SYSTEMIC RESISTANCE IN *ARABIDOPSIS THALIANA* INDUCED BY BIOCONTROL BACTERIA

Corné M.J. PIETERSE, Saskia C.M. VAN WEES, Johan A. VAN PELT
Annemiek TRIJSSENAAR, Yvonne A.M. VAN 'T WESTENDE
Esther M. BOLINK & Leendert C. VAN LOON.
Department of Plant Ecology and Evolutionary Biology
Section Plant Pathology, Utrecht University
P.O. Box 800.84, 3508 TB Utrecht, the Netherlands

SUMMARY

Systemic acquired resistance (SAR) is a pathogen-inducible defense mechanism in plants effective against a broad spectrum of plant pathogens. The resistant state is dependent on endogenous accumulation of salicylic acid (SA) and is associated with the activation of a specific set of genes encoding pathogenesis-related (PR) proteins. Recently, selected nonpathogenic, root-colonizing bacteria with disease suppressive properties have been shown to trigger a systemic resistance response as well, without provoking any symptoms themselves. To study the molecular and mechanistic basis underlying this type of induced resistance, we developed a model system using *Arabidopsis thaliana* as the host plant and the fungal root pathogen *Fusarium oxysporum* f.sp. *raphani* and the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* as challenging pathogens. Colonization of the rhizosphere of *A. thaliana* ecotypes Columbia and Landsberg *erecta* by biocontrol strain *Pseudomonas fluorescens* WCS417r resulted in a plant-mediated resistance response that significantly reduced symptoms elicited by both *F. oxysporum* and *P. syringae*. Moreover, proliferation of *P. syringae* in infected leaves was strongly inhibited in WCS417r-treated plants. Treatments with cell wall preparations of WCS417r and a WCS417r-mutant lacking the O-antigenic side chain of the extracellular lipopolysaccharide (LPS), revealed that LPS is one of the bacterial determinants involved in the elicitation of the induced systemic resistance (ISR) response. Two other biocontrol strains, *P. putida* WCS358r and *P. fluorescens* WCS374r, appeared to be intermediate and noninducers of ISR, respectively. Interestingly, *A. thaliana* ecotype RLD showed no resistance response upon treatment with WCS-417r. These results suggest that elicitation of ISR involves specific recognition mechanisms between *Arabidopsis* and inducing rhizobacteria. Transgenic *Arabidopsis* NahG plants, unable to accumulate SA, and wild-type plants were equally responsive to WCS417r-induction. Furthermore, WCS417r-mediated induction of systemic resistance did not coincide with the accumulation of PR mRNAs prior to challenge inoculation. These results demonstrate that WCS417r induces a signalling pathway different from that controlling classical SAR, leading to a form of systemic resistance that is independent of SA accumulation and PR gene expression.

INTRODUCTION

Induced resistance is the phenomenon in which a plant acquires a higher level of resistance to pathogen infection after it has been appropriately stimulated. This resistance response is expressed systemically throughout the plant and is effective against a broad spectrum of pathogens (Hammerschmidt and Kuć, 1995). The classical way of inducing systemic resistance is by a predisposing infection with a necrotizing pathogen (Ross, 1961; Kuć, 1982). The resulting state of systemic acquired resistance (SAR) is characterized by an early increase in endogenously synthesized salicylic acid (SA) which appears to be critical for the induction of the SAR signalling 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), some of which exhibit limited antifungal activity. Exogenous application of the chemicals SA or 2,6-dichloroisonicotinic acid (INA) has been reported to mimic pathogen-induced SAR (Métraux *et al.*, 1991; White, 1979) as they induce resistance to the same spectrum of pathogens and concurrently activate the SAR genes (Uknes *et al.*, 1992; 1993; Ward *et al.*, 1991).

A relatively novel way of inducing systemic resistance in plants is through colonization of the rhizosphere with selected plant growth-promoting rhizobacteria (PGPR). This type of systemic

resistance is generally referred to as induced systemic resistance (ISR; Kloepper *et al.*, 1992). Selected PGPR, mainly fluorescent *Pseudomonas* spp., have been demonstrated to effectively control plant diseases by suppressing pathogens and deleterious microorganisms through competition for substrate, siderophore-mediated competition for iron, or antibiosis (Schippers, 1992; Thomashow and Weller, 1995). Research on mechanisms of biological control by PGPR revealed that some biocontrol bacteria protect plants against pathogen infection through induction of systemic resistance, without provoking any symptoms themselves (Alström, 1991; van Peer *et al.*, 1991; Wei *et al.*, 1991). To date, PGPR-mediated ISR has been demonstrated in several plant-pathogen systems (Table 1). Maurhofer *et al.* (1994) described that ISR induced by *P. fluorescens* CHA0 in tobacco against tobacco necrosis virus was accompanied by an increase in PR pro-

Table 1. Reports on systemic resistance induced by plant growth-promoting rhizobacteria.

Reference	PGPR strain	Challenging pathogen	Plant
Alström, 1991	*Pseudomonas fluorescens* S97	*P. syringae* pv. *phaseolicola*	bean
van Peer *et al.*, 1991	*P. fluorescens* WCS417r	*Fusarium oxysporum* f.sp. *dianthi*	carnation
Wei *et al.*, 1991	*P. fluorescens* G8-4 *P. aureofaciens* 25-33 *P. aureofaciens* 28-9 *P. aureofaciens* 36-5 *P. putida* 34-13 *Serratia plymuthica* 2-67	*Colletotrichum orbiculare*	cucumber
Buysens *et al.*, 1994	*P. aeruginosa* 7NSK2	*Botrytis cinerea*	bean
Maurhofer *et al.*, 1994	*P. fluorescens* CHA0	Tobacco necrosis virus	tobacco
Zhou and Paulitz, 1994	*P. fluorescens* isolate 15 *P. corrugata* isolate 13	*Pythium aphanidermatum*	cucumber
Leeman *et al.*, 1995a	*P. fluorescens* WCS417 *P. fluorescens* WCS374	*F. oxysporum* f.sp. *raphani*	radish
Liu *et al.*, 1995	*P. putida* 89B-27 *S. marcescens* 90-166	*P. syringae* pv. *lachrymans*	cucumber
de Meyer *et al.*, 1996	*P. aeruginosa* 7NSK2	Tobacco mosaïc virus	tobacco
Hoffland *et al.*, 1996	*P. fluorescens* WCS417r	*F. oxysporum* f.sp. *raphani* (root) *F. oxysporum* (leaf) *P. syringae* pv. *tomato* *Alternaria brassicicola*	radish
Pieterse *et al.*, 1996	*P. fluorescens* WCS417r	*F. oxysporum* f.sp. *raphani* *P. syringae* pv. *tomato*	*Arabidopsis*
van Wees *et al.*, 1996	*P. putida* WCS358r	*F. oxysporum* f.sp. *raphani* *P. syringae* pv. *tomato*	*Arabidopsis*

tein accumulation, suggesting that PGPR-mediated ISR and pathogen-induced SAR are manifestations of a common defense mechanism. In contrast, Hoffland *et al.* (1995) demonstrated *P. fluorescens* WCS417r-mediated ISR in radish against *Fusarium oxysporum* in absence of PR protein accumulation. Hence, it is unclear in how far PGPR-mediated ISR and pathogen-induced SAR share a common signal transduction pathway.

With the goal to study the molecular basis underlying ISR mediated by biocontrol bacteria, two bioassays were developed using *Arabidopsis* as the host plant and *F. oxysporum* f.sp. *raphani* and *P. syringae* pv. *tomato* as challenging pathogens. Here we describe that *Pseudomonas* spp.-mediated ISR can be induced in *Arabidopsis* against both pathogens and that elicitation of

the resistance response involves specific recognition mechanisms.Moreover, we provide evidence that extracellular lipopolysaccharides (LPS) of the inducing bacteria are involved in eliciting the resistance response. We also demonstrate that, in contrast to pathogen-induced SAR, PGPR-mediated ISR is independent of both endogenous SA accumulation and PR gene activation.

MATERIALS AND METHODS

Cultivation of PGPR and pathogens

Rifampicin-resistant mutants of PGPR strains *Pseudomonas fluorescens* WCS417 (Lamers *et al.*, 1988), *P. fluorescens* WCS374 (Geels and Schippers, 1983) and *P. putida* WCS358 (Geels and Schippers, 1983), hereafter called WCS417r, WCS374r and WCS358r, respectively, and a spontaneous mutant of WCS417r lacking the O-antigenic side chain of the LPS (WCS417rOA$^-$) were grown on King's medium B agar plates (King *et al.*, 1954) for 24 hours at 28°C. The bacterial leaf pathogen *P. syringae* pv. *tomato* DC3000 (Whalen *et al.*, 1991) was cultured overnight in liquid King's medium B at 28°C. The bacterial cells were collected and resuspended in 10 mM $MgSO_4$. *Fusarium oxysporum* f.sp. *raphani* WCS600 was grown as described by Leeman *et al.* (1995a) and mixed through sterile peat.

Cultivation of plants

Seedlings of *Arabidopsis thaliana* ecotypes Columbia (Col-0), Landsberg *erecta* (L*er*), and RLD and transgenic Col-0 plants harbouring the bacterial *nahG* gene (NahG plants; Delaney *et al.*, 1994) were grown in sterile quartz sand for two weeks. For the *F. oxysporum* bioassay, seedlings were then transferred to a rock wool system consisting of two spatially separated compartments, allowing an induction treatment and a challenge inoculation on different parts of the same root system (Leeman *et al.*, 1995a; Pieterse *et al.*, 1996). For the *P. syringae* bioassay, seedlings were transferred to pots (60 mL) containing a sand/potting soil mixture that had been autoclaved twice before application of PGPR or 10 Mm $MgSO_4$. Plants were cultivated in a growth chamber with a 9-hour day (200 $\mu E\ m^{-2}\ sec^{-1}$ at 24°C) and 15-hour night (20°C) cycle and 65% relative humidity.

The *Arabidopsis-F. oxysporum* bioassay

After transfer of the *Arabidopsis* seedlings to the rock wool separate inoculation system, the lower part of the roots was covered with 1 ml of either a 1:1 (v/w) PGPR/talcum suspension (final density 5×10^8 cfu/g), or a 1:1 (w/v) mixture of talcum with 1 mM SA (pH 6) or 10 mM $MgSO_4$. Three days after the induction treatment, the plants were inoculated with *F. oxysporum* by applying about 0.25 g of peat inoculum (4×10^6 cfu/g) to the upper parts of the roots. Control plants were mock inoculated with about 0.25 g of sterile peat. For each treatment, a set of 30 individual plants was used. Protection against *F. oxysporum* was analyzed at 22-28 days postinoculation by determining the proportion of leaves showing fusarium wilt symptoms. The data were statistically analyzed using analysis of variance followed by Fisher's least significant differences (LSD) test at $\alpha=0.05$.

The *Arabidopsis-P. syringae* bioassay

Prior to transfer of the *Arabidopsis* seedlings to the pots, a PGPR 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 by applying a 1 mM SA solution (pH 6) as a soil drench 7 and 4 days prior to challenge inoculation or by spraying a 5 mM SA solution (pH 6) on the leaves to the point of imminent runoff 4 days before challenge. INA (325 μM), formulated as a 25% active ingredient in a wettable powder carrier (Ciba-Geigy), was suspended in water and sprayed on the leaves in a similar manner. Crude PGPR cell wall preparations were obtained as described by Leeman *et al.* (1995b) and applied as a soil drench at a similar density as present in soil treated with living PGPR. One day before challenge inoculation, the plants were placed at 100% relative humidity.

Five-week-old plants were inoculated by dipping the leaves in a *P. syringae* suspension containing 10^8 cfu/mL (L*er*), 2.5×10^7 cfu/mL (Col-0 and NahG), or 10^7 cfu/mL (RLD) in 10 mM $MgSO_4$, 0.01% (v/v) Silwet L-77. Mock inoculations were performed with the same solution without bacteria. Four or 5 days after inoculation, the proportion of leaves with symptoms was determined per plant (20-25 plants per treatment). Data were statistically analyzed using analysis of variance followed by Fisher's LSD test at $\alpha = 0.05$.

To monitor growth of *P. syringae* in inoculated leaves, 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 50 mg/L rifampicin and 100 mg/L cycloheximide. After an incubation time of 48 hours at 28°C, the number of rifampicin-resistant cfu/g of infected leaf tissue was determined.

Rhizosphere colonization

Per treatment, roots were harvested (2×0.5 g fresh weight) and shaken vigorously for 1 minute in 5 mL of 10 mM $MgSO_4$ containing 0.5 g of glass beads (0.17 mm). Dilutions were plated onto King's medium B agar supplemented with 100 mg/L cycloheximide, 50 mg/L ampicillin, 13 mg/L chloramphenicol, and 150 mg/L rifampicin. After overnight incubation at 28°C, the number of rifampicin resistant cfu/g of root fresh weight was determined.

Competitive RT-PCR

Analysis of PR-1 gene expression was performed using competitive RT-PCR as described by Siebert and Larrick (1992). A PR-1-specific primer pair (GTAGGTGCTCTTGTTCTTCC and TTCACATAATTCCCACGAGG), 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 nanogram of poly(A)$^+$ RNA, isolated from frozen leaves using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech), was converted into first-strand cDNA 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 using the PR-1-specific primer pair. The products were then resolved on an agarose gel stained with ethidium bromide.

RESULTS

PGPR-mediated ISR against *F. oxysporum* and *P. syringae*

Arabidopsis was susceptible to infection by the fungal root pathogen *F. oxysporum* f. sp. *raphani* and the bacterial leaf pathogen *P. syringae* pv. *tomato*. Inoculation of the roots with *F. oxysporum* resulted in a relatively long latent period of 2-3 weeks in which no wilt symptoms were visible. Thereafter, leaves turned yellow rapidly, beginning at the veins, then wilted, and finally died. Plants inoculated with *P. syringae* developed necrotic lesions that were surrounded by extensive spreading chlorosis 4 to 5 days after challenge inoculation.

To study PGPR-mediated ISR in *Arabidopsis* against these pathogens, PGPR strains WCS417r, WCS374r and WCS358r were tested for their ability to induce ISR in comparison to SA, which is known to be an effective inducer of SAR (Malamy and Klessig, 1992; Uknes *et al.*, 1993). Table 2 shows that treatment of Col-0 and L*er* plants with WCS417r and WCS358r resulted in a significant reduction of disease symptoms caused by *F. oxysporum* and *P. syringae*, respectively, whereas treatment with WCS374r did not. WCS417r induced a relatively high level of resistance against both pathogens, whereas the level of protection induced by WCS358r was moderate. In both bioassays, the level of protection induced by WCS417r was comparable to that obtained by treatment of the roots with SA.

To examine whether the observed reduction of symptoms in *P. syringae*-infected plants was associated with diminished pathogen growth, the increase of *P. syringae* cells was monitored in infected leaves. The number of rifampicin-resistant cells per gram of inoculated leaf tissue was assessed at 15 minutes after leaf dipping to determine the number of *P. syringae* bacteria that entered the leaves (typically about 10^6/g of leaf tissue). Four days after inoculation, the number of cfu of *P. syringae* in the leaves was determined and the extent of bacterial multiplication calculated. Table 2 shows that proliferation of *P. syringae* in plants treated with WCS417r, WCS358r and SA was inhibited. In WCS374r-treated plants growth of the pathogen was similar to that observed in control plants.

Table 2. Quantification of induced systemic resistance against *F. oxysporum* in *A. thaliana* ecotype Col-0 and against *P. syringae* in *A. thaliana* ecotype L*er* after treatment with PGPR or SA.

Treatment	Disease incidence *F. oxysporum* (%)[1,2]	Disease incidence *P. syringae* (%)[1,2]	Relative growth of *P. syringae* in leaves (%)[3]
Control	100[a]	100[a]	100
P. fluorescens WCS417r	38[bc]	60[c]	14
P. fluorescens WCS374r	106[a]	100[a]	119
P. putida WCS358r	72[b]	81[b]	38
SA[4]	42[c]	65[c]	13

[1] Proportion of leaves with symptoms compared to the control (100%), 4 days after challenge inoculation.
[2] Different letters in the same column indicate statistically significant differences (Fisher's LSD test, α=0.05).
[3] Relative growth of *P. syringae* in leaves compared to the control (100%), 4 days after challenge inoculation.
[4] SA (1 mM) was applied to the roots.

Differential induction of WCS417r-mediated ISR in different *A. thaliana* ecotypes

To investigate whether WCS417r induces ISR against *F. oxysporum* and *P. syringae* in different *A. thaliana* ecotypes, the responsiveness of ecotypes Col-0, L*er* and RLD to WCS417r treatment was tested. Table 3 shows that treatment of the roots of ecotype L*er* with WCS417r reduced symptoms caused by *F. oxysporum* and *P. syringae* infection to a similar extent as in Col-0 plants. In contrast, ecotype RLD did not exhibit ISR against either pathogen upon treatment of the roots with WCS417r. Nevertheless, RLD plants showed significant protection against both pathogens after treatment with SA.

Table 3. Quantification of ISR against *F. oxysporum* and *P. syringae* in *A. thaliana* ecotypes Col-0, L*er* and RLD after treatment with *P. fluorescens* WCS417r or SA.

Ecotype (treatment)	Disease incidence *F. oxysporum* (%)[1,2]	Disease incidence *P. syringae* (%)[1,2]	Relative growth of *P. syringae* in leaves (%)[3]
Col-0 (control)	100[a]	100[a]	100
Col-0 (WCS417r)	38[b]	54[b]	28
Col-0 (SA)[4]	42[b]	35[b]	12
L*er* (control)	100[a]	100[a]	100
L*er* (WCS417r)	55[b]	60[b]	14
L*er* (SA)[4]	52[b]	65[b]	13
RLD (control)	100[a]	100[a]	100
RLD (WCS417r)	116[a]	102[a]	95
RLD (SA)[4]	68[b]	55[b]	34

[1] Proportion of leaves with symptoms compared to the control (100%), 4 days after challenge inoculation.
[2] Different letters within an ecotype/pathogen combination indicate statistically significant differences (Fisher's LSD test, $\alpha=0.05$).
[3] Relative growth of *P. syringae* in leaves compared to the control (100%), 4 days after challenge inoculation.
[4] SA (1 mM) was applied to the roots.

Involvement of outer membrane LPS in WCS417r-mediated ISR

Previously, van Peer and Schippers (1992) and Leeman *et al.* (1995b) demonstrated that the extracellular lipopolysaccharide (LPS) of WCS417r elicits ISR in carnation and radish, respectively. To investigate whether the LPS of WCS417r is also involved in elicitation of ISR in *Arabidopsis*, bioassays were performed with living cells and cell wall preparations of WCS417r and the mutant WCS417rOA$^-$ lacking the O-antigenic side chain of the LPS. Cell walls of the noninducing strain WCS374r were used for comparison. Table 4 shows that treatment of roots of L*er* with cell walls of WCS417r resulted in a significant reduction of symptoms elicited by *P. syringae* infection, whereas cell walls of WCS417rOA$^-$ did not, indicating that the LPS of WCS417r is involved in the induction of ISR. As with living WCS374r bacteria, cell walls of this strain did not induce ISR. Interestingly, living cells of WCS417rOA$^-$ were able to induce ISR to some extent, suggesting that factors other than LPS can be involved in elicitation of ISR as well.

Table 4. Quantification of ISR against *P. syringae* in *A. thaliana* ecotype L*er* after treatment with *P. fluorescens* WCS417r or WCS417rOA$^-$ or crude cell walls of *P. fluorescens* WCS417r, WCS417rOA$^-$ or WCS374r.

Treatment	Disease incidence *P. syringae* (%)[1,2]
Control	100[a]
Cell wall extracts of *P. fluorescens* WCS417r	84[b]
Cell wall extracts of *P. fluorescens* WCS417rOA$^-$	114[a]
Cell wall extracts of *P. fluorescens* WCS374r	109[a]
P. fluorescens WCS417r	58[c]
P. fluorescens WCS417rOA$^-$	81[b]

[1] Proportion of leaves with symptoms compared to the control (100%), 4 days after challenge inoculation.
[2] Different letters indicate statistically significant differences (Fisher's LSD test, α=0.05).

WCS417r-mediated ISR is independent of endogenous SA accumulation

Recently, Gaffney *et al.* (1993) and Delaney *et al.* (1994) demonstrated that transgenic tobacco and *Arabidopsis* plants expressing the bacterial salicylate hydroxylase (*nahG*) gene are unable to accumulate SA following 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 NahG and wild-type Col-0 plants treated with WCS417r, SA, or INA. WCS417r was applied to the soil prior to planting, whereas SA and INA were sprayed onto the leaves 4 days before challenge inoculation. Table 5 shows that treatment of Col-0 plants with WCS417r, SA and INA resulted in a significant level of protection against *P. syringae* infection. INA-treated NahG plants showed reduced disease severity and inhibition of growth of the pathogen, to a similar extent as INA-treated wild-type plants. This is consistent with previous findings (Delaney *et al.*, 1994; Vernooij *et al.*, 1995) demonstrating that INA acts downstream of SA in the SAR signalling pathway. 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 to similarly treated wild-type plants. Five days after challenge inoculation, 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 WCS417r-treated Col-0 and NahG plants, the level of induced protection was similar, indicating that WCS417r induced resistance in wild-type and NahG plants to similar levels. These results demonstrate that, contrary to pathogen-induced SAR, WCS417r-mediated ISR is independent of SA accumulation.

Table 5. Quantification of ISR against *P. syringae* in wild-type Col-0 and SA-nonaccumulating NahG plants upon treatment with *P. fluorescens* WCS417r, INA and SA.

Treatment	Disease incidence *P. syringae* (%)[1,2]	Relative growth of *P. syringae* in leaves (%)[3]	PR-1 gene expression[4]
Col-0 (control)	100[a]	100	-
Col-0 (WCS417r)	48[b]	5	-
Col-0 (INA)[5]	13[c]	6	+
Col-0 (SA)[5]	2[d]	1	+
NahG (control)	100[a]	100	-
NahG (WCS417r)	43[c]	12	-
NahG (INA)[5]	8[d]	1	+
NahG (SA)[5]	53[b]	122	-

[1] Proportion of leaves with symptoms compared to the control (100%), 5 days after challenge inoculation.
[2] Different letters within data of Col-0 or NahG plants indicate statistically significant differences (Fisher's LSD test, α=0.05).
[3] Relative growth of *P. syringae* in leaves compared to the control (100%), 5 days after challenge inoculation.
[4] PR-1 mRNA was detected in leaves harvested just prior to challenge inoculation by competitive RT-PCR using *A. thaliana* PR-1 specific primers.
[5] INA (325 µM) and SA (5 mM) were sprayed onto the leaves until imminent runoff.

Expression of PR genes

Induction of SAR 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 by competitive RT-PCR showed that INA induced PR-1 mRNA accumulation in both NahG and wild-type Col-0 plants, whereas SA induced PR-1 gene expression in wild-type plants only (Table 5). This is in agreement with the expression of SAR in these plants. In contrast, WCS417r-treated Col-0 and NahG plants did not show accumulation of PR-1 mRNA, whereas systemic resistance was clearly induced. Northern blot analyses revealed that the expression patterns of the PR-2 and PR-5 genes were similar to that of the PR-1 gene (data not shown), indicating that WCS417r-mediated ISR is not associated with the activation of PR genes.

Colonization of the rhizosphere by PGPR

In all bioassays performed, the PGPR and the challenging pathogens were applied at spatially separated sites to prevent direct interaction between the two microorganisms. To verify whether the PGPR and the pathogens remained spatially separated for the duration of the assays, the presence of PGPR was evaluated in PGPR-treated parts of the roots and in pathogen-treated parts of the roots or leaves at the end of each experiment. In all bioassays performed, PGPR were recovered from the treated roots at mean population densities ranging between 10^4-10^6 cfu/g of root fresh weight. From nontreated roots and pathogen-inoculated plant parts, no rifampicin-resistant PGPR were recovered, demonstrating that the inducing rhizobacteria remained spatially separated from the pathogens throughout the experiments.

DISCUSSION

With the goal of developing *Arabidopsis* as a model for studying PGPR-mediated ISR, we demonstrated that *P. fluorescens* WCS417r and *P. putida* WCS358r effectively protect *A.*

thaliana ecotypes Col-0 and L*er* against infection by *F. oxysporum* f. sp. *raphani* and *P. syringae* pv. *tomato*. As inducing bacteria and challenging pathogens remained spatially separated throughout the experiments, antagonism by direct interactions could be ruled out, indicating that WCS417r- and WCS358r-induced protection is plant-mediated. The PGPR-mediated resistance response is effective against a fungal root pathogen as well as a bacterial leaf pathogen, demonstrating that, like pathogen-induced SAR, PGPR-mediated ISR is effective against different types of pathogens.

In contrast to WCS417r and WCS358r, strain WCS374r of *P. fluorescens*, which has been demonstrated to induce systemic resistance against *F. oxysporum* in radish (Leeman *et al.*, 1995a), did not elicit ISR in *Arabidopsis*. Moreover, *A. thaliana* ecotype RLD, which is able to express a SA-induced SAR response, was not responsive to WCS417r-mediated induction of ISR. Colonization of the rhizosphere in these plant/PGPR combinations was not impaired which strongly suggests that specific recognition mechanisms between *Arabidopsis* and inducing PGPR are involved in the elicitation of ISR. Among the bacterial determinants implicated in eliciting metabolic events in plants is the outer membrane LPS (Graham *et al.*, 1977; Newman *et al.*, 1995). Previously, it was demonstrated that the LPS of WCS417r is sufficient to elicit ISR in carnation (van Peer and Schippers, 1992) and radish (Leeman *et al.*, 1995b). By using cell wall preparations of WCS417r and WCS417rOA$^-$ we showed that the LPS of WCS417r is also involved in the elicitation of the ISR response in *Arabidopsis*. However, other factors seem to play a role as well because WCS417rOA$^-$ is still able to induce systemic resistance to some extent.

SA is an essential intermediate in the signal transduction pathway leading to pathogen-induced SAR as SA-nonaccumulating NahG plants do not develop a SAR response when infected with a pathogen (Delaney *et al.* 1994; Gaffney *et al.*, 1993). The nonpathogen WCS417r is equally capable of inducing ISR in *Arabidopsis* NahG and wild-type plants. This demonstrates that, in contrast to pathogen-induced SAR, endogenous accumulation of SA is not required for induction of WCS417r-mediated ISR. Another important feature of SAR is the systemic activation of PR genes (Uknes *et al.*, 1993; Ward *et al.*, 1991;). In contrast to inducers of SAR in *Arabidopsis*, WCS417r induces systemic resistance without activating PR gene expression. It must therefore be concluded 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) demonstrating that WCS417r-mediated ISR against *F. oxysporum* is not associated with PR protein accumulation in roots and leaves of radish. WCS417r thus appears to be a biological inducer of systemic resistance that acts without simultaneously activating PR gene expression.

Upon colonization of the roots by WCS417r, systemic resistance is evident in *Arabidopsis* in the absence of the two major characteristics of SAR, *i.e.* endogenous accumulation of SA and PR gene activation. It can thus be concluded that WCS417r induces a SA-independent signalling pathway, different from that controlling SAR. Whether the signalling pathways regulating WCS417r-mediated ISR and pathogen-induced SAR converge to elicit a similar phenotypic effect remains to be determined.

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