Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*

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The plant-signaling molecules salicylic acid (SA) and jasmonic acid (JA) play an important role in induced disease resistance pathways. Cross-talk between SA- and JA-dependent pathways can result in inhibition of JA-mediated defense responses. We investigated possible antagonistic interactions between the SA-dependent systemic acquired resistance (SAR) pathway, which is induced upon pathogen infection, and the JA-dependent induced systemic resistance (ISR) pathway, which is triggered by nonpathogenic *Pseudomonas* rhizobacteria. In *Arabidopsis thaliana*, SAR and ISR are effective against a broad spectrum of pathogens, including the foliar pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*). Simultaneous activation of SAR and ISR resulted in an additive effect on the level of induced protection against *Pst*. In *Arabidopsis* genotypes that are blocked in either SAR or ISR, this additive effect was not evident. Moreover, induction of ISR did not affect the expression of the SAR marker gene *PR-1* in plants expressing SAR. Together, these observations demonstrate that the SAR and the ISR pathway are compatible and that there is no significant cross-talk between these pathways. SAR and ISR both require the key regulatory protein NPR1. Plants expressing both types of induced resistance did not show elevated *Npr1* transcript levels, indicating that the constitutive level of NPR1 is sufficient to facilitate simultaneous expression of SAR and ISR. These results suggest that the enhanced level of protection is established through parallel activation of complementary, NPR1-dependent defense responses that are both active against *Pst*. Therefore, combining SAR and ISR provides an attractive tool for the improvement of disease control.

Recent advances in research on plant defense-signaling pathways have shown that plants are capable of differentially activating distinct defense pathways depending on the type of invader encountered (1–5). The plant-signaling molecules salicylic acid (SA), jasmonic acid (JA), and ethylene play an important role in this signaling network: blocking the response to either of these signals can render plants more susceptible to pathogens (6–10) and insects (11). Evidence is accumulating that components from SA-, JA-, and ethylene-dependent defense pathways can affect each others signaling. For instance, JA and ethylene have been shown to act in concert in activating genes encoding defensive proteins, such as proteinase inhibitors and plant defensins (12, 13). Negative interactions have been reported as well: SA and its functional analogues 2,6-dichloroisonicotinic acid and benzothiadiazole suppress JA-dependent defense gene expression (14–19), possibly through the inhibition of JA synthesis and action (20). In some cases, the latter has been shown to negatively affect JA-dependent defense against insect herbivory (21). Conversely, JA and ethylene have been shown to stimulate SA action (22–24), although antagonistic effects have been described as well (18). Cross-talk between different signal transduction pathways is thought to provide great regulatory potential for activating multiple resistance mechanisms in varying combinations, and may help the plant to prioritize the activation of a particular defense pathway over another (1, 5).

Plants possess various inducible defense mechanisms to protect themselves against pathogen attack. A classic example is systemic acquired resistance (SAR) that is activated after infection by a necrotizing pathogen. SAR has been demonstrated in many plant species and confers resistance against a broad spectrum of plant pathogens in distant, uninfected plant parts (25). Selected nonpathogenic, rhizosphere-colonizing *Pseudomonas* bacteria trigger a phenotypically similar form of resistance, called rhizobacteria-mediated induced systemic resistance (ISR) (26). *Pseudomonas fluorescens* strain WCS417r (WCS417r) has been shown to activate ISR in several plant species (27–29) including *Arabidopsis thaliana* (30). In *Arabidopsis*, WCS417r-mediated ISR is active against the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani* (30, 31), the oomycetous leaf pathogen *Peronospora parasitica* (J. Ton and C.M.J.P., unpublished results), and the bacterial leaf pathogens *Xanthomonas campestris* pv. *campestris* (J. Ton and C.M.J.P., unpublished results) and *Pseudomonas syringae* pv. *tomato* (*Pst*) (30, 31), indicating that, like SAR, WCS417r-mediated ISR is effective against different types of pathogens.

In *Arabidopsis*, SAR and ISR are regulated by distinct signaling pathways. As in many other plant species, pathogen-induced SAR is associated with local and systemic increases in endogenously synthesized SA and a coordinate expression of genes encoding pathogenesis-related (PR) proteins (32, 33). SA is a necessary intermediate in the SAR signal transduction pathway because SA-nonaccumulating NahG plants, expressing the bacterial SA hydroxylase gene *NahG*, are impaired in SAR (32). In contrast, WCS417r-mediated ISR functions independently of SA and *PR* gene activation (30, 31) but requires JA and ethylene signaling. The JA response mutant *jar1* and the ethylene response mutant *etr1*, that express normal levels of pathogen-induced SAR (32, 34, 35), did not express ISR upon treatment with WCS417r, indicating that the ISR-signaling pathway requires components of the JA and ethylene response (35, 36).

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Abbreviations: SAR, systemic acquired resistance; ISR, induced systemic resistance; SA, salicylic acid; JA, jasmonic acid; PR, pathogenesis-related; WCS417r, *Pseudomonas fluorescens* strain WCS417r; *Pst*, *Pseudomonas syringae* pv. *tomato*; *Pst(avrRpt2)*, *Pseudomonas syringae* pv. *tomato* carrying the *avrRpt2* gene; cfu, colony-forming unit.

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Although SAR and ISR follow distinct signaling pathways, they are both blocked in the regulatory mutant *npr1* (for *n*onexpresser of *PR* genes) of *Arabidopsis* (35, 37). NPR1 (also called NIM1 or SAI1) was originally discovered as a key regulatory protein that functions downstream of SA in the SAR pathway (37–39). Recently, Zhang *et al.* (40) provided evidence that, upon induction of SAR, NPR1 activates *PR-1* gene expression by physically interacting with a subclass of basic leucine zipper protein transcription factors that bind to promoter sequences required for SA-inducible *PR* gene expression. Elucidation of the sequence of ISR-signaling events revealed that NPR1 also functions downstream of the JA and ethylene response in the ISR pathway (35). Evidently, NPR1 is not only required for the SA-dependent expression of *PR* genes that are activated during SAR, but also for the JA- and ethylene-dependent activation of so far unidentified defense responses resulting from rhizobacteria-mediated ISR. The mechanism underlying the divergence of the SAR and the ISR pathway downstream of NPR1 is not known. Possibly, interactions of pathway-specific proteins with NPR1 are involved.

The requirement of the same regulatory component NPR1 for both SAR and ISR, combined with possible cross-talk between the SA- and JA-dependent signaling pathways, raises the question whether the SA-dependent SAR pathway and the JA-dependent ISR pathway interact negatively. Here, we demonstrate that the SAR and ISR pathway are fully compatible, resulting in an additive effect on the level of induced protection. Furthermore, we provide evidence that there is no significant cross-talk between the two pathways, suggesting that the additive effect on the level of induced protection is caused by the induction of complementary, NPR1-dependent defense responses that are both active against *Pst*.

Materials and Methods

Bacterial Strains, Plant Material, and Growth Conditions. Nonpathogenic, ISR-inducing *P. fluorescens* WCS417r rhizobacteria (WCS417r) (27) were grown on King's medium B agar plates (41) for 24 h at 28°C. Subsequently, bacterial cells were collected and resuspended in 10 mM $MgSO_4$.

The avirulent pathogen *P. syringae* pv. *tomato* DC3000 with the plasmid pV288 carrying the avirulence gene *avrRpt2* [*Pst(avrRpt2)*] (42) was used for induction of SAR. *Pst(avrRpt2)* bacteria were cultured overnight at 28°C in liquid King's medium B, supplemented with 25 mg/liter kanamycin to select for the plasmid. Bacterial cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$.

The virulent pathogen *P. syringae* pv. *tomato* strain DC3000 (*Pst*) (43), used for challenge inoculations, was grown overnight in liquid King's medium B at 28°C. After centrifugation, the bacterial cells were resuspended in 10 mM $MgSO_4$ containing 0.01% (vol/vol) of the surfactant Silwet L-77 (van Meeuwen Chemicals BV, Weesp, The Netherlands).

Seeds of wild-type *A. thaliana* ecotype Col-0, transgenic NahG plants harboring the bacterial *NahG* gene (44), and mutant *cpr1* (45), *jar1* (46), *etr1* (47), and *npr1* (37) plants were sown in quartz sand. Two-week-old seedlings were transferred to 60-ml pots containing a mixture of sand and potting soil that had been autoclaved twice for 20 min 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 $MgSO_4$. Plants were cultivated in a growth chamber with a 9-h day (200 $\mu E/m^2/sec$ 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 modified one-half strength Hoagland's nutrient solution, as described (31).

Induction Treatments. Plants were treated with ISR-inducing rhizobacteria by transferring the seedlings to soil that was mixed with a suspension of WCS417r rhizobacteria to a final density of 5×10^7 colony-forming units (cfu)/g of soil. The plants were grown in this soil for 3 wk before they were challenged with *Pst*. 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-wk-old plants, using a 1-ml syringe without a needle. Alternatively, the leaves of 5-wk-old plants were dipped in a solution containing 1 mM SA and 0.01% (vol/vol) Silwet L-77. Control plants were treated with 0.01% (vol/vol) Silwet L-77 only. Plants were challenged with *Pst* 3 days later.

Challenge Inoculation and Disease Assessment. Five-week-old plants were challenge inoculated by dipping the leaves for 2 sec in a suspension of the virulent pathogen *Pst* at 2.5×10^7 cfu/ml in 10 mM $MgSO_4$ and 0.01% (vol/vol) Silwet L-77. Because NahG plants are highly susceptible to *Pst* infection, a 10-fold lower bacterial density was used for inoculation of these plants. Before challenge (1 day), the plants were placed at 100% relative humidity. After challenge (4 days), disease severity was assessed by determining the percentage of diseased leaves per plant and by examining growth of the challenging pathogen in the leaves. Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. With the number of diseased and nondiseased leaves, the disease index was calculated for each plant (20 plants per treatment). The number of *Pst* bacteria in challenged leaves was assessed in three (Table 1) or five (Fig. 1*D*) samples per treatment. Each sample consisted of the leaves of one (Fig. 1*D*) or six (Table 1) whole plants. The leaf tissue was weighed and homogenized in 10 mM $MgSO_4$. Subsequently, appropriate dilutions were plated onto King's medium B agar supplemented with 50 mg/liter rifampicin and 100 mg/liter cycloheximide. After incubation at 28°C for 2 days, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined.

RNA Blot Analysis. Leaves were collected just before the challenge inoculation. Total RNA was extracted by homogenizing 2 g of frozen leaf tissue in 2 ml of extraction buffer [0.35 M glycine/0.048 M NaOH/0.34 M NaCl/0.04 M EDTA/4% (wt/vol) SDS]. The homogenates were extracted with phenol and chloroform, and the RNA was precipitated using LiCl, as described by Sambrook *et al.* (48). For RNA analysis, 15 μg of RNA was denatured by using glyoxal and DMSO as described (48). Subsequently, samples were electrophoretically separated on 1.5% agarose gels and blotted onto Hybond-N^+ membranes (Amersham) 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 *PR-1* or *Npr1* gene-specific probes as described previously (31). To check for equal loading, the blots were stripped and hybridized with a probe for the constitutively expressed β-tubulin (*Tub*) gene. The α-[^{32}P]dCTP-labeled cDNA probes were synthesized by random primer labeling (48). The *PR-1* probe was derived from an *Arabidopsis PR-1* cDNA clone (49). Probes for the detection of *Npr1* and *Tub* transcripts were prepared by PCR with primers based on sequences of *Arabidopsis* obtained from GenBank accession nos. U76707 and M21415, respectively.

Results

Simultaneous Activation of SAR and ISR Results in an Enhanced Level of Protection. The effect of simultaneous activation of the SA-dependent SAR pathway and the JA-dependent ISR pathway on the level of systemically induced protection was examined in *Arabidopsis* ecotype Col-0. SAR was induced 3 days before challenge by pressure infiltrating three lower leaves with avirulent *Pst(avrRpt2)* bacteria or dipping the leaves in a solution

Table 1. Number of *Pst* bacteria in challenged leaves of different *Arabidopsis* genotypes pretreated with WCS417r, *Pst(avrRpt2)*, SA, or a combination of inducers

	cfu/g ($\times 10^6$)*				cfu/g ($\times 10^8$)*			
	Exp. 1	Exp. 2	Exp. 3		Exp. 4			
Treatment	Col-0	Col-0	Col-0	*cpr1*	*jar1*	*etr1*	NahG	*npr1*
Ctrl	24 ± 4^a	17 ± 2^a	122 ± 17^a	30 ± 2^a	138 ± 6^b	116 ± 31^a	156 ± 22^a	72 ± 9^a
WCS417r	15 ± 2^b	10 ± 2^b	55 ± 3^b	22 ± 2^b	181 ± 21^a	112 ± 5^a	81 ± 9^b	80 ± 12^a
Pst(avrRpt2)	11 ± 1^b				31 ± 3^c	54 ± 3^b	173 ± 48^a	68 ± 28^a
Pst(avrRpt2) + WCS417r	6 ± 2^c				34 ± 5^c	60 ± 19^b	73 ± 7^b	68 ± 10^a
SA		9 ± 2^b						
SA + WCS417r		5 ± 1^b						

*Values presented are average numbers ($\pm$ SE) of cfu/g fresh weight, each from three sets of six whole shoots harvested 4 days after challenge inoculation with virulent *Pst*, and correspond to the bioassays shown in Fig. 1*A* (Exp. 1), Fig. 1*B* (Exp. 2), Fig. 1*C* (Exp. 3), and Fig. 2 (Exp. 4). The number of *Pst* bacteria present in the leaves just after challenge ranged from 2×10^5 to 10^6 cfu/g but was similar for all treatments within single experiments. Within each genotype, different letters indicate statistically significant differences between treatments (Fisher's Least Significant Differences test, $\alpha = 0.05$). Similar results were obtained in repeated experiments, except for the difference between control- and WCS417r-treated *jar1* plants, which was not apparent in the repeats nor in similar experiments in other studies (35).

containing 1 mM SA. ISR was induced by growing the plants in soil containing WCS417r bacteria for 3 wk. Four days after challenge with virulent *Pst* bacteria, the plants had developed typical bacterial speck disease symptoms, consisting of necrotic or water-soaked spots surrounded by extensive chlorosis. Disease severity was assessed by determining the percentage of leaves with symptoms per plant. Pretreatment with either SAR-inducing *Pst(avrRpt2)* or SA, or with ISR-inducing WCS417r resulted in a significant reduction in the proportion of diseased leaves compared with noninduced control plants (Fig. 1 *A* and *B*). Plants induced by a combination of the WCS417r treatment and either the *Pst(avrRpt2)* or SA treatment showed a statistically significant higher reduction in disease severity than plants treated with either inducer alone. Determination of the number of *Pst* bacteria in challenged leaves revealed that proliferation of *Pst* was significantly inhibited in plants treated with either WCS417r, *Pst(avrRpt2)*, or SA (Table 1, experiments 1 and 2). Plants treated with both inducers showed an even more pronounced inhibition of pathogen growth, although for the SA/WCS417r combination this was not statistically significant at the $\alpha = 0.05$ level ($P < 0.15$). These results demonstrate that simultaneous induction of pathogen-induced SAR and rhizobacteria-mediated ISR results in further enhancement of the level of protection compared to that obtained by activation of either SAR or ISR alone.

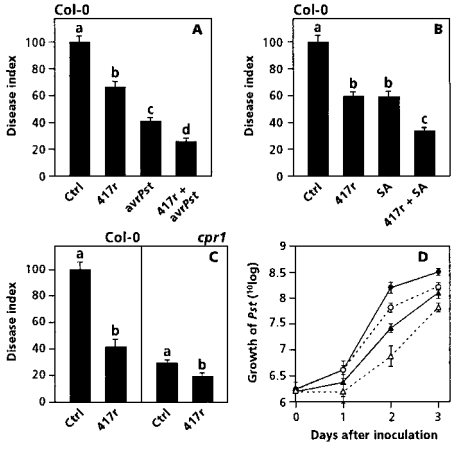

Fig. 1. Induced protection against *Pst* in *Arabidopsis* plants expressing ISR, SAR, or both types of induced resistance. ISR was induced by growing the plants in soil containing WCS417r (417r) at 5×10^7 cfu/g. SAR was induced in wild-type Col-0 plants by preinfecting three leaves per plant with avirulent *Pst(avrRpt2)* (avr*Pst*) at 10^7 cfu/ml (*A*), or by exogenous application of 1 mM SA (*B*) 3 days before challenge inoculation. Mutant *cpr1* constitutively expressed SAR (*C*). The disease index is the mean $\pm$ SE ($n = 20$ plants) of the proportion of leaves with symptoms per plant relative to that of control-treated (Ctrl) Col-0 plants (set at 100%), 4 days after challenge with virulent *Pst*. The absolute proportions of diseased leaves of the control-treated Col-0 plants depicted in *A*, *B*, and *C* were 55%, 52, and 71%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fischer's Least Significant Differences test; $\alpha = 0.05$). Corresponding bacterial growth data are given in Table 1. The data presented are from representative experiments that were repeated at least twice with similar results. In *D*, growth curves of *Pst* in Col-0 plants expressing ISR and in *cpr1* plants expressing either SAR or both SAR and ISR. Values presented are average numbers ($\pm$SE) of cfu/g fresh weight, each from five whole shoots harvested 0, 1, 2, or 3 days after challenge with *Pst*. For experimental details see text and Table 1 legend. The additive effect on inhibition of pathogen growth in the combination treatment was statistically significant at all time points tested (Fisher's Least Significant Differences test; $\alpha = 0.05$). Circles, Col-0 plants; triangles, *cpr1* plants; solid lines with closed symbols, control treatment; and dotted lines with open symbols, WCS417r treatment.

In the experiment described above, ISR was induced first and the level of protection could be enhanced by subsequent induction of SAR. To investigate whether a similar enhancement of resistance could be achieved when instead of ISR, SAR was induced first, we examined the ISR-inducibility of mutant *cpr1* of *Arabidopsis*. Mutant *cpr1* exhibits high levels of SA and *PR* gene expression, leading to constitutive expression of SAR (45). Compared to control-treated wild-type plants, control-treated *cpr1* plants showed a 70% lower proportion of leaves with symptoms after inoculation with *Pst* and a 4-fold decrease in growth of *Pst* in the challenged leaves, confirming that the *cpr1* plants expressed SAR (Fig. 1*C* and Table 1, experiment 3). By growing *cpr1* plants in soil containing WCS417r, a statistically significant higher level of protection was evident in these plants. In an additional experiment, we monitored growth of *Pst* at different time points after infection. Again, proliferation of *Pst* was significantly inhibited in WCS417r-treated *cpr1* plants at all time points tested (Fig. 1*D*). The ability of mutant *cpr1* to express ISR provides another demonstration that the level of induced resistance can be increased by concurrent expression of SAR and ISR, irrespective of the sequence in which SAR and ISR are induced.

Cross-Talk Between the SAR and the ISR-Signaling Pathway Is Absent. The enhanced state of induced protection after simultaneous activation of SAR and ISR may result from additive effects of two complementary defense responses, or from synergistic ef-

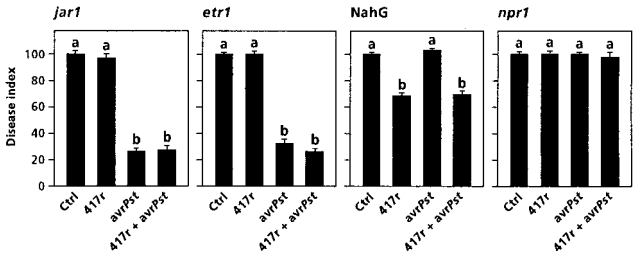

Fig. 2. Quantification of protection against *Pst* in *Arabidopsis* genotypes *jar1*, *etr1*, NahG, and *npr1* after treatment with the ISR inducer WCS417r, the SAR inducer *Pst(avrRpt2)*, or a combination of both inducers. For experimental details see the text and Fig. 1 legend. The absolute proportions of diseased leaves of control-treated *jar1*, *etr1*, NahG, and *npr1* plants were 82%, 75%, 89%, and 70%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fischer's Least Significant Differences test; $\alpha = 0.05$). The data presented are from a representative experiment that was repeated twice with similar results.

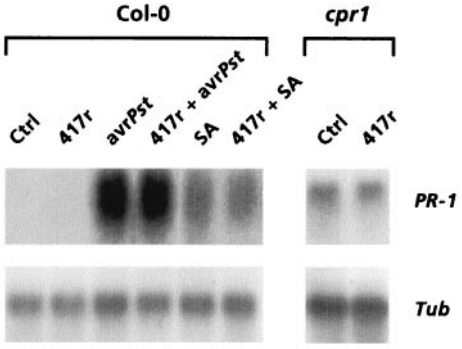

Fig. 3. RNA blot analysis of the expression of the SAR response gene *PR-1* in *Arabidopsis* plants expressing ISR, SAR, or both. ISR was induced by growing the plants in soil containing WCS417r (417r). SAR was induced in wild-type Col-0 plants either by preinfecting three leaves per plant with *Pst(avrRpt2)* (avr*Pst*) or by dipping the plants in 1 mM SA 3 days before harvest, or was constitutively expressed in mutant *cpr1*. Of the *Pst(avrRpt2)*-induced plants, the systemic, noninoculated tissue was collected. A *PR-1* gene-specific probe was used to detect *PR-1* transcripts. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β-tubulin (*Tub*).

fects on either the SAR or ISR response. Such synergistic effects might result from cross-talk between both pathways, leading to stimulation of either the SAR or the ISR response, or both. To assess whether early signaling steps in the SAR and the ISR pathway influence the expression of ISR and SAR, respectively, we investigated whether *Pst(avrRpt2)* stimulates the ISR response in genotypes that are impaired in the expression of SAR and whether WCS417r stimulates the SAR response in mutants that are impaired in the expression of ISR. Consistent with previous findings (32, 34, 35, 37), WCS417r-mediated ISR was blocked in the JA response mutant *jar1*, the ethylene response mutant *etr1*, and the SAR and ISR regulatory mutant *npr1*, whereas SAR was abolished in the SA-nonaccumulating NahG transgenic and the *npr1* mutant (Fig. 2). In contrast to wild-type plants (Fig. 1*A*), treatment with both *Pst(avrRpt2)* and WCS417r did not result in an enhanced level of protection in *jar1*, *etr1*, NahG, and *npr1* plants (Fig. 2). In the mutants *jar1* and *etr1*, the combination of treatments induced the same level of protection as *Pst(avrRpt2)* alone, whereas in NahG plants the combination of treatments protected the plants to the same extent as WCS417r alone. Mutant *npr1*, which is blocked in both the SAR and the ISR pathway, showed no induced protection at all. As in wild-type plants, the observed reduction in symptoms through induction of either SAR or ISR was associated with inhibition of growth of *Pst* in challenged leaves, but treatment with both inducers did not further reduce the number of *Pst* bacteria in the mutants (Table 1, experiment 4). These results indicate that components of the ISR pathway, acting upstream of the JA and the ethylene response, have no effect on the level of SAR attained. Moreover, components of the SAR pathway acting upstream of SA do not influence the expression of ISR.

Furthermore, we studied whether the induction of ISR affects SAR-associated *PR* gene expression. Therefore, we studied the effect of WCS417r treatment on *PR-1* gene activation in SAR-expressing plants. RNA blot analysis demonstrated that the *PR-1* gene was not expressed in WCS417r-treated wild-type Col-0 plants (Fig. 3). Significant amounts of *PR-1* mRNA were detected in Col-0 plants expressing *Pst(avrRpt2)*- or SA-induced SAR and in control-treated *cpr1* mutants constitutively expressing SAR. In plants simultaneously expressing SAR and ISR, the level of *PR-1* transcript accumulation was similar to that observed in plants expressing SAR only (Fig. 3). This indicates that the SAR pathway is neither stimulated nor suppressed in plants expressing both SAR and ISR.

Basal *Npr1* Transcript Levels Are Sufficient to Facilitate Simultaneous Expression of both SAR and ISR. Overexpression of the SAR- and ISR-regulatory gene *Npr1* in *Arabidopsis* has been demonstrated to increase resistance against *P. syringae* pv. *maculicola* and *Peronospora parasitica* (50). To investigate whether the increased level of induced protection observed in plants expressing both SAR and ISR can be explained by an increase in *Npr1* gene expression, transcript levels of this gene were assessed in plants expressing SAR, ISR, or both. Fig. 4 shows that the expression level of *Npr1* was not elevated in tissues expressing either WCS417r-mediated ISR, *Pst(avrRpt2)*-induced SAR, or both types of induced resistance, suggesting that the enhanced level of induced resistance in plants simultaneously expressing SAR and ISR is not related to an increased availability of the NPR1 protein.

Discussion

Plants are capable of differentially activating distinct defense pathways, depending on the inducing agent. SA, JA, and ethylene play an important role in this signaling network. Cross-talk between SA-, JA-, and ethylene-dependent signaling pathways is thought to play an important role in fine-tuning complex defense responses (1–5). Previously, it was shown that SA is a potent inhibitor of JA-dependent defense responses (14–21). There is also evidence for inhibition of salicylate action by JA (18). Therefore, the SA-dependent SAR pathway and the JA-

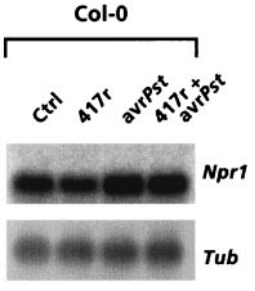

Fig. 4. RNA blot analysis of the expression of the SAR and ISR regulatory gene *Npr1* in *Arabidopsis* Col-0 plants expressing ISR, SAR, or both types of induced resistance. ISR was induced by growing the plants in soil containing WCS417r (417r). SAR was induced 3 days before harvest by preinfecting three leaves per plant with *Pst(avrRpt2)* (avr*Pst*). Of the *Pst(avrRpt2)*-induced plants, the systemic, noninoculated tissue was collected. A *Npr1* gene-specific probe was used to detect *Npr1* transcripts. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β-tubulin (*Tub*).

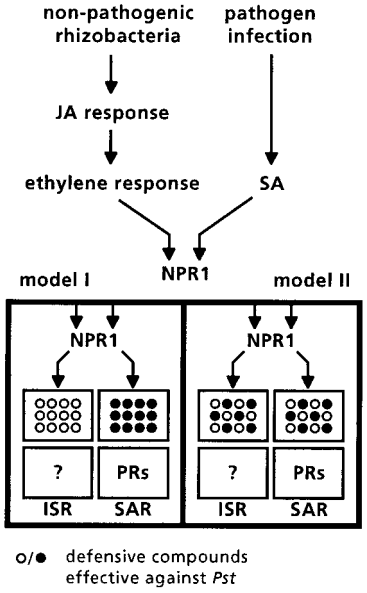

Fig. 5. Model for the enhanced level of induced protection against *Pst* in *Arabidopsis* plants simultaneously expressing SAR and ISR. Pathogen-induced SAR is dependent on SA, requires NPR1, and is associated with *PR* gene expression (25, 54). WCS417r-mediated ISR requires responsiveness to JA and ethylene, also is dependent on NPR1 but is not associated with *PR* gene expression (35), indicating that downstream of NPR1 the pathways diverge. PR proteins that accumulate in plants expressing SAR are unlikely to contribute to induced resistance against *P. syringae* pathogens (53). Cross-talk between the SAR and the ISR pathway is absent. Simultaneous activation of SAR and ISR results in an additive effect on the level of protection against *Pst*. This is not accompanied by an increase in the expression of the *Npr1* gene. Therefore, the enhanced level of induced protection against *Pst* must be accomplished through the parallel activation of so far unidentified defense responses that are all effective against *Pst*. The complementary effects on the level of protection could be achieved through the production of distinct (model I) or more of the same antibacterial gene products (model II). In both cases, simultaneous induction of SAR and ISR leads to enhanced levels of defensive components that are active against *Pst*. Question marks indicate unidentified defensive components.

dependent ISR pathway might have an impact on each other's performance. However, we demonstrated that the SA-dependent SAR pathway is fully compatible with the JA-dependent ISR pathway. Simultaneous activation of both pathways resulted in an additive effect on the level of induced protection against *Pst*. This additive effect was established irrespective of whether SAR was expressed constitutively, as in *cpr1* plants, or was induced by predisposal infection with *Pst(avrRpt2)* or exogenous application of SA (Fig. 1). A single inoculation of *Arabidopsis* with *Pst(avrRpt2)* has been shown to be sufficient for induction of the maximum level of SAR (51). Indeed, we observed no elevated levels of SAR in plants that were treated with both *Pst(avrRpt2)* and SA (unpublished data). WCS417r-mediated ISR was expressed at a maximum level as well, because a 100-fold lower density of WCS417r in the rhizosphere than that used in our experiments is already sufficient to induce the maximum level of protection (unpublished data). Therefore, the additive effect on the level of induced protection must be accomplished through complementary effects of SAR- and ISR-specific defense responses that are both effective against *Pst*.

In plants expressing either ISR or SAR, protection was typically manifested as a relative reduction in symptoms of 40–60%. This reduction was enhanced up to 80% when ISR and SAR were expressed simultaneously. Although in most cases statistically significant, the effects on pathogen growth were less pronounced (up to 4-fold). Nevertheless, these reductions in pathogen growth are in the range of what is found in most studies on biologically induced resistance against *P. syringae* in *Arabidopsis*. Except for Cao *et al.* (37) who found a 200-fold reduction of growth of *P. syringae* pv. *maculicola* upon induction of SAR by *Pst(avrRpt2)*, others have found growth reductions ranging between 2- and 10-fold (32, 51, 52). Despite the relatively low effect of induced resistance on pathogen proliferation, inhibition of *Pst* growth was consistently found at different days after inoculation and to a higher extent in the combination treatments (Fig. 1*D*; Table 1). The incongruity between the magnitude of the effect of induced resistance on symptom development on the one hand and pathogen growth on the other hand, might be caused by the possibility that induced resistance has an effect on both growth and activity of the pathogen. If this is the case, then one would expect a stronger reduction of disease symptoms than could be expected from the bacterial growth data alone. This is exactly what we observed. However, whether induced resistance exerts an effect on the activity of bacterial pathogens needs to be elucidated.

The enhanced level of protection was absent in *Arabidopsis* genotypes NahG, *jar1*, and *etr1* that are affected in either SAR or ISR. Transgenic NahG plants that are impaired in the SAR response showed a similar level of ISR when treated with either WCS417r or both WCS417r and *Pst(avrRpt2)* (Fig. 2). Moreover, mutants *jar1* and *etr1*, which are blocked in the ISR response, developed similar levels of SAR after receiving the *Pst(avrRpt2)* treatment or the combination treatment (Fig. 2). This indicates that, upstream of the perception of either SA, JA, or ethylene, cross-talk between the pathways does not occur or, at least, has no influence on the outcome of the induced resistance effective against *Pst*. Moreover, in plants expressing SAR, either constitutively or after induction by *Pst(avrRpt2)*, the magnitude of *PR-1* gene expression was unaltered when ISR was expressed as well (Fig. 3). This demonstrates that activation of the ISR pathway does not sensitize the tissue for SAR expression. Thus, the additive effect on the level of protection in plants expressing both SAR and ISR is unlikely to be caused by cross-talk between the signaling pathways, but rather results from a parallel activation of defense responses with complementary effects against *Pst*.

NPR1 is a key regulator of both SAR and ISR. In the SAR pathway, NPR1 regulates the SA-dependent expression of *PR* genes (37–39), whereas in the ISR pathway it is required for the expression of the JA- and ethylene-dependent enhanced defensive capacity (35). Our finding that simultaneous expression of SAR and ISR results in an enhanced level of protection indicates that the SAR and the ISR pathway do not compete for NPR1. Apparently, the pool of NPR1 protein is sufficient to allow simultaneous expression of SAR and ISR. Recently, Cao *et al.* (50) demonstrated that elevated levels of NPR1 protein, through overexpression of the *Npr1* gene, leads to enhanced resistance against *P. syringae* pv. *maculicola* and *Peronospora parasitica*. This raised the question whether the enhanced level of induced resistance observed in plants expressing both SAR and ISR is based on elevated levels of NPR1. We did not observe an increase in the expression of the *Npr1* gene in leaves expressing either SAR, ISR, or both types of induced resistance (Fig. 4). This strongly suggests that the constitutive level of NPR1 is sufficient to facilitate the expression of both types of induced resistance.

Recently, Clarke *et al.* (53) demonstrated that the enhanced resistance against *P. syringae* pv. *maculicola*, observed in the constitutively SAR-expressing mutant *cpr6*, is blocked in the *cpr6 npr1* double mutant, despite unaltered constitutive expression of *PR* genes. This indicates that induced resistance against *P. syringae* is independent of PR proteins and must be accomplished through so far unidentified antibacterial factors that are regulated through NPR1 (53). Whether the same compounds are

involved in ISR against *Pst* is currently unknown. The mechanism underlying the additive effect on the level of induced protection in plants expressing both SAR and ISR can be hypothesized in different ways. The SAR and the ISR pathway may generate distinct defensive compounds that are both effective against *Pst* (Fig. 5, model I). Alternatively, activation of both the ISR and the SAR pathway may lead to the production of the same antibacterial compounds, but these compounds do not accumulate to maximal levels when only SAR or ISR is induced (Fig. 5, model II). In both scenarios, concurrent activation of SAR and ISR leads to higher levels of defensive compounds that are active against *Pst*.

In conclusion, this study demonstrates that plants are capable of expressing SA-, JA-, and ethylene-dependent defense responses at the same time without antagonistic effects; leading to an elevated level of protection against pathogen attack. Therefore, simultaneous activation of ISR and SAR provides an attractive tool for the improvement of disease control.

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