A Novel Signaling Pathway Controlling Induced Systemic Resistance in Arabidopsis

Corné M. J. Pieterse,a,1 Saskia C. M. van Wees,a Johan A. van Pelt,a Marga Knoester,a Ramon Laan,a Han Gerrits,a Peter J. Weisbeek,b and Leendert C. van Loona

aSection of Plant Pathology, Department of Plant Ecology and Evolutionary Biology, Utrecht University, Sorbonnelaan 16, 3584 CA, Utrecht, The Netherlands
bDepartment of Molecular Cell Biology, Utrecht University, Padualaan 8, 3584 CH, Utrecht, The Netherlands

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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1To whom correspondence should be addressed. E-mail c.m.j. pieterse@bio.uu.nl; fax 31-30-2518366.
SAR develop normal levels of ISR after treatment of the roots with ISR-inducing rhizobacteria (Pleterse 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 (Bredereode 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 (MeJ A), leading to a decrease in MeJ A-inducible inhibition of primary root growth and MeJ A-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 sal1 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 IxBα, 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. tomento 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. tomento. 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. tomento carrying avrRpt2 (Whalen et al., 1991) 3 days before challenge inoculation with a virulent strain of P. s. tomento. 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. tomento carrying avrRpt2 resulted in a significant reduction of symptoms 4 days after challenge inoculation with P. s. tomento. Moreover, in Col-0 plants pretreated with avirulent P. s. tomento 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. tomento 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. tomento 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
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 pathogen *P. s. tomato*. 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.

**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,
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-aminoacyclopropane-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. H2O- 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 non-responsive 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

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

<table>
<thead>
<tr>
<th>Plant Type</th>
<th>cfu/g Fresh Weight (×10^6)a</th>
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<tbody>
<tr>
<td>Col-0</td>
<td>8.4 ± 0.4</td>
</tr>
<tr>
<td>NahG</td>
<td>7.5 ± 1.1</td>
</tr>
<tr>
<td>jar1</td>
<td>9.7 ± 0.8</td>
</tr>
<tr>
<td>etr1</td>
<td>6.6 ± 0.6</td>
</tr>
<tr>
<td>npr1</td>
<td>7.3 ± 1.1</td>
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*aValues 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).*

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 H2O, 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 H2O, 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; α = 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 Pdfl2, encoding a small protein with antifungal activity (Peninckx 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 Pdfl2 transcript accumulation in the leaves. However, in roots and leaves of P. fluorescens WCS417r-induced plants, no increase in Atvsp, Hel, or Pdfl2 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
signaling pathway as P. fluorescens WCS417r-mediated ISR. First, P. fluorescens WCS417r, Mej A, 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, Mej A- 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, Mej A, 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, Mej A, or ACC is reached by activating the same defense pathway.

Using Mej A 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 Mej A-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, Mej A- 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 Pdfl1.2 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, Mej A, 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 Mej A 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 Pdfl1.2 gene expression (S.C.M. van Wees, unpublished results). Hence, P. fluorescens WCS417r bacteria trigger a novel signaling pathway 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 Pdfl1.2 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 Pdfl1.2 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 Pfd1.2 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 Pfd1.2 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 Pfd1.2 transcript levels (Figure 3). Moreover, signal transduction leading to Pfd1.2 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 Pfd1.2 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 MgSO4, and adjusted to a concentration of 10⁵ colony-forming units (cfu) per mL (OD₆₀₀ = 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 MgSO4, and adjusted to a concentration of 10⁷ 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 × 10⁷ cfu/mL in 10 mM MgSO4 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 jα1 (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 μE 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 KNO₃, 5 mM Ca(NO₃)₂, 1 mM KH₂PO₄, 1 mM MgSO₄, 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 × 10⁷ 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 10⁷ cfu/mL in 10 mM MgSO₄ 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 (Mej A; 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. Mej A was purchased from Serva, Brunschwig Chemie (Amsterdam, The Netherlands), ACC from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands), and SA from Malinkrodt 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 \times 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. 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 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 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’-GAGTGTCGCTTTGTTCTCC-3’ and 5’-AGGGATTCTCTCGACGTTG-3’), yielding RT-PCR products of 242 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-[32P]-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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