Salicylic Acid Is Important for Basal Defense of *Solanum tuberosum* Against *Phytophthora infestans*

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The importance of the signaling compound salicylic acid for basal defense of potato (*Solanum tuberosum* L. cv. Désirée) against *Phytophthora infestans*, the causal agent of late blight disease, was assessed using transgenic *NahG* potato plants which are unable to accumulate salicylic acid. Although the size of lesions caused by *P. infestans* was not significantly different in wild-type and transgenic *NahG* plants, real-time polymerase chain reaction analyses revealed a drastic enhancement of pathogen growth in potato plants depleted of salicylic acid. Increased susceptibility of *NahG* plants correlated with compromised callose formation and reduced early defense gene expression. *NahG* plants pretreated with the salicylic acid analog 2,6-dichloro-isonicotinic acid allowed pathogen growth to a similar extent as did wild-type plants, indicating that salicylic acid is an important compound required for basal defense of potato against *P. infestans*.

Salicylic acid (SA), jasmonic acid (JA), and ethylene activate and mediate defense responses against pathogens in a number of plant species (Shah 2003). In *Arabidopsis thaliana*, SA is important for both basal defense and resistance (*R*) gene-mediated resistance, as well as for either the establishment or maintenance, or both, of systemic acquired resistance (SAR) (Durrant and Dong 2004). SA accumulation induced in response to pathogen infection requires de novo biosynthesis via the isochlorismate pathway in *Arabidopsis* (Wildermuth et al. 2001), which is controlled by EDS1 and PAD4, proteins with homology to lipases (Wiermer et al. 2005; Zhou et al. 1998). Although the mechanisms of SA perception are still unclear, the components of the SA signal transduction chain consist of an oxidative burst (Overmyer et al. 2003), activation of MAP kinase cascades (Zhang and Klessig 1997), and the induction of defense gene expression (Ward et al. 1991). As a central regulator required for SA signaling, the ankyrin-repeat-containing protein NPR1 (nonexpressor of PR1) was identified in mutant screens of *Arabidopsis* (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). NPR1, present in the cytoplasm presumably as an oligomer, translocates to the nucleus in its monomeric form upon pathogen-induced alterations in the cellular redox state (Dong 2004; Fobert and Despres 2005). Activation of defense gene expression is mediated by interaction of

NPR1 with distinct TGA transcription factors in the nucleus (Despres et al. 2000; Niggeweg et al. 2000; Zhou et al. 2000). Moreover, the NPR1-interacting proteins NIMIN1 and NIMIN2 modulate SA-induced NPR1-mediated responses (Weigel et al. 2005).

Based on studies in *Arabidopsis*, it appears that, in a number of cases, the life style of a pathogen determines which signal transduction pathway becomes activated, with biotrophic pathogens inducing defense responses via the SA signaling pathway (McDowell and Dangl 2000). JA-dependent defense responses, on the other hand, are considered to be activated in *Arabidopsis* in response to infection with necrotrophic pathogens which require host cell death to obtain nutrients (Glazebrook 2005).

The oomycete *Phytophthora infestans* is a hemibiotrophic pathogen which causes late blight disease of potato. Susceptible potato plants are infected by penetration of the epidermal cells or by direct entry of the pathogen into the leaf tissue via the stomata. The biotrophic phase is characterized by formation of haustoria-like feeding structures inside penetrated cells, whereas intercellular growth of the pathogen as well as active killing of plant cells during the necrotrophic phase result in the rapid destruction of the tissue as well as the whole plant. Basal defense responses are activated in susceptible plants; however, they are not sufficient to arrest pathogen growth. Enhanced resistance can be induced in susceptible plants by pre-inoculation with the heterologous pathogen *Pseudomonas syringae* (Kombrink et al. 1996, 1997) or by treatment with elicitors such as arachidonic acid (Coquoz et al. 1995).

Compared with tobacco and *Arabidopsis*, SA is present at elevated levels in uninfected potato plants (Yu et al. 1997). Interestingly, its role in pathogen defense in potato appears to be different from that in *Arabidopsis* and tobacco. First, application of exogenous SA does not lead to SAR (Coquoz et al. 1995). Second, induction of SAR by arachidonic acid (Coquoz et al. 1995) or infiltration of *Pseudomonas* spp. (P. Landgraf, D. Scheel, and S. Rosahl, *unpublished*) does not lead to systemic accumulation of SA. Third, disease lesion size is unaltered in transgenic potato plants unable to accumulate SA due to the expression of the *NahG* gene encoding a salicylate hydroxylase, suggesting that the signaling compound SA does not play a role in defense against *Phytophthora infestans* (Yu et al. 1997).

In order to elucidate the role of SA for defense responses in potato, we analyzed transgenic potato plants expressing the *NahG* gene for alterations in their response to infection with *P. infestans*. In susceptible potato plants with reduced levels of SA, *P. infestans* can grow to higher extents than in control plants, suggesting that SA is important for basal defense in potato.

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RESULTS

Impaired SA accumulation in *NahG* potato plants in response to infection by *P. infestans*.

We previously have reported the generation of potato (*Solanum tuberosum* cv. Désirée) plants expressing the *NahG* gene encoding a salicylate hydroxylase (Halim et al. 2004). To analyze whether SA accumulation is efficiently reduced after pathogen infection, the levels of SA and its glucoside, salicylic acid glucoside (SAG), were determined in the wild type and the two *NahG* lines (*NahG A* and *NahG D*) after infection with *P. infestans* (Fig. 1). SA and SAG were present in untreated wild-type plants at approximately 0.1 and 1 µg/g fresh weight, respectively (data not shown). Upon infection with *P. infestans*, both compounds increased in wild-type plants approximately twofold, whereas plants expressing the salicylate hydroxylase gene were unable to accumulate SA (Fig. 1A) or SAG (Fig. 1B) to detectable levels.

Increased pathogen biomass in *NahG* potato plants.

Upon infection of susceptible potato cultivars with *P. infestans*, the oomycete generally enters the leaf tissue via stomata or by direct penetration of epidermal cells. Intercellular growth and the formation of feeding structures are followed by host defense responses such as cell death, formation of callose, and accumulation of autofluorescent material (Vleeshouwers et al. 2000). Lesion development usually is observed 2 to 3 days after infection. Interestingly, it was reported that SA is not important in potato for defense against *P. infestans*, based on measurements of lesion size which indicated that disease severity was not increased in transgenic potato *NahG* plants (Yu et al. 1997). Indeed, our determinations of the size of *P. infestans*-induced lesions in Désirée and Désirée *NahG* plants did not reveal significant differences 3 days after infection (Fig. 2A and B). However, microscopic examinations of infected leaves after staining with trypan blue revealed increased

mycelial growth in *NahG* compared with wild-type plants (Fig. 3A). In particular, in wild-type plants, infection sites were characterized by a large number of dead cells, with few incidences of emanating hyphal-like structures. In contrast, a higher number of sites with escaping hyphal-like structures were observed in *NahG* plants, suggesting that plants unable to accumulate SA cannot contain pathogen growth to the same extent as wild-type plants. Consequently, *P. infestans* biomass determinations were carried out. Using primers derived from highly repetitive sequences from the *P. infestans* genome (Judelson and Tooley 2000), real-time polymerase chain reaction (PCR) with TaqMan probes was performed and revealed significantly higher pathogen biomass in infected leaves of *NahG* plants compared to wild type plants (Fig. 3B). Thus, despite the lack of differences in disease symptoms, growth of *P. infestans* on *NahG* potato plants was enhanced, leading to a 10-fold difference in pathogen biomass 3 days after infection.

Increased pathogen growth on *NahG* plants correlates with reduced callose formation.

To study differences in oomycete growth during the early stages of infection of wild-type and *NahG* plants, microscopic analyses were carried out. In response to pathogen infection, plant cells deposit callose at the penetration sites. Staining of callose with aniline blue revealed striking differences between wild-type plants and those unable to accumulate SA (Fig. 4). Thus, whereas whole cells were stained for aniline blue in the wild type (Fig. 4A), only minor callose depositions could be found in *NahG* plants (Fig. 4B). These observations indicate that callose deposition is dependent on SA accumulation and that the lack of callose deposition is correlated with increased growth of the oomycete in potato plants.

Moreover, electron microscopic studies demonstrated massive depositions of callose around the sites of oomycete growth in wild-type plants (Fig. 4C) whereas, in *NahG* plants, *P. infestans* structures were observed close to parenchyma cells that did not react with callose deposition at all, or that reacted to a lesser extent compared with wild-type cells (Fig. 4D).

Increased pathogen growth on *NahG* plants correlates with reduced early defense gene expression.

The electron microscopic analyses suggested that *P. infestans* could spread further in *NahG* compared with wild-type plants, possibly due to a reduced response of attacked cells. To substantiate this observation, *PR* gene expression was analyzed at different time points after infection by Northern analyses (Fig. 5). Indeed, whereas wild-type and empty-vector-transformed plants showed induction of *PR1* gene expression 1 day after infection, *NahG* plants did so only after 2 days. Differences in *PR1* transcript levels were not visible 3 days after infection. Thus, the reduction of SA levels in *NahG* potato plants correlated with decreased early defense responses and higher pathogen growth.

Pretreatment of *NahG* plants with 2,6-dichloroisonicotinic acid restores wild-type growth of *P. infestans*.

2,6-Dichloroisonicotinic acid (INA) induces a spectrum of defense responses similar to that of SA and is considered to be an analog of SA. To address the question of whether enhanced susceptibility of *NahG* potato plants against *P. infestans* is based on the inability to accumulate SA, a solution of 0.3 mM INA was sprayed onto leaves of *NahG* and control plants 24 h before infection with *P. infestans*. Pathogen biomass at 3 days after infection was determined by real-time PCR (Fig. 6). Pretreatment of *NahG* potato plants with INA resulted in pathogen growth similar to that observed on wild-type plants. Thus,

Fig. 1. Accumulation of **A**, salicylic acid (SA) and **B**, salicylic acid glucoside (SAG) in potato leaves after infection with *Phytophthora infestans*. SA and SAG levels were determined in leaves of wild-type (wt) and *NahG* potato plants (lines *A* and *D2*) after infection with *P. infestans* at the time points indicated (dpi = days after infection). Error bars represent standard deviation.

Fig. 2. A, Phenotype and **B**, diameter of disease lesions 3 days after infection of wild-type (wt) and transgenic *NahG* plants (lines A and D2) with *Phytophthora infestans*. Error bars represent standard deviation. Same letters indicate non-significant difference using one-way analysis of variance with Tukey's post hoc test ($P = 0.01$, $n = 10$, two independent experiments).

Fig. 3. A, Microscopic analysis of disease lesions and oomycete structures, visualized by trypan blue staining in wt and *NahG* potato plants 3 days after infection with *P. infestans* (bars represent 500 μm). **B**, Determination of pathogen biomass by real-time polymerase chain reaction (PCR) after infection of wt and *NahG* plants at the time points indicated (dpi = days post inoculation). Real-time PCR was performed with *P. infestans*-specific primers using DNA isolated from leaf disks of infected plants. Error bars represent standard deviation. Different letters indicate significant difference using one-way analysis of variance with Tukey's Post Hoc Test ($P = 0.01$, $n = 12$, two independent experiments).

these results suggest that reduced basal defense against *P. infestans* in *NahG* plants is due to the lack of SA because it can be overcome by supplying the SA analog INA.

DISCUSSION

NahG plants are unable to accumulate SA due to the expression of a salicylate hydroxylase which catalyzes the conversion of SA to catechol. They have been widely used to demonstrate a role for SA in mediating defense responses in *Arabidopsis* and tobacco (AbuQamar et al. 2006; Chen et al. 2004; Delaney et al. 1994). In contrast, in potato, expression of the *NahG* gene was reported to have no effect on infection by *P. infestans* (Yu et al. 1997). These results were based on measuring lesion size which, indeed, is very similar in wild-type and *NahG* plants infected with *P. infestans* (Fig. 2). However, using real-time PCR to determine pathogen biomass, we observed a significantly higher growth of *P. infestans* on *NahG* plants (Fig. 3). These results suggest that the inability to accumulate SA

might be responsible for enhanced susceptibility. However, phenotypes of *NahG* plants might result, on the one hand, from the inability to accumulate SA or, on the other hand, from the accumulation of catechol or a product thereof (Heck et al. 2003; van Wees and Glazebrook 2003). To address the question of whether catechol could enhance susceptibility to *P. infestans*, wild-type plants were sprayed with 1 mM catechol solution 24 h prior to infection with *P. infestans*. Real-time PCR assays did not show significant differences in pathogen biomass between plants sprayed with water or with catechol (data not shown), suggesting that catechol itself does not induce susceptibility to *P. infestans*. Moreover, it was possible to restore the low level of pathogen growth observed on wild-type plants when *NahG* plants were sprayed with INA. As an SA analog, INA is able to overcome the loss of SA accumulation and to activate SA-dependent responses (Uknes et al. 1992).

Activation of the SA and JA defense signaling pathways in *Arabidopsis* has been proposed to be dependent on the life style of the pathogen (McDowell and Dangl 2000). In response

Fig. 4. Microscopic analyses of **A** and **C**, wild-type (wt) and **B** and **D**, *NahG* plants after infection with *Phytophthora infestans*. **A** and **B**, Infected leaves were stained with aniline blue for callose depositions (fluorescing areas). Bar represents 100 μm. **C** and **D**, Electron microscopy of mesophyll cells 3 days after infection with *P. infestans*. Oomycete structures are characterized by unstained cell walls (white in appearance) and their lack of plastids. They are visible both within and between cells due to intra- and intercellular growth at this time point of infection. Ca = callose, ic = intercellular space, Pi = *P. infestans*, and v = vacuole. Bar represents 2 μm.

to infection with biotrophic pathogens, which depend on obtaining nutrients from living host cells, plants activate the SA pathway, which leads to expression of *PR* genes and hypersensitive cell death. On the other hand, JA-dependent defense responses are necessary for successful defense against necrotrophic pathogens such as *Alternaria brassicicola* or *Botrytis cinerea* (Thomma et al. 1998). However, exceptions to this model include, for example, the enhanced susceptibility toward *Botrytis* spp. of *NahG* plants (Ferrari et al. 2003) and the enhanced resistance toward biotrophs in *Arabidopsis* mutants with constitutive activation of the JA signaling pathway (Ellis et al. 2002). Moreover, because many pathogens which are considered to be necrotrophic undergo a biotrophic phase during the initial invasion of the plant, a clear distinction between biotrophic and necrotrophic life style is not always possible (Thaler et al. 2004).

Infection of potato plants with the hemibiotrophic oomycete *P. infestans* generally starts via germination of encysted zoospores on the leaf surface and appressorium formation. The initial phases of infection, comprising penetration of epidermal cells and formation of infection structures, resemble those of biotrophic pathogens. The plant reaction to infection by biotrophic pathogens includes cell wall appositions, callose formation, and activation of defense genes (Latijnhouwers et al. 2003). Our data suggest that, in potato, SA is required for maximal defense responses of potato during this biotrophic phase of *P. infestans* infection, because the inability to accumulate SA results in a loss of callose deposition and defense gene expression at early time points (Figs. 4 and 5). Moreover, hyphal structures emerge from the site of primary infection in *NahG* plants, whereas wild-type plants still are able to contain pathogen growth at this point to a higher extent (Fig. 3A). Thus, it appears that SA is important in potato for defense against *P. infestans* during the early phase of infection.

Callose deposition, which generally is considered to be an effective barrier against fungal ingress, is an SA-dependent process (Donofrio and Delaney 2001). In accordance with this,

reduced callose formation is observed in *NahG* potato plants after infection with *P. infestans*. However, the recent isolation of mutant *Arabidopsis* lines defective in callose synthase genes or the generation of transgenic plants compromised in callose synthase gene expression revealed that loss of callose deposition actually enhances resistance against *Erysiphe cichoracearum* and *Golovinomyces orontii* (Jacobs et al. 2003; Nishimura et al. 2003). Possibly, callose formation by the plant enables virulent pathogens to avoid recognition. Interestingly, SA responses are hyperinduced in the *pmr4* mutant, which is defective in the gene encoding the pathogen-induced callose synthase (Nishimura et al. 2003). Pathogen susceptibility can be restored in double mutants impaired in SA accumulation or signaling, indicating that callose might function as a suppressor of SA-dependent defense responses (Nishimura et al. 2003). Thus, there is a discrepancy between the absence of callose in *NahG* plants (Donofrio and Delaney 2001) (Fig. 4) and enhanced SA responses in callose-deficient plants, suggesting the existence of a complex network of SA-dependent defense signaling, possibly including negative feedback loops (Nishimura et al. 2003).

The necrotrophic phase of infection by *P. infestans* is characterized by disease lesions. The fact that lesion size is not altered in *NahG* compared with wild-type plants (Fig. 2) suggests that the same number of cells undergo cell death. At this time point, however, more hyphae have escaped from the primary infection site. Interestingly, *PR1* gene expression is not different at later stages of infection (Fig. 5). These observations suggest that differences in the strength of defense responses are eliminated at later time points due to the infection of more cells in *NahG* plants, where the oomycete can spread further. Thus, our data suggest that lack of SA increases susceptibility in compatible interactions of potato with *P. infestans* and that SA is required in susceptible potato cultivars for defense responses such as *PR* gene expression and callose deposition at early time points after infection.

MATERIALS AND METHODS

Treatment of plants and analysis of defense responses.

Sterile potato plants (*S. tuberosum* L. cv. Désirée) were grown in tissue culture in a phytochamber with 16 h of light (140 μ E) at 22°C. Plants were transferred to soil and grown in a phytochamber with 16 h of light (140 μ E) at 20°C and 60% humidity for 4 weeks. For *P. infestans* infections, lower leaves

Fig. 5. *PR1A* expression in wild-type (wt) and *NahG* plants in response to infection with *Phytophthora infestans*. RNA was isolated from leaves of wt and transgenic plants carrying an empty vector construct (ev) or the *NahG* gene (*NahGA* and *NahGD2*) and subjected to Northern analyses. Hybridization was carried out with a radioactively labeled cDNA fragment of *StPR1A*. As a loading control, the agarose gel was stained with ethidium bromide to visualize rRNA.

Fig. 6. Pathogen biomass in *NahG* plants sprayed with INA. Wild-type (wt) and *NahG* plants were treated with 0.3 mM 2,6-dichloroisonicotinic acid (+INA) or mock treated (-INA) 24 h prior to infection with *Phytophthora infestans*. Pathogen biomass was determined 3 days after infection. Pooled data obtained from two lines of *NahG* plants are shown. Error bars represent standard deviation. The experiment was repeated twice with similar results.

were drop inoculated with a zoospore suspension in water (1×10^5 zoospores/ml) on the abaxial leaf surface and kept at 100% humidity for the duration of the experiment. As controls, water was pipetted onto the leaves. RNA was isolated from leaves using trizol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Northern analyses were carried out as described (Geerts et al. 1994).

To assess the influence of INA and catechol on pathogen growth, 0.3 or 1 mM solutions, respectively, were sprayed onto the adaxial side of detached potato leaves 24 h before inoculation with *P. infestans*.

Determination of *P. infestans* biomass.

Quantitative real-time PCR was applied to determine the growth of *P. infestans* on wild-type and transgenic potato plants (Eschen-Lippold et al. 2007). Leaf disks, 6 mm in diameter, were cut from the inoculation sites. DNA was isolated from six leaf disks using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), after addition of external standard DNA. The primers PIO8-3-3F (5'-CAATTCGCCACCTTCTTCGA-3') and PIO8-3-3R (5'-GCCTTCCTGCCCTCAAGAAC-3') (MWG Biotech, Ebersberg, Germany) as well as the Taqman-MGB-NFQ probe PIO8-3-3M1: 6-FAM-5'-CGTACGGCCAATGTA-3'-MGB-NFQ (Applied Biosystems, Darmstadt, Germany) were used to amplify and detect a *P. infestans*-specific repetitive DNA sequence (Judelson and Tooley 2000) with the ABI Prism Sequence Detection System (Applied Biosystems). For standard curves, a larger DNA fragment was amplified from *P. infestans* genomic DNA with the primers O8-3 (5'-TAACCGACCAAGTAGTAAA-3') and O8-4 (5'-GAAAGGCATAGAAGGTAGA-3') (Judelson and Tooley 2000) and cloned into the vector pCR2.1 (Invitrogen). To detect the external standard plasmid DNA, appropriate primers and a second Taqman-MGB-NFQ probe were designed. PCR was carried out using the Taqman Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) with 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C each. Relative amounts of *P. infestans* DNA in the samples was calculated using the ABI Prism 7000 Sequence Detection-Software (version 1.1) by including the external standard and the data from the standard curve. Therefore, pathogen biomass is given as "relative units."

Determination of SA.

Determination of SA was performed as described (Halim et al. 2004).

Microscopy.

Trypan blue staining was carried out as described (Lipka et al. 2005). Callose staining was performed with aniline blue according to Adam and Somerville (1996).

Electron microscopy.

Necrotic regions of the potato leaves were prefixed with 3% (vol/vol) glutaraldehyde/phosphate buffer, pH 7.4, for 2 h, fixed with 1% (wt/vol) OsO₄/Palade buffer for 1 h, and dehydrated in series of acetone. After being embedded in an epoxide resin, ultrathin sections (50 to 70 nm) were stained with lead and viewed using a transmission electron microscope (EM 912 OMEGA LEO Elektronenmikroskopie, Oberkochen, Germany).

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