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RESEARCH ARTICLE

Systemic Resistance in Arabidopsis Induced by Biocontrol Bacteria Is Independent of Salicylic Acid Accumulation and Pathogenesis-Related Gene Expression

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Systemic acquired resistance is a pathogen-inducible defense mechanism in plants. The resistant state is dependent on endogenous accumulation of salicylic acid (SA) and is characterized by the activation of genes encoding pathogenesis-related (PR) proteins. Recently, selected nonpathogenic, root-colonizing biocontrol bacteria have been shown to trigger a systemic resistance response as well. To study the molecular basis underlying this type of systemic resistance, we developed an Arabidopsis-based model system using *Fusarium oxysporum* f sp *raphani* and *Pseudomonas syringae* pv *tomato* as challenging pathogens. Colonization of the rhizosphere by the biological control strain WCS417r of *P. fluorescens* resulted in a plant-mediated resistance response that significantly reduced symptoms elicited by both challenging pathogens. Moreover, growth of *P. syringae* in infected leaves was strongly inhibited in *P. fluorescens* WCS417r-treated plants. Transgenic Arabidopsis NahG plants, unable to accumulate SA, and wild-type plants were equally responsive to *P. fluorescens* WCS417r-mediated induction of resistance. Furthermore, *P. fluorescens* WCS417r-mediated systemic resistance did not coincide with the accumulation of PR mRNAs before challenge inoculation. These results indicate that *P. fluorescens* WCS417r induces a pathway different from the one that controls classic systemic acquired resistance and that this pathway leads to a form of systemic resistance independent of SA accumulation and PR gene expression.

INTRODUCTION

Induced disease resistance is the phenomenon by which a plant exhibits an increased level of resistance to infection by a pathogen after appropriate stimulation. This resistance response, first characterized by Ross (1961a, 1961b), is expressed systemically throughout the plant and is effective against a broad spectrum of viral, bacterial, and fungal pathogens (reviewed in Hammerschmidt and Kuć, 1995).

In general, induced resistance can be triggered in three ways: (1) by a predisposing infection with a necrotizing pathogen (Ross, 1961a, 1961b; Kuć, 1982); (2) by treatment with certain chemicals, such as salicylic acid (SA; White, 1979; Malamy and Klessig, 1992) or 2,6-dichloroisonicotinic acid (INA; Métraux et al., 1991); or (3) by colonization of the rhizosphere with selected plant growth–promoting rhizobacteria (PGPR; Alström, 1991; van Peer et al., 1991; Wei et al., 1991). Systemic resistance induced by the first two types of inducers is termed systemic acquired resistance (SAR; Ross, 1961b), whereas PGPR-mediated protection is generally referred to as induced systemic resistance (ISR; Kloepper et al., 1992).

Pathogen-induced SAR has been studied most extensively in tobacco and cucumber (Kuć, 1982; Ward et al., 1991) and was recently demonstrated in Arabidopsis as well (Uknes et al., 1993; Cameron et al., 1994; Mauch-Mani and Slusarenko, 1994). The resistant state is characterized by an increase in endogenously synthesized SA at the onset of SAR (Malamy et al., 1990; Métraux et al., 1990). Accumulation of SA appears to be critical for the induction of the SAR-signaling pathway because transgenic plants unable to accumulate SA are incapable of developing SAR (Gaffney et al., 1993). Furthermore, SAR is associated with the coordinate expression of a set of so-called SAR genes (Ward et al., 1991). These SAR genes include genes encoding pathogenesis-related (PR) proteins (van Loon, 1985), of which some exhibit limited antifungal activity in vitro (Mauch et al., 1988; Roberts and Selitrennikoff, 1988; Vigers et al., 1991; Woloshuk et al., 1991) as well as in vivo (Broglie et al., 1991; Alexander et al., 1993; Liu et al., 1994). Exogenous application of the chemicals SA or INA has been reported to mimic pathogen-induced SAR because they induce resistance to the same spectrum of pathogens and concurrently activate the expression of SAR genes (Ward et al., 1991; Uknes et al., 1992, 1993). Although these observations

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are highly suggestive of a causal relationship between accumulation of PR proteins and SAR, definitive evidence that they are responsible for broad-spectrum resistance is still lacking.

Selected PGPR, mainly fluorescent *Pseudomonas* spp, have been demonstrated to control plant diseases effectively by suppressing pathogens and deleterious microorganisms through siderophore-mediated competition for iron, or antibiosis (reviewed in Schippers, 1992; Thomashow and Weller, 1995). Recently, research on mechanisms of biological control by PGPR revealed that some PGPR strains protect plants against pathogen infection through induction of systemic resistance, without provoking any symptoms themselves. Alström (1991) demonstrated *P. fluorescens*–mediated ISR in bean against halo blight caused by *P. syringae* pv *phaseolicola*, van Peer et al. (1991) in carnation against fusarium wilt, and Wei et al. (1991) in cucumber against *Colletotrichum orbiculare* infection. More recently, PGPR-mediated induction of ISR has been reported for several other plant–pathogen systems (Maurhofer et al., 1994; Zhou and Paulitz, 1994; Leeman et al., 1995a; Liu et al., 1995).

Maurhofer et al. (1994) showed that ISR induced by strain CHA0 of *P. fluorescens* in tobacco against tobacco necrosis virus was accompanied by an increase in PR protein accumulation, suggesting that PGPR-mediated ISR and pathogen-induced SAR are manifestations of a similar defense mechanism. However, Hoffland et al. (1995) were unable to establish an accumulation of PR proteins in radish displaying substantial ISR against *Fusarium oxysporum* when plants were treated with strain WCS417r of *P. fluorescens*. Therefore, it is unclear whether PGPR-mediated ISR and pathogen-induced SAR share a common signal transduction pathway. With the goal of addressing whether a common pathway is shared, two bioassays for PGPR-mediated ISR were developed by using Arabidopsis as the host plant and a rifampicin-resistant mutant of the nonpathogenic, root-colonizing PGPR strain WCS417 of *P. fluorescens* (*P. fluorescens* WCS417r) as an inducer. *P. fluorescens* WCS417 is an effective biocontrol agent of the take-all disease in wheat caused by *Gaeumannomyces graminis* pv *tritici* (Lamers et al., 1988) and has been demonstrated to be a strong inducer of ISR against vascular wilt caused by *F. oxysporum* in carnation and radish (van Peer et al., 1991; Leeman et al., 1995a). Previously, van Peer and Schippers (1992) and Leeman et al. (1995b) demonstrated that the extracellular lipopolysaccharide (LPS) of *P. fluorescens* WCS417r is sufficient to elicit ISR in carnation and radish, respectively, indicating that this type of resistance is not based on microbial antagonism but is plant mediated. In this study, we describe *P. fluorescens* WCS417r–mediated ISR in Arabidopsis against the fungal root pathogen *F. oxysporum* f sp *raphani* and the bacterial leaf pathogen *P. syringae* pv *tomato*. In addition, we provide evidence that, in contrast to classic SAR, induction of *P. fluorescens* WCS417r–mediated ISR is independent of both endogenous SA accumulation and PR gene activation.

RESULTS

PGPR-Mediated ISR against *F. oxysporum*

The soil-borne fungus *F. oxysporum* is the causal agent of vascular wilt in a wide range of host plants, including economically important crops. The fungus invades the roots and colonizes the vascular tissue. Arabidopsis is susceptible to infection by *F. o. raphani*. To study PGPR-mediated ISR against this pathogen in Arabidopsis, a system ensuring spatial separation of the inducing agent and the challenging root pathogen was used as described by Leeman et al. (1995a). For induction, a rifampicin-resistant mutant of the PGPR strain WCS417 of *P. fluorescens* (*P. fluorescens* WCS417r; van Peer et al., 1991) was used. A suspension of *P. fluorescens* WCS417r in 10 mM $MgSO_4$, mixed with talcum powder as a carrier, was applied to the lower part of the root system. As a control, 10 mM $MgSO_4$ was applied in a similar manner. Three days later, the upper parts of the roots were inoculated with *F. oxysporum* or mock-inoculated with sterilized peat. After inoculation, a relatively long latent period (2 weeks) during which no fusarium wilt symptoms were visible was followed by a phase of rapid development of symptoms. Thereafter, leaves turned yellow, beginning with the veins, then wilted, and finally died. Occasionally, wilting of the leaves started before yellowing became apparent. Progressive development of disease eventually led to death of the plant.

Figures 1A and 1B show typical differences in symptom expression of fusarium wilt between nontreated Arabidopsis ecotype Columbia (Col-0) plants and *P. fluorescens* WCS417r–treated Col-0 plants. As shown in Figure 2A, in control plants the percentage of leaves per plant showing yellowing and wilting increased markedly between 17 and 24 days after inoculation. In *P. fluorescens* WCS417r–treated plants, disease progressed substantially more slowly, and the number of plants with 76 to 100% diseased leaves was reduced by 80% at 24 days after inoculation. Thus, colonization of the rhizosphere by *P. fluorescens* WCS417r resulted in a delay in symptom development and reduction of disease severity. Similar results were obtained with ecotype Landsberg *erecta* (Ler) (data not shown).

To examine the inducing potential of *P. fluorescens* WCS417r in comparison with SA, which is known to be an effective inducer of SAR (Malamy and Klessig, 1992; Uknes et al., 1993), induction treatments with 1 mM SA and *P. fluorescens* WCS417r were performed. Both agents caused a statistically significant reduction of symptoms compared with the control treatment (Figure 2B). There were no statistically significant differences in disease severity between the SA- and *P. fluorescens* WCS417r–treated plants. In all experiments, mock-inoculated control plants remained healthy, and no apparent differences in the growth and development of control, *P. fluorescens* WCS417r–, and SA-treated plants were observed.

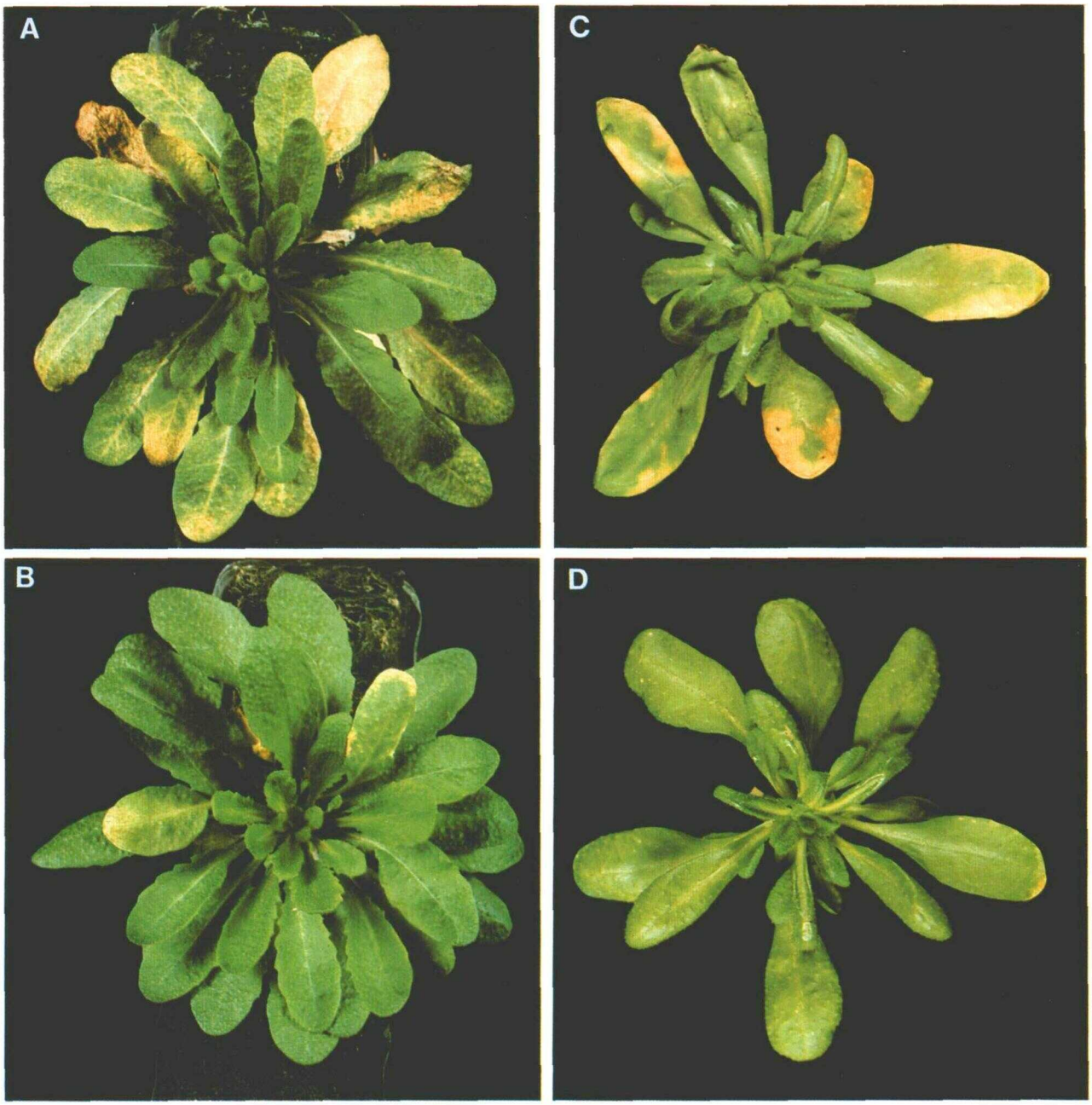

Figure 1. Response of Arabidopsis to *F. oxysporum* and *P. syringae* Infection after *P. fluorescens* WCS417r–Mediated Induction of ISR.

(A) and **(B)** Fusarium wilt symptoms on Col-0 plants pretreated with 10 mM $MgSO_4$ **(A)** or *P. fluorescens* WCS417r **(B)** 19 days after challenge inoculation with *F. oxysporum*. Inoculations were performed by applying 10^6 conidia of *F. oxysporum* in 0.25 g of peat to the upper parts of the roots that were spatially separated from the treated lower parts of the roots.

(C) and **(D)** Symptoms caused by *P. syringae* infection on L*er* plants grown in soil supplemented with 10 mM $MgSO_4$ **(C)** or *P. fluorescens* WCS417r **(D)** 4 days after challenge inoculation. Leaves were inoculated with *P. syringae* by dipping them into a 10^8 cfu/mL bacterial suspension in 10 mM $MgSO_4$, 0.01% Silwet L-77.

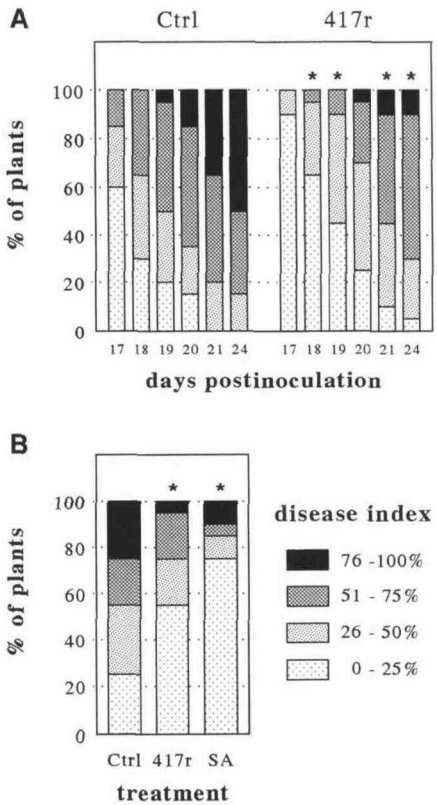

Figure 2. Classification of Diseased Plants in the Arabidopsis–*F. oxysporum* Bioassay.

(A) Time course of fusarium wilt symptom development in control and *P. fluorescens* WCS417r–treated Arabidopsis plants 17 to 24 days after inoculation.

(B) Disease severity in SA-treated, *P. fluorescens* WCS417r–treated, and noninduced Arabidopsis plants 19 days after inoculation with *F. oxysporum*.

The lower parts of the roots, which were spatially separated from the inoculated upper parts of the roots, were treated with 10 mM $MgSO_4$ (Ctrl), *P. fluorescens* WCS417r (417r), or 1 mM SA (SA). The disease index is based on the percentage of leaves per plant showing fusarium wilt symptoms. Asterisks indicate statistically significant differences in frequency distribution compared with the noninduced control plants (**[A]** Wilcoxon's two sample test, $\alpha = 0.05$, $n = 20$; **[B]** Kruskal–Wallis multiple comparison test, $\alpha = 0.05$, $n = 20$).

PGPR-Mediated ISR against *P. syringae*

P. s. tomato causes bacterial speck disease of tomato and has been demonstrated to be virulent on Arabidopsis as well (Dong et al., 1991; Whalen et al., 1991). Previously, Arabidopsis was shown to develop SAR against infection with *P. syringae* when induced by necrogenic pathogens (Uknes et al., 1993) or chemical agents (Uknes et al., 1992). To test for PGPR-mediated ISR in this pathosystem, Arabidopsis seedlings were planted in soil with or without *P. fluorescens* WCS417r. Five-week-old plants were challenge-inoculated with *P. syringae*. As a positive control, a solution of 1 mM SA was applied to the plants as a soil drench 7 and 4 days before challenge inoculation.

Four days after inoculation with *P. syringae*, leaves of control L*er* plants displayed necrotic lesions surrounded by extensive spreading chlorosis (Figure 1C), whereas L*er* plants pretreated with *P. fluorescens* WCS417r (Figure 1D) or SA showed significantly fewer symptoms. Mock-inoculated control plants remained symptomless. Plants grown in sand/potting soil mixture supplemented with *P. fluorescens* WCS417r bacteria were slightly larger than control and SA-treated plants, indicating that this PGPR strain can stimulate plant growth in Arabidopsis as observed in other species (van Peer and Schippers, 1989).

Protection against *P. syringae* was quantified by assessing the proportion of leaves per plant showing symptoms 4 or 5 days after challenge inoculation. Figure 3A shows that treatment of L*er* plants with *P. fluorescens* WCS417r or SA resulted in a statistically significant reduction of the percentage of leaves with symptoms. The level of protection against *P. syringae* infection induced by *P. fluorescens* WCS417r was similar to that induced by SA (a disease severity 60 and 65%, respectively, of that observed in the control).

To examine whether the observed reduction of symptoms was associated with diminished pathogen growth in the leaves, the number of *P. syringae* cells was monitored in inoculated leaves of control, *P. fluorescens* WCS417r–, and SA-treated plants. In mock-inoculated plants, no rifampicin-resistant bacteria were detected (data not shown). The number of rifampicin-resistant cells per gram of infected leaf tissue was assessed at 15 min after inoculation to determine the number of *P. syringae* bacteria that entered the leaves (typically $\sim 10^6$ per gram of leaf tissue). Subsequently, at two time points after inoculation, the number of colony-forming units (cfu) of *P. syringae* in the leaves was determined. Figure 3B illustrates that treatment with *P. fluorescens* WCS417r or with SA resulted in inhibition of growth of *P. syringae* in the leaves compared with control treated plants. Growth of *P. syringae* in *P. fluorescens* WCS417r– and SA-treated plants was inhibited up to 10-fold by day 1 after inoculation. Three days later, differences in bacterial proliferation between control and protected plants increased up to 22-fold.

P. fluorescens WCS417r-Induced Protection Is Plant Mediated

In the bioassays performed, *P. fluorescens* WCS417r and the challenging pathogens were spatially separated to prevent direct interaction. To verify whether *P. fluorescens* WCS417r and *F. oxysporum* remained spatially separated for the duration of the assay, the presence of *P. fluorescens* WCS417r in both root zones was evaluated at the end of each experiment. Table 1 shows that *P. fluorescens* WCS417r bacteria were recovered from the induction treatment zone at mean population densities

of 5.4 × 10^5 cfu/g of root fresh weight. No rifampicin-resistant bacteria were recovered from the *F. oxysporum*–inoculated zone; this observation showed that *P. fluorescens* WCS417r remained spatially separated from the inoculated zone throughout the experiment. In addition, possible spreading of *P. fluorescens* WCS417r from roots to leaves was assessed by plating leaf extracts from root-induced plants onto selective King's medium B agar plates (King et al., 1954). Table 1 shows that in all bioassays performed, *P. fluorescens* WCS417r bacteria were absent from leaves of root-induced plants, indicating

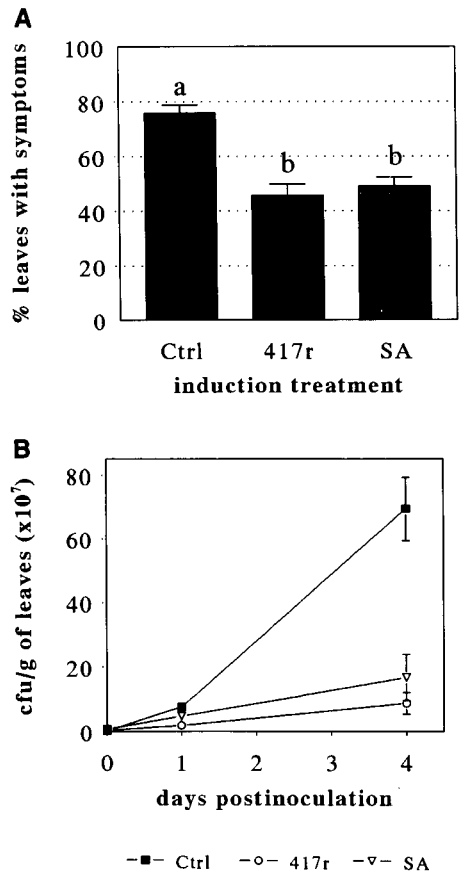

Figure 3. Quantification of Induced Resistance in the Arabidopsis–*P. syringae* Bioassay.

(A) Percentage of leaves per plant with symptoms 4 days after challenge inoculation with *P. syringae*. Different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha = 0.05$). Error bars are ±SE from 20 to 25 replicate plants that received the same treatment.

(B) Time course of growth of *P. syringae* in leaves. Data points are means (colony-forming units per gram) with standard errors from two sets of 20 randomly selected leaves. The values presented are the averages of two independent experiments.

Induction treatments were performed by culturing the plants in soil supplemented with 10 mM $MgSO_4$ (Ctrl) or *P. fluorescens* WCS417r bacteria (417r), or by applying a solution of 1 mM SA (SA) as a soil drench on days 4 and 7 before challenge inoculation.

Table 1. Colonization of Arabidopsis by *P. fluorescens* WCS417r

Bioassay	cfu/g Fresh Weight (× 10^5)[a] Control Treatment	WCS417r Treatment
F. oxysporum–Col-0		
Induction treatment root zone[b]	0	5.4 ± 0.5
Inoculated root zone[b]	0	0
Leaves[c]	ND[d]	0
P. syringae–Ler		
Roots[b]	0	2.6 ± 0.4
Leaves[c]	ND	0
P. syringae–Col-0		
Roots[b]	0	2.8 ± 0.6
Leaves[c]	ND	0
P. syringae–NahG		
Roots[b]	0	3.3 ± 0.6
Leaves[c]	ND	0

[a] Detection limit was 10^3 cfu/g.
[b] Roots were harvested at the end of the bioassays.
[c] Leaves were harvested at different time points to check for systemic colonization by *P. fluorescens* WCS417r.
[d] ND, not determined.

that *P. fluorescens* WCS417r remained localized at the roots. These data clearly demonstrate that *P. fluorescens* WCS417r–mediated protection is not due to microbial antagonism but results from increased resistance in the plant.

In spite of the demonstrated spatial separation of the inducing *P. fluorescens* WCS417r bacteria and the challenging pathogens, indirect interaction between both microorganisms would be possible if PGPR-mediated production of antibiotics antagonized the pathogen at distant sites. However, such antagonism did not play a role. First, *P. fluorescens* WCS417r did not inhibit growth of *F. oxysporum* and *P. syringae* in vitro. Second, treatment of Arabidopsis roots with cell wall extracts of *P. fluorescens* WCS417r resulted in induced protection, whereas cell wall extracts from *P. fluorescens* WCS417rOA$^-$, a mutant lacking the O-antigenic side chain of the lipopolysaccharide (LPS), showed no inducing activity. This distinction indicates that bacterial LPS is involved in eliciting the ISR response in Arabidopsis (S.C.M. van Wees, C.M.J. Pieterse, A. Trijssenaar, Y.A.M. van 't Westende, J.A. van Pelt, and L.C. van Loon, manuscript in preparation).

PGPR-Mediated ISR Is Expressed in NahG Plants

Previously, Gaffney et al. (1993) demonstrated that transgenic tobacco plants expressing the bacterial salicylate hydroxylase (*nahG*) gene are unable to accumulate SA after pathogen infection. Consequently, NahG plants do not develop SAR when induced by a pathogen. To investigate whether PGPR-mediated

ISR is also dependent on SA production, Arabidopsis–*P. syringae* bioassays were performed using transgenic Col-0 plants expressing the *nahG* gene (NahG plants; Delaney et al., 1994) and wild-type Col-0 plants. Both were treated with *P. fluorescens* WCS417r, INA, or SA. *P. fluorescens* WCS417r was applied to the soil before planting, whereas INA and SA were sprayed onto the leaves 4 days before challenge inoculation. Protection against *P. syringae* infection was assessed by determining the percentage of leaves with symptoms 5 days after challenge inoculation and by monitoring growth of the pathogen in the leaves.

Figures 4A and 4B show that in Col-0 plants, *P. fluorescens* WCS417r induced resistance against *P. syringae* to an extent similar to that in L*er* plants (disease severity, 48% of that observed in the control). Spraying Col-0 plants with INA and SA resulted in a significantly higher level of protection (disease severity, 13 and 3%, respectively, of that observed in the control). Control NahG plants were more sensitive to *P. syringae* infection than were wild-type Col-0 plants in that symptoms elicited by *P. syringae* were more severe and the proliferation rate of this pathogen was considerably higher (166-fold). This result agrees with those of others (Gaffney et al., 1993; Delaney

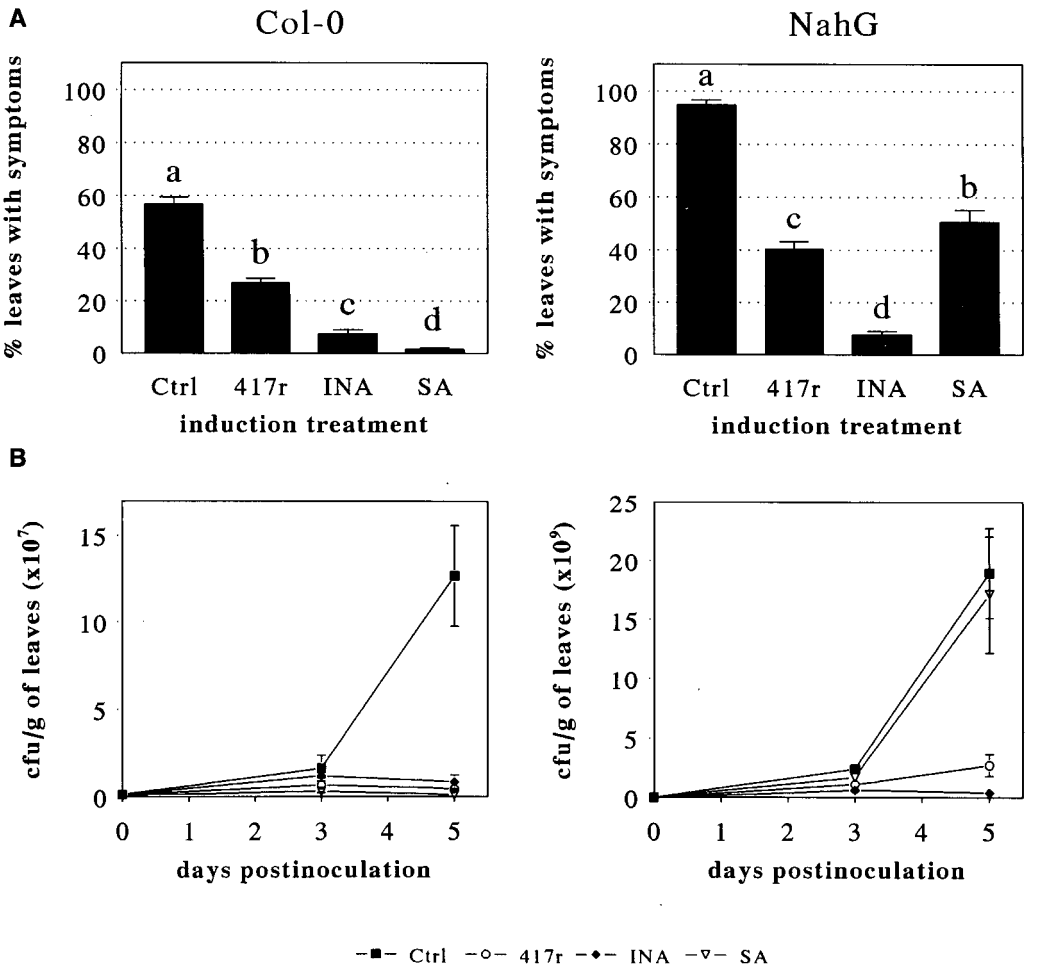

Figure 4. Quantification of Induced Resistance against *P. syringae* in Arabidopsis Col-0 and NahG Plants Treated with *P. fluorescens* WCS417r, INA, or SA.

(A) Percentage of leaves with symptoms 5 days after challenge inoculation with *P. syringae*. Different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha = 0.05$). Error bars are ±SE from 20 to 25 replicate plants that received the same treatment.
(B) Time course of growth of *P. syringae* in leaves. Data points are means (colony-forming units per gram) with standard errors from two sets of 20 randomly selected leaves.
Induction treatments were performed by culturing the plants in soil supplemented with 10 mM $MgSO_4$ (Ctrl) or *P. fluorescens* WCS417r bacteria (417r), or by spraying the leaves until imminent runoff with a solution of 325 μM INA (INA) or 5 mM SA (SA) on days 4 and 7 before challenge inoculation.

et al., 1994; Vernooij et al., 1994, 1995), who found that the disease symptoms in tobacco and Arabidopsis NahG plants were significantly more severe than in wild-type plants. Based on these results, the authors postulated that SA has a role not only in SAR but also in the primary resistance response to pathogen infection.

INA-treated NahG plants showed reduced disease severity and inhibition of growth of the pathogen to an extent similar to INA-treated wild-type plants. This observation is consistent with previous findings (Delaney et al., 1994; Vernooij et al., 1995) demonstrating that INA induction is not affected by the *nahG* gene. In contrast, SA-treated NahG plants showed no inhibition of growth of *P. syringae.* Moreover, a considerably lower level of protection was observed in SA-treated NahG plants compared with similarly treated wild-type plants (disease severity, 60 and 2%, respectively, of that observed in the controls). However, SA-treated NahG plants were clearly less diseased than control NahG plants, possibly because NahG plants need time to convert the excess of exogenously applied SA (5 mM) and as a result show a delay in symptom development.

Notably, in *P. fluorescens* WCS417r–treated Col-0 and NahG plants, the level of induced protection was similar (disease severity was 48 and 43%, respectively, of that observed in the controls). Table 1 shows that the roots of Col-0 and NahG plants harbored similar levels of *P. fluorescens* WCS417r bacteria and that the inducing bacteria remained confined to the rhizosphere. Thus, in contrast to SA but like INA, *P. fluorescens* WCS417r induced resistance to similar levels in wild-type and NahG plants, indicating that *P. fluorescens* WCS417r–mediated induction of ISR is independent of SA accumulation.

Expression of PR Genes

Induction of SAR by necrogenic pathogens or selected abiotic agents in Arabidopsis is associated with the coordinated accumulation of PR-1, PR-2, and PR-5 mRNAs (Uknes et al., 1992, 1993). Analysis of PR-1 mRNA levels in treated Col-0 and NahG plants using the competitive reverse transcriptase–polymerase chain reaction (RT-PCR) (Figure 5) shows that INA induced PR-1 mRNA accumulation in both wild-type Col-0 plants and NahG plants, whereas SA induced PR-1 gene expression in Col-0 plants but not in NahG plants. This result agrees with the expression of SAR in these plants. In contrast, *P. fluorescens* WCS417r–treated Col-0 and NahG plants do not show increased accumulation of PR-1 mRNA, whereas systemic resistance was clearly induced (Figures 4A and 4B).

To compare induction of PR gene expression by different inducers of systemic resistance, the expression of the PR-1, PR-2, and PR-5 genes was studied in *P. fluorescens* WCS417r–, *P. syringae*–, and SA-induced plants. Induction of ISR in L*er* plants through colonization of the rhizosphere by *P. fluorescens* WCS417r was performed as described above for the *P. syringae* bioassay. At the same time, induction treatments were performed by pressure infiltration with 10 mM $MgSO_4$ (control), *P. fluorescens* WCS417r (10^7 cfu/mL), *P. syringae* (10^7 cfu/mL), or SA (0.1 and 1 mM) into three lower leaves of 4-week-old L*er* plants. *P. syringae*–infiltrated leaves showed heavy necrosis after 4 days, whereas leaves infiltrated with 10 mM $MgSO_4$, *P. fluorescens* WCS417r, or SA remained symptomless. Figures 6A and 6B show that infiltration of the leaves with *P. fluorescens* WCS417r, *P. syringae*, or SA and colonization of the rhizosphere by *P. fluorescens* WCS417r resulted in a significant level of systemic protection after challenge inoculation of upper leaves with *P. syringae.*

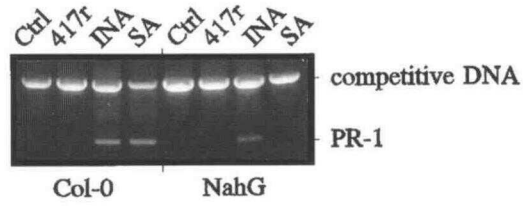

Figure 5. Competitive RT-PCR Analysis of PR-1 Gene Expression in Arabidopsis Col-0 and NahG Plants after Treatment with *P. fluorescens* WCS417r, INA, and SA.

Shown is an ethidium bromide–stained agarose gel with competitive RT-PCR products obtained after amplification of equal portions of first-strand cDNA and 500 pg of heterologous competitor DNA by using PR-1–specific primers. First-strand cDNA was synthesized on mRNA isolated from leaves of the indicated plant and treatment combinations. Leaves were harvested just before challenge inoculation. Induction treatments were performed by culturing the plants in soil supplemented with 10 mM $MgSO_4$ (Ctrl) or *P. fluorescens* WCS417r bacteria (417r), or by spraying the leaves until imminent runoff with a solution of 325 μM INA (INA) or 5 mM SA (SA) on days 4 and 7 before challenge inoculation. Competitive DNA, 900-bp heterologous competitor DNA amplified from 500 pg; PR-1, 422-bp DNA fragment amplified from the PR-1 cDNA.

PR gene expression was studied in infiltrated as well as in nontreated leaves of induced and noninduced plants harvested at different time points after induction treatment. RNA gel blot analyses (Figure 7) show that *P. syringae* infection caused a high, local accumulation of PR-1, PR-2, and PR-5 transcripts 2 days after infiltration. Later time points could not be studied because of the formation of heavy necroses. Systemic accumulation of PR mRNAs was observed up to 5 days after induction by *P. syringae* infiltration. At 8 days, the day of challenge inoculation, PR mRNA levels had dropped to undetectable levels. In contrast, no local accumulation of PR mRNA was observed in leaves infiltrated with *P. fluorescens* WCS417r or 0.1 mM SA, whereas the increase in PR transcript levels after infiltration with 1 mM was moderate. Moreover, neither colonization of the rhizosphere nor infiltration of leaves with *P. fluorescens* WCS417r bacteria or SA resulted in a systemic induction of PR-1, PR-2, and PR-5 gene expression. Nonetheless, these treatments clearly induced systemic resistance (Figure 6). To rule out the possibility that in protected plants PR proteins accumulated from low mRNA levels, proteins were extracted from leaves and roots of control plants and from plants protected

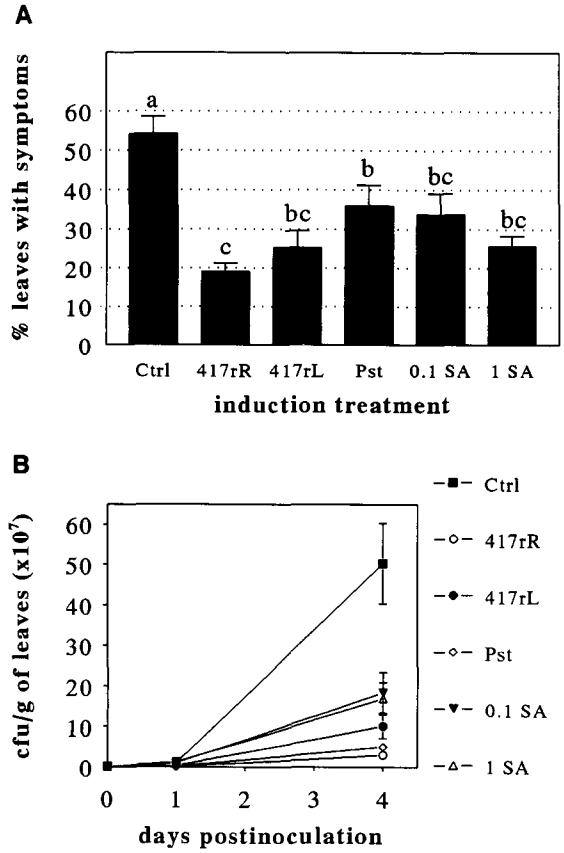

Figure 6. Quantification of Resistance against *P. syringae* in Arabidopsis Induced by Pressure Infiltrating Leaves with *P. fluorescens* WCS417r, *P. syringae*, and SA or through Colonization of the Rhizosphere by *P. fluorescens* WCS417r.

(A) Percentage of leaves with symptoms 4 days after challenge inoculation with *P. syringae*. Different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha = 0.05$). Error bars are ±SE from 20 to 25 replicate plants that received the same treatment.

(B) Time course of growth of *P. syringae* in the leaves. Data points are means (colony-forming units per gram) with standard errors from two sets of 20 randomly selected leaves.

Induction treatment of the roots was performed by culturing the plants in soil supplemented with *P. fluorescens* WCS417r bacteria (417rR; root treatment). Treatment of the leaves was performed by pressure infiltrating three leaves with a solution of 10 mM $MgSO_4$ (Ctrl), 0.1 mM SA (0.1 SA), or 1 mM SA (1 SA) or with a bacterial suspension of 10^7 cfu/mL of *P. fluorescens* WCS417r (417rL; leaf treatment) or *P. syringae* (Pst). Leaves were treated 8 days before challenge inoculation.

by colonization of the rhizosphere with *P. fluorescens* WCS417r. Protein gel blot analyses showed no specific accumulation of PR-1, PR-2, or PR-5 proteins in leaves or roots from protected plants compared with control plants (data not shown). These results clearly demonstrate that *P. fluorescens* WCS417r–mediated induction of systemic resistance is not associated with the activation of PR genes and subsequent accumulation of PR proteins before challenge inoculation.

DISCUSSION

Systemic Resistance in Arabidopsis Induced by Biocontrol Bacteria

With the goal of developing Arabidopsis as a model system for studying PGPR-mediated ISR, we demonstrated that *P. fluorescens* WCS417r effectively protects Arabidopsis against infection by *F. o. raphani* as well as *P. s. tomato*. Root colonization by *P. fluorescens* WCS417r resulted in a marked delay in symptom development and reduction of disease severity after challenge inoculation with *F. oxysporum* (Figure 2). Likewise, *P. fluorescens* WCS417r reduced both the visible symptoms caused by *P. syringae* infection and the growth of this pathogen in the leaves (Figures 3, 4, and 6). Because inducing bacteria and challenging pathogens remained spatially separated throughout the experiment, antagonism by direct interactions could be ruled out, demonstrating that *P. fluorescens* WCS417r–induced protection is plant mediated. Among the bacterial determinants implicated in eliciting metabolic events in plants is the outer membrane LPS (Graham et al., 1977; Mazzuchi et al., 1979; Dazzo et al., 1991; Newman et al., 1995). Previously, it was demonstrated that the LPS of *P. fluorescens* WCS417r is involved in eliciting systemically enhanced resistance in carnation (van Peer and Schippers, 1992), radish (Leeman et al., 1995b), and Arabidopsis (S.C.M. van Wees, C.M.J. Pieterse, A. Trijssenaar, Y.A.M. van 't Westende, J.A. van Pelt, and L.C. van Loon, manuscript in preparation), indicating that PGPR-mediated protection is accomplished by induction of ISR in the plant. In Arabidopsis, this resistance response is effective against a fungal root pathogen as well as a bacterial leaf pathogen. This demonstrates that, like pathogen-induced SAR, *P. fluorescens* WCS417r–mediated ISR is effective against different types of pathogens.

P. fluorescens WCS417r-Mediated ISR Is Independent of SA Accumulation and PR Gene Expression

Endogenous accumulation of SA is one of the characteristics of pathogen-induced SAR (Malamy et al., 1990; Métraux et al., 1990). Transgenic NahG plants that are unable to accumulate SA do not develop a SAR response when treated with a pathogen, indicating that SA accumulation is a crucial step in the signal transduction pathway leading to SAR (Gaffney et al., 1993). *P. fluorescens* WCS417r is equally capable of inducing ISR in Arabidopsis NahG and wild-type plants (Figure 4). This demonstrates that, in contrast to pathogen-induced SAR, endogenous accumulation of SA is not required for induction of *P. fluorescens* WCS417r–mediated ISR. It can thus be con-

cluded that an SA-independent signaling pathway is involved in the elicitation of *P. fluorescens* WCS417r–mediated ISR.

Another important feature of pathogen-induced SAR is the activation of PR gene expression in nontreated leaves (Ward et al., 1991; Uknes et al., 1993). Accordingly, we observed systemic accumulation of PR-1, PR-2, and PR-5 mRNAs when using *P. syringae* as a pathogenic inducer of SAR (Figure 7). Exogenous application of SA or INA is reported to induce SAR and PR gene expression as well (Ward et al., 1991; Malamy and Klessig, 1992; Uknes et al., 1992). In Arabidopsis leaves sprayed with SA or INA, we indeed observed an induced accumulation of PR-1 mRNA (Figure 5). However, we never observed a detectable systemic activation of PR gene expression after application of SA as a soil drench (data not shown) or upon pressure infiltration of leaves with SA (Figure 7), whereas systemic protection was clearly induced in these plants (Figures 2, 3, and 6, respectively). Hence, SA is acting only locally on the expression of PR genes in Arabidopsis, as has also been observed in tobacco (van Loon and Antoniw, 1982; Vernooij et al., 1994).

In contrast to inducers of SAR in Arabidopsis, *P. fluorescens* WCS417r induces systemic resistance without activating PR gene expression (Figures 5 and 7) and subsequent accumulation of PR proteins (data not shown). This demonstrates that activation of PR genes is not a prerequisite for the induction of ISR in Arabidopsis. This result supports and extends recent findings of Hoffland et al. (1995), who demonstrated that *P. fluorescens* WCS417r–mediated ISR against *F. oxysporum* is not associated with accumulation of PR protein in roots and leaves of radish. *P. fluorescens* WCS417r thus appears to be a definitive biological inducer of systemic resistance that does not simultaneously activate PR gene expression. These results differ from those of Maurhofer et al. (1994), who showed that systemic resistance induced by PGPR strain CHA0 of *P. fluorescens* in tobacco against tobacco necrosis virus coincides with the accumulation of PR-2 and PR-3 proteins. However, those authors suggested that induction of resistance may be due to enhanced stress caused by strain CHA0. On the contrary, we observed stimulation of growth rather than stress effects in *P. fluorescens* WCS417r–treated Arabidopsis plants. This observation suggests that different mechanisms are involved.

PR Proteins and Systemic Resistance

The ability of Arabidopsis to develop a systemic resistance response without concomitant PR gene activation sheds new light on the role of PR proteins in systemic resistance. Although not required for *P. fluorescens* WCS417r–mediated ISR, accumulation of PR proteins seems to be essential for the expression of pathogen-induced SAR. In their attempt to genetically dissect the signal transduction pathway regulating SAR in Arabidopsis, Cao et al. (1994) showed that mutant *npr1*, which is a nonexpresser of PR genes, is nonresponsive to induction of SAR by SA, INA, or avirulent pathogens. Similarly, Delaney et al. (1995) showed that the noninducible immunity mutant *nim1*, which is insensitive to chemical and biological induction of SAR, is also unable to activate PR genes upon treatment with inducers of SAR. Moreover, mutant *cpr1*, which is a constitutive expresser of PR genes, also expresses SAR constitutively (Bowling et al., 1994). Although these

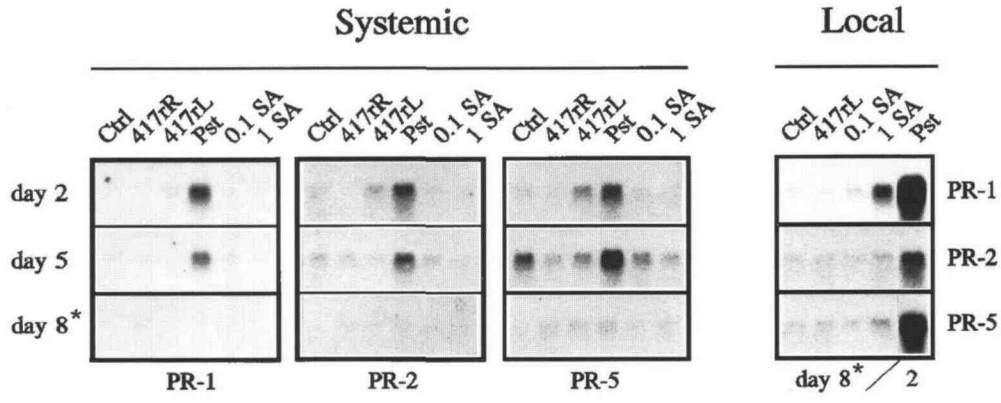

Figure 7. RNA Gel Blot Analyses of PR Gene Expression in Arabidopsis in Response to Induction of Resistance by *P. fluorescens* WCS417r, *P. syringae*, and SA.

A subset of plants from the Arabidopsis–*P. syringae* bioassay (the results of which are presented in Figure 6) was used for RNA extractions. RNA was isolated from pressure-infiltrated leaves (Local) and from noninfiltrated leaves (Systemic) that were harvested at indicated time points after infiltration. The asterisks indicate that leaves were harvested just before challenge inoculation. Pressure infiltration was performed by infiltrating three leaves with a solution of 10 mM $MgSO_4$ (Ctrl), 0.1 mM SA (0.1 SA), or 1 mM SA (1 SA) or with a bacterial suspension of 10^7 cfu/mL of *P. fluorescens* WCS417r (417rL; leaf treatment) or *P. syringae* (Pst). Leaves infiltrated with *P. syringae* were sampled at day 2, whereas the other infiltrated leaves were harvested at day 8. Plants induced by the application of *P. fluorescens* WCS417r to the soil before planting (417rR; root treatment) were harvested at the same time points as plants induced by pressure infiltration. Arabidopsis PR-1, PR-2, and PR-5 gene-specific probes were used for RNA gel blot hybridizations.

findings suggest a critical role of PR proteins in SAR, the evidence is circumstantial because other factors in the signaling pathway downstream of *npr1*, *nim1*, and *cpr1* may contribute to the induced resistant state as well. Furthermore, in other plant species, a causal relationship between accumulation of PR proteins and SAR has never been convincingly demonstrated. Nevertheless, the fact that transgenic tobacco plants, constitutively expressing one or more PR genes, show enhanced resistance to a limited number of fungal pathogens (Broglie et al., 1991; Alexander et al., 1993; Liu et al., 1994) demonstrates that PR proteins can contribute to resistance. However, it is unlikely that they are responsible for the broad spectrum–induced resistance characteristic of SAR.

P. fluorescens WCS417r-Mediated ISR versus Pathogen-Induced SAR

Disease reduction in plants expressing *P. fluorescens* WCS417r–mediated ISR typically ranged from 40 to 60%. Significantly higher levels of protection (up to 97%) were achieved when using chemical inducers of SAR as a spray (Figure 4). Nevertheless, our results clearly demonstrate that, when using *P. fluorescens* WCS417r as inducing agent, systemic resistance manifested effectively in the absence of both endogenous accumulation of SA and expression of the PR genes. This indicates that *P. fluorescens* WCS417r induces a SA-independent signaling pathway different from that controlling SAR. Whether the signaling pathways regulating *P. fluorescens* WCS417r–mediated ISR and pathogen-induced SAR converge to elicit a similar or partially similar phenotypic effect remains to be determined. It is possible that certain defense-related activities induced in both pathways are responsible for enhancing resistance to an intermediate level. Necrogenic and chemical inducers of PR gene expression might further enhance resistance through activity of PR proteins. In this scenario, SAR and the observed PGPR-mediated ISR may share a part of the signal transduction pathway(s) controlling systemic resistance. Alternatively, *P. fluorescens* WCS417r could trigger a completely different, parallel pathway unconnected with accumulation of SA and activation of PR genes. Whatever the case may be, further study of the Arabidopsis–*P. fluorescens* WCS417r system will provide important information on the regulation of ISR.

METHODS

Fungal Cultures

Fusarium oxysporum f sp *raphani* WCS600 was isolated initially from the tuber of a naturally infected radish plant (Leeman et al., 1995a). The fungus was maintained on potato–dextrose agar. A suspension of 10^8 conidia per mL in 10 mM $MgSO_4$ was prepared from a culture grown for 7 days at 22°C on aerated 2% malt extract. This conidial suspension (0.1 mL/g) was added to sterile peat (Agrifutur s.r.l., Alfianello, Italy) and allowed to germinate and grow for 2 days at 24°C. The final density of colony-forming units (cfu) in the peat was determined by plating dilutions of a peat suspension on potato–dextrose agar. An inoculum density of 4×10^6 cfu/g of peat was used in the Arabidopsis–*F. oxysporum* bioassays.

Bacterial Cultures

A rifampicin-resistant mutant of the biocontrol strain *Pseudomonas fluorescens* WCS417 (*P. fluorescens* WCS417r), which was originally isolated from the rhizosphere of wheat grown in a field that suppressed the take-all disease caused by *Gaeumannomyces graminis* pv *tritici* (Lamers et al., 1988), was used throughout this study. *P. fluorescens* WCS417r was grown on King's medium B agar plates (King et al., 1954) for 24 hr at 28°C. The bacterial cells were collected and resuspended in 10 mM $MgSO_4$.

The necrogenic leaf pathogen *P. syringae* pv *tomato* DC3000 (Whalen et al., 1991) was cultured overnight in liquid King's medium B at 28°C. After we collected the cells by centrifugation, bacterial cells were resuspended in 10 mM $MgSO_4$.

Cultivation of Plants

For the *Arabidopsis thaliana–F. oxysporum* bioassay, ecotype Columbia (Col-0) or ecotype Landsberg *erecta* (Ler) seeds were sown singly in 1-mL pipette tips filled with sterile quartz sand to stimulate root elongation. The tips were drenched daily in water for 30 min and once a week in modified half-strength Hoagland 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 (CIBA-Geigy, Basel, Switzerland). After 2 weeks, seedlings were gently washed out of the pipette tips and transferred to a rock wool system containing two spatially separated compartments, allowing an induction treatment and a challenge inoculation of the same root system (Leeman et al., 1995a). Briefly, rock wool cubes (rock wool; Grodan B.V., Roermond, The Netherlands) were drenched in nutrient solution (see above) and placed in two polyethylene bags. Three-week-old seedlings with roots 2–3 cm in length were positioned horizontally on two separated rock wool cubes through a vertical incision in the bags; the lower part of the roots on one cube and the upper part, along with the rosette, on the other. Both parts of the roots were covered with small rock wool cubes to prevent desiccation and exposure to light.

For the Arabidopsis–*P. syringae* bioassay, seeds of Ler, Col-0, and Col-0 plants harboring the bacterial *nahG* gene (Delaney et al., 1994) were sown in sterile quartz sand. Two weeks later, seedlings were transferred to pots (60 mL) containing a sand/potting soil mixture that had been autoclaved twice before application of either *P. fluorescens* WCS417r bacteria or 10 mM $MgSO_4$.

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 65% relative humidity. Plants were watered or supplied with modified Hoagland nutrient solution twice a week.

Arabidopsis–*F. oxysporum* ISR Bioassay

After transfer of the Arabidopsis seedlings to the rock wool separate inoculation system, the lower parts of the roots were covered with either 1 mL of a 1:1 (v/w) *P. fluorescens* WCS417r/talcum suspension (final

concentration, 5×10^8 cfu/g), a 1:1 (w/v) mixture of talcum with 1 mM salicylic acid (SA), pH 6, or 10 mM $MgSO_4$. Three days after induction treatment, the plants were inoculated with *F. oxysporum* by applying ~0.25 g of peat inoculum (4×10^6 cfu/g) to the upper parts of the roots. Control plants were mock-inoculated with ~0.25 g of sterile peat. A set of 20 individual plants was used for each treatment. Protection against *F. oxysporum* was analyzed at different time points after inoculation by determining the frequency distribution of plants over four distinct disease severity classes, that is, 0 to 25, 26 to 50, 51 to 75, and 76 to 100% of the leaves showing symptoms of fusarium wilt. Data were statistically analyzed using Wilcoxon's two-sample test at $\alpha = 0.05$ or the Kruskal–Wallis multiple comparison test at $\alpha = 0.05$.

Arabidopsis-*P. syringae* ISR Bioassay

Before transfer of the Arabidopsis seedlings to the sand/potting soil mixture, a *P. fluorescens* WCS417r suspension (10^9 cfu/mL) was mixed thoroughly through the sterile sand/potting soil mixture to a final density of 5×10^7 cfu/g. Nontreated soil was supplemented with an equal volume of sterile 10 mM $MgSO_4$. SA induction treatment was performed either by applying 20 mL of a 1 mM SA solution, pH 6, as soil drench to the plants on days 7 and 4 before challenge inoculation, or by spraying a 5 mM SA solution on the leaves to the point of imminent runoff 4 days before inoculation. 2,6-Dichloroisonicotinic acid (INA) (325 μM), formulated as a 25% active ingredient in a wettable powder carrier (Ciba-Geigy AG), was suspended in water and sprayed on the leaves in a similar manner.

Five-week-old plants were challenge-inoculated with *P. syringae* by dipping the leaves in a bacterial suspension containing 10^8 cfu/mL (*Ler*) or 2.5×10^7 cfu/mL (Col-0 and NahG) in 10 mM $MgSO_4$, supplemented with 0.01% (v/v) of the surfactant Silwet L-77 (van Meeuwen Chemicals BV, Weesp, The Netherlands). Mock inoculations were performed with a solution of 10 mM $MgSO_4$, 0.01% Silwet L-77 without bacteria. One day before challenge inoculation, the plants were placed at 100% relative humidity. Growth of *P. syringae* was assessed in inoculated leaves of control and treated plants at different time points after inoculation. Two sets of 20 randomly selected leaves per treatment were weighed, rinsed thoroughly in sterile water, and homogenized in 10 mM $MgSO_4$. Subsequently, dilutions were plated onto King's medium B agar supplemented with rifampicin (50 mg/L) and cycloheximide (100 mg/L). After incubation for 48 hr at 28°C, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined.

Four or 5 days after inoculation, the proportion of leaves per plant with symptoms was determined (20 to 25 plants per treatment). Data were analyzed using analysis of variance followed by Fisher's test for LSDs at $\alpha = 0.05$.

Pressure Infiltration of Leaves

Three fully expanded leaves of 4-week-old *Ler* plants were pressure infiltrated on the lower side of the leaf with *P. syringae* (10^7 cfu/mL), *P. fluorescens* WCS417r (10^7 cfu/mL), SA (0.1 or 1 mM), or 10 mM $MgSO_4$ by using a 1-mL syringe without a needle as described by Swanson et al. (1988). Two, 5, and 8 days later, pressure-infiltrated as well as noninfiltrated leaves were harvested from a subset of plants of each treatment. Subsequently, the leaves were frozen in liquid nitrogen and stored at −80°C until RNA extractions were performed. Eight days after induction, another subset of plants from each treatment was challenge-inoculated with *P. syringae*, and induced resistance was quantified as described above.

Rhizosphere Colonization

Roots (±0.5 g fresh weight) were harvested in duplicate and shaken vigorously for 1 min in 5 mL of 10 mM $MgSO_4$ containing 0.5 g of glass beads (0.17 mm). Subsequently, 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.

RNA Extraction and RNA Gel Blot Analysis

Total RNA was isolated from frozen leaf samples by 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 electrophoresed on denaturing formaldehyde–agarose gels and blotted onto Hybond-N^+ membranes (Amersham) 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), using Pharmacia Biotech's Oligolabeling Kit. The PR-2 probe was derived from plasmid A-2237 carrying the Arabidopsis β-1,3-glucanase gene (Dong et al., 1991). Probes for PR-1 and PR-5 were derived from an Arabidopsis PR-1 and a PR-5 cDNA clone, respectively (Uknes et al., 1992).

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 by using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech), was converted into first-strand cDNA by using a T-Primed First-Strand Kit (Pharmacia Biotech). 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.

Protein Extraction and Immunoblot Analyses

Proteins were extracted 3, 10, and 20 days (leaves) or 7 and 14 days (roots) after induction treatment. Protein extraction and immunoblot analyses, using specific antisera against tobacco PR-1, PR-2, and PR-5 proteins, were performed as described previously (Hoffland et al., 1995).

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