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A Novel Signaling Pathway Controlling Induced Systemic Resistance in Arabidopsis

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Plants have the ability to acquire an enhanced level of resistance to pathogen attack after being exposed to specific biotic stimuli. In Arabidopsis, nonpathogenic, root-colonizing *Pseudomonas fluorescens* bacteria trigger an induced systemic resistance (ISR) response against infection by the bacterial leaf pathogen *P. syringae* pv *tomato.* In contrast to classic, pathogen-induced systemic acquired resistance (SAR), this rhizobacteria-mediated ISR response is independent of salicylic acid accumulation and pathogenesis-related gene activation. Using the jasmonate response mutant *jar1*, the ethylene response mutant *etr1*, and the SAR regulatory mutant *npr1*, we demonstrate that signal transduction leading to *P. fluorescens* WCS417r–mediated ISR requires responsiveness to jasmonate and ethylene and is dependent on NPR1. Similar to *P. fluorescens* WCS417r, methyl jasmonate and the ethylene precursor 1-aminocyclopropane-1-carboxylate were effective in inducing resistance against *P. s. tomato* in salicylic acid–nonaccumulating NahG plants. Moreover, methyl jasmonate–induced protection was blocked in *jar1*, *etr1*, and *npr1* plants, whereas 1-aminocyclopropane-1-carboxylate–induced protection was affected in *etr1* and *npr1* plants but not in *jar1* plants. Hence, we postulate that rhizobacteria-mediated ISR follows a novel signaling pathway in which components from the jasmonate and ethylene response are engaged successively to trigger a defense reaction that, like SAR, is regulated by NPR1. We provide evidence that the processes downstream of NPR1 in the ISR pathway are divergent from those in the SAR pathway, indicating that NPR1 differentially regulates defense responses, depending on the signals that are elicited during induction of resistance.

INTRODUCTION

Plants of which the roots have been colonized by selected strains of nonpathogenic fluorescent *Pseudomonas* spp develop an enhanced level of protection against pathogen attack (reviewed in van Loon et al., 1998). Strain WCS417r of *P. fluorescens* is a biological control strain that has been shown to trigger an induced systemic resistance (ISR) response in several plant species, including carnation (van Peer et al., 1991), radish (Leeman et al., 1995), tomato (Duijff et al., 1996), and Arabidopsis (Pieterse et al., 1996). In Arabidopsis, *P. fluorescens* WCS417r–mediated ISR has been demonstrated against the bacterial leaf pathogen *P. syringae* pv *tomato*, the fungal root pathogen *Fusarium oxysporum* f sp *raphani* (Pieterse et al., 1996; van Wees et al., 1997), and the fungal leaf pathogen *Peronospora parasitica* (J. Ton and C.M.J. Pieterse, unpublished data), indicating that this type of biologically induced resistance is effective against different types of pathogens.

ISR-inducing rhizobacteria show host specificity in regard to eliciting resistance (Leeman et al., 1995; van Wees et al., 1997), which indicates that specific recognition between protective bacteria and the plant is a prerequisite for the activation of the signaling cascade leading to ISR. The downstream signaling events in the rhizobacteria-mediated ISR pathway clearly differ from those in the pathway leading from pathogen infection to classic systemic acquired resistance (SAR). SAR is a form of systemically induced disease resistance that is triggered upon infection by a necrotizing pathogen (reviewed in Ryals et al., 1996). The state of SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA; Malamy et al., 1990; Métraux et al., 1990) and the concomitant activation of genes encoding pathogenesis-related (PR) proteins (Ward et al., 1991). SA-nonaccumulating NahG plants expressing the bacterial salicylate hydroxylase (*nahG*) gene are incapable of developing SAR and do not show *PR* gene activation upon pathogen infection, indicating that SA is a necessary intermediate in the SAR signaling pathway (Gaffney et al., 1993; Delaney et al., 1994). In contrast to pathogen-induced SAR, rhizobacteria-mediated ISR is not associated with the activation of *PR* genes (Hoffland et al., 1995; Pieterse et al., 1996; van Wees et al., 1997). Moreover, NahG plants that are unable to express

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SAR develop normal levels of ISR after treatment of the roots with ISR-inducing rhizobacteria (Pieterse et al., 1996; Press et al., 1997; van Wees et al., 1997). This demonstrates that biologically induced disease resistance can be controlled by at least two pathways that diverge in their requirement for SA accumulation.

Besides SA, the plant growth regulators jasmonic acid and ethylene have been implicated in plant defense responses (Boller, 1991; Wasternack and Parthier, 1997). Jasmonic acid and derivatives, collectively referred to as jasmonates, induce the expression of genes encoding defense-related proteins, such as thionins (Epple et al., 1995) and proteinase inhibitors (Farmer et al., 1992), whereas ethylene activates several members of the *PR* gene superfamily (Brederode et al., 1991; Potter et al., 1993). Jasmonate and ethylene also have been shown to act synergistically in stimulating elicitor-induced *PR* gene expression (Xu et al., 1994). Moreover, both regulators are implicated in the activation of genes encoding plant defensins (Penninckx et al., 1996) and enzymes involved in phytoalexin biosynthesis (Ecker and Davis, 1987; Gundlach et al., 1992). Both jasmonate and ethylene have been reported to be involved in systemically induced defense responses (van Loon, 1977; Farmer and Ryan, 1992; Penninckx et al., 1996), although their role is in many cases still unclear.

Several Arabidopsis mutants affected in their response to the signaling molecules jasmonate, ethylene, or SA have been characterized in the past years. To gain more insight into the signaling pathway controlling nonpathogenic rhizobacteria-mediated ISR, we examined whether the jasmonate response mutant *jar1* (Staswick et al., 1992), the ethylene response mutant *etr1* (Bleecker et al., 1988), and the SAR regulatory mutant *npr1* (Cao et al., 1994) are able to express ISR after colonization of the roots by *P. fluorescens* WCS417r. Mutant *jar1* exhibits reduced sensitivity to methyl jasmonate (MeJA), leading to a decrease in MeJA-inducible inhibition of primary root growth and MeJA-inducible accumulation of a vegetative storage protein (Staswick et al., 1992). Mutant *etr1* (Bleecker et al., 1988) is altered in its ability to perceive and react to ethylene due to a mutation in the *ETR1* gene, encoding an ethylene receptor (Chang et al., 1993; Schaller and Bleecker, 1995). Arabidopsis *jar1* plants, as well as ethylene-insensitive tobacco plants expressing the mutant Arabidopsis *ETR1* gene, are susceptible to opportunistic microorganisms (http://www.sheridan.com/aspp/abs/60/1458.html; Knoester et al., 1998), whereas wild-type plants show a resistant phenotype, indicating that both mutations affect signaling events leading to disease resistance. Arabidopsis mutants *npr1*, *nim1*, and *sai1* are affected downstream of SA in the SAR signaling pathway and as a result are blocked in the SAR response (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). The genes involved are allelic and code for an ankyrin repeat–containing protein with homology to the mammalian signal transduction factor IκBα, which is implicated in disease resistance responses in a wide range of higher organisms (Cao et al., 1997; Ryals et al., 1997).

Using the Arabidopsis mutants *jar1*, *etr1*, and *npr1*, we demonstrate that *P. fluorescens* WCS417r–mediated ISR against *P. s. tomato* in Arabidopsis follows a novel signaling pathway that is dependent on responsiveness to both jasmonate and ethylene. Moreover, we show that similar to SAR, the regulatory protein NPR1 plays a crucial role in the expression of ISR.

RESULTS

Rhizobacteria-Mediated ISR Requires Components of the Jasmonate and Ethylene Response

To investigate whether jasmonate and/or ethylene play a role in rhizobacteria-mediated ISR, we tested the jasmonate response mutant *jar1* and the ethylene response mutant *etr1* for their ability to develop biologically induced resistance against infection by *P. s. tomato.* Wild-type Columbia (Col-0) plants, transgenic SA-nonaccumulating NahG plants, and mutant *jar1* and *etr1* plants were grown in soil containing ISR-inducing *P. fluorescens* WCS417r bacteria. Another subset of plants received SAR treatment by inoculating three lower leaves with the avirulent pathogen *P. s. tomato* carrying *avrRpt2* (Whalen et al., 1991) 3 days before challenge inoculation with a virulent strain of *P. s. tomato.* Control plants received no treatment before challenge. Figure 1 shows that in wild-type Col-0 plants, colonization of the roots by *P. fluorescens* WCS417r and predisposing infection with *P. s. tomato* carrying *avrRpt2* resulted in a significant reduction of symptoms 4 days after challenge inoculation with *P. s. tomato.* Moreover, in Col-0 plants pretreated with avirulent *P. s. tomato* or *P. fluorescens* WCS417r, growth of the challenging pathogen was inhibited (Table 1), indicating that *P. fluorescens* WCS417r–mediated ISR and pathogen-induced SAR were triggered in these plants.

Figure 1 and Table 1 show that SA-nonaccumulating NahG plants mounted resistance against *P. s. tomato* infection after *P. fluorescens* WCS417r treatment but not after preinfection with the avirulent pathogen. Furthermore, only plants expressing SAR concomitantly showed accumulation of *PR-1* transcripts (Figure 1), whereas plants expressing ISR did not, confirming that ISR and SAR are controlled by distinct signaling pathways that diverge in their requirement for SA. Both *jar1* and *etr1* plants developed SAR after preinoculation with the avirulent *P. s. tomato* strain and showed activation of *PR-1* gene expression (Figure 1), supporting previous findings (Lawton et al., 1995, 1996) that SAR signal transduction in Arabidopsis does not require components of the jasmonate or ethylene response. However, neither *jar1* nor *etr1* plants developed ISR when roots were colonized by *P. fluorescens* WCS417r, indicating that an intact response to both jasmonate and ethylene is required for the development of rhizobacteria-mediated ISR. Evidently, both of the

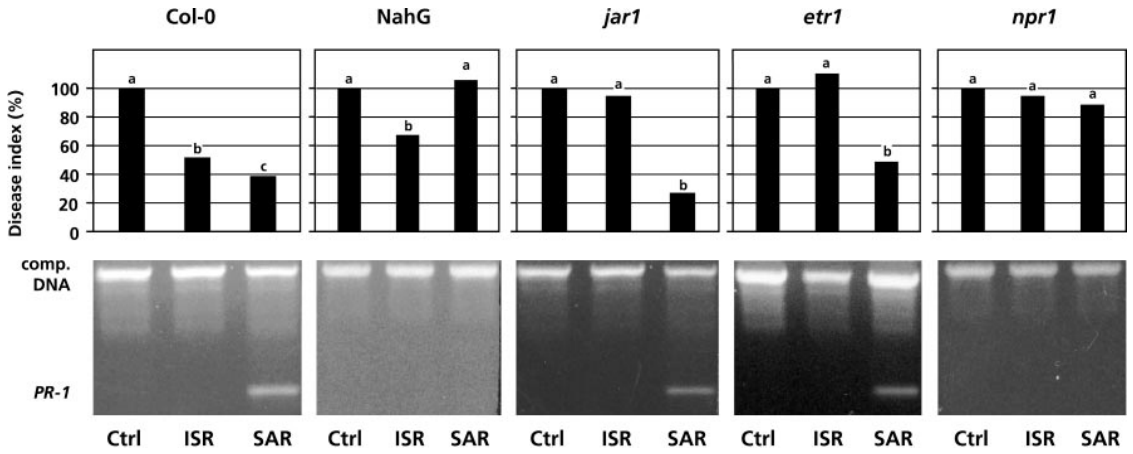

Figure 1. Quantification of ISR and SAR against *P. s. tomato* Infection in Arabidopsis Col-0, NahG, *jar1*, *etr1*, and *npr1* Plants and Analysis of *PR-1* Gene Expression.

Shown at top is the disease index of control plants (Ctrl) and plants that received an ISR or SAR treatment. The disease index is the mean (n = 20 plants) of the percentage of leaves with symptoms relative to control plants (100%) 4 days after challenge inoculation with the virulent pathogen *P. s. tomato.* The absolute proportions of diseased leaves of control-treated Col-0, NahG, *jar1*, *etr1*, and *npr1* plants were 60.1, 82.9, 80.6, 60.2, and 68.0%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fisher's LSD test; $\alpha = 0.05$). Plants treated with ISR-inducing bacteria were grown in soil containing *P. fluorescens* WCS417r. SAR was induced 3 days before challenge inoculation by infiltrating three leaves per plant with the avirulent pathogen *P. s. tomato* carrying *avrRpt2.* Shown below are ethidium bromide–stained agarose gels with competitive RT-PCR products obtained after amplification of equal portions of first-strand cDNA and 500 pg of heterologous competitor DNA (comp. DNA) by using *PR-1*–specific primers. First-strand cDNA was synthesized on mRNA that was isolated from leaves of the indicated plant and treatment combinations that were harvested just before challenge inoculation. The data shown are taken from representative experiments that were repeated at least twice with similar results.

phytohormones jasmonate and ethylene play a crucial role in the ISR signaling pathway but SA does not.

Rhizobacteria-Mediated ISR Is Dependent on NPR1

NPR1 has been shown to be an important regulatory factor in the SA-dependent SAR response (Cao et al., 1994). To investigate whether NPR1 is involved in the SA-independent ISR response as well, we tested the Arabidopsis mutant *npr1*. Figure 1 and Table 1 show that *npr1* plants failed to develop SAR and did not show *PR-1* gene activation after predisposing infection with the avirulent *P. s. tomato* strain, confirming that the SAR response was effectively blocked in these plants. Surprisingly, *npr1* plants were also affected in the expression of *P. fluorescens* WCS417r–mediated ISR, indicating that both types of biologically induced disease resistance are dependent on NPR1.

Colonization of the Rhizosphere by *P. fluorescens* WCS417r

To investigate whether the inability to express ISR in the mutants was caused by insufficient colonization of the rhizosphere by *P. fluorescens* WCS417r, we determined the number of rifampicin-resistant *P. fluorescens* WCS417r bacteria per gram of root fresh weight at the end of each bioassay. Table 2 shows that *P. fluorescens* WCS417r colonizes the rhizosphere of Col-0, NahG, *jar1*, *etr1*, and *npr1* plants with equal efficiency. Thus, the loss of the capacity to express *P. fluorescens* WCS417r–mediated ISR in *jar1*, *etr1*,

Table 1. Number of *P. s. tomato* Bacteria in Challenged Leaves of Control Plants and *P. fluorescens* WCS417r– and Avirulent *P. s. tomato*–Treated Arabidopsis Plants

	cfu/g Fresh Weight ($\times 10^7$)*		
Plant Type	Control	WCS417r	*P. s. tomato avrRpt2*
Col-0	2.5 ± 0.2[a]	0.6 ± 0.2[b]	0.5 ± 0.2[b]
NahG	143.0 ± 21.6[a]	91.7 ± 6.5[b]	161.7 ± 27.3[a]
jar1	31.1 ± 2.9[a]	27.6 ± 1.3[a]	1.9 ± 0.7[b]
etr1	28.1 ± 1.2[b]	43.0 ± 1.7[a]	9.6 ± 0.8[c]
npr1	138.8 ± 17.3[a]	110.5 ± 26.5[a]	147.3 ± 39.6[a]

*Values presented are average numbers of colony-forming units (cfu; ±SE) per gram of infected leaf tissue. Leaves were harvested 4 days after challenge inoculation with the virulent *P. s. tomato* strain.
[a,b,c]Within each row, different letters (a to c) indicate statistically significant differences between treatments (Fisher's LSD test; $\alpha = 0.05$).

Table 2. Colonization of the Rhizosphere of Arabidopsis Col-0, NahG, *jar1*, *etr1*, and *npr1* Plants by *P. fluorescens* WCS417r

Plant Type	cfu/g Fresh Weight ($\times 10^6$)[a]
Col-0	8.4 ± 0.4
NahG	7.5 ± 1.1
jar1	9.7 ± 0.8
etr1	6.6 ± 0.6
npr1	7.3 ± 1.1

[a]Values presented are average population densities ±SE. Roots were harvested at the end of the bioassays. On nontreated roots, no rifampicin-resistant bacteria were detected (detection limit, 10^3 cfu/g).

and *npr1* plants is not caused by changes in bacterial root colonization but must be the result of alterations in properties of the mutants.

Sequence of Signaling Events

To elucidate the sequence of the signaling events involved in the jasmonate-, ethylene-, and NPR1-dependent ISR response, we tested the resistance-inducing ability of MeJA and 1-aminocyclopropane-1-carboxylate (ACC), the natural precursor of ethylene, in Col-0, NahG, *jar1*, *etr1*, and *npr1* plants. Figure 2A shows that applied ACC was readily converted to ethylene by endogenous ACC oxidase activity. H_2O- and MeJA-treated plants showed basal levels of ethylene production, whereas in ACC-treated plants, a 10- to 25-fold increase in ethylene production was observed (Figure 2B). As shown in Figure 2C, pretreatment of Col-0 plants with MeJA or ACC resulted in a 50% reduction of the symptoms caused by *P. s. tomato* infection. Table 3 shows that growth of *P. s. tomato* was inhibited as well, indicating that the observed reduction of symptoms is caused by the activation of a resistance response.

Application of MeJA or ACC to NahG plants also resulted in a reduction of symptoms, although the level of protection was somewhat lower than that observed in wild-type Col-0 plants. In *jar1* plants, application of MeJA did not elicit a resistance response, whereas application of ACC resulted in wild-type levels of protection. Mutant *etr1* plants were nonresponsive to ACC treatment but also failed to respond to MeJA-treatment, indicating that components of the ethylene response act downstream of jasmonate in the signaling

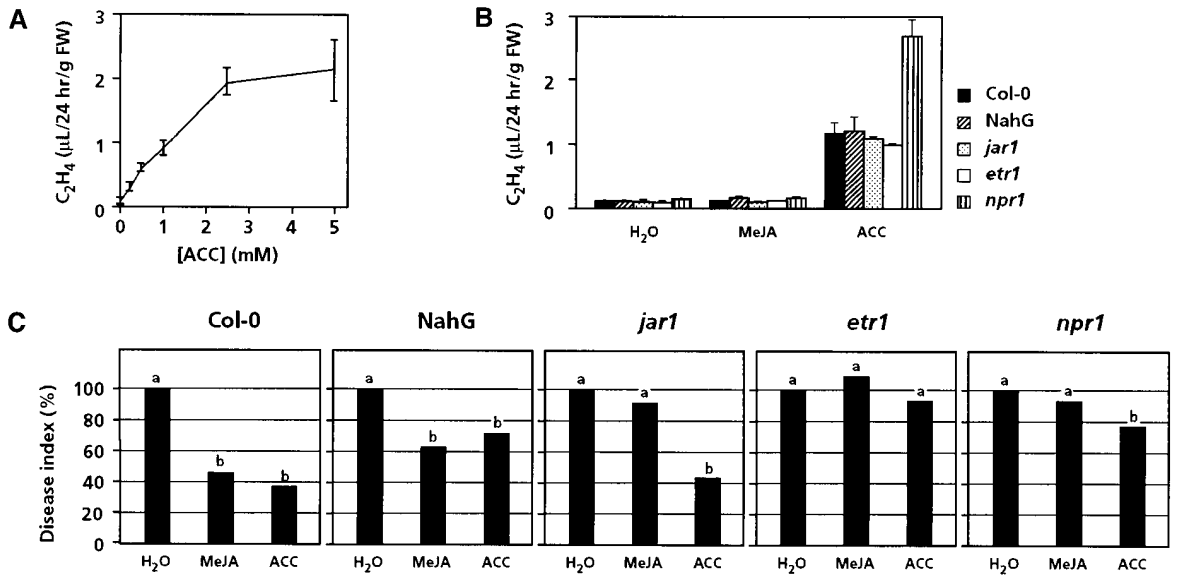

Figure 2. Ethylene Production and Quantification of Induced Protection in MeJA- and ACC-Treated Arabidopsis Plants.

(A) Ethylene production in leaves of Arabidopsis ecotype Col-0 after treatment of the leaves with different concentrations of ACC. Data points are means (microliters of ethylene produced per gram fresh weight [FW] of leaf tissue in the first 24 hr after treatment) with standard errors from three independent samples that received the same treatment.

(B) Ethylene production in leaves of Col-0, NahG, *jar1*, *etr1*, and *npr1* plants in the first 24 hr after treatment with H_2O, 100 μM MeJA, or 1 mM ACC. Bars represent standard errors from six independent samples that received the same treatment.

(C) MeJA- and ACC-mediated protection against *P. s. tomato* in Col-0, NahG, *jar1*, *etr1*, and *npr1* plants. Plants were pretreated with H_2O, 100 μM MeJA, or 1 mM ACC 3 days before challenge inoculation with *P. s. tomato.* Four days after challenge inoculation, the disease index was determined (see legend to Figure 1). The absolute proportions of diseased leaves of control-treated Col-0, NahG, *jar1*, *etr1*, and *npr1* plants were 49.9, 78.2, 74.8, 61.0, and 70.0%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fisher's LSD test; $n = 20$; $\alpha = 0.05$).

Table 3. Number of *P. s. tomato* Bacteria in Challenged Leaves of Control Plants and MeJA- and ACC-Treated Arabidopsis Col-0 Plants

Treatment	cfu/g Fresh Weight ($\times 10^6$)*
H_2O	6.0 ± 0.4^a
MeJA	2.4 ± 0.3^b
ACC	2.6 ± 0.2^b

*Values presented are average numbers of colony-forming units ($\pm$SE) per gram of infected leaf tissue. Leaves were harvested 4 days after challenge inoculation with *P. s. tomato*.

[a,b] Different letters (a and b) indicate statistically significant differences between treatments (Fisher's LSD test; $\alpha = 0.05$).

pathway leading to protection against *P. s. tomato.* In *npr1* plants, responsiveness to MeJA or ACC was blocked and strongly reduced, respectively, indicating that components of the jasmonate and ethylene response act upstream of NPR1 in regulating the expression of induced resistance against *P. s. tomato.*

ISR Is Not Associated with Jasmonate- and Ethylene-Responsive Gene Activation

The involvement of components from the jasmonate and ethylene response in rhizobacteria-mediated ISR suggests that ISR might be associated with jasmonate- and ethylene-induced processes. To investigate whether treatment with *P. fluorescens* WCS417r stimulates known jasmonate- or ethylene-inducible responses, we studied the expression of the jasmonate-inducible gene *Atvsp*, encoding a vegetative storage protein (Berger et al., 1995), the ethylene-inducible *Hel* gene, encoding a hevein-like protein with antifungal activity (Potter et al., 1993), and the jasmonate- and ethylene-inducible plant defensin gene *Pdf1.2*, encoding a small protein with antifungal activity (Penninckx et al., 1996). Figure 3 shows that application of MeJA or ACC to the leaves activated the expression of the *Atvsp* or *Hel* gene, respectively, demonstrating that both MeJA and ACC triggered their corresponding response pathway specifically. As expected, both MeJA and ACC induced *Pdf1.2* transcript accumulation in the leaves. However, in roots and leaves of *P. fluorescens* WCS417r–induced plants, no increase in *Atvsp*, *Hel*, or *Pdf1.2* transcript levels was detected, indicating that the expression of *P. fluorescens* WCS417r–mediated ISR does not coincide with a strong stimulation of the jasmonate and ethylene response.

DISCUSSION

We demonstrated previously that plants expressing pathogen-induced SAR or rhizobacteria-mediated ISR against *P. s. tomato* infection develop significantly fewer symptoms compared with noninduced plants and show a strong inhibition of pathogen growth in the leaves (Pieterse et al., 1996; van Wees et al., 1997). Despite these phenotypical similarities, the signaling pathways leading to both biologically induced resistance responses diverge in their requirement for SA. Moreover, the expression of SAR is accompanied by the activation of *PR* genes, whereas this response is lacking during expression of ISR (Pieterse et al., 1996). In this study, we used well-characterized Arabidopsis mutants in our attempt to elucidate the steps involved in the SA-independent signaling pathway controlling rhizobacteria-mediated ISR. Systemic resistance induced by nonpathogenic rhizobacteria was blocked in the Arabidopsis mutants *jar1*, *etr1*, and *npr1* (Figure 1 and Table 1), indicating that components of the jasmonate and ethylene response as well as NPR1 play a crucial role in the ISR signaling pathway. Consistent with our observations, Lawton et al. (1995, 1996) previously demonstrated that both *jar1* and *etr1* are not impaired in their ability to develop SAR. Thus, the rhizobacteria-mediated ISR and pathogen-induced SAR signaling pathways diverge in their requirement for SA, on the one hand, and for jasmonate and ethylene, on the other hand.

Several lines of evidence indicate that MeJA- and ACC-induced protection against *P. s. tomato* follow the same

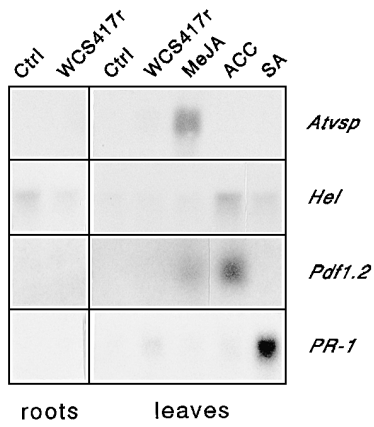

Figure 3. Expression of Jasmonate-, Ethylene-, and SA-Inducible Genes in Response to *P. fluorescens* WCS417r, MeJA, ACC, and SA Treatment.

The results of RNA gel blot analysis of *Atvsp*, *Hel*, *Pdf1.2*, and *PR-1* gene expression are shown. Roots and leaves of plants that were grown in soil supplemented with 10 mM $MgSO_4$ (Ctrl) or *P. fluorescens* WCS417r bacteria (WCS417r) were harvested when the plants were 5 weeks old. Chemical treatments were performed by dipping the leaves of 5-week-old plants in a solution containing 0.01% (v/v) Silwet L-77 and MeJA (100 μM), ACC (1 mM), or SA (5 mM). Control leaves were treated with 0.01% (v/v) Silwet L-77 only. Chemically treated leaves were harvested 2 days after the application of the chemicals. Arabidopsis *Atvsp*, *Hel*, *Pdf1.2*, and *PR-1* gene-specific probes were used for RNA gel blot hybridizations.

signaling pathway as *P. fluorescens* WCS417r–mediated ISR. First, *P. fluorescens* WCS417r, MeJA, and ACC induce resistance against *P. s. tomato* in NahG plants (Figures 1 and 2C), indicating that these agents activate an SA-independent resistance mechanism. This is supported by the fact that *P. fluorescens* WCS417r–, MeJA- and ACC-treated plants do not show an increase in SA-inducible *PR-1* gene expression (Figure 3). The level of protection in NahG plants after induction by these agents was lower compared with that observed in wild-type Col-0 plants. This may be due to the fact that NahG plants are more susceptible to pathogen infection (Delaney et al., 1994; Figures 1 and 2, and Table1), resulting in a lower efficacy of the ISR-inducing agents. Nevertheless, a modulating role of SA in the ISR response cannot be ruled out. The second line of evidence indicating that *P. fluorescens* WCS417r, MeJA, and ACC trigger the same signaling pathway controlling induced resistance against *P. s. tomato* is the observation that resistance induced by these three agents requires responsiveness to ethylene and is dependent on NPR1 to be fully expressed. All together, this strongly suggests that resistance induced by *P. fluorescens* WCS417r, MeJA, or ACC is reached by activating the same defense pathway.

Using MeJA and ACC as inducing agents, we determined the sequence of signaling events involved in the pathway leading to resistance against *P. s. tomato.* Figure 2C clearly shows that MeJA-mediated protection against *P. s. tomato* requires an intact response to ethylene, whereas ACC is fully active in *jar1* plants. Hence, components of the ethylene response act downstream of jasmonate. Moreover, MeJA- and ACC-induced protection are blocked or highly diminished in *npr1* plants, indicating that NPR1 acts downstream of jasmonate and ethylene in the signaling pathway leading to resistance against *P. s. tomato.* Therefore, we postulate that during signal transduction leading to *P. fluorescens* WCS417r–mediated ISR, the jasmonate and ethylene responses are engaged successively to trigger a defense response that is regulated by NPR1 (Figure 4).

The observation that ACC-mediated protection was not completely blocked in *npr1* plants (Figure 2C) suggests the existence of a parallel ethylene-inducible defensive pathway that does not require NPR1. A candidate pathway might be the ethylene-inducible pathway leading to *Pdf1.2* gene expression that has been shown to be NPR1 independent (Penninckx et al., 1996). Alternatively, this low level of protection in *npr1* plants may be caused by the twofold higher production of ethylene after ACC treatment (Figure 2B). However, the latter possibility seems unlikely because a twofold increase in ethylene production in wild-type Col-0 plants, by applying 2.5 mM ACC to the leaves instead of 1 mM, does not result in a higher level of protection against *P. s. tomato* infection (S.C.M. van Wees, unpublished results). In itself, the enhanced level of ethylene production in ACC-treated *npr1* plants is intriguing because it demonstrates that *npr1* plants show twofold higher ACC oxidase activity than do wild-type plants. Interestingly, pathogen infection also causes a significantly higher increase in ethylene production in *npr1* plants (C.M.J. Pieterse, unpublished results), suggesting that not only SA responsiveness but also ethylene metabolism is altered by the *npr1* mutation.

Elicitation of a similar SA-independent defense pathway against *P. s. tomato* infection by *P. fluorescens* WCS417r, MeJA, and ACC implies that ISR is associated with an increase in the production of jasmonate or ethylene. However, *P. fluorescens* WCS417r–mediated ISR does not coincide with jasmonate- and ethylene-responsive gene expression (Figure 3), suggesting that the production of jasmonate and ethylene is not strongly stimulated. When plants were treated with lower concentrations of MeJA or ACC (25 μM and 0.25 mM rather than 100 μM and 1 mM, respectively), they clearly developed enhanced protection against *P. s. tomato*, without activating *Atvsp*, *Hel*, or *Pdf1.2* gene expression (S.C.M. van Wees, unpublished results). Hence, *P.*

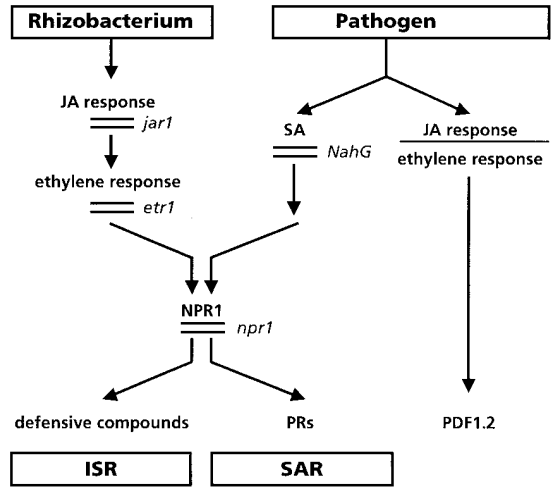

Figure 4. Proposed Model for the Nonpathogenic Rhizobacteria-Mediated ISR Signaling Pathway as Part of the Network of Pathways Controlling Biologically Induced Systemic Resistance.

P. fluorescens WCS417r bacteria trigger an SA-independent pathway in which components from the jasmonate (JA) and ethylene response act in sequence to activate a systemic resistance response that is dependent on the regulatory protein NPR1. The ISR pathway shares signaling events that are initiated upon pathogen infection but is not associated with *PR* or *Pdf1.2* gene expression. This indicates that *P. fluorescens* WCS417r bacteria trigger a novel signaling pathway and that resistance induced by these nonpathogenic rhizobacteria involves the production of thus far unidentified defensive compounds that are active against *P. s. tomato.* The NPR1-dependent pathway controlling *PR* gene expression and the NPR1-independent pathway leading to *Pdf1.2* gene expression are according to Ryals et al. (1996) and Penninckx et al. (1996), respectively.

fluorescens WCS417r–mediated ISR may involve a moderate or localized stimulation of the jasmonate and ethylene response that is below the threshold level needed for *Atvsp*, *Hel*, and *Pdf1.2* gene activation. Nevertheless, it cannot be ruled out that simply the availability of jasmonate and ethylene signaling intermediates is sufficient to facilitate induction of ISR. Recently, Schweizer et al. (1997) demonstrated that during infection of rice with the fungal pathogen *Magnaporthe grisea*, jasmonate-inducible genes are activated without an increase in endogenous jasmonate levels. Moreover, Tsai et al. (1996) provided evidence that an increase in ethylene sensitivity rather than ethylene production is the initial event to trigger jasmonate-enhanced senescence in detached rice leaves. Thus, ethylene- and jasmonate-dependent plant responses can be triggered without a concomitant increase in the levels of these phytohormones. Whether enhanced sensitivity to either jasmonate or ethylene plays a role in rhizobacteria-mediated ISR needs to be elucidated.

Pathogen-induced systemic activation of the Arabidopsis plant defensin gene *Pdf1.2* is independent of SA and requires components from both the jasmonate- and the ethylene-response pathways (Penninckx et al., 1996). Therefore, this defense reaction appears to share specific signaling events with *P. fluorescens* WCS417r–mediated ISR. However, the latter is not associated with an increase in *Pdf1.2* transcript levels (Figure 3). Moreover, signal transduction leading to *Pdf1.2* gene activation was reported to be independent of NPR1 (Penninckx et al., 1996), whereas *P. fluorescens* WCS417r–mediated ISR requires NPR1 (Figure 1). Thus, the corresponding signaling pathways must be dissimilar (Figure 4). Recently, analysis of the SAR signal transduction mutant *cpr5* revealed that the signaling pathways controlling NPR1-dependent SAR and NPR1-independent *Pdf1.2* gene expression are connected in early signal transduction steps and branch upstream of SA (Bowling et al., 1997). Here, we show that the ISR pathway is connected with that of SAR as well in that they both require NPR1. Apparently, biologically induced systemic resistance responses in plants are connected via a complex network of signaling pathways that involve that not only SA but also the concerted action of jasmonate and ethylene (Figure 4).

Mutant *npr1* was originally isolated in a screen for SAR mutants that are blocked in the response pathway leading from SA to *PR* gene activation (Cao et al., 1994). Although ISR is independent of SA accumulation and is not associated with *PR* gene activation, this resistance response is blocked in mutant *npr1* as well. Hence, NPR1 is not only required for the SA-dependent expression of *PR* genes that are activated during SAR but also for the jasmonate- and ethylene-dependent activation of thus far unidentified defense responses that are involved in rhizobacteria-mediated ISR. Thus, NPR1 differentially regulates defense gene expression, depending on the signaling pathway that is activated upstream of it. Future research should reveal the molecular basis underlying this phenomenon.

METHODS

Bacterial Cultures

Induced systemic resistance (ISR)–inducing *Pseudomonas fluorescens* WCS417r bacteria (van Peer et al., 1991) were grown on King's medium B agar plates (King et al., 1954) for 24 hr at 28°C. The bacterial cells were collected, resuspended in 10 mM $MgSO_4$, and adjusted to a concentration of 10^9 colony-forming units (cfu) per mL ($OD_{600} = 1.0$) before mixing throughout the soil.

The avirulent pathogen *P. syringae* pv *tomato* DC3000 carrying a plasmid with avirulence gene *avrRpt2* (Whalen et al., 1991) was used for induction of systemic acquired resistance (SAR). Bacteria were cultured overnight at 28°C in liquid King's medium B (King et al., 1954) supplemented with 20 mg/L tetracycline to select for the plasmid. The bacterial cells were collected by centrifugation, resuspended in 10 mM $MgSO_4$, and adjusted to a concentration of 10^7 cfu/mL before pressure infiltration into the leaves.

The virulent pathogen *P. s. tomato* DC3000 without the plasmid carrying *avrRpt2* (Whalen et al., 1991) was used for challenge inoculations. *P. s. tomato* bacteria were grown overnight in liquid King's medium B at 28°C. After centrifugation, bacterial cells were resuspended to a final concentration of 2.5×10^7 cfu/mL in 10 mM $MgSO_4$ containing 0.01% (v/v) of the surfactant Silwet L-77 (van Meeuwen Chemicals BV, Weesp, The Netherlands).

Cultivation of Plants

Seeds of wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) plants, transgenic NahG plants harboring the bacterial *nahG* gene (Delaney et al., 1994), and mutant *jar1* (Staswick et al., 1992), *etr1* (Bleecker et al., 1988), and *npr1* plants (Cao et al., 1994) 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 hr. Plants were cultivated in a growth chamber with a 9-hr day (200 $\mu E\ m^{-2}\ sec^{-1}$ at 24°C) and 15-hr night (20°C) cycle and 70% relative humidity. Plants were watered on alternate days and once a week were supplied with modified half-strength Hoagland's nutrient solution (2 mM KNO_3, 5 mM $Ca[NO_3]_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, and trace elements, pH 7; Hoagland and Arnon, 1938) containing 10 μM Sequestreen (Novartis, Basel, Switzerland).

Induction Treatments

Plants were treated with nonpathogenic, ISR-inducing rhizobacteria by mixing a suspension of *P. fluorescens* WCS417r bacteria throughout the soil to a final density of 5×10^7 cfu/kg just before the seedlings were planted as described by Pieterse et al. (1996).

SAR was induced 3 days before challenge inoculation by pressure infiltrating three lower leaves per plant with the avirulent pathogen *P. s. tomato* carrying *avrRpt2* at 10^7 cfu/mL in 10 mM $MgSO_4$ by using a 1-mL syringe without a needle, as described by Swanson et al. (1988).

Chemical treatments were performed 3 days before challenge inoculation by dipping the leaves of 5-week-old plants in a solution containing 0.01% (v/v) Silwet L-77 and either methyl jasmonate (MeJA; 100 μM), salicylic acid (SA; 5 mM), or 1-aminocyclopropane-1-carboxylate (ACC; 0.25, 0.5, 1.0, 2.5, or 5.0 mM), pH 6. Control

plants were treated with 0.01% (v/v) Silwet L-77 only. MeJA was purchased from Serva, Brunschwig Chemie (Amsterdam, The Netherlands), ACC from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands), and SA from Malinckrodt Baker BV (Deventer, The Netherlands).

Challenge Inoculation and Disease Assessment

Challenge inoculations were performed by dipping the leaves of 5-week-old plants in a bacterial suspension of the virulent pathogen *P. s. tomato* at 2.5×10^7 cfu/mL in 10 mM $MgSO_4$, 0.01% (v/v) Silwet L-77. Four days after challenge, disease severity was assessed by determining the percentage of leaves with symptoms per plant (20 plants per treatment) and by examining the growth of the challenging pathogen in leaves. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. The number of *P. s. tomato* bacteria in inoculated leaves was assessed in three sets of 20 randomly selected leaves per treatment. Leaves were weighed, rinsed thoroughly in sterile water, and homogenized in 10 mM $MgSO_4$. Subsequently, appropriate dilutions were plated onto King's medium B agar supplemented with 50 mg/L rifampicin and 100 mg/L cycloheximide. After an incubation time of 48 hr at 28°C, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined.

Rhizosphere Colonization

Colonization of the rhizosphere of wild-type, transgenic, and mutant plants by rifampicin-resistant *P. fluorescens* WCS417r bacteria was examined at the end of each bioassay. In duplicate, roots of six plants per treatment were harvested, weighed, and shaken vigorously for 1 min in 5 mL of 10 mM $MgSO_4$ containing 0.5 g of glass beads (0.17 mm diameter). Appropriate dilutions were plated onto King's medium B agar supplemented with cycloheximide (100 mg/L), ampicillin (50 mg/L), chloramphenicol (13 mg/L), and rifampicin (150 mg/L), which is selective for rifampicin-resistant, fluorescent *Pseudomonas* spp (Geels and Schippers, 1983). After overnight incubation at 28°C, the number of rifampicin-resistant colony-forming units per gram of root fresh weight was determined.

Competitive Reverse Transcriptase–Polymerase Chain Reaction

Analysis of *PR-1* gene expression was performed using the competitive reverse transcriptase–polymerase chain reaction (RT-PCR) as described by Siebert and Larrick (1992). A *PR-1*–specific primer pair (5′-GTAGGTGCTCTTGTTCTTCC-3′ and 5′-TTCACATAATTCCCACGAGG-3′), yielding RT-PCR products of 422 bp, was prepared based on the Arabidopsis *PR-1* cDNA sequence described by Uknes et al. (1992). A 900-bp heterologous competitor DNA fragment, competing for the same set of primers, was obtained as described by Siebert and Larrick (1992). Fifty nanograms of poly(A)$^+$ RNA, isolated from frozen leaves, was converted into first-strand cDNA. Subsequently, equal portions of cDNA were amplified in the presence of 500 pg of competitive DNA by using the *PR-1*–specific primer pair. The products were then resolved on an agarose gel stained with ethidium bromide.

Ethylene Measurement

Thirty minutes after the application of the chemicals, leaves were detached, weighed, and placed in 25-mL gas-tight serum flasks that subsequently were incubated for 24 hr under climate chamber conditions. Ethylene accumulation was measured by gas chromatography as described by de Laat and van Loon (1982).

RNA Gel Blot Analysis

Total RNA was extracted from roots and leaves of 5-week-old control and ISR-expressing plants and from leaves collected 2 days after chemical application, using the guanidine–hydrochloride RNA extraction method as described by Logemann et al. (1987). For RNA gel blot analysis, 15 μg of total RNA was electrophoretically separated on denaturing formaldehyde–agarose gels and blotted onto Hybond-N$^+$ membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer, as described by Sambrook et al. (1989). RNA gel blots were hybridized and washed as described previously (Pieterse et al., 1994) and exposed to a Kodak X-OMAT AR film. DNA probes were labeled with α-^{32}P-dCTP by random primer labeling (Feinberg and Vogelstein, 1983). Probes for the detection of *Atvsp* and *Hel* transcripts were prepared by PCR with primers based on sequences obtained from GenBank accession numbers Z18377 and U01880, respectively. Probes for *Pdf1.2* and *PR-1* were derived from an Arabidopsis *Pdf1.2* and a *PR-1* cDNA clone, respectively (Uknes et al., 1992; Penninckx et al., 1996).

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