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Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge

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Abstract

Selected strains of nonpathogenic rhizobacteria from the genus *Pseudomonas* are capable of eliciting broad-spectrum induced systemic resistance (ISR) in plants that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). In *Arabidopsis*, the ISR pathway functions independently of salicylic acid (SA) but requires responsiveness to jasmonate and ethylene. Here, we demonstrate that known defense-related genes, i.e. the SA-responsive genes *PR-1*, *PR-2*, and *PR-5*, the ethylene-inducible gene *Hel*, the ethylene- and jasmonate-responsive genes *ChiB* and *Pdf1.2*, and the jasmonate-inducible genes *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2*, are neither induced locally in the roots nor systemically in the leaves upon induction of ISR by *Pseudomonas fluorescens* WCS417r. In contrast, plants infected with the virulent leaf pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) or expressing SAR induced by preinfecting lower leaves with the avirulent pathogen *Pst(avrRpt2)* exhibit elevated expression levels of most of the defense-related genes studied. Upon challenge inoculation with *Pst*, *PR* gene transcripts accumulated to a higher level in SAR-expressing plants than in control-treated and ISR-expressing plants, indicating that SAR involves potentiation of SA-responsive *PR* gene expression. In contrast, pathogen challenge of ISR-expressing plants led to an enhanced level of *Atvsp* transcript accumulation. The other jasmonate-responsive defense-related genes studied were not potentiated during ISR, indicating that ISR is associated with the potentiation of specific jasmonate-responsive genes.

Introduction

To combat invasions by micro-organisms, plants have evolved several lines of defense. Besides preexisting physical and chemical barriers (Osbourn, 1996), inducible resistance mechanisms can be activated upon pathogen infection. An example of activated resistance is the hypersensitive response (HR) that is induced when the invading pathogen carries an avirulence (*avr*) gene of which the product is directly or indirectly recognized by the product of the corresponding resistance (*R*) gene of the host plant. In cells adjacent to the HR lesion, transcription of defense-related genes is triggered, resulting in the accumulation of compounds that are involved in cell wall reinforcement or possess antimicrobial activity (reviewed by Hammond-Kosack and Jones, 1996). When the pathogen is virulent, it is not specifically recognized by the plant. Nevertheless, nonspecific elicitors activate plant defense mechanisms, though possibly too late or too weakly to definitively restrict the pathogen, which escapes and successfully infects the plant (van Loon, 1997).

Particularly avirulent pathogens provoke accumulation of the signalling molecules salicylic acid (SA) (Malamy *et al.*, 1990; Métraux *et al.*, 1990), jasmonate (Penninckx *et al.*, 1996), and ethylene (Boller, 1991),

which coordinate the activation of defense responses, and when applied exogenously can induce resistance themselves (Boller, 1991; Cohen *et al.*, 1993; Ryals *et al.*, 1996). In *Arabidopsis*, all three signalling molecules have been shown to activate specific sets of pathogen-inducible defense-related genes. SA controls the induction of genes encoding pathogenesis-related proteins (PRs) of the families PR-2 (β-1,3-glucanases), PR-5 (thaumatin-like proteins), and PR-1 with unknown biochemical properties (Uknes *et al.*, 1992). Several PRs have been shown to possess antimicrobial activity *in vitro* or *in vivo*, primarily against fungal pathogens (Kombrink and Somssich, 1997). Ethylene is involved in the expression of the pathogen-inducible genes *Hel* (encoding a hevein-like protein) (Potter *et al.*, 1993), *ChiB* (encoding a basic chitinase) (Samac *et al.*, 1990), and *Pdf1.2* (encoding a plant defensin) (Penninckx *et al.*, 1996), all with potential antifungal activity. Jasmonate has been shown to activate the *Hel*, *ChiB*, and *Pdf1.2* genes as well (Penninckx *et al.*, 1996; Thomma *et al.*, 1998). For full expression of *Pdf1.2*, both ethylene and jasmonate are required, indicating that these hormonal signals act in concert (Penninckx *et al.*, 1998). Jasmonate is also implicated in the regulation of other pathogen-induced genes. *Lox1* and *Lox2* (encoding two lipoxygenases) (Bell and Mullet, 1993; Melan *et al.*, 1993) control a feed-forward loop in jasmonate synthesis, but may also cause irreversible membrane damage leading to plant cell death (reviewed by Siedow, 1991). *Pal1* (encoding phenylalanine ammonia-lyase) controls the synthesis of phenylpropanoids such as lignin, and of SA in *Arabidopsis* (Mauch-Mani and Slusarenko, 1996), and has been demonstrated to be jasmonate-inducible (McConn *et al.*, 1997). Furthermore, jasmonate has emerged as an important signal in the expression of wound-induced genes, such as *Pin* (encoding proteinase inhibitor) in tomato (Farmer and Ryan, 1992). Proteinase inhibitors help protect the plant against herbivory (Heitz *et al.*, in press) and have been reported to be induced upon pathogen infection in tobacco plants (Linthorst *et al.*, 1993). Jasmonate also activates expression of the *Atvsp* gene (encoding vegetative storage protein) in *Arabidopsis* (Berger *et al.*, 1995). VSPs accumulate in the vacuoles of developing reproductive structures and young leaves, and function as temporary deposits of amino acids (reviewed by Creelman and Mullet, 1997). Upon wounding, VSPs are induced also in older plant parts (Berger *et al.*, 1995). Defense activity of VSPs has not been established but has been suggested (Creelman and Mullet, 1997).

Resistance responses activated in primary pathogen-infected leaves are often extended to distant, non-infected tissue, conferring an elevated level of protection. This phenomenon is referred to as systemic acquired resistance (SAR) (reviewed by Ryals *et al.*, 1996). SAR is effective against a broad spectrum of pathogens, and is tightly correlated with the induction of *PR* genes. NahG transformants of *Arabidopsis* and tobacco that do not accumulate SA fail to express SAR and *PR* genes, indicating that SA is required for the SAR signalling pathway (Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Lawton *et al.*, 1995). Independent mutant screens of *Arabidopsis* led to the discovery of an important SAR regulatory protein, called NPR1, NIM1, or SAI1 (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). This factor acts downstream of SA and is required for both the expression of pathogen-induced *PR* genes and SAR. Ethylene and jasmonate response mutants of *Arabidopsis* express normal levels of pathogen-induced SAR, but the systemic activation of the ethylene- and jasmonate-regulated defense-related genes *Hel*, *ChiB*, and *Pdf1.2* is blocked in these mutants. This demonstrates that SAR can function independently of ethylene- and jasmonate-mediated defense responses (Lawton *et al.*, 1995, 1996; Penninckx *et al.*, 1996; Pieterse *et al.*, 1998; Thomma *et al.*, 1998). Moreover, the pathway controlling ethylene- and jasmonate-dependent expression of the *Hel*, *ChiB*, and *Pdf1.2* genes functions independently of the SAR regulators SA and NPR1 (Penninckx *et al.*, 1996; Thomma *et al.*, 1998).

Besides pathogen infection, colonization of the rhizosphere by selected non-pathogenic *Pseudomonas* bacteria can trigger a systemic resistance as well. This form of induced resistance is referred to as rhizobacteria-mediated induced systemic resistance (ISR) (reviewed by van Loon *et al.*, 1998). In *Arabidopsis*, *P. fluorescens* WCS417r-mediated ISR has been shown to be effective against different types of pathogens, including the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*), the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani* (Pieterse *et al.*, 1996; van Wees *et al.*, 1997), and the oomycetous leaf pathogen *Peronospora parasitica* (J. Ton and C.M.J. Pieterse, unpublished result). ISR signal transduction follows an SA-independent pathway that confers resistance in the absence of *PR* gene expression (Pieterse *et al.*, 1996; van Wees *et al.*, 1997). Instead, WCS417r-mediated ISR requires responsive-

ness to both jasmonate and ethylene (Pieterse *et al.*, 1998). However, like SAR, rhizobacteria-mediated ISR is dependent on NPR1, indicating that NPR1 is not only required for the expression of *PR* genes that are activated during SAR, but also functions in the jasmonate- and ethylene-dependent activation of thus far unidentified defense responses implicated in rhizobacteria-mediated ISR (Pieterse *et al.*, 1998).

In addition to defense reactions already apparent upon induction of resistance, other responses are manifested only after challenge inoculation. 'Potentiation' is expressed as a faster and greater activation of defense-related genes after infection of induced plants with a challenging pathogen. For instance, tobacco plants exhibiting pathogen-induced SAR show enhanced expression of *PR-10* and *Pal* genes upon challenge with a pathogen (Mur *et al.*, 1996). SA, ethylene, and jasmonate have been shown to act as potentiating signals of defense-related gene expression. SA was reported to be a potentiator of pathogen-induced defense responses such as *Pal* gene expression and phytoalexin accumulation that do not respond directly to SA (Kauss *et al.*, 1992, 1993; Mur *et al.*, 1996). In contrast to resistance-inducing derivatives of SA, biologically inactive anologues of SA failed to potentiate elicitor-induced *Pal* gene expression in cultured parsley cells, indicating that the resistance inducers may act in part by augmenting the activation of certain defense-related genes (Katz *et al.*, 1998). Jasmonate has been shown to potentiate the elicitor-induced accumulation of active oxygen species in parsley cells (Kauss *et al.*, 1994). In rice, jasmonate has been demonstrated to potentiate the induction of *PR-1* gene expression and resistance against the fungal pathogen *Magnaporthe grisea* by low doses of the resistance-inducing synthetic compound 2,6-dichloroisonicotinic acid (INA) (Schweizer *et al.*, 1997). Ethylene acts as a potentiator of SA- and pathogen-induced *PR-1* gene expression in *Arabidopsis* (Lawton *et al.*, 1994, 1995). Moreover, in regard to the expression of the *Pin* gene in tomato and the *PR-5* gene in tobacco, ethylene has been reported to have a potentiating effect on the action of jasmonate, and vice versa (Xu *et al.*, 1994; O'Donnell *et al.*, 1996). In *Arabidopsis*, a marked synergy between ethylene and jasmonate was demonstrated for the induction of *Pdf1.2* (Penninckx *et al.*, 1998).

WCS417r-mediated ISR in carnation was reported to be associated with potentiation of phytoalexin accumulation, resulting in higher phytoalexin levels after infection by the fungal pathogen *F. oxysporum* f. sp. *dianthi* (van Peer *et al.*, 1991). The molecular mechanisms underlying rhizobacteria-mediated ISR in *Arabidopsis* are to a large extent unknown. In this study, we investigated whether the ISR-inducing rhizobacterium WCS417r activates any of a set of known defense-related genes locally in the roots and/or systemically in the leaves at different time points after treatment. Moreover, the possibility of potentiation of defense-related gene activation in ISR-expressing tissue challenged with *Pst* was assessed.

Materials and methods

Bacterial cultures

Non-pathogenic, ISR-inducing *Pseudomonas fluorescens* WCS417r rhizobacteria (van Peer *et al.*, 1991) were grown on King's medium B (KB) agar plates (King *et al.*, 1954) for 24 h at 28 °C. Subsequently, bacterial cells were collected and resuspended in 10 mM $MgSO_4$.

The virulent pathogen *P. syringae* pv. *tomato* strain DC3000 (*Pst*) (Whalen *et al.*, 1991), used for challenge inoculations, was grown overnight in liquid KB at 28 °C. After centrifugation, the bacterial cells were resuspended in 10 mM $MgSO_4$ and supplemented with 0.01% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals, Weesp, Netherlands).

The avirulent pathogen *P. syringae* pv. *tomato* strain DC3000 with the plasmid pV288 carrying avirulence gene *avrRpt2* (*Pst(avrRpt2)*) (Kunkel *et al.*, 1993) was used for induction of SAR. *Pst(avrRpt2)* bacteria were cultured overnight at 28 °C in liquid KB, supplemented with 25 mg/l kanamycin to select for the plasmid. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$.

Cultivation of plants

Seeds of wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) were sown in quartz sand. Two-week old seedlings were transferred to 60 ml pots containing a sand and potting soil mixture that had been autoclaved twice for 1 h with a 24 h interval. Before transfer of the seedlings, the potting soil was supplemented with either a suspension of ISR-inducing WCS417r rhizobacteria or an equal volume of a solution of 10 mM $MgSO_4$. For the analysis of defense-related gene expression during the first 7 days after induction of ISR, the seedlings were

transferred to rock wool drenched with nutrient solution, and WCS417r bacteria were applied in talcum to the roots, as described previously (Pieterse *et al.*, 1996; van Wees *et al.*, 1997). This rock wool system was previously proven to be suitable for the analysis of rhizobacteria-mediated ISR against the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani* (Pieterse *et al.*, 1996; van Wees *et al.*, 1997). Plants were cultivated in a growth chamber with a 9 h day (200 μE/m^2·s at 24 °C) and a 15 h night (20 °C) cycle at 70% relative humidity. Plants were watered on alternate days and once a week supplied with a modified half-strength Hoagland's nutrient solution, as described previously (Pieterse *et al.*, 1996).

Induction treatments and inoculations

Plants were treated with ISR-inducing rhizobacteria by transferring 2-week old seedlings to soil that was mixed with a suspension of WCS417r rhizobacteria to a final density of 5×10^7 colony-forming units (cfu) per gram of soil. For induction of ISR in the rock wool system, the seedlings were placed horizontally on rock wool cubes and the root systems were covered with 1 ml of a 1:1 (w/v) mixture of talcum powder and a suspension of WCS417r bacteria (final density 5×10^8 cfu per gram of talcum), as described previously (Pieterse *et al.*, 1996; van Wees *et al.*, 1997).

Pathogen-induced SAR was triggered by pressure-infiltrating a suspension of the avirulent pathogen *Pst(avrRpt2)* at 10^7 cfu/ml into three lower leaves of 5-week old plants, using a 1 ml syringe without a needle.

Chemical treatments were performed by dipping the leaves of 5-week old plants in a solution containing 0.01% (v/v) of Silwet L-77 and either salicylic acid (SA), 1-aminocyclopropane-1-carboxylate (ACC), methyl jasmonate (MeJA), or a combination of these chemicals. Control plants were treated with 0.01% (v/v) Silwet L-77 only. One day before application of the chemicals, the plants were placed at 100% relative humidity. SA was purchased from Mallinckrodt Baker, Deventer, Netherlands, ACC from Sigma-Aldrich Chemie, Zwijndrecht, Netherlands, and MeJA from Serva, Brunschwig Chemie, Amsterdam, Netherlands.

Plants were challenge-inoculated with the virulent pathogen *Pst* by dipping them in a suspension of *Pst* at 2.5×10^7 cfu/ml in 10 mM $MgSO_4$, 0.01% (v/v) Silwet L-77. One day before challenge, the plants were placed at 100% relative humidity.

RNA blot analysis

Leaf and root tissues were collected for RNA analysis. Soil or talcum covering the root system was removed by rinsing the roots with water. Total RNA was extracted by homogenizing at least 2 g of frozen tissue in an equal volume of extraction buffer (0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS). The homogenates were extracted with phenol and chloroform and the RNA was precipitated with LiCl, as described (Sambrook *et al.*, 1989). For RNA analysis, 15 μg of RNA was denatured using glyoxal and DMSO, according to Sambrook *et al.* (1989). Subsequently, samples were electrophoretically separated on 1.5% agarose gels and blotted onto Hybond-N$^+$ membranes (Amersham, 's-Hertogenbosch, Netherlands) by capillary transfer. The electrophoresis buffer and blotting buffer consisted of 10 mM and 25 mM sodium phosphate (pH 7), respectively. RNA blots were hybridized with *Arabidopsis* gene-specific probes as described previously (van Wees *et al.*, 1997). To check for equal loading, the blots were stripped and hybridized with a probe for the constitutively expressed β-tubulin (*Tub*) gene. ^{32}P-labelled *Arabidopsis* gene-specific probes to detect *PR-1*, *PR-2*, *PR-5*, *Pdf1.2*, or *Pal1* were synthesized by random primer labelling (Feinberg and Vogelstein, 1983) with *PR-1*, *PR-2*, *PR-5*, *Pdf1.2*, and *Pal1* cDNA clones as template (Uknes *et al.*, 1992; Wanner *et al.*, 1995; Penninckx *et al.*, 1996). Probes for the detection of *Arabidopsis Hel*, *Atvsp*, *Lox1*, *Lox2*, *Pin2*, and *Tub* transcripts were prepared using templates that were generated by PCR with primer sets based on sequences obtained from GenBank accession numbers U01880, Z18377, L04637, L23968, X69139, and M21415, respectively. Template for the *ChiB* probe was prepared by PCR with primers based on the genomic sequence published by Samac *et al.* (1990). The following primer sets were used: (*Hel*) 5′-ATCTGCTGCAGTCAGTACGG-3′/5′-TGAGCTCATTGCCACAGTCG-3′, (*Atvsp*) 5′-GTGTTGGTGTGACAAAATGG-3′/5′-GATGCGGTGAAGATATATGC-3′, (*Lox1*) 5′-AACTTAGGGATCTGCTTCCC-3′/5′-CTCTATACTTGAGCAGCGTC3′, (*Lox2*) 5′-TAGAGAGTCCTTGTCGAGTC-3′/5′-CATACACGTTGTGCGTAGTC-3′, (*Pin2*) 5′-TCTATGCGTTTGATCTCAGC-3′/5′-GCAATGTCTGGTGCAGAAGC-3′, (*Tub*) 5′-AATACGTCGGCGAT-

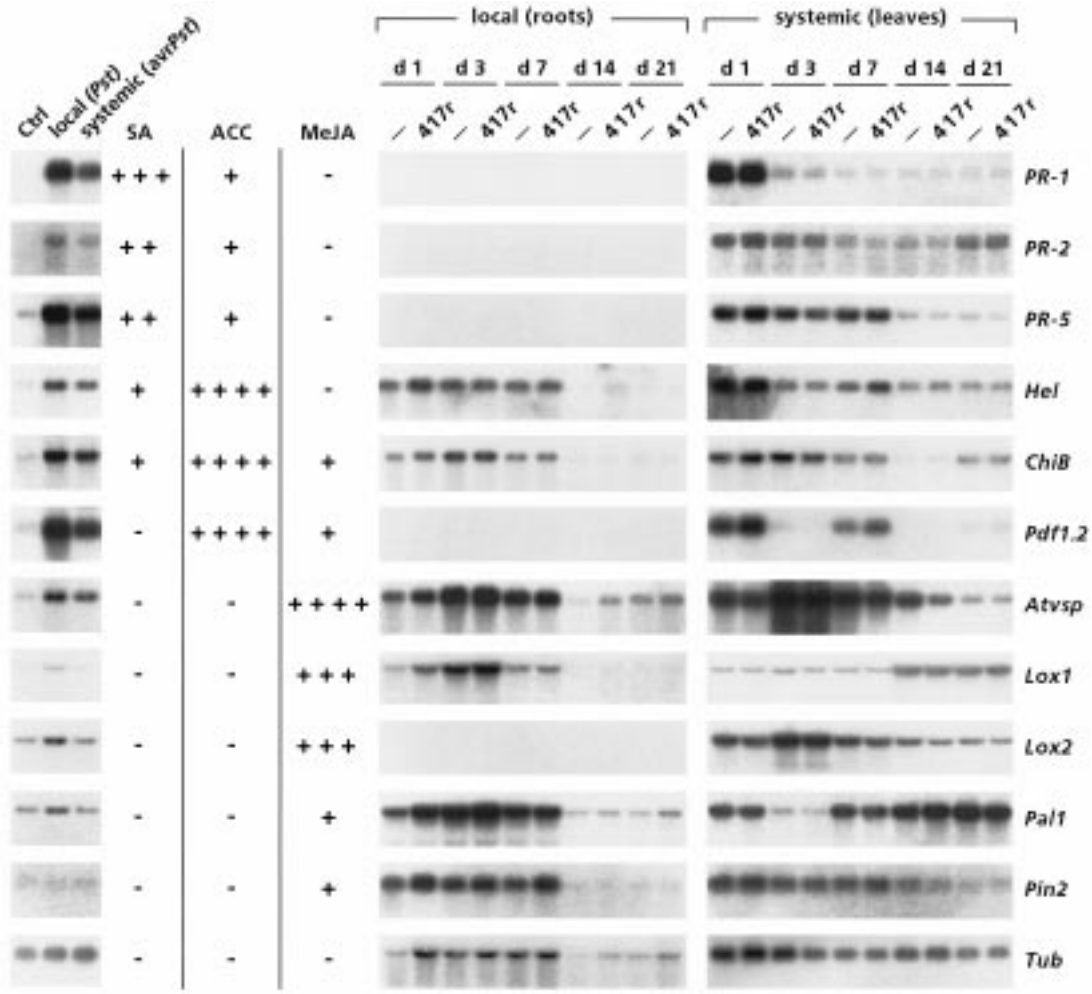

Figure 1. Local and systemic expression of defense-related genes in response to infection with *Pst*, or treatment with SA, ACC, MeJA, or WCS417r. RNA was isolated from leaves of 5-week old *Arabidopsis* plants harvested 2 days after either infection with *Pst* (local (*Pst*)), or application of 1 mM SA, 1 mM ACC, or 100 μM MeJA. SAR-expressing upper leaves were harvested 2 days after preinfection of three lower leaves with *Pst(avrRpt2)* (systemic (avr*Pst*)). Roots and leaves of WCS417r-treated plants (417r) were harvested at the indicated days after transferring 2-week-old seedlings to either rock wool (days 1, 3 and 7) or soil (days 14 and 21). Treatment with WCS417r bacteria consisted of application to the roots only. Control-treated plants are indicated above the lanes as Ctrl or $-$. *Arabidopsis* gene-specific probes were used for the RNA blot analyses as indicated. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β-tubulin (*Tub*). $-$, no induction; $+$ to $++++$, increasing degrees of induction, with $+++$ representing transcript levels comparable to that observed in *Pst*-infected leaves 2 days after inoculation.

TCTCCG-3′/5′-CACAGACACTGGACTTGACG-3′, (*ChiB*) 5′-GCTTCAGACTACTGTGAACC-3′/5′-TCCACCGTTAATGATGTTCG-3′.

Disease assessment

Four days after challenge inoculation with *Pst*, disease severity was assessed by determining the percentage of leaves with symptoms per plant, and by examining growth of the challenging pathogen in the leaves (20 plants per treatment). Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. The number of *Pst* bacteria in challenged leaves was assessed in three samples per treatment. Each sample consisted of the leaves from 6 plants. The leaf tissue was weighed and homogenized in 10 mM $MgSO_4$. Subsequently, appropriate dilutions were plated onto KB agar supplemented with 50 mg/l rifampicin and 100 mg/l cycloheximide. After incubation at 28 °C for 2 days, the number of

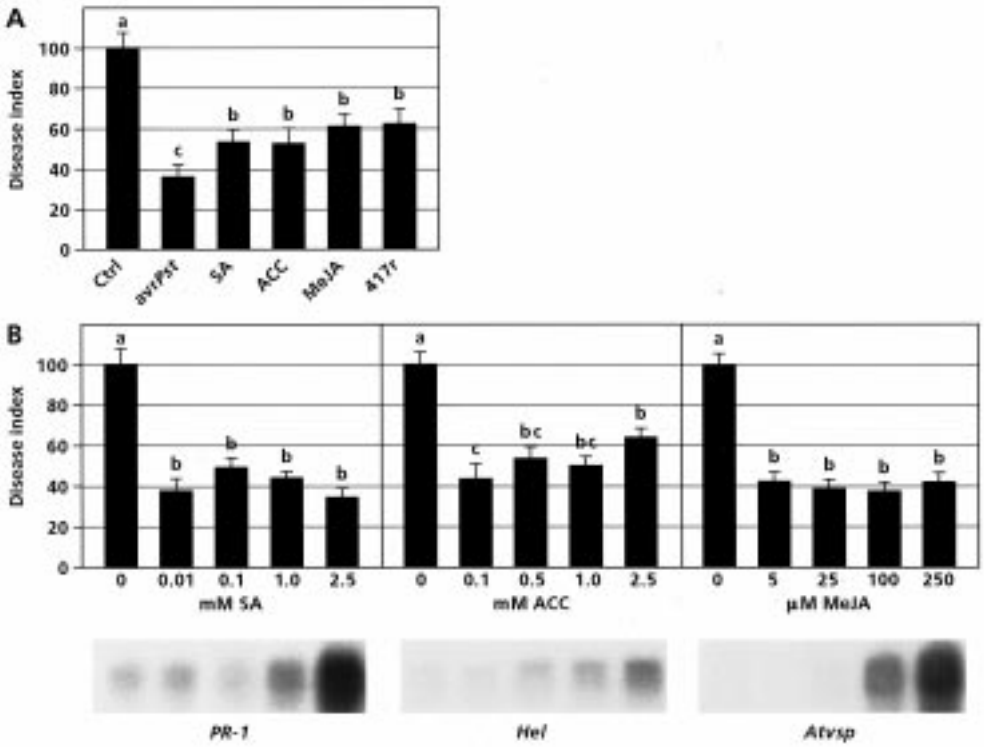

Figure 2. Quantification of induced protection against *Pst* triggered by different inducers and the effect of exogenous application of a concentration series of the chemicals SA, ACC, and MeJA on the level of protection against *Pst*, and the induction of *PR-1*, *Hel*, and *Atvsp* gene expression, respectively. *Pst(avrRpt2)*, SA, ACC, and MeJA were applied 3 days before challenge inoculation with *Pst*. ISR was induced by growing the plants in soil containing WCS417r for 3 weeks. In A, 1 mM SA, 1 mM ACC, and 100 μM MeJA were applied. In B, the chemicals were applied at the concentrations indicated. The disease index is the mean ($n = 20$ plants) of the percentage of leaves with symptoms per plant, compared to control-treated plants (set at 100%), 4 days after challenge with virulent *Pst*. The absolute proportion of diseased leaves of control-treated plants depicted in A was 72.1%, and in B was 51.6% (for the SA range) and 44.7% (for the ACC and MeJA ranges). Within each panel, different letters indicate statistically significant differences between treatments (Fischer's LSD test; $\alpha = 0.05$). The data presented are from representative experiments that were repeated at least twice. For experimental details on the RNA blot analyses, see legend to Figure 1.

rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined.

Results

Defense-related gene expression

To gain more insight into the defense mechanisms involved in rhizobacteria-mediated ISR, we studied the expression of a large set of well-characterized defense-related genes of *Arabidopsis*. Gene expression was investigated locally, as well as systemically, by analyzing mRNA accumulation in roots and leaves of ecotype Col-0 plants whose roots were treated with the ISR-inducing rhizobacterial strain WCS417r. ISR is triggered within the first 7 days after bacterization of the roots with WCS417r (J. Ton and C.M.J. Pieterse, unpublished results) and lasts until at least 21 days after bacterization (van Wees *et al.*, 1997). Therefore, leaves and roots were collected over an extended time period, between 1 and 21 days after transplanting 2-week old seedlings to rock wool or soil, with or without WCS417r bacteria. A parallel set of plants were challenged with *Pst* and shown to express ISR (data not shown). Gene expression studies were performed with *Arabidopsis* gene-specific probes for the defense-related genes *PR-1*, *PR-2*, *PR-5*, *Hel*, *ChiB*, *Pdf1.2*, *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2*. As a control, the expression of these genes was analyzed in *Pst*-infected leaves (local). Moreover, as a comparison, gene expression was assessed in uninfected leaves of plants that expressed SAR upon pretreatment of three lower leaves with *Pst(avrRpt2)*. Responsiveness of the genes to the defense signalling molecules SA, ethylene, and jasmonate was verified by analyzing their expression in leaves treated with SA, the ethylene precursor 1-

aminocyclopropane-1-carboxylate (ACC), or methyl jasmonate (MeJA).

Infection of the leaves with the virulent pathogen *Pst* resulted in local induction of nearly all of the genes, with *Pin2* as an exception (Figure 1). SAR was accompanied by a systemic accumulation of transcripts of most of the genes studied (from *PR-1* up to and including *Atvsp* in Figure 1), indicating that SAR is associated with activation of these genes. *PR-1*, *PR-2*, and *PR-5* genes were strongly induced by SA, and slightly activated by ACC. *Hel*, *ChiB*, and *Pdf1.2* were prominently induced by ACC, and the latter two were also responsive to MeJA. The genes *Atvsp*, *Lox1*, *Lox2*, *Pal1*, and *Pin2* were all induced by MeJA only, although the level of induction varied greatly. These results are in agreement with the findings of other research groups (Samac *et al.*, 1990; Farmer and Ryan, 1992; Uknes *et al.*, 1992; Bell and Mullet, 1993; Melan *et al.*, 1993; Potter *et al.*, 1993; Berger *et al.*, 1995; Penninckx *et al.*, 1996; McConn *et al.*, 1997), and indicate that these genes indeed serve as markers for the activation of either SA-, ethylene-, or jasmonate-responsive defense pathways. Gene expression analyses of plants expressing WCS417r-mediated ISR revealed that the state of ISR differs greatly from SAR in regard to the expression of defense-related genes. Although variation in the expression of most genes was apparent, roots and leaves of WCS417r-treated plants never showed an enhanced expression of any of the genes, at any of the time points tested (Figure 1). Thus, elicitation of the ISR pathway by WCS417r has no direct effect on the local or systemic expression of the defense-related genes studied.

Induced protection in the absence of activation of known defense-related genes

Pst is the causal agent of bacterial speck disease, which is characterized by the development of necrotic or water-soaked spots surrounded by spreading chlorosis. Preinfection with an avirulent pathogen, exogenous application of SA, ACC, or MeJA, or colonization of the rhizosphere with WCS417r results in induced protection against this pathogen, as manifested by a reduction in both the percentage of leaves showing disease symptoms and growth of *Pst* bacteria in the challenged leaves (Figure 2A and Table 1). Typically, induction of SAR by preinfection with an avirulent pathogen led to the largest suppression of disease, whereas the level of protection triggered by WCS417r was comparable to that induced by SA, ACC, or MeJA. As shown in Figure 1, all these resistance-inducing agents, except for WCS417r, triggered the expression of specific sets of the defense-related genes studied. We investigated whether there is a quantitative relationship between the expression of these defense-related genes and induced protection. Therefore, we determined the effect of a concentration range of SA, ACC, and MeJA on the induction of resistance against

Table 1. Number of *Pst* bacteria in challenged leaves of plants pretreated with *Pst(avrRpt2)*, WCS417r, or a concentration range of SA, ACC, and MeJA.

Treatment	cfu per gram fresh weight ($\times 10^6$)[1]
Ctrl	37.02 ± 5.25^{a}
Pst(avrRpt2)	2.38 ± 1.40^{c}
SA (1 mM)	6.68 ± 0.72^{b}
ACC (1 mM)	5.21 ± 1.07^{b}
MeJA (100 μM)	6.42 ± 1.84^{b}
WCS417r	7.05 ± 2.01^{b}
SA (mM)	
0	12.61 ± 1.43^{a}
0.01	4.09 ± 0.92^{b}
0.1	4.66 ± 1.66^{b}
1.0	5.45 ± 2.29^{b}
2.5	5.58 ± 1.23^{b}
ACC (mM)	
0	6.01 ± 0.35^{a}
0.25	1.56 ± 0.36^{c}
0.5	2.63 ± 0.42^{c}
1.0	2.71 ± 0.19^{c}
2.5	4.12 ± 0.49^{b}
MeJA (μM)	
0	6.01 ± 0.35^{a}
5	2.45 ± 0.30^{b}
25	2.43 ± 0.34^{b}
100	1.21 ± 0.12^{b}
250	2.59 ± 0.51^{b}

[1]Values presented are average numbers of cfu/g ($\pm$ SE) from three sets of 6 plants that were harvested 4 days after challenge inoculation with virulent *Pst*. Within each set of data, different letters indicate statistically significant differences between treatments (Fischer's LSD test, $\alpha = 0.05$). Data sets are from representative experiments performed at least twice with similar results. Values correspond to the bioassays shown in Figure 2. Variation in the number of *Pst* bacteria in the controls is due to variation in disease severity between experiments (see also legend to Figure 2).

Pst and activation of the genes *PR-1*, *Hel*, and *Atvsp*, which are SA-, ethylene-, and jasmonate-responsive marker genes, repectively. Resistance against *Pst* was induced maximally already at the lowest concentrations tested (Figure 2B and Table 1). Concentrations of ACC above 1 mM were significantly less effective in inducing resistance compared to the lowest concentration. This may be due to induced senescence examplified by chlorotic spots on the leaves before challenge, resulting in a higher susceptibility to *Pst*. Although resistance was clearly induced after application of the lowest concentrations of SA, ACC, and MeJA, activation of the marker genes *PR-1*, *Hel*, and *Atvsp* was apparent only when 5- to 100-fold higher concentrations were applied (Figure 2B). Moreover, transcript levels increased in a dose-dependent manner, whereas resistance did not (Figure 2B). This indicates that, similar to WCS417-mediated ISR, chemically induced resistance against *Pst* is not based on a direct activation of these defense-related genes.

Potentiation of defense-related gene expression

Prior to challenge inoculation, none of the genes tested showed enhanced expression in ISR-expressing plants. Nevertheless, the expression of these genes might be potentiated, resulting in a faster or greater activation after challenge inoculation with a pathogen. To investigate this possibility, leaves from WCS417r-treated plants were analyzed for defense-related gene expression at 0, 1, and 2 days after challenge inoculation with *Pst*. For comparison, gene expression was studied in SAR-expressing leaves that were exposed to challenge with *Pst*. Figure 3A shows that challenge inoculation of SAR-expressing tissue results in enhanced expression of all SA-inducible *PR* genes in comparison to challenged control tissue. This potentiation effect was specific for the SA-inducible genes, because the transcript levels of the ethylene- and/or jasmonate-responsive marker genes *Pdf1.2* and *Atvsp* were not enhanced in SAR-expressing tissue (Figure 3A). Challenge inoculation of ISR-expressing plants was not accompanied by a greater expression of *PR* genes (Figure 3B). However, when the same RNA blots were hybridized to the *Atvsp* probe, an enhanced accumulation of *Atvsp* transcripts in challenged ISR-expressing plants was detected (Figure 3B; box with one asterisk). Potentiation of *Atvsp* by ISR was repeatedly observed in independent experiments, whereas none of the other defense-related genes showed a stimulated expression. Thus, colonization of the rhizosphere by WCS417r can potentiate systemic expression of the jasmonate-inducible gene *Atvsp*, leading to a higher level of transcript accumulation after challenge.

Orchestration of SA- and jasmonate-responsive genes during pathogen infection

Pathogen infection results in the production of the signalling molecules SA, ethylene, and jasmonate, which in turn activate SA-, ethylene-, and jasmonate-responsive defense-related genes, respectively. Figure 3B shows that the jasmonate-inducible genes *Atvsp*, *Pdf1.2*, *Lox2*, and *Pal1* were induced 1 day after infection with *Pst*. Although jasmonate levels continued to increase to the next day (Penninckx *et al.*, 1996; C.M.J. Pieterse, unpublished results), the expression of these jasmonate-responsive genes was strongly reduced; *Atvsp* and *Pdf1.2* transcripts were even undetectable 2 days after infection (Figure 3B; box with two asterisks). During the first 2 days after inoculation, SA levels increase dramatically (Uknes *et al.*, 1993), leading to an increase in transcript levels of SA-inducible *PR* genes (Figure 3B). Previously, Doares *et al.* (1995) demonstrated that SA has an antagonistic effect on jasmonate-induced *Pin* gene expression in tomato, while MeJA has no effect on the expression level of the SA-induced *PR-3* gene. To investigate whether the increased SA levels in infected tissue might be responsible for the inhibition of jasmonate-responsive gene expression as observed 2 days after infection with *Pst*, we tested the effect of SA on MeJA-induced expression of *Atvsp*. Indeed, as shown in Figure 4, SA inhibited MeJA-induced expression of *Atvsp*, but MeJA did not affect the expression of the SA-inducible gene *PR-1*. This suggests that the expression of jasmonate-responsive genes is orchestrated during infection by changes in the endogenous levels of SA.

The timing of *Pdf1.2* and *Atvsp* suppression varied between experiments (data not shown). This is probably caused by variations in disease severity. For instance, in Figure 1 high transcript levels of *Pdf1.2* and *Atvsp* are detectable at 2 days after *Pst* infection. The plants used for gene expression analysis in this experiment were less severely diseased than the plants used for Figure 3 (data not shown) resulting in a slower increase in SA accumulation and differences in timing and magnitude of inhibition of JA-inducible gene expression.

Discussion

Rhizobacteria-mediated ISR and pathogen-induced SAR are phenotypically similar in that they provide protection against various pathogens. However, in *Arabidopsis*, both types of biologically induced disease resistance follow a distinct signalling pathway that either requires responsiveness to jasmonate and ethylene, or is dependent on SA, respectively (Lawton *et al.*, 1995; Pieterse *et al.*, 1998). Induction of SAR by infection with a necrotizing pathogen is accompanied by local and/or systemic production of SA, ethylene, and jasmonate, leading to the activation of a large set of SA-, ethylene-, and jasmonate-responsive defense-related genes. We previously showed that rhizobacteria-mediated ISR in *Arabidopsis* is not associated with changes in the expression of *PR* genes (Pieterse *et al.*, 1996, 1998; van Wees *et al.*, 1997). In those studies, gene expression was analyzed only in the leaves and at a single time point, i.e. just prior to challenge inoculation of 5-week old plants that had been grown for 3 weeks in soil containing ISR-inducing WCS417r bacteria. In search for genes that are specifically activated during ISR, in the present study we analyzed the expression of 11 well-characterized *Arabidopsis* defense-related genes at different times after induction of ISR, before as well as after challenge inoculation with *Pst*. All defense-related genes studied showed responsiveness to either SA, ethylene or jasmonate, and in most cases were activated upon pathogen infection in locally infected tissue and in systemic, SAR-expressing tissue (Figure 1). Before challenge inoculation with the pathogen, none of the genes studied were induced in ISR-expressing plants, neither locally in the roots, nor systemically in the leaves, and at none of the time points tested (Figure 1). Thus, elicitation of the ISR pathway has no direct effect on the expression of these defense-related genes.

The fact that none of the ethylene- or jasmonate-responsive genes studied was induced in ISR-expressing tissue prior to challenge inoculation, is in line with recent observations that ISR-expressing *Arabidopsis* plants do not produce enhanced levels of ethylene and jasmonate (C.M.J. Pieterse, unpublished results). Nevertheless, ISR is dependent on intact responses to both signalling compounds. This requirement for responsiveness to ethylene and jasmonate suggests that induced tissues may have become more sensitive to these regulators. Sensitization of plant cells to SA, ethylene or jasmonate action has been described. For instance, in *Arabidopsis*, both the SA-induced expression of *PR-1* (Lawton *et al.*, 1994) and the jasmonate-induced expression of *Pdf1.2* (Penninckx *et al.*, 1998) are sensitized by ethylene. Conversely, the ethylene-induced expression of *Pdf1.2* is sensitized by jasmonate (Penninckx *et al.*, 1998). We found that in SAR-expressing *Arabidopsis* leaves, the SA-inducible *PR-1*, *PR-2*, and *PR-5* genes are potentiated, leading to enhanced expression of these genes after challenge inoculation with *Pst* (Figure 3A). The ethylene- and/or jasmonate-responsive genes *Pdf1.2* and *Atvsp* showed no potentiated expression in SAR-induced tissue after challenge (Figure 3A). In ISR-expressing leaves, none of the SA- and ethylene-responsive genes was potentiated, and neither were most of the jasmonate-responsive genes (Figure 3B). However, the jasmonate-inducible gene *Atvsp* showed increased transcript levels in challenged ISR-expressing tissue compared to challenged control leaves, indicating that the expression of *Atvsp* is potentiated (Figure 3B). The jasmonate levels in challenged ISR-expressing plants were not enhanced (C.M.J. Pieterse, unpublished results), which is in line with our observation that the other jasmonate-responsive genes studied, i.e. *Pdf1.2*, *ChiB*, *Lox1*, *Lox2*, and *Pal1*, were not potentiated and even showed a somewhat reduced level of expression in challenged, ISR-expressing plants (Figure 3B). Therefore, the potentiated expression of *Atvsp* cannot be explained by accelerated jasmonate production in challenge-inoculated ISR-expressing tissue. A possible explanation for the potentiated *Atvsp* expression is that ISR-expressing tissue is sensitized only for the expression of specific jasmonate-responsive genes. The differential potentiation of jasmonate-responsive genes might be regulated by an ISR-induced transcription factor that binds to specific genes, such as *Atvsp*. In combined action with increased levels of endogenous jasmonate, as observed during pathogen attack, this could lead to an enhanced gene activation.

Vsp genes encode vegetative storage proteins that function as temporary deposits of amino acids, and accumulate in the vacuoles of young developing leaves and reproductive structures (reviewed by Creelman and Mullet, 1997). Upon wounding, *Atvsp* gene expression is induced in local and systemic tissue of *Arabidopsis* (Berger *et al.*, 1995). Proteinase inhibitors (PINs) behave similarly to VSPs in regard to vacuolar localization in young apical sinks and being induced upon wounding (Creelman and Mullet, 1997). However, whereas defensive activity of PINs has clearly

been demonstrated, this is not the case for VSPs (Creelman and Mullet, 1997).

Although SA, ACC, and MeJA are capable of inducing both defense-related gene expression and resistance against *Pst*, we found no quantitative relationship between these two phenomena. Resistance against *Pst* was induced already to the highest level at the lowest concentrations tested, whereas the marker genes *PR-1*, *Hel* and *Atvsp* were not activated at these concentrations of SA, ACC and MeJA, respectively (Figure 2B). This demonstrates that the absence of enhanced levels of SA-, ethylene-, or jasmonate-responsive defense-related gene transcripts does not automatically imply that no SA-, ethylene-, or jasmonate-dependent defense responses are elicited. The resistance against *Pst* induced by low concentrations of SA, ACC, or MeJA more likely results from either the accumulation of other defensive compounds prior to challenge inoculation, and/or from potentiation of defense-related gene expression after challenge with a pathogen. These mechanisms could be the same as the ones controlling WCS417r-mediated ISR.

To date, the nature of the defense responses that contribute to the induced resistance against *Pst* is unclear. The role of *PR* genes in the expression of SAR against *P. syringae* has been questioned earlier (Clarke *et al.*, 1998). The constitutively SAR- and *PR* gene-expressing mutant *cpr6* of *Arabidopsis* was shown to loose its elevated level of resistance against *P. syringae* pv. *maculicola* in the mutant *npr1* background, whereas the enhanced level of *PR* gene expression was not affected by the mutation in *Npr1*. This indicates that *PR* gene activation and bacterial resistance are not causally related (Clarke *et al.*, 1998). The increased resistance against *P. syringae* in *cpr6* mutants must therefore be accomplished through other, as yet unidentified, resistance responses that are regulated through NPR1.

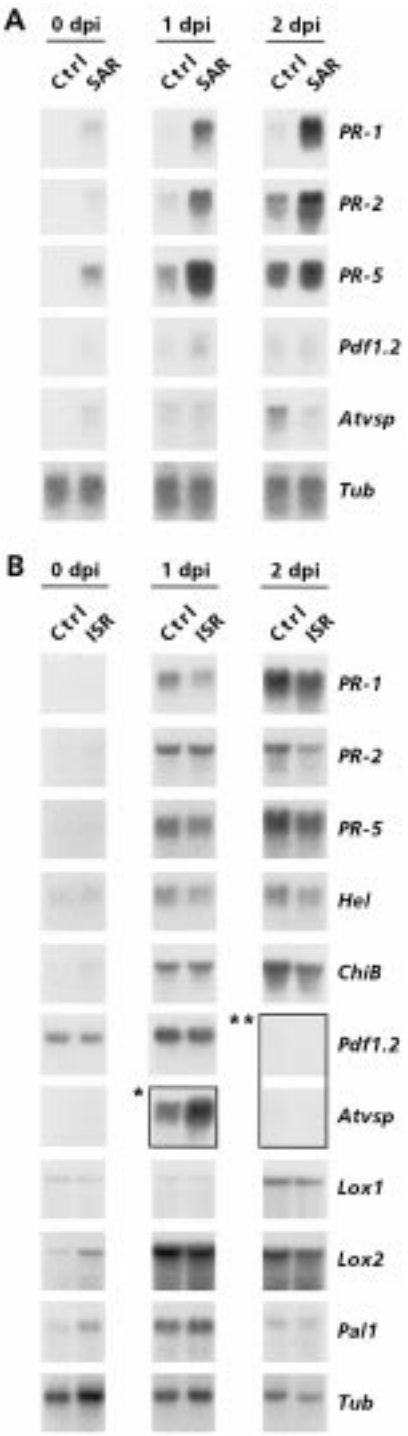

Figure 3. Expression of defense-related genes in SAR- and ISR-expressing tissue challenge-inoculated with *Pst*. SAR was induced by preinfecting three lower leaves with *Pst(avrRpt2)* 3 days before challenge. ISR was induced by growing the plants in soil containing WCS417r. Leaves of control-treated plants, and leaves expressing SAR (A) or ISR (B) were harvested at 0, 1, and 2 days after inoculation (dpi) with *Pst*. The box with one asterisk shows potentiation of *Atvsp* in ISR-expressing plants; the box with two asterisks demonstrates reduced levels of *Pdf1.2* and *Atvsp* transcripts at 2 days after challenge with *Pst*. The data presented are from a representative experiment that was repeated with similar results. For experimental details on the RNA blot analyses, see legend to Figure 1.

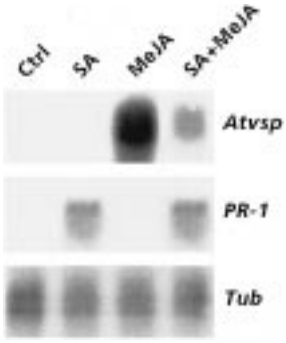

Figure 4 Expression of the jasmonate-inducible *Atvsp* gene and the SA-inducible *PR-1* gene in plants treated with SA, MeJA, or a combination of SA and MeJA. SA (1 mM) and MeJA (100 μM) were applied exogenously, 2 days before harvest of the leaves. For experimental details on the RNA blot analyses, see legend to Figure 1.

Defense mechanisms of the plant are regulated by a complex network of SA-, ethylene-, and jasmonate-dependent signalling pathways. Recent investigations have proven the existence of cross-talk between these signalling pathways (reviewed by Dong, 1998; Reymond and Farmer, 1998; Pieterse and van Loon, 1999). In the experiment shown in Figure 3B, the expression of the jasmonate-responsive genes *Atvsp*, *Pdf1.2*, *Lox2*, and *Pal1* was reduced to insignificant or moderate levels 2 days after challenge with *Pst* compared to the expression levels induced 1 day after challenge. The jasmonate levels, however, continued to increase (C.M.J. Pieterse, unpublished results). In contrast, transcript levels of the SA-responsive *PR* genes continued to increase between 1 and 2 days after challenge (Figure 3B). SA inhibited the induction of *Atvsp* by MeJA, whereas MeJA did not affect the induction of *PR-1* by SA (Figure 4). This suggests that the strong decrease in the expression of jasmonate-reponsive genes observed 2 days after challenge inoculation might be due to elevated levels of SA, which exert an inhibitory effect on the action of jasmonate. Varying results have been presented on cross-talk between SA- and jasmonate-regulated defense pathways. In accordance with our observation, all studies to date show an inhibitory effect of SA on jasmonate action (Doares *et al.*, 1995; O'Donnell *et al.*, 1996; Niki *et al.*, 1998). In contrast, for jasmonate, different effects on SA action have been reported, i.e. stimulating, inhibiting, and no effect (Xu *et al.*, 1994; Doares *et al.*, 1995; Niki *et al.*, 1998).

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