The Role of Salicylic Acid and Octadecanoids for Pathogen Defense in Potato

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Contents

List of abbreviations

ACX	Acyl-coA Oxidase
AOC	Allene Oxide Cyclase
AOS	Allene Oxide Synthase
Avr	Avirulence gene
BA2H	Benzoic Acid-2-hydroxylase
BSA	Bovine Serum Albumin
BTH	Benzothiadiazole
CA	Colneleic Acid
CnA	Colnelenic Acid
COI1	Coronatin Insensitive
DAB	Diamino Benzidine
DAPI	4'-6-diamidine-2-phenylindole
DDE	Delayed Dehiscence
DEF	Defenseless
DES	Divinyl Ether Synthase
DNA	Deoxyribo Nucleic Acid
DND	Defense No Death
DNTP	Deoxyribonucleotide
EDTA	Ethylendiaminetetraaceticacid
EM	Electron Microscopy
EST	Expressed Sequence Taq
FAD	Fatty Acid Desaturase
FDA	Fluorescein di acetate
GC	Gas Chromatography
GSL	Glucan Synthase Like
GUS	β-glucuronidase
HOD	Hydroxyoctadecadienoic acid
HOT	Hydroxyoctadecatrienoic acid
HPLC	High Performance/ Pressure Liquid Chromatography
HR	Hypersensitive Reaction / Response
ICS	Isochorismate synthase
IDP	Diphenyliodonium
INA	2,6-dichloroisonocotinic acid
IPL	Isochorismate Pyruvate Lyase
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
JAI	Jasmonic Acid Insensitive
JAR	Jasmonic Acid Resistant
JIN	Jasmonic Acid Insensitive
LOX	Lipoxygenase
MAPK	Mitogen-Activated Protein Kinase
MS	Mass Spectrometry
NaCl	Sodium Chloride
NO	Nitric Oxide
NPR	Non expressor *PR1* gene
OD	Optical Density
OPC8	(9S, 13S)-12-oxophytodienoic acid to 3-2(2'(Z)-pentenyl) cyclopentane-1-octanoic acid

OPDA	Oxo-phytodienoic acid
OPR3	12-oxo-phytodienoate reductase
ORCA	Octadecanoid-derivative Responsive *Catharanthus* AP2-domain
PAL	Phenylalanine Amonia Lyase
PAMP	Pathogen Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PDF	Plant Defensin
Pep-13	Peptide elicitor from *Phytophthora* cell wall glycoprotein
PI	Propidium Iodide
Pi	*Phytophthora infestans*
Pin2	Proteinase Inhibitor 2
PR	Pathogenesis Related
Prp	Proline Rich Protein
Psm	*Pseudomonas Syringae* pv. *maculicola*
Pst	*Pseudomonas Syringae* pv. *tomato*
R	Resistance gene
Rboh	Respiratory burst oxydase homologue
RNA	Ribo Nucleic Acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase HPLC
rRNA	Ribosomal Ribo Nucleic Acid
RT-PCR	Reverse Transcript PCR
SA	Salicylic Acid
SAG	Salicylic Acid Glucoside
SAR	Systemic Acquired Resistance
SDS	Sodium Dodecyl Sulfate
SID	Salicylic Acid Induction Deficient
SPE	Solid Phase Extraction
SPR	Suppressed in 35S: prosystemin-mediated responses
SSC	Standard Saline Citrate
TCA	Tri Chloro Acetic Acid
TE	TRIS-EDTA buffer
THT	Hydroxycinnamoyl-CoA:tyramine N-(hydroxycinnamoyl)transferase
TIGR	The Institute of Genome Research
TUNEL	Terminal deoxynucleotidyl transferase UTP nick end labeling
UV	Ultra Violet
TMV	Tobacco Mosaic Virus
VSP	Vegetative Storage Protein
W2A	Inactive analogon of Pep-13
WRKY	Transcription factor
YEB	Yeast Extract Broth

I. Introduction

Due to their sessile nature, plants have to stay at the same place for their whole life span. To anticipate different environmental challenges, such as abiotic and biotic stresses, plants developed different kinds of strategies. In comparison to animals, at the cellular level, plants cells are not as specialized as animal cells. For example, against different kind of biotic stress such as pathogens, there are no phagocyte cells, T cells or B cells that function as active defenses. As a consequence, all plant cells should be able to defend themselves in many ways. Plants equip themselves with preformed barriers as well as inducible defenses. Preformed barriers vary from physical structures such as thorns, trichomes, the cuticle or the cell wall, to preformed chemical barriers such as saponins and alkaloids. The glycoalkaloid α-tomatine and the saponin avenacin are examples of preformed anti-microbial compounds in tomato and oat (Osbourn, 1996). Additional defense mechanisms are activated upon stress application. Cell wall depositions, oxidative burst, phytoalexin production, PR protein accumulation and finally localized cell death to restrict pathogen growth are examples of inducible defenses upon pathogen attack. Despite the existence of preformed barriers, the effective inducible defenses often play an important role to restrict the pathogen growth. The output of pathogen challenges, disease or resistance, is defined by how fast and how strong the inducible defenses are activated by the plants (Dong, 1998). The successful defense normally depends on specific recognition of the pathogen, effective signal transmission and finally, the ability to mount efficient responses.

Recognition can be at the species level, for example recognition of pathogen-associated molecular patterns (PAMPs) or at the cultivar level, which is marked by recognition of avirulence (*avr*) gene products of the microorganism by corresponding resistance (*R*) genes products in the plant (Nurnberger and Scheel, 2001). Recognition of PAMPs by plants leads to defense responses both in non-host and host plants. The defense responses in non-host plants lead to non-host resistance, which is defined as resistance of an entire plant species to all isolates of a microbial species (Nurnberger and Lipka, 2005). This type of resistance is normally very effective to stop the pathogen growth. However, defense responses in host plant due to the recognition of PAMPs are not as effective as in non-host plant to stop the growth of pathogen. This type of defense is defined as basal defense. Although basal defense is not effective to completely stop pathogen growth, it is able to reduce the growth of pathogens. To deal with the resistant

plants, some races of pathogens might develop virulence factor to suppress the plant defense and cause disease. However, some plants acquired the ability to recognize the virulence factor of the pathogen and became resistant to this certain race of pathogen. This type of resistance is defined as race-cultivar-specific resistance (Hammond-Kosack and Jones, 1996; Nurnberger and Lipka, 2005).

Signal transmission leading to defense is triggered upon perception of a pathogen. Defense signaling is normally conserved in plants, regardless of the interaction, non-host- or host-pathogen interaction. Transient changes in the ion permeability of the plasma membrane apparently initiates the signaling pathways, followed by the activation of a mitogen-activated protein kinase (MAPK) cascade and the oxidative burst. The MAPK cascade is the best example for converging signals upon perception. Recognition of PAMPs such as flagellin by its receptor, *FLS2* in *Arabidopsis* (Gomez-Gomez and Boller, 2002) and Pep-13 by parsley cells (Kroj et al., 2003) trigger downstream defense responses mediated by a MAPK cascade. Pep-13 is a 13-amino-acid peptide, which is conserved in a *Phytophthora* cell-wall-glycoprotein. It is able to elicit a wide range of defense responses in parsley and potato cells, (Nurnberger et al., 1994; Geiler, 2001; Brunner et al., 2002). Interestingly, MAPK cascades also mediate defense responses that are mounted by tobacco plants upon recognition of the race-specific elicitor Avr9 from *Cladosporium fulvum* by the corresponding *R* gene product in tomato, Cf-9 (Hammond-Kosack et al., 1994; Romeis et al., 2001). These data clearly show conserved signaling pathways from different resistance mechanisms. Many microbial elicitors and many attempted infections by avirulent pathogens also cause a rapid oxidative burst that triggers programmed cell death in challenged cells (Levine et al., 1994). Later experiments showed that this programmed cell death is triggered mainly by nitric oxide (NO) that acts synergistically with reactive oxygen species from the oxidative burst (Delledonne et al., 1998). Rapid cell death at the site of infection might have a role to restrict biotrophic pathogens. Reactive oxygen species may also have a direct anti-microbial effect and may serve as a signal for the activation of other defense responses (Glazebrook, 2005).

Salicylic acid (SA), jasmonic acid (JA), and ethylene have been shown as signaling molecules that have a central role in signaling networks (Dong, 1998). Other defense pathways independent from SA, JA, and ethylene signaling might exist and await discovery (McDowell et al., 2000; Zimmerli et al., 2000; Zipfel et al., 2004). Using *NahG* plants that are unable to accumulate SA, various mutants of *Arabidopsis*, and exogenous SA application, SA has been shown to be important in controlling a wide range of

downstream defense responses such as callose deposition, pathogenesis related (PR) protein accumulation, phytoalexin production, oxidative burst, and finally hypersensitive cell death (HR). Experiments on the role of JA have been done by exogenous application of JA and its methyl ester, by wounding and herbivore attack on various JA-deficient and JA-insensitive tomato and *Arabidopsis* mutants. In wounded and herbivore-attacked plants, JA has been shown to be important for the induction of downstream defense responses, such as the oxidative burst, proteinase inhibitor accumulation, plant defensin gene expression (*AtPDF1.2*) and plant volatile production (Lou et al., 2005), (Schweizer et al., 1997), (Tamogami et al., 1997). Studies of pathogens from different classes such as viruses, bacteria, fungi, oomycetes and various pathogens from different lifestyles such as biotrophs and necrotrophs on different *Arabidopsis* defense signaling mutants have shown the importance of distinct SA or JA signaling pathway to control the growth of pathogen from certain lifestyle regardless of the class of the pathogen. Biotrophic pathogens normally grow and feed on living cells. The SA pathway in plants is normally up-regulated upon attack by biotrophic pathogens. Since SA is important for HR formation, activation of the SA pathway leads to a rapid cell death formation around the infection site and abrogates the growth of the pathogen due to the lack of living cells to feed them. Necrotrophic pathogens normally kill the cells and feed on them. To prevent this, plants normally up-regulate the JA/ethylene pathway to prevent cell death and thus reduce the growth of the pathogen. Inactivation of the corresponding pathway normally leads to susceptibility (Glazebrook, 2005). Because of the central role of SA, JA and ethylene in controlling a wide range of downstream defenses against pathogens, the ability of plants to fine-tune the signaling through the SA, JA and ethylene pathways is very important for the survival of plants against pathogens. Fine-tuning will lead to an optimal mixture of defense responses to resist the intruder. It can be done by controlling basal levels of the signals and the changes of these levels upon infection. The other example of fine-tuning is the cross-talk between signaling pathways (Pieterse, 2001).

A. Basal defense

Basal defense is defined as a rapid-activated defense upon infection by almost all microbes due to recognition of general elicitors from microbes by plants (Boller et al., 2005). This general defense mechanism can be activated after an HR in gene-for-gene interactions or during a successful infection to prevent an existing infection from spreading further or to combat secondary infections from a broad spectrum of

pathogens (Dong, 1998). Inhibiting this defense often leads to even higher susceptibility of the plant and higher growth of the pathogen. The phenomena of increasing susceptibility in susceptible plants by inhibiting basal defense showed that resistance and susceptibility are not binary alternatives. Resistance and susceptibility should be seen as a continuum of possible interactions, ranging from complete resistance to extreme susceptibility (Glazebrook, 2005). An example of basal defense is the interaction between *Arabidopsis* and *Hyaloperonospora parasitica*. Loss of resistance of *Arabidopsis* to both avirulent and virulent *H. parasitica* can be induced by diminishing SA levels in the plant (Delaney et al., 1994).

The importance of downstream defense responses, such as the accumulation of callose, pathogenesis related proteins, stimulation of the oxidative burst and hypersensitive cell death for basal resistance has been studied. *In vivo* correlation studies using wildtype, *npr1* (mutant that is unable to express PR1a) and *NahG Arabidopsis* plants infected with *H. parasitica* showed that callose might be important for basal defense of *Arabidopsis* against *H. parasitica* (Donofrio and Delaney, 2001). Knocking out *GLUCAN SYNTHASE LIKE5* (*GSL5*) in *Arabidopsis* using a T-DNA insertion line or dsRNAi, that caused loss of wound as well as papillary callose, resulted in enhanced penetration of the grass powdery mildew fungus *Blumeria graminis* on the non-host *Arabidopsis* (Jacobs et al., 2003). Paradoxically, the absence of callose in papillae or haustorial complexes correlated with the effective growth cessation of several normally virulent powdery mildew species and of *H. parasitica* (Jacobs et al., 2003). The ability of virulent powdery mildew to exploit the interaction among defense responses for their ends including callose formation has been shown. In an *Arabidopsis* mutant that is unable to form callose (*pmr4*), the SA-induced gene expression is higher upon powdery mildew infection compared to wildtype. This correlates with increased resistance of *pmr4* against powdery mildew (Nishimura et al., 2003). Overexpression of *PR1a* in tobacco and *PR5* (osmotin) in potato resulted in higher resistance to virulent pathogens (Alexander et al., 1993; Liu et al., 1994). However, overexpression of *PR5* (osmotin) in tobacco did not make tobacco plants more resistant to *Phytophthora parasitica* var. *nicotinae*. Although it has been correlated with defense for a long time because of its role in HR formation, the role of the oxidative burst for plant defense is more problematic. A recent publication reported that two *Atrboh D* and *Atrboh F* genes are important for full oxidative burst formation in *Arabidopsis* upon incompatible interaction with bacterial pathogen

Pseudomonas syringae pv. *tomato* (*Pst*) DC3000 (*avrRPM1*) (Torres et al., 2002). However, those mutants showed enhanced cell death after infection with *H. parasitica*. The enhanced cell death in these mutants later on can be correlated to the enhanced resistance to *H. parasitica* (Torres et al., 2002). These data showed the importance of cell death instead of oxidative burst for the resistance of *Arabidopsis* against *H. parasitica*. The importance of quick and strong hypersensitive cell death to restrict hyphal growth of *P. infestans* during *R* gene-mediated resistance in potato has been shown (Vleeshouwers et al., 2000).

B. Systemic acquired resistance (SAR)

Systemic acquired resistance is defined as a general resistance mechanism, which is induced after an HR or during a successful infection to combat secondary infection from a broad spectrum of pathogens or to prevent an existing infection from spreading further (Dong, 1998). The mechanism of SAR is a general strategy that is used by plants to defend themselves and has been shown for different plant species such as tobacco, cucumber, potato, and rice. For example, infection of potato plants with *Pseudomonas syringae* pv. *maculicola* (*Psm*) induces SAR which is effective against *P. infestans* (Kombrink et al., 1994). However, SAR can also be induced by SA. Chemical analogues of SA, such as benzothiadiazole (BTH) and 2,6-dichloroisonicotinic acid (INA) are also active in inducing SAR in tobacco and *Arabidopsis*. Upon infection, SA levels increase systemically in tobacco and cucumber (Rasmussen et al., 1991). SA could also be found in their phloem exudates (Rasmussen et al., 1991). Analysis of the phloem exudates showed that an unknown signal leading to SAR is produced before SA accumulation (Rasmussen et al., 1991). Using grafting experiments between wildtype and *NahG* tobacco plants, SA was shown not to be the transported signal for SAR. However, it is important to perceive the signal in the systemic leaves (Vernooij et al., 1994). The importance of ethylene perception to generate the systemic signal has also been demonstrated (Verberne et al., 2003). The JA pathway has been shown to be important for wound-induced defense responses and induced systemic resistance (ISR) by root-colonizing bacteria (Pieterse et al., 1998). Induced resistance in systemic leaves against *Magnaporthe grisea* was also shown in wounded or JA-treated rice plants (Schweizer et al., 1998). However, the possible role of JA for systemic acquired resistance (SAR) is not well studied. Previously, our lab showed that there was no increase of JA in systemic leaves after

Psm infection. Unlike JA, the levels of OPDA in systemic leaves increased upon *Psm* infection (Landgraf et al., 2002). Although the primary signal for SAR in plants is still elusive, it is known that this signal should be mobile. The importance of the protein secretory pathway, extracellular protease function and lipid transfer protein was demonstrated recently (Maldonado et al., 2002; Xia et al., 2004; Wang et al., 2005).

C. SA-signaling

SA is a naturally occurring phenolic compound in many plants. It is produced through the phenylpropanoid pathway, with benzoic acid as an intermediate (Shah, 2003). However, an alternative pathway exists. Like in some bacteria, *Arabidopsis* was shown to produce SA through the isochorismate pathway upon infection with the virulent pathogen *Erysiphe* and the avirulent pathogen *Pseudomonas syringae* pv *maculicola (Psm)*. In *sid2* mutants, that have a defect in the gene encoding isochorismate synthase, total SA accumulation is about 5-10% of the wildtype levels in response to infection (Wildermuth et al., 2001). More details on the SA biosynthesis pathway in plants are shown in Figure 1.

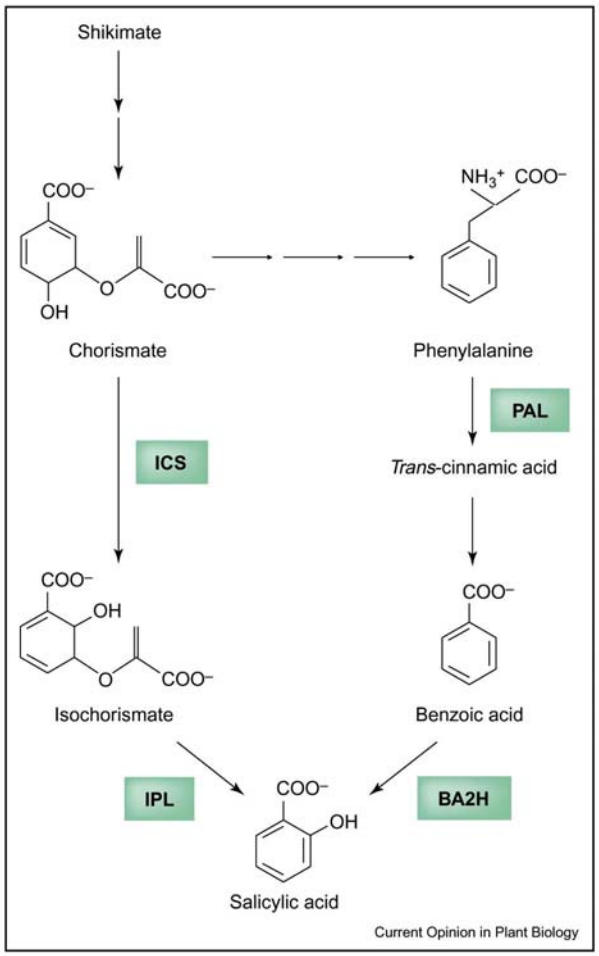


Fig. 1. In plants, SA can be synthesized from two different pathways, the phenylpropanoid pathway with benzoic acid as an intermediate, as well as the newly identified isochorismate pathway. Isochorismate synthase (ICS), Isochorismate pyruvate lyase (IPL), Phenylalanine ammonia lyase (PAL), Benzoic-acid-2-hydroxylase (BA2H) are the important enzymes for SA biosynthesis (Shah, 2003).

In plants, SA can be found in a free as well as a conjugated form. Glucosides, esters and amide conjugates have been identified in different plants, but the majority is usually 2-O-β-glucopyranosyl-SA (Coquoz et al., 1998). It was suggested that free SA is active, while conjugated SA acts as storage form of SA which can be cleaved to free SA by glucosidase when needed. Both forms are referred to as total SA. Interestingly, the levels of total SA are different among plants. *Arabidopsis* and tobacco contain small amounts of SA (<50 ng/ g fresh weight). Upon pathogen attack the amounts increase. However, only a small increase of SA level in systemic leaves (1.2- to 4- fold) is sufficient for the establishment of SAR (Vernooij et al., 1994). Higher basal levels of SA were observed in potato and rice (Yu et al., 1997).

The role of SA for plant defense was first studied by the observation of toxic effects of SA on many pathogens and by correlating SA levels in bark extract from different poplar cultivars with the ability of the cultivars to resist infection (Malamy and Klessig, 1992). Exogenous application of SA to tobacco induced many responses, which are associated with viral attack. Comparing SA levels in resistant and susceptible tobacco cultivars upon TMV infection provided more information. Higher SA levels in a TMV-resistant tobacco cultivar (at least 20 fold) were observed. However, when the infected plant was incubated at higher temperature (>28 ^{o}C), the resistance was blocked. Interestingly, the block in resistance is concomitant with the block in SA accumulation and PR gene expression (Malamy and Klessig, 1992). The role of endogenous SA for plant defense was studied in more detail using *NahG* plants that contain almost no SA. *NahG* plants were obtained by transforming wildtype plants with the *NahG* gene from *Pseudomonas putida*. This gene encodes an SA hydroxylase which converts SA to catechol. *NahG Arabidopsis* and tobacco plants were more susceptible to viral and bacterial pathogens (Delaney et al., 1994). Moreover, *NahG* tobacco plants were also unable to develop SAR (Gaffney et al., 1993). Enhanced susceptibility and lack of inducible SAR in *NahG* plants indicate that SA accumulation is essential for the expression of multiple types of plant disease resistance.

In the past ten years, more detailed studies have been performed using different *Arabidopsis* mutants which have defects in SA biosynthesis such as *sid2*, *eds5* and in SA signaling (*npr1)*. *SID2* encodes an isochorismate synthase, the *eds5* mutant possibly has a defect in the transport of intermediates in SA biosynthesis (Wildermuth et al., 2001; Nawrath et al., 2002), and *NPR1* encodes an ankyrin repeat protein (Cao

et al., 1997). These mutants are more susceptible to biotrophic pathogens such as *H. parasitica* but not necrotrophic pathogens such as *Alternaria brassicicola* and *Botrytis cinerea* (Thomma et al., 1998; Nawrath and Metraux, 1999). The importance of the SA pathway to restrict biotrophic pathogen growth has been correlated with the role of SA to cause HR. SA induction was associated with HR formation (Hammond-Kosack and Jones, 1996). Cell death in the *Arabidopsis lsd1* mutant also depend on SA (Aviv et al., 2002). Together with an increase in reactive oxygen species (ROS), SA was postulated to induce cell death after ozone treatment (Overmyer et al., 2003). The importance of other signaling compound, nitric oxide (NO) for cell death formation has also been shown (Delledonne et al., 1998). However, the study on *Arabidopsis dnd1* mutant revealed that SA accumulation can be uncoupled from HR (Yu et al., 1998). Therefore, it has been hypothesized that SA may work together with other signals, such as H_2O_2 and NO to cause HR in plant (Shirasu et al., 1997; Delledonne et al., 2001).

The importance of SA for development of systemic acquired resistance (SAR) was revealed by comparing SAR development in wildtype and *NahG* tobacco plants (Gaffney et al., 1993). As mention before, grafting experiments between wildtype and *NahG* tobacco plants revealed that SA is not the transported signal for SAR. However, it is important to perceive the signal in the systemic leaves (Vernooij et al., 1994).

D. JA-signaling

JA is a lipid-derived compound. JA biosynthesis is started from linolenic acid (Fig. 2). Introduction of molecular oxygen into linolenic acid which is catalyzed by a 13-lipoxygenase marks the first step of the oxylipin pathway. Sequential enzymatic reactions catalyzed by allene oxide synthase (AOS), allene oxide cyclase (AOC), 12-oxo-phytodienoic acid reductase (OPR3), followed by three cycles of β-oxidation lead to JA formation. In the last couple of years, additional enzymes of JA biosynthetic pathway were identified. *In vitro* studies have shown that peroxisomal acyl-coenzyme A synthetases from *Arabidopsis thaliana* could use OPDA as the substrate. This finding and localization study showed the possibility that this enzyme contributes to JA biosynthesis by initiating the beta-oxidative chain shortening of its precursors (Schneider et al., 2005). Analysis of tomato mutants that lack local and systemic expression of proteinase inhibitor in response to wounding led to the identification of

acyl-coA oxidase (ACX1A) as an important enzyme in JA biosynthesis, performing β-oxidation (Li et al., 2005).

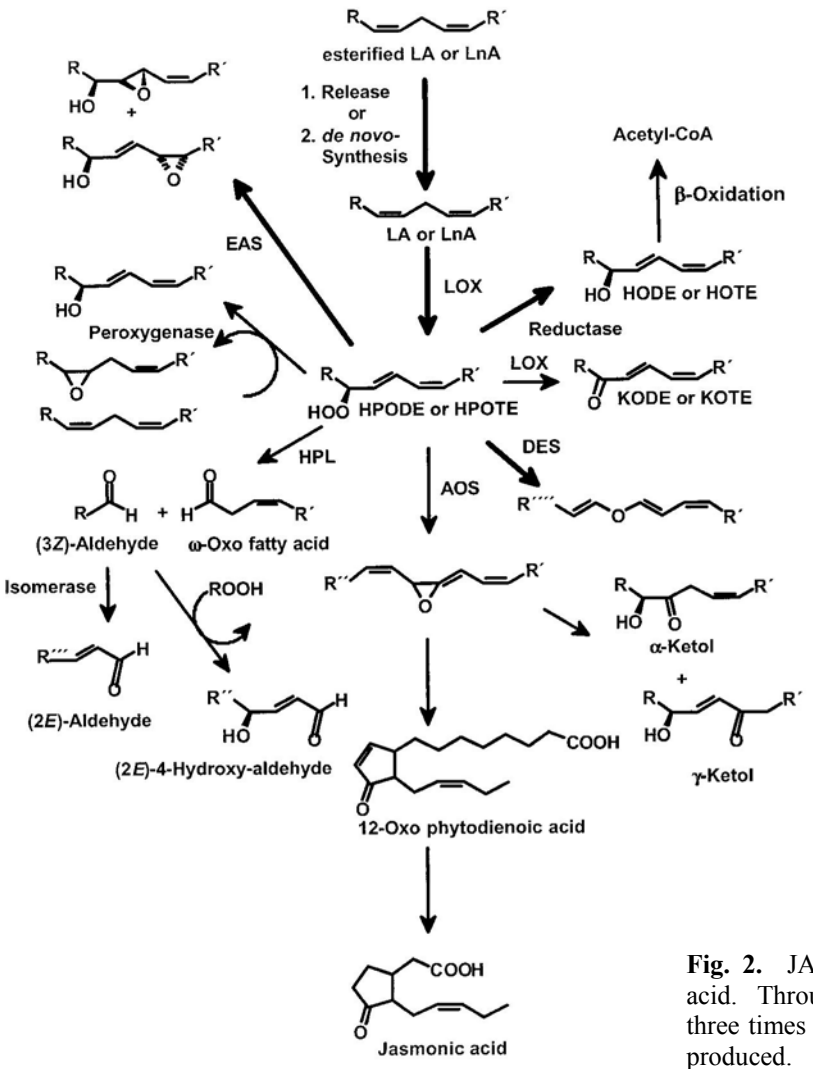


Fig. 2. JA originates from linolenic acid. Through the LOX pathway and three times β-oxidation reaction, JA is produced.

Most researches on JA were performed to study its role in development, wounding and herbivore defense. JA plays a role in developmental processes such as seed germination, root growth, pollen development and tendril coiling (Turner et al., 2002). Upon wounding or herbivore attack, JA levels increase. This increase induces the expression of genes, such as those encoding proteinase inhibitor (*Pin2*) in *Solanaceae,* plant defensin (*Pdf1.2*) and a vegetative storage protein (*Vsp*) in *Arabidopsis* (Leon et al., 2001), (Berger et al., 1995). JA-methyl ester was shown to be as active as JA in inducing defense gene expression. Precursors of JA biosynthesis are also able to induce proteinase inhibitor accumulation in tomato plants (Farmer and Ryan, 1992). Levels of a related compound derived from hexadecatrienoic acid, dinor-oxophytodienoic acid (dinor-OPDA), also increase upon wounding. Dinor-OPDA is

able to regulate its own pathway and might also be active (Weber et al., 1997). Together, the levels of dinor-OPDA, OPDA and JA in the plant were defined as oxylipin signature (Weber et al., 1997). Genetic screening of *Arabidopsis* led to the identification of mutants with defects in JA biosynthesis, such as the *fad3 fad7 fad8* "triple" mutant, *dde1, dde2-2, acx1* and in JA perception, such as *coi1*, *jar1*, and *jin1*. While the biosynthetic mutants have defects in JA biosynthetic enzymes such as AOS for *dde2-2* (von Malek et al., 2002), OPR3 for *dde1* (Sanders et al., 2000), and acyl-CoA oxidase for *acx1* (Li et al., 2005), the perception mutants are mostly disturbed in regulatory mechanisms related to JA such as the F box protein COI1 (Xie et al., 1998), the JA-aminosynthetase JAR1 (Staswick and Tiryaki, 2004) and the MYC transcription factor JIN1 (Lorenzo et al., 2004). Little is known about downstream response regulation of JA leading to defense responses. While the mechanism of JA to modulate defense reactions through the transcription regulator, *ORCA3*, has already been shown (van der Fits and Memelink, 2000), yet the JA receptor awaits to be discovered.

The role of JA for plant defense against pathogens was shown earlier by exogenous application of JA and Me-JA which protected potato plants locally and systemically against *P. infestans* (Cohen et al., 1993). Later on, with different tomato and *Arabidopsis* mutants, JA was shown to be important for plant defenses against necrotrophic pathogens such as *A. brassicicola* and *B. cinerea* (Thomma et al., 1998). Ozone has been implicated in the studies of pathogen-induced cell death due to its similarity in apoplastic reactive oxygen species production and cell death formation (Overmyer et al., 2003). The importance of JA for antagonizing cell death upon ozone treatment has been shown (Overmyer et al., 2003).

Stintzi *et al*. (2001) showed that the *fad3 fad7 fad8* "triple" mutant that fails to synthesize linolenate, the precursor of JA, is more susceptible to the dipteran insect *Bradysia impatiens* and the fungus *Alternaria brassicicola*. The *coi1* mutant that is defective in JA signaling also shows susceptibility. Surprisingly, the *opr3* mutant that lacks JA shows the same resistance to both insect and fungus as wildtype plants. This leads to the conclusion that in the absence of JA, the upstream precursor, OPDA is the active compound (Stintzi et al., 2001). Further gene expression analyses of wounded and hormone-treated wild type and *opr3* plants showed that in the absence of JA, some JA- and wound-induced genes can still be up-regulated. The authors hypothesized that OPDA can induce gene expression via two distinct pathways. One pathway is similar

to the JA pathway while the other is activated via the electrophile effect of the cyclopentenone ring (Stintzi et al., 2001). The ability of OPDA to induce expression of several genes independent from JA biosynthesis and perception has also been reported (Taki et al., 2005).

The importance of JA for systemic wound responses was demonstrated by grafting experiments of wildtype, JA biosynthetic mutant (*spr2*) and JA response mutant (*jai1*) tomato plants (Li et al., 2002). Analysis of the tomato mutant *acx1*, which has a defect in β-oxidation revealed that JA, but not its precursors, is important for the generation of the mobile signal for systemic defense gene expression upon wounding (Li et al., 2005). As discussed before, JA signaling is also important for systemic gene expression in induced systemic resistance (ISR) by root-colonizing bacteria (Pieterse et al., 1998). The possible role of JA for systemic acquired resistance (SAR) is not well studied. In potato, there was no increase in JA levels in systemic leaves after *Psm* infection, whereas the level of OPDA in systemic leaves increased upon *Psm* infection (Landgraf et al., 2002).

E. Cross-talk in plant defense signaling pathways

Cross-talk between defense signaling pathways is well documented in plant systems. Exogenous application of SA inhibits wound- and JA-induced proteinase inhibitor gene expression and protein accumulation in tomato (Penacortes et al., 1993; Doares et al., 1995). Tomato *coi1* mutants which are unable to accumulate transcripts of JA-responsive genes are more resistant to *Pst* DC3000 compared to wildtype (Zhao et al., 2003). Interestingly, this resistance correlates with higher SA-responsive gene expression in the *coi1* mutant compared to that in wildtype plants. These data show the antagonistic effect between SA and JA pathways in tomato. In *Arabidopsis*, cross-talk between SA and JA is also mutual repression. Infection of *Arabidopsis* plants, which are unable to accumulate SA leads to much higher JA accumulation and JA-responsive gene expression such as *LOX2, PDF1.2* and *VSP* (Spoel et al., 2003). An effect of light on SA and JA accumulation after *Psm* (*avrRPM1*) infection also shows an antagonistic correlation. Whereas JA accumulation was higher in the dark, SA accumulation was higher under the high light condition (Zeier et al., 2004). Enhanced SA levels and reduced JA responses in the *Arabidopsis mpk4* mutant (Petersen et al., 2000) indicate the regulation of cross-talk upstream of SA and of JA accumulation. However, downstream components like *NPR1* and *WRKY70* have also been shown to

modulate the cross-talk between SA and JA signaling pathway (Spoel et al., 2003; Li et al., 2004).

A close relationship between JA and ethylene signaling pathways to induce defense gene expression has been shown. Infection of *Arabidopsis* with *A. brassicicola* led to increased ethylene production and *PDF1.2* expression. No increase in *PDF1.2* expression was observed in the ethylene-insensitive mutant, *ein2*, and the JA-insensitive mutant *coi1,* suggesting that concomitant activation of JA and ethylene response pathways is important for *PDF1.2* induction (Penninckx et al., 1998).

Less data are available on the relationship between SA and ethylene signaling pathways. Ethylene is important for the development of disease symptoms. The synergistic effect between SA and ethylene to cause cell death after ozone treatment has been reported (Overmyer et al., 2003). The role of ethylene for SA accumulation under ozone treatment has also been shown (Ogawa et al., 2005). However, more detailed studies are required to further elucidate the relationship between these two pathways.

The complexity of the cross-talk among SA, JA and ethylene signaling pathways suggests that fine-tuning is important to get an optimal mixture of defense responses to resist the intruder. Therefore, inducing defense pathways for improved resistance should be considered carefully. It is possible that inducing a certain pathway for creating resistant plants against certain pathogens by genetic engineering or application of defense signal-mimicking plant protectants can end up in susceptibility to other pathogens (Pieterse, 2001).

F. Potato defense against *Phytophthora infestans* (*P. infestans*): The role of SA and JA signaling for basal defense and systemic acquired resistance (SAR)

P. infestans is the classic pathogen of potato plants that causes the devastating late blight disease. US$ 2.75 billion annual loss is caused by the late blight disease (source: The International Potato Center). Using resistant varieties and application of the fungicide metalaxyl are the common solution for the problem. However, the high frequency of mutations in *P. infestans* and the emerging of new mating types of *P. infestans* amplifies the problems. Previously resistant varieties become susceptible and fungicides become less potent against this disease. Therefore, new approaches are needed to improve the defense of potato plants against *P. infestans.*

Sensitive recognition and effective signaling pathways are important factors for plant defense against pathogens. Recognition of *P. infestans* by introduction of an *R* gene which recognizes an *Avr* gene in *P. infestans* results in highly resistant potato plants against certain isolates of *P. infestans* (Ballvora et al., 2002). However, this approach encounters some problems. An *R* gene is only related to a single *Avr* gene, therefore it is difficult to generate resistance to a wide range of *P. infestans* in this way. If avirulent *P. infestans* strains can evolve to overcome the genetic resistance that has been introgressed by conventional breeding from wild potato species into commercial varieties, it is possible with a simple mutation they can avoid *R* gene mediated recognition. Effective signaling is the other possibility to increase resistance against *P. infestans.* As mentioned previously, SA, JA and ethylene are important signaling components in various plants species. In potato, there is a positive correlation between SA levels and field resistance against *P. infestans* (Coquoz, 1995). Unfortunately, the role of SA against *P. infestans* could not be confirmed using *NahG* potato plants. Pathogenicity studies based on the lesion size on wildtype and *NahG* potato plants showed that *NahG* potato plants were not more susceptible than wildtype (Yu et al., 1997). However, over-expression of the SA-induced gene *PR5* resulted in resistance of potato plants against *P. infestans* (Liu et al., 1994). Detailed studies to clarify the role of SA are needed. Exogenous application of JA and MeJA resulted in protection of potato plants to *P. infestans* (Cohen et al., 1993). Further studies provided conflicting data on the role of this compound. Thus, JA was shown to be effective against a wide range of pathogens in tomato (Thaler et al., 2004). Analysis of the growth of eight different pathogens in jasmonate-deficient tomato mutants (*def1*) was done in comparison to that in wildtype tomato plants. JA-deficient tomato mutants were more susceptible to five pathogens including two bacteria (*Pseudomonas syringae* and *Xanthomonas campestris*), two fungi (*Verticillium dahliae* and *Fusarium oxysporum* f. sp. *lycopersici*) and one oomycete (*P. infestans*). However susceptibility to three fungi (*Cladosporium fulvum*, *Oidium neolycopersici*, and *Septoria lycopersici*) was unaffected (Thaler et al., 2004). Therefore, it is interesting to see the role of JA in the potato – *P. infestans* pathosystem. Recent finding on the possible role of OPDA, the precursor of JA, for defense against pathogens and herbivores (Stintzi et al., 2001) and systemic accumulation of OPDA in systemic leaves of potato upon primary challenge with *Psm* (Landgraf et al., 2002) makes it also interesting to be studied.

Basal levels of signaling compounds are different among different leaf stages within a plant. The levels also differ among varieties. The SA levels are higher in the certain varieties, such as Mathilda and Panda, while it is lower in Bintje and Sirtema (Coquoz, 1995). The levels of SA are also different among the plant species. The basal SA levels are low in tobacco and *Arabidopsis*, 40 to 100 times higher in potato and hundreds times higher in rice. In tobacco and *Arabidopsis*, SA acts as a signaling compound. Increase of SA upon pathogen attack, elicitor treatment as well as exogenous SA application induces downstream defense responses, such as PR gene expression. Cross-talk between SA and JA pathways was also demonstrated in tobacco and *Arabidopsis*. It has been discussed in section E of this chapter. In rice, the SA level is not increased upon pathogen treatment. Also, rice plants do not appear to react to exogenous SA treatment, since PR genes and induced resistance are also poorly activated. Instead, high SA levels in rice are important to modulate the redox balance and to protect rice plants from oxidative stress (Yang et al., 2004). In rice, the importance of JA as a signaling compound is more pronounced. Exogenous JA application also induced some PR gene expression, phytoalexin accumulation and volatile emission (Schweizer et al., 1997; Tamogami et al., 1997; Lou et al., 2005). Furthermore, JA is an effective inducer of systemic acquired resistance (SAR) in rice seedlings against infection of blast fungus (Lee et al., 2001). No detailed studies on the cross-talk between SA and JA in rice has been reported.

The aim of the project is to elucidate the role of SA, JA and OPDA for basal defense of potato plants against *P. infestans*. Modulation of the levels of these compounds should be achieved by loss-of-function approaches using *NahG*, *StOPR3*-RNAi and *StAOS2* co-suppressed plants. The importance of each compound should be reflected by the growth of *P. infestans* in the corresponding transgenic potato plants compared to that in wild type potato plants.

In addition to the basal defense study, the role of SA, JA and OPDA for Pep-13-induced defense responses in potato should be evaluated using the transgenic plants. The ability of SA and JA to affect the downstream defense responses such as oxidative burst, defense gene expression, and hypersensitive cell death will be analyzed using wildtype, *NahG*, *StOPR3*-RNAi and *StAOS2* co-suppressed potato plants. The relationship between these two signaling pathways should be studied indirectly by analyzing downstream defense responses of each pathway. Data from Pep-13-treated potato cell cultures and *Psm*-treated potato leaves will be incorporated when relevant.

To further study SA and JA defense signaling in potato, the ability of Pep-13 in inducing SAR in potato and the importance of SA and JA for establishment of SAR should be studied. Identification of marker gene for Pep-13-induced SAR will be important to study the mechanism of Pep-13-induced SAR in potato.

II. Materials and methods

A. Cloning of the *StOPR3*-RNAi construct

Sequence comparison of *AtOPR3* and *LeOPR3* revealed regions of high homology, from which the primers **5'-AATCCACTCAGCCTTGGCTTAGCAG-3'** and **5'-GTCCATTGC TTCCATTTCCTTGAA-3'** were generated. These primers later on were used to make a partial *StOPR3*-cDNA from potato leaf RNA using a one step RT-PCR kit (Invitrogen, Carlsbad, USA). This partial *StOPR3*-cDNA was ligated to the pCR 2.1 vector. To use gateway system, new attB-containing primers **5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAATCCACTCAGCCTTGGC-3'** and **5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGTCCATTGCTTCCATTTC-3'** were generated. These primers were used to generate an attB-containing *StOPR3* fragment. Using the BP reaction, the fragment was cloned into pDONR 201 (Invitrogen, Carlsbad, USA). Using the LR reaction, the fragment was further transferred to the binary vector pHellsgate 8 (Wesley et al., 2001), http://www.pi.csiro.au/tech_licensing_biol/licensing_pdfs/Hell28maps.pdf). It carries a 35S promoter, a spectinomycin resistance gene, a kanamycin resistance gene and two insertion sites to form an inverted repeat that is the prerequisite for silencing. Fragment-containing pHellsgate 8 was later on transferred to *Agrobacterium tumefaciens* LBA 4404 which has no spectinomycin resistance gene (Wesley et al., 2001). Fifty micromolar spectinomycin was normally used for selection. Potato plants were transformed with the recombinant agrobacteria (Feltkamp et al., 1995). For transformation, potato leaves from four-week-old potato plants grown in the sterile solid 2MS medium and 20 ml two-day-old agrobacteria culture in the antibiotic-containing YEB medium are needed. Agrobacteria was centrifuged 4200 (g) for 10 min, washed with 10 mM $MgSO_4$ and finally resuspended in 10 ml 3MS medium by gentle mixing. The detached potato leaves were then put on the surface of 100x diluted agrobacteria suspension for 2 days in the dark at 20-25^{o}C. After 2 days, potato leaves were taken and transferred to the root-inducing medium and after 1 week

the leaf was moved to the shoot-inducing medium. The leaves produced callus and shoots and after 4 to 6 weeks the shoots were big enough to be moved to an agar pot.

B. DNA extraction and southern analysis

After kanamycin selection, the transformed plants were analyzed for the presence of the *StOPR3* fragment. For this purpose, DNA from the plants was extracted. Frozen leaf material was ground in the presence of liquid nitrogen, 1 ml extraction buffer containing 100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl and 1,5 % SDS, was added and the extract was incubated at 65^{o}C for 10 minutes. After addition of 300 µl 5 M potassium acetate solution the extract was incubated on ice for 10 minutes. The extract was centrifuged at 18900 *g* for 5 minutes and the supernatant was mixed gently with 800 µl phenol/ chloroform/ isoamylalkohol (25:24:1, v:v:v). The extract was centrifuged at 12100 *g* for 5 minutes and the aqueous phase was gently mixed with isopropanol (1:1, v:v). The extract was centrifuged at 15000 rpm and the pellet was washed with 70% (v/v) ethanol. The pellet was dried and dissolved in 50 µl TE buffer. This DNA was ready for analysis. Prior to gel separation, the DNA was digested with a restriction enzyme which does not have a recognition site inside the fragment. After digestion, DNA sample marker was added and the mixture was run in 0.8% (w/v) agarose gel with 20-50 volt for 4-8 hours. The picture of the gel with a scale was taken under UV light. After denaturation and neutralization, the gel was ready to be blotted to nylon membrane with 20x SSC solution. The DNA extraction and southern analysis procedures were adapted from standard molecular biology manual (Sambrook et al., 1989).

C. Northern analysis

Prior to northern analysis, RNA was extracted using the Trizol-RNA extraction procedure (Chomczynski and Sacchi, 1987). 0.1-0.2 g leaf material was ground in the presence of liquid nitrogen. One ml trizol solution (50% v/v phenol, 0.8 M guanidium thiocyanate, 0.4 M ammonium thiocyanate and 0.1 M sodium acetate) was added and mixed for 1 minute. After 5 minutes incubation at room temperature the mixture was mixed again for 1 minute. 0.2 ml chloroform was added, the sample was mixed strongly for 20 seconds, and was incubated at room temperature for 5 minutes; After centrifugation at 12100 *g* for 15 minutes at 4^{o}C, 0.5 ml of aqueous phase was gently mixed with 0.5 ml isopropanol. The mixture was incubated for 10 minutes at

room temperature and centrifuged at 12100 *g* for 10 minutes at 4°C. The pellet was washed with 70% ethanol, dried and finally dissolved in 20-30 µl RNase-free water. For analysis, 20µg RNA in 10 µl water were used. The RNA solution was mixed with buffer containing ethidium bromide. The mixture was heated for 10 minutes at 65°C and was put on ice directly afterwards. The RNA was separated on a 1% formaldehyde gel using 80 volt. The gel was photograph under UV light to get the picture of rRNA. The gel was blotted to a nylon membrane with 20x SSC solution.

D. Labeling and hybridization

Prior to radioactive labeling, nylon membranes from southern and northern blot were UV cross-linked using UV Stratalinker (Stratagene, La Jolla, USA). The membranes were then pre-hybridized in hybridization buffer for 1 hour. Hybridization buffer contained 0.1% PVP, 0.1% Ficoll, 0.1% BSA in 0.9 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 0.1% SDS pH 7.0, freshly heat-denatured herring sperm DNA (50µg/ml) and 50% formamide. The probe was prepared by digestion of the DNA-probe-containing vector with appropriate restriction enzymes, separation of the DNA-probe using gel electrophoresis and extraction of the DNA-probe from gel (Qiagen, Hilden, Germany). Using this probe as a template, random primers and Klenow enzyme from Amersham Megaprime Kit (Amersham, Bukinghamshire, UK), a DNA fragment containing radioactively labeled α-^{32}P-dATP was synthesized at 37°C for 10 minutes. The excessive amount of radioactive dATP was removed using ProbeQuant G-50 Micro columns (Amersham, Bukinghamshire, UK). Labeled fragments were then denatures at 95-100°C for 5 minutes and cooled on ice before they were used for hybridization. Hybridization was done overnight in hybridization buffer at 42°C. After hybridization, the membrane was washed 3 times with 3x SSC buffer containing 0.1% SDS at 60°C for 15 minutes each time. Finally, the membrane was washed with 3x SSC buffer at room temperature for 2 minutes. The membrane was dried, covered with plastic foil, and exposed to a phosphor screen overnight. The signal was read at 200 micron pixel size resolution using a Typhoon Scanner 9410 series (Amersham, Bukinghamshire, UK).

E. RT-PCR analysis

RNA was extracted as described above. The PCR reaction was performed using one step RT-PCR (Invitrogen, Carlsbad, USA). Specific primers for the gene

were used. Checking the expression of the gene in RNAi plants was done by amplifying a fragment of the gene outside the RNAi fragment. *StEF1α* was used as the RNA extraction control (Wolf, 2005).

F. *P. infestans* infection

P. infestans isolate 208 m2 was kindly provided by Felix Mauch (Fribourg, Switzeland) (Si-Ammour et al., 2003). *P. infestans* was grown at 18^{o}C on Oat-Bean-Medium in dark condition. The medium contained 3.4% (w/v) bean flour, 1.7% (w/v) oat flour, 0.85% (w/v) sucrose, 1.5 % (w/v) bacto-agar, and 5 (µg/ml) geneticin for selection of transgenic *P. infestans*. Ten to twelve-day-old *P. infestans* cultures were used. To prepare spore suspensions, 10 ml sterile water were added to the culture. The culture was kept at 4^{o}C for 3 hours; It was shaken, filtered, and diluted 10 times. The concentration was determined using a Fuchs-Rosenthal-hemocytometer. *P. infestans* spore solution was diluted to a concentration of 1x10^{5} spores/ml; This spore solution was kept on ice until use. From the agar, the sterile potato explants were transferred to soil and they were grown under tightly controlled condition in a phytochamber with 16 h of light, 18^{o}C and 60% humidity for 4 weeks. Well expanded leaves were used. Using a dispenser micro pipette, 10 µl of the spore suspension were put on the abaxial surface of the leaf, 10 drops on each leaf. After drop inoculation, the leaf was covered with a plastic bag to create the high humidity that is essential for *P. infestans* germination. Three days after infection the leaves were detached and the infected areas were isolated using a cork borer with 0.7 cm diameter. *P. infestans* growth later on was measured with Real-Time PCR.

G. *P. infestans* biomass measurement

Total DNA was extracted from the sample using the plant DNA extraction kit (Qiagen, Hilden, Germany). *P. infestans* biomass was analyzed indirectly by measuring the level of a repetitive element in the *P. infestans* genome using the TAQ MAN Real-Time PCR system (Eschen-Lippold, 2004). Using primers that were reported before (Judelson and Tooley, 2000), the fragment was generated and cloned into pCR 2.1 vector. From this fragment, new primers and a TAQ MAN probe were derived. The sequence of the two primers and the probe: **5'-CAATTCGCCACCTTCTTCGA-3', 5'-GCCTTCCTGCCCTCAAGAAC-3', 5'-CGTACGGCCAATGTA-3'**. As an internal standard for extraction, plasmids containing *Strboh* were added during DNA extraction and amplified separately using the SYBR

GREEN system (Applied Biosystem, Warrington, UK) and specific primers (**5'-CACTAAGACTGATGATCTCTTTCACCTC-3', 5'-GACAAAGATGCAGATGGGAGAATTACTC-3'**). Series of diluted *P. infestans* fragment containing vector and *Strboh* containing vector were used as external standard to get quantitative data.

H. Stereo microscopic analysis

The stereo microscope MZFL III (Leica, Cambridge, UK) with GFP I filter was used to analyze the *P. infestans* structures on infected leaves. UV light was used to analyze GFP containing *P. infestans* structure.

I. Trypan-blue staining

This staining method was adapted from the standard method (Peterhansel et al., 1997). Trypan-blue staining was done by boiling the potato leaf in trypan-blue solution until the green color disappeared. The solution contained 0.033% (w/v) trypan-blue, 8% lactate (v/v), 8% glycerol (v/v), 8% phenol (v/v), 8% water (v/v) and 67% ethanol (v/v). After boiling, the leaves were washed with water and transferred to the saturated chloralhydrate solution (2.5 g chloralhydrate in 1 ml water) to remove unspecific staining. The leaves were kept in the 50% (v/v) glycerol solution until analysis.

J. Aniline-blue staining

This staining method was adapted from (Adam and Somerville, 1996). Leaves were boiled in ethanol to remove the chlorophyll. The cleared leaf was rinsed in water and stained for 30 minutes in 150 mM KH_2PO_4 (pH 9.5) containing 0.01% (w/v) aniline-blue. Aniline-blue was purchased from Fluka as water-blue. The staining was performed in the dark. Leaves were finally mounted in 50% (v/v) glycerol and examined by epifluorescent illumination.

K. Diaminobenzidine (DAB) staining

This staining method was adapted from (ThordalChristensen et al., 1997). 3, 3'-diaminobenzidine was dissolved in water with a concentration of 1 mg/ ml (w/v). HCl was used to adjust the pH to 3.8. The solution was kept in the dark to avoid oxidation by light. Leaves were incubated in freshly prepared DAB solution for 2 hours before it was boiled with ethanol to remove the chlorophyll. After boiling, the leaves were washed with water and transferred to the saturated chloralhydrate solution (2.5 g

chloralhydrate in 1 ml water) to remove the unspecific staining. Leaves were kept in 50% (v/v) glycerol solution until analysis.

L. Light and fluorescence microscopic analysis

A microscope (Axioskop 2, Zeiss, Jena, Germany) was used to analyze *P. infestans* structure and leaf's cell after different staining methods. Micrographs were taken with a CCD camera (Sony, Tokyo, Japan) and processed through the Axio Vision 2.0.5 (Zeiss, Jena, Germany). Normal light was used to analyze the *P. infestans* structures and cell death upon trypan-blue staining. Fluorescence light with appropriate filter was used to analyze callose formation after aniline blue staining.

M. Electron microscopic analysis

Potato leaf disks were prefixed with 3% (v/v) glutaraldehyde/phosphate buffer pH 7.4 for 2 hours, fixed with 1% (w/v) OsO_4/Palade buffer for 1 hour and dehydrated in a series of acetone. Finally, it was embedded in ERL (Epoxy Resin Low Viscosity). The sample was then polymerized at 60^{o}C for 2 days. The polymerized sample was then sliced using ultramicrotom (Ultracut S, Reichert Leica, Germany) with 50-70 nm thickness. Those thin section were then stained with Pb and viewed using transmission electron microscope (EM 912 OMEGA LEO electron microscope, Oberkochen, Germany). All electron microscopic analysis were performed by Dr. Mandy Birschwilks.

N. SA analysis

Leaf material (0.5 g) was ground in the presence of liquid nitrogen. One milliliter of 90% methanol was added and the sample was mixed well. The sample was sonicated for 5 minutes and centrifuged 14200 *g* for 5 minutes. The supernatant was kept and the pellet was resuspended in 0.5 ml 100% methanol. Again the mixture was sonicated and centrifuged. The supernatant was taken and combined with the previous one. Twenty micro liter of 2 M NaOH was added to the pooled supernatant, mixed well, and evaporated in a speedvac concentrator with high temperature (circa 2 hours). The dried sample was resuspended in 250 µl of 5% (w/v) tri-chloro acetic acid (TCA) and partitioned twice with ethyl acetate/cyclohexane (1:1, v:v), 800 µl each time. The organic phase was taken each time and combined. Fifty micro liter HPLC mobile phase (described later) was added to the sample (organic phase). The sample was

dried until all of the organic phase had evaporated and the mobile phase was left (circa 30 minutes). One half milliliter HPLC mobile phase was added. The solution was mixed and centrifuged 14200 *g* for 5 minutes. The sample was ready for HPLC analysis to determine free SA content.

To measure conjugated SA levels, 250 µl of 8 M HCl was added to the leftover TCA fraction. The sample was mixed well and incubated in a water bath at 80^{o}C for 1 hour. This suspension was partitioned twice with ethyl acetate/cyclohexane (1:1, v:v), 800 µl each time. Each time, the organic phase was taken and combined. Fifty micro liter HPLC mobile phase was added to the sample (organic phase). The sample was dried until all of the organic phase evaporated and the mobile phase was left (circa 30 minutes). One milliliter HPLC mobile phase was added. The solution was mixed and centrifuged 14200 *g* for 5 minutes. The sample was ready for HPLC analysis.

The HPLC system consisted of Phenomenex column type Luna 3µ C18(2) 150 x 4.60 mm. The eluent contained 60% acidified water (pH 2.8, using acetic acid) and 40 % methanol. The flow rate was 0.7 ml/min. Twenty micro liters of the extract were injected. Salicylic acid was detected with a Jasco FP-920 spectrofluorometric detector, using an excitation wavelength of 300 nm and an emission wavelength of 410 nm.

O. JA and OPDA analysis

Leaf material (0.5 g) was ground in the presence of liquid nitrogen. Deuterated JA and OPDA were added as the internal standards, 100 ng each. Ten milliliter methanol was added and the sample was mixed directly. The sample was homogenized with an ultraturrax for 1 minute and filtered by Whatman paper. The cleaned eluate was applied to a cleaned DEAE-sephadex column that had been rinsed with 5 ml methanol previously. The flow through was discarded. The column was washed with 3 ml methanol and 3ml methanol containing 0.1 N acetate sequentially. The flow through was discarded. The JA and OPDA containing fraction were eluted from the column using 3 ml methanol containing 1 N acetate and 3 ml methanol containing 1.5 N acetate sequentially. The eluate was dried and further purified using HPLC.

The sample was dissolved in 100 µl methanol and injected into the HPLC, which consisted of HP 1100 system (Agilent, Palo Alto, USA) with Phenomenex column type Luna 3µ C18(2) 150 x 4.60 mm. The eluent were 100 % methanol (solvent A) and 0.2 % acetic acid in H_2O (solvent B). The mobile phase flowed at 0.7 ml/ min with a gradient 40% A to 100% A within 30 minutes. The eluate was detected with a HP diodearray detector at 210 nm and 235 nm. The fractions of the eluate containing

JA and OPDA were collected using an Agilent fraction collector. Both fractions were combined and dried. The dried sample was dissolved in 200 µl $CHCl_3$/N,N-diisopropylethylamine (1:1) and derivatized using 10 µl pentafluorobenzylbromide at 20°C overnight. The evaporated sample was dissolved in 5 ml n-hexane and passed through a SiOH-column (Macherey-Nagel, USA). The pentafluorebenzyl esters were eluted with 7 ml n-hexane/diethylether (2:1, v:v), evaporated, dissolved in 70 µl MeCN and finally analyzed by GC-MS.

GC-MS analysis was performed using GCQ Finnigan, 70 eV, NCI, ionization gas NH_3, source temperature 140°C, column Rtx-5w/ Integra Guard (Restek, Germany) (5 m inert precolumn; 30m x 0.25 mm, 0.25 µm film thickness, injection temperature 250°C, interface temperature 275°C, helium 40 cm/ second, split less injection, column temperature program: 1 minute 100° C, 25° C/ minute to 200° C, 5°C/ minute to 300° C, 20 minute 300° C; Rt of ($^2H_6$)JA-pentafluorobenzyl ester 11.92 minute, JA-pentafluorobenzyl ester 11.98 min and ($^2H_5$)OPDA-pentafluorobenzyl ester 21.31 minute and OPDA-pentafluorobenzyl ester 21.39 minute. Fragments *m/z* 209.215 (standard) and 291.296 (standard) were used for quantification of JA and OPDA, respectively. GC-MS analysis was performed by Dr. Otto Miersch

P. Exogenous INA application

INA (2,6-dichloroisonicotinic acid) was dissolved in 1% ethanol to a final concentration of 0.33 mM. The solution was sprayed onto the adaxial side of detached potato leaf. The leaf was put into a wet chamber. After 24 hours, the abaxial side of the leaf was challenged with *P. infestans*. The leaf was incubated in a wet chamber for 3 days. Growth of *P. infestans* was determined by the Real-Time PCR. Water sprayed potato leaves were used as control treatments in this experiment.

Q. Pep-13 infiltration

Pep-13 was synthesized with 70% minimal purity (kindly provided by J. Elster). Pep-13 was dissolved in water to 100 µM concentration. Infiltration was done by pressure infiltration using 1 ml plastic syringe. Water as well as inactive analogue, W2A were used as the control treatments.

R. Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) analysis

TUNEL labeling of free 3'-OH groups of DNA was performed using Dead End TM Fluorometric TUNEL System (Promega, Madison, USA) according to the manufacturer's protocol. Potato leaves were treated with 100µM Pep-13 or sterile ddH_2O (control), harvested 24 h after infiltration and fixed for 24 hours in 4% paraformaldehyde (Merck, Germany) plus 0.1% Triton X-100 (Sigma-Aldrich) according to the standard protocol (Promega, USA). Sections of 10 µm were dewaxed by immersing the slide twice in xylol (Sigma) for 5 minutes and rehydrated in a series of ethanol. Following TUNEL labeling, slides were counterstained with 1 µg/ml DAPI (Sigma) for 15 minutes to visualize nuclei and washed with PBS. Control experiments were performed with Dnase I. Section was analyzed with epifluorescence microscope. TUNEL analysis was performed by Dr. Violetta Macioszek.

S. IDP feeding

Diphenyliodonium (IDP) was dissolved in water to a concentration of 250 µM. The potato leaves were cut at petiole. The leaves petioles were dipped into IDP solution for 3 and 5 hours. The leaves were subsequently infiltrated with Pep-13 and W2A and transferred to water. The infiltrated leaves were kept on water for 24 hours for HR formation analysis. As a control treatment, the leaves were pretreated with water.

T. Cell culture generation and elicitation

Suspension cultures were initiated from leaves of potato plants (*Solanum tuberosum* cv désirée) grown under sterile condition. First, callus growth was induced on the solid MS medium containing 3% sucrose and 2 mg/l of 2,4-D. The cell suspensions were obtained by growing callus tissue in the liquid MS medium containing 3% sucrose and 2 mg/ml 2,4-D. The cell cultures were grown by shaking at room temperature. Subculturing was done in the dark for several weeks. Cultures were obtained from different wildtype and empty vector containing plants as well as from 2 independent transformants carrying the *NahG* transgene. Dark-grown, five-day-old suspension-cultured potato cells were elicited by addition of the aqueous solution of Pep-13 and subsequently incubated for the times indicated. Control cells were incubated with equal amounts of W2A.

U. Oxidative burst analysis

Formation of hydrogen peroxide after addition of elicitor to the cultured potato cells was measured by determining the increase in luminol-mediated chemiluminescence. Fifty micro litter medium of elicited cell was taken and mixed with 750 µl phosphate buffer pH 7.9 (50 mM KH_2PO_4, pH was adjusted with KOH). This mixture was combined with 200 µl luminol (0.3 mM) and 100 µl $K_3Fe(CN)_6$ (14 mM) before analyzed with a Bertholt lumat. Dilution series of H_2O_2 (30% H_2O_2 = 8.82M) from 0.2 µM to 100 µM was prepared to make a standard curve.

V. *Pseudomonas syringae* pv. *maculicola* (*Psm*) infiltration

A glycerol stock of *Psm* M2 (Debener et al., 1991) was streaked on King's B medium plates containing 50 µg/ml rifampicin. The plates were incubated at 28°C for 2-3 days. A single colony was taken, incubated in 3 ml King's B medium containing 50 µg/ml rifampicin and shaken overnight at 28°C. This culture was transferred to 50 ml King's B medium containing 50 µg/ml rifampicin and shaken 3–5 hours at 28°C until the OD_{600} reached 0.2. This culture was centrifuged at 3400 *g* at 4°C for 5 minutes. The pellet was washed with 10 mM $MgCl_2$ and centrifuged again 3400 *g* at 4°C for 5 minutes. The pellet was resuspended into 25 ml $MgCl_2$ 10 mM and diluted to concentration of 10^8 colony-forming units per ml in 10 mM $MgCl_2$. The bacterial suspension was kept on ice until use. Infiltration into leaves was done by pressure infiltration using 1 ml plastic syringe. Infiltration of 10 mM $MgCl_2$ were used as control treatments.

W. SAR analysis

The following system was used to study SAR in potato plant (Kombrink et al., 1994). Four weeks old potato plants were used. Four to five leaves were infiltrated with inducers. Four to six infiltration spots were made on each leaf depending on the leaf size. Three days later, the adjacent upper leaves were challenged with *P. infestans.* Spore solution ($1x10^5$ spores/ml) was used. The infection was done by drop inoculation. Ten drops, 10 µl each, were applied on the abaxial surface of the leaf. After drop inoculation, the leaf was covered with plastic bag to create high humidity that is essential for *P. infestans* growth. Three days after infection the leaves were detached and *P. infestans* in leaf was harvested using cork bore. *P. infestans* growth later on was measured with Real-Time PCR as described previously. As a

negative control in each experiment, plants were pre-treated with water or W2A, inactive analogue of Pep-13.

X. Macroarray analysis

Extraction of mRNA from total RNA was done by oligo dT using dynabeads according to the manufacturer's protocol (Dynal, Oslo, Norway). Typically, 30 µg total RNA in 50 µl and 100 µl dynabeads were used. Synthesis of single strand DNA was done using Superscript II RT (Invitrogen, Carlsbad, USA). Labeling and hybridization were done as described by Veß (2004). After the hybridization, the membranes were washed in three steps at 60^{o}C and 10 minutes each step. In the first step, membranes were washed with 2x SSC + 0.1% SDS. After that, they were washed in 1x SSC + 0.1% SDS. A final washing step was done using 0.2x SSC + 0.1% SDS. After washing and drying, the membranes were exposed on a phosphor screen for 2 days. The signal was read at 50 micron resolution using Typhoon Scanner 9410 series from Amersham. Further data analysis and normalization was done by AIDA software with TIFF as input file.

Y. Microarray analysis

Three wildtype potato plants (*Solanum tuberosum* cv. Désirée) for each treatment were used. Plants were infiltrated with *Pseudomonas syringae* pv. *maculicola* and Pep-13. Twenty four hours after treatments, local and systemic leaves were harvested. Seventy two hours after treatments, systemic leaves from Pep-13 infiltrated plants were also harvested. This material was used for RNA extraction. One array was used for each material and treatment. In total, five arrays were used in this experiment.

RNA isolation was done using Rneasy Plant Mini Kit from Qiagen. For each hybridization, cDNA was generated from 130 µg total RNA. cDNA from treated plants was labeled with one fluorescence dye either Cy3 or Cy5, whereas the cDNA from control treated plants was labeled with the other fluorescence dye.

The 7680-clone potato cDNA array (with each element spotted twice) generated by TIGR (Rockville, USA) was used in these experiments (Restrepo et al., 2005). The clones were selected from EST sequence that were generated from a series of potato cDNA libraries used in the NSF potato EST sequencing project (TIGR, Rockville, USA). All clones were validated through resequencing and agarose gel electrophoresis prior to printing to confirm the sequence of the clone and the presence

of the insert (TIGR, Rockville, USA). All data on the EST sequences, the clones on the array, and annotation of the clones can be found at the TIGR web site (http://www.tigr.org/tdb/potato/microarray_comp.shtml).

Hybridization was done following the hybridization method from TIGR. The arrays were prehybridized in order to block the non-specific background during hybridization. The slides were blocked in 5x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 1% bovine serum albumin and 10 mM EDTA at 42°C for 45 minutes. The slides were washed in sterile double-distilled-water, followed by isopropanol, and were dried. Cy3 and Cy5 probes were combined, placed on the slide, and covered using a glass cover slip washed in 1% SDS. The arrays were put into hybridization chambers (ArrayIt, TeleChem International, Sunnyvale, USA), wrapped in light-tight foil and hybridized 16-20 hours at 42°C in a water bath. The slides were removed from the chamber and washed first in 2x SSC, 0.1% SDS at 42°C for 5 minutes, then in 0.05x SSC, 0.1% SDS at room temperature for 5 minutes, twice in 0.05x SSC at room temperature for 5 minutes, and finally dried by shaking in isopropanol and blowing with pressurized air. The slides were scanned with a laser, and read at 580BP30 florescence emission for Cy3 and 670BP30 florescence emission for Cy5. the resolution was adjust to 10 micron pixel size. Those were done using Typhoon Scanner 9410 series from Amersham (Buckinghamshire, UK) equipped with microarray slide holder.

To determine fluorescence and background intensity, 16-bit scanned TIFF images were analyzed using the Spot Finder software from TIGR (Rockville, USA) and the results were exported to Mev file. Those data were further normalized and processed using the MIDAS software from the TIGR (Rockville, USA). Total intensity, standard deviation, regularization and low intensity factor were chosen as normalization mode. Finally, the data were read and displayed using the TMEV software from the TIGR (Rockville, USA) by choosing correct data and annotation file.

III. Results

A. The role of salicylic acid (SA) and jasmonic acid (JA) for defense of potato against *P. infestans*

NahG potato plants were used to study the role of SA for basal defense responses against *P. infestans.* Transgenic plants were kindly provided by A. Veß and P. Landgraf. Using agrobacteria, wild type potato plants (*Solanum tuberosum* cv. Désirée) were transformed with the *NahG* gene under the control of the 35S promoter. Selection was done using kanamycin and Southern blot analysis. Two lines of *NahG* potato plants, line A and D2, were chosen for further studies. Figure 3 shows the increase of SA in wildtype potato leaves (free and conjugated SA) after *P. infestans* infection. The inability of *NahG* potato plants to accumulate SA is also shown.

In order to address the importance of SA for defense of potato against *P. infestans*, pathogen growth in wildtype and *NahG* potato plants was measured. Lesion size was used as an indicator of *P. infestans* growth. Three days after infection, lesion size on the infected leaves was compared. No significant difference in lesion size between wildtype and *NahG* potato leaves was observed (Fig. 4.). Based on lesion size after 6 to 7 days of *P. infestans* infection, Yu *et al*. (1997) also reported that there was no difference in *P. infestans* growth in *NahG* potato plants compared to wildtype potato plants. However, more detailed investigations showed that lesions on *NahG* potato leaves contain more mycelium compared to wildtype. Using stereo-fluorescence microscopy with UV light, *P. infestans* mycelium that contain green fluorescence protein was detected as yellow ramified objects around the lesion sites. This escaping mycelia around the lesion sites was undetectable in infected wildtype potato plants (Fig. 5). This observation suggests that there was no linear correlation between lesion size and *P. infestans* growth in *NahG* potato plants.

To obtain more reliable quantitative data on *P. infestans* growth in potato plants, a Real-Time PCR method, which had been established in our laboratory, was used (Eschen-Lippold, 2004). A repetitive element in the *P. infestans* genome was used for quantification (Judelson and Tooley, 2000).

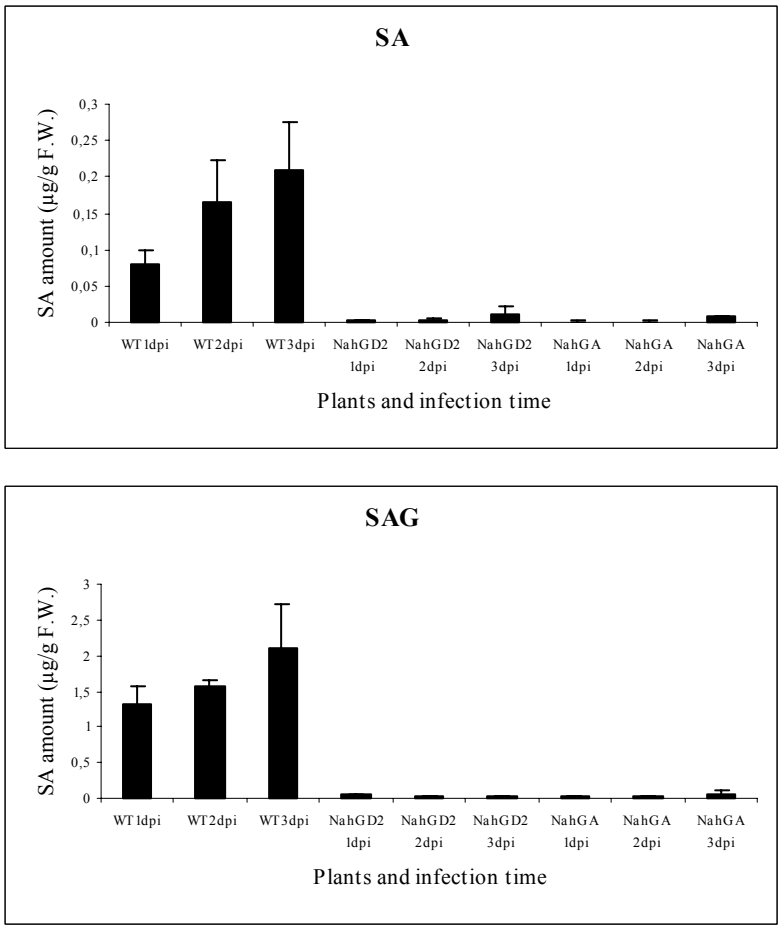


Fig. 3. The amount of free salicylic acid (SA) (upper panel) and conjugated salicylic acid (SAG) (lower panel) of wildtype and *NahG* potato leaves after *Pi* infection. Each column represents the mean from 2 different plants. Error bars represent the standard deviation for the samples. (dpi: day post infection).

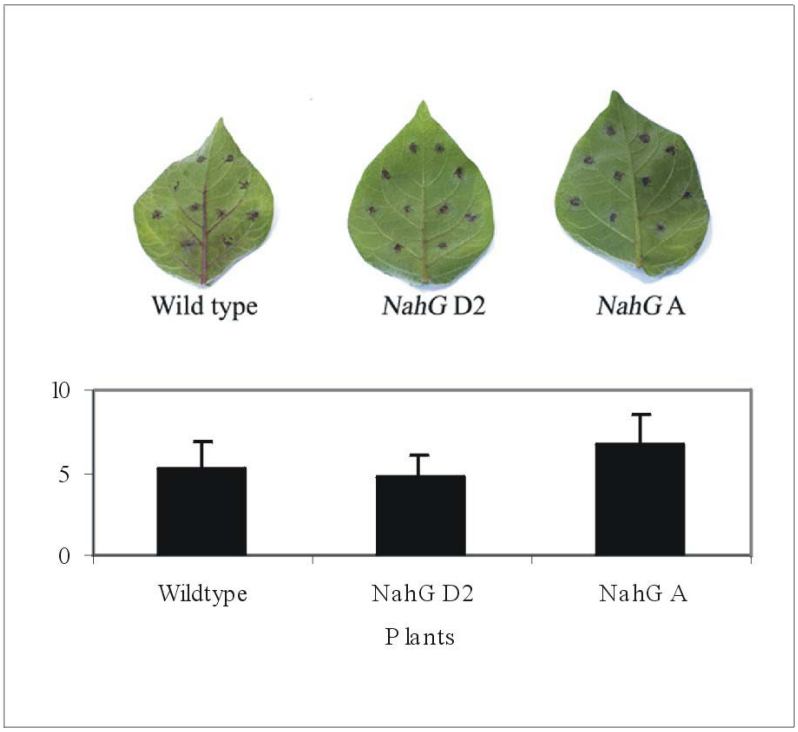


Fig. 4. The comparison of disease lesion on wildtype and *NahG* potato leaves after 3 days of *Pi* infection (upper panel). Lesion size (in mm diameter) was also quantitatively determined. Each column represents the mean from 2 different plants. Error bars represent the standard deviation for the samples (lower panel).

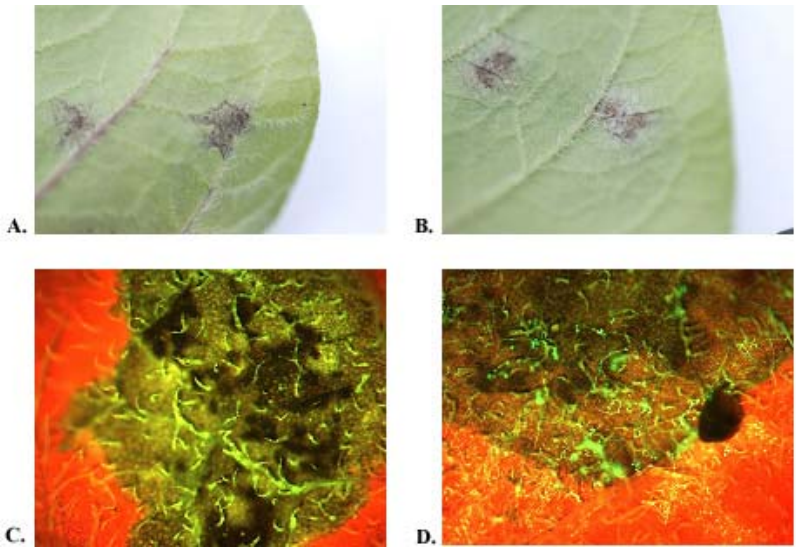


Fig. 5. Magnification of the site of *Pi* infection on a wildtype potato leaf (A) and a *NahG* potato leaf (B). No *Pi* mycelium was observed around the lesion in the wildtype potato leaf (C) In contrast, a lot of *Pi* mycelia were observed around the lesion of a *NahG* potato leaf (D). *Pi* mycelia looks yellowish and ramified on the surface next to lesion sites under UV light (C, D). Observation was done 3 days after *Pi* infection.

Using Real-Time PCR, the growth of *P. infestans* can be quantified even at earlier time points. Much stronger *P. infestans* growth in both lines of *NahG* potato plants was observed compared to that in wildtype potato plants (Fig. 6.). Three days after infection, *P. infestans* growth in *NahG* potato plants was around 10 times stronger than that in wildtype. This result was different from that reported before by Yu *et al*. (1997).

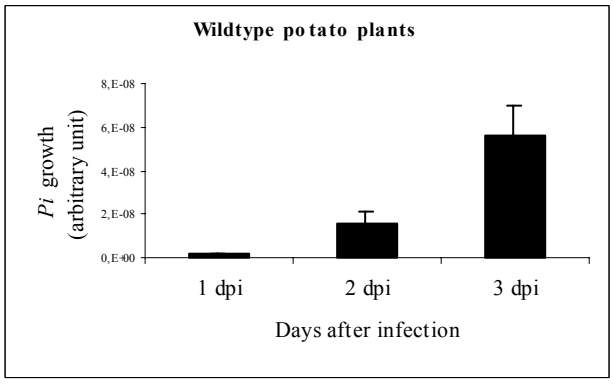
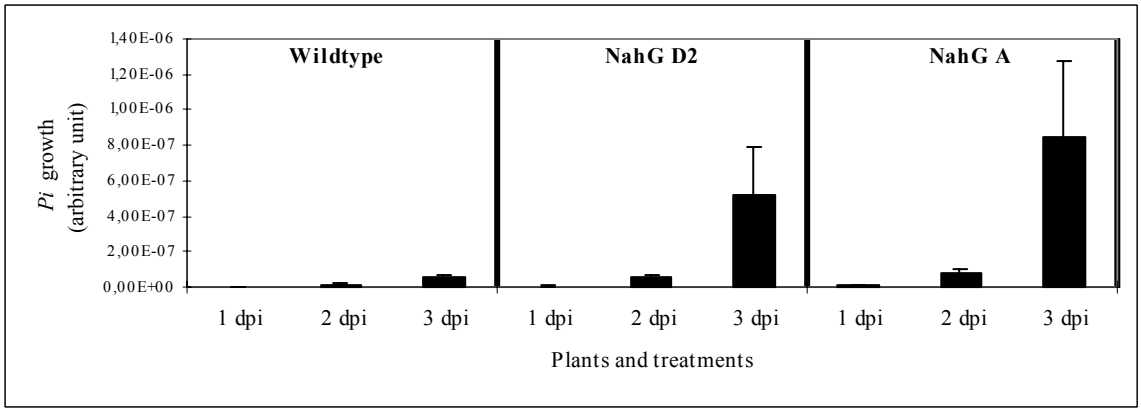


Fig. 6. *Pi* growth in wildtype potato leaves within 3 days of infection (upper panel). *Pi* growth in *NahG* potato leaves, line D2 and A, compared to *Pi* growth in wildtype potato leaves (lower panel). *Pi* biomass was determined by Real-Time PCR.



In order to understand the action of SA in promoting basal defense against *P. infestans* in potato plants, several defense responses that have been reported to be important to reduce *Phytophthora* growth in plants were analyzed (Alexander et al., 1993; Liu et al., 1994; Kamoun et al., 1999; Vleeshouwers et al., 2000; Donofrio and Delaney, 2001).

Using aniline-blue staining, *NahG* potato plants were shown to have a reduced ability to form callose around the infection site. As shown in Figure 7A, three days after infection of wildtype potato plants, callose surrounded the infected area. A different reaction was observed in *NahG* potato plants. Although callose was still formed, the deposition was scattered around parenchyma cells and there was no evidence of encasing the infected area (Fig. 7B). Cellular analysis using electron microscopy did not only confirm aniline-blue staining analysis but also showed that callose was formed in the wildtype potato cells surrounding of *P. infestans* structures (Fig. 7C). In contrast, *NahG* potato cells at the surrounding of *P. infestans* structures formed less or even no callose at all (Fig. 7D). Therefore, callose deposition might be one component of basal defense that is controlled by SA.

Another factor that might be important for basal defense is pathogenesis related (PR) gene expression. PR proteins, the products of PR genes, might have direct effects on *Phytophthora* growth as has been shown for PR1a and PR5 (Alexander et al., 1993; Liu et al., 1994). In this experiment, the expression of *PR1a* and *PR5* in wildtype and *NahG* potato plants was checked after *P. infestans* infection. Three days after infection, no differences in *PR1a* and *PR5* expression between wildtype and *NahG* potato plants were observed. However, lower *PR1a* and *PR5* expression in *NahG* potato plants compared to wildtype potato plants were observed after 1 day of infection (Fig. 8.). This experiment was repeated with the same result. The high expression levels of *PR1a* and *PR5* after three days of infection probably occurred because more cells were affected by the stronger growth of *P. infestans.* From this experiment, we conclude that both PR1a and PR5 expression might be related to reduced *P. infestans* growth in wildtype potato plants. Again, the expression was controlled by SA.

The importance of quick and strong hypersensitive cell death to restrict the growth of *P. infestans* hyphae during *R* gene-mediated resistance in potato has been shown (Vleeshouwers et al., 2000). Here, the importance of cell death to reduce escaping hyphae from the sites of infection was shown (Fig. 9).

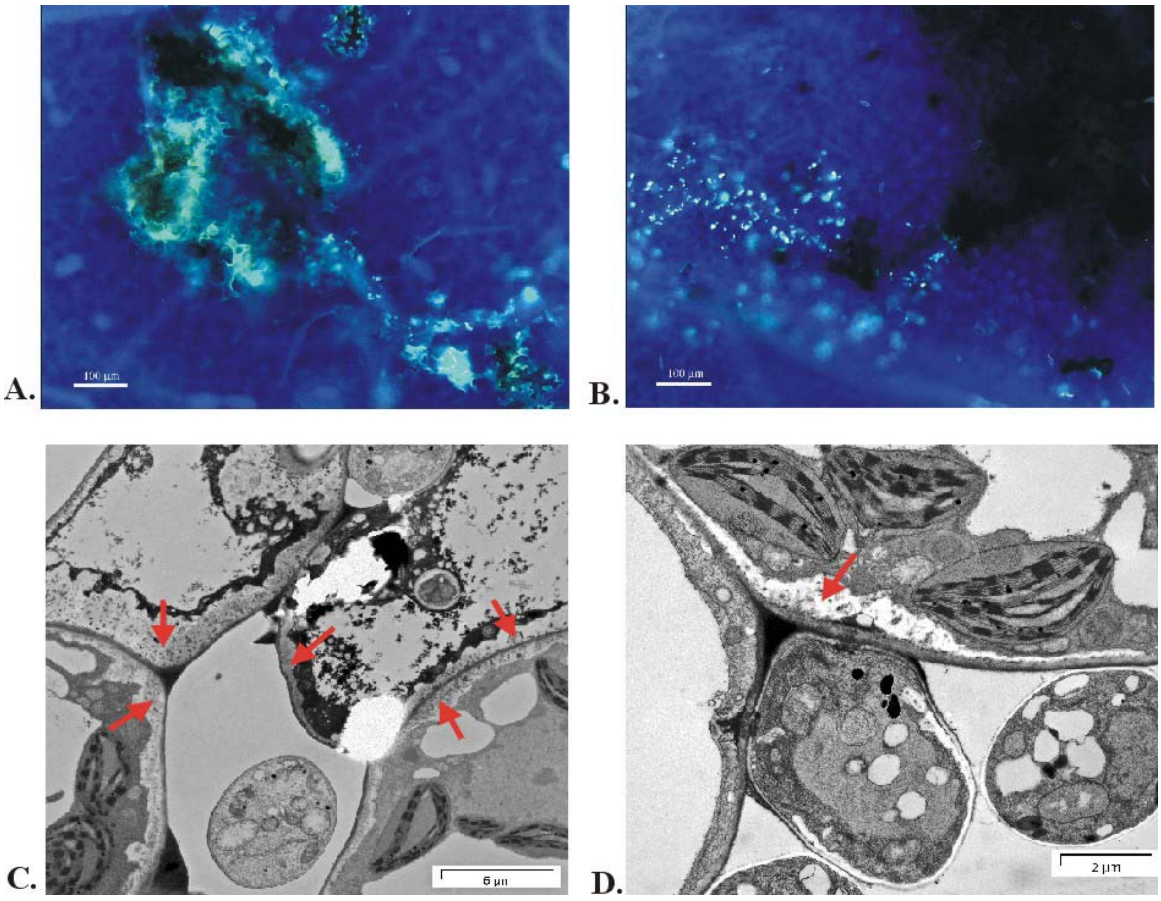


Fig. 7. *Pi*-infected potato leaves were stained with aniline blue (A, B). Wildtype potato leaves showed massive callose accumulation at the infection site (A). *NahG* potato leaves showed reduced callose accumulation (B). This aniline blue experiment was repeated several times with similar results. This finding was also shown by cellular analysis using electron microscopy. More callose accumulated around *Pi* structures in a wildtype potato leaf (C). Little amount of callose was observed in *NahG* potato leaves (D). Higher amount of spores were used for electron microscopic analysis ($5x10^5$ spores/ml). Electron microscopic analysis was kindly performed by Dr. Mandy Birschwilks.

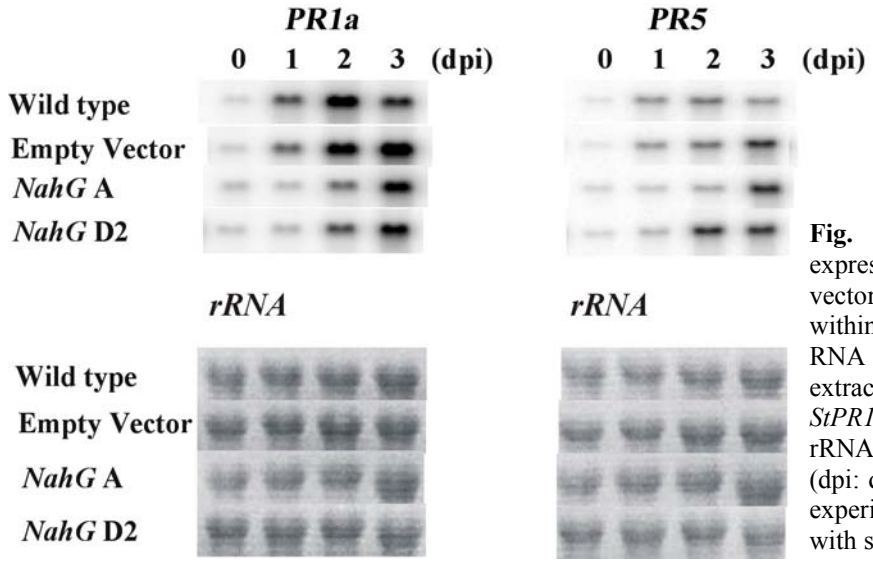


Fig. 8. *PR1a* and *PR5* expression in wildtype, empty vector, and *NahG* potato leaves within 3 days of *Pi* infection. RNA from infected tissue was extracted and probed with *StPR1a* and *StPR5* fragments. rRNA served as loading control (dpi: days post infection). This experiment was repeated twice with similar results.

Thirty six hours after infection, wildtype and *NahG* potato leaves were stained with trypan-blue, which stains dead cells and *P. infestans* structures. As shown in a wildtype leaf, there was more intense blue color of plant cells around *P. infestans* structures and not many hyphae escaped from that area. In contrast, a *NahG* potato leaf showed less intense blue color of plant cells around *P. infestans* structures with a lot of hyphae escaping the area. From this result, we conclude that cell death is an important component for basal defense of potato plants against *P. infestans*, which is controlled by SA.

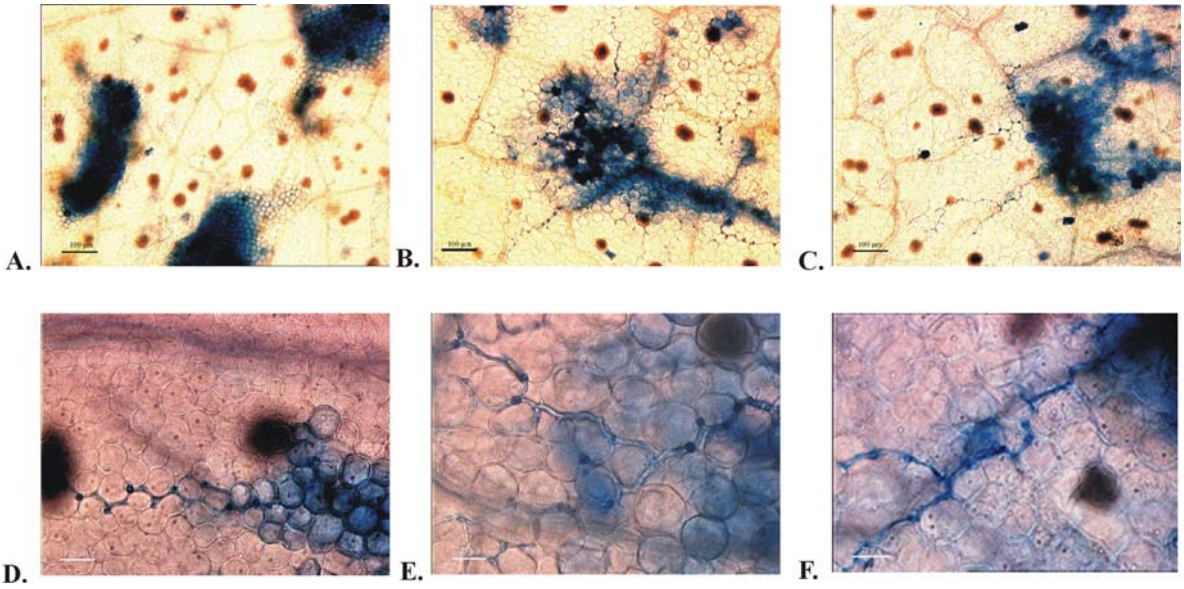


Fig. 9. One-week-old potato plant was infected with *Pi.* Wildtype potato leaves reacted with cell death as shown by intense blue colour at the site of infection (A, D). *NahG* potato leaves of line A (B, E)and D2 (C, F) show less cell death and more escaping hyphae. The leaves were harvested 36 hpi and were stained with trypan-blue. Both dead cells and *Pi* structures will be stained blue in this treatment.

To confirm that SA and its downstream defense responses play a role for basal defense of potato plants against *P. infestans*, gain of function experiments were done. The synthetic analog of SA, 2,6-dichloroisonicotinic acid (INA), was sprayed on *NahG* potato leaves. The adaxial side of the detached wildtype and *NahG* potato leaves were sprayed with 0.33 mM INA solution. Twenty four hours after spraying, the abaxial side of the leaf was infected with *P. infestans* spore solution. The leaves were incubated in a closed and humid chamber for three days before *P. infestans* growth analysis. As a control treatment, wildtype and *NahG* potato leaves were sprayed with water. A preliminary experiment showed that INA was able to restore basal resistance in *NahG* potato plants to the same level as wildtype potato plant (Fig. 10).

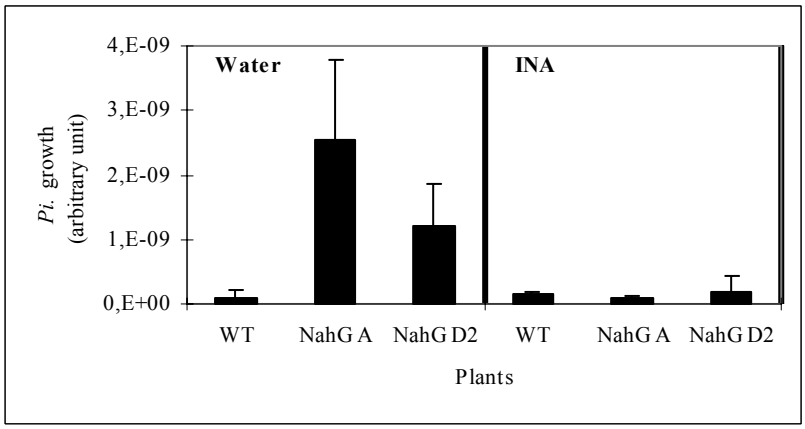


Fig. 10. Exogenous application of 2,6-dichloro isonicotinic acid (INA) rescued the resistance of *NahG* potato plants against *P. infestans* to the same level as wildtype potato plant. No increase of resistance in *NahG* potato plants is observed after water treatment. Detached leaves were used in this experiment. Leaves were sprayed with water or INA 24 hours prior to *P. infestans* infection.

OPR3 (12-oxophytodienoate reductase 3) is an important enzyme in the JA biosynthetic pathway. It converts (9S, 13S)-12-oxophytodienoic acid to 3-2(2'(Z)-pentenyl) cyclopentane-1-octanoic acid (OPC-8:0) (Schaller et al., 2000). Detailed functional studies of *OPR3* have been done in *Arabidopsis* and tomato. After wounding, *Arabidopsis OPR3* knock-out plants almost completely lack JA (Stintzi et al., 2001).

In this experiment, *StOPR3*-RNAi potato plants were used to study the role of JA for basal defense response against *P. infestans*. Efficiency of silencing with different constructs was also studied. Since there was no potato *OPR3* sequence available at the time, tomato and *Arabidopsis OPR3* sequences were aligned. A sequence of four hundred bases within high homology area was determined and primers were generated based on it. Using potato RNA, an *StOPR3* cDNA fragment was generated. This fragment was constructed in an inverted repeat position using pHellgate 8 that contains an intron in between (Fig. 11A) and pTR-GUS that contains a truncated *GUS* gene in between. The intron in pHellsgate 8 later on will be spliced to form a double-stranded RNA, while truncated GUS construct will be kept and form a double-stranded RNA with hairpin formation. pHellsgate 8 was used because of its compatibility with the Gateway system that allows fast and high throughput silencing. However, there was no available data on the successful silencing using this vector in *Solanaceae*. The pTR-GUS vector was chosen because it has been used successfully to silence *9-LOX* in potato (Gobel et al., 2003). Successful agrotransformations were done for those two constructs. Southern blot analysis of some transgenic lines carrying *StOPR3*-RNAi construct was shown (Fig. 11B). Because of the low expression levels of endogenous *StOPR3* in normal potato leaves, wounding experiments were done for expression analysis. RT-PCR analyses using primers which amplify an *StOPR3* fragment outside the RNAi region was performed to measure the expression. Comparison of *StOPR3* expression in wounded potato leaves

showed the effective silencing of *StOPR3* leading to undetectable transcripts upon wounding (Fig. 11C). *EF-1α* transcript levels were analyzed as RNA extraction control. Finally, loss of *StOPR3* can be correlated with low levels of JA after wounding (Fig. 12). Less than 5% of JA levels of wildtype potato leaves was detectable in *StOPR3*-RNAi potato plants after wounding. No significant difference in OPDA levels between wildtype and *StOPR3* potato plants. Analysis of different *StOPR3*-RNAi potato plants transformed with the two different constructs showed that both constructs were effective to silence endogenous *StOPR3*, leading to reduced *StOPR3* transcripts and JA levels (data not shown). Since using the same cDNA fragment leading to similar efficiency of silencing, pHellsgate 8 is the better silencing vector in potato, due to its compatibility with Gateway system which allows high throughput and faster construction. Two *StOPR3*-RNAi potato lines, A3 and Z2, were chosen for further studies. Figure 12 shows that reduction of *StOPR3* transcript levels affected JA but not OPDA accumulation. After wounding, *StOPR3*-RNAi potato plants were still able to accumulate OPDA to a level similar to that of wildtype plants while JA levels of the transgenic plants were much lower compared to JA levels in wildtype plants. Furthermore, the reduction of JA levels correlates with reduced *StPin2* expression (Fig. 13). *StPin2* encodes a proteinase inhibitor that is important for plant defense against herbivore attack (Royo et al., 1999).

In order to study the regulation of JA levels in potato plants upon *P. infestans* infection, JA levels were determined. Healthy wildtype potato plants contain low amounts of JA (30-50 pmol/ g F.W.) (Fig. 14A). This amount is similar in different stages of leaf development. No significant increase of JA levels after *P. infestans* infection. Three days after infection, JA levels of wildtype and empty vector control potato plants were only slightly higher compared to both lines of *StOPR3*-RNAi potato plants (Fig. 14B).

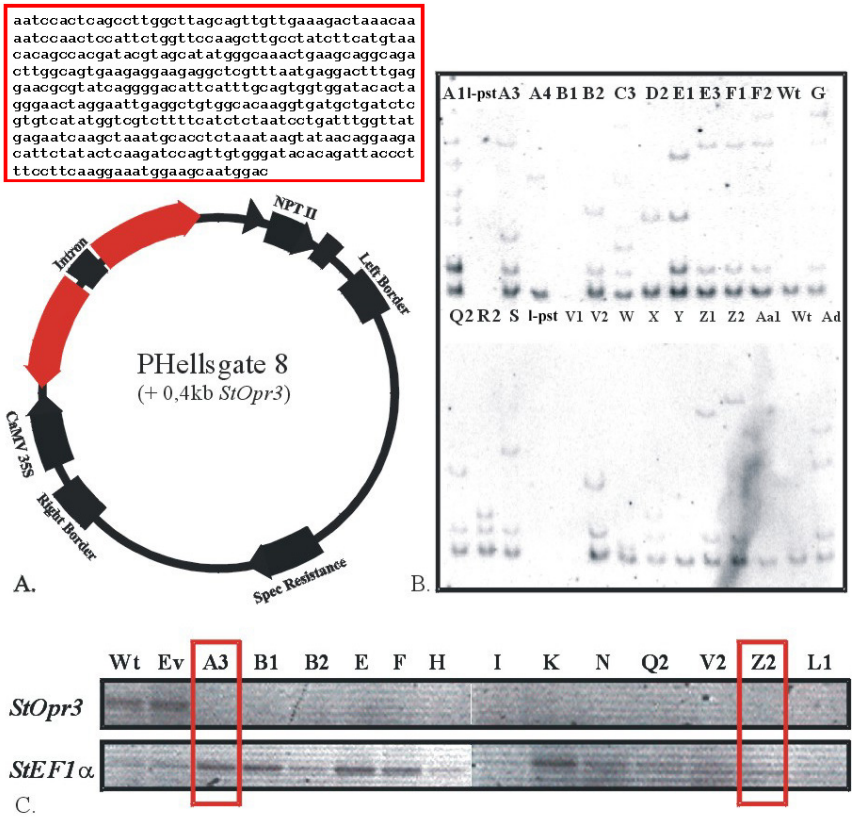


Fig. 11. The pHellsgate8 construct contains inverted repeats of a 0.4 kb *StOPR3* fragment. The sequence can be seen inside the box (A). For Southern analysis of transformants, plant DNA was extracted, digested with EcoRI and EcoRV, and probed with the *StOPR3* fragment (B). Lower expression of *StOPR3* in transgenic potato plants compared to wildtype potato plants was shown using RT-PCR analysis (C). *StEF1α* was used as the control.

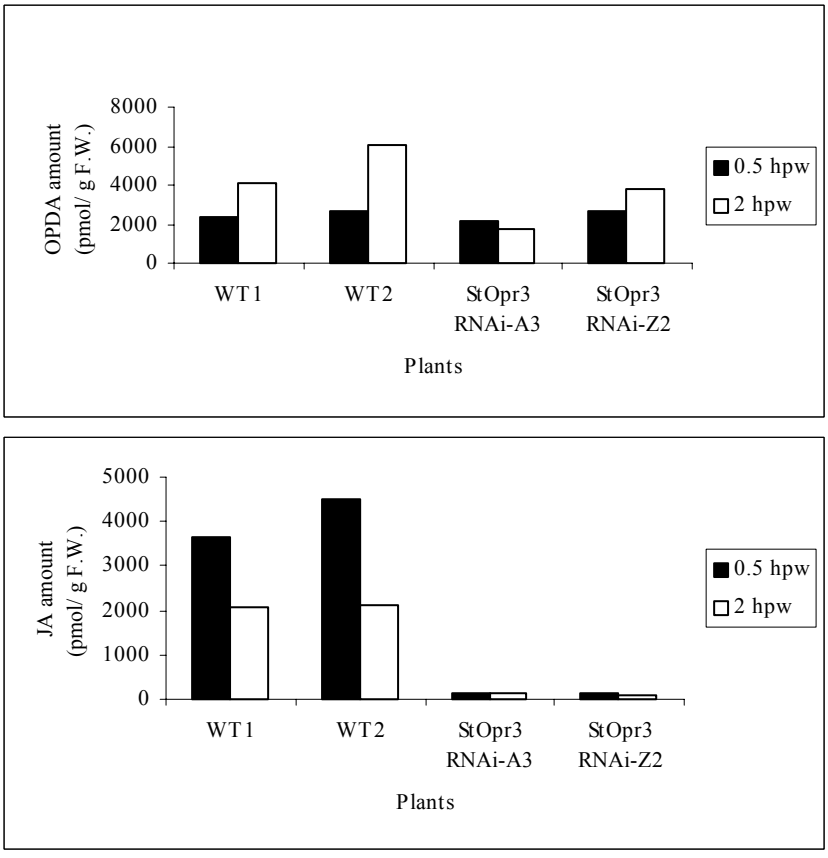


Fig. 12. The amount of OPDA (upper panel) and jasmonic acid (JA) (lower) of wildtype and *StOPR3*-RNAi potato leaves after wounding. (hpw: hours post wounding).

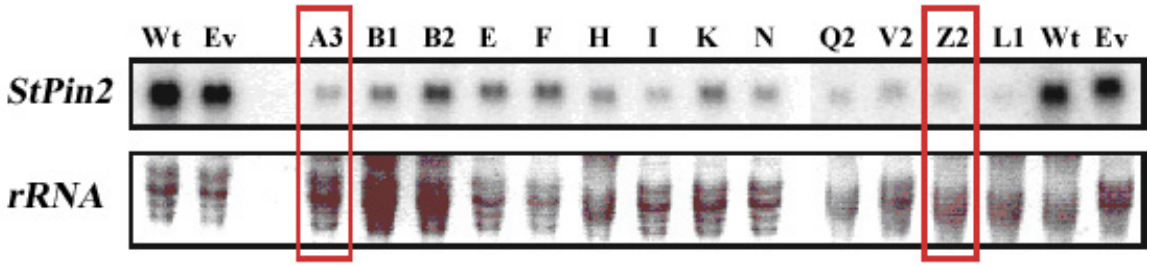


Fig. 13. Level of *StPin2* expression in *StOPR3*-RNAi compared to wildtype potato leaves upon wounding (2 hour post wounding). rRNA was used as a loading control.

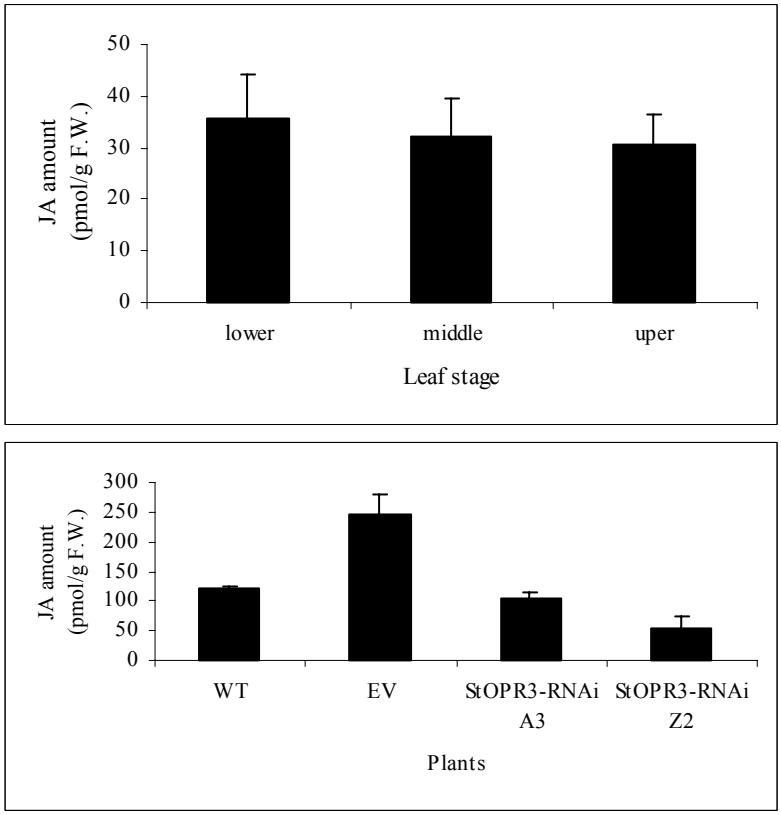


Fig. 14. JA amounts in different leaf stages of healthy wildtype potato plant. Each column represents the mean from 4 different plants and error bars represent the standard deviation for the samples (upper panel). JA amounts in wildtype and *StOPR3*-RNAi potato leaves, 3 days after *Pi* infection. Each column represents the mean from 2 different plants and error bar represents the standard deviation for the samples (lower panel).

To functionally study the importance of JA for basal defense in potato, *P. infestans* growth in wildtype and *StOPR3*-RNAi was measured. Three days after infection, around 5 times more *P. infestans* growth in *StOPR3*-RNAi potato plants than in wildtype potato plants was observed. Statistical analysis of eight data points from three different experiments showed the growth was significantly different. A P-value smaller than 0.05 was obtained when *P. infestans* growth in wildtype potato plants was compared with *P. infestans* growth in both lines of *StOPR3*-RNAi potato plants (Fig. 15). From these experiments, we conclude that in addition to SA, JA also plays a role in basal defense responses of potato plants against *P. infestans.*

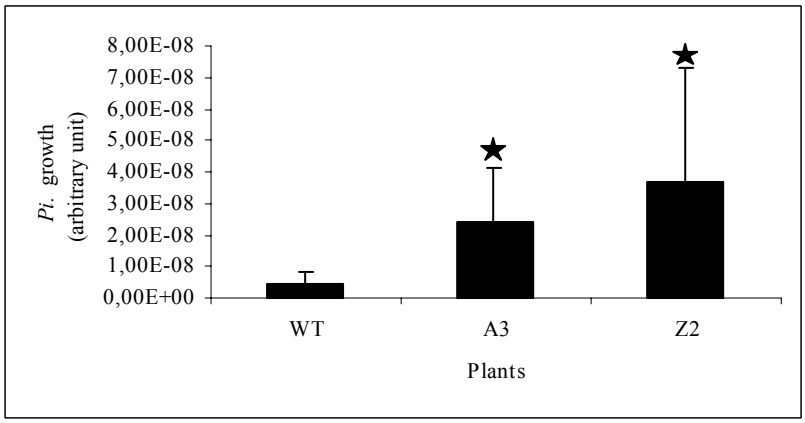


Fig. 15. *Pi* growth in *StOPR3*-RNAi compared to wildtype potato leaves. *Pi* growth was measured 3 days after infection (★ : significantly different by Paired T-test at $P<0.05$ with n=8).

To identify downstream defense responses of JA signaling that might contribute to the basal defense of potato plants against *P. infestans*, callose formation and PR gene expression after *P. infestans* infection in *StOPR3*-RNAi potato plants were examined. After *P. infestans* infection, callose formation in *StOPR3*-RNAi potato plants was not different from that in wildtype potato plants (Fig. 16). It was difficult to compare PR gene expression of wildtype and *StOPR3*-RNAi potato plants. Comparison of PR gene expression in 3 different experiments showed inconsistent differences (data is not shown). We concluded PR gene expression in *StOPR3*-RNAi potato plants was not significantly different from that in wildtype potato plant.

In addition to JA, the role of OPDA for plant defense against pathogens has been reported (Stintzi et al., 2001). To study this possibility, plants with reduced OPDA levels are needed. AOS (allene oxide synthase) is an enzyme that converts fatty acid hydroperoxides to unstable allene epoxides, which either spontaneously or through action of an allene oxide cyclase form 12-OPDA, the precursor of JA (Laudert et al., 1996). *StAOS2* co-suppressed potato plants contain less OPDA upon wounding (kindly provided by J. Sanchez-Serrano, Madrid, Spain). Two lines of potato plants, 18 and 19 showed a co-suppression effect of *StAOS2*. Wounding experiments were performed to analyze how effective the suppression was. Two and four hours after wounding, expression of *StAOS2* in wildtype potato plants could be seen. However, the expressions of *StAOS2* in both lines of *StAOS2* co-suppressed potato plants was hardly detectable (Fig. 17). Lower expression of *StAOS2* in turn correlated qualitatively but not quantitatively with reduced OPDA and JA levels. Table 1 shows JA and OPDA levels of both lines of *StAOS2* co-suppressed potato plants in comparison to wildtype potato plants upon Pep-13 treatment (24 hpi).

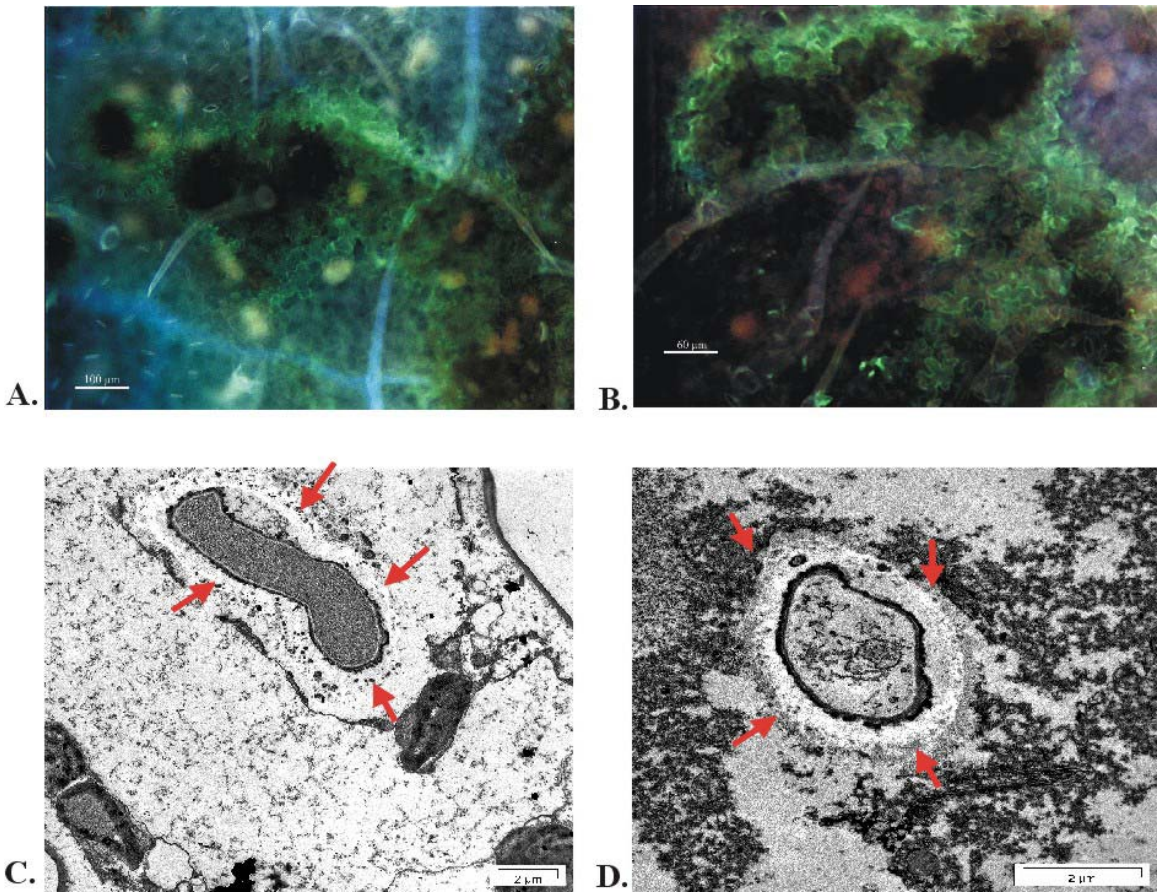


Fig. 16. *Pi* infected potato leaves were stained with aniline blue. Callose formation in wildtype (A, C) and *StOPR*-RNAi (B, D) potato leaves after 3 days of *Pi.* infection. A and B, aniline blue staining. C and D, cellular analysis using electron microscopy. Higher amounts of spores were used for electron microscopic analysis ($5x10^5$ spores/ml). Electron microscopic analysis was kindly performed by Dr. Mandy Birschwilks.

Incomplete reduction of OPDA in *StAOS2* co-suppressed potato plants might be explained by the existence of other AOS homologues in potato. Pep-13 is an oligopeptide elicitor from a cell wall glycoprotein of *Phytophthora* species. Pep-13 is able to induce a wide range of defenses and to increase JA levels in potato leaves upon infiltration (Halim et al., 2004). From two independent experiments, there were consistent differences of OPDA and JA levels among wildtype and both lines of *StAOS2* co-suppressed potato plants (Table 1.). However, reduction of OPDA to the level lower than 50 percent convinced us to use *StAOS2* co-suppressed potato plants to study the role of OPDA.

To investigate the role of OPDA for basal defense of potato plants against *P. infestans*, wildtype and both lines of *StAOS2* co-suppressed potato plants were infected with *P. infestans*. Growth of *P. infestans* was measured using Real-Time PCR.

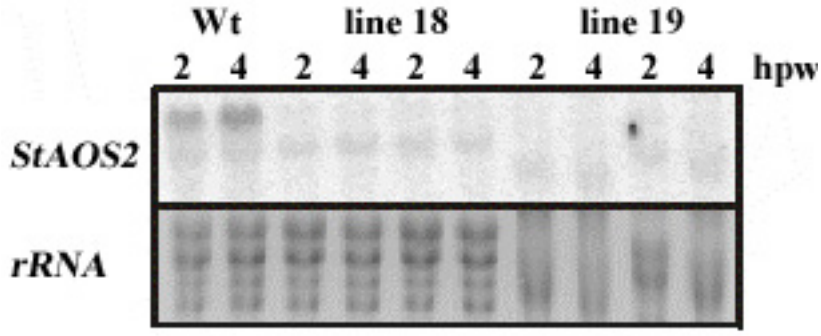


Fig. 17. *StAOS2* expression in wildtype and both lines of *StAOS2* co-suppressed potato plants after wounding; hpw: hours post wounding.

	JA level (pmol/g F.W.)	
	exp1	**exp2**
WT	416	101
AOS18	179	80
AOS19	97	28
	OPDA level (pmol/g F.W.)	
	exp1	**exp2**
WT	2503	1589
AOS18	554	210
AOS19	994	799

Table 1. JA and OPDA levels in both lines of *StAOS2* co-suppressed potato plants compared to JA and OPDA levels in wildtype potato plants. The leaves were infiltrated with Pep-13. After 24 hours, the leaves were harvested, JA and OPDA levels were analysed.

There was different susceptibility between the two lines of *StAOS2* co-suppressed potato plants (Fig. 18). Line 19, which had 50 to 60 percent less OPDA and 70 to 80 percent less JA compared to wildtype, showed significantly more *P. infestans* growth compared to wildtype potato plants. In contrast, line 18, which had 80 to 90 percent less OPDA and 20 to 50 percent less JA compared to wildtype, showed no significant difference in *P. infestans* growth compared to wildtype potato plants. This data suggests that JA instead of OPDA might be important for basal defense of potato plant against *P. infestans.* However, it should be kept in mind that the line 19 showed an alteration in leaf morphology.

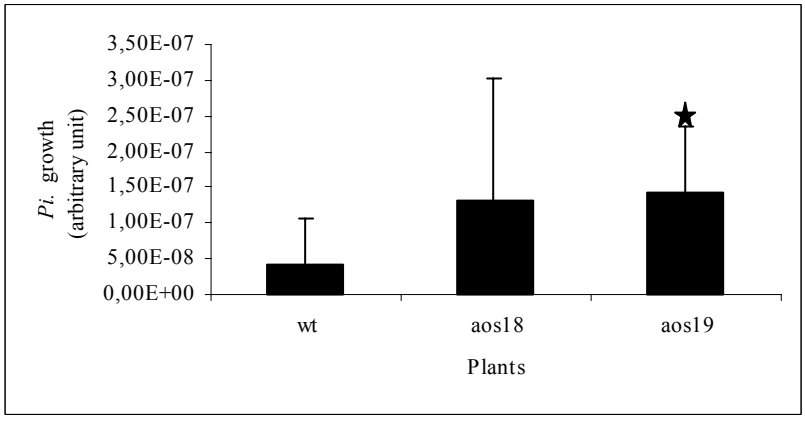


Fig. 18. *Pi* growth in wildtype and two different lines of *StAOS2* co-suppressed potato plants. *Pi* growth was measured 3 days after infection (★ : significant different by Paired T-test at $P<0.05$ with n=7).

In summary, the importance of SA and JA for basal defense of potato plants against *P. infestans* can be shown in this study. Many defense responses such as callose formation, PR gene expression and HR cell death are controlled by SA. Together, they might be important to promote basal defense of potato plant and to slow down *P. infestans* growth in the plant. In *NahG* potato plants that have almost no SA, all of these defense responses were impaired or reduced. This might explain why *NahG* potato plants were more susceptible to *P. infestans* compared to wildtype potato plant (around 10 times higher). Upon *P. infestans* infection, *StOPR3*-RNAi potato plants can form callose and express PR genes to the same level as in wildtype potato plants. Lack of JA in *StOPR3*-RNAi potato plants did not change callose formation and PR gene expression in the plant. However, the *StOPR3*-RNAi potato plants are unable to mount an oxidative burst and to initiate HR upon Pep-13 infiltration (section B). Therefore, it is interesting to see the oxidative burst and HR formation of *StOPR3*-RNAi potato plants upon *P. infestans* infection. These defects might cause increased susceptibility of the plants to *P. infestans* compared to wildtype potato plants (around 3 to 5 times higher).

B. Pep-13 elicits defense responses in potato plants

To study SA and JA signaling in potato plants in more detail, the potato-Pep-13 system was used. It was known that Pep-13 is able to elicit defense responses in potato cell cultures (Geiler, 2001; Brunner et al., 2002). In potato plants, Pep-13 can elicit defense responses only when infiltrated into the leaf (Geiler, 2001).

In this study, defense responses of potato plants after Pep-13 infiltration are further characterized. The defense response was accompanied by the accumulation of SA and JA. Twenty four hours after Pep-13 infiltration, wildtype potato leaves contained 2-3 times higher SA levels compared to control, wildtype potato leaves treated with the inactive analogon of Pep-13 (W2A) (Fig. 19). Not only SA but also JA levels were increased. Twenty four hours after Pep-13 infiltration, wildtype potato leaves contained 5 times higher JA levels compared to control, wildtype potato leaves treated with W2A (Fig. 19). An increase in defense gene expression was also observed (Fig. 22). Twenty four hours after Pep-13 infiltration, expression of several defense genes, such as *PR1a, PR10, chitinase and LOX3* was increased. There was no increase of defense gene expression in control, wildtype potato plant treated with W2A.

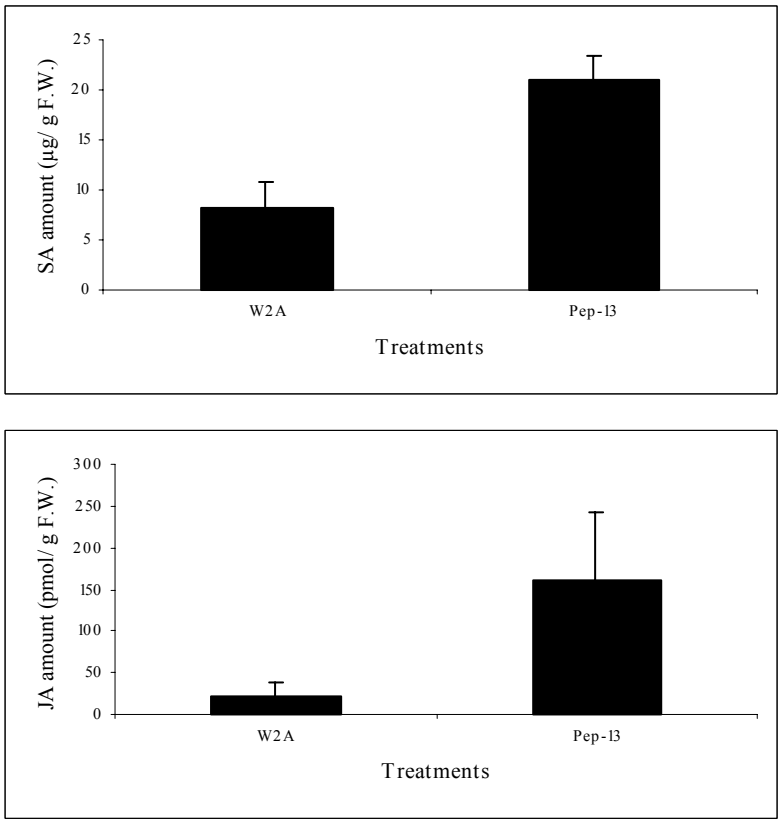


Fig. 19. Twenty four hours after Pep-13 infiltration, an increase in both free and conjugated SA (total SA) levels was observed. Each column represents the mean from 3 different plants and error bars represent the standard deviation for the samples (upper panel). JA levels were also increased upon Pep-13 treatment compared to control, wildtype potato plant treated with W2A. Each column represents the mean from 2 different plants and error bars represent the standard deviation for the samples (lower panel).

H_2O_2 as one component of the oxidative burst has been shown to be important to orchestrate the plant hypersensitive disease resistance response. Its functions in cell wall cross-linking, inducing expression of genes encoding cellular protectants and triggering programmed cell death have been shown (Levine et al., 1994). Twenty four hours after Pep-13 infiltration, the leaves were detached and stained with diamino benzidine (DAB). DAB reacts with H_2O_2 in the cell, polymerizes and precipitates at the site of formation. As a result, brown color around the infiltrated area is observed. There was highly reduced formation of H_2O_2 in the control wildtype plants treated with W2A or water (Fig. 20). Finally, HR-like cell death was observed at Pep-13 infiltration sites. The cells contained condensed and fragmented DNA (Fig. 21A-21F), which is characteristic of programmed cell death. This necrotic lesion continued to grow for 1 to 2 days. Dose response studies showed that 100 µM Pep-13 solution was needed to get a good and reproducible response (data not shown). W2A, an inactive analogon of Pep-13 and water as a medium did not cause any HR. Another analogon, P5A, caused reduced HR (Fig. 21G).

From these experiments, we conclude that Pep-13 is able to induce a wide range of defense reactions in potato plants and can be used as an experimental system to study defense mechanism in potato plants.

Fig. 20. Twenty four hours after Pep-13 infiltration, wildtype potato leaves accumulated H_2O_2 as shown by brown color after DAB staining (B). As a control we treated wildtype potato leaves with W2A (A). Leaves were stained with DAB 24 hours after infiltration.

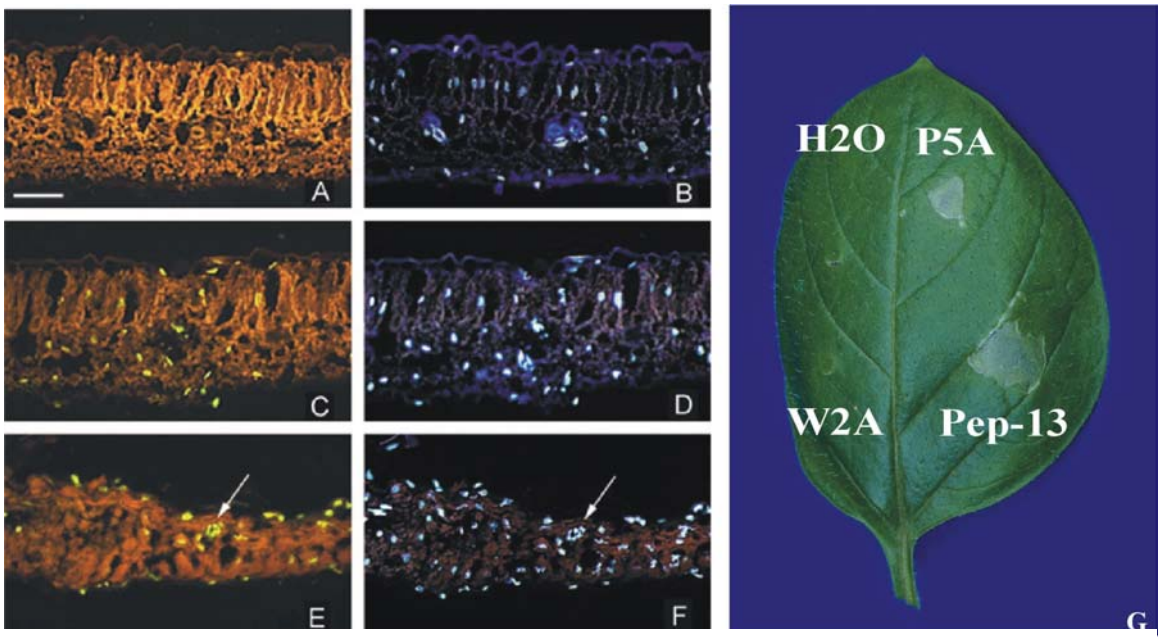


Fig. 21. Infiltration of water (A,B) and Pep-13 (E,F) into wildtype potato leaves led to tissue collapse and DNA fragmentation at Pep-13 infiltration sites. As a positive control, leaf sections were treated with DNAse I (C,D). A, C, E show TUNEL staining; B, D, F show DAPI staining. The arrowhead marks fragmented nuclei. The scale bar represents 100 µm. Picture G shows HR formation after water, W2A, P5A and Pep-13 infiltration (24 hpi). TUNEL and DAPI analyses were kindly performed by Dr. Violetta Macioszek.

The increase of SA and JA levels in potato leaves upon Pep-13 elicitation may infer a role in plant defense. In order to analyze the importance of SA and JA signaling for defense responses of potato plants and possible cross-talk between them to control downstream defense responses, *NahG* and *StOPR3*-RNAi potato plants were analyzed and compared to wildtype.

Upon Pep-13 infiltration, there was an increase in defense gene expression, as known for *PR1a*, *PR10*, *ChtA* and *LOX3* in wildtype potato plants (Fig. 22). The increase could be observed as early as 8 hours after infiltration. However, different expression of defense genes was observed in *NahG* potato plants upon Pep-13 infiltration. *PR1a* expression was highly reduced while other defense genes were expressed to the same level as in wildtype. The low expression of *PR1a* can be correlated with the low levels of SA in *NahG* potato plants after Pep-13 infiltration (Fig. 23). Moreover, other defense responses, such as the oxidative burst and HR formation were also compromised in *NahG* potato plants after Pep-13 infiltration (Fig. 24B,D). Extension of incubation time from 20 to 48 hours did not increase oxidative burst and HR formation in *NahG* potato plants (data not shown).

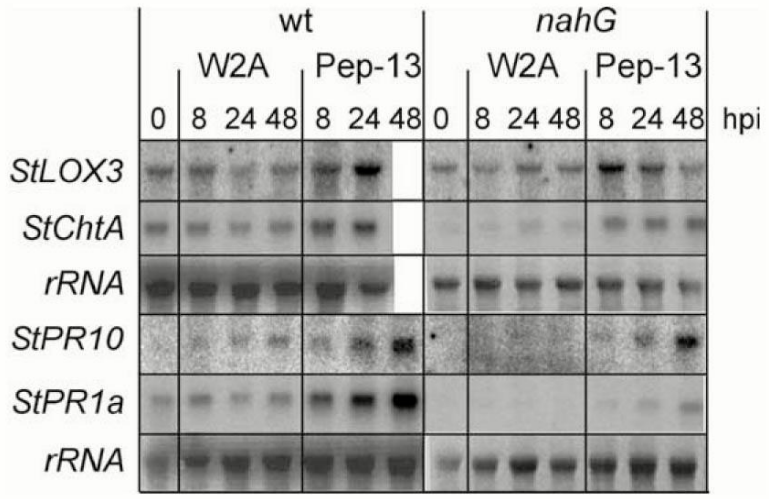


Fig. 22. Comparison of gene expression in wildtype and *NahG* potato plants upon Pep-13 infiltration. W2A was used as control treatment.

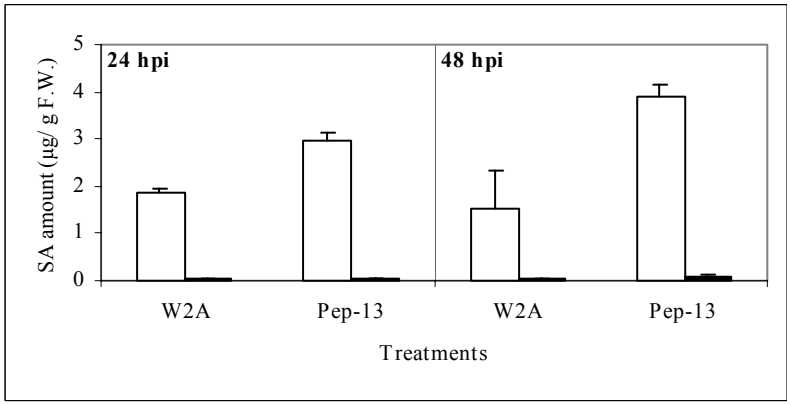


Fig. 23. Pep-13 infiltration led to an increase of SA levels in infiltrated wildtype potato leaves (white bars). No SA accumulation was observed in Pep-13-infiltrated *NahG* potato leaves (black bars). As a control, both wildtype and *NahG* potato leaves were infiltrated with W2A. Each column represents the mean from 2 different plants and error bars represent the standard deviation for the samples.

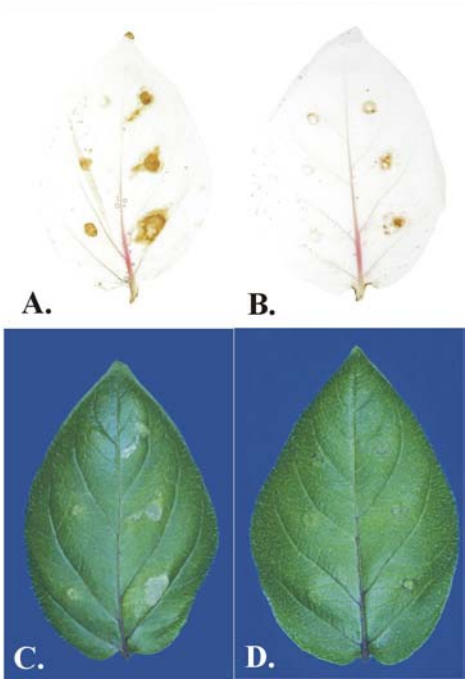


Fig. 24. Potato leaves were infiltrated with Pep-13 in the right half and W2A in the left half. HR pictures were taken 20 hours after infiltration (C,D). Subsequently, leaves were stained with DAB for oxidative burst analysis (A,B). Wildtype potato leaves (A,C) are compared to *NahG* potato leaves (B,D).

Interestingly, the lack of SA accumulation in *NahG* potato plants also affected JA accumulation in the plants upon Pep-13 infiltration (Fig. 25). This experiment was repeated several times with similar results. We conclude that SA signaling is needed to mount complete defense responses upon Pep-13 infiltration. Expression of some defense genes in potato is induced by Pep-13 independently from SA. JA accumulation in potato plants after Pep-13 infiltration depends on SA accumulation.

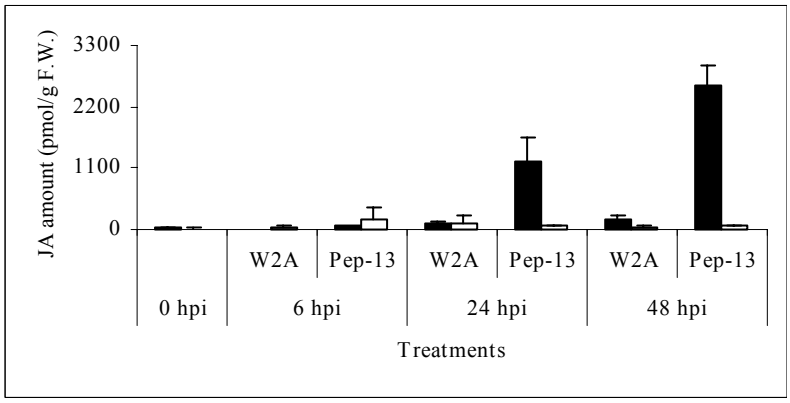


Fig. 25. Pep-13 infiltration led to an increase of JA levels in infiltrated wildtype potato leaves (black bars). No increase of JA levels is observed in Pep-13-infiltrated *NahG* potato leaves (white bars). As controls, both wildtype and *NahG* potato leaves were infiltrated with W2A. Each column represents the mean from 2 different plants and error bars represent the standard deviation for the samples.

The importance of SA for oxidative burst and HR formation in potato plants has been shown by infiltrating wildtype and *NahG* potato plants with Pep-13. However no information is available on the direct relation between SA and oxidative burst. Upon Pep-13 elicitation, cultured potato cells could mount defense responses, such as defense gene expression (Geiler, 2001; Brunner et al., 2002). Cultured potato cells were able to

produce H_2O_2 upon Pep-13 elicitation. Treatment of the cells with 10nM Pep-13 caused the production of H_2O_2 as early as 15 minutes and reached the peak of accumulation between 1 to 2 hours after elicitation (Fig. 26). This H_2O_2 production decreased to basal levels after 6 hours. No H_2O_2 was detected upon elicitation with the inactive analogon of Pep-13, W2A. Interestingly, *NahG* potato cells also reacted upon Pep-13 elicitation with similar pattern of H_2O_2 production as wildtype cells (Fig. 26). These data indicate the existence of a negative regulator of H_2O_2 production and cell death in *NahG* potato plants, which is absent in *NahG* potato cell cultures. In contrast to plants, twenty four hours after elicitation, cell death was found in Pep-13-treated wildtype and *NahG* potato cell cultures (Fig. 27).

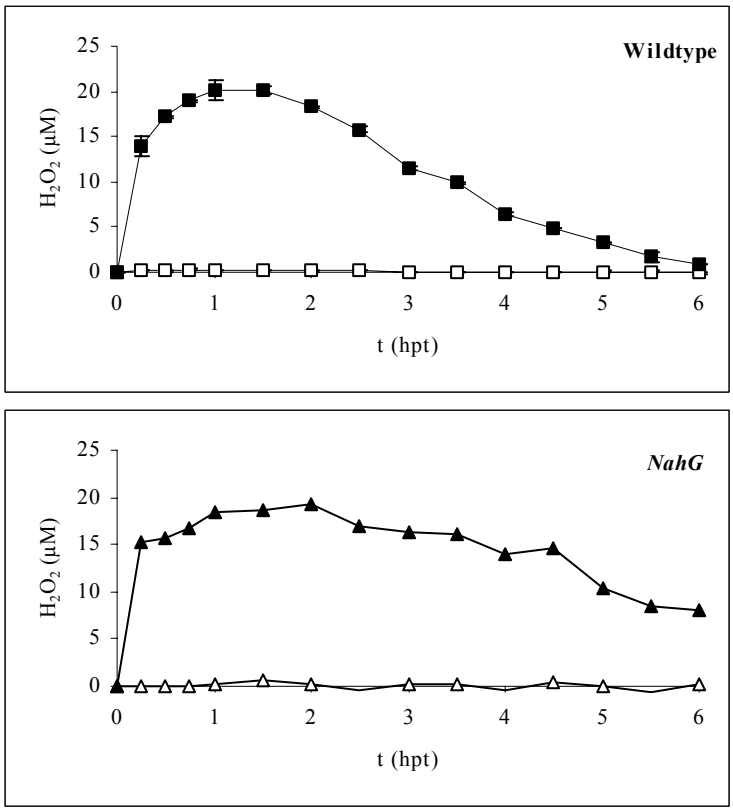


Fig. 26. H_2O_2 accumulation upon Pep-13 elicitation of wildtype cell cultures (upper panel) are compared to *NahG* cell cultures (lower panel). As control for Pep-13 elicitation (filled symbols), cell cultures were elicited with W2A (open symbols). Elicitors concentration were 10 nM. Each point represents the mean from 2 different samples and error bars represent the standard deviation for the samples.

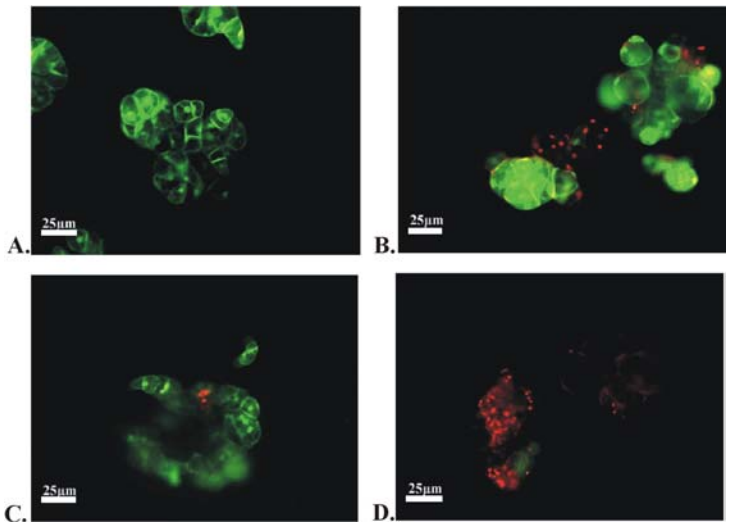


Fig. 27. Wildtype cell cultures (A,B) and *NahG* cell cultures (C,D) were elicited with Pep-13 (B,D). As control both cell cultures were elicited with W2A (A,C). Elicitor concentration is 10 nM. Those cell cultures were stained with fluorecein diacetate (FDA) for viability test and propidium iodide (PI) for cell death.

It is important to answer the question whether accumulation of H_2O_2 or formation of HR in potato plants can take place in the absence of SA. *Pseudomonas syringae* pv. *maculicola* (*Psm*) is a non-host pathogen for potato. Upon *Psm* infiltration, potato leaves reacted with quick and strong HR (Fig. 28). This HR formation correlated with increased SA levels (Fig. 29). Upon *Psm* infiltration, *NahG* potato leaves contained very low amount of SA. However, *NahG* potato leaves were still able to form HR upon *Psm* infiltration. *Psm* was also able to induce JA accumulation in the absence of SA (Fig. 30). Moreover, wounding of *NahG* potato plant also induced H_2O_2 accumulation to the same level with wounded wildtype potato plant (Altmann, 2005). Based on these data, we concluded that H_2O_2 production and HR-like necrosis formation can be mounted independently from SA.

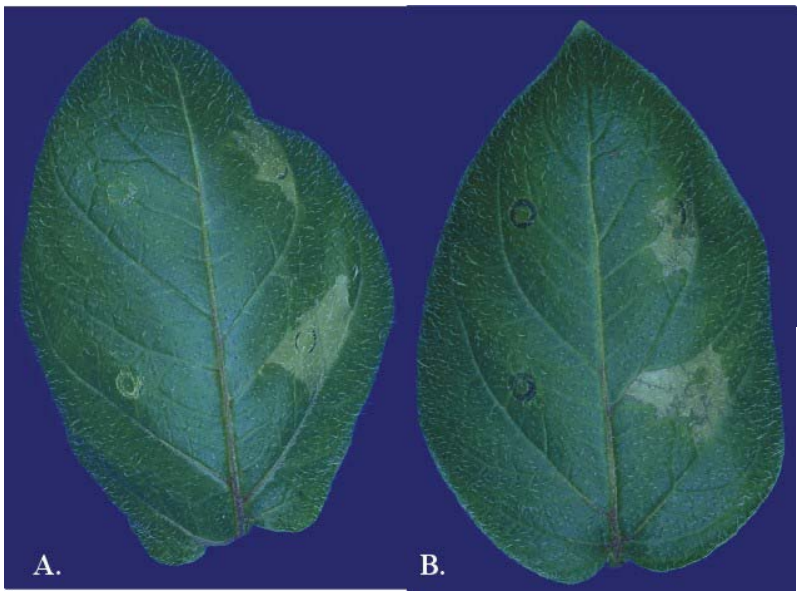


Fig. 28. Twenty four hours after *Psm* infiltration, HR-like necroses were observed in wildtype (A) as well as *NahG* (B) potato leaves. Potato leaves were infiltrated with *Psm* in the right half and $MgCl_2$ in the left half.

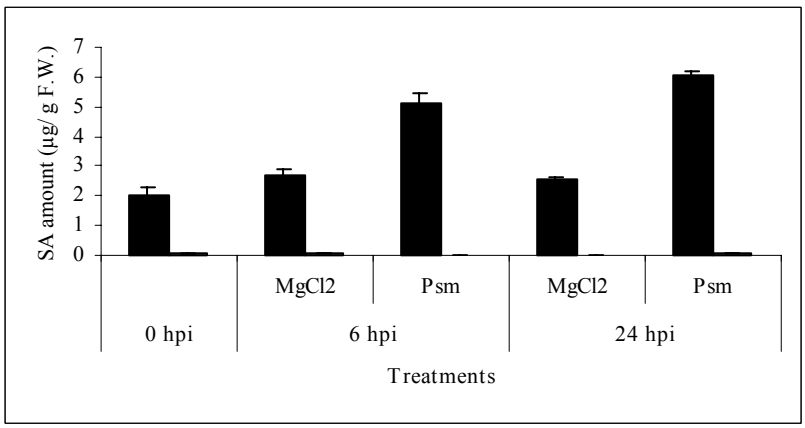


Fig. 29. Total SA in wildtype (black bars) and *NahG* (white bars) potato leaves upon *Psm* infiltration. Each column represents the mean from 2 different plants and error bars represent the standard deviation for the samples.

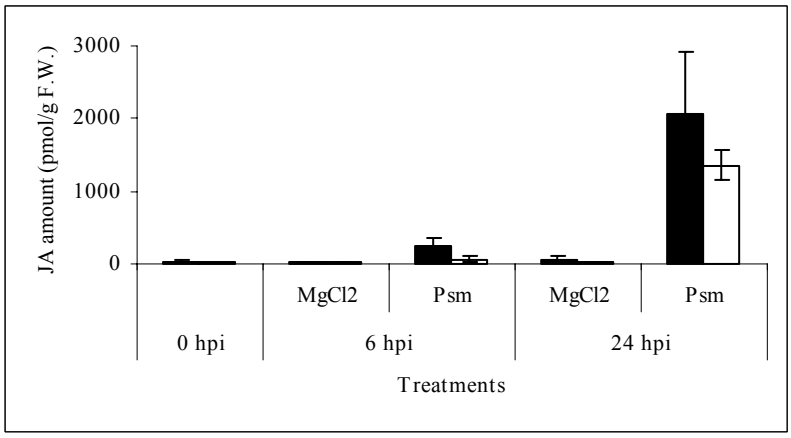
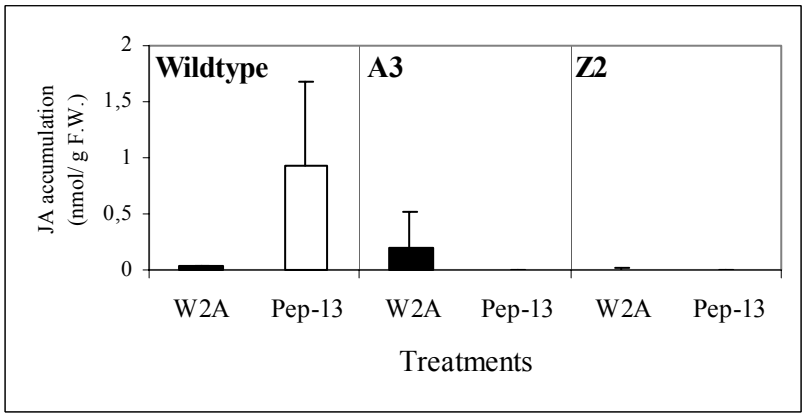


Fig. 30. JA levels in wildtype (black bars) and *NahG* (white bars) potato leaves upon *Psm* infiltration. Each column represents the mean from 2 different plants and error bars represent the standard deviation for the samples.

To address the possible role of JA signaling for defense responses in potato plants after Pep-13 infiltration, defense responses of *StOPR3*-RNAi potato plants upon Pep-13 infiltration were analyzed.



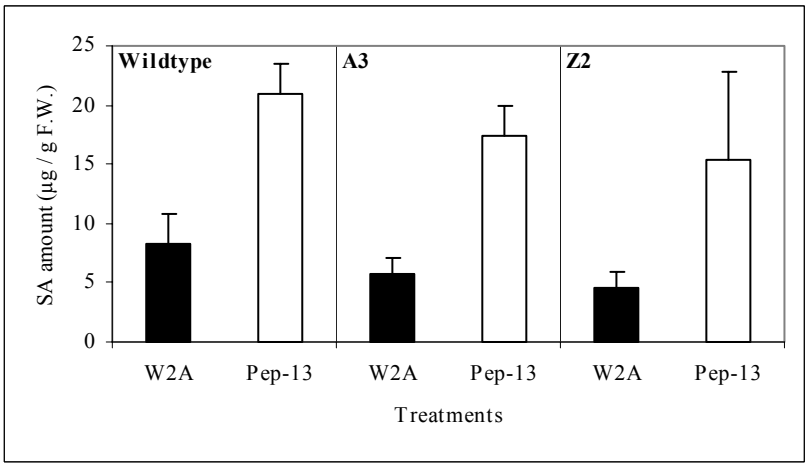


Fig. 31. JA levels in wildtype and 2 lines of *StOPR3*-RNAi potato plants upon Pep-13 infiltration (upper panel). Total SA levels in wildtype and 2 lines of *StOPR3*-RNAi potato plants upon Pep-13 infiltration (lower panel). As controls, both wildtype and *StOPR3*-RNAi potato leaves were infiltrated with W2A (black bars). Leaves were harvested 24 hours after infiltration for JA and SA measurements. Each column represents the mean from 3 different plants and error bars represent the standard deviation for the samples.

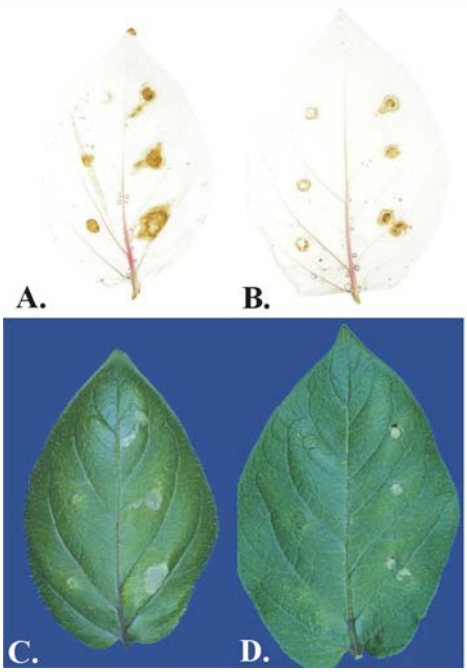


Fig. 32. Potato leaves were infiltrated with Pep-13 in the right half and W2A in the left half. Twenty hours after infiltration, HR pictures were taken (C,D). Leaves were stained with DAB for oxidative burst analysis (A;B). In this picture, wildtype potato leaves (A,C) are compared to *StOPR3*-RNAi potato leaves (B,D).

Twenty hours after Pep-13 infiltration, the *StOPR3*-RNAi potato leaves showed an oxidative burst and HR formation (Fig. 32B,D). However, the response of the *StOPR3*-RNAi leaves to Pep-13 treatment was notably weaker than that of the wildtype potato leaves. This experiment shows that both *NahG* and *StOPR3*-RNAi potato plants were compromised in oxidative burst and HR formation. However, *StOPR3*-RNAi potato plants can accumulate SA to the same level as wildtype potato plants (Fig. 31). This experiment also showed that JA is important for potato plant defense upon Pep-13 infiltration and acts downstream of SA.

NahG and *StOPR3*-RNAi potato plants produced less H_2O_2 and less HR after Pep-13 infiltration than wildtype potato plants (Fig. 24, 32). This implies that SA and JA are important for both oxidative burst and HR formation.

Many studies that were conducted in *Arabidopsis* showed that the oxidative burst is important for HR formation. In order to study the importance of H_2O_2 for HR formation in potato plants upon Pep-13 infiltration, diphenyliodonium (IDP) was used. IDP is a weak inhibitor of NADPH oxidases, which are an important source of H_2O_2 in many organisms including plants. Indeed, feeding IDP for 3 to 5 hours to wildtype potato leaves before Pep-13 infiltration reduced HR formation (Fig. 33). However, the amount of IDP should be controlled carefully. Too much IDP absorption will lead to unspecific cell death around the midrib of the leaves. We conclude that the oxidative burst is important for HR formation in potato leaf upon Pep-13 infiltration.

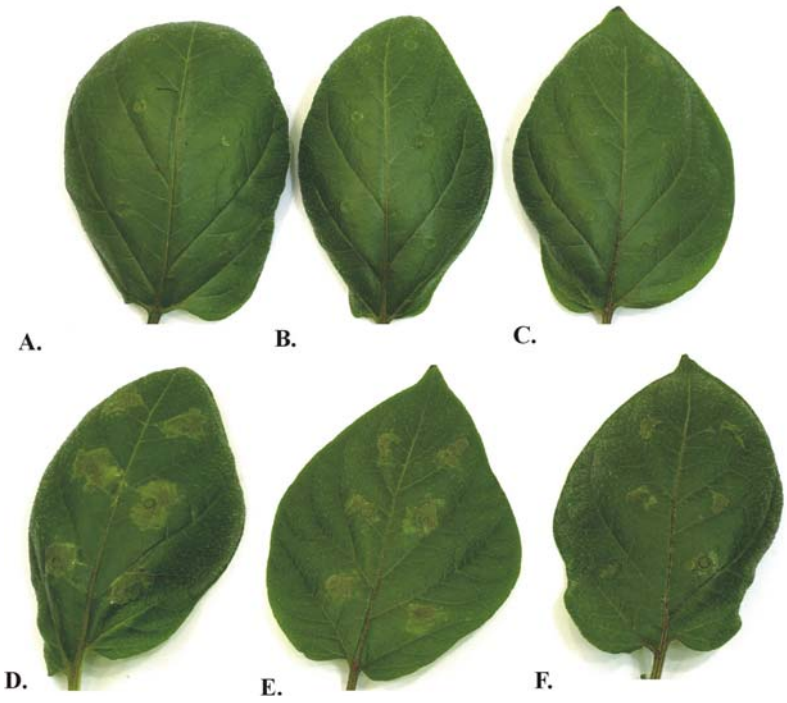


Fig. 33. Pep-13 infiltration into wildtype potato leaves led to clear HR formation (D). Feeding of 250 µM IDP, NADPH inhibitor, into potato leaves for 3 hours (E) and 5 hours (F) significantly reduced HR formation compared to water-fed leaves (D). No HR was observed on W2A infiltrated wildtype potato leaves (A-C) despite of feeding with IDP (B,C). This experiment was repeated with a similar result.

C. Pep-13-induced systemic acquired resistance (SAR) in potato plants; the role of jasmonic acid (JA) and salicylic acid (SA)

In a variety of plant species, the development of necrotic lesions in response to pathogen infection leads to the induction of generalized disease resistance in uninfected tissues (Ward et al., 1991). This mechanism of disease resistance is known as SAR. Local infection of potato leaves with the bacterial pathogen *Psm* also leads to increased resistance of the whole plant towards subsequent infection by virulent races of *P. infestans* (Kombrink et al., 1994). Treatment of the lower leaves of potato plants with arachidonic acid, a major fatty acid in the mycelial cell membrane and spores of *P. infestans*, protected the plants to subsequent *P. infestans* infection (Coquoz, 1995).

To test the experimental system and verify the published work, lower leaves of potato plants were infected with *Psm* before upper untreated leaves were challenged with *P. infestans*. *Psm*-pretreated potato plants were clearly more resistant to *P. infestans* than $MgCl_2$-pretreated potato plants (Fig. 34). This experiment verified the published work (Kombrink et al., 1994) and showed that the experimental system can be used to analyze SAR in potato plants.

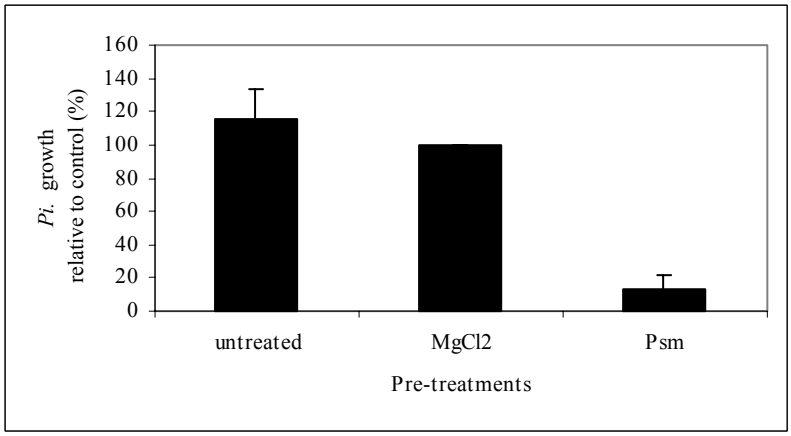


Fig. 34. Potato plants were pretreated with *Psm* 48 hours before *Pi* infection. Plants with *Psm* pretreatment showed more resistance to subsequent *Pi* infection. $MgCl_2$ pretreatment did not increase the resistance of potato plants. *Pi* growth in $MgCl_2$-pretreated potato plants was regarded as 100%. Each column represents the mean from 2 different experiments and error bars represent the standard deviation for the samples.

To test whether Pep-13 was also able to induce SAR in potato plants, the same experimental system was used. Figure 35 shows significantly lower *P. infestans* growth in Pep-13- than in W2A-pretreated potato plants. The experiment was repeated five times with similar results. This experiment shows that Pep-13 is able to induce SAR in potato plants.

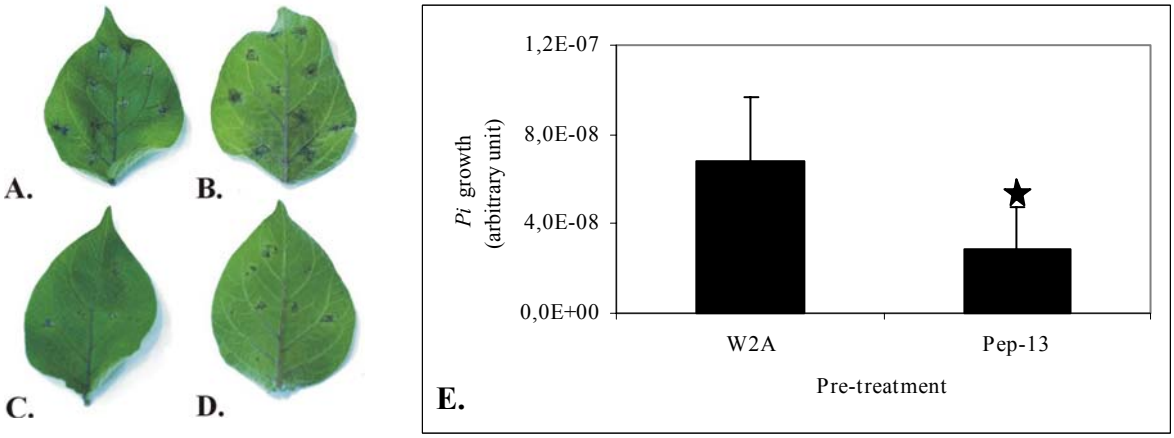


Fig. 35. Disease lesions after *Pi* infection are depicted in picture A – D. W2A-pretreated potato plants showed big disease lesion (A,B). Pep-13-pretreated potato plants showed smaller disease lesion (C,D). A and C show adaxial leaves. B and D show abaxial leaves. Real-Time PCR analysis showed that *Pi* growth in Pep-13-pretreated potato plants was significantly lower than *Pi* growth in W2A-pretreated potato plants (E). Statistical analysis was done using one-tailed Paired T-test; $P < 0.05$; $n = 6$.

To study the importance of SA for SAR establishment in potato plants, SA levels of both Pep-13-infiltrated and remote uninfiltrated potato leaves were measured. Twenty four hours after Pep-13 infiltration, an increase of free and conjugated SA in Pep-13-infiltrated potato leaves was observed. However, there was no increase of SA in uninfiltrated potato leaves even after 2 days (Fig. 36). To further study the role of SA in

SAR formation in potato plants against *P. infestans*, *NahG* potato plants were analyzed. There was no increase of both free and conjugated SA in local and systemic leaves of *NahG* potato plants 24 hour after Pep-13 infiltration. This picture did not change after 48 hours (Fig. 36).

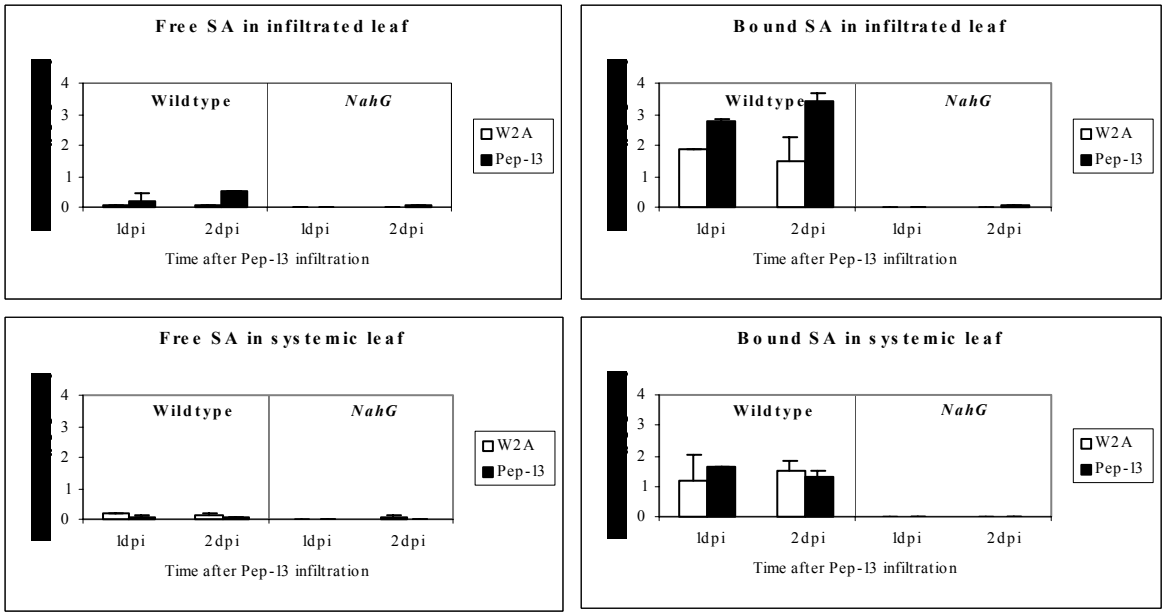


Fig. 36. Wildtype potato plants contain certain amounts of free and conjugated SA. Upon Pep-13 infiltration, both free and conjugated SA levels were increased. *NahG* potato plants contained very small amounts of free and conjugated SA. Free and conjugated SA levels in *NahG* potato plants stayed low even after Pep-13 infiltration. W2A infiltrated plants served as control. Each column represents the mean from 2 different plants and error bars represent the standard deviation for the samples.

Analysis of SAR in *NahG* potato plants showed that pre-treatment with Pep-13 did not increase the resistance of its upper leaves against *P. infestans*. *P. infestans* grew to the same level in control, W2A pre-treated *NahG* potato plant (Fig. 37). We conclude that accumulation of SA in the plants and increase of SA in the infected leaves was important for SAR against *P. infestans* in wildtype potato plant.

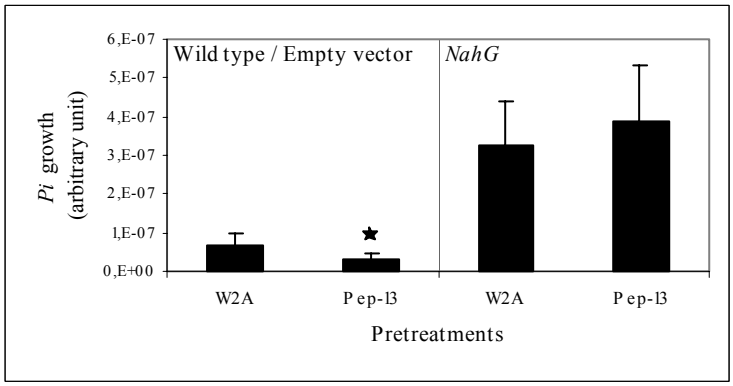


Fig. 37. *Pi* growth in Pep-13-pre-treated *NahG* potato leaves was not significantly different than in W2A pre-treated *NahG* potato leaves. There was a clear difference between *Pi* growth in *NahG* potato leaves and *Pi* growth in wildtype potato leaves. Statistical analysis was done using one-tailed Paired T-test; $P < 0.05$; $n = 6$. These samples consisted of 2 independent lines of *NahG* potato plants.

To address the role of JA for SAR, JA levels were measured. Figure 38 showed that infiltration of wildtype potato plants with Pep-13 led to JA accumulation in the infiltrated leaves but not in the remote uninfiltrated (systemic) leaves. The role of JA for SAR was further analyzed using S*tOPR3*-RNAi potato plant. After Pep-13 infiltration, *StOPR3*-RNAi potato leaves contained lower amounts of JA than wildtype potato leaves (Fig. 39). Further analyses showed that *StOPR3*-RNAi potato plants could not develop SAR. There was no significant difference between *P. infestans* growth in Pep-13-treated and W2A-treated *StOPR3*-RNAi potato plants (Fig. 40).

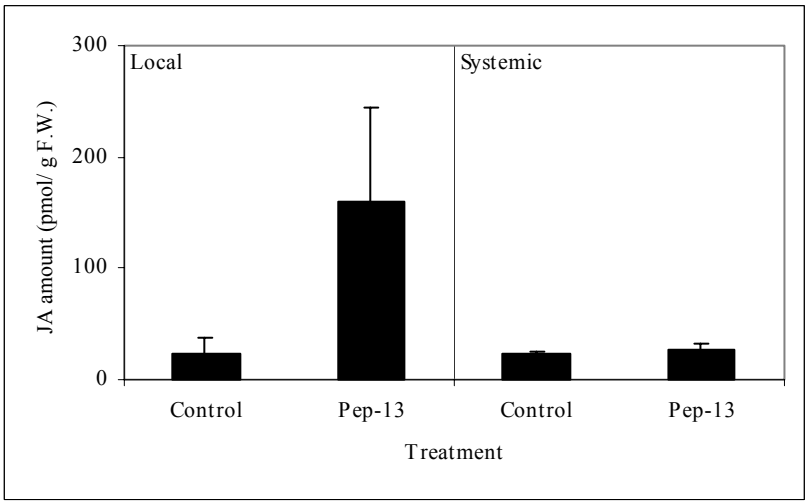


Fig. 38. Pep-13 infiltration caused an increase in JA levels in the local infiltrated leaves, but not in the systemic uninfiltrated leaves. There was no increase in JA levels in local or systemic wildtype potato leaves after water or W2A infiltration (control). JA levels were analysed 24 hours after Pep-13 infiltration. Each column represents the mean from 2 different plants and error bars represent the standard deviation for the samples. The experiment was repeated with similar result.

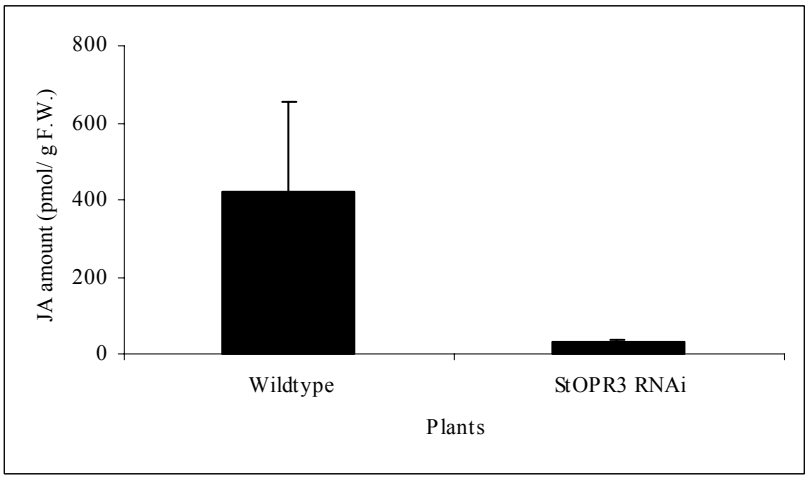


Fig. 39. Twenty four hour after Pep-13 infiltration, JA levels in *StOPR3*-RNAi potato leaves were much lower than in wildtype potato leaves. JA levels from 2 independent *StOPR3*-RNAi potato lines, A3 and Z2 were analysed. Each column represents the mean from 2 different plants and error bars represent the standard deviation for the samples. The experiment was repeated with similar result.

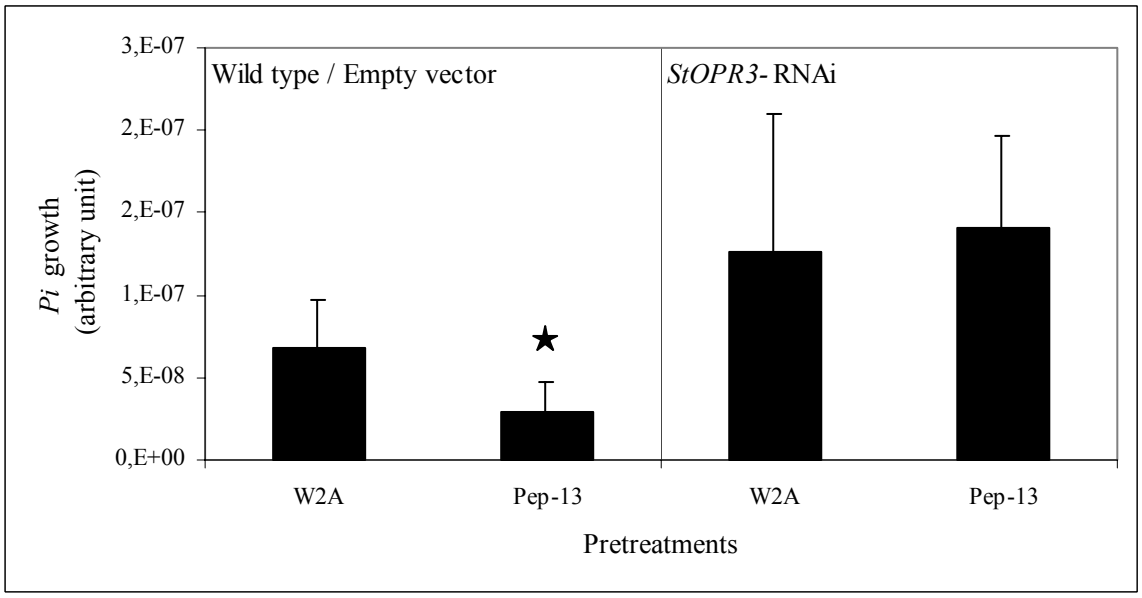


Fig. 40. *Pi* growth in Pep-13 pre-treated *StOPR3*-RNAi potato leaves was not significantly different than in W2A pre-treated *StOPR3*-RNAi potato plant. There was a slight difference between *Pi* growth in *StOPR3*-RNAi potato plant and *Pi* growth in wildtype potato plant. Statistical analysis was done using one-tailed Paired T-test; $P < 0.05$; $n = 6$. Combined data from lines A3 and Z2 are shown.

To analyze the importance of the HR at the primary site of infection for SAR development, *StAOS2* co-suppressed potato plants were incorporated in our analysis. In spite of different degrees of reduction in OPDA and JA levels after Pep-13 infiltration, both lines of *StAOS2* co-suppressed potato plants showed no reduction of HR size compared to wildtype potato plants. Figure 41 shows that despite the normal HR formation in *StAOS2* co-suppressed potato plant, there was no SAR formation in these plant. Therefore we conclude that the signal that was generated during the HR formation instead of the HR it self was important for SAR development.

To study the mechanism of SAR in potato plant, microarray experiments were conducted. These were carried out using the 10K chip from The Institute of Genome Research (TIGR) that contained 10.000 different potato ESTs, both complete and partial (7680-clones are validated). More details on the potato microarray can be found on the TIGR potato microarray web page (http://www.tigr.org/tdb/potato/microarray_comp.shtml). Due to the limited number of slides available, one experiment was carried out. In this experiment, five potato slides were hybridized with potato leaf cDNA from *Psm*-and Pep-13-treated plants. For local response studies, cDNA from treated leaves was used. To study SAR, cDNA from the upper uninfiltrated (systemic) leaves was used. As controls, cDNA from $MgCl_2$-or W2A-

treated plants was used, which was hybridized together with cDNA from *Psm*-or Pep-13-treated plants using different dye.

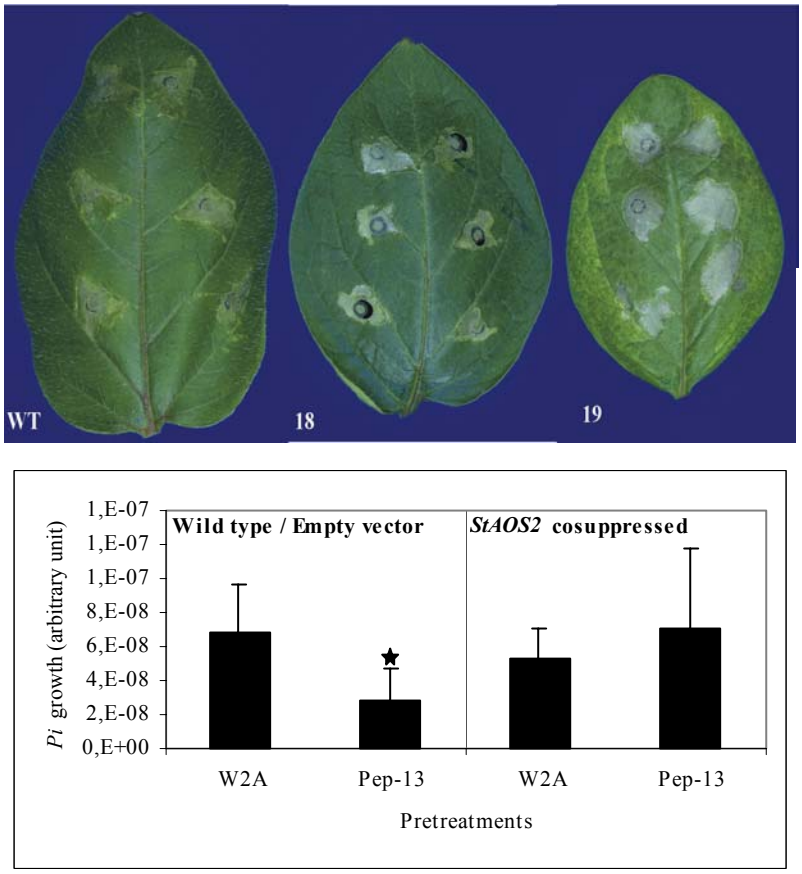


Fig. 41. Twenty four hours after Pep-13 infiltration, both lines of *StAOS2* co-suppressed potato plants, line 18 and 19, showed normal HR formation as wildtype potato plant (upper panel). *Pi* growth in Pep-13 pre-treated *StAOS2* co-suppressed potato leaves was not significantly different than *Pi* growth in W2A pre-treated *StAOS2* co-suppressed potato leaves (lower panel). Statistical analysis was done using one-tailed Paired T-test; $P < 0.05$; $n = 6$. Data are from 2 independent lines StAOS2 co-suppressed potato plants, 18 and 19.

Data analysis was done using a TIGR software package consisting of TIGR Spotfinder, TIGR Microarray Data Analysis System and TIGR Multi Experiment Viewer. These softwares can be downloaded from the TIGR web page. General information about the treatments of samples and regulation of the gene expression can be seen in Table 2. Expression of the genes was described as the ratio of intensity between treatment and control treatment, for example the ratio of intensity between Pep-13 treatment to W2A treatment. A gene was grouped as repressed gene when the intensity was 2.5 times lower compared to the intensity of control treatment. Similarly, a gene was considered as activated gene if the intensity of treatment was 2.5 times higher compared to control.

Tbl. 2. Number of genes that were regulated in different treatments and samples.

Expression	Psm		Pep-13		
	local (1dpi)	systemic (2dpi)	local (1dpi)	systemic (1dpi)	systemic (3dpi)
repressed genes	478	34	347	23	45
induced genes	1542	288	671	224	341

To study SAR, we concentrated only on genes, the expression of which is induced systemically after *Psm* and Pep-13 pretreatments. Interestingly, increased expression of some JA-induced genes in systemic tissue, such as LOX H1, polyphenol oxidase, and proteinase inhibitor was observed.

Macroarray analysis was also performed to study SAR in potato plant. The macroarray contained 250 cDNA fragments from *Solanaceae* species that have different functions, including some defense-related genes (prepared by Petra ten Hoopen, IPK) (Ten Hopen, 2002). The macroarray membranes were hybridized with cDNA from Pep-13 treated plants (local and systemic) as well as *Psm* treated plants (local and systemic). From this macroarray experiment, 10 genes were identified, the expression of which was induced in both Pep-13-and *Psm*-treated leaves. Eight genes were defense-related genes such as *PR 1a*; *PR b1b*, *PR 5*, *chitinase A*, *glucanase*, *THT*, *Prp 1*, and *Proteinase Inhibitor II*. A few genes were induced in systemic untreated leaves upon Pep-13- or *Psm*-pretreatment of the plants. Interestingly, among five genes that were induced systemically, such as *LOX 2*, *Proteinase Inhibitor II*, *Cystein Proteinase Inhibitor*, *Cathepsin Inhibitor*, and *OS 91*, most of them except *OS 91* belong to jasmonate-induced genes that have already been identified in the microarray analysis. More details on systemically induced genes can be found in Figure 42.

To verify the induction of JA-induced genes in systemic leaves after Pep-13 infiltration, expression of *StPin2* was analyzed using northern blot. Indeed, 3 days after Pep-13 infiltration of lower leaves, a significant increase of *StPin2* expression in systemic leaves was observed (Fig. 43). There was no increase of *StPin2* expression in systemic leaves after W2A infiltration. The experiment was repeated three times with similar results.

The increased expression of *StPin2* in systemic leaves correlated with the development of SAR in systemic leaves. Therefore *StPin2* can be used as a marker gene to study Pep-13-induced SAR in potato. However, the increase of *StPin2* expression and other JA-induced gene expression did not correlate with JA levels in systemic leaves. Upon Pep-13 pretreatment of potato plants, no increase of JA was observed in systemic potato leaves (Fig. 38). Therefore, the role of other compounds of the JA biosynthesis pathway in inducing expression of those genes was hypothesized.

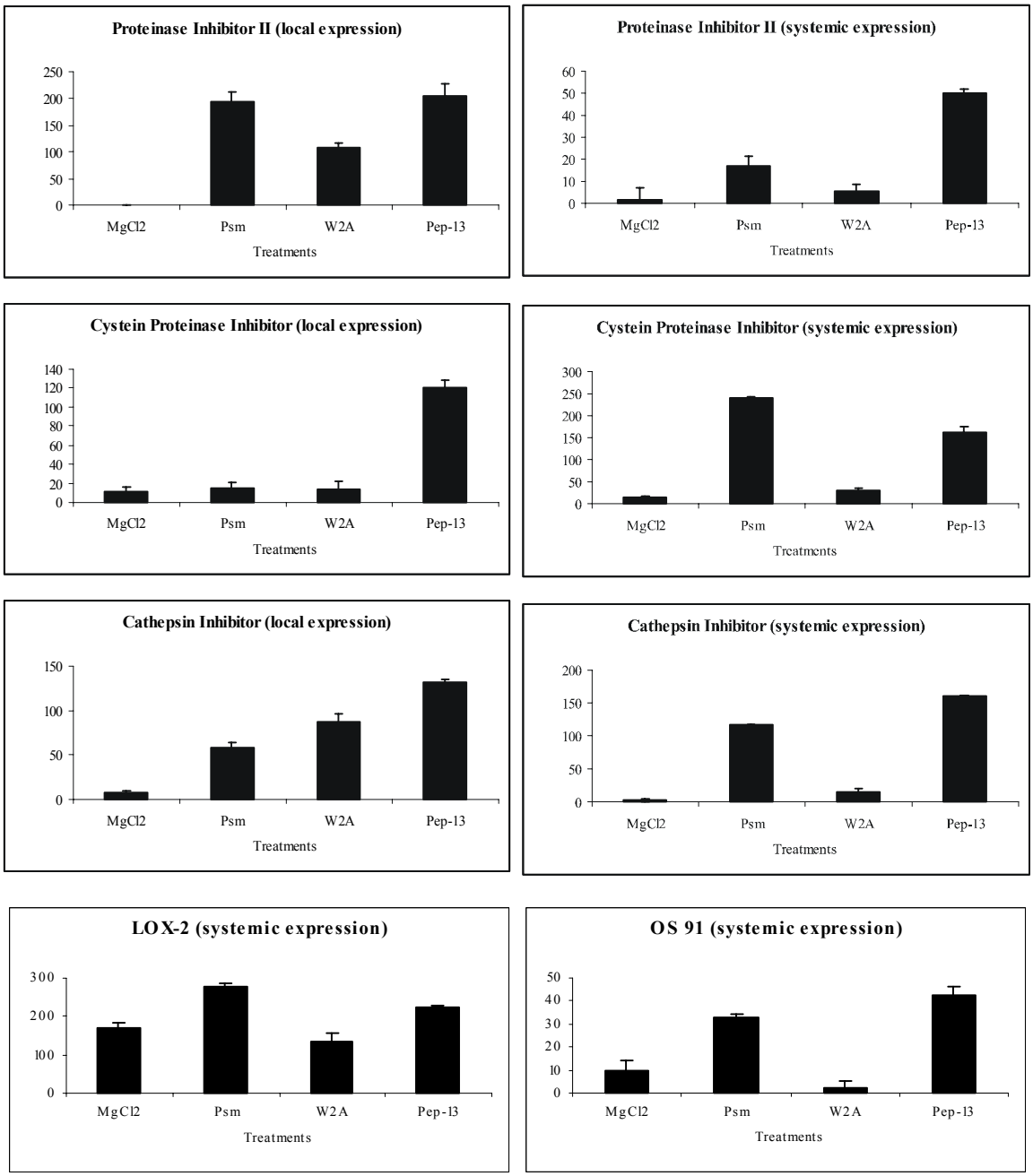


Fig. 42. Local and systemic gene expression in response to *Psm* and Pep-13 treatments. Gene expression was analysed by Macroarray. Each cDNA was spotted twice on the membrane. Local leaves of *Psm*- and Pep-13-treated plants were harvested 24 hours after infiltration. Systemic leaves of *Psm*-treated plant were harvested 48 hours after infiltration. Systemic leaves of Pep-13-treated plants were harvested 72 hours after infiltration.

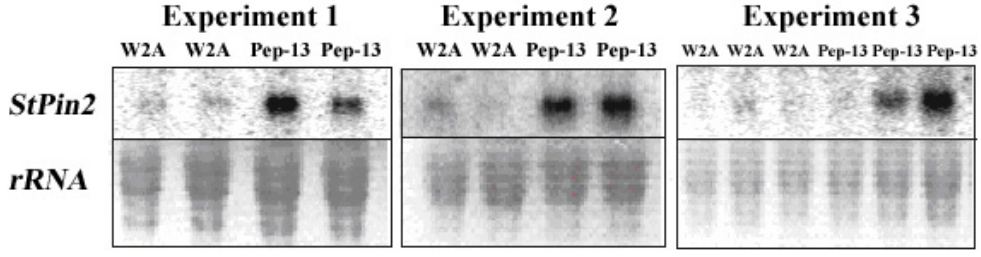


Fig. 43. Systemic increase of *StPin2* gene expression in Pep-13-treated potato plants. W2A infiltration did not increase *StPin2* gene expression systemically. rRNA was used as loading control. Systemic leaves were harvested 72 hours after infiltration.

OPDA levels and *P. infestans* growth in systemic leaves from experiment 1 and experiment 2 were measured. These experiments were performed within the same set of plants to reduce the variation between plants and to get a direct correlation between *StPin2* expression, OPDA levels and SAR against *P. infestans.* Indeed, the increase of *StPin2* expression was concomitant with the increase of OPDA level and reduced *P. infestans* growth (Fig. 45). OPDA was also increased in local leaves after Pep-13 infiltration (Fig. 44). However the possible role of OPDA as mobile signal for SAR in potato still has to be proven.

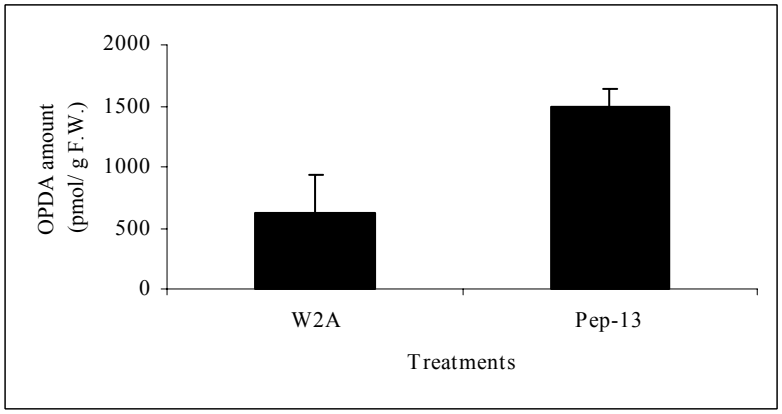
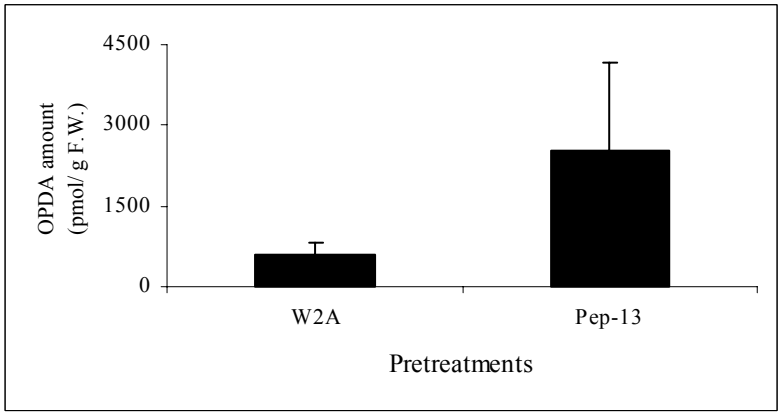


Fig. 44. Pep-13 infiltration caused increase of OPDA levels in local infiltrated wildtype potato leaves. There was no increase of OPDA levels in local or systemic wildtype potato leaf after water or W2A infiltration (control). OPDA level was analysed 24 hours after Pep-13 infiltration.



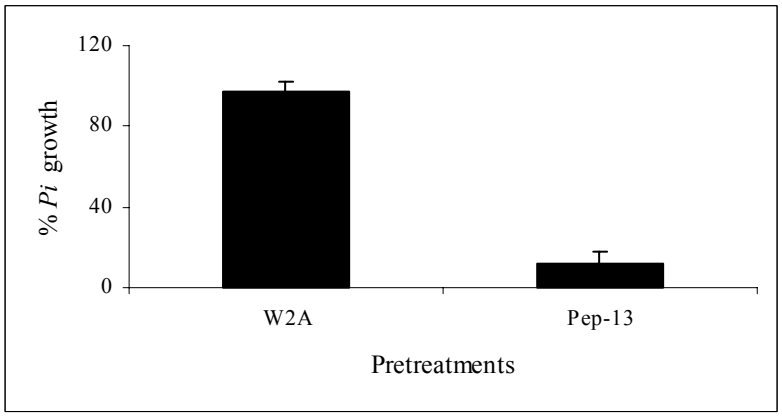


Fig. 45. Seventy two hours after Pep-13 infiltration into lower leaves, an increase of OPDA levels in upper uninfiltrated / systemic leaves was observed (upper panel). Seventy two hours after Pep-13 infiltration into lower leaf, we infected upper uninfiltrated /systemic leaves with *Pi.* Less *Pi* growth was observed in Pep-13-pretreated plants compared to W2A (lower panel). This analysis were done in 2 different experiments.

IV. Discussion

A. The role of salicylic acid (SA) and jasmonic acid (JA) for defense of potato against *Phytophthora infestans* (*P. infestans*)

The role of SA and JA for defense against pathogens has been studied in various pathosystems. Currently, there is a widely accepted opinion on the role of SA in plant defense against biotrophic pathogens and of JA against necrotophic pathogens (McDowell and Dangl, 2000). This opinion is regarded valid based on different kinds of interactions of pathogens with *Arabidopsis* (Glazebrook, 2005). Studies using other plant-pathogen systems are obviously important to prove whether these mechanisms are employed by plants in general to defend themselves against various pathogens. Studies on various systems demonstrated already that plants developed different defense signaling mechanisms. The constitutive high levels of SA in rice correlates with its insensitivity towards endogenous increases and exogenous application of SA (Yang et al., 2004). Also in rice, the contribution of JA to plant defense, such as PR gene expression, phytoalexin production and volatile emission was shown (Schweizer et al., 1997; Tamogami et al., 1997; Lou et al., 2005). Moreover, JA is an effective inducer of systemic acquired resistance (SAR) in rice seedlings against infection by the blast fungus (Lee et al., 2001). Potato contains high basal levels of SA. The levels are higher than in *Arabidopsis* but still lower than in rice (Yu et al., 1997). Therefore, detailed studies on the role of JA for pathogen defense in potato will provide a better understanding of defense response regulation in plants.

NahG potato plants were used to study the role of SA. They were used in our experiments for several reasons. It has been shown that the phenylpropanoid pathway is the main source of SA in potato plants upon elicitation with arachidonic acid (Coquoz et al., 1998). In contrast to potato, in *Arabidopsis*, the isochorismate pathway is the main source of SA for defense against pathogens (Wildermuth et al., 2001). Because of the possibility of SA production from these two pathways, the approach of expressing *NahG* genes to degrade SA in plant is the best choice to get SA-depleted potato plants. Important data on the role of SA in plant defense were obtained from studies using *NahG* plants. In potato, the role of SA for basal and systemic defense of potato plants against *P. infestans* was studied using *NahG* potato plants (Yu et al., 1997). The role of SA for basal resistance and SAR of *Arabidopsis* against *H. parasitica* was also studied using *Arabidopsis* expressing the *NahG* gene (Delaney et

al., 1994), Moreover, cross talk between SA and JA in *Arabidopsis* was nicely shown by analysis of *Pst*-infected *NahG* plants (Spoel et al., 2003). Therefore, studies using *NahG* potato plants helps to integrate our data into established plant defense models. However, some studies in *Arabidopsis* showed side effects of the *NahG* gene, which did not result from lack of SA (Heck et al., 2003; van Wees and Glazebrook, 2003). So far, this side effect has only been reported for *Arabidopsis*, even more specific in an incompatible interaction with *Pseudomonas syringae* pv *phaseolicola* (van Wees and Glazebrook, 2003) or an interaction with avirulent *Pseudomonas syringae* pv *tomato* (Heck et al., 2003),. Studies in rice did not show similar effects like that in *Arabidopsis* (Yang et al., 2004). Still, conclusions based on experiments with *NahG* plants should be considered with precaution. Therefore, these experiments were complemented with experiments in which the synthetic analog of SA was applied exogenously.

P. infestans is the pathogen of Potato, that causes the devastating late blight disease. *P. infestans* is a hemibiotrophic pathogen that needs living plant cells to support growth at early phase, but kills plant cells in the later stages (Thaler et al., 2004), (Smart et al., 2003).

Upon *P. infestans* infection, SA levels in potato plants increase (Fig. 3) (Coquoz, 1995). This increase was correlated with elevated *PR1a* and *PR5* expression (Fig 8). However, the increase in SA levels did not correlate with reduced susceptibility of potato against *P. infestans*, since continued growth of *P. infestans* was observed after infection. This finding was interesting, since a correlation had been demonstrated between endogenous levels of SA in different potato cultivars and their susceptibility to *P. infestans* (Coquoz, 1995). A Loss-of-function approach using *NahG* potato plants was used to further investigate the role of SA for potato defense against *P. infestans*. Microscopic observation showed that infected *NahG* potato leaves contained more mycelium around the infected area (Fig. 5). Growth determination by Real-Time PCR revealed *P. infestans* growth in *NahG* potato leaves being tenfold higher than in wildtype (Fig. 6). Therefore, the importance of SA for defense of potato plants against *P. infestans* can be confirmed. Measuring pathogen growth based on symptoms such as lesion size is not sufficient. Based on lesion size after 6 to 7 days of *P. infestans* infection, Yu et al. (1997) reported that there was no difference in *P. infestans* growth in *NahG* potato plants compared to wildtype potato plants. Different sensitivity of the methods might explain the differences.

The potato cv. *Désirée* is susceptible to *P. infestans*. It contains no known R proteins that would recognize the product of known *Avr* genes from *P. infestans* (Armstrong et al., 2005). It allows *P. infestans* to grow. Reducing SA levels by overexpression of SA hydroxylase in *NahG* potato plants increases the susceptibility against *P. infestans*. Therefore, we conclude that SA is important for basal defense of potato against *P. infestans.* Lack of SA in *NahG* potato plants leads to stronger growth of *P. infestans* in the plant.

INA is a synthetic analogue of SA. In initial experiments, application of INA to *NahG* potato leaves complemented the resistance of the plant to the wildtype level of resistance against *P. infestans* (Fig. 10). Direct effects of INA to *P. infestans* growth was excluded because INA was applied at the adaxial side of the leaves while *P. infestans* infection was done at the abaxial side of the leaves. This complementation study shows the importance of SA for basal defense of potato plants against *P. infestans*.

Studies on several defense responses in wildtype and *NahG* potato plants upon *P. infestans* infection revealed that reduced SA levels resulted in compromised callose formation (Fig. 7), defense gene expression (Fig. 8) and cell death (Fig. 9).

The importance of callose in providing a physical barrier has been shown in many plant pathogen interaction. The full encasing callose around the structure of *H. parasitica* has been correlated with reduced growth in *Arabidopsis* (Donofrio and Delaney, 2001). The importance of callose formation to stop penetration of downy mildew on grapevine leaves has also been shown (Hamiduzzaman et al., 2005). The deposition of callose on the wall of cells adjacent to HR cells or in papillae has been shown in potato-*P. infestans* interactions (Vleeshouwers et al., 2000). However, some pathogens employ callose formation to avoid recognition by the plant. In *Arabidopsis* mutants that are unable to form callose (*pmr4*), SA-induced gene expression is higher upon powdery mildew infection than in wildtype plants. This correlates with an increase of resistance against powdery mildew in *pmr4* (Nishimura et al., 2003). In contrast, the susceptibility of *NahG* potato plants to *P. infestans* can be correlated with reduced callose formation at the site of infection. However, it is difficult to conclude how important callose formation alone is for basal resistance of potato plants against *P. infestans*, since callose is not the only defense response that is impaired in *P. infestans* infected *NahG* potato plants. Pharmacological approaches, such as the application of the callose inhibitor, 2-deoxy-D-glucose, might be an alternative to

answer this question. It has been shown that treatment of ml-o (convers resistance to powdery mildew) barley coleoptiles with 2-deoxy-D-glucose reduces and delays papilla formation upon powdery mildew infection and increases penetration efficiency (Bayles et al., 1990). Initial studies applying 2-deoxy-D-glucose failed to reduce callose formation in wildtype potato leaves upon *P. infestans* infection. Optimization of the delivery method and incubation time might help to further study the importance of callose formation for basal resistance of potato plants against *P. infestans*.

The role of PR proteins for plant defense has been studied in many plant pathosystem. Overexpression of the PR protein, PR1a, in tobacco has no effect on virus infection (Linthorst et al., 1989). However, transgenic tobacco expressing the PR1a protein showed increased resistance to two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander et al., 1993). Transgenic potato plants expressing PR5 (osmotin) exhibited delayed disease symptoms after *P. infestans* infection, whereas PR5 overexpression did not confer resistance against *Phytophthora parasitica* var. *nicotianae* in tobacco plants (Liu et al., 1994). Effectiveness of a protein with similarity to osmotin for lyses of sporangia and growth inhibition of *P. infestans* has been shown (Woloshuk et al., 1991). The effectiveness of PR proteins might be different against various pathogens. Multiple expression of PR proteins might also be more potent to confer resistance against certain pathogens. In *NahG* potato plants, lower *PR1a* and *PR5* expression at one day post infection can be correlated with stronger *P. infestans* growth three days post infection (Fig. 8). Thus, *PR1a* and *PR5* expression in potato plants might contribute to their basal defense against *P. infestans*. However, stronger growth of *P. infestans* in turn effects more cells in *NahG* leaves. Accumulation effects might explain why after three days of infection, *PR1a* and *PR5* expression in *NahG* potato plants is similar to wildtype potato plants.

Recent advances in the genetic, biochemical and cytological characterization of disease resistance suggests that HR is associated with all forms of resistance to *Phytophthora* and downy mildews (Kamoun et al., 1999). Using trypan-blue staining, which stains dead cells and fungal structures, the relationship between intensity of blue staining of dead cells around *P. infestans*-infected sites and escaping hyphae from these sites was demonstrated. In comparison to wildtype potato leaves, *NahG* potato leaves contained less dead cells as shown by less intense blue staining color around the site of infection. This observation correlates with more and longer escaping

hyphae from the site of infection compared to that in wildtype potato leaves (Fig. 9). The inability of *NahG* potato plants to mount cell death in comparison to wildtype was also shown after infiltration with Pep-13 elicitors (Fig. 24). Thus, this study shows a correlation between cell death and reduced growth of *P. infestans* in potato.

Studies on the function of JA for defense of potato plants against *P. infestans* was initiated for following reasons. Previous reports were unable to demonstrate the role of SA for basal defense of potato plants against *P. infestans* (Yu et al., 1997). It is known that JA is important for plant defense against necrotrophic pathogen (Glazebrook, 2005). Due to the fact that *P. infestans* has a necrotrophic life style in the late infection stage, it is possible that JA is important for the defense of potato againsts this pathogen. The ability of exogenous JA and me-JA to protect potato plants against *P. infestans* and induce systemic protection (Cohen et al., 1993) indicate the important role of JA for defense of potato plants against *P. infestans*. A recent report about the importance of endogenous JA for defense of tomato against different pathogens including *P. infestans* (Thaler et al., 2004), provides evidence of the importance of JA for plant defense against *P. infestans*. The importance of OPDA for defense of *Arabidopsis* against the pathogen *Alternaria brassicicola*, in the absence of JA (Stintzi et al., 2001) and local and systemic accumulation of OPDA in potato plants after *Psm* infection (Landgraf et al., 2002) are the reasons why we were also interested in studying the role of OPDA.

AOC and OPR3 are two enzymes in the JA biosynthetic pathway. While AOC is the important enzyme to convert labile allene oxide to OPDA, OPR3 has the function to convert OPDA to OPC8 ((9S, 13S)-12-oxophytodienoic acid to 3-2(2'(Z)-pentenyl) cyclopentane-1-octanoic acid), the intermediate of JA. These enzymes have been well studied in tomato and *Arabidopsis* (Ziegler et al., 2000; Strassner et al., 2002; Stenzel et al., 2003). By an RNAi approach on *AOC*, we wanted to reduce OPDA levels and study its role for defense of potato plants against *P. infestans*. In a similar way, the function of JA was studied using *OPR3*-RNAi potato plants. Loss-of-function experiments were done to get information on the importance of OPDA and JA for defense of potato plants. Potato and tomato AOC share 95% identity at nucleotide level and 70% identity at amino acid level. Transforming potato plants with the tomato *AOC*-RNAi construct resulted in 60% reduction of OPDA and JA levels upon wounding compared to wildtype potato plants (data not shown). No difference in defense responses, such as oxidative burst and HR cell death were observed upon Pep-

13 infiltration. Susceptibility against *P. infestans* in *AOC*-RNAi plants was also not affected. Incomplete reduction of OPDA levels might explain the lack of phenotype of these transgenic plants.

Comparison of *P. infestans* growth in *StOPR3*-RNAi potato plants to that in wildtype potato plants represent the first *in vivo* study of the importance of JA biosynthesis for defense of potato plants against *P. infestans*. Compared to wildtype potato plants, two lines of *StOPR3*-RNAi potato plants are more susceptible (Fig. 15). Three days after infection, three to five times more *P. infestans* growth was observed in *StOPR3*-RNAi potato plants. This indicates that JA is also important for basal defense of potato plants against *P. infestans*. This result confirms the *in vitro* study in which exogenous application of JA and me-JA protected potato plants against *P. infestans* (Cohen et al., 1993). This result is also in agreement with Thaller *et al*. (2004). Using JA-deficient tomato mutants (*def1*) they showed the importance of JA for defense of tomato plants against *P. infestans*. In rice, which has high SA levels, the role of JA for plant defense is more prominent. It has also been shown that JA is important for defense of rice against the blast fungus. More studies are necessary to investigate whether the importance of JA for basal defense of potato plants against *P. infestans* is related to the high SA levels. However, our result supports the growing evidence on the important role of JA for plant defense against pathogen.

StOPR3-RNAi potato plants were not impaired in PR gene expression and callose formation (Fig. 16) after *P. infestans* infection. However, less oxidative burst and HR formation was observed on *StOPR3*-RNAi potato leaves compared to wildtype potato leaves in response to Pep-13 infiltration (Fig. 32). Therefore, the importance of JA for promoting basal resistance of potato against *P. infestans* may be mediated through oxidative burst and HR formation. The importance of HR formation for resistance of potato plants against *P. infestans* has been shown in R gene-mediated resistance (Vleeshouwers et al., 2000). HR is also correlated with all resistance against *Phytophthora* and downy mildews (Kamoun et al., 1999).

Stintzi et al. (2001) showed the importance of OPDA instead of JA for resistance of *Arabidopsis* against *Alternaria brassicicola*. Using *AOS2* co-suppressed potato plants we were able to study the role of OPDA for resistance of potato plants against *P. infestans*. In both lines of *AOS2* co-suppressed potato plants, the OPDA levels were less than 50% than that of wildtype potato plants (Table 1). The growth of *P. infestans* in 2 lines of *StAOS2* co-suppressed potato plants were compared to that in

wildtype potato plants. Interestingly, the susceptibility of two lines *AOS2* co-suppressed potato plants against *P. infestans* were different (Fig. 18). While line 19 was significantly more susceptible compared to wildtype, the susceptibility of line 18 was not different compared to that in wildtype. More detailed comparison showed that line 19 contains less JA compared to line 18 (Table 1). However, indirect comparison shows none of those *AOS2* co-suppressed potato plants are more susceptible to *P. infestans* than *StOPR3*-RNAi potato plants. While *P. infestans* growth in *StOPR3*-RNAi potato plants is around 5 times higher than that in wildtype potato plants, *P. infestans* growth in *StAOS2* co-suppressed potato plant is not more than 3 times. In this context it is interesting to note that these data support our previous conclusion on the importance of JA, suggesting that lower JA levels rather than OPDA correlate with increase susceptibility of potato plants against *P. infestans*. Our conclusion on the role of OPDA is different from that observed by Stintzi et al. (2001). The role of OPDA might be different in different pathosystem. Chlorosis was also observed in *AOS2* co-suppressed potato plants line 19 although cellular analysis using electron microscopy did not show the difference.

In summary, the importance of SA and JA for basal defense of potato plants against *P. infestans* can be shown in this experiment. Collectively, callose formation, PR proteins, oxidative burst and HR might act as basal defenses in potato plants against *P. infestans*.

B. Characterization of Pep-13 induced defense responses in potato and its signaling mechanism

Mutual antagonism between SA and JA signaling pathways has been shown in *Arabidopsis* (Dong, 1998). The importance of both SA and JA in promoting basal defense of potato plants against *P. infestans* indicates the existence of different defense signaling regulation in potato. It was known that Pep-13 elicitation is able to induce defense gene expression in potato cell culture (Geiler, 2001; Brunner et al., 2002). Further studies also showed that Pep-13 is also able to induce defense gene expression and necrotic cell death in potato plants (Geiler, 2001; Veß, 2004).

Analyses of defense signaling and responses in potato shall be achieved using Pep-13 as elicitor. The potato - Pep-13 interaction represents a system of reduced complexity when compared to the phatosystem. Upon infiltration, Pep-13 induced accumulation of defense signals, such as SA and JA (Fig. 19). The increases of SA

and JA levels were correlated with the expression of several PR genes, such as *PR1a*, *PR10*, *chitinase* and *LOX3* (Fig. 22). While increases in defense gene expression can be detected as early as 8 hours after Pep-13 infiltration and still increase after 24 or 48 hours, oxidative burst and necrotic cell death were detected 24 hours after Pep-13 infiltration (Fig. 20, 21). Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay showed that this necrotic cell death results in fragmented DNA (Fig. 21). Together with oxidative burst around the cells undergoing necrotic cell death, this reaction exhibited features of programmed cell death leading to HR. Here, characterization of Pep-13-induced defense responses and signaling mechanism leads to defense responses, which are similar to those in *Arabidopsis* leaves upon treatment with the necrosis-inducing *Phytophthora* protein (NPP1) (Fellbrich et al., 2002).

While some information is available on the mechanism of defense gene activation by SA and JA, their role in the stimulation of oxidative burst and HR is unclear. Reduced oxidative burst and HR in Pep-13-infiltrated *NahG* potato leaves compared to wildtype showed the importance of SA for both reactions (Fig. 24). A different result was obtained when *NahG* potato cells elicited with Pep-13, since similar levels of oxidative burst and cell death were observed in *NahG* and wildtype cells (Halim et al., 2004). H_2O_2 accumulation was observed as early as 15 minutes after Pep-13 elicitation and peaked within 1 to 2 hours after elicitation. The absence of a negative regulator of H_2O_2 accumulation in cultured cells may explain this difference. Another possibility is that the early burst is needed to trigger programmed cell death. Possibly, this HR is important for the secondary burst that is detected by DAB staining of the leaves. The disruption of organelles and cell membranes during HR might trigger the release of H_2O_2 from various organelles within a cell. The application of NADPH inhibitor may act on the early burst leading to loss of HR (Fig. 33). Loss of HR may lead to loss of secondary oxidative burst as shown in Pep-13-infiltrated *NahG* potato leaves. Although no HR and oxidative burst formation were observed in Pep-13-infiltrated *NahG* potato leaves, H_2O_2 formation was observed in *NahG* potato leaves upon wounding (Wolf, 2005). Therefore, one might also speculate that the disruption of cells may be the source of H_2O_2 in the wounding response.

In addition to the defects in various defense responses, *NahG* potato plants were also unable to accumulate JA after Pep-13 infiltration (Fig.25). Therefore, it is interesting to study the role of JA for defense responses of potato and possible cross-

talk with SA. *StOPR3*-RNAi potato plants were used to study the role of JA signaling in pathogen defense of potato plants. In comparison to wildtype, lower levels of JA were observed in Pep-13-infiltrated *StOPR3*-RNAi potato leaves (Fig. 31). In addition, reduced oxidative burst and HR were found in Pep-13-treated *StOPR3*-RNAi potato leaves compared to wildtype (Fig. 32).

The importance of both, SA and JA for potato defense against *P. infestans*, the simultaneous increases of SA and JA levels in Pep-13-treated potato plants, and the similar defects of defense responses in SA- and JA-deficient potato plants upon Pep-13 treatment suggest a connection between SA and JA signaling. Interestingly, lack of JA in Pep-13-treated *NahG* potato leaves showed a positive instead of a negative correlation that has been shown in *Arabidopsis* (Spoel et al., 2003; Zhao et al., 2003). No significant effect on SA levels in Pep-13-infiltrated *StOPR3*-RNAi potato leaves (Fig. 31) suggests that in potato, JA accumulation is downstream of SA signaling. The conclusion that there is no negative correlation between SA and JA signaling in potato plants is also supported by experiments with *Psm*. *NahG* potato leaves are unable to accumulate SA after *Psm* infiltration (Fig. 29). However, infiltration of *Psm* suspension resulted in JA accumulation and HR formation in *NahG* potato leaves to a similar level as in wildtype (Fig. 28, 30). This is different from what was observed in *Arabidopsis*. Infiltration of *NahG Arabidopsis* plants with *Pst* DC3000 led to JA accumulation to levels that were almost 10 times higher than that in wildtype (Spoel et al., 2003). Upon *Pst* DC3000 infection, tomato *coi1* mutants, which are unable to accumulate transcripts of JA-responsive genes show higher expression levels of SA-responsive genes than wildtype plants (Zhao et al., 2003). The fact that both JA accumulation and HR were found in *Psm*-infiltrated *NahG* potato leaves, raised the question of the importance of JA for HR. Although the importance of JA for HR has been shown in Pep-13-treated potato leaves, analysis of HR in *Psm*-treated *StOPR3*-RNAi leaves will extend our knowledge on defense signaling mechanism in potato plant.

Based on this study, a tentative model of defense signaling in Pep-13-treated potato plants is suggested, which is depicted in Figure 46. Both SA and JA signaling pathways exist in potato plants. The activation of these signaling pathways depends on the particular stress applied. Upon Pep-13 infiltration, SA and JA levels increase. Increase of JA levels is SA-dependent. JA accumulation is important for H_2O_2 production. Finally, accumulation of H_2O_2 will initiate HR.

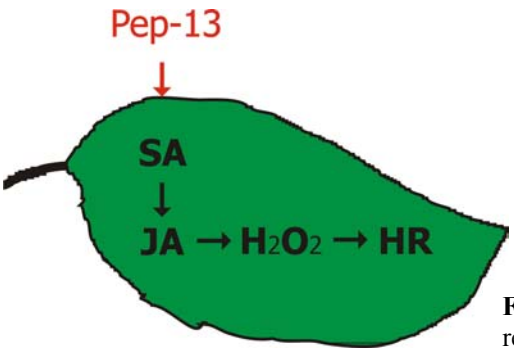


Fig. 46. Model of potato defense signaling in response to Pep-13 treatment.

Defense signaling in plant is very complex and the interaction between plant and pathogen involves mixtures of effectors and PAMPs. Therefore, this model can not be generalized for all plant pathogen interaction. Fine-tuning of defense signaling must exist to create the effective defense. *Psm*, for instance, is able to induce JA accumulation and HR in potato in the absence of SA. However, the importance of both SA and JA signaling for downstream defense responses has been demonstrated in this study. Interestingly, JA acts downstream of SA accumulation. This mechanism is different from what that has been shown for *Arabidopsis*.

C. Pep-13-induced systemic acquired resistance (SAR) in potato plants; the role of jasmonic acid (JA) and salicylic acid (SA)

SAR is a defense mechanism that is developed by plants after the formation of necrotic lesions, either as apart of a hypersensitive response (HR) or as a symptom of disease. SAR can be distinguished from other disease resistance responses by both, the pathogen non-specific protection and the associated changes in gene expression (Ryals et al., 1996). SAR activation can be useful to combat secondary infection from a broad spectrum of pathogens or to prevent an existing infection from spreading further (Dong, 1998). In addition to intact organisms, some chemicals and pathogen-derived molecules have been identified as SAR-inducers (Uknes et al., 1992; Coquoz, 1995; Lawton et al., 1996). Because Pep-13 is a peptide elicitor from *Phytophthora* species and causes necrotic lesions upon infiltration into potato leaves, it was interesting to study the ability of Pep-13 to induce SAR against *P. infestans* in potato. Using an improved system that was able to analyze SAR in the potato-*Psm* system (Fig. 34), SAR establishment in Pep-13-infiltrated potato plants was analyzed. Indeed, Pep-13 is able to induce SAR against *P. infestans* in potato as shown in Figure 35. Coquoz et al. (1995) demonstrated the ability of arachidonic acid, a major fatty acid in

the mycelial cell membrane and spores of *P. infestans*, to induce SAR in potato against *P. infestans*. This demonstrated, the ability of potato plants to recognize PAMPs from its potential pathogen and mount a general defense response, such as SAR.

Although no increase in SA and JA was detectable in systemic leaves (Fig. 36, 38), SA and JA were important for Pep-13-induced SAR in potato plants. Both, *NahG* and *StOPR3*-RNAi potato plants that are unable to accumulate SA and JA after Pep-13 infiltration, respectively (Fig. 36,39), were unable to mount SAR against *P. infestans* (Fig. 37, 40). We do not know exactly in which step SA and JA are important for potato plants to develop SAR. It is possible that SAR can not be established in *NahG* and *StOPR3*-RNAi potato plants because of loss of defense responses in local leaves, such as HR, oxidative burst and defense gene expression. However, SAR analysis of grafted tobacco plants (wildtype and *NahG*) demonstrated the importance of basal SA level in systemic leaves for SAR of tobacco plants against tobacco mosaic virus (Gaffney et al., 1993). In wounded tomato plant, JA but not OPDA accumulation in local leaves was needed for systemic defense gene expression (Li et al., 2005). Moreover, it was shown that JA accumulation in systemic leaves was not important for systemic defense gene expression.

When *P. infestans* growth in W2A pre-treated *NahG* and *StOPR3*-RNAi potato plants is compared with that in wildtype plants, different levels of *P. infestans* growth were observed (Fig. 37, 40). This indicates a role of SA in basal resistance. More details on the role of SA for basal defense of potato plants against *P. infestans* were discussed previously.

HR is the hallmark of SAR development. In most of the cases, SAR is preceded by HR at the primary site of infection. This was also the case in Pep-13-induced SAR. Twenty four hours after Pep-13 infiltration, wildtype potato plants developed an HR that was expanding within three days. Three days post infiltration, the upper uninfiltrated leaf became more resistant to subsequent *P. infestans* infection. Interestingly, loss of SAR in *NahG* and *StOPR3*-RNAi potato plant was accompanied by loss and reduced HR, respectively. The importance of HR for SAR development was studied using *StAOS2* co-suppressed potato plants. Despite the reduced OPDA and JA levels, those plants were still able to form normal size HR (Fig. 41). However, SAR analysis showed that *StAOS2* co-suppressed potato plants were unable to mount a SAR against *P. infestans*. This experiment suggests that there is no correlation

between HR and SAR formation. The signal that was generated during the HR must be the critical point for SAR formation. Activation of defense in the absence of HR has been shown in the *dnd1 Arabidopsis* mutant (Yu et al., 1998). The level of SA and the expression of some PR genes were constitutively high. This could be correlated with resistance of the plants against a wide range of pathogens. However, in most of the cases, necrotic cell death is the hallmark of SAR formation either as part of HR or as a symptom of disease (Ryals et al., 1996). Increase of several PR genes such as *PR1a*, *PR2* and *PR5* can be used as marker for SA-induced SAR.

Characterization of SAR in potato in more detail using microarray analysis resulted in identification of several genes that are consistently upregulated in local and systemic leaves of Pep-13-treated plants. Several genes that have been reported as JA-responsive were upregulated in both, local and systemic leaves of Pep-13-treated plants. Genes encoding LOX H1, polyphenol oxidase, and proteinase inhibitor were among them. Separately, macroarray analysis showed the systemic induction of several JA-responsive genes that encode proteinase inhibitor 2, cystein protease inhibitor, cathepsin inhibitor and LOX2. Confirmation by northern blot analysis showed a significant increase of *StPin2* expression in systemic leaves (Fig. 43). Therefore, *StPin2* can be used as a marker gene to study Pep-13-induced SAR. Increases of *Pin1* and *Pin2* transcript levels in local tomato leaves after *Pst* infection has been shown (Pautot et al., 1991). Interestingly, systemic increase of *Pin1* was also observed after *Pst* infection for both, resistant and susceptible tomato plants (Pautot et al., 1991).

Whether Pin2 has a direct effect against *P. infestans* or its increased expression is only a secondary effect of SAR signaling still has to be investigated. So far, a correlation can be seen between systemic *StPin2* expression, OPDA levels and SAR against *P. infestans* in Pep-13-treated potato plants (Fig. 45). Similarly, an earlier study in our lab showed that there were local and systemic increases of OPDA levels in response to *Psm* infection (Landgraf et al., 2002). Further studies on the importance of OPDA will be necessary, since it might be the mobile signal that stimulates *StPin2* expression, while no increase in JA levels were detected systemically.

Our study on Pep-13-induced SAR in potato plants can be summarized in a model (Fig. 47). Pep-13 is able to induce SAR in potato plants. SA and JA are the important components for Pep-13-induced SAR. *StPin2* can be used as a marker gene of Pep-13-induced SAR. Infiltration of Pep-13 into the lower leaves will induce SA and JA

accumulation. SA and JA are important for HR formation. SA and JA might have a role to generate the mobile signal. Transport of a mobile signal to upper infiltrated leaves will induce *Pin2* expression and SAR formation. With the help of *StPin2* as a marker gene, further studies using grafting will provide more information on the mobile signal of SAR in potato plants.

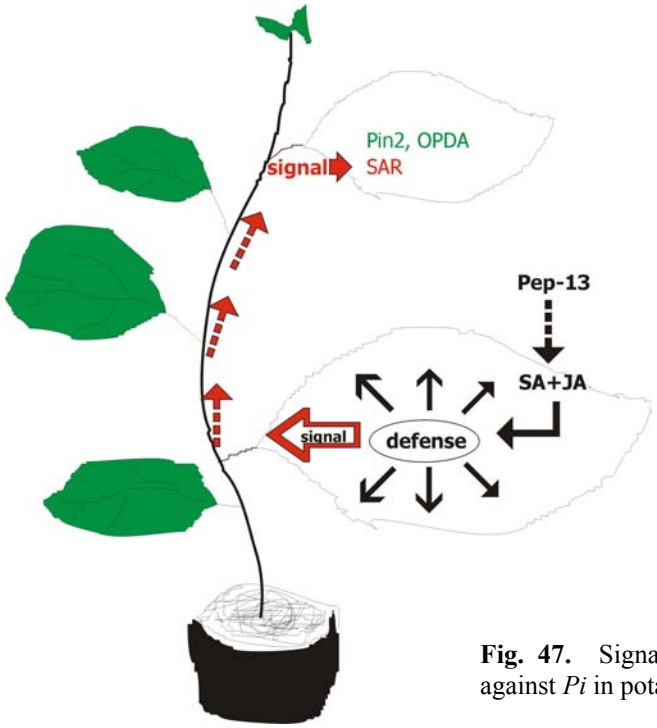


Fig. 47. Signaling model of Pep-13-induced SAR against *Pi* in potato plants.

V. Conclusion

Our study using *Solanum tuberosum* cv. *Désirée* as plant and *Phytophthora infestans* as pathogen shows the importance of both SA and JA signaling pathways for basal defense of potato plants against *P. infestans*. Both *NahG* and *StOPR3*-RNAi potato plants are significantly more susceptible to *P. infestans* than wildtype potato plants. Callose deposition, PR proteins, oxidative burst and cell death might collectively be important for basal defense of potato plants against *P. infestans*.

Using the peptide elicitor Pep-13, from a glycoprotein of *Phytophthora* cell walls, the importance of SA and JA for a wide range of defense responses can also be shown. Furthermore, JA action downstream of SA accumulation can also be shown in this study. Extended studies using *Psm* show JA accumulation in potato plants can happen independently from SA. This shows different interactions can switch on different defense signaling pathways. However, our studies and data did not show any proof on negative cross-talk between SA and JA signaling in potato plants as has been shown in *Arabidopsis*.

Finally, this study also shows the ability of Pep-13 to induce the development of SAR against *P. infestans* in potato. This SAR development depends on SA and JA. StPin2 has been identified as a marker gene for Pep-13-induced SAR. Further studies of Pep-13-induced SAR shall be facilitated using this marker.

As a whole, the importance of JA signaling in addition to SA signaling for various pathogen defense mechanisms in potato can be shown.

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