1993). Upon transfection of p35S::*avrBs3* and p35S::*avrBs4,* we were able to detect AvrBs3- and AvrBs4-specific bands in immunoblot analysis. By contrast, *Bs4* promoter-driven Avr levels were not detectable by Western analysis (Fig. 6). Thus, immunoblot analysis demonstrates that 35S-driven *avr* constructs in planta generate substantially higher protein levels than the *Bs4*-promoter driven constructs. To study the impact of Avr abundance on recognition specificity, *Bs4*-promoter and 35S-driven *avr* genes subsequently were agroinfiltrated together with p3516$_{Bs4}$::*Bs4* in *N. benthamiana.* We observed HR upon agro-coinfiltration of p3516$_{Bs4}$::*Bs4* with p35S::*avrBs4*, p604$_{Bs4}$::*avrBs4,* and p302$_{Bs4}$::*avrBs4,* but not with p0::*avrBs4* (Fig. 7A). Remarkably, *avrBs3* containing T-DNA constructs (p35S::*avrBs3*, p604$_{Bs4}$::*avrBs3,* and p302$_{Bs4}$::*avrBs3*) triggered a *Bs4*-dependent HR only when driven by the strong 35S promoter, but not when fused to *Bs4* promoter fragments (p604$_{Bs4}$ and p302$_{Bs4}$) (Fig. 7A). This suggests that the p35S::*avrBs3*-triggered and *Bs4*-mediated HR is due to higher expression levels of AvrBs3. We also tested whether overexpressed *Bs4* (p35S::*Bs4*) would allow detection of low-level expressed *avrBs3* (p604$_{Bs4}$::*avrBs3* and p302$_{Bs4}$::*avrBs3*). However, in *N. benthamiana,* p3516$_{Bs4}$::*Bs4* and p35S::*Bs4* yielded identical reactions when coexpressed with 35S- or *Bs4*-promoter driven *avrBs3* and *avrBs4*, respectively (data not shown). The same results also were observed upon agroinfiltration of *Bs4*-resistant tomato genotypes (Fig. 7B). Thus, *Bs4*-resistant tomato and *N. benthamiana* plants agroinfiltrated with p3516$_{Bs4}$::*Bs4* or p35S::*Bs4* constructs showed identical reactions with all tested *avr* constructs (Fig. 7A and B; data not shown). None of the agroinfiltrated *avr*-constructs triggered HR in the tomato *bs4* genotype MM^{bs4}-BC4 (Ballvora et al. 2001; data not shown), thus confirming *Bs4* dependency of the observed reactions. In summary, only *avrBs4* but not *avrBs3* triggered a *Bs4*-dependent HR when expressed from the weak *Bs4* promoter.

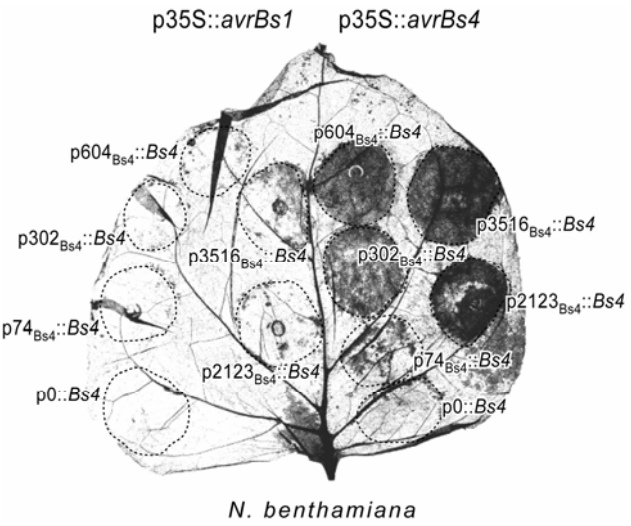

Fig. 4. Functional analysis of the Bs4 gene under transcriptional control of the native promoter. In all, 3,516 bp of *Bs4* upstream sequence (p3516$_{Bs4}$::*Bs4*) and truncations thereof (p2123$_{Bs4}$::*Bs4*, p604$_{Bs4}$::*Bs4*, p302$_{Bs4}$::*Bs4*, p74$_{Bs4}$::*Bs4*, and p0::*Bs4*) were transcriptionally fused to *Bs4* and tested for function. *Agrobacterium* strains harboring the different *Bs4* fusion constructs were mixed prior infiltration with *Agrobacterium* strains harboring p35S::*avrBs1* (left side of leaf) or p35S::*avrBs4* (right side of leaf). At 8 days postinfiltration, the leaf was cleared in ethanol.

35S-driven expression of *avrBs4* in planta does not trigger *Bs3* resistance.

Previously, we demonstrated that pepper *Bs3* mediates recognition of AvrBs3- but not AvrBs4-delivering *X. campestris* pv. *vesicatoria* strains (Ballvora et al. 2001). Here, we used agroinfiltration assays to study the interdependency of *avr* expression levels and recognition specificity in *Bs3*-mediated resistance. Promoterless *avrBs3* (p0::*avrBs3*) failed to trigger the HR, whereas all other transcriptional fusions to *avrBs3* (p35S::*avrBs3*, p604$_{Bs4}$::*avrBs3,* and p302$_{Bs4}$::*avrBs3*) triggered HR in pepper *Bs3* plants (genotype ECW-30R), demonstrating functionality of these constructs. In contrast, pepper *Bs3* plants did not show HR upon *Bs4* promoter-driven nor

Table 1. DNA sequence identities between tomato *Bs4* and *Bs4* homologous sequences of *Solanum demissum*

***Bs4* homologs analyzed**	**Identity (%)**		
	mTERF	**Noncoding region**	***R* gene exon1**
Bs4-SdBs4H1	95.1	73.2	92.9
Bs4-SdBs4H2	95.0	73.3	94.5
SdBs4H1-SdBs4H2	97.9	90.3	94.4

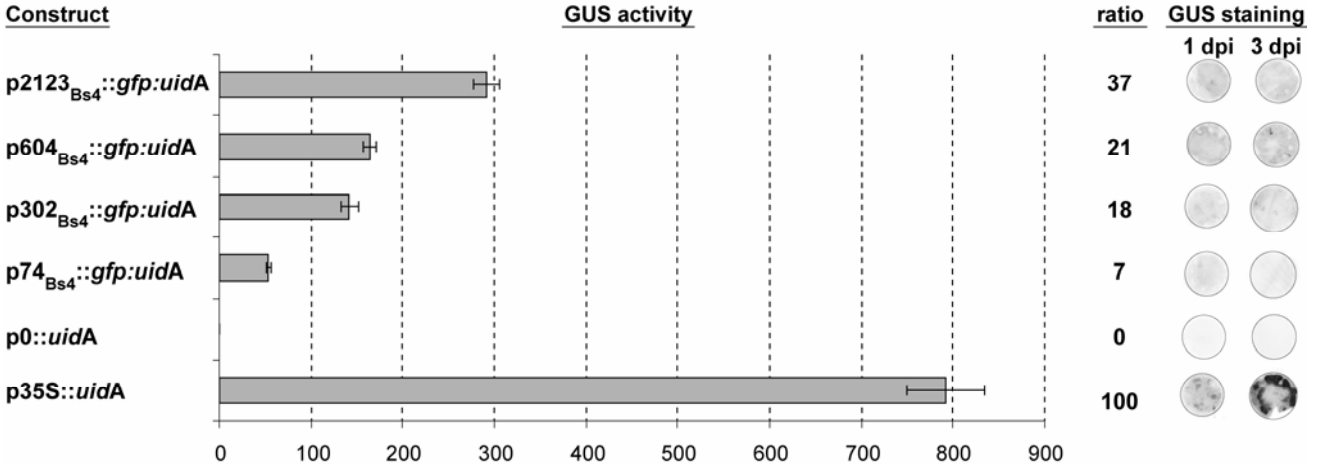

Fig. 5. Promoter activity of *Bs4* upstream sequences. Fragments of 2,123, 604, 302, and 74 bp (p2,123$_{Bs4}$::*gfp*:*uid*A, p604$_{Bs4}$::*gfp*:*uid*A, p302$_{Bs4}$::*gfp*:*uid*A, and p74$_{Bs4}$::*gfp*:*uid*A, respectively) of *Bs4* 5′ sequence were fused to the *gfp:uidA* reporter gene, agroinfiltrated into *Nicotiana benthamiana,* and analyzed for β-glucuronidase (GUS) activity. p0::*uidA* is a promoterless construct. p35S::*uid*A is a 35S-driven *uid*A reporter. Activities (pmol of methylumbelliferone per milligrams of protein per hour) are the mean values of two separate agroinfiltrations. GUS activities are defined relative to the activity of the p35S::GUS construct (100%). Stained infiltration sites at 1 and 3 days postinfiltration (dpi) are shown on the far right side of the figure.

35S-driven expression of *avrBs4* (Fig. 7C). None of the *avrBs3* and *avrBs4* constructs triggered HR in pepper *bs3* plants (genotype ECW) (data not shown), confirming the *Bs3* dependency of recognition. Taken together, pepper *Bs3* mediates specifically recognition of AvrBs3 and not the sequence-related AvrBs4 protein even if *avrBs4* was expressed via the strong 35S promoter.

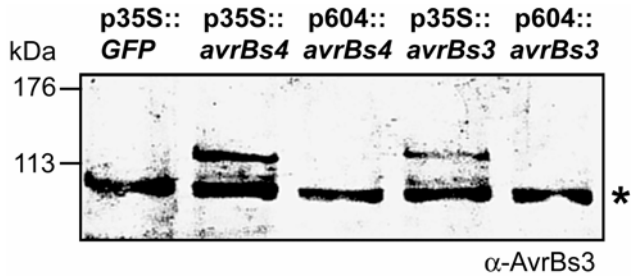

Fig. 6. In planta expression levels of AvrBs3 and AvrBs4 upon agroinfiltation of corresponding 35S or *Bs4* promoter-driven T-DNA constructs. Agroinfiltrated *Nicotiana benthamiana* leaf patches were harvested 36 h postinfiltration and subjected to immunoblot analysis using anti-AvrBs3 antiserum which also cross-reacts with AvrBs4. An asterisk indicates a background band that served as a loading control.

Low-level in planta expression of the *avrBs3*-like *hax3* and *hax4* genes triggers a *Bs4*-dependent HR.

As described above, only *avrBs4* but not *avrBs3* is capable of triggering a *Bs4*-dependent HR when expressed from the weak *Bs4* promoter. To determine whether *avrBs4* is the only *avr* gene that can trigger the *Bs4* HR when driven by the weak *Bs4* promoter, we extended our analysis to the *avrBs3*-like *hax3* and *hax4* genes which recently were isolated from the Brassicaceae pathogen *X. campestris* pv. *armoraciae* (Kay et al. 2005). The overall sequence identity of Hax3 and Hax4 with respect to AvrBs4 is 96.1 and 96.2%, respectively. Previous analyses showed that Hax3 and Hax4 trigger a *Bs4*-dependent HR when they are (i) delivered by the *X. campestris* pv. *vesicatoria* TTSS (Kay et al. 2005) or (ii) if the corresponding genes are expressed in planta under control of the 35S promoter (Fig. 7D) (Kay et al. 2005). We expressed *hax3* and *hax4* under control of the strong 35S promoter or the weak $p604_{Bs4}$ and $p302_{Bs4}$ promoter fragments and agroinfiltrated these together with $p3516_{Bs4}$::*Bs4*. We found that $p3516_{Bs4}$::*Bs4* mediated an HR when codelivered

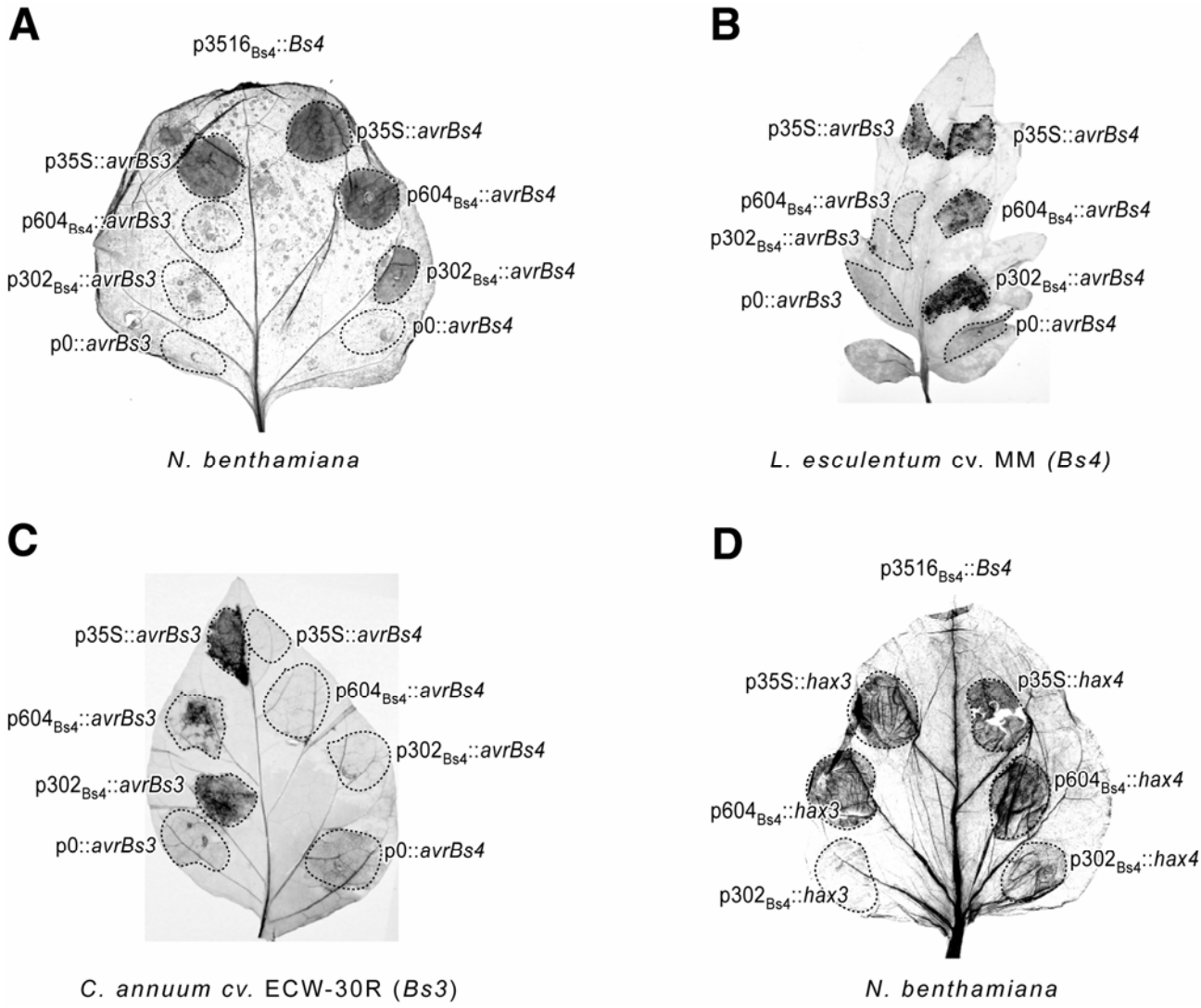

Fig. 7. Functional analysis of the tomato *Bs4* and pepper *Bs3* genes upon 35S or *Bs4* promoter-driven expression of *avrBs3*-like genes. *Agrobacterium* strains carrying T-DNAs with *avrBs3*-like genes under control of different promoters were infiltrated into **A** and **D,** *Nicotiana benthamiana,* **B,** *Bs4*-resistant tomato (*Lycopersicon esculentum* cv. Moneymaker), and **C,** *Bs3*-resistant pepper (*Capsicum annuum* cv. ECW-30R). For infiltration of *N. benthamiana,* avirulence (*avr*)-expressing *Agrobacterium* strains were mixed with equal amounts of *Agrobacterium* carrying $p3516_{Bs4}$::*Bs4*. Tomato and pepper leaves were harvested 3 days postinfiltration (dpi) and *N. benthamiana* leaves 6 dpi. All leaves were cleared in ethanol for better visualization of the hypersensitive response.

with nearly all constructs, namely p35S::*hax3*, p35S::*hax4*, $p604_{Bs4}$::*hax3*, $p604_{Bs4}$::*hax4*, and $p302_{Bs4}$::*hax4* (Fig. 7D). Only the $p302_{Bs4}$::*hax3* T-DNA did not trigger *Bs4*-dependent HR. Given that GUS assays showed minor but significant differences between the promoter strength of $p302_{Bs4}$ and $p604_{Bs4}$ (Fig. 5), our data indicate that subtle differences in expression levels of highly homologous Avr proteins are crucial in *Bs4*-mediated recognition.

DISCUSSION

Tomato *Bs4* and tobacco *N* genes differ in their regulation of transcript abundance.

Analysis of the tobacco *N* gene, which encodes a protein that is 54% sequence identical to tomato Bs4, revealed that *N* transcript levels increased approximately 40-fold upon delivery of its cognate viral Avr protein (Levy et al. 2004). By contrast, we showed that *Bs4* transcript levels are not elevated upon *Xanthomonas* spp.-mediated delivery of AvrBs4, indicating constitutive expression of *Bs4*. As for any promoter study, it is not excluded that *Bs4* transcript levels increase transiently or can be induced by a yet unknown stimulus. However, comparative studies of *Bs4* under control of the constitutive 35S promoter (p35S::*Bs4*) or its own promoter ($p3516_{Bs4}$::*Bs4*) in transient assays (Fig. 2) and stable transformants (Schornack et al. 2004) did not uncover any differences with respect to the timing or intensity of the *Bs4*-mediated HR. In contrast, the tobacco *N* gene is not functional when constitutively expressed from the 35S promoter (Dinesh-Kumar and Baker 2000). Thus, the regulation and importance of transcript abundance seem to be fundamentally different in tobacco *N*- and tomato *Bs4*-mediated resistance.

Threshold levels are crucial for the function of Bs4 and other NB-LRR type R proteins.

Progressive 5′ deletions of the *Bs4* upstream sequence resulted in the identification of a 302-bp fragment that was sufficient to promote functionality of *Bs4* (Fig. 4). In contrast, a 74-bp fragment was not sufficient, although it gave detectable GUS reporter activity (Fig. 5). This suggests that Bs4 expression must reach a certain threshold level in order to fulfill its function in pathogen resistance. Is this finding unique to Bs4 or a more general feature of NB-LRR proteins? Mutations in *HSP90*, a gene that encodes a molecular chaperone (Sangster and Queitsch, 2005), result in significantly decreased levels of the *Arabidopsis* NB-LRR protein RPM1 (Hubert et al. 2003), and silencing of *HSP90* in *N. benthamiana* reduces the levels of the potato NB-LRR protein Rx (Lu et al. 2003). In both studies, a decrease in NB-LRR protein abundance was linked to a loss of resistance. Further evidence comes from comparative analysis of the 92% identical barley Mla1 and barley Mla6 NB-LRR proteins (Halterman et al. 2001; Zhou et al. 2001). Mutations in *Rar1*, which has been postulated to encode a chaperone-like protein (Shirasu and Schulze-Lefert 2003), reduce abundance of both Mla isoforms, but compromises only the Mla6-mediated resistance and not the resistance that is mediated by the fourfold more abundant Mla1 protein (Bieri et al. 2004). Thus, the barley Mla proteins may represent another example of NB-LRR proteins that require a certain threshold level to trigger a resistance reaction. Therefore, threshold levels may be crucial not only to Bs4 but also for the activity of other NB-LRR proteins.

Bs4 promoter-fragments direct low in planta expression levels.

One of our aims was to establish a system that allows analysis of recognition specificity in the context of varying amounts of Avr protein. Strong, constitutive promoters such as the 35S promoter have been used extensively to drive transgene expression in plants, whereas complementary weak promoters only rarely have been implemented into commonly used vector backbones; one notable exception is described by Chen and associates (2000). The *Bs4* promoter is constitutively active but gives approximately 100-fold lower transcript levels than the 35S promoter (Schornack et al. 2004). Thus, the *Bs4* promoter provides a suitable low-level expression alternative to 35S-driven expression. Therefore, we engineered GATEWAY-based binary vectors in which the T-DNA is under transcriptional control of a 604- or 302-bp *Bs4* promoter fragment. Agroinfiltration-based analysis of various *avr-R* combinations showed that both *Bs4* promoter-driven binary constructs are functional in tomato, *N. benthamiana,* and pepper (Fig. 7). We also fused these two *Bs4* upstream sequences to a *uidA* reporter and demonstrated functionality in the solanaceous species pepper, tomato, *N. benthamiana*, *Nicandra physalodes*, and a *Physalis* sp., and the nonsolanaceous species *Lactuca sativa* (data not shown). Hence, the *Bs4* promoter-based, low-level expression vectors are applicable to a broad range of plant species.

Although we used promoters of different strengths, others have applied agroinfiltration dilution series to study *R* gene function in the context of varying amounts of Avr protein (Van der Hoorn et al. 2000). We anticipate that a particular dilution of an *Agrobacterium* strain carrying a 35S construct may generate the same total amount of protein in a tissue patch as in the case of a *Bs4* promoter construct. However, on the single-cell level, the situation will be quite different. Reduced expression levels in titration experiments most likely are due to a patchwork of transformed and nontransformed plant cells. In contrast, our experiments with GUS-reporter assays show that the low-level *Bs4* promoter results in homogenously transformed tissue (data not shown). Consequently, the Avr threshold levels that are crucial to HR elicitation by a given R protein may be different when determined by agroinfiltration dilution series or promoters of different strengths.

Inducible promoters that act in a dose-dependent manner (Aoyama and Chua 1997; McNellis et al. 1998; Zuo et al. 2000) are another option to modulate transgene expression levels and should allow nongradual expressional changes. However, when using inducible promoters, it might be difficult to reproducibly generate identical and homogenous concentrations of the inducer. Furthermore, most inducible systems will cause only a transient increase of a given transgene in contrast to the constitutive low-level *Bs4* promoter.

In summary, the GATEWAY-compatible binary expression vectors carrying a *Bs4* promoter-driven T-DNA presented here provide a new tool to generate low-level in planta expression. In this context, one also might question whether $p604_{Bs4}$, $p302_{Bs4}$, or 35S promoter-driven in planta expression resemble most closely the amounts of protein injected by the bacterial TTSS system. The fact that *Bs4*-mediated recognition of *avrBs3*-like genes is expression-level dependent provides a basis for such estimates. For example, 35S-driven in planta expression of *avrBs3* but not infection of *avrBs3*-containing *X. campestris* pv. *vesicatoria* strains triggers *Bs4* resistance (Schornack et al. 2004), suggesting that the *X. campestris* pv. *vesicatoria* TTSS translocates less AvrBs3 than produced upon agroinfiltration of corresponding 35S constructs. In a reciprocal situation, *hax3*-containing *X. campestris* pv. *vesicatoria* strains but not a $p302_{Bs4}$-driven *hax3* transgene triggers *Bs4* HR (Fig. 7D), suggesting that *X. campestris* pv. *vesicatoria*-delivered amounts of Hax3 exceed the levels that are produced upon $p302_{Bs4}$-driven expression. Finally, $p604_{Bs4}$-driven expression of *avrBs3*, *avrBs4*, *hax3,* and *hax4* resulted in identical reactions, as observed upon in-

fection with *X. campestris* pv. *vesicatoria* strains that contain the corresponding *avr* gene. Together, these findings suggest that $p604_{Bs4}$-driven *avr* expression and TTSS-mediated Avr delivery result in similar amounts of Avr proteins in planta, whereas the 35S and $p302_{Bs4}$ promoter direct the generation of higher and lower Avr quantities, respectively, compared with TTSS-mediated delivery of Avr proteins.

Tomato *Bs4* but not pepper *Bs3* exerts an *avr* dose-dependent mode of recognition.

The pepper *Bs3* gene mediates HR only upon delivery of a p35S::*avrBs3* T-DNA and not upon 35S-driven expression of any other *avrBs3*-like gene, such as *avrBs4*, *hax3,* or *hax4* (Ballvora et al. 2001; Kay et al. 2005). In contrast, a *Bs4*-dependent HR is triggered not only upon agroinfiltration of a p35S::*avrBs4* T-DNA but also upon 35S-driven expression of *avrBs3* (Schornack et al. 2004), *hax3,* and *hax4* (Kay et al. 2005), which share 96.6, 96.1, and 96.2% identity, respectively, with *avrBs4* at the amino acid level. Thus, overexpression of *avrBs3*-like genes causes a loss of recognition specificity in *Bs4*- but not in *Bs3*-mediated perception. However, 35S-driven expression of *hax2*, another *avrBs3*-like gene that is 92% identical with *avrBs4* at the amino acid level, does not trigger a *Bs4*-dependent HR (Kay et al. 2005), indicating that 35S expression causes only a partial but not complete loss of recognition specificity.

Here, we extended our studies of the recognition specificity and determined whether *avrBs4* and the highly similar *avrBs3*, *hax3,* and *hax4* genes retain elicitor activity when expressed from the weak $p604_{Bs4}$ or the even weaker $p302_{Bs4}$ promoter. In contrast to the previously observed loss of *Bs4* recognition specificity upon 35S-driven *avrBs3* expression, *Bs4* retains its specificity if *avrBs3* is expressed under transcriptional control of the weak $p604_{Bs4}$ or $p302_{Bs4}$ promoters (Fig. 7A and B). AvrBs3 is more similar to AvrBs4 than to Hax3 and Hax4. Thus, one might expect that Hax3 and Hax4 would not trigger a *Bs4*-dependent HR when expressed under control of the weak $p604_{Bs4}$ promoter. However, *hax3* and *hax4* triggered a *Bs4*-dependent HR, even when expressed under control of the weak $p604_{Bs4}$ promoter (Fig. 7D). These data indicate that overall sequence homology to AvrBs4 is not the only factor affecting elicitor activity of a given AvrBs3-like protein. Thus, we speculate that short peptide motifs may play a crucial role in *Bs4*-mediated recognition.

Because *avrBs4*, *hax3,* and *hax4* triggered a *Bs4*-dependent HR when expressed from the weak $p604_{Bs4}$ promoter, it raised the question of whether these are, to the same extent, elicitors of *Bs4* resistance. Transcriptional fusions of the *hax3* gene to $p302_{Bs4}$, a fragment that results in even lower expression levels than the $p604_{Bs4}$ promoter, revealed that *hax3* no longer was capable of triggering a *Bs4*-dependent HR, whereas *avrBs4* and *hax4* retained their elicitor activity. Availability of further promoters that direct even lower expression levels than $p302_{Bs4}$ possibly would allow further hierarchical ordering of the elicitor activity of *avrBs4* and *hax4* in the context of *Bs4* recognition. However, it also seems possible that other not-yet-identified *avrBs3-like* genes have even stronger elicitor activity in *Bs4*-resistant plants than *avrBs4* and *hax4*.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Escherichia coli Top10 and DB3.1 (Invitrogen GmbH, Karlsruhe, Germany) were cultivated at 37ºC and *Agrobacterium tumefaciens* GV3101 (Van Larebeke et al. 1974) at 30ºC in lysogeny broth (LB) (Bertani 1951). Transconjugants of *X. campestris* pv. *vesicatoria* 75-3 that carries pDS200F (*avrBs4*) (Schornack et al. 2004) were grown on NYG agar plates as described (Daniels et al. 1984).

Plant material and infiltration tests.

Plants were grown as described (Kay et al. 2005) with the exception that all plants were kept in the greenhouse. To determine *Bs4* transcript levels, tomato leaves were infiltrated with a suspension of *X. campestris* pv. *vesicatoria* 75-3 carrying pDS200F (*avrBs4*) in 10 mM $MgCl_2$ (optical density at 600 nm [OD_{600}] of 0.4) and vacuum infiltrated into tomato leaves. Coinfiltrations of *avr*- and *Bs4*-harboring *Agrobacterium* strains were carried out in *N. benthamiana.* For *Bs4* specificity tests, *Bs4* tomato (*L. esculentum* cv. MM), *Bs3* pepper (*C. annuum* ECW-30R), and *bs3* pepper (*C. annuum* cv. ECW) plants were used. *Agrobacterium* strains were grown overnight in LB medium, resuspended in inoculation medium (10 mM $MgCl_2$, 5 mM MES, pH 5.3, 150 µM acetosyringone) adjusted to an OD_{600} of 0.8 and mixed if necessary prior to infiltration into the abaxial side of leaves using a blunt syringe. Leaves were harvested 3 to 8 days postinfiltration. For better visualization of the HR, leaves were cleared by incubation in ethanol at 60ºC and were dried and photographed.

Immunoblot analysis.

Protein extracts from leaf tissue were prepared by grinding five leaf discs (1 cm in diameter) in liquid nitrogen. Extraction buffer (400 µl; 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol [DTT]), plant protease inhibitor cocktail (Roche, Mannheim, Germany) and 2% polyvinylpolypyrrolidone were added and extracts were spun at 12,000 × *g* at 4ºC for 15 min. Immunoblots were reacted with polyclonal anti-AvrBs3 antibody and detected as described (Kay et al. 2005).

Binary vector constructs containing *Bs4*.

Southern hybridization of digested cosmid T2-2 (Schornack et al. 2004) with a probe derived from *Bs4* sequence of exon1 resulted in an approximately 4-kb *Bam*H1 fragment. *Bam*H1-digested cosmid T2-2 was *Bam*H1 subcloned in pBSKII+ (Stratagene, Heidelberg, Germany) and screened by PCR. Positive clones were sequenced using the BigDye Terminator Kit (Applied Biosystems GmbH, Weiterstadt, Germany) and analyzed using Sequencher 4.0 (GeneCodes Corp., Ann Arbor, MI, U.S.A.). The resulting sequence (3,516 bp) showed 100% identity to the *Bs4* upstream sequence (AY438027). The *Bam*H1 fragment encompassing exon 1 and the *Bs4* promoter was used to substitute a shorter *Bam*H1 fragment in pVTSB1 (Schornack et al. 2004) resulting in $p3516_{Bs4}$::*Bs4.*

Promoter fragments harboring 2,123, 604, 302, and 74 bp of *Bs4* upstream sequence were generated by PCR using $p3516_{Bs4}$::*Bs4* as template and primers Bs4-PRA03, Bs4-PRA06, Bs4-PRA08, Bs4-PRA07, and Bs4-PRA09 (sequences available upon request) that allow amplification with (Bs4ORF-rev) or without *Bs4* ORF (Bs4-PRB01), respectively. PCR amplicons were cloned into pENTR/D-TOPO (Invitrogen GmbH) and transferred to promoterless binary vector pBGWFS7 (Karimi et al. 2002) using GATEWAY recombination (Invitrogen GmbH) to obtain *Bs4* ORF containing constructs $p2123_{Bs4}$::*Bs4*, $p604_{Bs4}$::*Bs4*, $p302_{Bs4}$::*Bs4*, $p74_{Bs4}$::*Bs4,* and p0::*Bs4*. Constructs $p2123_{Bs4}$::*gfp:uidA*, $p604_{Bs4}$::*gfp:uidA*, $p302_{Bs4}$::*gfp:uidA*, $p74_{Bs4}$::*gfp:uidA,* and $p0_{Bs4}$::*gfp:uidA* lack the *Bs4* ORF.

Binary vector constructs containing *avr* genes.

For comparison of 35S versus $p3516_{Bs4}$::*Bs4* and delimitation of *Bs4* promoter, we employed the following constructs: p35S::*avrBs1* (Ronald and Staskawicz 1988), p35S::*avrBs4*

(pVS200F) (Ballvora et al. 2001), p35S::*avrBs4*Δ227 (pVS227) (Schornack et al. 2004), and p35S::*avrBs3* (Van den Ackerveken et al. 1996). For *avr* fusions to *Bs4* promoter fragments, we replaced the 35S promoter of pGWB20 (T. Nakagawa, Shimane University, Izumo, Japan) by a *Hin*dIII-*Sma*I-*Xba*I cassette. Subsequently, PCR-amplified fragments of 604 and 302 bp of *Bs4* promoter sequence were cloned into *Sma*I and *Xba*I sites using restriction enzyme recognition sites introduced into the primers to yield pBs4P6 and pBs4P3.

avrBs4, *avrBs3*, *hax3,* and *hax4* were transferred into both vectors using GATEWAY recombination and entry clones pENTR256, pENTR356 (Gürlebeck et al. 2005), pAGH3, and pAGH4 (Kay et al. 2005), respectively. pENTR256 was generated by substituting the *Stu*I-*Age*I-flanked *avrBs3* repeat region of pENTR356 by the corresponding *avrBs4* fragment.

Sequence analysis.

The DNA sequence of the 3.5-kb *Bs4* ORF upstream region was determined using the dideoxy-termination method (Sanger et al. 1977) and vector- and insert-specific primers. Sequences were run on an ABI PRISM 310 genetic analyzer and processed using Sequencher 4.1.2 (GeneCodes Corp.). Annotations and homologous sequences were obtained from National Center for Biotechnology Information (NCBI) GenBank database using BLASTX and BLASTN (Altschul et al. 1990). Sequences were aligned and sequence distances were calculated using MegAlign Jotun Hein method (DNASTAR, Madison, WI, U.S.A.) or Softberry MaliN algorithm (Softberry Inc.).

Genome-wide virtual mapping in *Arabidopsis* with the m*TERF* as query was carried out using the *A. thaliana* section of the NCBI MapViewer. The 35 identified *mTERF* genes were inspected for the presence of adjacent TIR-NB-LRR encoding sequences. Putative *cis*-regulatory sequence elements like the TATA-, W-, and CAAT- box elements were identified and designated according to the PLACE (Plant *cis*-acting regulatory DNA elements) database (Higo et al. 1999).

GUS activity measurements.

GUS measurements were carried out according to Jefferson and associates (1987) with the following modifications. For qualitative GUS staining, leaf discs were immersed in GUS staining solution (10 mM sodium phosphate, pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.1% 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 1 mM potassium ferricyanide, and 1 mM potassium ferrocyanide), incubated at 37ºC for 24 h and then cleared in ethanol.

For quantitative GUS measurements, two leaf discs (1 cm in diameter) per sample were ground in liquid nitrogen. Proteins were extracted using 150 µl of GUS extraction buffer (50 mM sodium phosphate, pH 7, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton-X100, 0.1% sodium dodecyl sulfate). After centrifugation at full speed and 4ºC in a tabletop centrifuge, 100 µl of supernatant was collected. Proteins were quantified by Bradford assay (BioRad, Hercules, CA, U.S.A.) and diluted for equal protein concentrations. For the fluorometric assay, 90 µl of assay buffer (GUS extraction buffer supplied with 5 mM 4-methyl-umbelliferyl β-D-glucuronide) was mixed with 10 µl of sample and incubated for 60 min at 37ºC. Reaction was stopped by adding 900 µl of 0.2 M sodium carbonate, pH 9.5. Measurements were done in a plate reader at 360 nm (excitation) and 465 nm (emission) using 4-methyl-umbelliferon dilutions as standard.

Quantitative real-time PCR.

Plants of the tomato cultivar *L. esculentum* cv. MM were infiltrated with either *X. campestris* pv. *vesicatoria* transconjugants of strain 75-3 carrying pDS200F (*avrBs4*) (Schornack et al. 2004) at an OD_{600} of 0.4 or with infiltration medium. Two leaf discs (1 cm in diameter) of two biological replicates were harvested at 0, 20, and 44 hpi and frozen in liquid nitrogen. RNA was extracted using the RNAeasy plant miniprep kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Subsequently, 2.5 µg of cDNA was synthesized by reverse transcription using the Revert Aid First Strand Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Real-time PCR was carried out on an iCycler (Bio-Rad Laboratories, München, Germany) with a reaction mixture containing SYBR Green as the fluorescent dye and 2 µl of template cDNA in dilutions of 1:5, 1:10, and 1:20. Each dilution was replicated twice, resulting in six technical replicates. Primer sequences and the PCR profile are available upon request. Amplicons were subjected to melting curve analysis (60 to 95ºC, 0.5ºC increments). Tomato EF1α (GenBank accession number X53043) was used as constitutive standard. The level of *Bs4* cDNA was normalized using the level of EF1α cDNA as described in the ABI user bulletin 2.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Softberry Inc.: www.softberry.com
NCBI MapViewer: www.ncbi.nlm.nih.gov/mapview
PLACE database: www.dna.affrc.go.jp/htdocs/PLACE

2.3.3 Zusätzliche Ergebnisse

2.3.3.1. Bs4- und AvrBs4-Strukturvorhersagen über *in silico* 3D-Modellierung

Bs4 ist in der Lage, neben AvrBs4 auch die Erkennung anderer AvrBs3-ähnlicher Proteine in Abhängigkeit von ihrem Expressionsniveau zu vermitteln (siehe Manuskript 2). Außerdem induzieren auch stark verkürzte AvrBs4-Derivate, denen NLSs und AD fehlen, eine Bs4-HR (siehe Manuskript 1). Die Daten sprechen für die Erkennung einer konservierten Avr-Struktur, möglicherweise über direkte Interaktion von AvrBs4-Fragmenten mit Bs4-Domänen. Um kritische AS-Reste gezielt experimentell testen zu können, ist die 3D-Struktur beider Partner sehr hilfreich. Da bislang keine Kristallstruktur von AvrBs3-ähnlichen Proteinen oder NB-LRR-Proteinen verfügbar ist, wurden über Sequenz- bzw. Sekundärstruktur-Homologien zu bereits kristallisierten Proteinen 3D-Modelle abgeleitet. Dazu wurde Bs4 in einzelne Domänen unterteilt. Über die Internet-Server 3D-Jury (www.bioinfo.pl, Ginalski *et al.*, 2003) und PHYRE (www.sbg.bio.ic.ac.uk/phyre/) wurden für die Bs4-Domänen und für AvrBs4 Strukturmodelle generiert.

2.3.3.2. 3D-Modell der TIR-Domäne von Bs4

Das Bs4-TIR-Modell besteht aus einem von α-Helices umgebenen zentralen β-Blatt und zeigte strukturelle Übereinstimmungen zur TIR-Domäne des menschlichen Toll-like-Rezeptor1 (pdb-Datenbankeintrag: 1fyva, Bustin, ; pdb-Datenbankeintrag: 1fyva, Xu *et al.*, 2000). Im Modell wird deutlich, dass AS-Reste, die beim TIR-NB-LRR Protein N aus Tabak als kritisch für die Funktion identifiziert wurden (Dinesh-Kumar *et al.*, 2000) im Bs4-TIR-Modell alle auf der gleichen Seite der globulären Struktur positioniert sind, an

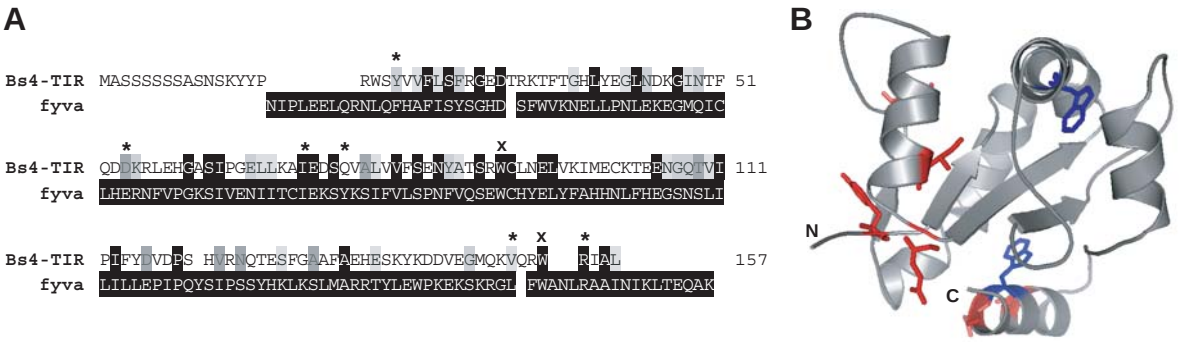

Abb. 9. 3D-Modell der Bs4-TIR-Domäne. (A) Sequenzvergleich des Bs4-TIR zur TIR-Domäne von TLR1 (pdb-Eintrag: 1fyva). Zu TLR1 sequenzidentische AS-Reste sind schwarz unterlegt, ähnliche AS-Reste durch verschieden starke Schattierung. AS-Reste, deren Mutation zum Verlust (*) bzw. zu dominant negativen Effekten (x) der Tabak-N-Protein-Funktion führen, sind über der Sequenz angegeben. **(B)** 3D-Modell des Bs4-TIR auf der Basis von TLR1. Kritische AS-Reste im N Protein aus Tabak sind als rote (Funktionsverlust) bzw. blaue (dominant negativ) Seitenketten hervorgehoben. N, N-Terminus; C, C-Terminus.

der auch die Verbindung zum NB (C-Terminus) liegt (Abb. 9B). Die zwei Tryptophan-Reste deren Mutation im N-Protein zu dominant negativen Effekten führten, zeigen ins Innere des Bs4-TIR. Die Auswirkungen solcher Mutationen auf die Bs4-Funktion müssen aber noch geprüft werden.

2.3.3.3. Die NB-Domäne von Bs4 weist eine ARC3-ähnliche Region auf

Sequenzvergleiche und 3D-Modelle der NB-Domäne anderer NB-LRR-Proteine haben strukturelle Ähnlichkeiten zu APAF1 (*Apoptotic protease-activating factor1*) offengelegt (Van der Biezen und Jones, 1998a; Riedl *et al.*, 2005; Albrecht und Takken, 2006). Deshalb wurde diese Kristallstruktur auch für ein 3D-Modell der Bs4-NB-Domäne verwendet. Der Bs4-NB besteht aus einer N-terminalen AAA+ATPase mit den konservierten Kinase1a, Kinase2 und Kinase3a-Motiven, gefolgt von ARC1, ARC2, und ARC3 (Abb. 10). Die ATPase-Domäne enthält ein β-Blatt, welches zwischen α-Helix-Bündeln gepackt liegt (Sandwich-Struktur, Abb. 10B). ARC1 und ARC3 bestehen ausschließlich aus α-Helices, während ARC2 eine *winged helix*-Struktur aus α-Helix- und β-Strängen ausbildet. Die ARC-Domänen stehen der ATPase-Domäne gegenüber. Für APAF1 ist bekannt, dass ATP zwischen der ATPase und den ARC-Regionen gebunden wird (Riedl *et al.*, 2005). In Übereinstimmung damit konnte ATP-, bzw. ADP-Bindung und Hydrolyse für die NB-LRR-Proteine I-2 und Mi-1 gezeigt werden (Tameling *et al.*, 2002). AS-Reste der ATPase und des ARC2, die an der ATP-Bindung und Hydrolyse beteiligt sind, sind auch im Bs4-NB konserviert und weisen ins Innere zur ATP-Bindestelle (Abb. 10B). Diese Informationen bilden den Ausgangspunkt für die Identifizierung von Bs4-Autoaktivator-Mutanten.

Im Gegensatz zu APAF1 haben NB-LRR-Proteine keine ARC3-Domäne (Albrecht und Takken, 2006). Der Bs4-NB weist jedoch einen Sequenzabschnitt mit struktureller Ähnlichkeit zur ARC3-Domäne von APAF1 auf (Abb. 10A,B). Vergleicht man korrespondierende AS-Sequenzabschnitte von Bs4 mit anderen NB-LRR-Proteinen, so findet sich kein vergleichbarer Abschnitt (Abb. 10C, siehe auch Abb. M1-6, AS-Reste 585-603). Bei BLAST-Analysen mit diesem Abschnitt wurde kein weiteres bekanntes R-Protein identifiziert, wohl aber Proteine aus *Dictyostelium*, Hefe, *Xenopus*, Maus und Luzerne, mit 81-94% identischem Motiv, deren Funktion aber unbekannt ist (Daten nicht dargestellt). Die gezielte Mutagenese dieser Region könnte zukünftig Aufschluss über seine Relevanz und Funktion geben.

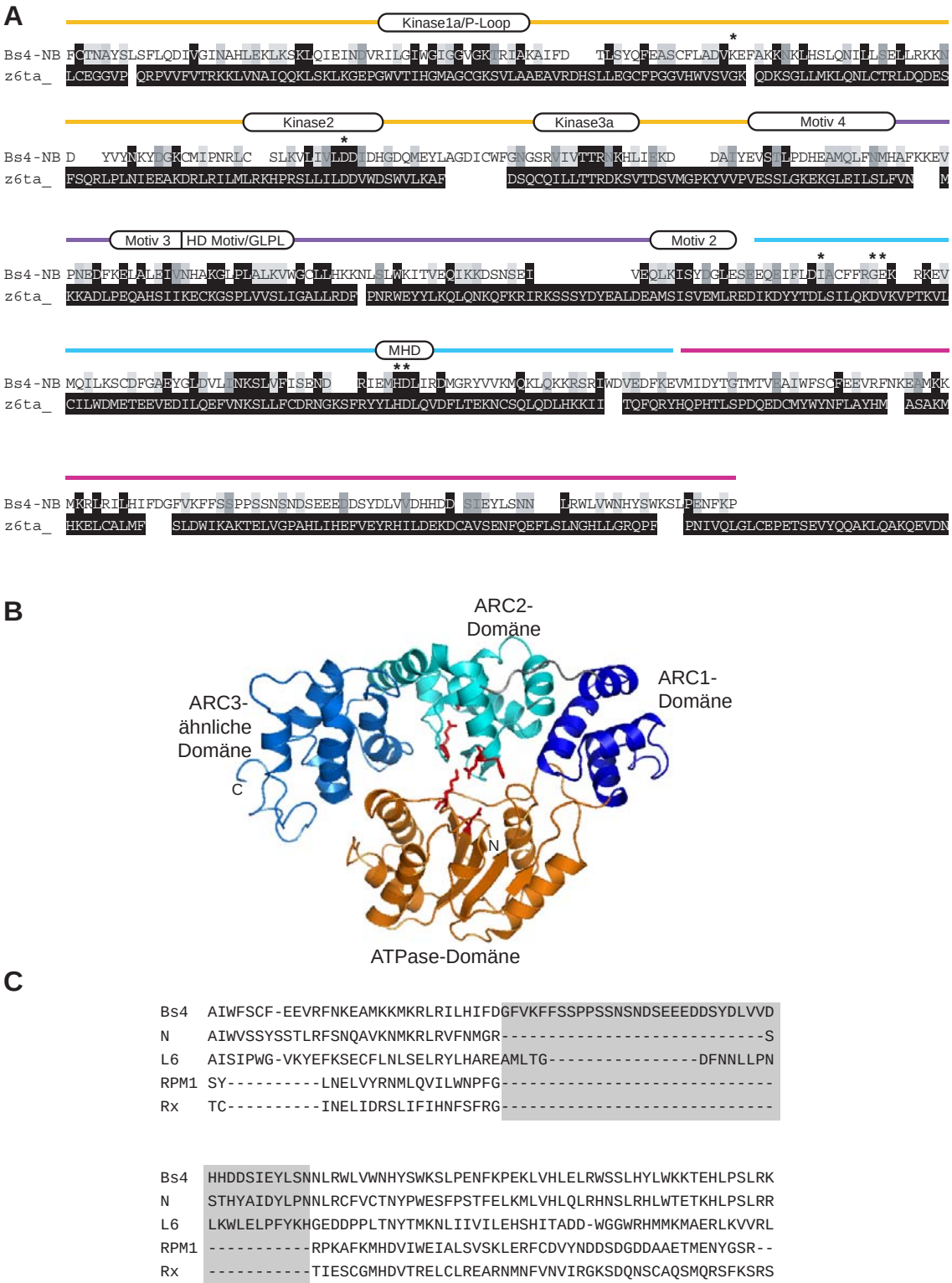

Abb. 10. 3D-Modell der Bs4-NB-Domäne. (A) Sequenzvergleich der NB-Domäne von Bs4 zu APAF1 (pdb-Eintrag: 1z6ta_). Die Sequenzkonservierung ist durch Schattierung hervorgehoben. AS-Reste, deren Mutation zur Autoaktivierung anderer NB-LRR-Proteine führten, sind durch * markiert. Zwischen NB-LRR-Proteinen und APAF1 konservierte Motive (Van der Biezen und Jones, 1998a) sind bezeichnet. **(B)** 3D-Modell der NB-Domäne auf Basis von 1z6ta_. Kritische AS-Reste für die Autokativierung sind rot hervorgehoben. Strukturelle Abschnitte sind passend zu (A) unterlegt. **(B)** Partieller Sequenzvergleich zwischen Bs4 und weiteren NB-LRR-Proteinen. Der ARC3-ähnliche Bereich ist grau unterlegt und fehlende Sequenzabschnitte durch „---“ repräsentiert.

2.3.3.4. 3D-Strukturmodell der LRR-Domäne von Bs4

Das auf der 3D-Struktur des Ribonuklease-Inhibitors aus dem Schwein (pdb-Eintrag: 2bnh, Kobe und Deisenhofer, 1995) basierende Modell der Bs4-LRR-Domäne zeigt eine hufeisenförmige Struktur aus 15 Wiederholungen, wobei sich β-Stränge auf der konkaven Innenseite und α-Helix-Abschnitte auf der konvexen Außenseite abwechseln. (Abb. 11A). Das β-Faltblatt-Rückgrat wird durch ins Protein orientierte konservierte Leucin-Reste im xxLxLxx-AS-Motiv ausgebildet (Abb. 11B). Die dazwischen liegenden AS-Reste (x) sind hypervariabel und zum Lösungsmittel ausgerichtet (*solvent exposed*). Vergleicht man die einzelnen Repeats, so wird die Konservierung des β-Faltblatts deutlich (Abb. 11C). Dagegen enthalten die Repeats 2, 3, 5, 9, 11 und 13-15 keine α-Helixbereiche. Im Rahmen einer Diplomarbeit wurden vier Einzel- und vier Doppelmutanten identifiziert, die keine

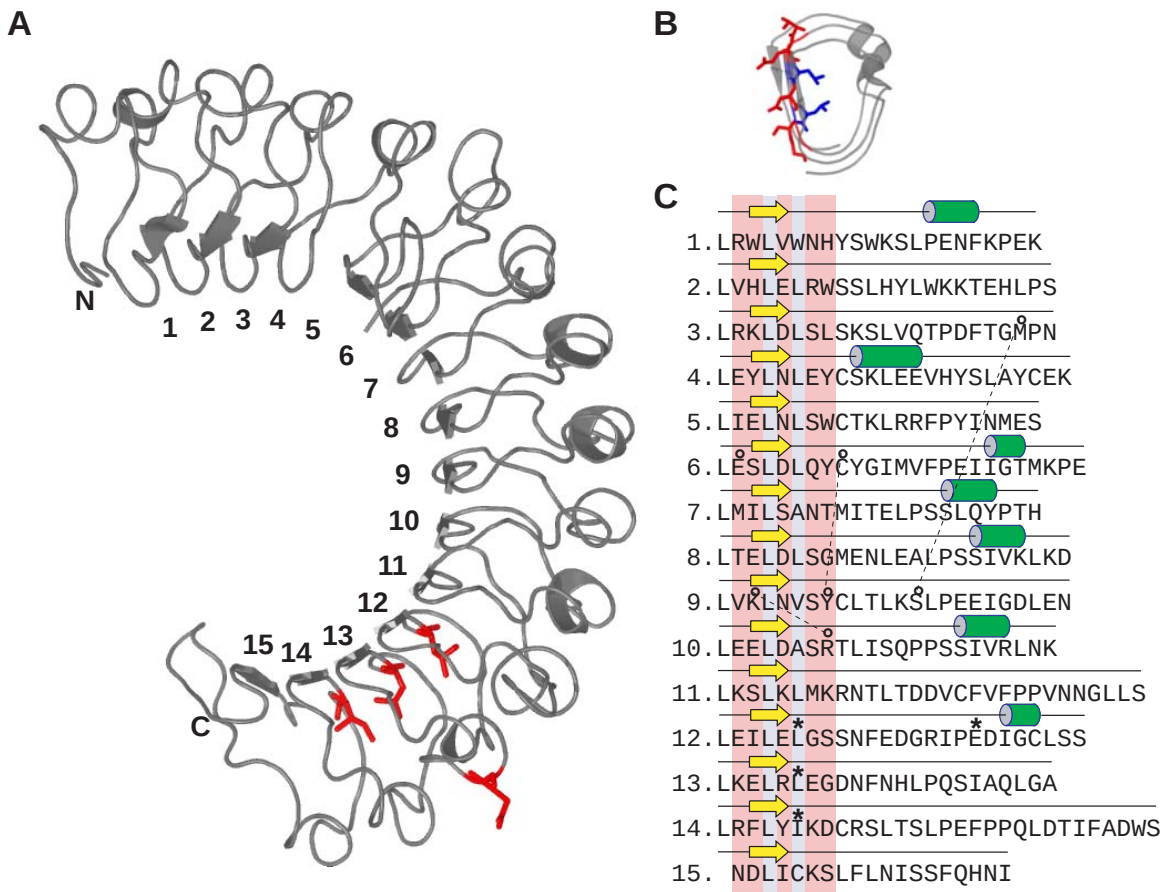

Abb. 11. 3D-Modell der Bs4-LRR-Domäne. (A) 3D-Modell des Bs4-LRR auf Basis von 2bnh. LRR-Einzelmutanten sind rot unterlegt. **(B)** Einzelne leucinreiche Wiederholungen bestehen aus einem β-Strang und einer α-Helix, die durch unstrukturierte Abschnitte verbunden sind. Konservierte Leucin-Reste (blau) im β-Strang sind nach innen orientiert. Dagegen sind dazwischen liegende hypervariable AS-Reste (rot) zum Lösungsmittel ausgerichtet und könnten zusammen eine variable Interaktionsoberfläche ausbilden. **(C)** AS-Sequenz und Sekundärstruktur (β-Strang gelb, α-Helix grün) der Bs4-LRRs. Konservierte Leucine sind blau, hypervariable AS-Reste rot unterlegt. Die Position identifizierter Einzel- (*) bzw. Doppelmutanten (durch unterbrochene Linien verbundene °) ist über der Sequenz angegeben.

Avr-Erkennung zeigten (A. Fick & T. Lahaye, unveröffentlicht). Interessanterweise betreffen drei der vier Einzelmutanten das LRR-Rückgrat, während alle AS-Substitutionen der Doppelmutanten außerhalb dieser strukturgebenden Region liegen (Abb. 11C). Messungen der Proteinmenge von Bs4-LRR-Mutanten könnte Aufschluss darüber geben, ob die Einzelmutanten im LRR-Rückgrat mit einer Protein-Instabilität korrelieren und ob die Doppelmutanten einen Effekt auf die Erkennungsspezifität haben.

2.3.3.5. 3D-Modell einer möglichen AvrBs4-Bs4-Interaktion

Die *in silico* Analysen von AvrBs4 zeigten, dass die Repeat-Region von AvrBs4 strukturelle Ähnlichkeit zu Tetratricopeptid (TPR)-Repeats anderer Proteine aufweist (Abb. 12). Das TPR-Motiv besteht, ebenso wie die AvrBs4-Repeat Region, aus 3-16 Wiederholungen einer 34-AS Sequenz, die Protein-Protein-Interaktionen vermittelt (Blatch

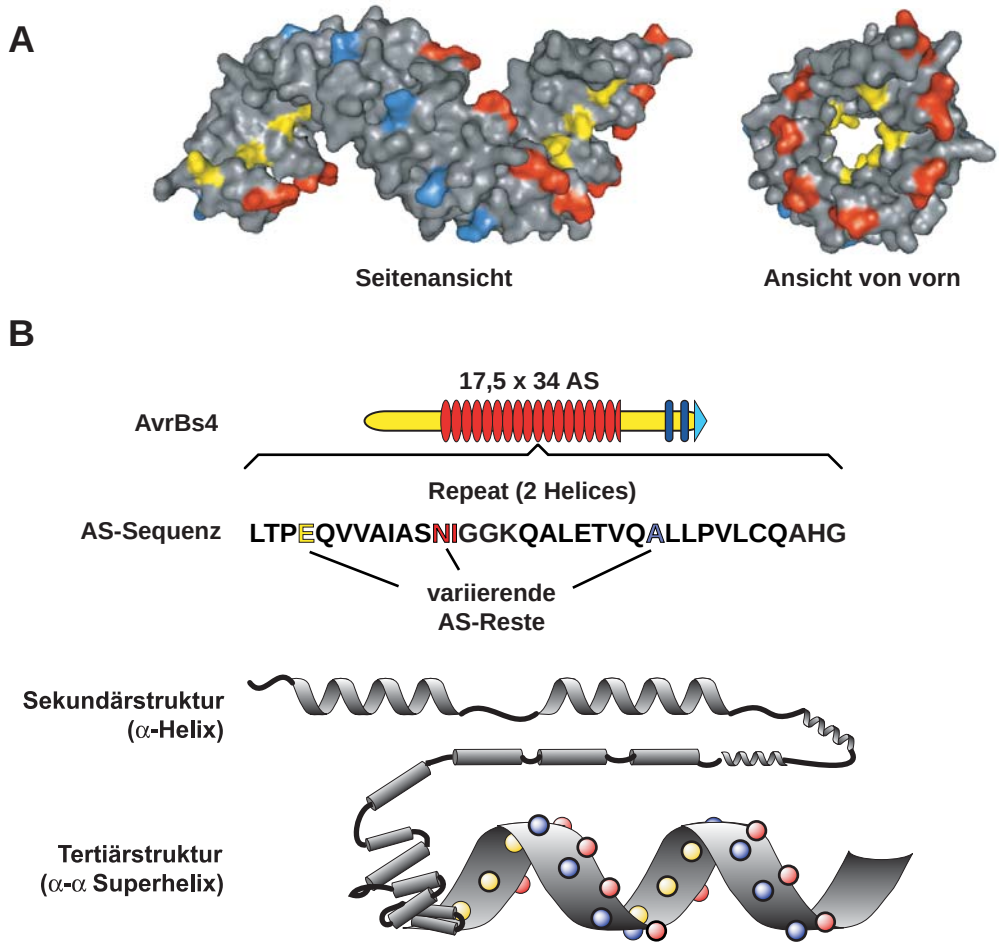

Abb. 12. AvrBs4-Strukturvorhersage. AvrBs3-ähnliche Proteine bilden über ihre Repeat-Region wahrscheinlich eine superhelikale, Tetratricopeptid (TPR)-ähnliche Struktur (aus Schornack *et al.*, 2006, modifiziert). **(A)** Oberflächen-Modell der AvrBs4-Repeat-Region basierend auf der Kristallstruktur der TPR-Domäne der GLCNAC Transferase (1w3bA, Jinek *et al.*, 2004), in Seiten- und Frontalansicht. **(B)** Schematische Darstellung der vorhergesagten rechtsgewundenen α–α-Superhelix der AvrBs4 Repeat-Region und seine strukturelle Hierarchie. Variierende AS-Reste 4 (gelb), 12+13 (rot) und 24 (blau) innerhalb der Repeats sind hervorgehoben.

und Lässle, 1999). Jede 34-AS-Sequenzwiederholung bildet, basierend auf TPR-Homologie, zwei antiparallele α-Helices aus. Der gesamte Bereich bildet eine rechts gewundene α-α-Superhelix-Struktur. In dieser Struktur winden sich die hypervariablen AS-Reste 12 und 13 entlang einer Linie um die Superhelix-Achse.

In Übereinstimmung mit der Funktion des TPR-Motivs als Protein-Protein-Interaktionsmotiv wurde gezeigt, dass die TPR-ähnliche Repeat-Region notwendig für die Dimerisierung von AvrBs3 ist (Gürlebeck *et al.*, 2005).

Basierend auf den Dimensionen des Bs4-LRR und der AvrBs4-Repeat Region besteht die Möglichkeit, dass die Bs4-LRR-Domäne die AvrBs4-Struktur im Bereich von drei TPR-Repeats umschließt (Abb. 13). In Übereinstimmung mit dieser Hypothese ist ein 3,5-Repeats enthaltendes AvrBs4-Derivat noch in der Lage, eine Bs4-HR zu induzieren (Schornack *et al.*, 2005). Trotz der sterischen Kompatibilität von AvrBs4 und Bs4 konnte aber bisher keine Interaktion zwischen beiden Proteinen nachgewiesen werden. Interessanterweise wurde für den LRR des R-Proteins I-2 aus Tomate eine Interaktion mit der TPR-Region von Protein Phosphatase 5 (PP5) gezeigt. Diese LRR-TPR-Interaktion deutet darauf hin, dass auch eine Bs4-LRR Interaktion mit AvrBs4 grundsätzlich möglich wäre. Die Interaktion von I-2 mit PP5 war mit dem gesamten I-2 Protein als Interaktionspartner nicht detektierbar (de la Fuente van Bentem *et al.*, 2005). Dies könnte erklären, warum eine direkte Interaktion zwischen AvrBs4 und Bs4 bisher nicht gezeigt werden konnte (Schornack, 2004, siehe auch folgendes Manuskript).

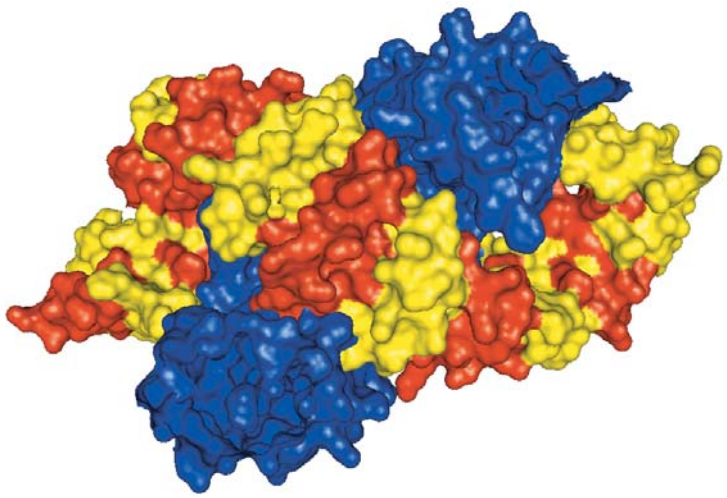

Abb. 13. Modell einer Bs4-AvrBs4-Assoziation. Dargestellt ist die mögliche Assoziation des Bs4-LRRs (blau) und der AvrBs4-Repeat-Region (Repeats abwechselnd gelb und rot; aus Schornack et al., 2006, modifiziert). Die Oberflächen-Modelle sind zueinander maßstabsgerecht dargestellt. Relative Größe und Anordnung beider Interaktionspartner wären kompatibel mit einem Rezeptor-Ligandenmodell. Der Bs4-LRR würde dabei einen Bereich von 2,5-3 einzelnen Repeats abdecken.

2.4. Die Erkennung des AvrBs4-Proteins aus *Xanthomonas campestris* pv. *vesicatoria* wird durch miteinander interagierende Bs4-TIR-NB- und LRR-Domänen vermittelt, durch Überexpression oder Silencing von *Hsp90* aufgehoben und geht außerdem mit dem Abbau von Bs4 einher

2.4.1 Zusammenfassung

Bs4 kodiert für ein R-Protein der TIR-NB-LRR-Klasse, das eine AvrBs4-induzierte HR auslöst. Konfokale Laserscanning-Mikroskopie und Proteinanalysen belegen, dass Bs4 ein lösliches, cytoplasmatisches Protein ist, welches die Erkennung von kernlokalisierten und cytoplasmatischen AvrBs4-Derivaten vermittelt. weder durch Bimolekulare Fluoreszenz-Komplementation noch durch Koimmunopräzipitation konnte eine AvrBs4-Bs4-Interaktion nachgewiesen werden. Bs4-TIR-NB- und LRR-Domänen immunopräzipitieren gemeinsam und vermitteln eine AvrBs4-induzierte HR in *N. benthamiana*. Die Bs4-Domänen-Interaktion ist jedoch bei Koexpression von AvrBs4 nicht detektierbar, was auf eine Aufhebung der Domänen-Interaktion bei AvrBs4-Erkennung hindeutet. Außerdem sind bei Anwesenheit von AvrBs4 reduzierte Bs4-, bzw. Bs4-NB- und Bs4-LRR-Domänen-Mengen nachweisbar. AvrBs4 beeinflusst aber nicht die TIR-Domänen-Menge. Demnach sind spezifische Bs4-Regionen ein Ziel des AvrBs4-verursachten Abbaus.

Bs4 assoziiert *in planta* mit Hsp90 und Virus-induziertes *Hsp90*-Silencing resultierte im Verlust der *Bs4*-HR und niedrigeren Bs4-Mengen. Die *Hsp90*-Überexpression führte dagegen zu höherer Bs4-Menge, verzögertem Bs4-Abbau, aber auch reduzierter HR. Außerdem sind bei *Hsp90*-Überexpression auch die N-Protein- und Bs2-vermittelten HRs abgeschwächt, jedoch nicht die AvrBsT-induzierte HR in *N. benthamiana*. Größere Hsp90-Mengen interferieren also mit der NB-LRR-vermittelten HR, aber nicht generell mit der HR.

Die Bs4-HR wurde auch durch die Expression von *ubR48* supprimiert, einer Ubiquitin-Mutante, die den Ubiquitin-vermittelten Proteinabbau blockiert. Interessanterweise hemmte *ubR48* auch die *N*-Gen- und *Bs2*-vermittelten HRs. Trotz Inhibierung des Proteinabbaus führte die *ubR48*-Überexpression zu reduzierten Bs4-Mengen, d. h. eine blockierte Bs4-Ubiquitinierung ist nicht die Ursache für die *ubR48*-vermittelte Reduzierung der HR-Intensität. Wahrscheinlich sind ausbalancierte Hsp90-Mengen notwendig für die Funktion von Bs4 und weiteren NB-LRR-Proteinen.

2.4.2 Manuskript 3

Recognition of the *Xanthomonas campestris* pv. *vesicatoria* AvrBs4 protein is mediated by interacting Bs4 TIR-NB and LRR domains, abolished by overexpression or silencing of *Hsp90*, and coincides with disappearance of Bs4

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Running title: Functional analysis of Bs4 signaling

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ABSTRACT

The tomato *Bs4* gene encodes a member of the Toll Interleukin 1 receptor - like / nucleotide binding / leucine rich repeat (TIR-NB-LRR) class of resistance (R) proteins and triggers a hypersensitive response (HR) upon recognition of the *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) AvrBs4 protein. Confocal microscopy and subcellular fractionation studies demonstrate that Bs4 is a soluble, cytoplasmatic protein that mediates recognition of nuclear-targeted AvrBs4 as well as cytoplasmatically-localized AvrBs4 derivatives. Coexpressed Bs4 TIR-NB and LRR domains coimmunoprecipitate and can functionally complement one another *in planta*. However, association of the Bs4 domains is not detectable upon coexpression of AvrBs4, suggesting that AvrBs4 recognition is linked to a change in the interaction status of Bs4 domains. Furthermore AvrBs4 was found to cause reduced levels of Bs4, the Bs4 NB and LRR domains but not of the Bs4 TIR domain, indicating that AvrBs4-dependent degradation targets specific Bs4 subdomains. BIFC and coimmunoprecipitation assay demonstrate association of Bs4 and Hsp90 *in planta*. Silencing of *Hsp90* causes a reduced Bs4 HR and reduced Bs4 levels. Conversely, overexpression of *Hsp90* led to increased Bs4 levels, delayed disappearance of Bs4 and a reduced HR. Overexpression of *Hsp90* also reduced the HRs mediated by the TIR-NB-LRR tobacco protein N and the coiled coil (CC)-NB-LRR pepper protein Bs2 but not the AvrBsT induced HR. Thus increased Hsp90 levels interfere with NB-LRR mediated controlled HR but not with the HR as such. The Bs4 HR was also suppressed by ubR48, a ubiquitin mutant that inhibits ubiquitination-dependent protein degradation. Surprisingly, ubR48 overexpression resulted in reduced Bs4 levels, suggesting that ubR48-mediated HR suppression is not due to blocked polyubiquitination of Bs4. Interestingly, ubR48 expression also suppressed HRs mediated by tobacco N, and pepper Bs2. We propose that fine-tuned levels of Hsp90 are crucial to the function of Bs4 and other NB-LRR proteins.

INTRODUCTION

Plants have evolved multiple ways to defend themselves against potentially pathogenic microbes. A basal defense layer mediates perception of conserved pathogen-associated molecular patterns (PAMPs) such as flagellin and lipopolysaccharides (Nürnberger et al., 2004; Boller, 2005; Zipfel and Felix, 2005; Chisholm et al., 2006; da Cunha et al., 2006). A second defense layer is dedicated to protection of individual cultivars against pathogens that suppress or evade the basal host defense system (Jones and Takemoto, 2004). Cultivar-specific resistance relies on the expression of plant resistance (*R*) genes that mediate recognition of corresponding pathogen avirulence (*avr*) genes (Flor, 1971). This gene-for-gene resistance frequently is associated with a programmed cell death, termed the hypersensitive response (HR), which is concomitant with halt of pathogen spread (Lam, 2004).

Over the last decade, gene-for-gene resistance has been extensively studied resulting in the molecular isolation of more than 40 *R* genes (Hammond-Kosack and Parker, 2003; Martin et al., 2003). Most *R* genes encode intracellular proteins with nucleotide-binding (NB) and leucine-rich repeat (LRR) domains. NB-LRR proteins have been classified, according to their N-termini, into those that share homology with the Toll and the Interleukin-1 receptor (TIR) or those that contain a putative coiled-coil (CC) domain. Although collectively designated as NB-LRR proteins, TIR- and CC-type NB-LRR proteins can be distinguished solely by the level of sequence similarity between their NB domains (Meyers et al., 1999; Pan et al., 2000; Young, 2000) reflecting different evolutionary histories or a degree of functional specialization.

NB-LRR proteins display a modular architecture, which is in agreement with their dual function in signal recognition and transduction. The N-terminal domain of NB-LRR proteins influences the requirement for downstream signaling components (Feys and Parker, 2000; Glazebrook, 2001), indicating a function in signal transduction. Domain-swapping experiments, mutational analysis and extensive sequence comparisons of R proteins, indicate that recognition specificity is governed predominantly by the LRR domain (Ellis et al., 2000; Jones, 2001). Solvent-exposed residues in the LRR are also subject to diversifying selection (Meyers et al., 2005). This is believed to render the NB-LRR proteins instable (Schulze-Lefert, 2004; Sangster and Queitsch, 2005) and in support of this postulate, the molecular chaperone Hsp90 known to buffer metastable proteins was

found to be required for the function of several NB-LRR proteins (Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Zhang et al., 2004; de la Fuente van Bentem et al., 2005).

Biochemical studies demonstrated that the NB domains of the I-2 and Mi CC-NB-LRR proteins bind to and hydrolyze ATP *in vitro* (Tameling et al., 2002). Analysis of potato Rx, another CC-NB-LRR protein, revealed functional complementation and physical interaction of coexpressed CC and NB-LRR domains (Moffett et al., 2002). Notably, physical interaction of CC and NB-LRR was abolished by a mutation in the NB domain. These data inspired formulation of a model in which ATP hydrolysis induces a conformational change leading to an activated CC-NB-LRR protein (Rathjen and Moffett, 2003; Belkhadir et al., 2004; Takken et al., 2006). Given that the NB domains of TIR- and CC-NB-LRR R proteins are distinct (Meyers et al., 1999; Pan et al., 2000; Young, 2000), it remains to be seen if the findings on CC-NB-LRR proteins can be extrapolated to TIR-NB-LRR-type R proteins.

Based on the strictly genetic concept of gene-for-gene resistance, many scientists envisioned plant R proteins as receptors that mediate a coordinated defense response upon physical interaction with a matching Avr ligand (Gabriel and Rolfe, 1990; Keen, 1990). In agreement with this model, experimental evidence generally shows a spatial interdependency between the locations of the Avr and R proteins (Nimchuk et al., 2001; Bonas and Lahaye, 2002; Martin et al., 2003; Maurer-Stroh and Eisenhaber, 2004). Four R proteins have indeed been shown to interact physically with their corresponding Avr proteins (Scofield et al., 1996; Tang et al., 1996; Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). However, further empirical evidence for the receptor-ligand model is scarce, which stimulated formulation of an alternative model, in which R proteins act as guardians of host proteins, which themselves complex with or are modified by pathogen Avr proteins (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). Indeed, molecular analysis of several *Pseudomonas syringae* Avr proteins and their matching R proteins provided experimental evidence in favor of the guard model (Innes, 2004).

The analysis of phytopathogenic bacteria and their plant hosts revealed that these pathogens deposit their Avr proteins into the host cytoplasm via a type-III secretion system (TTSS) (Chang et al., 2004). Considering evolutionary forces, the presence of the TTSS system indicates that the primary function of bacterial Avr proteins is in virulence rather than avirulence, an assumption that has been confirmed for many Avr proteins (Chang et al., 2004). Most Avr proteins share little or no homology to each other, with the notable

exception of the AvrBs3 family. Members of the AvrBs3 family, named after its first sequenced member, from *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) (Bonas et al., 1989), are highly conserved (> 90% identity) and have been identified in several *Xanthomonas* pathovars and *Ralstonia solanacearum* (White et al., 2000; Ponciano et al., 2003; Schornack et al., 2006). The most striking feature of AvrBs3-like proteins is a variable number of tandemly-arranged nearly perfect copies of a 34-amino-acid (aa) motif that are located in the center of the polypeptide chain. Domain swapping experiments demonstrated that the repeat domain defines both virulence and avirulence specificity (Lahaye and Bonas, 2001). The C-terminus of AvrBs3-homologues contains functional nuclear localization signals (NLSs) and an acidic transcriptional activation domain (Szurek et al., 2001). Functional assays revealed that (i) the AvrBs3 family member AvrXa7 binds dsDNA (Yang et al., 2000) and (ii) that AvrBs3 is capable of altering plant gene expression (Marois et al., 2002). Taken together, these findings suggest that AvrBs3-like proteins are targeted to the plant nucleus where they modulate transcription of host genes in order to generate conditions favorable to the pathogen.

We study cultivar-specific resistance towards the bacterial pathogen *Xcv*, the causal agent of bacterial spot disease in tomato and pepper (Jones et al., 1998). More specifically, we are interested in the recognition of the *Xcv* effector proteins AvrBs3 and AvrBs4, which share 97% sequence identity (Bonas et al., 1993) but are recognized specifically by the pepper *Bs3* and tomato *Bs4* genes, respectively (Ballvora et al., 2001b). Bs3 and Bs4 mediate not only recognition of full-length, *Xcv* type III-secreted effector proteins but also of *Agrobacterium tumefaciens*-delivered AvrBs3- and AvrBs4-deletion derivatives lacking the residues 2 - 153, demonstrating that the N-terminal region is dispensable for *Bs3*- and *Bs4*-mediated recognition (Szurek et al., 2002; Schornack et al., 2004). Intriguingly, NLS-deletion derivatives of AvrBs4 and AvrBs3 trigger *Bs4*- but not *Bs3*-resistance, respectively suggesting that the corresponding *R*-gene products are located in the cytoplasm and nucleus, respectively (Ballvora et al., 2001b). In agreement with these studies the Bs4 protein was predicted to be cytoplasmic (Schornack et al., 2004).

In order to gain further insight into the molecular basis of the *Bs4*-mediated resistance, we studied the *in planta* localization of Bs4, AvrBs4 and derivatives thereof. Furthermore we tested if the TIR-NB-LRR protein Bs4 would show domain *trans*-complementation as shown previously for the CC-NB-LRR type R proteins Rx and Bs2 (Moffett et al., 2002; Leister et al., 2005). We found that Bs4 TIR-NB and LRR domains are able to interact and to complement one another *in trans*. Gene silencing and

overexpression of *Hsp90* was found to negatively and positively regulate Bs4 levels, respectively, and to suppress the Bs4 HR. Finally we show that Bs4 and Bs4 domains disappear upon expression of AvrBs4 prior to HR symptoms and that this disappearance of Bs4 is most likely independent of polyubiquitination.

RESULTS

AvrBs4-derivatives that trigger a Bs4*-dependent HR localize to the plant cytoplasm and/or the plant nucleus*

To define the localization of the *Xcv* AvrBs4 protein and derivatives thereof *in planta* we generated T-DNA constructs encoding GFP-tagged fusion proteins (Figure 1A). *A. tumefaciens*-mediated transient expression (agroinfiltration) in *N. benthamiana* and tomato demonstrated that GFP-tagged AvrBs4-derivatives retain their avirulence specificity (data not shown) and are readily detectable by protein gel blot analysis (Figure 1B). Confocal laser-scanning microscopy (CLSM) was used to determine the subcellular localization of GFP-tagged AvrBs4-derivatives in *N. benthamiana* (Figure 1C). Because C-terminal GFP fusion proteins did not produce fluorescence we focused on the analysis of N-terminally tagged AvrBs4 derivatives. Agroinfiltration of *GFP:avrBs4Δ152*, a construct encoding a GFP-tagged AvrBs4-derivative that lacks the N-terminal type-III secretion signal (deletion of residues 2 - 153) (Bonas et al., 1989), but which contains the AvrBs4 repeat region and the C-terminal NLSs, led to GFP-fluorescence exclusively in the nucleus (Figure 1C). Inspection at higher magnification revealed increased fluorescence at distinct subnuclear foci. Expression of *GFP:avrBs4Δ152Δ1-3*, a construct that encodes an NLS-deletion derivative of AvrBs4Δ152 resulted in cytoplasmic GFP-fluorescence. In contrast, expression of *GFP:avrBs4Δ227*, which encodes a severely truncated AvrBs4-derivative that contains only 5.5 of 17.5 repeat units and no NLSs produced cytoplasmic and nuclear fluorescence. Analysis at higher magnification did not reveal accumulation at distinct subnuclear foci as observed upon expression of *GFP:avrBs4Δ152* (Figure 1C).

In summary we found that different AvrBs4-derivatives, which are all recognized by the tomato *Bs4* gene, localize to the nucleus (AvrBs4Δ152), the cytoplasm (AvrBs4Δ152Δ1-3) or both cellular compartments (AvrBs4Δ227).

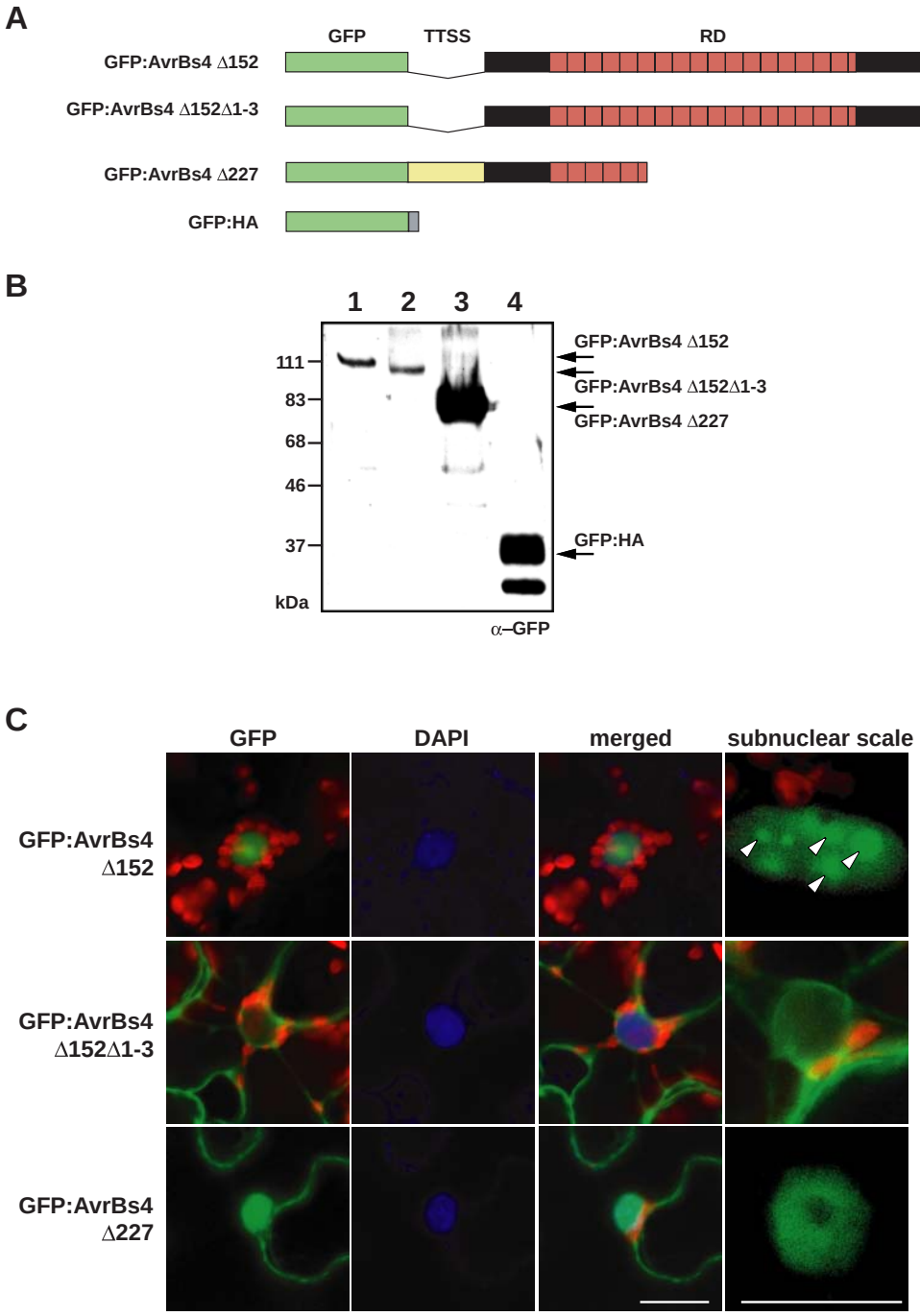

Figure 1. Structure, expression levels and compartimentation of AvrBs4 derivatives.

(A) Domain architecture of green fluorescent protein (GFP) tagged AvrBs4-derivatives. The N-terminally located EGFP (GFP) epitope and type III secretion signal (TTSS) are depicted as green and yellow boxes, respectively. The 17.5 tandemly-arranged repeat units that collectively define the repeat domain (RD) are depicted as red boxes. C-terminal nuclear localization signals (NLSs), activation domain (AD) and hemagglutinin (HA) epitope are displayed as blue, white and gray boxes, respectively.

(B) Protein gel blot analysis of *in planta* expressed AvrBs4 derivatives. *N. benthamiana* was agroinfiltrated with 35S-driven *GFP:avrBs4Δ152* (lane 1), *GFP:avrBs4Δ152Δ1-3* (lane 2), *GFP:avrBs4Δ227* (lane 3), and *GFP:HA* (lane 4). All T-DNA constructs were agro-coinfiltrated with *35S-p19*. AvrBs4 derivatives of predicted size (arrows) were detected with α-GFP antibody in crude extracts of *N. benthamiana* leaves two days after agroinfiltration. Numbers at the left indicate positions of molecular mass standards in kilodalton (kDa).

(C) Subcellular localization of AvrBs4 derivatives. Confocal images showing GFP fluorescence (green) and chloroplast auto fluorescence (red), DAPI-stained nuclei (blue) and a merged image. Subnuclear foci that are visible in the close-up view (subnuclear scale) are marked with arrowheads. Agroinfiltrated AvrBs4 derivatives are indicated at the left side. Leaf patches were inspected two days after agroinfiltration. Scale bar, 20 μm.

The tomato Bs4 protein localizes to the cytoplasm

To determine the location of the Bs4 protein we generated T-DNA constructs encoding GFP:Bs4 (N-terminal GFP) or Bs4:GFP (C-terminal GFP) fusion proteins under control of the 35S promoter. Agroinfiltration of *GFP:Bs4* or *Bs4:GFP* mediated an HR in *N. benthamiana* when coexpressed with *avrBs4*Δ*152* or *avrBs4*Δ*227* but not when coexpressed with *avrBs1* (Ronald and Staskawicz, 1988) (AvrBs1 shares no sequence homology with AvrBs4) thus suggesting that the GFP epitope does not interfere with Bs4 function. To determine if *GFP:Bs4* and *Bs4:GFP* are translated into corresponding full-length fusion proteins, we performed protein gel blot analysis using crude protein extracts from agroinfiltrated *N. benthamiana* leaf tissue. We detected a 158-kDa band, the expected molecular mass of full-length Bs4:GFP and GFP:Bs4 fusion proteins (131 kDa Bs4 + 27 kDa GFP), which was absent in the GFP-only control (Figure 2A; arrow). Notably, *in planta* expression of *GFP:Bs4* but not *Bs4:GFP* produced a prominent signal at a position that is diagnostic for GFP (Figure 2A; arrow).

CLSM-based analysis showed that expression of *GFP:Bs4* produced approximately equal fluorescence intensities in the cytoplasm and the nucleus while *Bs4:GFP* constructs generated a weaker but exclusively cytoplasmic fluorescence (Figure 2B). As *in planta* expression of *GFP:Bs4* generates high levels of a protein that is similar in size to GFP (Figure 2A; black arrow) and since the observed fluorescence patterns matched well to the GFP control (Figure 2B) we infer that the detected nuclear fluorescence is primarily due to GFP expression but not due to expression of the GFP:Bs4 fusion protein.

Subcellular localization of tomato Bs4 was also studied by differential centrifugation of cell extracts and a 10xMYC epitope-tag. Agroinfiltration with *Bs4:MYC* and *MYC:Bs4* T-DNA constructs mediated an HR in *N. benthamiana* upon coexpression with *avrBs4*Δ*152* or *avrBs4*Δ*227* but not upon coexpression with *avrBs1*, indicating that the MYC-epitope does not interfere with Bs4 function (data not shown). The α-MYC antibody detected signals corresponding to the predicted molecular mass of full-length Bs4 fusion proteins upon agroinfiltration with *Bs4:MYC* but not upon agroinfiltration with *MYC:Bs4* T-DNA constructs (data not shown). After separation of soluble and membrane-bound proteins by centrifugation Bs4:MYC was found to be present in the soluble- but not in the membrane-fraction of *N. benthamiana* and tomato cells (Figure 2C; arrow).

In summary the analysis of functional epitope-tagged Bs4 fusion proteins by confocal microscopy and differential centrifugation, respectively indicated that Bs4 is a cytoplasmatic protein.

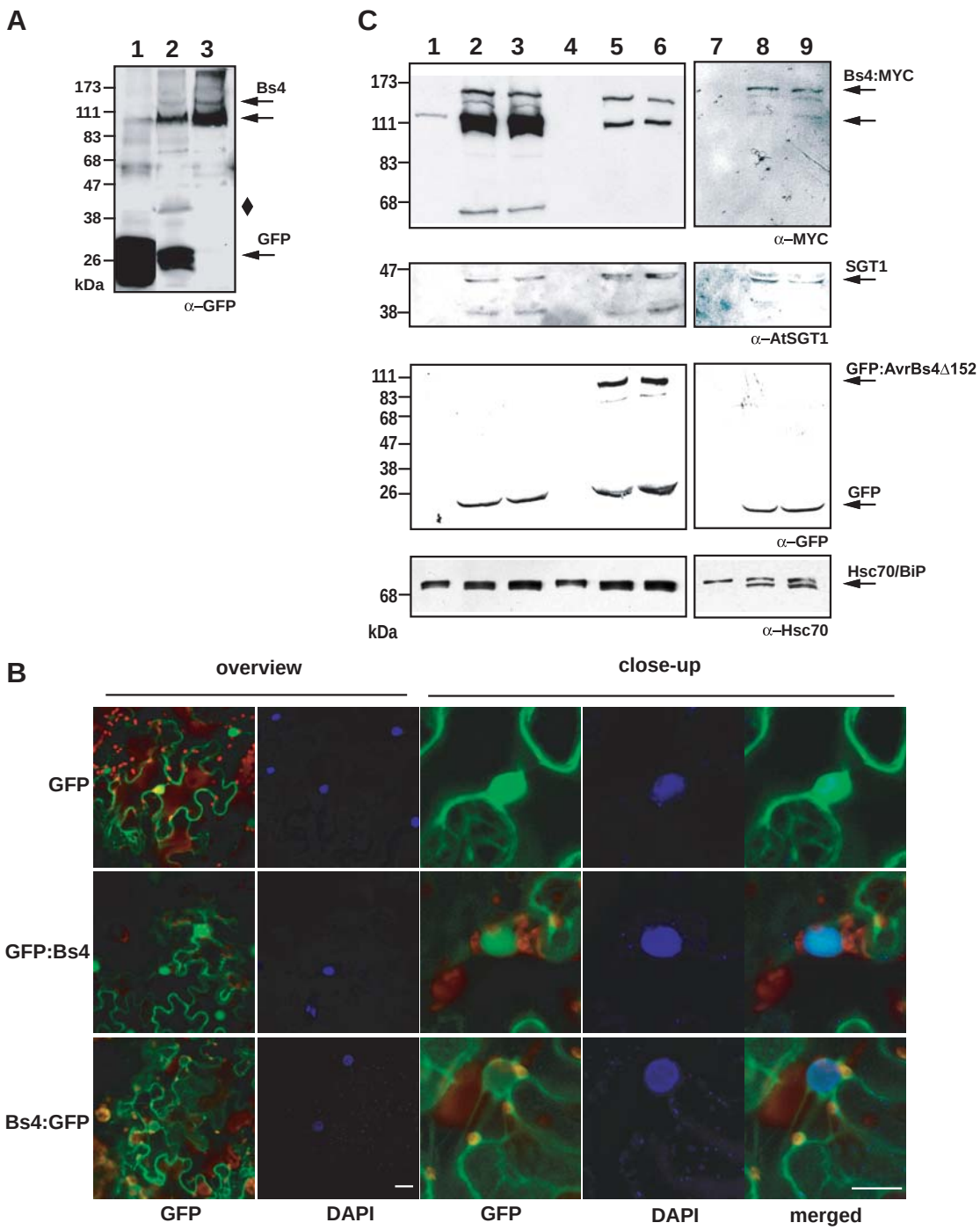

Figure 2 (for legend see next page)

Figure 2 (previous page). Subcellular localization of tomato Bs4.

(A) Protein gel blot analysis of N- and C-terminally GFP-tagged Bs4. An α-GFP antibody detected proteins of predicted size (arrows) in crude extracts of *N. benthamiana* leaves one day after agroinfiltration of *GFP* (lane 1), *GFP:Bs4* (lane 2), or *Bs4:GFP* constructs (lane 3), respectively. All T-DNA constructs are under control of the 35S promoter and were agro-coinfiltrated with *35S-p19*. A highly abundant protein of about 100 kDa that is present in N- and C-terminally GFP-tagged Bs4 is marked (arrow). A 40-kDa protein that is specific to GFP:Bs4 is marked with a black diamond. Numbers at the left indicate positions of molecular mass standards in kilodalton (kDa).

(B) Confocal imaging of GFP-tagged Bs4. Low (overview) and high (close-up) magnification views of agroinfiltrated *N. benthamiana* leaf tissue. Confocal images showing GFP (green) and chloroplast autofluorescence (red), DAPI-stained nuclei (blue) and a merged image. Proteins corresponding to agroinfiltrated T-DNA constructs are indicated at the left side. Note that the length of exposure was the same for Bs4:GFP and GFP:Bs4 images but shorter for the GFP image. All T-DNA constructs were agro-coinfiltrated with *35S-p19*. If not stated, the constructs are driven by the 35S promoter. Scale bar, 20 μm. Measurements were done two days after agroinfiltration.

(C) Localization of MYC-tagged Bs4 by differential centrifugation and subsequent protein gel blot analysis. Expression levels corresponding to a *Bs4:MYC* construct in agroinfiltrated *N. benthamiana* (lanes 1-6) and tomato plants (lanes 7-9) were studied two dpi with an α-MYC antibody. Bs4:MYC levels were determined in the absence (lanes 1-3 and lanes 7-9) and the presence (lanes 4-6) of transiently expressed AvrBs4Δ152 (arrow). Lanes 1, 4 and 7, Pellet (100,000 g). Lanes 2, 5 and 8, soluble fraction. Lanes 3, 6 and 9, total protein. All T-DNA constructs are under control of the 35S promoter and were agro-coinfiltrated with *35S-p19*. A highly abundant protein of about 100 kDa is marked (arrow). A protein that is specific to Bs4:MYC is marked with a black diamond. The blots were reprobed with antisera against endogenous SGT1 and transiently expressed GFP proteins to control purity of the pellet fractions. Equal protein loading was confirmed by successive reprobing with an α-Hsc70/BiP antibody, which crossreacts with cytoplasmatic and membrane-associated members of the Hsc70 protein family (arrow) (Anderson et al., 1994).

No evidence for a direct interaction between Bs4 and AvrBs4 in planta

Previous yeast two-hybrid (Y2H) studies did not reveal a Bs4-AvrBs4 interaction (Schornack et al., 2004), which might be due to lack of the appropriate plant-specific cellular context. We therefore tested the potential Bs4-AvrBs4 interaction *in planta.* Firstly, we used confocal microscopy and fractionation studies to determine if transient coexpression of *Bs4* and *avrBs4* would affect the subcellular localization of the corresponding proteins, which would be indicative of a direct interaction. Nuclear targeting of GFP:AvrBs4 was not significantly affected by coexpression of cytoplasmatic Bs4 suggesting that Bs4 does not retain AvrBs4 in the cytoplasm (data not shown). Likewise, expression of AvrBs4 had no visible effects on the subcellular distribution of Bs4:GFP or Bs4:MYC (Figure 2C and data not shown). We also employed an AvrBs3-specific antibody (Knoop et al., 1991) that was shown previously to cross-react with AvrBs4 (Bonas et al., 1993; Ballvora et al., 2001a) to test if immunoprecipitated AvrBs4 protein would coimmunoprecipitate coexpressed Bs4:MYC protein. However, coimmunoprecipitation (CoIP) studies provided no indication for a Bs4-AvrBs4 interaction although protein gel blot analysis confirmed that both proteins were expressed (data not shown).

Although colocalization and CoIP studies did not provide any evidence for a direct interaction of Bs4 and AvrBs4 we cannot exclude the possibility of a transient interaction. We therefore utilized bimolecular fluorescence complementation (BIFC, "split-YFP")(Bracha-Drori et al., 2004; Walter et al., 2004). In this approach physical interaction of proteins that are fused to non-fluorescing N- and C-terminal YFP fragments (YN, YC) causes proximity of YN and YC and results in reconstitution of YFP fluorescence. As the association of YN and YC is essentially irreversible (Magliery et al., 2005) BIFC traps weak interaction partners, thus increasing the sensitivity of detection (Bhat et al., 2006).

Transient coexpression of YN- and YC-tagged derivatives of the *Arabidopsis* TGA5 transcription factor, which is known to homodimerize in the nucleus (Jakoby et al., 2002), produced nuclear fluorescence and thus demonstrated functionality of our BIFC assay (Figure 3). Because expression of *GFP:Bs4* and *avrBs4*Δ152*:GFP* produced truncated or non-fluorescing proteins, respectively (Figure 2A; lane 2 and data not shown), we generated *YC:avrBs4*Δ152 and *Bs4:YN* constructs for our BIFC studies. Transient coexpression of *YC:avrBs4*Δ152 and *Bs4:YN* elicited an HR in *N. benthamiana* indicating that both fusion proteins are biologically functional (data not shown). To avoid interference from HR-associated protein degradation we conducted our BiFC studies at 2 dpi, which is prior to the onset of HR (3-4 days post infiltration [dpi]). However, coexpression of *YC:avrBs4*Δ152 and *Bs4:YN* fusion proteins did not result in detectable YFP fluorescence suggesting that Bs4 and AvrBs4 do not interact (Figure 3). As AvrBs3 was previously shown to interact with pepper importin α (Szurek et al., 2001; Szurek et al., 2002) it seemed likely that the 97% identical AvrBs4 protein would interact with tomato importin α (*L. esculentum* karyopherin α; LeKAP*α*). Indeed, *YC:avrBs4Δ152* produced nuclear fluorescence when coexpressed with *LeKAPα:YN* (Figure 3) thus demonstrating functionality of *YC:avrBs4Δ152* construct in the BIFC assay. We can also exclude that the lack of interaction between Bs4 and AvrBs4 is due to non-functionality of the *Bs4:YN* transgene since this construct produced fluorescence in combination with other YC fusion proteins (see below).

We also tested if the lack of detectable interaction between AvrBs4 and Bs4 was due to efficient nuclear targeting of AvrBs4. Therefore, we fused the YC sensorpeptide to an NLS-deletion derivative of AvrBs4 that remains in the cytoplasm (AvrBs4Δ152Δ1-3, see Figure 1) were the Bs4 protein is also located. However, coexpression of *YC:avrBs4Δ152Δ1-3* and *Bs4:YN* produced no fluorescence (Figure 3), suggesting that the

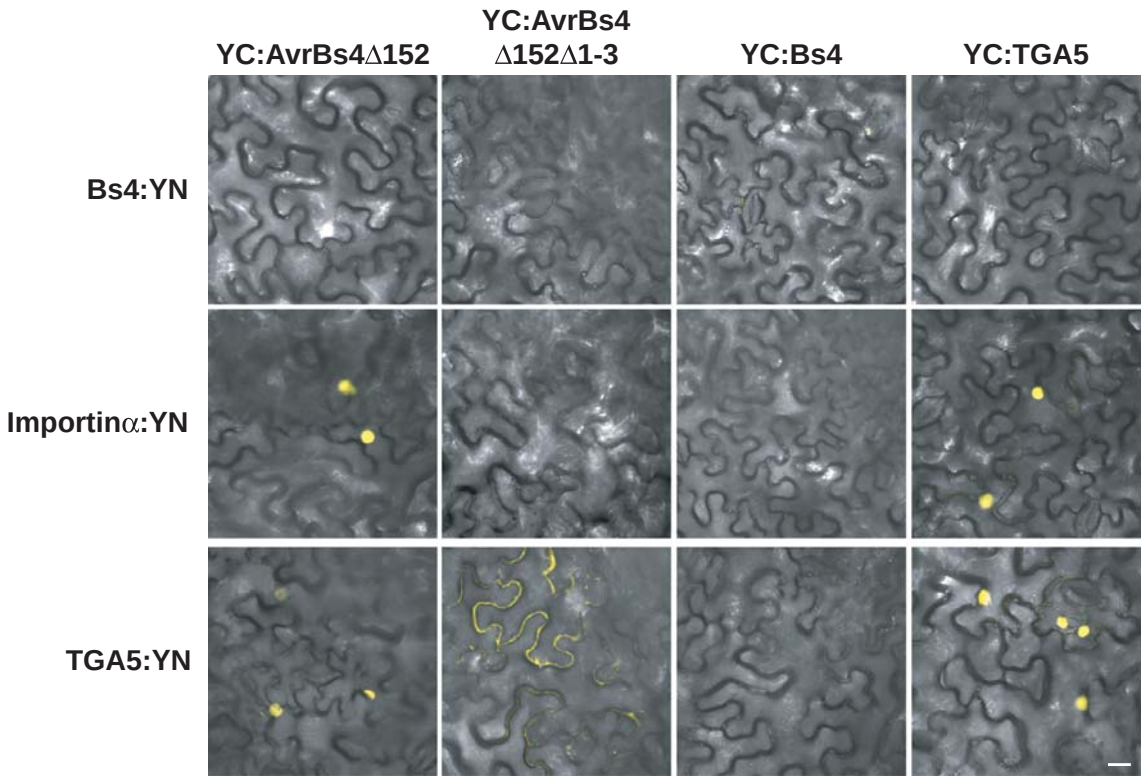

Figure 3. Confocal images of bimolecular fluorescence complementation studies with Bs4 and AvrBs4.

N. benthamiana leaves were agro-coinfiltrated with *35s-p19* and constructs encoding the indicated YC- and YN-fusion proteins. Images were taken two days post inoculation which is before the onset of the AvrBs4-triggered and Bs4-mediated HR in *N. benthamiana*. Note that exposure time is identical for all images. Confocal images show YFP (yellow) and bright field (grey). Scale bar, 20 μm.

lack of a detectable interaction between AvrBs4 and Bs4 is not due to their different locations.

Taken together, CoIP, BIFC and localization studies of coexpressed AvrBs4 and Bs4 suggest that Bs4 and AvrBs4 do not interact *in planta*.

Coexpressed Bs4 TIR-NB and LRR domains mediate recognition of AvrBs4

Recent analysis of the potato Rx and the pepper Bs2 protein has shown that coexpressed CC-NB plus LRR domains or coexpressed CC plus NB-LRR domains mediate an Avr-dependent HR (Moffett et al., 2002; Leister et al., 2005). To test whether domains of the TIR-NB-LRR type R protein Bs4 could also functionally complement each other *in trans*, we analyzed different Bs4 domain combinations by *Agrobacterium*-mediated transient expression in *N. benthamiana*. As shown in Figure 4, coexpression of the TIR-NB ($TIR\text{-}NB_{Bs4}$) and LRR (LRR_{Bs4}) domains of Bs4 resulted in recognition of AvrBs4 and its C-terminal deletion derivative AvrBs4Δ227 but not of AvrBs1 suggesting that *trans* complementing domains have the same recognition specificity as the full-length Bs4 protein. The HR was strictly dependent on coexpression of both the $TIR\text{-}NB_{Bs4}$ and the LRR_{Bs4} domains since expression of these domains on their own did not mediate

recognition of AvrBs4 or AvrBs4Δ227 (Figure 4). In contrast to the TIR-NB_{Bs4} and LRR_{Bs4} combination, coexpression of TIR_{Bs4} and NB-LRR_{Bs4} domains did not result in functional *trans*-complementation (data not shown).

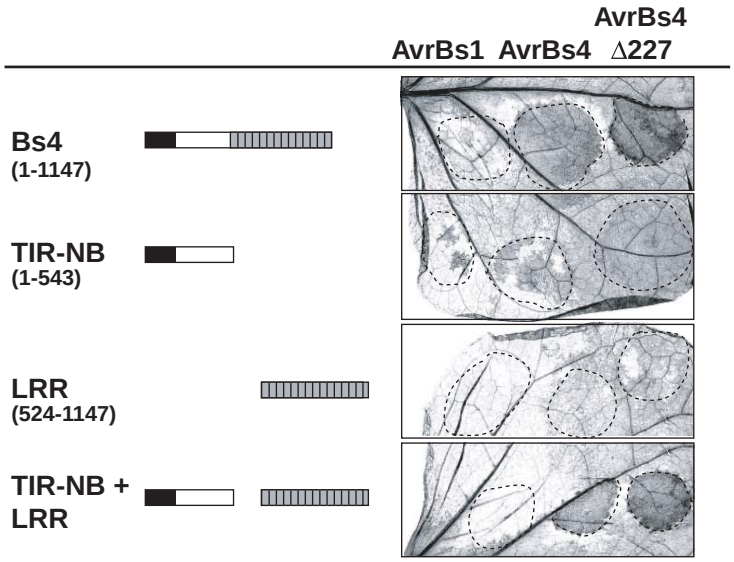

Figure 4. Functional complementation of the Bs4 TIR-NB and LRR regions.

Bs4 constructs are displayed schematically. The TIR, NB and LRR regions of Bs4 are represented by black, white and grey boxes, respectively. *Bs4* and *Bs4* regions were coexpressed together with the depicted *avr* constructs via agroinfiltration in *N. benthamiana* leaves. Images were taken eight days post infiltration. For better visualization of the HR, leaves were destained by incubation in ethanol.

2.4.3 The Bs4 NB-LRR and the corresponding tobacco N domain show low expression levels

Because TIR_{Bs4} and NB-LRR_{Bs4} domains did not functionally *trans*-complement each other we wondered if these Bs4 domains are intrinsically instable or mislocalized. Indeed, a C-terminally GFP-tagged NB-LRR_{Bs4} fusion was not detectable by fluorescence microscopy (Figure 5A) whereas the complementary TIR_{Bs4} domain produced bright fluorescence in the cytoplasm and the nucleus. GFP-tagged TIR-NB_{Bs4} and LRR_{Bs4} domains generated similar signal intensities as Bs4:GFP but were detected in the cytoplasm and the nucleus while Bs4:GFP was found exclusively in the cytoplasm.

Abundance of GFP-tagged Bs4 domains was also studied by protein gel blot analysis of crude protein extracts from agroinfiltrated *N. benthamiana* tissue. Since only the TIR_{Bs4} domain was readily detectable with α-GFP antibodies (data not shown) we expressed MYC-tagged Bs4-domains instead. Relative abundance of MYC-tagged Bs4 domains (Figure 5B) was generally similar as to that observed by CLSM-based quantification of GFP-tagged domains (Figure 5A) and confirmed the very low expression

levels of the NB-LRR$_{Bs4}$ fusion. We also studied expression levels in yeast and found that the relative abundance of different Bs4 domains was similar to those observed in *N. benthamiana* (Figure 5C).

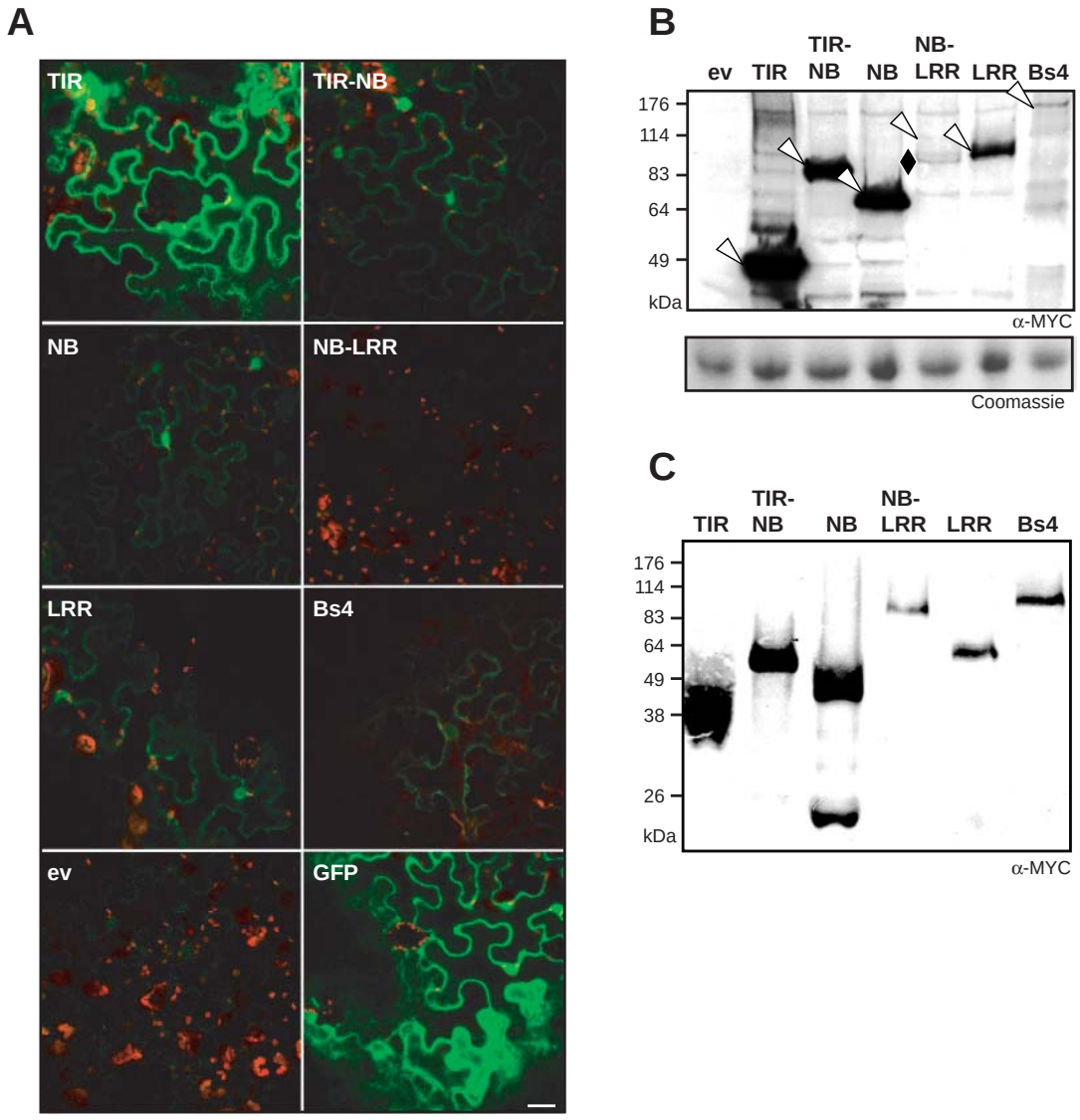

Figure 5. Compartmentation and expression levels of Bs4 and Bs4 domains.

(A) Confocal images of GFP-tagged Bs4 and Bs4 domains. Fusion proteins were expressed together with p19 via agroinfiltration in *N. benthamiana* leaves and studied by confocal microscopy. Confocal images show GFP (green) and chloroplast autofluorescence (red) and were taken 2 days post infiltration (dpi). Ev, empty vector control.

(B) Expression levels of C-terminally MYC-tagged Bs4 and Bs4 domains in agroinfiltrated *N. benthamiana* leaves. All T-DNA constructs were agro-coinfiltrated with *35S-p19*. Proteins of expected size (white arrowheads) were detected two dpi in crude extracts by protein gel blot analysis with an α-MYC antibody. The most abundant NB-LRR degradation product is labeled (black diamond). Total extracts were stained with Coomassie to demonstrate equal loading.

(C) Expression levels of N-terminally MYC-tagged Bs4 and Bs4 domains in yeast. Crude yeast lysates were studied with an α-MYC antibody. Note that size differences of corresponding fusion proteins in yeast and *in planta* are due to translational fusion of ten tandemly arranged MYC epitopes (B) or a single MYC epitope tag (C).

We wondered whether the high and low abundance of the TIR and NB-LRR domains, respectively are Bs4 specific or a more general phenomenon of TIR-NB-LRR proteins and tested the expression levels of tobacco N domains in crude extracts from agroinfiltrated *N. benthamiana*. Protein gel blot analysis of MYC-tagged N-domains revealed that the steady-state levels of tobacco N-derived TIR and NB-LRR domains were substantially higher and lower, respectively than full-length N-protein and all other tested N-domains (Figure 6). Thus, the relative abundance of the tobacco N protein and the respective tomato Bs4 domains was similar when corresponding constructs were expressed *in planta* or in yeast, which might suggest common regulatory mechanisms in the control of Bs4 and N abundance.

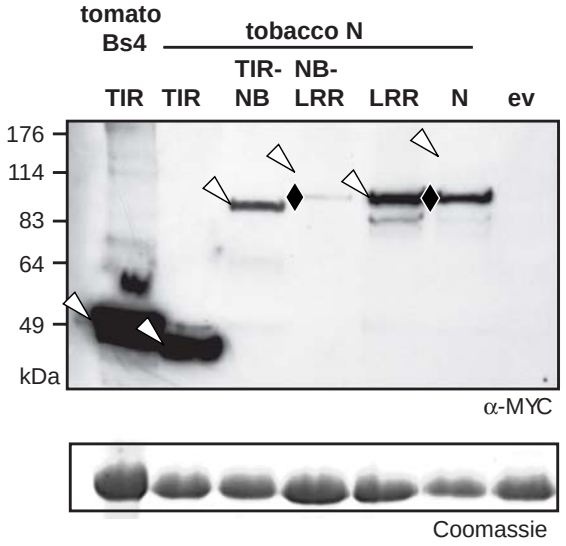

Figure 6. In planta expression levels of tobacco N and N domains.

MYC-tagged N and N domains were expressed in *N. benthamiana* leaves via agroinfiltration. Proteins of expected size (white arrowheads) were detected two dpi in crude extracts by protein gel blot analysis with an α-MYC antibody. The most abundant NB-LRR degradation product is labeled (black diamond). Total extracts were stained with Coomassie to demonstrate equal loading.

Physical interactions between domains of Bs4

In previous studies describing the CC-NB-LRR proteins Rx and Bs2 it was shown that the LRR domain interacted *in planta* with the CC-NB domain fusion, as did the CC with the NB-LRR (Moffett et al., 2002; Leister et al., 2005). To analyze if domains of the TIR-NB-LRR protein Bs4 would also interact with themselves *in planta*, we coexpressed hemagglutinin- (HA) tagged Bs4 LRR and NB-LRR domains together with MYC-tagged TIR-NB and TIR domains in *N. benthamiana*, respectively and performed CoIP experiments. Immunoprecipitation of HA-tagged Bs4-domains, followed by protein gel blot analysis of MYC-tagged Bs4 domains, revealed association between TIR-NB and LRR domains (Figure 7A; lane 3) as well as association between the TIR and NB-LRR domains (Fig 7B; lane 3). The domain associations were not detectable upon coexpression

of *avrBs4*, (Figure 7A; lane 4 and Figure 7B; lane 4) suggesting that activation of Bs4 is linked to a change in its domain interaction status. We can rule out that this loss of association is due to protein degradation because (i) the transfected tissue was harvested one dpi, which is prior to HR formation (3-4 dpi) and (ii) Bs4 domains were equally abundant either in the presence or the absence of AvrBs4 (Figure 7A; lanes 3 and 4; Figure 7B; lanes 3 and 4). We also studied association between Bs4 domains by Y2H. However, although expression of appropriately-sized bait and prey proteins could be demonstrated, Bs4 domains seem not to interact in yeast (data not shown).

Taken together, *in planta*-expressed combinations of Bs4 TIR plus NB-LRR as well as TIR-NB plus LRR domains were shown to coimmunoprecipitate in the absence,

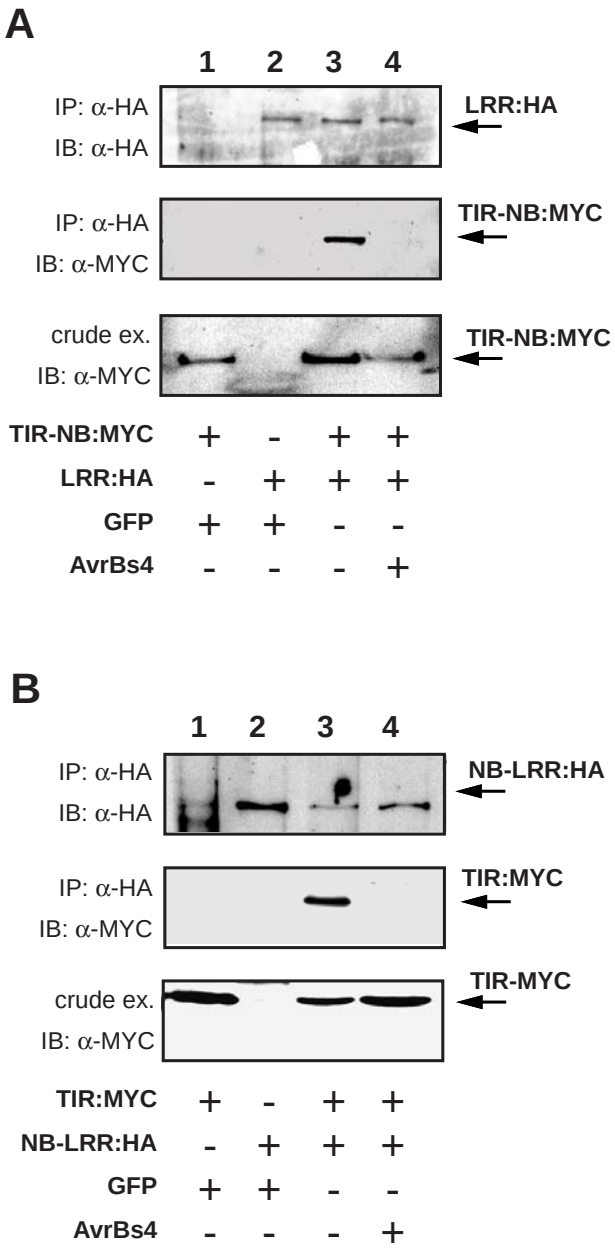

Figure 7. Association of Bs4 domains in planta.

(A) Coimmunoprecipitation of Bs4 TIR-NB and LRR domains. *TIR-NB:MYC* and *LRR:HA* constructs were agroinfiltrated into *N. benthamiana* either separately with *GFP* (lanes 1, 2) or together without (lane 3) or with *avrBs4* (lane 4). All T-DNA constructs were agro-coinfiltrated with *35S-p19*. Total protein extracts were immunoprecipitated with α-HA antibodies and subjected to protein gel blot analysis with either α-HA or α-MYC antibodies. Expression of MYC-tagged proteins was studied in crude extracts. Arrows indicate predicted sizes of proteins.

(B) Coimmunoprecipitation of Bs4 TIR and NB-LRR domains. *TIR:MYC* and *NB-LRR:HA* were agroinfiltrated into *N. benthamiana* either separately with *GFP* (lanes 1, 2) or together without (lane 3) or with *avrBs4* (lane 4). Protein gel blot analysis was conducted as described in (A).

but not in the presence, of AvrBs4. In contrast, the same domain combinations did not interact in Y2H, suggesting that additional plant proteins are crucial for this interaction.

Expression of* avrBs4 *triggers Bs4 disappearance

Since it has been suggested that stability and activity of NB-LRR proteins is interconnected (Boyes et al., 1998) we studied Bs4 abundance in a time course experiment. Upon agroinfiltration of a *35S-Bs4:MYC* construct into *N. benthamiana* Bs4:MYC abundance peaked at about 1-2 dpi and declined at 4 dpi (data not shown). Co-transfection of *Bs4:MYC* with *p19*, which encodes a potent inhibitor of post-transcriptional gene silencing (PTGS) (Voinnet et al., 2003) led to a constant gradual increase of Bs4:MYC levels within the time frame studied (1, 2 and 4 dpi; data not shown) and allowed us to study Bs4:MYC abundance without interference from PTGS. Although Bs4:MYC accumulated to higher levels when coexpressed with than without p19 the AvrBs4-triggered HR was not visibly altered by p19 coexpression (data not shown). Thus our data indicate that the speed and extent of the Bs4-mediated HR reaction are not significantly changed by raising Bs4 levels.

Next we analyzed Bs4:MYC abundance during the course of the Bs4-mediated HR. Protein gel blot analysis showed that Bs4:MYC levels declined upon coinfiltration with a *35S-avrBs4* construct (Figure 2C; compare lanes 2 and 3 with lanes 5 and 6). The AvrBs4-triggered decline of Bs4 was visible in the presence and absence of p19 (Figure 2C and data not shown). Thus, the decline of Bs4:MYC is unlikely to be related to PTGS. If the AvrBs4-triggered disappearance of Bs4 is causally linked to its activation, we would expect that not only AvrBs4 but any elicitor that triggers the Bs4 mediated HR would induce the decline of Bs4 levels. Indeed, Bs4:MYC levels dropped not only upon agro-coinfiltration with *35S-avrBs4* (Figures 2C and 8A) but also upon agro-coinfiltration with a *35S-avrBs3* construct (Figure 8A), which had previously been shown to trigger a *Bs4*-dependent HR (Schornack et al., 2004). When driven by the weak *Bs4* promoter ($p604_{Bs4}$), *avrBs4* but not *avrBs3* triggers *Bs4*-dependent HR (Schornack et al., 2005) and decline of Bs4:MYC levels. Thus, only *avr*-constructs that triggered a *Bs4*-dependent HR triggered disappearance of Bs4:MYC. Notably, the levels of endogenous NbSGT1 did not change upon coexpression with AvrBs4 or its derivatives (Figure 8A), suggesting that the disappearance of Bs4:MYC is not a consequence of general protein degradation at the onset of HR (Figure 8A).

Since AvrBs4 triggers disappearance of the full-length Bs4 protein we wondered if individual Bs4 domains or domain combinations could be targeted by this mechanism. Coexpression of AvrBs4 together with TIR, NB, LRR, TIR-NB or NB-LRR domains of Bs4 led to decreased levels of all domains except the TIR domain (Figure 8B). However, the decrease of TIR-NB and NB-LRR domains was more severe than that of the NB or LRR domains of Bs4. Thus our data suggest that AvrBs4 does not trigger disappearance of all Bs4 domains to the same extent. Furthermore these data suggest that the AvrBs4-induced decline does not require a functional Bs4 protein, since the tested Bs4 domains were not capable of triggering an HR when expressed by themselves.

To study the specificity of the AvrBs4-induced Bs4 decline we also analyzed the tobacco N protein, which is 54% identical to Bs4. Coexpression of a MYC-tagged N protein with various *avrBs4*- and *avrBs3*-derivatives that trigger the Bs4 HR did not

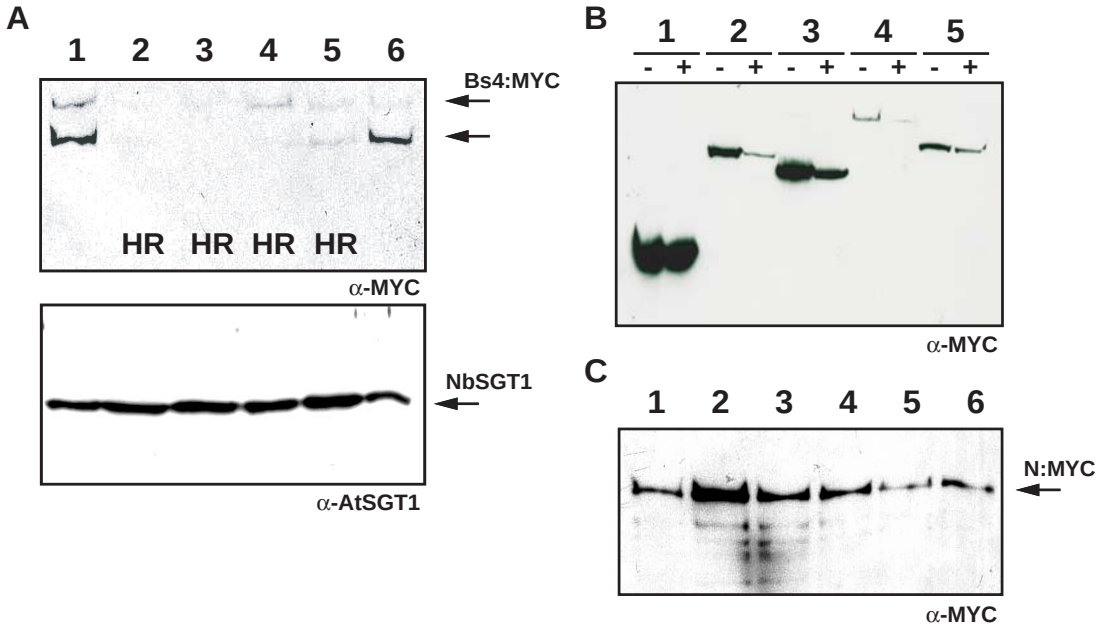

Figure 8. AvrBs4 induces disappearance of Bs4 and Bs4 domains but not of tobacco N.

(A) Bs4 levels decline upon coexpression of HR eliciting Avr constructs. A *35S-Bs4:MYC* construct was agroinfiltrated in *N. benthamiana* together with empty vector (lane 1), *35S-avrBs4* (lane 2), *35S-avrBs4Δ227* (lane 3), *avrBs4* under control of the weak $p604_{Bs4}$ promoter (4) and *avrBs3* driven by 35S- and $p604_{Bs4}$-promoter (lanes 5, 6). Samples were harvested 1.5 dpi. Constructs, that triggered HR response at 4-6 dpi are labeled (HR). Expression levels of Bs4:MYC were studied in crude leaf extracts by α-MYC protein gel blot analysis. The protein gel blot was re-probed with α-AtSGT1 antiserum to demonstrate equal loading of total protein. Arrows indicate predicted sizes of proteins.
(B) AvrBs4 induced disappearance of Bs4 domains. C-terminally MYC-tagged TIR (lane 1), TIR-NB (lane 2), NB (lane 3), NB-LRR (lane 4) and LRR (lane 5) domains of Bs4 were transiently expressed in *N. benthamiana* together with GFP (-) or AvrBs4 (+). Expression levels of Bs4 domains were studied by protein gel blot analysis with α-MYC antibody. Leaf samples were harvested at 1.5 dpi.
(C) Steady-state levels of tobacco N do not decline upon coexpression of AvrBs4 or AvrBs3. *35S-N:MYC* was agroinfiltrated in *N. benthamiana* together with the *avr*-constructs as defined in (A). N:MYC fusions were detected with an α-MYC antibody. Leaf samples were harvested at 1.5 dpi. The expected size of tobacco N is indicated (arrow).

cause a visible change of N:MYC abundance (Figure 8C). Thus elicitors that trigger the decline of Bs4 and Bs4 domains do not affect the structurally-related tobacco N protein.

Hsp90 is required for Bs4 mediated HR in tomato and N. benthamiana *and interacts with Bs4* in planta

Mutations in and silencing of *Hsp90*, which encodes a molecular chaperone (Sangster and Queitsch, 2005) affect the abundance and the function of multiple NB-LRR proteins (Hubert et al., 2003; Lu et al., 2003; Liu et al., 2004; Holt et al., 2005). Furthermore Hsp90 associates with the tobacco N protein which is structurally related to tomato Bs4 (Liu et al., 2004). We therefore wondered if Hsp90 is involved in the AvrBs4-triggered Bs4 HR and disappearance.

Upon tobacco rattle virus (TRV)-based virus induced gene silencing (VIGS) (Liu et al., 2002a) of *NbHsp90-2* (> 89% identical with tomato and *N. benthamiana Hsp90* genes) (Liu et al., 2004) neither AvrBs3- nor AvrBs4-delivering *Xcv* strains triggered an HR in

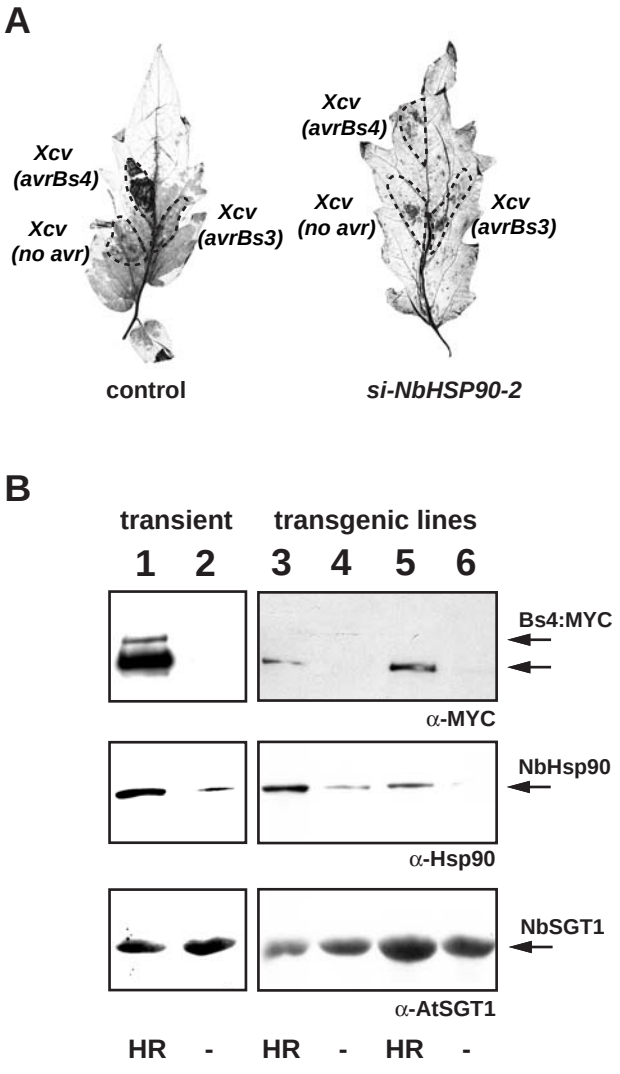

Figure 9. Hsp90 is required for Bs4 function and stability.

(A) *Bs4* mediated recognition of *avrBs4* expressing *Xanthomonas* strains is suppressed in *Hsp90* tomato knockdown plants. Bs4 tomato plants (*L. esculentum* cv. Moneymaker) were agroinfiltrated with *NbHsp90-2*- or *GFP*- (negative control) silencing constructs. About 19 days post infiltration (dpi) leaves were challenge inoculated with *Xcv* expressing *avrBs4*, *avrBs3* or none of the both. Leaves were harvested 3 dpi and destained by incubation in ethanol for better visualization of the HR.

(B) VIGS of *Hsp90* results in reduced Bs4 levels. *N. benthamiana* wildtype plants (lanes 1, 2) and two independent *35S*-Bs4:MYC transgenic lines (lanes 3+4, 5+6) were agroinfiltrated with silencing constructs *GFP* (lanes 1, 3, 5) or *NbHsp90-2* (lanes 2, 4, 6). About 14 dpi *35S-Bs4:MYC* was agroinfiltrated into wildtype plants (lanes 1, 2). Three days later leaf patches were harvested and crude extracts were subjected to protein gel blot analysis using α-MYC, α-Hsp90 and α-AtSGT1 antisera. Below response to infiltration of AvrBs4 is listed. Arrows indicate predicted sizes of proteins.

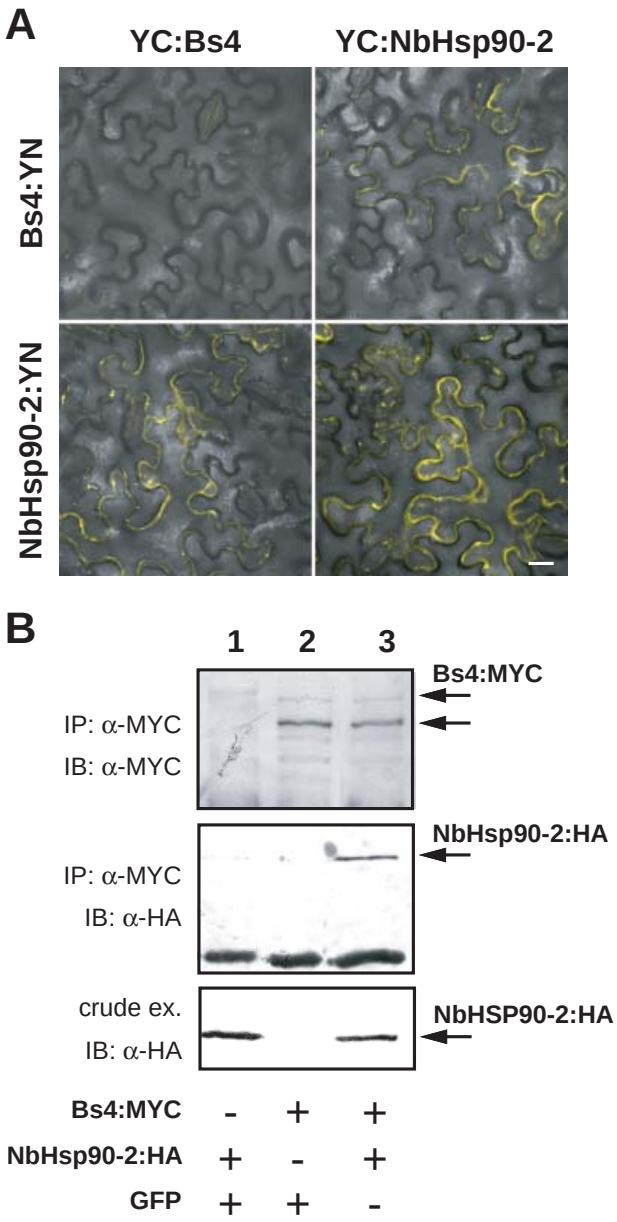

Figure 10. Hsp90 associates with Bs4 in planta.

(A) Bimolecular fluorescence complementation of Hsp90 and Bs4. T-DNA constructs expressing *NbHsp90-2* and *Bs4* fused to both, N- and C-terminal YFP halves (YN, YC) were agroinfiltrated into *N. benthamiana*. Two days post infiltration leaf patches were inspected for YFP fluorescence. Bar = 20 µm.

(B) Coimmunoprecipitation of Bs4 and Hsp90. T-DNA constructs expressing 35S-*NbHsp90-2:HA* and *35S-Bs4:MYC* were agroinfiltrated either separately with *35S-GFP* (lanes 1, 2) or together (lane 3) into *N. benthamiana*. All T-DNA constructs were agro-coinfiltrated with *35S-p19*. Crude plant proteins were extracted 24 h post infiltration and immunoprecipitated using α-MYC (IP). IP fractions and crude extracts were subjected to α-MYC (IP) and α-HA (CoIP and crude extracts) protein gel blot analysis. Arrows indicate predicted sizes of proteins.

Lycopersicon esculentum cv. Moneymaker, a *Bs4*-resistant tomato cultivar (Figure 9A). In contrast AvrBs4-, but not AvrBs3-containing *Xcv* strains triggered HR in control plants that were infiltrated with the empty TRV-VIGS vector (Figure 9A). Likewise, in *N. benthamiana* transient coexpression of *Bs4* and *avrBs4* triggered HR in control plants that were infiltrated with the empty TRV-VIGS vector but not in *NbHsp90-2* silenced plants (data not shown). Protein gel blot analysis demonstrated that *Hsp90* knockdown plants had not only reduced Hsp90 but also reduced Bs4 levels when compared to the non-silenced control plants (Figure 9B).

In summary, silencing of *Hsp90* leads to reduced Bs4 levels and abrogates the Bs4 HR in tomato and *N. benthamiana*.

Using BIFC we tested whether Hsp90 would interact with the tomato Bs4 protein *in planta*. Coexpression of *Bs4:YN* and *YC:NbHsp90-2* or the reciprocal combination, *NbHsp90-2:YN* and *YC:Bs4* yielded YFP fluorescence in the cytoplasm (Figure 10A) indicating that Bs4 and NbHsp90-2 interact *in planta.* Furthermore this finding demonstrates that our *YC:Bs4* construct is functional in the BIFC assay and is likely to be suitable for studying interaction of Bs4 and AvrBs4 (see Figure 3).

As a complementary approach to BIFC we used CoIP to study the Bs4-Hsp90 interaction. We agroinfiltrated *N. benthamiana* with constructs encoding MYC-tagged Bs4 and HA-tagged NbHsp90-2. Immunoprecipitation of a MYC-tagged Bs4, followed by protein gel blot analysis with a HA-specific antibody resulted in identification of a band corresponding to the molecular weight of HA-tagged NbHsp90-2 (Figure 10B). Thus both, CoIP and the BIFC assay demonstrated that Bs4 and NbHsp90-2 interact *in planta*.

Overexpression of NbHsp90-2 suppresses AvrBs4-induced decline of Bs4 and activation of HR

Given that the Bs4-mediated HR is *Hsp90* dependent (Figure 9A) and associated with the disappearance of Bs4 (Figure 8A) we speculated that *Hsp90* overexpression would stabilize Bs4 and thus possibly interfere with its function. Indeed, the AvrBs4-triggered and Bs4-mediated HR in *N. benthamiana* was reduced by coexpression of a 35S-driven *NbHsp90-2:HA* construct but not by coexpression of *35S-GFP* (Figure 11A). Notably, coexpression of *NbHsp90-2:HA* and *AvrBsT* (encodes a *Xcv* avirulence protein that was shown to trigger an HR in *N. benthamiana*) (Orth et al., 2000) triggered HR, demonstrating that these plants were in principle capable of executing an HR. To quantify the effects of *Hsp90* overexpression, we measured the leakage of electrolytes from leaf discs, a physiological response that is associated with the HR (Baker et al., 1991). In agreement with the reduced HR phenotype, the conductance was significantly reduced upon *NbHsp90-2:HA* but not upon *GFP* overexpression (Figure 11B). To account for potential effects caused by *Bs4* overexpression we replaced *35S-Bs4:MYC* with the native promoter driven *Bs4* construct $p3516_{Bs4}$*-Bs4* (Schornack et al., 2005) and obtained similar results (Figure 11B). Subsequent protein gel blot analysis of crude *N. benthamiana* protein extracts demonstrated that overexpression of *NbHsp90-2:HA* seemed to suppress the AvrBs4 triggered Bs4 decline (Figure 11C).

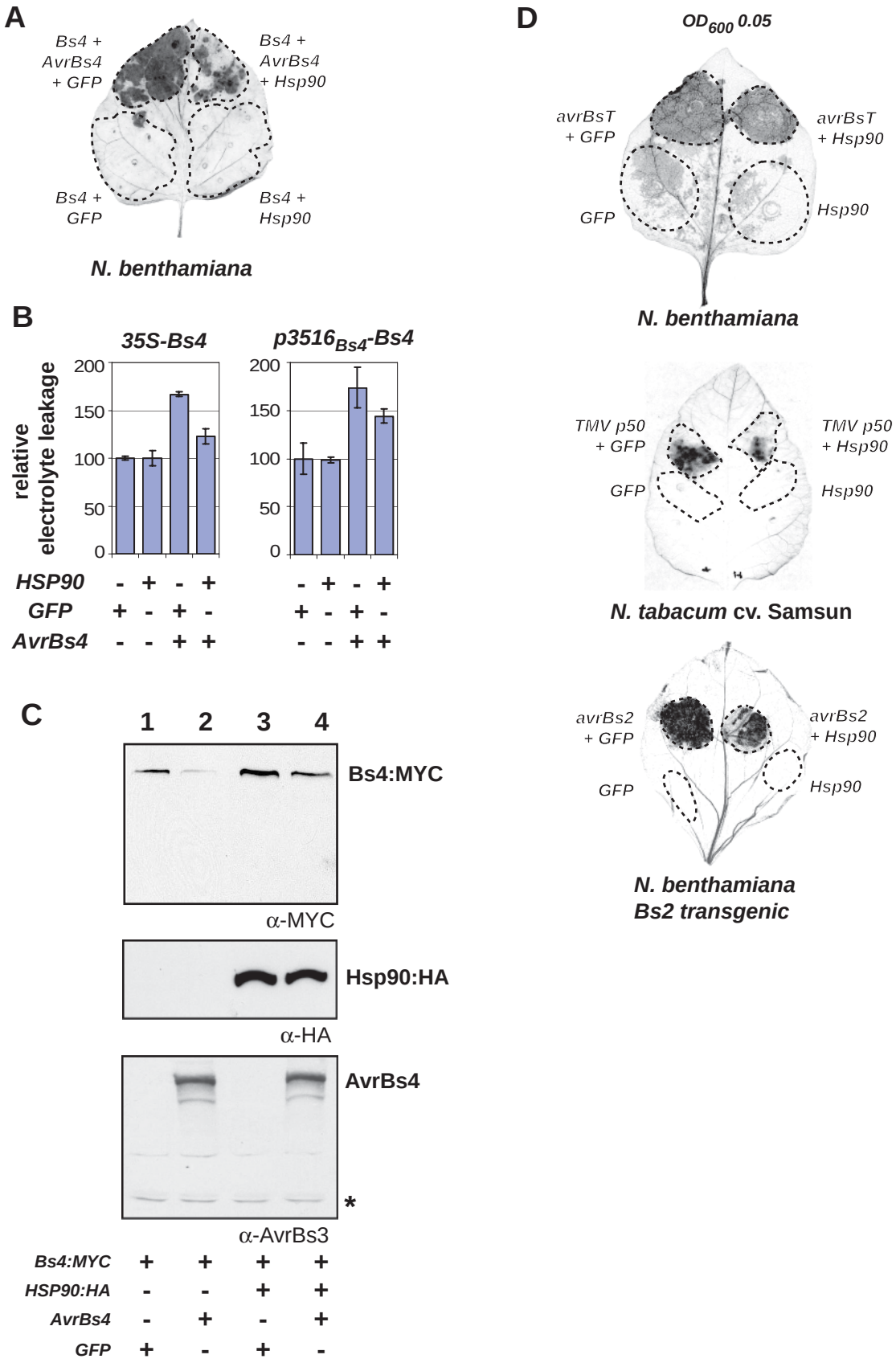

Figure 11 (for legend see next page)

Figure 11 (previous page). Hsp90 overexpression affects the function of several NB-LRR proteins.
(A) HR intensity is reduced upon *Hsp90* overexpression. *35S-Bs4:MYC* was agroinfiltrated into *N. benthamiana* leaf either with or without *35S-GFP:avrBs4*Δ152. The left leaf half was additionally infiltrated with *35S-GFP*, the right half with *35S-Hsp90:HA*.
(B) *Hsp90* overexpression reduces AvrBs4-Bs4 specific electrolyte leakage. *N. benthamiana* leaf patches were infiltrated with *35S-Bs4:MYC* (left panel) or *Bs4* under control of its own promoter ($p3516_{Bs4}$-*Bs4*, right panel). Additionally, 35S-*GFP*, *35S-Hsp90-2:HA* and *35S-GFP:avrBs4*Δ152 were infiltrated as indicated below the diagrams. Leaf discs were harvested 1 dpi for electrolyte leakage measurements.
(C) Bs4 protein levels are affected upon *Hsp90* overexpression. *35S-Bs4:MYC* was agroinfiltrated together with *35S-GFP* (lanes 1, 2) or *35S-Hsp90-2:HA* (lanes 3, 4). Additionally *35S-GFP:avrBs4*Δ152 was infiltrated (lanes 2, 4). Bs4 and Hsp90 were detected using α-MYC and α-HA antisera on crude plant protein extracts, respectively.
(D) Intensity of N and Bs2 mediated HR, but not of AvrBsT triggered HR is reduced upon *Hsp90* overexpression. The upper leaf halves of *N. benthamiana* wildtype, *N. tabacum* cv. Samsun NN or *N. benthamiana Bs2* transgenic plants were infiltrated with *35S-*A*vrBsT, 35S-TMV p50* or *35S-avrBs2*, respectively. Additionally, left halves were infiltrated with *35S-GFP* and right halves with *35S-Hsp90-2:HA*.
In all infiltrations omitted specific T-DNA constructs were substituted by empty vector to maintain equal *Agrobacterium* optical density. Leaves were harvested 4 dpi and cleared in ethanol.

Taken together, overexpression of *NbHsp90-2:HA* counteracts the AvrBs4-induced Bs4 disappearance and suppresses the Bs4 mediated HR.

NbHsp90-2 *overexpression perturbs the function of the pepper Bs2 and the tobacco N protein*

Effects of *NbHsp90-2* overexpression on the functionality of the tobacco N and the pepper Bs2 proteins were also tested. We agroinfiltrated T-DNAs encoding TMV p50 (Avr protein corresponding to the tobacco N protein) or AvrBs2 into *N. tabacum* cv. *Samsun* NN (contains the tobacco *N* gene) (Whitham et al., 1994) or a *Bs2* transgenic *N. benthamiana* plant (Leister et al., 2005), respectively. Agro-coinfiltration of a *35S-NbHsp90-2:HA* but not of a *35S-GFP* T-DNA significantly reduced the intensity of the tobacco N and the pepper Bs2 HRs (Figure 11D) indicating that high levels of Hsp90 perturb the function of many, if not all NB-LRR proteins.

Polyubiquitination may play a role in Bs4 resistance response

The ubiquitin system is a major pathway for regulated protein degradation in eukaryotes (Welchman et al., 2005). To test whether the AvrBs4-induced decline of Bs4 involves ubiquitination we took advantage of the dominant-acting ubiquitin mutant ubR48 (Schlögelhofer et al., 2005) which terminates extension of the nascent ubiquitin chain by preventing linkages with additional ubiquitin monomers via lysine 48. Since efficient proteasome binding and degradation requires a ubiquitin chain of at least four subunits (Hicke, 2001) ubR48 suppresses ubiquitin-dependent protein degradation of a given target protein (Schlögelhofer et al., 2005).

Coexpression of an eight-repeat-*ubR48* construct (*35S-BW8*) with *Bs4* (*35S-Bs4:MYC*) and *avrBs4* (*35S-GFP:AvrBs4*Δ152) in *N. benthamiana* resulted in severe reduction of the HR and electrolyte leakage (Figures 12A and 12B). Notably, HR was reduced more drastically upon coexpression with ubR48 than with Hsp90 (Figures 11A and 12A). Identical phenotypic results were observed when the 35S-driven *Bs4* construct (*35S-Bs4:MYC*) was replaced with the native promoter *Bs4* construct ($p3516_{Bs4}$-*Bs4*; data not shown). The AvrBsT elicited HR was not attenuated by coexpression of *35S-ubR48* (Figure 12C) indicating that ubR48 does not interfere with cell death in general.

To address whether ubR48-mediated HR reduction is not only restricted to Bs4 we agroinfiltrated stably transformed *N. benthamiana* plants carrying potato *Rx* (Peart et al., 2002), pepper *Bs2* or tobacco *N* with T-DNAs encoding their cognate Avr proteins (all driven by 35S promoter). In addition we agro-coinfiltrated either a *35S-GFP* or a *35S-ubR48* T-DNA construct. Coexpression of ubR48 but not GFP significantly reduced the Rx-, Bs2- and N-mediated HRs (Figure 12D) indicating that polyubiquitination is important for functionality of many NB-LRR proteins.

If Bs4 itself would undergo ubiquitin-dependent proteolysis *ubR48* overexpression should result in increased Bs4 levels. However, protein gel blot analysis revealed that Bs4:MYC levels in crude *N. benthamiana* extracts were decreased upon *ubR48* overexpression relative to overexpression with GFP (Figure 12E) suggesting that the AvrBs4-dependent decline of Bs4 is not ubiquitin dependent.

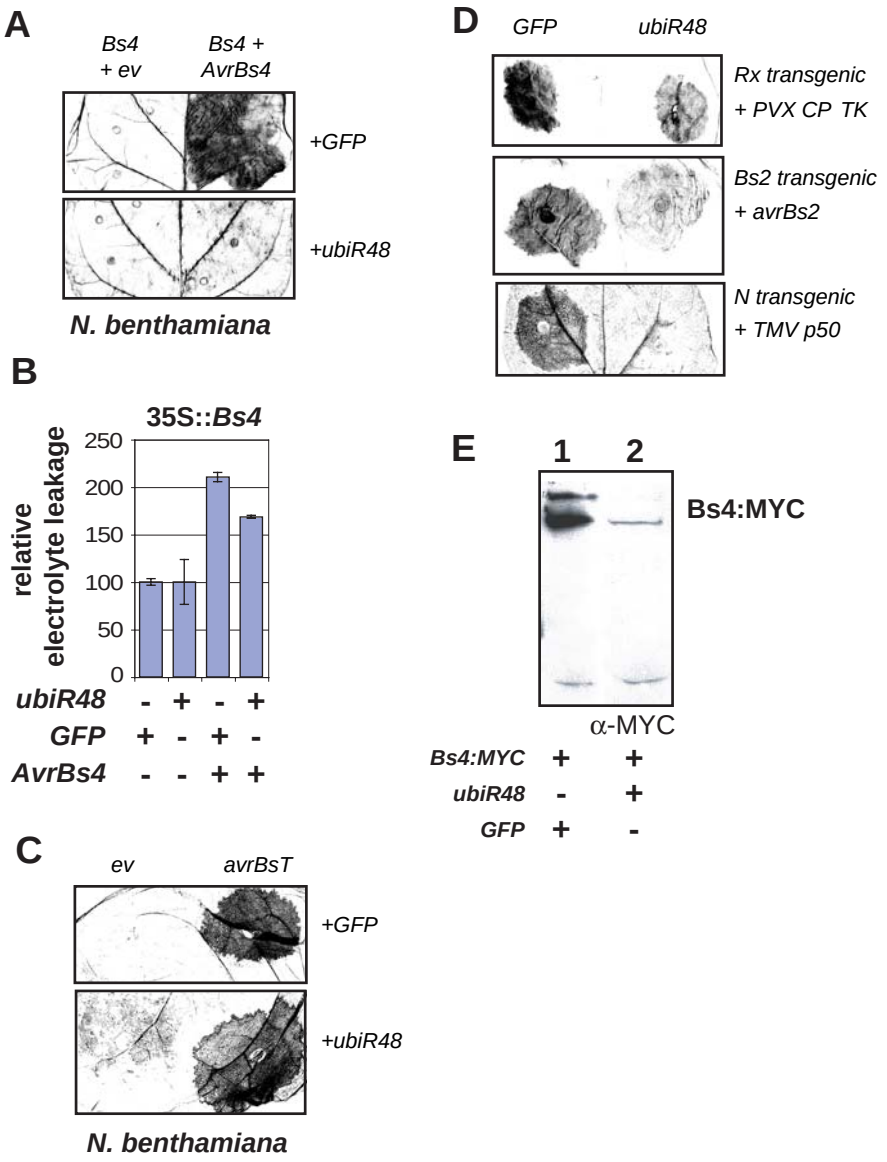

Figure 12. Overexpression of ubR48 ubiquitin mutant attenuates HR associated with several resistance specificities.

(A) Overexpression of *ubR48* reduces HR intensity. *35S-Bs4:MYC* was agroinfiltrated into *N. benthamiana* wildtype leaf either with *35S-GFP* (upper panel) or with an ubiR48-expressing construct (*35S-BW8*, lower panel). The left leaf halves were additionally infiltrated with empty vector (ev), the right half with *35S-GFP:avrBs4*Δ152.

(B) *ubR48* overexpression reduces AvrBs4-Bs4 specific electrolyte leakage. *N. benthamiana* leaf patches were infiltrated with *35S-Bs4:MYC*. Additionally, *35S-GFP*, *35S-BW8* and *35S-GFP:avrBs4*Δ152 were infiltrated as indicated below the diagrams. Leaf discs were harvested 1 dpi for electrolyte leakage measurements.

(C) AvrBsT-triggered HR in *N. benthamiana* is not affected by *ubR48* overexpression. *N. benthamiana* wildtype leaf patches were infiltrated either with *35S-GFP* (upper panel) or with *35S-BW8* (lower panel). The left leaf halves were additionally infiltrated with empty vector (ev), the right half with *35S-avrBsT*.

(D) HRs elicited by Rx – PVX coat protein (CP TK), Bs2 – AvrBs2, N – TMV p50 are all affected by overexpression of *ubR48*. Leaf patches of several *N. benthamiana* lines harboring *Rx*, *Bs2* or *N*, respectively, were infiltrated with the corresponding *avr* genes (*35S-PVX CP TK*, *35S-avrBs2*, *35S-TMV p50*). Additionally left leaf halves were infiltrated with *35S-GFP* and right halves with the ubiR48-expressing construct *35S-BW8*.

(E) Bs4 protein levels are affected upon *ubR48* overexpression. *35S-Bs4:MYC* was agroinfiltrated together with *35S-GFP* (lane 1) or *35S-ubR48* (lane 2). Bs4 was detected using α-MYC antiserum on crude plant protein extracts.

DISCUSSION

No evidence for AvrBs4-Bs4 association

Y2H, CoIP and BIFC studies failed to detect Bs4-AvrBs4 interaction or association. Lack of a Bs4-AvrBs4 interaction in Y2H (Schornack et al., 2004) could be due to the absence of necessary plant proteins or due to unsuitable cellular context in yeast. We also failed to detect AvrBs4-Bs4 associations using CoIP from plant extracts (data not shown) indicating that AvrBs4 and Bs4 are not part of a common high-molecular weight complex. As, the Bs4-AvrBs4 interaction might be transient and thus difficult to detect by CoIP we used BIFC, as YFP reconstitution is irreversible and thus traps transient interactions (Magliery et al., 2005). However, our BIFC studies provided no indication for an AvrBs4-Bs4 interaction which might be due to insufficient flexibility of AvrBs4- or Bs4-fusion proteins that possibly hinders YFP reconstitution (Bracha-Drori et al., 2004) Altogether, the lack of an AvrBs4-Bs4 interaction in our assays might be due to limitations of the methods or the experimental design and still does not exclude formation of such interaction complexes.

From studies on AvrBs3, which shares 97% sequence identity with AvrBs4 (Bonas et al., 1993) we can infer that AvrBs4 exerts its virulence activity via transcriptional reprogramming of the host and that the NLS and AD domains are crucial to this function (Schornack et al., 2006). The fact that deletion derivatives of AvrBs4 lacking the NLS and/or the AD (NLS/AD) retain their avirulence activity towards Bs4 (Ballvora et al., 2001b; Schornack et al., 2004; Schornack et al., 2006) indicates that recognition of AvrBs4 by Bs4 does not involve transcriptional reprogramming of the host. Bs4 mediates not only recognition of AvrBs4 NLS/AD deletion derivatives but also of NLS/AD deletion derivatives of Hax3 and Hax4, two recently identified AvrBs3-like proteins (Kay et al., 2005). Given that structural conservation rather than virulence activity of the Avr component is crucial to Bs4 mediated recognition we assume that Bs4 and corresponding AvrBs3-like proteins interact directly, despite the fact that we have not been able to validate this model experimentally.

What is the functional relevance of Bs4 disappearance?

The observation that AvrBs4 triggers disappearance of Bs4 raises the question of the functional relevance of this process? In one scenario AvrBs4-induced Bs4 degradation could be the initiating event that links recognition and downstream signaling. However, AvrBs4 triggered dissociation of Bs4 domains occurs already at 1 dpi, while reduction in abundance of Bs4 or Bs4 domains was not detectable by that time point (Figure 7 and data

not shown). Yet, low levels of Bs4 degradation products that are not detectable by protein gel blot analysis and that may arise immediately after or even before Bs4 undergoes a change in its domain interaction status might be sufficient to trigger HR. Alternatively, Bs4 disappearance might limit the HR, preventing it from continuing or spreading beyond when and where it is needed, a model that has been proposed previously for the *Arabidopsis* NB-LRR protein RPM1 (Boyes et al., 1998). Like Bs4, RPM1 levels decline rapidly upon elicitation (Boyes et al., 1998; Kawasaki et al., 2005). While the RPM1 studies were limited to the full-length protein we showed that not only the full-length Bs4 protein but also the levels of all Bs4 domain fusions except the TIR domain decline after the introduction of AvrBs4 (Figure 8B). AvrBs4-triggered decline of Bs4 domains that are expressed in combinations that do not trigger HR demonstrates that Bs4 disappearance is not sufficient to activate HR.

Stability of Bs4 domain constructs affects* trans*-complementation

We could show that coexpressed Bs4 TIR-NB and LRR domains interact physically and complement functionally. We also demonstrated CoIP of the Bs4 TIR with the Bs4 NB-LRR domains but failed to detect functional complementation for this combination (Figure 7B and data not shown). How can we explain the lack of complementation for the combination of Bs4 TIR and NB-LRR domains despite the fact that these domains interact physically (Figure 7B)? Inspection of relative steady-state levels showed that the Bs4 NB-LRR is only poorly expressed (Figure 5). Since the domain expressed at the lowest level determines the abundance of the complex, it is reasonable to propose that overall levels of Bs4 reconstituted from the TIR and NB-LRR domains were too low to mediate AvrBs4 recognition. A requirement for a threshold level of NB-LRR protein for activation of the HR has been described for the barley Mla protein (Bieri et al., 2004). Support for a Bs4 threshold model is also provided by the fact that transcriptional fusion of a 74 bp *Bs4* promoter in front of the *Bs4* ORF does not mediate AvrBs4 recognition although the same promoter still generates low but measurable GUS activities (Schornack et al., 2005). It would therefore be interesting to see whether overexpression of the NB-LRR domain using a promoter much stronger than 35S (Ni et al., 1995) would enable functional complementation of the TIR domain.

Disruption of domain interactions by AvrBs4

Pioneering work by Moffett et al. (2002) demonstrated that complementing protein fragments of potato Rx or pepper Bs2 interact physically in the absence but not in the presence of the corresponding elicitor. These studies were carried out in *SGT1*-silenced

plants in order to suppress HR related protein degradation without knowledge of the chaperone-like activity of SGT1 (Muskett and Parker, 2003; Schulze-Lefert, 2004; Holt et al., 2005; Azevedo et al., 2006). More recent analysis of pepper Bs2 in non-silenced plants showed no change in domain interaction status upon coexpression of the cognate AvrBs2 protein (Leister et al., 2005). We conducted our studies as well in the wildtype background prior to any visible HR symptoms and detected interaction of Bs4 domains in the absence but not in the presence of AvrBs4 (Figure 7). In this context it should be noted that Bs4 differs from Rx and Bs2 in being a member of the TIR subclade of NB-LRR proteins. Thus structural differences in TIR- and non-TIR NB-LRR proteins might give rise to different results in the context of domain-interaction studies. When comparing Bs4 to other plant NB-LRR proteins and the NB containing apoptotic protease activating factor 1 (APAF1) we found a stretch of amino acids that is present in Bs4 and the ARC3 domain of APAF1 but not detectable in other TIR- or CC-NB-LRR proteins. Whether this observation has functional relevance remains to be determined.

How does Hsp90 affect Bs4 function?

We used silencing and overexpression of *N. benthamiana Hsp90* to address its relevance in Bs4 function. Previous studies had shown that *Hsp90* silencing abolishes the function of several TIR- and CC-NB-LRR proteins (Liu et al., 2002b; Liu et al., 2002c; Lu et al., 2003; Zhang et al., 2004; de la Fuente van Bentem et al., 2005). In agreement with these studies we found that *Hsp90* silencing abolishes the Bs4-mediated HR in tomato and *N. benthamiana* (Figure 9 and data not shown). This suggests that Hsp90 is a component that is crucial to the function of many or possibly all NB-LRR proteins. Notably, *in planta* expression of the *Xanthomonas* AvrBsT protein triggered an HR in *Hsp90 N. benthamiana* knockdown plants (Figure 11) demonstrating that silencing of *Hsp90* does not inhibit plant cell death response in general. In addition these data suggest that AvrBsT recognition in *N. benthamiana* is somewhat unique and may not involve NB-LRR proteins.

While *Hsp90* knockdown and *Hsp90* mutant plants had been studied in the context of several gene-for-gene interactions, the molecular consequences of *Hsp90* overexpression on NB-LRR function had to our knowledge not been addressed so far. A reason for that may be that overexpression of *Hsp90* in stable transgenics affects plant viability, probably because proteins chaperoned by Hsp90 are also involved in many processes. We studied the effects of *Hsp90* overexpression on Bs4 function by *Agrobacterium*-mediated transient expression. In this assay overexpression of *Hsp90* can be obtained in a fully developed leaf and is restricted to the infiltrated tissue patch.

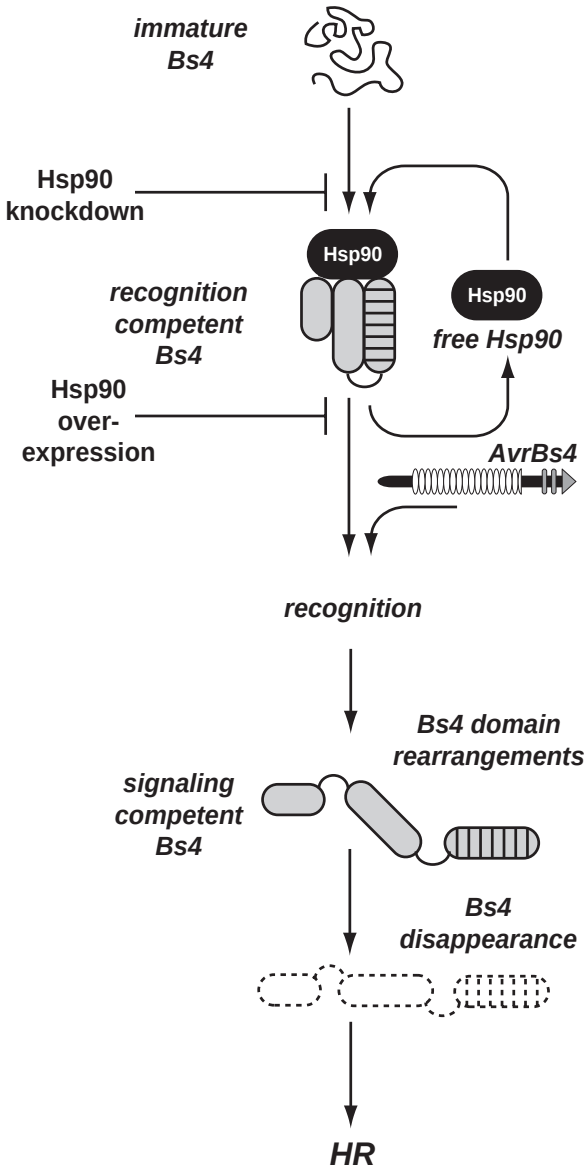

Figure 13. Model of Hsp90 controlled Bs4 regulation.

A working model for Bs4-mediated recognition of AvrBs4.
Hsp90 is required for maturation of recognition competent Bs4 protein. AvrBs4 triggers the transition from recognition into signaling competent Bs4. In the course of this activation the interaction of Bs4 with Hsp90 is abolished. Subsequently Bs4 undergoes intramolecular domain rearrangements that lead to Bs4 disappearance and finally to HR elicitation.

Overexpression of *Hsp90* led to reduction of the tomato Bs4, tobacco N and pepper Bs2 mediated HR but had no influence on the AvrBsT triggered HR (Figure 11). Given that Bs4 and N both interact with Hsp90 (Figure 10) (Liu et al., 2004), it seems plausible that the observed *Hsp90* overexpression effects are the consequence of a changed interaction status of Hsp90 and the given NB-LRR protein.

In addition to the studies on HR we tested Bs4 levels upon silencing and overexpression of *Hsp90*. *Hsp90* silencing resulted in reduced Bs4 steady-state levels, which is in agreement with its supposed role as a chaperone. Previous studies revealed that the levels of the CC-NB-LRR proteins RPM1 and Rx were also reduced upon mutation and silencing of Hsp90, respectively. By contrast, levels of the TIR-NB-LRR protein RPS4 were not affected upon silencing of *Hsp90* in *N. benthamiana* although HR was attenuated in these plants (Hubert et al., 2003; Lu et al., 2003; Zhang et al., 2004). Therefore, there

may be only a portion of NB-LRR proteins that are regulated in their levels by Hsp90. As Bs4 and RPS4 both are TIR-NB-LRR proteins and their steady-state levels differ in response to Hsp90 it seems unlikely that the TIR domain defines Hsp90 dependence.

Hsp90 silencing or overexpression led to reduced and increased Bs4 levels, respectively (Figures 9B and 11C), but both attenuated the Bs4 HR. How can we explain these seemingly contradictory results? It was already proposed that Hsp90 supports NB-LRR functionality in two ways: it chaperones NB-LRRs and it regulates their transition into a signaling competent form upon recognition of the cognate Avr protein (Schulze-Lefert, 2004). In accordance with this model we postulate that Bs4 needs to interact with Hsp90 in order to maturate and possibly to remain recognition competent. In *Hsp90* knockdown plants, Hsp90 levels may be too low to support generation and maintenance of sufficient recognition-competent Bs4. This may be why these plants are not able to mount an AvrBs4-triggered HR (Figure 13).

Increased availability of Hsp90 (e.g. overexpression of Hsp90) may shift the equilibrium of its interactions towards the client-bound state (Sangster et al., 2004). The observation that *Hsp90* overexpression abolishes the Bs4 HR might therefore suggest that transition into signaling competent Bs4 involves the release of its interaction with Hsp90. If we assume that AvrBs4-triggered decline of Bs4 occurs only upon disruption of the Hsp90-Bs4 complex, this would explain why *Hsp90* overexpression suppresses not only the Bs4 HR but also the disappearance of Bs4 (Figure 13).

Bs4 decline is most likely not ubiquitin-dependent

We addressed the relevance of polyubiquitin-mediated proteolysis for Bs4 HR by transient overexpression of a ubiquitin mutant (ubR48) in which lysine 48 is replaced by arginine (Schlögelhofer et al., 2005). When linked to a protein substrate, ubR48 prevents attachment of additional ubiquitin monomers and thus acts as a chain-terminator. Since polyubiquitin-dependent proteolysis requires at least a tetra-ubiquitin tag attached to the target protein (Thrower et al., 2000) ubR48 inhibits polyubiquitin-dependent proteolysis.

We found that overexpression of ubR48 attenuates not only tomato Bs4 HR but also potato Rx, pepper Bs2 and tobacco N mediated HRs (Figures 12A and 12D). Importantly, we can exclude that overexpression of ubR48 suppresses HR in general, because the AvrBsT triggered HR was not affected (Figure 12C). These data suggest that disappearance of Bs4 involves the ubiquitin-proteasome pathway, a mechanism that was already suggested for RPM1. Like Bs4, Arabidopsis RPM1 disappears coincident with the onset of HR (Boyes et al., 1998). Surprisingly, Arabidopsis transgenic lines that

overexpress ubR48 are not affected in RPM1 mediated resistance (Schlögelhofer et al., 2005), although the effect of ubR48 on RPM1-HR progression was not addressed.

It is tempting to speculate that Bs4 and NB-LRR proteins in general are ubiquitinated and proteolytically degraded. If this hypothesis is correct, overexpression of ubR48 should suppress ubiquitin-dependent degradation of Bs4. However, we observed the opposite, as overexpression of ubR48 strongly reduced Bs4 levels while at the same time the level of transiently expressed GFP increased (data not shown). Thus proteolysis of Bs4 seems unlikely to be mediated *via* the polyubiquitin pathway, and may instead be degraded by the proteasome in an ubiquitin-independent manner (Orlowski and Wilk, 2003) or degraded proteasome independently (Schmitz and Herzog, 2004). Evidence for the latter scenario in RPM1 disappearance was provided by the lack of an apparent change in RPM1 steady-state levels upon chemical inhibition of the proteasome (Holt et al., 2005).

It seems likely that ubR48 overexpression causes accumulation of misfolded proteins that recruit Hsp90 and thus lower the levels of free Hsp90. Since Bs4 needs to exceed a certain threshold level to trigger HR (Schornack et al., 2005) and since Hsp90 positively regulates Bs4 levels (Figure 11C), the effects of ubR48 on Bs4 function and Bs4 levels are possibly a consequence of reduced levels of free Hsp90. If our model is correct, we should be able to compensate the effects observed upon overexpression of ubR48 by overexpression of Hsp90. Indeed we could show that Hsp90 overexpression, which itself suppresses the Bs4 HR, compensates the negative effects of ubR48 overexpression on Bs4 function (S. Schornack & T. Lahaye, unpublished results). Taken together our data suggest that suppression of the Bs4 HR by ubR48 is not linked to ubiquitination of Bs4 but rather due to reduced availability of Hsp90.

METHODS

Gateway entry clones

A Gateway entry clone containing *avrBs4Δ152* (pENTR256) was generated by transferring the repeat containing *Stu*I-*Age*I restriction fragment of pBS256F (Schornack et al., 2004) into pENTR356NC which contains the N- and C-terminal coding regions of AvrBs3 (Gürlebeck et al., 2005). *avrBs4Δ152Δ1-3* which does not encode NLSs was generated by introducing an *Aat*II-*Sac*I fragment from pBS356ΔNLS (Szurek et al., 2001) into *Aat*II/*Sac*I digested pENTR256 to yield pENTR256ΔNLS.

An entry clone for AvrBs4 carrying the N-terminus and 5.5 repeats (pAG227) was generated by transferring AvrBs4Δ227 from pBS227F (Schornack et al., 2004) via flanking *Eco*RI-*Xho*I sites to pAGROMAC KS (S. Schornack & T. Lahaye, unpublished results) to obtain pAC227 where *avrBs4Δ227* is flanked by *att*B1 and *att*B2 sites (S. Köthke & U. Bonas, unpublished results). Gateway-LR recombination was used to transfer the insert into pAG35P (K. Peter & T. Lahaye, unpublished results) to obtain pAG227 where *avrBs4Δ227* is flanked by *att*L1 and *att*L2 sites (S. Köthke & U. Bonas, unpublished results).

An entry clone harboring the full Bs4 ORF was generated by PCR amplification of genomic *Bs4* using primers Bs4-ORF-ATG-*att*B1 and Bs4-ORF-*att*B2 thereby introducing *att*B1 and *att*B2 sites before the ATG and after the last codon of Bs4, respectively. Subsequently the amplicon was Gateway-BP-recombined into pAG35P (K. Peter & T. Lahaye, unpublished results) to obtain the binary construct pAGSB8.3 harboring *Bs4* ORF flanked by *att*L1- and *att*L2 sites, respectively, under control of the 35S promoter.

Entry clones encoding Bs4 domains were generated by PCR using *Bs4* cDNA as template and sense primers TIR-CACC-F, NBS-CACC-F and LRR-CACC-F in combination with antisense primers TIR-R-NOSTOP-BLUNT, NBS-R-NOSTOP-BLUNT and Bs4-ORF-NOSTOP-BLUNT, respectively. Amplicons were transferred into pENTR/D-TOPO (Invitrogen, Karlsruhe, Germany) resulting in pENTR-TIR(Bs4), pENTR-TN(Bs4), pENTR-NB(Bs4), pENTR-NL(Bs4), and pENTR-LRR(Bs4), respectively.

The corresponding tobacco N domain encoding entry clones were generated by PCR using primers derived from the part of the *N* gene corresponding to the *Bs4* primers. Amplicons were transferred into pENTR/D-TOPO (Invitrogen, Karlsruhe, Germany) resulting in pENTR-TIR(N), pENTR-TN(N), pENTR-NL(N), pENTR-LRR(N), and pENTR-TNL(N), respectively.

Entry clones harboring the coding sequence of tomato *importinα* (*LeKAPα*, AF017252) or *Arabidopsis TGA5* (Q39163) were generated by amplification from cDNA using primers that bypass the stop codon and facilitate subsequent cloning into pENTR/D-TOPO.

An entry clone (pENTR-Hsp90DK) containing the coding sequence of *N. benthamiana Hsp90-2* (AY368905) was generated by PCR amplification using pYL737 as template (kind gift of S.P.Dinesh-Kumar, Yale University, New Haven, USA). The identities of all entry clones were verified by sequencing.

Bimolecular fluorescence complementation

To study BIFC the two proteins of interest were fused C-terminally to an N-terminal MYC epitope and 154 aa of YFP (gene:YN) and N-terminally to a C-terminal YFP-half (YC:gene) plus HA epitope using LR recombination into destination vectors. To this end, we created GATEWAY compatible vectors by transferring a GATEWAY-conversion cassette (rfB) that was *EcoR*V-inserted into pBSKII+, into pSPYNE 35S (Walter et al., 2004) using an *Xba*I and *Xho*I digest and ligation to obtain pSPYNE 35S GW (gene:YN). The pGWB735/1 vector (YC:gene) was generated by insertion of a PCR-generated *Afl*III-*Sac*I flanked GATEWAY cassette (rfB, Invitrogen, Karlsruhe, Germany) into *Xho*I and *Sac*I sites of pSY735 (Bracha-Drori et al., 2004) and subsequent transfer of a *Hind*III-*Sac*I-fragment into pGWB1 (kindly provided by T. Nakagawa, Shimane University, Izumo, Japan).

Gateway expression constructs

Entry clones pENTR256, pENTR256ΔNLS and pAG227 were transferred by an LR Clonase reaction into pK7WGF2 (Karimi et al., 2002) to yield GFP:AvrBs4Δ152, GFP:AvrBs4Δ152Δ1-3 and GFP:AvrBs4Δ227, respectively.

Entry clones that encode Bs4 or Bs4 domains were transferred by an LR Clonase reaction into pK7WGF2, pK7FWG2 (Karimi et al., 2002) to generate N- and C-terminal GFP fusions or into pGWB14, pGWB20, and pGWB21 (T. Nakagawa, Shimane University, unpublished) to generate C-terminal 3xHA-, C-terminal 10xMYC and N-terminal 10xMYC fusions, respectively. The transfer of *GFP* by an LR Clonase reaction into pGWB14 and pGWB20 was used to generate *GFP:HA* and *GFP:MYC* constructs.

Agrobacterium *mediated transient expression*

Agrobacterium tumefaciens GV3101 (Van Larebeke et al., 1974; Ballvora et al., 2001b) was used to deliver T-DNA constructs into three-week-old *Nicotiana benthamiana* or *N. tabacum* cv. Samsun NN plants. Expression of AvrBs1 (Escolar et al., 2001), AvrBs4 (Ballvora et al., 2001b), AvrBs4Δ227 (Schornack et al., 2004) together with Bs4 constructs were used in functional trans-complementation assays. We obtained *N. benthamiana* plants expressing tobacco *N* or potato *Rx* under their own promoters and binary vectors harboring *35S-TMV p50* (Erickson et al., 1999) *35S-PVX CP* (pBIN35-TK) (Bendahmane et al., 1999) from J. Peart and D. Baulcombe. *Bs2* transgenic *N. benthamiana* and *35S-avrBs2* were obtained from P. Moffett. If not otherwise stated, cells harboring all constructs were diluted to an OD_{600} of 1.0 and mixed equally for Agro-coinfiltration of Avr

and Bs4 domains. In GFP fluorescence assays constructs were agro-coinfiltrated together with silencing inhibitor p19 (Voinnet et al., 2003) at OD_{600} of 1.0. Samples were taken 1-3 days post infiltration.

Virus-induced gene silencing

NbHsp90-2 from pENTR-Hsp90DK was transferred into pYL279a (an ampicillin resistant derivative of pTRV2-*att*R2-*att*R1; details available upon request) by LR recombination and infection of plants with TRV derivatives was performed by agroinfiltration as described previously (Liu et al., 2002a). Two weeks later *N. benthamiana* leaves were infiltrated with *Agrobacterium* strains delivering *avr* constructs and *Bs4* (see above). Tomato was challenged with *Xcv* 75-3 strains expressing *avrBs4*, *avrBs3* or neither *avr* gene (Bonas et al., 1989; Minsavage et al., 1990). HR development was monitored 2-8 days post-infiltration.

DAPI staining and fluorescence imaging

Agroinfiltrated tissue was DAPI (4' 9,6-diamidino-2-phenylindole) stained by infiltration of DAPI in a phosphate buffered saline solution into the tissue and incubation at room temperature for one hour. Cut leaf patches were mounted in water and analyzed for EGFP/EYFP fluorescence using a Zeiss Confocal Laser Scanning Microscope (LSM 510, Carl Zeiss, Jena, Germany) and lasers and filter settings suitable for DAPI and EGFP or EYFP fluorescence.

Protein gel blot analysis from crude plant protein extracts

Protein extracts from leaf tissue were prepared by grinding two leaf discs (1 cm in diameter) in liquid nitrogen and adding 120 µl of 8M urea. Protein sample buffer was added and extracts were spun at 12,000 x g. 40 µl of the supernatant was separated on 10% PAGE gels and blotted onto nitrocellulose membranes. For protein gel blot analysis α-MYC antibody (9E10, Roche Molecular Biochemicals, Mannheim, Germany), α-HA antibody (3F10, Roche Molecular Biochemicals, Mannheim, Germany), polyclonal α-GFP antiserum (Invitrogen/Molecular Probes, Karlsruhe, Germany), α-AvrBs3 antiserum (Knoop et al., 1991), α-SGT1 antiserum (Azevedo et al., 2002), α-Hsp90 antiserum (de la Fuente van Bentem et al., 2005), α-Hsc70/BiP and α-Hsp90 antibody (Stressgen, Victoria, Canada), were used at dilutions of 1:2000 to 1:10.000 in Tris-buffered saline supplemented with Triton X100, using incubation times of one to three days at 4 °C.

Immunoprecipitation and protein gel blot analysis

Protein extracts from leaf tissue were prepared by grinding two leaf discs (1 cm in diameter) in liquid nitrogen. Extraction buffer [1 ml; 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol (DTT)] containing plant protease inhibitor cocktail (Roche) and 2% polyvinylpolypyrrolidone was added and extracts were spun at 12,000 x g at 4°C for 15 min. Immunoprecipitation and protein gel blot analysis were carried out essentially as described (Moffett et al., 2002).

Cell extract fractionation

Total extracts from leaf tissue were prepared by grinding eight leaf discs (1 cm in diameter) in liquid nitrogen. Extraction buffer [1 ml; 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol (DTT)] containing plant protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and 2% polyvinylpolypyrrolidone was added and extracts were spun at 100,000 x g at 4°C for 15 min.

Yeast expression assay

Bs4 domain encoding yeast bait and prey constructs were generated by PCR amplification using *Bs4* cDNA as template and primers corresponding to primers used for generation of *Bs4* entry clones (see above) but introducing *Mun*I- and *Sal*I-sites flanking the amplicons, and subsequent transfer into *Eco*RI/*Sal*I cleaved pBGKT7 and *Eco*RI/*Xho*I cleaved pGADT7 yeast vectors (Clontech, Palo Alto, CA, USA). Constructs represent fusions of Bs4 domains to GAL4-binding (bait) and GAL4 activation domain (prey), respectively. Crude extracts were generated from yeast overnight cultures at identical OD by centrifugation and resuspension of the pellet in protein sample buffer. Protein gel blot analysis of fusion proteins was carried out using α-MYC antibody (9E10, Roche) and α-HA antibody (3F10, Roche) as described for plant extracts (see above).

Electrolyte leakage measurements

One day after *Agrobacterium* infiltration, four leaf discs (1 cm in diameter) were shaken head-over-head in 6 ml deionized water for 24 h, and the solution measured for sample conductivity. Leaves were then boiled in the same solution for 5 min, and the solution was measured for the total conductivity. Membrane leakage is represented by the relative conductivity, which was calculated as described (Kim et al., 2003). Conductivity was measured with a conductivity meter (model 703, Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany).

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3. Diskussion

3.1. Die *Bs4*-vermittelte Erkennung von AvrBs4 weicht vom generellen Erkennungsprinzip AvrBs3-ähnlicher Proteine ab

Die AvrBs3-ähnlichen Proteine PthXo1 (entspricht Avrxa13), AvrXa27, AvrBs3, AvrBs4 und das wahrscheinlich AvrBs3-ähnliche Avrxa5 (Bai *et al.*, 2000) induzieren alle eine Resistenzreaktion in Pflanzenlinien mit den korrespondierenden *R*-Genen *xa5, xa13, Xa27, Bs3* und *Bs4*. Die Isolierung dieser *R*-Gene zeigte jedoch, dass trotz der strukturellen Ähnlichkeit der Avr-Proteine unterschiedliche Mechanismen der Erkennung angewandt werden. Das R-Protein der *xa5*-basierten Resistenz gegen *Xanthomonas oryzae pv. oryzae* (*Xoo*) ist eine Unterheit des transkriptionellen Präinitiationskomplexes (PIC) (Iyer und McCouch, 2004). Die durch Avrxa5 kontrollierte und von dessen AD abhängige PIC-vermittelte Regulierung pathogenitätsfördernder Wirts-Gene ist wahrscheinlich in *xa5*-Linien nicht mehr möglich. Die *xa13*-vermittelte Resistenz in Reis folgt einem ähnlichen Prinzip. PthXo1 induziert in suszepiblen Reispflanzen über seine AD die Transkription von *Xa13*, einem für die Pollenentwicklung notwendigen Gen. Diese Induktion ist für das *Xoo*-Wachstum notwendig. In resistenten *xa13*-Linien erfolgt dagegen keine Induktion von *xa13* und *Xoo* kann sich nicht vermehren (Chu *et al.*, 2006; Yang *et al.*, 2006). Beide Mechanismen basieren also nicht auf der Wirkung eines Resistenzproteins, sondern auf einem Verlust der Anfälligkeit gegenüber dem Pathogen. Dies wird unterstützt durch die vom dominanten Vererbungstyp nahezu aller *R*-Gene abweichende rezessive Vererbung der *xa5*- und *xa13*-vermittelten Resistenz.

Die *Xa27*-vermittelte Detektion von AvrXa27 in Reis basiert auf einer Aktivierung des *Xa27*-Promotors. AvrXa27 induziert über seine AD spezifisch das resistente *Xa27*-, aber nicht das suszeptible *xa27*-Allel. Beide Allele weisen signifikante Unterschiede in ihren Promotor-Sequenzen auf, die wahrscheinlich die Spezifität der Aktivierung vermitteln (Gu *et al.*, 2005).

Auch bei AvrBs3 ist die AD notwendig für die *Bs3*-vermittelte HR in Paprika (Van den Ackerveken *et al.*, 1996; Szurek *et al.*, 2001; Marois *et al.*, 2002). Dies deutet auf eine dem *Xa27*-Mechanismus ähnliche AvrBs3-induzierte *Bs3*-Induktion hin. Allen genannten Resistenzmechanismen ist gemeinsam, dass die AD des Avr-Proteins notwendig ist für seine Avr-Aktivität.

Die AD-vermittelte Transkriptregulation ist aber auch die Grundlage bekannter Virulenz- oder virulenzähnlicher Effekte AvrBs3-ähnlicher Proteine in der Pflanze wie z. B.

Hypertrophie oder verstärktes bakterielles Wachstum und Läsionsbildung (Bai *et al.*, 2000; Marois *et al.*, 2002). Unlängst wurde ein AvrXa7-Derivat identifiziert, das keine Vir-Funktion mehr hat, jedoch weiterhin *Xa7*-spezifisch detektiert wurde (Yang *et al.*, 2005). Die Avr-Aktivität dieses Konstruktes hatte nur 40% der Aktivität von AvrXa7, d. h. der Verlust der Vir-Aktivität führte auch zum partiellen Verlust der Avr-Aktivität (Yang 2005). In Übereinstimmung damit zeigten frühere Arbeiten, in denen NLS- und AD-Deletionen von AvrXa7 getestet wurden, dass diese Deletionsderivate keine Avr-Aktivität haben (Yang *et al.*, 2000). Auch bei anderen untersuchten AvrBs3-ähnlichen Proteinen führen Deletionen der AD- oder NLS-Sequenzen sowohl zum Verlust der Erkennung, als auch messbarer Vir-Effekte wie z. B. AvrBs3-induzierter Hypertrophie (Van den Ackerveken *et al.*, 1996; Szurek *et al.*, 2001; Marois *et al.*, 2002). Die Erkennung von AvrBs3-ähnlichen Proteinen in resistenten Pflanzen ist also gekoppelt an ihre Vir-Aktivität (Lahaye und Bonas, 2001).

Im Gegensatz zu AvrBs3 induziert AvrBs4 keine im Labor messbaren Vir-Effekte. Aktuelle Daten belegen jedoch einen Beitrag von *avrBs4* zum bakteriellen Wachstum von *Xcv in planta*. Dieser Virulenzeffekt von AvrBs4 war jedoch nur in *avrBs2*-Mutanten detektierbar (Wichmann und Bergelson, 2004). Ob AvrBs4-Derivate ohne NLS und AD zum *Xcv*-Wachstum beitragen, wurde bisher nicht adressiert. In dieser Arbeit wurde gezeigt, dass AvrBs4 in der Pflanzenzelle in subnukleären Foci akkumuliert. Dies könnte auf eine transkriptionelle Aktivität hinweisen (Taddei *et al.*, 2004). Das AvrBs4Δ227-Derivat, dem die AD fehlt, ist zwar auch im Zellkern nachweisbar, zeigt aber keine solchen Foci (Manuskript3, Abb. M3-1C). Die Tatsache, dass AvrBs4-Derivate aber auch ohne AD eine HR in *Bs4*-Tomaten induzieren, weist darauf hin, dass sich die Bs4-Erkennung durch ihre Unabhängigkeit von AD-bedingter Transkriptregulation von den anderen Erkennungsmechanismen unterscheidet.

Die Erkennungsspezifität von *Xa27*, *Bs3* sowie den bisher nicht isolierten R-Genen *Xa7* und *Xa10* wird durch die Repeat-Regionen von *avrXa27, avrBs3, avrXa7* und *avrXa10* definiert (Herbers *et al.*, 1992; Yang *et al.*, 1994; Zhu *et al.*, 1998; Gu *et al.*, 2005), während die N- und C-Termini verschiedener AvrBs3-ähnlicher Proteine funktionell austauschbar sind und keinen Einfluß auf die Spezifität haben (Zhu *et al.*, 1998; Ballvora *et al.*, 2001; Kay *et al.*, 2005). Im Gegensatz dazu vermittelt *Bs4* die Erkennung auch stark verkürzter AvrBs4-Derivate, denen ein Großteil der Repeat-Region fehlt. Außerdem

induziert neben AvrBs4 auch AvrBs3 bei Überexpression eine *Bs4*-spezifische HR, obwohl sich die Repeat-Regionen beider Proteine unterscheiden (Abb. M1-4, M1-9).
Interessanterweise weist das AvrBs4Δ227-Derivat sogar eine stärkere Avr-Aktivität auf als AvrBs4 (Bonas *et al.*, 1993; Ballvora *et al.*, 2001). Ein Grund für diese verstärkte Aktivität könnte eine verbesserte Proteinstabilität von kürzeren Konstrukten sein (Abb. M3-1B). Die unterschiedlichen Proteinmengen von AvrBs4 und AvrBs4Δ227 nach Agroinfiltration machen deutlich, dass ein direkter Vergleich von Wildtyp und Derivaten ausschließlich anhand des Phänotyps nur bedingt möglich ist. Notwendig wäre neben dem Phänotyp auch die Quantifizierung der Proteinmengen über Epitop-spezifische Antikörper, da polyklonale Antiseren eine veränderte Affinität zu verkürzten Derivaten haben könnten. Außerdem muß geprüft werden, ob sich die Derivate in ihrer subzellulären Lokalisierung unterscheiden. So wurde z.B. AvrBs4 nach Deletion der NLS-Motive zwar nur noch im Cytoplasma und nicht mehr im Zellkern detektiert, ein stärker verkürztes Derivat (AvrBs4Δ227Δ152) lokalisierte jedoch wieder in beiden Kompartimenten (Abb. M3-1).

3.2. Die Fähigkeit zur Erkennung von verkürztem AvrBs4 ist auf die Gattung *Lycopersicon* beschränkt

Die Fähigkeit zur Erkennung von AvrBs4Δ227 ist typisch für *Bs4* und ermöglicht die Charakterisierung von Pflanzenlinien hinsichtlich eines *Bs4*-Erkennungsmechanismus.
Die *Agrobacterium*-vermittelte gemeinsame Expression von *Bs4* und *avrBs4*Δ227 zeigte, dass das Bs4-Protein nicht nur in Tomate, sondern auch in anderen Pflanzenarten funktionsfähig ist. Dies traf auch dann zu, wenn die Arten selbst keine Erkennung von AvrBs4 vermittelten. Sogar in Spinat (*Chenopodiaceae*) konnte die *Bs4*-Funktionalität gezeigt werden (Tabelle 2). Der Ausfall der *Bs4*-Funktion in einigen zu Tomate näher verwandten Arten (z. B. in Paprika und *S. nigrum*) könnte durch das Fehlen kritischer Komponenten der Bs4-Signalkaskade begründet sein.
Transiente *Agrobacterium*-Expressionsexperimente mit AvrBs4Δ227 führten nur in Tomate und anderen Arten der Gattung *Lycopersicon* zur HR-Induktion, jedoch in keiner anderen getesteten Spezies (Abb. 14, Tabelle 2). Dies deutet darauf hin, dass nur Tomaten ein funktionales *Bs4*-Gen besitzen. Obwohl sich *Lycopersicon* dadurch von den anderen getesteten *Solanum*-Arten abgrenzen lässt, ist es keine eigene taxonomische Gruppe mehr, sondern wurde in die Gattung *Solanum* integriert (Bohs und Olmstead, 1997). Grund dafür waren neuere Analysen, die zeigten, dass *Lycopersicon* keine monophyletische Gruppe ist,

weil einige Arten näher verwandt mit *Solanum* als mit anderen Arten der eigenen Gattung sind.

Die suszeptible *L. pennellii* LA2963-Linie ist bisher die einzige näher analysierte Linie, die keine AvrBs4Δ227-Erkennung vermittelt (Ballvora *et al.*, 2001). Vergleichende Sequenzanalysen hatten in dieser Linie jedoch ein *Bs4*-Sequenzhomolog identifiziert (bs4-pen2963, Schornack *et al.*, 2004). Da dieses nach gemeinsamer Expression mit AvrBs4Δ227 in *N. benthamiana* eine HR induziert, handelt es sich hier um ein funktionsfähiges Allel (S. Schornack und T. Lahaye, unveröffentlicht). Der suszeptible Charakter der *bs4*-Ursprungslinie *L. pennellii* LA2963 ist wahrscheinlich auf Unterschiede im Zeitverlauf der HR-Reaktion zurückzuführen. *L. pennellii* LA2963 reagierte auf *Xcv* (*avrBs4*) nicht mit einer schnellen HR-typischen Reaktion, sondern mit einer langsameren nekrotischen Reaktion (Daten nicht dargestellt). Dieser phänotypische Unterschied war ausreichend für die kartengestützte *Bs4*-Isolierung.

Weitere *Bs4*-homologe Sequenzen wurden in allen *Lycopersicon*-Arten detektiert, und alle induzieren bei *Agrobacterium*-vermittelte Expression mit *avrBs4*Δ227 eine HR in *N. benthamiana* (K. Peter, S. Schornack, L. Rose & T. Lahaye, unveröffentlicht). Das Vorhandensein funktionaler *Bs4*-Sequenzen in allen getesteten Spezies mit *Bs4*-typischer Erkennung deutet daraufhin, dass die Erkennung von AvrBs4Δ227 stets durch *Bs4* vermittelt wird. In *S. demissum* wurden auch zwei *Bs4*-Homologe (*SdBs4H1* und *SdBs4H2*; Manuskript 2) gefunden, die jedoch Leserastermutationen aufwiesen. Die

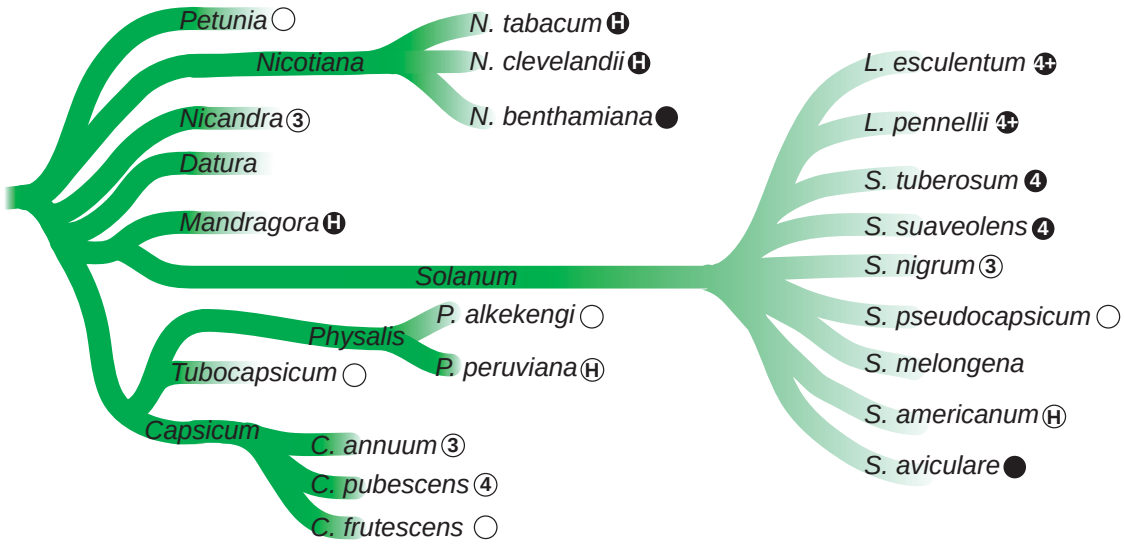

Abb. 14. Taxonomische Verbreitung der Erkennung AvrBs3-ähnlicher Proteine und der Bs4-Funktionalität innerhalb der getesteten Nachtschattengewächse. Analysierte Gattungen und Arten sind im Kladogramm (nach Knapp, 2002, erweitert) dargestellt. Getestete Linien mit Erkennung von AvrBs3 (3), AvrBs4 (4), AvrBs4Δ227 (+) oder Hax3 (H) sind angegeben. Pflanzen ohne bzw. mit *Bs4*-Funktionalität (HR nach Koexpression mit *avrBs4*Δ227) sind durch offene bzw. gefüllte Kreise markiert.

S. demissum-Linien zeigten auch keine HR bei *Agrobacterium*-vermittelter Expression von AvrBs4Δ227. Es könnte sich hier um inaktive *Bs4*-Homologe handeln.

Neben dem NLS-unabhängigen *Bs4*-Typ der Erkennung verkürzter Avr-Derivate existiert ein weiterer AvrBs4-Erkennungsmechanismus. In Kartoffel und *S. suaveolens* wird AvrBs4 NLS-abhängig detektiert. Ebenso war die Erkennung von AvrBs3 in den analysierten Arten stets abhängig von NLS-Sequenzen und entspricht damit dem *Bs3*-Erkennungstyp (Minsavage *et al.*, 1999; Ballvora *et al.*, 2001).

Rassen- bzw. Kultivarspezifische *R*-Gene geben nur einzelnen Genotypen einer Art die Fähigkeit zur Avr-Detektion. Ein Grund dafür ist die reduzierte biologische Fitness resistenter Genotypen (Dangl und Jones, 2001; Pink, 2002; Tian *et al.*, 2003). Sind ähnliche *R*-Gen-Sequenzen im mehreren Arten vorhanden, so weisen diese häufig eine abweichende Spezifität auf. Kartoffel *Rx* vermittelt z.B. Resistenz gegen PVX-Viren, während das homologe *Gpa2* aus Kartoffel ein Nematoden-Resistenzgen ist (Bendahmane *et al.*, 1999; Van der Vossen *et al.*, 2000). Die Tatsache, dass mehrere Arten der Familie *Solanaceae* zumindestens bei *Agrobacterium*-vermittelter Expression eine AvrBs4- bzw. AvrBs3-Erkennung aufweisen, deutet daraufhin, dass diese Erkennungsmechanismen nicht kultivar-spezifisch sind (Tabelle 2; Abb. 8; Abb. 14). Verläuft die NLS-abhängige Erkennung von AvrBs4 oder AvrBs3 über eine AD-vermittelte transkriptionelle Induktion des *R*-Gens, so wäre ohne das Avr-Protein das *R*-Gen nicht exprimiert und deshalb kein Fitnessverlust vorhanden. Dies könnte ein Grund für das Vorhandensein dieses Erkennungsprinzips in vielen Pflanzenspezies sein.

3.3. Die Bs4-Erkennungsspezifität ist abhängig von der Avr-Menge

Bekannterweise neigen Antikörper neben der Reaktion mit dem spezifischen Antigen auch zu Kreuzreaktionen mit strukturell ähnlichen Antigenen, besonders, wenn diese in hohen Mengen vorhanden sind. Es ist deshalb erstaunlich, dass *Bs3* aus Paprika und *Bs4* aus Tomate in der Lage sind, zwischen den 97% identischen AvrBs3- und AvrBs4-Proteinen zu differenzieren (Ballvora *et al.*, 2001).

Infiltrationen von *avrBs3*- und *avrBs4*-exprimierenden *Xcv* und die *Agrobacterium*-vermittelte Expression beider *avr*-Gene haben jedoch gezeigt, dass *Bs4* auch AvrBs3-Erkennung vermitteln kann, wenn dieses in der Pflanze überexprimiert wird (Abb. M1-4,

Abb. M2-7). Dagegen induziert AvrBs4 selbst bei Überexpression in *Bs3*-Paprika keine HR (Abb. M2-7C), d. h. die Erkennungsspezifitäten von *Bs3* und *Bs4* werden auf unterschiedliche Weise definiert. Auch die *R*-Gene *B4*, *b7* und *Bln* aus Baumwolle zeigen außer mit ihren korrespondierenden Avr-Proteinen keine HR-Aktivität gegenüber anderen überexprimierten *avrBs3*-ähnlichen Genen (De Feyter *et al.*, 1998). *Bs4* ist demnach bisher eine Ausnahme, da es zusätzlich in der Lage ist, auch mit Hax3 und Hax4, zwei weiteren AvrBs3-ähnlichen Proteinen, eine Resistenzreaktion auszulösen (Abb. M2-7D, Kay *et al.*, 2005). Hax3 und Hax4 haben im Gegensatz zu AvrBs3 eine andere Anzahl an Repeats (Abb. 3), werden aber trotzdem sowohl bei Translokation durch *Xanthomonas* als auch bei schwacher *Agrobacterium*-vermittelter Expression durch Bs4 erkannt. AvrBs3 muss dafür überexprimiert werden, obwohl es wie AvrBs4 17,5 Repeats hat. Dies weist darauf hin, dass nicht die Gesamtstruktur des Avr-Proteins, sondern eher ein kurzes Avr-Peptidmotiv als Spezifitätskomponente in Frage kommt. Zwar haben alle durch Bs4 erkannten Avr-Proteine eine andere Repeat-Reihenfolge, das Repertoire an unterschiedlichen 34-AS-Repeatsequenzen ist jedoch identisch. Das einzige bisher identifizierte AvrBs3-Homolog ohne *Bs4*-Erkennung ist Hax2. Allerdings hat Hax2 in jedem Repeat einen zusätzlichen AS-Rest und außerdem zwei in Hax3, Hax4, AvrBs4 und AvrBs3 nicht vorkommende Repeats (Kay *et al.*, 2005), die die Ursache für die fehlende *Bs4*-vermittelte Erkennung sein könnten. Zusätzlich beeinflusst sicherlich die Stabilität der Avr-Proteine in der Pflanze deren Avr-Aktivität. Bislang wurden keine Vergleiche der Avr-Proteinmengen über Epitop-markierte Derivate durchgeführt. In dieser Arbeit wurde der schwach exprimierende *Bs4*-Promotor im Vergleich zum stärkeren 35S-Promotor zur Adressierung quantitativer Aspekte der *Bs4*-vermittelten Erkennung verwendet.

Da *Bs4* sowohl unter Kontrolle des eigenen, als auch des 35S-Promotor ein identisches Erkennungsspektrum gegenüber AvrBs3-ähnlichen Proteinen besaß, haben *Bs4*-Transkript-Mengen keinen Einfluss auf die Erkennungsspezifität von Bs4 (Abb. M2-2, Schornack *et al.*, 2004). Im Gegensatz dazu beeinflusste aber das Expressionsniveau des Avr-Proteins die *Bs4*-Erkennungsspezifität. So löst AvrBs3 nur bei Überexpression, Hax3 und Hax4 jedoch auch noch bei Expression unter Kontrolle des schwachen *Bs4*-Promotors eine *Bs4*-HR aus. Wurden Promotorfragmente mit noch geringerer Aktivität vor Hax3- bzw. Hax4 geschaltet, so erfolgte zwar Hax4- und AvrBs4-Erkennung, jedoch keine Hax3-Erkennung mehr (Abb. M2-7). Geringe Unterschiede im Expressionsniveau von AvrBs3-ähnlichen Proteinen sind also bereits ausschlaggebend für ihre *Bs4*-vermittelte Erkennung. Schlussfolgerungen hinsichtlich Erkennungsspezifitäten von R-Proteinen sollten deshalb

immer dann vorsichtig interpretiert werden, wenn das *avr*-Gen unter Kontrolle starker Promotoren *in planta* exprimiert wurde.

3.4. Bs4 und AvrBs4: indirekte oder direkte Interaktion?

Bislang konnte experimentell keine Interaktion zwischen AvrBs4 und Bs4 nachgewiesen werden. Da dies auch für andere Avr-R-Interaktionen zutraf, wurde ein *guard*-Modell postuliert, das auf der R-Protein-vermittelten Detektion Avr-bedingter Veränderungen eines pflanzlichen Zielproteins basiert (Van der Biezen und Jones, 1998b; Dangl und Jones, 2001; Dangl und McDowell, 2006). Interaktionen treten demnach nicht direkt zwischen Avr- und R-Protein, sondern zwischen dem Zielprotein und dem Avr- bzw. R-Protein auf. Eine Interaktion von Avr-, R- und Zielprotein in einem trimeren Komplex, bei der auch Avr- und R-Protein miteinander interagieren, ist dabei nicht ausgeschlossen. Dagegen postuliert das Rezeptor-Liganden-Modell eine direkte Interaktion zwischen Avr- und R-Protein (Gabriel und Rolfe, 1990; Dangl und McDowell, 2006). Etwaige weitere Zielproteine des Avr-Proteins bleiben unberücksichtigt. Bislang ist jedoch keine direkte Interaktion eines R-Proteins mit einem AvrBs3-ähnlichen Protein identifiziert worden. Für andere Avr-Proteine wurde aber eine direkte Interaktion mit NB-LRR-Proteinen gezeigt (Jia *et al.*, 2000; Deslandes *et al.*, 2003; Dodds *et al.*, 2006).

Für eine direkte AvrBs4-Bs4-Interaktion ist eine Kolokalisation zwingend notwendig. Die voneinander abweichende Lokalisierung von AvrBs4 im Kern und Bs4 im Cytoplasma würde demnach eine direkte Interaktion ausschließen (Abb. M3-1B, M3-2B). Da aber auch cytoplasmatisch lokalisierte AvrBs4-Derivate ohne NLS durch Bs4 erkannt werden, muss die Erkennung im Cytoplasma stattfinden (Ballvora *et al.*, 2001; Schornack *et al.*, 2004). Ursache für den fehlenden Nachweis von AvrBs4 im Cytoplasma könnten zu geringe Protein-Mengen sein. Die subzelluläre Lokalisierung weiterer R-Proteine und ihrer korrespondierenden Avr-Proteine weist aber generell auf die Notwendigkeit einer räumlichen Beziehung beider Komponenten hin (Bonas und Lahaye, 2002). In *Arabidopsis* ist die RPM1-vermittelte Erkennung der *Pseudomonas syringae*-Proteine AvrRpm1 und AvrB abhängig von der Membranassoziation der beteiligten Proteine (Nimchuk *et al.*, 2000; Thompson und Okuyama, 2000). Ebenso membranlokalisiert sind RIN4 und NDR1 (*no disease resistance*), zwei weitere Komponenten der RPM1-Resistenz (Century *et al.*, 1997; Mackey *et al.*, 2002; Coppinger *et al.*, 2004). Potentielle membranassoziierende

Myristoylierungsmotive wurden auch in den interagierenden *Arabidopsis*-Proteinen RPS5 (R-Protein) und PBS1 (*AvrPphB susceptible protein1*) sowie dem korrespondierenden *Pseudomonas*-Avr-Protein AvrPphB gefunden (Puri *et al.*, 1997; Warren *et al.*, 1998; Swiderski und Innes, 2001; Shao *et al.*, 2003). Das durch *Cladosporium fulvum* in den Apoplasten von Tomaten sekretierte Avr2 wird durch membranständiges Cf-2 zusammen mit der ebenfalls extrazellulären Protease Rcr3 (*required for C. fulvum resistance*) erkannt (Rooney *et al.*, 2005). Sowohl die RPM1-, RPS5- als auch die Cf-2-Resistenz basieren auf Interaktionen von Avr- und R-Protein mit einem pflanzlichen Zielprotein im Sinne des *guard*-Modells.

Für direkte Avr-R-Interaktionen ist nur eine Kolokalisationsstudie publiziert. *Arabidopsis* RRS1 kolokalisiert mit dem *Ralstonia solanacearum* Avr-Protein PopP2 im Zellkern (Deslandes *et al.*, 2003). Die Interaktion der membranassoziierten Proteine AvrPto und Pto ist dagegen keine Avr-R-, sondern eine Avr-Zielprotein-Interaktion. Das korrespondierende R-Protein ist wahrscheinlich Prf (Salmeron *et al.*, 1996), dessen Interaktion mit AvrPto bisher nicht nachgewiesen wurde (Van der Biezen und Jones, 1998a).

Für RRS1 und PopP2 bzw. AvrL567 und L6 wurde eine direkte Interaktion im Hefe-System gezeigt (Deslandes *et al.*, 2003; Dodds *et al.*, 2006). Dagegen konnte für AvrBs4 keine Interaktion mit Bs4 nachgewiesen werden. Das verwendete Hefesystem erzwingt eine Lokalisierung beider zu testenden Partner im Zellkern, obwohl die AvrBs4-Erkennung wahrscheinlich im Cytoplasma stattfindet. Demnach entspricht der zelluläre Kontext nicht den Bedingungen in der Pflanze. Alternativ könnte ein kernunabhängiges Hefe-split-Ubiquitin-System (Johnsson und Varshavsky, 1994) verwendet werden. Zur Wahrung des zellulären Kontexts wurden in dieser Arbeit Co-IP-Analysen aus Pflanzenextrakt und Bimolekulare-Fluoreszenz-Komplementation (BiFC) *in vivo* durchgeführt, die jedoch auch keine AvrBs4-Interaktion mit Bs4 zeigen konnten. Der Erfolg dieser Methoden ist abhängig von nachweisbaren Protein-Mengen. Die Bs4-Menge in *N. benthamiana* ist gering und wird bei Anwesenheit von AvrBs4 noch reduziert, so dass die Sensitivität des experimentellen Nachweises der limitierende Faktor für den Nachweis einer Interaktion zwischen AvrBs4 und Bs4 sein könnte. Sollten nur Spaltprodukte eines oder beider Proteine in die Interaktion eingehen, ist ein Nachweis über fusionierte Epitope nicht mehr möglich. Alternativen wären ein polyklonales Antiserum mit mehreren über das Protein verteilten Antigen-Bereichen oder die radioaktive Markierung der Interaktoren.

BiFC-Experimente wurden auch durchgeführt, um temporäre Interaktionen in einem fluoreszierenden Komplex zu arretieren (Kerppola, 2006). So ist z.B. die im Cytoplasma und im Zellkern zu erwartende Interaktion zwischen dem Transport-Protein Importin α und AvrBs4 mittels BiFC ausschließlich im Zellkern detektierbar, weil die beiden Partner dort wahrscheinlich nicht dissoziieren können (Abb. M3-3). Mit AvrBs4 und Bs4 wurden keine fluoreszierenden Signale in BIFC-Experimenten detektiert. Ein Nachteil von BiFC ist, dass die Position der YFP-Hälften kritisch für den erfolgreichen Nachweis einer BiFC-Interaktion ist (Bracha-Drori *et al.*, 2004). Eine alternative Fixierung von Proteinkomplexen wäre das chemische Kreuzvernetzen (*cross-linking*) und die anschließende Aufreinigung der Proteinkomplexe.

Insgesamt könnten also experimentelle Ursachen den Nachweis einer AvrBs4-Interaktion mit Bs4 verhindert haben. Die durch Bs4 erkannten, verkürzten AvrBs4-Derivate haben keine NLS- und AD-Motive, die bei anderen AvrBs3-ähnlichen Proteinen die Grundlage für die Virulenz sind. Demnach besitzt auch AvrBs4 wahrscheinlich keine Vir-Funktion mehr, die die Basis einer indirekten Erkennung im Sinne des *guard*-Modells darstellt. Es existieren zum jetzigen Zeitpunkt demnach weder für das Rezeptor-Liganden-Modell, noch für das *guard*–Modell experimentelle Hinweise. Auf Basis der postulierten TPR-ähnlichen AvrBs4-Struktur wäre eine direkte Interaktion mit der Bs4-LRR-Domäne möglich (Abb. 13, Schornack *et al.*, 2006). In Übereinstimmung damit wurden bereits eine Interaktion von NB-LRR-Proteinen mit TPR-Domänen gezeigt (de la Fuente van Bentem *et al.*, 2003). Eine direkte Interaktion von AvrBs4 und Bs4 im Sinne des Rezeptor-Liganden-Modells kann also nicht ausgeschlossen werden.

3.5. Die Bs4-Menge wird posttranslational reguliert

Punktmutationen im C-terminalen Abschnitt der NB- und in der LRR-Domäne führten bei den R-Proteinen Rx aus Kartoffel und Mi-1 aus Tomate zur Avr-unabhängigen HR-Induktion (Autoaktivierung). Die Häufigkeit von NB-LRR-Autoaktivatoren bei Mutationsanalysen, deutet eher auf einen viel häufiger auftretenden Funktionsverlust, als auf den selteneren Funktionsgewinn hin. Es könnte sich um den Verlust einer Suppression handeln, die normalerweise das NB-LRR-Protein im inaktiven Zustand arretiert (Bendahmane *et al.*, 2002; Hwang und Williamson, 2003). So resultierte die Mutagenese des im NB lokalisierten konservierten MHD-Motivs z. B. bei L6 aus Flachs in einem Autoaktivierungsphänotyp (Howles *et al.*, 2005). Für Bs4 sind bisher keine Autoaktivator-

Mutanten bekannt. Die Mutagenese des Aspartats im MHD-Motiv hatte keine Auswirkung auf die Bs4-HR (S. Schornack & T. Lahaye, unveröffentlicht).

Neben Mutationen führt auch die Überexpression einiger NB-LRR-Gene zu deren Autoaktivierung (Mindrinos *et al.*, 1994; Oldroyd und Staskawicz, 1998; Dinesh-Kumar *et al.*, 2000; Tao *et al.*, 2000; Bendahmane *et al.*, 2002; Stokes *et al.*, 2002; Stokes und Richards, 2002; Tameling *et al.*, 2002; Grant *et al.*, 2003; Zhang *et al.*, 2004). Daraus folgt, dass die NB-LRR-Menge kritisch ist für die Funktion und deshalb Regulationsmechanismen unterliegt. Eine Überexpression von *Bs4* führte zwar zu erhöhten Transkriptmengen (diese Arbeit, Manuskript 1) und höheren Bs4-Mengen in der Pflanze (S. Schornack und T. Lahaye, unveröffentlicht), jedoch bei *Agrobacterium*-vermittelter Überexpressionen nicht zur Avr-unabhängigen Autoaktivierung von *Bs4*. Interessanterweise wird Bs4 trotz 35S-basierter konstitutiver Expression bereits 2-3 Tage nach Infiltration auch in Abwesenheit von AvrBs4 auf nicht detektierbare Mengen reduziert (Abb. M3-8). Dies deutet auf eine posttranslationelle Regulation der Bs4-Mengen hin. Nicht nur Bs4, sondern auch einzelne Bs4-Domänen wurden bei Überexpression in der Pflanze in ihrer Menge reguliert, wobei der TIR am stärksten und LRR-enthaltende Domänenkonstrukte am schwächsten nachweisbar sind (Abb. M3-5, M3-6). Dem LRR könnte also eine Rolle in der Regulierung der Bs4-Menge zukommen. In diesem Zusammenhang wäre es wichtig, zu prüfen, ob Punktmutationen im LRR, die zu einer veränderten Bs4-Funktionalität führen (A. Fick & T. Lahaye, unveröffentlicht) auch mit einer Veränderung der Bs4-Stabilität gekoppelt sind.

3.6. AvrBs4 induziert die Auflösung von Bs4-Domänen-Interaktionen und führt zum Verschwinden von Bs4

Experimente mit den CC-NB-LRR-Proteinen Rx aus Kartoffel und Bs2 aus Paprika zeigten, dass intramolekulare Interaktionen der CC- mit der NB-LRR-Domäne und der CC-NB- mit der LRR-Domäne an der Regulierung der Funktion beteiligt sind (Moffett *et al.*, 2002). Die für Bs4-Domänen hier experimentell gezeigten Interaktionen zwischen TIR und NB-LRR bzw. TIR-NB und LRR deuten darauf hin, dass dieses Regulationsprinzip auch auf TIR-NB-LRR-Proteine übertragbar ist (Abb. 3-4, 3-7). Im Hefe-Dihybrid-System konnte diese Bs4-Domänen-Interaktion jedoch nicht nachgewiesen werden. Dies lässt die Notwendigkeit weiterer pflanzlicher Komponenten für die intramolekularen Interaktionen vermuten. Interessanterweise sind Rx-Domänen-Interaktionen bei Expression des komplementären Avr-Proteins (PVX-Hüllprotein) nicht mehr nachweisbar (Moffett *et al.*,

2002). Das auf Basis dieser Daten entwickelte Schaltermodell postuliert eine Avr-induzierte Transition von einem durch intramolekulare Interaktionen inaktivierten Komplex in einen signalkompetenten, aktiven Komplex, bei dem intramolekulare Interaktionen nicht mehr nachweisbar sind (Rathjen und Moffett, 2003). In Übereinstimmung mit diesem Modell führt auch bei Bs4 die Anwesenheit von AvrBs4 zum Verlust der Bs4-Domänen-Interaktionen (Abb. M3-7). Die Bs4-Experimente wurden in *N. benthamiana* Wildtyp-Pflanzen durchgeführt. In vergleichbaren Arbeiten wurden Unterschiede bei der Avr-induzierten Aufhebung der Domänen-Interaktionen zwischen unbehandelten und *SGT1*-Silencing-Pflanzen festgestellt. Untersuchungen zu Bs2 aus Paprika bestätigten zwar die Domänen-Interaktion, konnten jedoch keine Avr-induzierte Aufhebung derselben finden (Leister *et al.*, 2005). Im Gegensatz zu den Arbeiten mit Rx wurden die neueren Bs2-Analysen nicht in *SGT1*-Silencing-Pflanzen, sondern in Wildtyp-Pflanzen durchgeführt. Aktuelle Arbeiten belegen eine Notwendigkeit von *SGT1* für die Stabilisierung und Funktion von NB-LRR-Proteinen (Azevedo *et al.*, 2001; Austin *et al.*, 2002; Azevedo *et al.*, 2002; Peart *et al.*, 2002; Tör *et al.*, 2002; Leister *et al.*, 2005). Tatsächlich führt Virus-induziertes *SGT1*-Silencing auch zu stark reduzierten Bs4-Mengen und zum Verlust der *Bs4*-HR (Abb. M1-10, Abb. M3-11; S. Schornack & T. Lahaye, unveröffentlicht). Im Gegensatz zu Bs4 sind Bs2-Domänen-Interaktionen möglicherweise stabiler und nur bei fehlender Stabilisierung von Bs2 durch SGT1 auflösbar.

Immunoblot-Analysen zeigten, dass die Bs4-Menge bei Koexpression mit AvrBs4 geringer ist als ohne AvrBs4. Dieser Effekt wurde auch für das NB-LRR Protein RPM1 aus *Arabidopsis* beschrieben und könnte der Pflanze dazu dienen, die Resistenz-Antwort zu limitieren (Boyes *et al.*, 1998; Kawasaki *et al.*, 2005). Das Verschwinden von Bs4 wird spezifisch durch alle AvrBs3-ähnlichen Proteine ausgelöst, die eine Bs4-HR induzieren. Jedoch handelt es sich nicht um eine generelle HR-bedingte Degradierung aller Proteine, da z.B. SGT1 in seiner Menge unverändert bleibt. Der Effekt ist zudem Bs4-spezifisch, da das 57% identische N-Protein bei Expression *avrBs3*-ähnlicher Gene nicht verschwindet (Abb. M3-8). Messbare Unterschiede in relativen Bs4-Mengen wurden 1,5-2 Tage nach Agroinfiltration detektiert. Damit ist das Verschwinden von Bs4 zeitlich nach der Auflösung der Bs4-Domänen-Interaktionen einzuordnen, die bereits einen Tag nach Agroinfiltration nicht mehr detektierbar waren. AvrBs4 induziert neben dem Verschwinden von Bs4 auch das von Bs4-Domänen (mit Ausnahme der TIR-Domäne), obwohl die Domänen alleine keine HR bei Koexpression mit AvrBs4 auslösen können (Abb. M3-8). Dies zeigt, dass die Verringerung der Bs4- bzw. Bs4-Domänen-Mengen

unabhängig von der HR ist. Möglich wäre ein Einfluss von AvrBs4 auf Hsp90, welches für die Stabilisierung der Bs4-Proteinmengen notwendig sind.

3.7. Hsp90 reguliert die Bs4-Aktivität

Sequenzvergleiche zwischen NB-LRR-Proteinen haben gezeigt, dass die AS-Sequenz im LRR stark variiert (*diversifying selection*). Diese hypervariablen AS-Reste definieren wahrscheinlich die Erkennungsspezifität. Es wird vermutet, dass diese Variabilität der LRR-Sequenz mit einem Stabilitätsverlust einhergeht, der durch Chaperone (z. B. Hsp90) kompensiert wird (Schulze-Lefert, 2004). Aus Silencing-Experimenten war bekannt, dass zahlreiche NB-LRR Proteine in ihrer Funktion von *Hsp90* abhängig sind (Lu *et al.*, 2003) (Liu *et al.*, 2004) (de la Fuente van Bentem *et al.*, 2005) (Zhang *et al.*, 2004). *Hsp90*-Silencing in *Bs4*-Tomatenpflanzen bzw. *Bs4*-transgenen *N. benthamiana* zeigten, dass *Hsp90* auch für die Bs4-HR notwendig ist (Abb. M3-9; Daten nicht dargestellt). Wird *Hsp90* durch Silencing reduziert, ist kein Bs4-Protein mehr nachweisbar, d. h. *Hsp90* ist an der Regulierung der Bs4-Menge beteiligt (Abb. M3-9). Darüber hinaus konnte eine Interaktion zwischen Bs4 und Hsp90 nachgewiesen werden. Ob ein funktionales Hsp90 für den Einfluss auf Bs4 notwendig ist, muss noch mit ATPase-inaktiven Hsp90-Mutanten getestet werden. Die Tatsache, dass Hsp90-Inhibition durch Geldanamycin in *Arabidopsis* zum Verlust der RPM1-Funktion führt (Holt *et al.*, 2005), deutet aber bereits darauf hin, dass nicht nur die Interaktion von Hsp90 mit RPM1 und anderen NB-LRR-Proteinen, sondern die Hsp90-Aktivität für deren Funktion notwendig ist.

Interessanterweise resultierte nicht nur *Hsp90*-Silencing, sondern auch die transiente Überexpression von *Hsp90* in einer signifikanten Reduktion der Bs4-HR, obwohl in diesem Fall die relativen Bs4-Mengen erhöht waren (Abb. M3-11). Im aktuellen Arbeitsmodell (Abb. M3-13) reguliert Hsp90 die Bs4-Funktion. Es kontrolliert die im Schaltermodell postulierte Transition des Bs4-Proteins von einem erkennungskompetenten in einen signalkompetenten Zustand, der für die HR notwendig ist (Abb. M3-13). Ein Hinweis für eine tatsächlich stattfindende Zustandsänderung sind nicht mehr nachweisbare Bs4-Domänen-Interaktionen und die reduzierte Bs4-Menge. *Hsp90*-Überexpression könnte das Gleichgewicht zwischen erkennungskompetentem und signalkompetentem Zustand in Richtung erkennungskompetenten Zustand verschieben und dadurch für höhere Bs4-Mengen verantwortlich sein. Die entsprechend geringere Menge an signalkompetentem Bs4 ist der Grund für die reduzierte HR bei *Hsp90*-Überexpression. Es bleibt zu prüfen, ob

ein veränderter Hsp90-Bedarf der Pflanze ein mögliche Ursache für den Funktionsverlust zahlreicher Resistenz-Reaktionen bei erhöhten Temperaturen ist (siehe z.B. Whitham *et al.*, 1996; siehe z.B. Erickson *et al.*, 1999). Die in dieser Arbeit zu anderen NB-LRR-Proteinen durchgeführten *Hsp90*-Expressionsanalysen belegen bereits, dass HR-Reduktion durch *Hsp90*-Überexpression kein Bs4-spezifisches Phänomen ist, sondern auch für andere NB-LRR-Proteine gilt (Abb. M3-11). Die Hsp90-kontrollierte HR-Induktion von NB-LRR-Proteinen könnte demnach ein generelles Regulationsprinzip sein.

3.8. Die Bs4-HR benötigt Polyubiquitinierung

Die Abhängigkeit der Bs4-HR von *SGT1* gab bereits Hinweise auf die Notwendigkeit des Ubiquitin- bzw. Proteasom-vermittelten Proteinabbaus, da SGT1 eine Komponente eines Ubiquitin-Ligase-Komplexes ist (Peart *et al.*, 2002). In dieser Arbeit wurden Experimente mit einer Ubiquitin-Mutante (*ubR48*) durchgeführt (Schlögelhofer *et al.*, 2006), um die Bedeutung der Ubiquitinierung für die Bs4-vermittelte HR zu prüfen. Die Expression von *ubR48* inhibiert die Polyubiquitinierung, da eine Ubiquitin-Ubiquitin-Verknüpfung über den Lysinrest 48 nicht mehr möglich ist (Abb. 15). Für die Proteasom-vermittelte Proteolyse sind jedoch mindestens vier aneinander gekoppelte Ubiquitin-Einheiten am Zielprotein notwendig (Thrower *et al.*, 2000). Die Überexpression dieses *ubR48*-Konstrukts führte zur Abschwächung der *Bs4*-vermittelten HR. Dies unterstreicht die Notwendigkeit der Polyubiquitinierung für die Bs4-HR. Wäre Bs4 selbst polyubiquitiniert, so wäre bei Überexpression von *ubR48* der Bs4-Abbau supprimiert und eine Bs4-Akkumulation zu erwarten. Interessanterweise waren aber die Bs4-Mengen reduziert (Abb. M3-12). Das bedeutet, dass Bs4

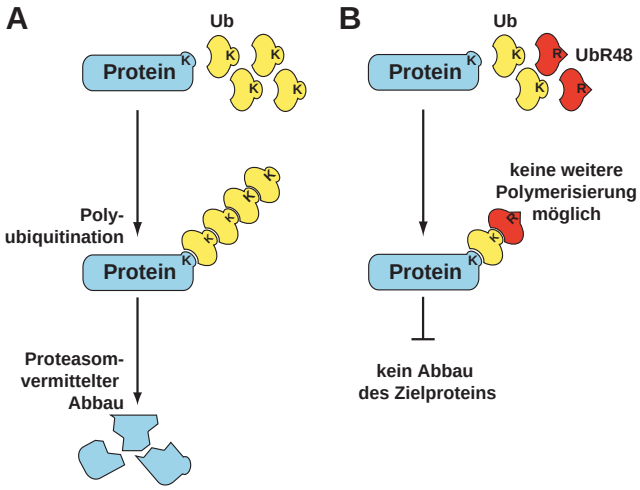

Abb. 15. Funktionsprinzip von ubR48 (A) Kovalente Bindung von Ubiquitin (Ubi) an Lysinreste (K) des Zielproteins und weitere Ubi-Ubi-Verknüpfung über K48 führt zur Polyubiquitinierung, einer Markierung für den Proteasom-vermittelten Abbau. **(B)** Bei ubR48 ist K48 durch Arginin (R) ersetzt. Eine Ubi-Ubi-Verknüpfung ist dadurch nicht mehr möglich. Wird ubR48 in die Poly-Ubi-Kette integriert, so kommt es zum Abbruch der Polymerisierung und damit je nach ubR48-Menge zur mehr oder weniger starken Inhibition des Proteasom-vermittelten Abbaus aller Ubi-markierten Proteine.

wahrscheinlich nicht selbst polyubiquitiniert wird. Da auch für RPM1 eine Elicitor-induzierte Reduktion der Proteinmenge beobachtet wurde, prüfte man dort den Einfluss des Ubiquitin/Proteasom-Abbaus auf die Resistenz mittels chemischer Inhibierung durch Geldanamycin. Es wurde zwar ein Funktionsverlust, aber keine Akkumulation von RPM1 nachgewiesen (Holt *et al.*, 2005). Demnach werden wahrscheinlich sowohl RPM1-, als auch Bs4-Mengen Ubiquitin-unabhängig reguliert.

3.9. Die Blockierung der Ubiquitinierung hat einen indirekten Effekt auf die Bs4-Funktion.

Die Überexpression von *ubR48* zeigte nicht nur einen Einfluss auf die Bs4-HR sondern beeinträchtigte auch die HRs aller anderen getesteten NB-LRR-Proteine (Abb. M3-12). Dies deutet auf eine Beeinflussung gemeinsamer Signalkomponenten der NB-LRR-vermittelten HR hin. Die Tatsache, dass *ubR48* keinen Einfluss auf die AvrBsT-induzierte HR in *N. benthamiana* hatte, lässt vermuten, dass das korrespondierende R-Protein nicht zum NB-LRR-Typ gehört.

Es kann angenommen werden, dass die *ubR48*-Überexpression zur Akkumulierung zahlreicher z. T. falsch gefalteter Proteine führt, die normalerweise über das Proteasom abgebaut werden. Die verstärkt vorhandenen Proteine sind zusätzliche Klienten des Hsp90-

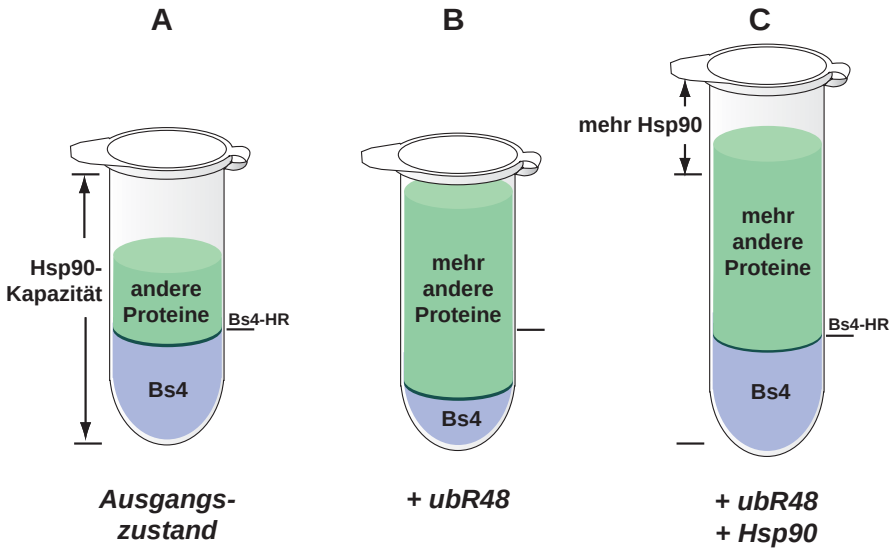

Abb. 16. Dosis-Modell des ubR48-Einflusses auf die Bs4-HR. (A) Viele pflanzliche Proteine benötigen Hsp90 als Chaperon. Bs4 erreicht durch Hsp90 eine für die Erkennung und HR kritische Menge. **(B)** Die *ubR48*-Expression blockiert die Polyubiquitinierung und führt dadurch zu Akkumulation fehlerhafter Proteine, die Hsp90-Klienten sind. Für Bs4 steht dann nicht genügend freies Hsp90 zur Verfügung. Bs4-Mengen und -HR sind reduziert. **(C)** Zusätzliche Hsp90-Überexpression stellt genügend freies Hsp90 bereit, um sowohl höhere Mengen an nicht abgebauten Proteinen abzudecken, als auch ausreichend erkennungskompetentes Bs4 zu generieren. Dadurch sollte die Bs4-HR wieder hergestellt sein.

Chaperons, so dass die Menge an verfügbarem Hsp90 für Bs4 reduziert ist (Abb. 16A,B). Demnach wäre Hsp90 der Mediator des *ubR48*-Expressionseffekts auf die Bs4-HR. Sollte diese Hypothese zutreffen, so müssten höhere Mengen an Hsp90 den *ubR48*-Effekt auf die Bs4-HR ausgleichen. Trotz Überexpression von *ubR48* wäre dann noch ausreichend Hsp90 vorhanden, um Bs4 zu stabilisieren und eine Bs4-HR auszulösen (Abb. 16C). Tatsächlich deuten vorläufige Daten daraufhin, dass bei gemeinsamer Überexpression von *Hsp90* und *ubR48* die ubR48-bedingte Bs4-HR-Reduktion nicht mehr nachweisbar ist (S. Schornack & T. Lahaye, unveröffentlicht).

Würde Bs4 nicht durch ausreichend Hsp90 stabilisiert, kann Bs4 keine AvrBs4-Erkennung und HR vermitteln. Diese postulierte Notwendigkeit einer Bs4-Mindestmenge für die HR wird unterstützt durch *Bs4*-Expressionsdaten unter Verwendung unterschiedlich starker Promotoren. Die *Bs4*-Expression unter Kontrolle eines schwachen Promotors war nicht ausreichend für eine Bs4-HR (Abb. M2-4, M2-5). Da *Hsp90* von einigen NB-LRR-Proteinen für die Funktion benötigt wird, lässt sich der nachgewiesene negative Einfluss der *ubR48*–Expression auf die HRs anderer NB-LRR-Proteine erklären (Abb. M3-12). Interessanterweise wurde bei erhöhten Temperaturen eine Mengenreduktion der R-Proteine Mla1 und Mla6 aus Gerste nachgewiesen (Bieri *et al.*, 2004). Dies könnte durch den temperaturbedingt höheren Bedarf an Hsp90 für andere Proteine und die dadurch reduzierte Menge an freiem Hsp90 für Mla1 und Mla6 verursacht worden sein.

3.10. Das Bs4-AvrBs4-System birgt einige ungelöste Fragen und bietet vielfältige Möglichkeiten zur weiteren Charakterisierung

Die in dieser Arbeit erhaltenen Daten bieten eine solide Basis für fortführende Experimente mit Bs4. Da Bs4 ein Vertreter der größten R-Protein-Klasse der NB-LRR Proteine ist, könnten die Ergebnisse einen Erkenntnisgewinn für andere *R*-Gene bringen.

Wichtige Aspekte wären z. B. proteinbiochemische Arbeiten zur Klärung der Bs4-Erkennungsspezifität und evolutionäre *Bs4*-Analysen. Die Verfügbarkeit von AvrBs3-ähnlichen Proteinen und zahlreichen davon abgeleiteten Derivaten bietet eine einmalige Ressource zur Eingrenzung des Avr-Erkennungsspektrums. Dieses könnte auch zusammen mit weiteren *Bs4*-sequenzhomologen Genen getestet werden, da die *Bs4*-vermittelte Resistenz und *Bs4*-ähnliche Sequenzen in allen bisher untersuchten *Lycopersicon*-Arten gefunden wurden. *Bs4* liegt als Einzelkopie im Tomaten-Genom vor (Schornack *et al.*, 2004). Dies erleichtert die PCR-basierte Amplifikation von *Bs4*-Allelen aus verschiedenen

Tomaten-Spezies. Zusammen mit transienten *Agrobacterium*-vermittelten Expressionen in *N. benthamiana* besteht die Möglichkeit, diese *Bs4*-Allele und ihre Sequenz-Funktions-Beziehungen zu analysieren. Dafür können auch verfügbare genetische Ressourcen in Tomate (z. B. Mutanten- und TILLING-Bibliotheken) verwendet werden, da die dort verwendeten Tomaten-Linien mit hoher Wahrscheinlichkeit ebenso ein funktionales *Bs4*-homologes Gen tragen.

Die PCR-basierte Mutagenese von *Bs4*-Abschnitten und *avrBs4*-Derivaten ist eine weitere Möglichkeit, um kritische AS-Reste für die Bs4-Autoaktivierung oder die AvrBs4-Erkennungsspezifität zu identifizieren. Aussagen zur funktionellen Relevanz generierter Mutationen können durch Analysen der Domänen-Interaktion und der Proteinstabilität getroffen werden. Reporter-Assays ohne arbeits- und zeitaufwendige Protein-Extraktion und Immunoblots sind langfristig für Hochdurchsatz-Sichtungen nach Mutanten notwendig. Eine Möglichkeit wären Fluoreszenz-basierte Reporter, z.B. YFP-Hälften an beiden Enden von Bs4, die nur bei intramolekularer Interaktion ein Fluoreszenzsignal abstrahlen. Auf diese Weise wäre auch die Suche nach weiteren pflanzlichen Proteinen möglich, die einen Einfluss auf die Regulationsmechanismen und die Bs4-vermittelte Erkennung von AvrBs4 haben könnten.

Die biochemische Aufreinigung von Bs4-haltigen Proteinkomplexen über Epitop-Markierung könnte zur Identifizierung von beteiligten Proteinen in der Pflanze verwendet werden. Die Charakterisierung solcher Komplexe könnte Einblicke in den, im Vergleich zur Bs4-Regulation, bisher noch unzureichend verstandenen Mechanismus der Avr-Erkennung zu geben.

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Verzeichnis der verwendeten Abkürzungen

3D	Dreidimensional
AD	Transkriptionelle Aktivierungsdomäne
AS	Aminosäure
ADP/ATP	Adenosin-Di-/Triphosphat
Avr	Avirulenz, avirulent
BiFC	*bimolecular fluorescence complementation*
C-	Carboxy-
CC	*coiled coil*
Co-IP	Co-Immunopräzipitation
d. h.	das heißt
d p.i.	Tage *postinfiltration* (nach Infiltration)
DNA	*desoxyribonucleic acid*, Desoxyribonucleinsäure
HR	hypersensitive Reaktion
hrp	hypersensitive Reaktion und Pathogenität
LRR	*leucine rich repeats*, Leucinreiche Wiederholungen
N-	Amino-
NB	Nukleotidbindestelle
NLS	Nukleäre Lokalisationssignale
NOD	*N-terminal oligomerisation domain*
PAMP	*Pathogenicity-associated molecular pattern*
p.i.	*post infiltration*, nach Infiltration
R	Resistenz, resistent
TILLING	*Targeted induced lesions in complex genomes*
TIR	Toll/Interleukin1-Rezeptor-ähnliche Domäne
Vir	Virulenz, virulent
WRKY	Tryptophan-Arginin-Lysin-Tyrosin-Motiv
WS	*watersoaked lesions*, Lässrige Läsionen
Xcv	*Xanthomonas campestris* pv. *vesicatoria*
z. B.	Zum Beispiel
Δ	Delta, Deletion

Lebenslauf

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende wissenschaftliche Arbeit selbstständig und ohne fremde Hilfe verfasst habe. Ich erkläre weiterhin, dass andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht wurden. Mit dieser Arbeit bewerbe ich mich erstmals um die Erlangung des Doktorgrades.

Halle (Saale), den 19.07.2006

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