

# Differential Induction of Systemic Resistance in *Arabidopsis* by Biocontrol Bacteria

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Selected nonpathogenic, root-colonizing bacteria are able to elicit induced systemic resistance (ISR) in plants. To elucidate the molecular mechanisms underlying this type of systemic resistance, an *Arabidopsis*-based model system was developed in which *Pseudomonas syringae* pv. *tomato* and *Fusarium oxysporum* f. sp. *raphani* were used as challenging pathogens. In *Arabidopsis thaliana* ecotypes Columbia and Landsberg *erecta*, colonization of the rhizosphere by *P. fluorescens* strain WCS417r induced systemic resistance against both pathogens. In contrast, ecotype RLD did not respond to WCS417r treatment, whereas all three ecotypes expressed systemic acquired resistance upon treatment with salicylic acid (SA). *P. fluorescens* strain WCS374r, previously shown to induce ISR in radish, did not elicit ISR in *Arabidopsis*. The opposite was found for *P. putida* strain WCS358r, which induced ISR in *Arabidopsis* but not in radish. These results demonstrate that rhizosphere pseudomonads are differentially active in eliciting ISR in related plant species. The outer membrane lipopolysaccharide (LPS) of WCS417r is the main ISR-inducing determinant in radish and carnation, and LPS-containing cell walls also elicit ISR in *Arabidopsis*. However, mutant WCS417rOA<sup>-</sup>, lacking the O-antigenic side chain of the LPS, induced levels of protection similar to those induced by wild-type WCS417r. This indicates that ISR-inducing bacteria produce more than a single factor that trigger ISR in *Arabidopsis*. Furthermore, WCS417r and WCS358r induced protection in both wild-type *Arabidopsis* and SA-nonaccumulating NahG plants without activating pathogenesis-related gene expression. This suggests that elicitation of an SA-independent signaling pathway is a characteristic feature of ISR-inducing biocontrol bacteria.

Induced resistance is defined as an enhancement of the plant's defensive capacity against a broad spectrum of pathogens that is acquired after appropriate stimulation (reviewed by Hammerschmidt and Kué 1995). The classic way of eliciting induced resistance is by a predisposal infection with a pathogen that causes a hypersensitive reaction. The resulting

elevated resistance response upon challenge inoculation of plant parts distant from the site of primary infection is known as systemic acquired resistance (SAR). SAR was first characterized in tobacco plants that expressed increased resistance systemically after infection by tobacco mosaic virus (Ross 1961). Pathogen-induced SAR is associated with an early increase in endogenously synthesized salicylic acid (SA) (Malamy et al. 1990; Métraux et al. 1990). Accumulation of SA is critical in the signaling pathway that controls SAR, since plants that do not accumulate SA are incapable of expressing induced resistance (Delaney et al. 1994; Gaffney et al. 1993). Furthermore, SAR is characterized by the activation of so-called SAR genes (Ward et al. 1991), including genes that encode pathogenesis-related (PR) proteins (Linthorst 1991; Van Loon 1985), which are often used as markers for the state of induced resistance. Both PR genes and induced resistance are expressed in plants treated with SA (Ward et al. 1991; White 1979). In addition, chemical agents such as 2,6-dichloroisonicotinic acid (Métraux et al. 1991) and benzothiadiazole (Lawton et al. 1996) have been shown to induce resistance to the same spectrum of pathogens and to concurrently activate expression of SAR genes.

In 1991, an alternative approach to inducing systemic resistance was reported by Alström (1991), Van Peer et al. (1991), and Wei et al. (1991). These authors independently demonstrated that selected strains of nonpathogenic plant growth-promoting rhizobacteria, which colonize the rhizosphere of the plant, are able to elevate plant resistance. Until then, these bacteria, mainly fluorescent *Pseudomonas* spp., had been studied for their ability to control soilborne pathogens through competition for nutrients, siderophore-mediated competition for iron, or antibiosis (Bakker et al. 1991; Schippers 1992; Thomashaw and Weller 1995). Induction of systemic resistance in the plant thus appeared to be an additional mechanism by which these bacteria could protect the plant against disease. To date, induced systemic resistance (ISR) (Kloepper et al. 1992) mediated by nonpathogenic rhizobacteria has been demonstrated in several plant species (Pieterse et al. 1996b) and shown to be effective against bacterial, viral, and fungal diseases. So far, little is known about the molecular basis underlying this type of ISR. Maurhofer et al. (1994) showed that ISR induced by *P. fluorescens* strain CHA0 in tobacco is associated with PR protein accumulation, suggesting that nonpathogen-induced

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ISR and pathogen-induced SAR share similar mechanisms. However, PR proteins did not accumulate in radish plants expressing ISR elicited by *P. fluorescens* strain WCS417r (Hoffland et al. 1995, 1996). Moreover, Pieterse et al. (1996a) demonstrated that in *Arabidopsis*, ISR induced by WCS417r was not associated with PR gene activation and was elicited in transgenic *Arabidopsis* plants unable to accumulate SA. This indicates that in contrast to pathogen-induced SAR, WCS417r-mediated ISR is controlled by an SA-independent signaling pathway.

Previously, Van Peer and Schippers (1992) and Leeman et al. (1995b) showed that the O-antigenic side chain of the outer membrane lipopolysaccharide (LPS) of strain WCS417r is the main determinant for the induction of ISR against Fusarium wilt disease in both carnation and radish. A bacterial mutant lacking the O-antigenic side chain did not induce resistance, whereas LPS-containing cell walls and purified LPS of WCS417r induced ISR to the same extent as living bacteria. Other bacterial determinants suggested to contribute to ISR are siderophores and SA (Leeman et al. 1996; Maurhofer et al. 1994).

The main objective of this study was to elucidate the basic mechanisms underlying nonpathogenic *Pseudomonas* spp.-mediated ISR in the *Arabidopsis* model system. Here, we demonstrate that ISR-inducing fluorescent *Pseudomonas* spp. are differentially active in eliciting ISR in *Arabidopsis*. Furthermore, we provide evidence that in contrast to what is observed in carnation and radish, the LPS of WCS417r plays only a minor role in the elicitation of ISR in *Arabidopsis*, indicating that WCS417r possesses more than a single ISR-inducing determinant.

## RESULTS

### Differential expression of *P. fluorescens* WCS417r-mediated ISR in *Arabidopsis*.

Recently, Pieterse et al. (1996a) demonstrated that colonization of the rhizosphere by strain WCS417r of *P. fluorescens* induces ISR in *Arabidopsis* against diseases caused by the bacterial leaf pathogen *P. syringae* pv. *tomato* (Whalen et al. 1991) and the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani* (Leeman et al. 1995a). To investigate whether different ecotypes of *A. thaliana* are equally able to express WCS417r-mediated ISR, ecotypes Columbia (Col), Landsberg erecta (*Ler*), and RLD were tested in bioassays in which *P. syringae* pv. *tomato* and *F. oxysporum* f. sp. *raphani* were used as challenging pathogens. In these bioassays, the resistance-inducing potential of WCS417r was compared with that of SA, an established inducer of SAR (Malamy and Klessig 1992). Leaves of noninduced control plants challenged with *P. syringae* pv. *tomato* developed necrotic lesions surrounded by extensive, spreading chlorosis. Upon challenge inoculation with *F. oxysporum* f. sp. *raphani*, control plants showed wilting and yellowing of the leaves after 3 to 4 weeks. Induced protection against either pathogen was quantified by determining the percentage of leaves with symptoms. In plants challenge inoculated with *P. syringae* pv. *tomato*, proliferation of the pathogen in the leaves was assessed also. Figure 1A and C shows that root treatment of ecotype Col with WCS417r resulted in a reduction of about 50% in the symptoms caused by either of the pathogens. The

level of protection induced by WCS417r was similar to or only slightly less than that induced by SA applied to the roots as a soil drench. As shown in Figure 1B, growth of *P. syringae* pv. *tomato* was significantly inhibited in WCS417r- and SA-treated Col plants, indicating that the reduction in symptoms is associated with inhibition of bacterial multiplication. Ecotype *Ler* responded similarly to WCS417r and SA treatments. Both inducers decreased disease symptoms to the same extent (Fig. 1D and F) and caused a 20-fold reduction in bacterial multiplication in leaves challenged with *P. syringae* pv. *tomato* (Fig. 1E). In ecotype RLD, however, WCS417r did not reduce symptoms provoked by either pathogen (Fig. 1G and I), nor did it inhibit growth of *P. syringae* pv. *tomato* in challenged leaves (Fig. 1H). In contrast, treatment with SA resulted in a significant reduction in symptoms caused by either pathogen, as in ecotypes Col and *Ler*. Moreover, proliferation of *P. syringae* pv. *tomato* in challenged leaves was clearly decreased. These results demonstrate that WCS417r induces ISR in ecotypes Col and *Ler* but fails to do so in ecotype RLD, whereas in all three ecotypes SAR can be induced by SA.

To determine whether the inability of RLD to exhibit WCS417r-mediated ISR might be attributed to a less effective colonization of the roots, the population density of WCS417r in the rhizosphere of treated Col, *Ler*, and RLD plants was examined. Table 1 shows that the numbers of rifampicin-resistant bacteria present in the rhizosphere of the three ecotypes were of the same order of magnitude ( $2.2\text{--}8.3 \times 10^5$  CFU/g of root, fresh weight). No rifampicin-resistant bacteria were detected on nontreated roots. Therefore, it can be concluded that WCS417r colonized the rhizosphere of the three ecotypes to comparable levels.

### Differential ability of strains of fluorescent *Pseudomonas* spp. to elicit ISR.

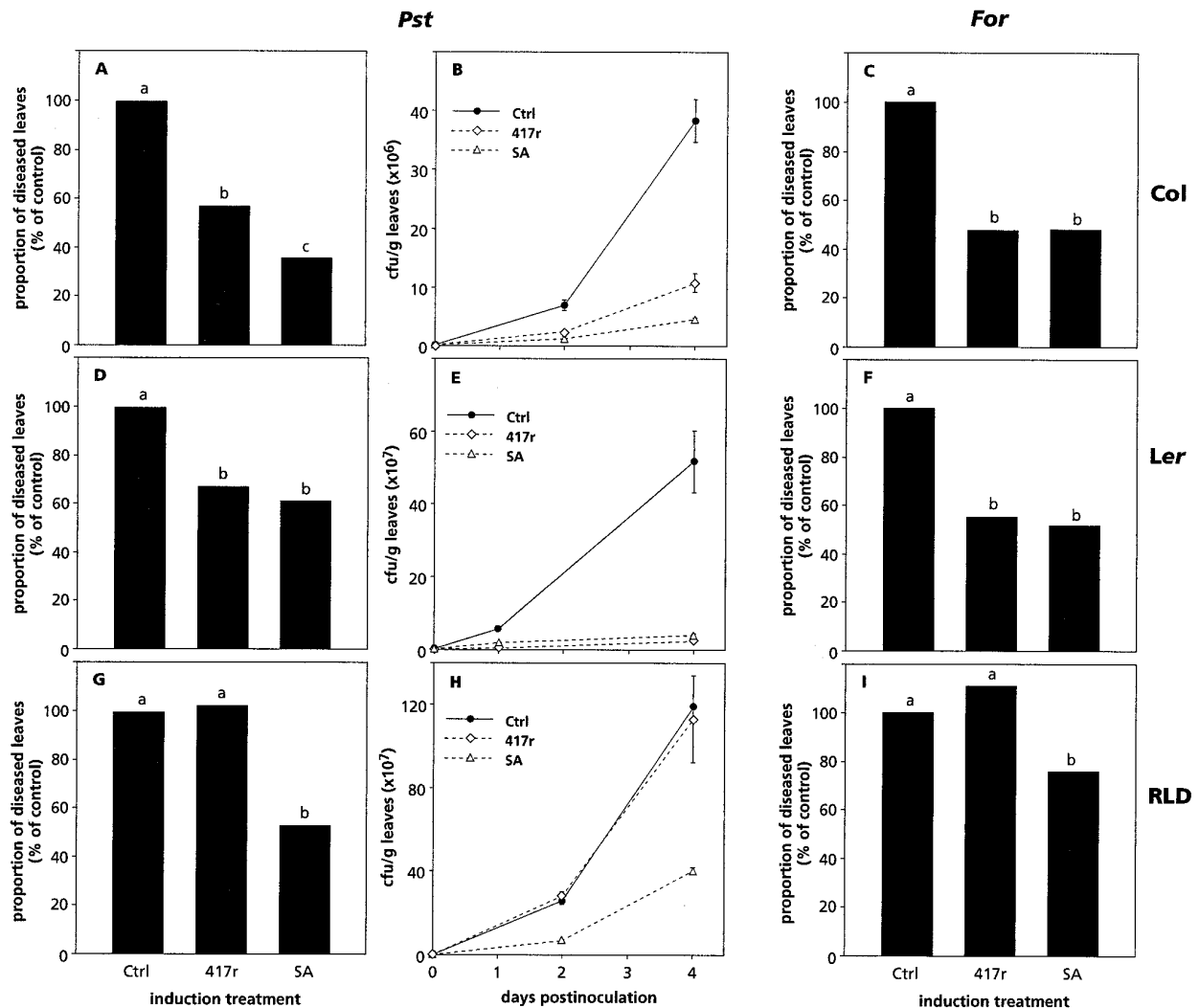
Previously, Leeman et al. (1995a) showed that in radish plants, strains WCS417r and WCS374r of *P. fluorescens* induce ISR against Fusarium wilt, whereas strain WCS358r of *P. putida* does not. To investigate whether *Arabidopsis* responds similarly, the ability of these strains to induce ISR against *P. syringae* pv. *tomato* or *F. oxysporum* f. sp. *raphani* infection was tested. In contrast to WCS417r, WCS374r did not reduce disease symptoms provoked by either *P. syringae* pv. *tomato* or *F. oxysporum* f. sp. *raphani* (Fig. 2A and C), nor did it inhibit proliferation of *P. syringae* pv. *tomato* in challenged leaves (Fig. 2B). WCS358r induced significant ISR against both pathogens. However, the extent of symptom reduction was less than that induced by WCS417r. On the other hand, multiplication of *P. syringae* pv. *tomato* upon challenge was reduced almost to the same level. Whereas WCS417r induced resistance in both radish and *Arabidopsis*, the resistance-inducing capacities of WCS374r and WCS358r clearly differed in these two species.

To exclude the possibility that the observed protection was caused by a direct effect of the inducing *Pseudomonas* strains on the pathogen, their spatial separation on the plant was verified. To this end, the population densities of the rhizobacterial strains on treated and nontreated plant parts were determined at the end of each bioassay by plating root washes or leaf extracts on selective King's medium B agar plates (King et al. 1954). Table 2 shows that from WCS417r- and WCS358r-treated roots

similar amounts of rifampicin-resistant *Pseudomonas* bacteria were recovered, whereas approximately 10-fold lower numbers were detected on WCS374r-treated roots. In the leaves used for challenge inoculation with *P. syringae* pv. *tomato* or on the root parts inoculated with *F. oxysporum* f. sp. *raphani*, rifampicin-resistant bacteria were never detected, demonstrating that for the duration of the bioassays, the inducing *Pseudomonas* strains remained spatially separated from the challenging pathogens. Moreover, in vitro antagonism assays showed no significant inhibition of growth of *P. syringae* pv. *tomato* or *F. oxysporum* f. sp. *raphani* by either of the three bacterial strains (data not shown), indicating that the induced protection is unlikely to be caused by accumulation of *Pseudomonas*-produced antibiotics in the plant.

### Both WCS417r- and WCS358r-mediated ISR are independent of SA.

Using transgenic *Arabidopsis* NahG plants that did not accumulate SA (Delaney et al. 1994), Pieterse et al. (1996a) demonstrated that in contrast to pathogen-induced SAR, WCS417r-mediated ISR is independent of endogenous SA accumulation and PR gene activation. To investigate whether WCS358r-mediated ISR is independent of SA as well, bioassays were performed with NahG plants and wild-type Col plants. In accordance with previous results, treatment of the roots with WCS417r resulted in a significant reduction in symptoms caused by *P. syringae* pv. *tomato* infection in both Col and NahG plants (Fig. 3). WCS358r similarly induced protection in both wild-type and NahG plants, whereas plants



**Fig. 1.** Quantification of induced resistance against *Pseudomonas syringae* pv. *tomato* (Pst) or *Fusarium oxysporum* f. sp. *raphani* (For) infection in Col (A–C), Ler (D–F), and RLD (G–I) plants treated with 10 mM MgSO<sub>4</sub> (Ctrl), *P. fluorescens* WCS417r (417r), or 1 mM salicylic acid (SA). The proportion of leaves with symptoms relative to control plants (100%) was determined 4 days after challenge inoculation with *P. syringae* pv. *tomato* (A, D, and G) or 3 to 4 weeks after challenge inoculation with *F. oxysporum* f. sp. *raphani* (C, F, and I). The absolute proportions of diseased leaves of the controls shown in A, C, D, F, G, and I were 58.9, 53.4, 80.9, 61.2, 54.4, and 35.8%, respectively. Different letters indicate statistically significant differences between treatments by Fisher's least significant difference test ( $\alpha = 0.05$ , and  $n = 30$ ). Growth of *P. syringae* pv. *tomato* in challenged leaves (B, E, and H) was assessed at indicated days after inoculation. Data points are means (CFU/g) with standard errors from two sets of 20 leaves randomly selected from plants of the bioassays shown in A, D, and G, respectively. The values presented are from representative experiments that were repeated at least twice with similar results.

treated with WCS374r did not show increased resistance. The level of protection induced by WCS417r and WCS358r is somewhat lower in NahG plants compared with that in wild-type plants, suggesting a modulating role for SA in the level of expression of ISR. Northern blot analyses demonstrated that none of the rhizosphere pseudomonads induced PR-1, PR-2, or PR-5 mRNAs (Fig. 4). In contrast, PR mRNA accumulated in noninoculated leaves of plants expressing SAR induced by a predisposal infection of primary leaves with pathogenic *P. syringae* pv. *tomato*. These results demonstrate that like WCS417r, WCS358r elicits an SA-independent signaling pathway leading to ISR without concomitant activation of PR genes.

#### Involvement of bacterial LPS in the elicitation of ISR in *Arabidopsis*.

In radish, purified LPS and LPS-containing cell wall preparations of WCS417r are as effective as living WCS417r bacteria in inducing ISR (Leeman et al. 1995b). To investigate

**Table 1.** Colonization of the rhizosphere of *Arabidopsis thaliana* ecotypes Col, *Ler*, and RLD by WCS417r<sup>a</sup>

Ecotype	CFU/g of root, fresh weight ( $\times 10^5$ ) <sup>b</sup>	
	<i>Pseudomonas syringae</i> pv. <i>tomato</i> <sup>c</sup>	<i>Fusarium oxysporum</i> f. sp. <i>raphani</i> <sup>d</sup>
Col	2.2 $\pm$ 0.2	6.4 $\pm$ 0.4
<i>Ler</i>	3.1 $\pm$ 0.3	3.9 $\pm$ 0.2
RLD	6.0 $\pm$ 1.0	8.3 $\pm$ 0.7

<sup>a</sup> Values presented are the average population densities  $\pm$  SE of multiple bioassays.

<sup>b</sup> Roots were harvested at the end of the bioassays. On nontreated roots or root parts, no rifampicin-resistant bacteria were detected (detection limit =  $10^3$  CFU/g).

<sup>c</sup> Number of WCS417r bacteria on the roots of plants from the *P. syringae* pv. *tomato* bioassays.

<sup>d</sup> Number of WCS417r bacteria on the treated root parts of plants from the *F. oxysporum* f. sp. *raphani* bioassays.

whether the LPS of WCS417r also elicits ISR in *Arabidopsis*, cell wall preparations of WCS417r and its mutant WCS417rOA<sup>-</sup> (Leeman et al. 1995b), which lacks the O-antigenic side chain of the LPS, were tested in *P. syringae* pv. *tomato* bioassays. Cell walls of the noninducing strain WCS374r were used as a control. Figure 5 shows that treatment of the roots with cell walls of WCS417r reduced symptoms by 20%, whereas the cell walls of WCS417rOA<sup>-</sup> or WCS374r were ineffective. The reduction was significantly less than the level of protection obtained with living bacteria, suggesting that the O-antigenic side chain of the LPS of

**Table 2.** Colonization of *Arabidopsis* by WCS417r, WCS374r, and WCS358r<sup>a</sup>

Treatment <sup>b</sup>	CFU/g, fresh weight ( $\times 10^5$ )		
	<i>Pseudomonas syringae</i> pv. <i>tomato</i> <sup>c</sup>	<i>Fusarium oxysporum</i> f. sp. <i>raphani</i> <sup>d</sup>	Challenged plant parts <sup>e</sup>
Control	b.d.	b.d.	b.d.
WCS417r	3.1 $\pm$ 0.2	5.8 $\pm$ 0.4	b.d.
WCS374r	0.3 $\pm$ 0.0	0.7 $\pm$ 0.1	b.d.
WCS358r	3.0 $\pm$ 0.3	6.7 $\pm$ 0.6	b.d.

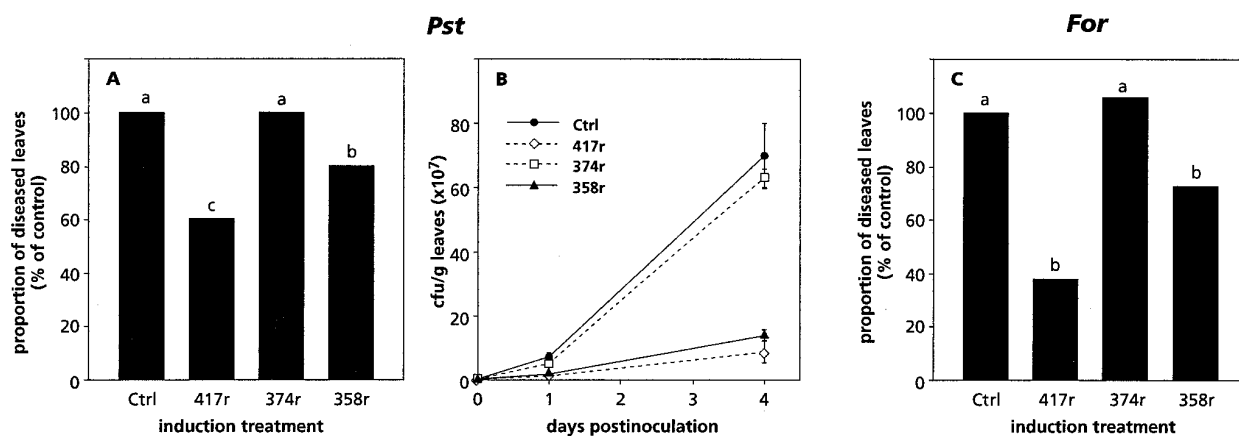
<sup>a</sup> Values presented are the average population densities  $\pm$  SE of multiple bioassays. b.d. = below detection (detection limit =  $10^3$  CFU/g).

<sup>b</sup> In the *P. syringae* pv. *tomato* bioassays, a solution of 10 mM MgSO<sub>4</sub> (control) or a suspension of *Pseudomonas* spp. strains in 10 mM MgSO<sub>4</sub> was mixed through the soil ( $5 \times 10^7$  CFU/g) prior to planting of *Ler*. In the *F. oxysporum* f. sp. *raphani* bioassays, talcum powder mixed with a solution of 10 mM MgSO<sub>4</sub> (control) or a suspension of *Pseudomonas* spp. strains in 10 mM MgSO<sub>4</sub> ( $5 \times 10^8$  CFU/g) was applied to the lower part of the roots of 2-week-old Col seedlings.

<sup>c</sup> Number of rhizobacteria at the end of the bioassays on the roots of plants challenged with *P. syringae* pv. *tomato*.

<sup>d</sup> Number of rhizobacteria at the end of the bioassays on the treated root parts of plants from the *F. oxysporum* f. sp. *raphani* bioassays.

<sup>e</sup> In the *P. syringae* pv. *tomato* bioassays, leaves were harvested just prior to challenge inoculation. In the *F. oxysporum* f. sp. *raphani* bioassays, inoculated upper root parts were harvested at the end of the bioassays.



**Fig. 2.** Quantification of induced systemic resistance against *Pseudomonas syringae* pv. *tomato* (*Pst*) or *Fusarium oxysporum* f. sp. *raphani* (*For*) infection in *Arabidopsis* plants treated with 10 mM MgSO<sub>4</sub> (Ctrl), *P. fluorescens* WCS417r (417r), *P. fluorescens* WCS374r (374r), or *P. putida* WCS358r (358r). The proportion of leaves with symptoms relative to control plants (100%) was determined 4 days after challenge inoculation of *Ler* plants with *P. syringae* pv. *tomato* (A) or 3 to 4 weeks after challenge inoculation of Col plants with *F. oxysporum* f. sp. *raphani* (C). The absolute proportions of diseased leaves of the controls shown in A and C were 75.9 and 39.0%, respectively. Different letters indicate statistically significant differences between treatments by Fisher's least significant difference test ( $\alpha = 0.05$ , and  $n = 30$ ). Growth of *P. syringae* pv. *tomato* in challenged leaves (B) was assessed at indicated days after inoculation. Data points are means (CFU/g) with standard errors from two sets of 20 leaves randomly selected from plants of the bioassay shown in A. The values presented are from representative experiments that were repeated at least twice with similar results.

WCS417r contributes to elicitation of ISR but is probably not sufficient for full induction.

Comparison of the resistance-inducing ability of living cells of WCS417r and its OA<sup>-</sup> mutant in Col and *Ler* plants revealed that in most experiments, wild-type and mutant bacteria induced similar levels of protection against both *P. syringae* pv. *tomato* and *F. oxysporum* f. sp. *raphani* infection (Fig. 6A, B, and D). However, in some bioassays, the mutant was significantly less effective (Fig. 6C). WCS417r and WCS417rOA<sup>-</sup> colonized the rhizosphere of *Arabidopsis* to similar levels (average of 3.6 and 3.4 × 10<sup>5</sup> CFU/g of root, fresh weight, respectively). These results demonstrate that in *Arabidopsis*, elicitation of ISR by WCS417r is not dependent upon the O-antigenic side chain of the LPS, although cell wall components can induce resistance and may contribute to the level of protection attained.

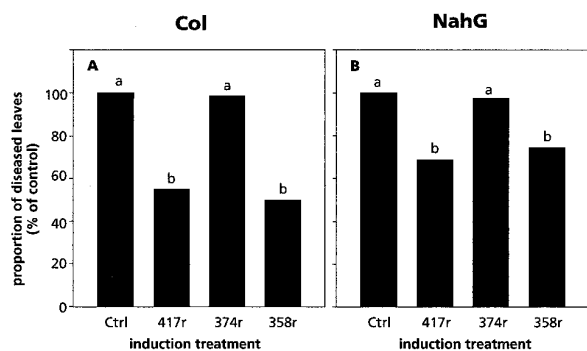
## DISCUSSION

Induction of systemic resistance is one of the mechanisms by which selected strains of nonpathogenic *Pseudomonas* spp. can reduce diseases. *P. fluorescens* WCS417r has been demonstrated to induce resistance in several plant species (Duijff et al. 1996; Leeman et al. 1995a; Van Peer et al. 1991). With the aim of studying the molecular and mechanistic basis underlying this type of systemic resistance, we recently developed *Arabidopsis* as a model host using WCS417r as the inducing agent and *P. syringae* pv. *tomato* and *F. oxysporum* f. sp. *raphani* as challenging pathogens (Pieterse et al. 1996a). ISR against *P. syringae* pv. *tomato* is manifested by both a reduction in the number of leaves showing symptoms and a decrease in the multiplication of the pathogen in the leaves. ISR against *F. oxysporum* f. sp. *raphani* was measured as a reduction in the percentage of leaves showing symptoms only.

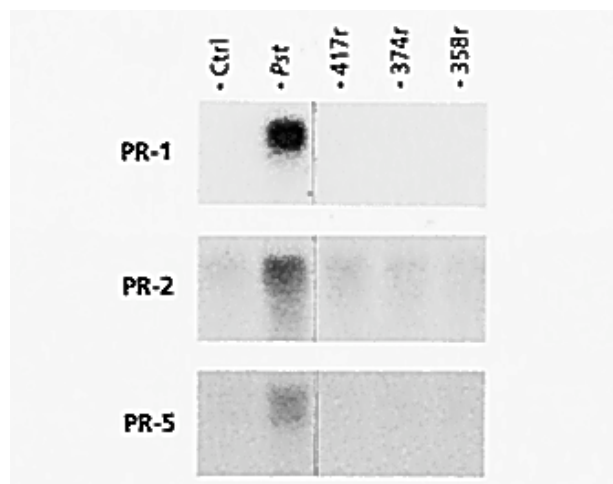
Using three *A. thaliana* ecotypes and three rhizobacterial strains, we now demonstrate that specific interactions between the bacterial strains and the plant ecotypes determine

induction of systemic resistance. On the one hand, ecotypes of *A. thaliana* were differentially responsive to WCS417r treatment. In contrast to ecotypes Col and *Ler*, ecotype RLD did not develop ISR upon treatment of the roots with WCS417r (Fig. 1). Nevertheless, all three ecotypes readily expressed SAR upon SA treatment. Colonization of the rhizosphere by WCS417r was similar in the three ecotypes (Table 1), suggesting that ecotype RLD either does not recognize elicitors of WCS417r or is impaired in the ISR signaling pathway. On the other hand, bacterial strains WCS417r, WCS374r, and WCS358r were differentially active in the induction of ISR. WCS417r and WCS358r triggered an ISR response in *Arabidopsis*, whereas WCS374r did not (Fig. 2). In contrast, in radish, Leeman et al. (1995a) demonstrated induction by WCS417r and WCS374r but not by WCS358r. Apparently, all three strains have the potential to induce ISR but do so only in selected plant species.

Compared with the ISR-inducing strains WCS417r and WCS358r, the noninducing strain WCS374r was present at a 10-fold lower level in the rhizosphere of *Arabidopsis* by the end of the bioassays (Table 2). Therefore, it cannot be ruled out that the inability of WCS374r to trigger ISR in *Arabidopsis* is caused by insufficient root colonization. However, in the *F. oxysporum* f. sp. *raphani* bioassay, in which plants were challenged as soon as 3 days after application of the rhizobacterial strains, only the initial density of bacteria applied to the roots appeared critical for the induction of ISR, and bacterial numbers often dropped to noninducing levels by the end of the bioassays (Leeman et al. 1995a; Raaijmakers et al. 1995). Since treatments constituted equal amounts of the different bacteria at a concentration 500-fold higher than the threshold for ISR in radish (Raaijmakers et al. 1995), the inability of WCS374r to induce ISR in this bioassay is more likely caused by a lack of response by the plant. This explanation is supported by our observation that cell wall preparations of WCS374r were ineffective in inducing ISR in *Arabidopsis*, in contrast to those of WCS417r,



**Fig. 3.** Quantification of induced systemic resistance against *Pseudomonas syringae* pv. *tomato* infection in *Arabidopsis* Col (A) or NahG (B) plants treated with 10 mM MgSO<sub>4</sub> (Ctrl), *P. fluorescens* WCS417r (417r), *P. fluorescens* WCS374r (374r), or *P. putida* WCS358r (358r). Proportion of leaves with symptoms relative to control plants (100%) was determined 4 days after challenge inoculation with the pathogen. The absolute proportions of diseased leaves of the controls shown in A and B were 58.5 and 80.1%, respectively. Different letters indicate statistically significant differences between treatments by Fisher's least significant difference test ( $\alpha = 0.05$ , and  $n = 30$ ). The values presented are from representative experiments that were repeated at least twice with similar results.



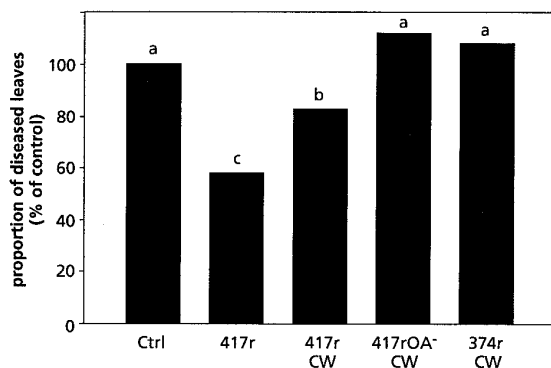
**Fig. 4.** Northern blot analyses of pathogenesis-related gene expression in leaves of 5-week-old *Arabidopsis* plants cultured in soil containing 10 mM MgSO<sub>4</sub> (Ctrl), *Pseudomonas fluorescens* WCS417r (417r), *P. fluorescens* WCS374r (374r), or *P. putida* WCS358r (358r), using PR-1, PR-2, and PR-5 gene-specific probes. Inoculation with *P. syringae* pv. *tomato* (Pst) was performed by pressure infiltrating three lower leaves 2 days before harvest of the noninoculated leaves.

whereas cell wall preparations from both strains were active in eliciting ISR in radish (Leeman et al 1995b).

The ecotype-specific induction of resistance in *Arabidopsis* by WCS417r further indicates that protection against *P. syringae* pv. *tomato* and *F. oxysporum* f. sp. *raphani* is dependent upon specific interactions between the bacteria and the plant. Direct suppression of the pathogen by bacterial antagonism can be ruled out, since the inducing pseudomonads and the challenging pathogens remained spatially separated (Table 2). Moreover, none of the bacterial strains significantly inhibited the pathogens in vitro, making it highly unlikely that accumulation of antibiotics produced by the rhizobacterial pseudomonads contributed to the increased protection.

A major bacterial trait implicated in the elicitation of resistance responses in plants by pathogens is the outer membrane LPS (Sequeira 1983). LPS-containing cell walls of WCS417r, which were able to elicit a full resistance response in radish and carnation (Leeman et al. 1995b; Van Peer and Schippers 1992), also induced protection in *Arabidopsis* (Fig. 5). However, the level of protection was significantly lower than that elicited by living bacteria. Moreover, the OA<sup>-</sup> mutant of WCS417r, which no longer induced ISR in radish (Leeman et al. 1995b), did reduce the disease symptoms in *Arabidopsis* in most experiments to the same extent as the wild-type (Fig. 6). This indicates that the LPS of WCS417r plays only a minor role in the elicitation of ISR in *Arabidopsis* and that other bacterial component(s) constitute the primary determinant.

SA produced by rhizosphere pseudomonads has been implicated in the activation of systemic resistance in radish (Leeman et al. 1996). However, bacterially produced SA is unlikely to be a determinant for eliciting ISR in *Arabidopsis*. First, both WCS417r and WCS358r induced ISR in *Arabidopsis*, but only WCS417r has the capacity to produce SA (Leeman et al. 1996). Moreover, WCS374r can produce the largest amount of SA but does not induce resistance in *Arabidopsis*. Second, inducing strains were equally effective

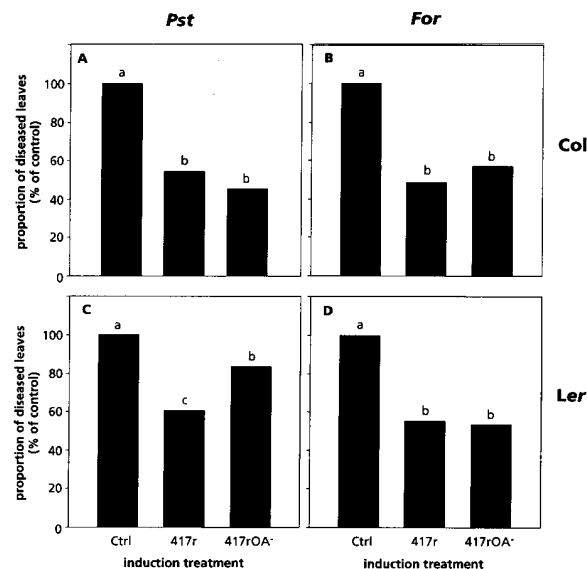


**Fig. 5.** Quantification of induced systemic resistance against *Pseudomonas syringae* pv. *tomato* infection in *Arabidopsis* plants treated with 10 mM MgSO<sub>4</sub> (Ctrl), *P. fluorescens* WCS417r (417r), or cell wall preparations of WCS417r (417r CW), WCS417rOA<sup>-</sup> (417rOA<sup>-</sup> CW), or *P. fluorescens* WCS374r (374r CW). The proportion of leaves with symptoms relative to control plants (100%) was determined 4 days after challenge inoculation of *Ler* plants with the pathogen. The absolute proportion of diseased leaves of the control was 65.5%. Different letters indicate statistically significant differences between treatments by Fisher's least significant difference test ( $\alpha = 0.05$ , and  $n = 30$ ). The values presented are from a representative experiment that was repeated twice with similar results.

in wild-type and NahG plants that readily inactivate SA. In addition, the OA<sup>-</sup> mutants of these strains had the same resistance-inducing capacity in NahG plants as the wild-type strains (data not shown), indicating that SA does not contribute to the ISR response elicited by the non-LPS determinant. Third, WCS417r did not trigger ISR in ecotype RLD, although this ecotype is responsive to induction by SA (Fig. 1).

Another metabolite implicated in ISR induction is the iron-regulated pyoverdine siderophore (Maurhofer et al. 1994). Leeman et al. (1996) demonstrated that the siderophore of WCS374r can act as an elicitor of ISR in radish, even though its effect is overridden by that of the LPS during the induction by living bacteria. We are currently investigating the involvement of siderophores in the elicitation of ISR in *Arabidopsis*.

As previously demonstrated for strain WCS417r (Pieterse et al. 1996a), WCS358r induces a plant-mediated resistance response in both wild-type and NahG plants without concomitant activation of genes encoding PR proteins (Figs. 3 and 4). These results indicate that both biocontrol strains induce a signaling pathway different from the one that controls classic SAR. Press et al. (1996) found that biocontrol strain *Serratia marcescens* 90-166 is able to induce protection in both wild-type and NahG tobacco plants against *P. syringae* pv. *tabaci* as well. Hence, it seems that the ability to trigger an SA-independent pathway controlling systemic resistance is a common trait of ISR-inducing biocontrol bacteria.



**Fig. 6.** Quantification of induced systemic resistance against *Pseudomonas syringae* pv. *tomato* (*Pst*) or *Fusarium oxysporum* f. sp. *raphani* (*For*) infection in Col (A and B) and *Ler* (C and D) plants treated with 10 mM MgSO<sub>4</sub> (Ctrl), *P. fluorescens* WCS417r (417r), or its LPS O-antigen mutant WCS417rOA<sup>-</sup> (417rOA<sup>-</sup>). The proportion of leaves with symptoms relative to control plants (100%) was determined 4 days after challenge inoculation with *P. syringae* pv. *tomato* (A and C) or 3 to 4 weeks after challenge inoculation with *F. oxysporum* f. sp. *raphani* (B and D). The absolute proportions of diseased leaves of the controls shown in A–D were 58.9, 57.6, 73.3, and 61.2%, respectively. Different letters indicate statistically significant differences between treatments by Fisher's least significant difference test ( $\alpha = 0.05$ , and  $n = 30$ ). The values presented are from representative experiments that were repeated at least twice with similar results.

## MATERIALS AND METHODS

### Microbial cultures.

*Pseudomonas fluorescens* strain WCS417 was initially isolated from the rhizosphere of wheat grown in a field suppressive to take-all disease caused by *Gaeumannomyces graminis* pv. *tritici* (Lamers et al. 1988) and *P. fluorescens* strain WCS374 and *P. putida* strain WCS358 were collected from the rhizosphere of potato (strains WCS374 and WCS358) (Geels and Schippers 1983). Rifampicin-resistant mutants of these strains (WCS417r, WCS374r, and WCS358r) were used throughout this study (Geels and Schippers 1983; Glandorf et al. 1992; Leeman et al. 1991). WCS417rOA<sup>-</sup> is a spontaneous phage-resistant mutant of WCS417r lacking the O-antigenic side chain of the outer membrane LPS (Leeman et al. 1995b). The bacteria were cultured for 24 h on King's medium B (KB) agar plates (King et al. 1954) at 28°C. Subsequently, the cells were collected and resuspended in 10 mM MgSO<sub>4</sub>.

The virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Whalen et al. 1991) was cultured in liquid KB at 28°C. After overnight incubation, the cells were collected by centrifugation and resuspended in 10 mM MgSO<sub>4</sub>.

The fungal pathogen *Fusarium oxysporum* f. sp. *raphani* WCS600 was initially isolated from tubers of a naturally infected radish plant (Leeman et al. 1995a), and a culture was maintained on potato-dextrose agar. The inoculum was prepared by incubating mycelial patches in aerated 2% malt extract at 22°C for 7 days. Subsequently, cultures were filtered and conidia were collected by centrifugation. Conidia were mixed with sterile peat (Agrifutur s.r.l., Alfianello, Italy) to a density of 10<sup>7</sup> conidia per gram and allowed to germinate and grow at 24°C for 2 days. The final density of colony-forming units in the peat was determined by dilution plating on potato-dextrose agar.

### Preparation of bacterial cell walls.

Cell walls of WCS417r, WCS417rOA<sup>-</sup>, and WCS374r were isolated from cultures grown overnight in liquid KB at 28°C, essentially as described by Leeman et al. (1995b). The bacteria were collected by centrifugation and resuspended in 50 mM Tris-HCl plus 2 mM EDTA (pH 8.5). The cells were then sonicated eight times for 15 s on ice at resonance amplitude. Intact cells were removed from the sonicated suspension by centrifugation at 600 × g for 20 min. After centrifugation of the supernatant at 8,000 × g for 60 min, the pellet of LPS-containing cell walls was resuspended in 10 mM phosphate-buffered saline (pH 7.2) plus 0.01% sodium azide and stored at -80°C until further use. The absence of living bacteria was verified by plating on KB agar plates.

### *P. syringae* pv. *tomato* bioassay.

Seeds of *Arabidopsis thaliana* ecotypes Columbia (Col), Landsberg *erecta* (Ler), RLD, and transgenic NahG plants harboring the bacterial *nahG* gene encoding salicylate hydroxylase (Delaney et al. 1994) were sown in sterile quartz sand. Once a day, the seedlings were supplied with modified half-strength Hoagland nutrient solution (2 mM KNO<sub>3</sub>, 5 mM Ca[NO<sub>3</sub>]<sub>2</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, and trace elements, pH 7) (Hoagland and Arnon 1938) containing 10 μM Sequestren (Fe-ethylenediamine-di[o-hydroxyphenylacetic acid]; CIBA-Geigy, Basel, Switzerland). Two-week-old seedlings

were transferred to 60-ml pots containing a sand and potting soil mixture that had been autoclaved twice for 1 h before it was mixed with either a suspension of pseudomonads to a final density of 5 × 10<sup>7</sup> CFU/g or an equal volume of a solution of 10 mM MgSO<sub>4</sub> (50 ml/kg). Treatment of the roots with bacterial cell walls was performed by applying 20 ml of a cell wall preparation as a soil drench 7 and 4 days before challenge inoculation (cell walls from 2.5 × 10<sup>6</sup> CFU/ml, resulting in an amount of cell walls equal to that present in soil containing 5 × 10<sup>7</sup> CFU/ml at the beginning of the bioassay). SA treatment was performed by applying 20 ml of a solution of 1 mM SA (pH 6) as a soil drench 7 and 4 days before challenge inoculation. Plants were cultivated in a growth chamber with a 9-h day (200 μE m<sup>-2</sup> s<sup>-1</sup> at 24°C) and a 15-h night (20°C) cycle at 70% relative humidity. The plants were watered on alternate days and once a week supplied with nutrient solution.

Plants were challenge inoculated when 5 weeks old. One day before challenge, the plants were placed at 100% relative humidity. Inoculation was carried out by dipping the leaves in a suspension of *P. syringae* pv. *tomato* in 10 mM MgSO<sub>4</sub> supplemented with 0.01% (vol/vol) of the surfactant Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands). Inoculation densities were chosen such that 4 days after challenge, approximately 70% of the leaves of the control plants showed symptoms (2.5 × 10<sup>7</sup> CFU/ml for Col and NahG, 1 × 10<sup>8</sup> CFU/ml for Ler, and 1 × 10<sup>7</sup> CFU/ml for RLD). At that time, the proportion of leaves with disease symptoms per plant was determined for 30 plants per treatment. Data were statistically analyzed by one-way analysis of variance (ANOVA) for a single experiment and two-way ANOVA for combined experiments followed by Fisher's test for least significant differences at α = 0.05.

Multiplication of *P. syringae* pv. *tomato* was assessed in challenged leaves at different time points after inoculation. Two pools of 1 g of randomly selected leaves (15 to 20) per treatment were rinsed thoroughly in sterile water and homogenized in a sterile solution of 10 mM MgSO<sub>4</sub>. Dilutions were plated onto KB agar supplemented with rifampicin (50 mg/liter) and cycloheximide (100 mg/liter). After incubation at 28°C for 2 days, the number of colony-forming units per gram of infected leaf tissue was determined.

### *F. oxysporum* f. sp. *raphani* bioassay.

Seeds of *A. thaliana* ecotypes Col, Ler, and RLD were sown singly in 1-ml pipette tips filled with sterile quartz sand to stimulate root elongation. The tips were drenched in water daily and in modified half-strength Hoagland nutrient solution once a week. After 2 weeks, seedlings were rinsed out of the pipette tips and placed horizontally on a system of rock wool cubes (Rock-wool/Grodan B.V., Roermond, the Netherlands), consisting of two spatially separated compartments. This system allows an induction treatment and a challenge inoculation of the same root system at different sites (Leeman et al. 1995a; Pieterse et al. 1996a). The lower part of the root system was covered with 1 ml of a 1:1 (wt/vol) mixture of talcum powder and either *Pseudomonas* bacteria in 10 mM MgSO<sub>4</sub> (final density 5 × 10<sup>8</sup> CFU/g), a solution of 1 mM SA (pH 6), or a solution of 10 mM MgSO<sub>4</sub> as a control. Three days after the induction treatment, the plants were challenge inoculated by applying approximately 0.25 g of the *F.*

*oxysporum* f. sp. *raphani* inoculum ( $4 \times 10^6$  CFU/g of peat) to the upper part of the roots. Subsequently, plants were cultivated as described above.

Thirty plants per treatment were analyzed for induced protection against *F. oxysporum* f. sp. *raphani* by determining the percentage of fully expanded leaves per plant with symptoms of Fusarium wilt at 3 to 4 weeks after challenge inoculation. The data were statistically analyzed as described above.

### Rhizosphere colonization.

Bacterial colonization of the root (parts) was determined by the time the bioassays were discontinued. The roots of six plants of each treatment were harvested, weighed, rinsed briefly in water, and shaken vigorously for 1 min in glass tubes containing 5 ml of 10 mM  $\text{MgSO}_4$  and 0.5 g of glass beads (0.17 mm). Appropriate dilutions were plated on KB agar supplemented with cycloheximide (100 mg/liter), ampicillin (50 mg/liter), chloramphenicol (13 mg/liter), and rifampicin (150 mg/liter), which is selective for rifampicin-resistant *Pseudomonas* spp. (Geels and Schippers 1983). After overnight incubation at 28°C, the number of colony-forming units per gram of root, fresh weight, was determined.

### RNA analysis.

For RNA extraction, leaves were harvested from 5-week-old plants that were either nontreated, treated with *Pseudomonas* rhizobacteria, or inoculated with *P. syringae* pv. *tomato*. Inoculation with *P. syringae* pv. *tomato* was performed by pressure infiltrating three lower leaves with a suspension of  $1 \times 10^7$  CFU/ml of 10 mM  $\text{MgSO}_4$  by using a syringe without a needle, as described by Swanson et al. (1988). Leaves were frozen in liquid nitrogen and stored at -80°C. RNA was extracted by the guanidine hydrochloride RNA extraction method as described by Logemann et al. (1987). Total RNA (15 µg) was electrophoretically separated on denaturing formaldehyde-agarose gels and blotted onto Hybond-N<sup>+</sup> membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer as described by Sambrook et al. (1989). Northern blots were hybridized and washed as described previously (Pieterse et al. 1994) and exposed to a Kodak X-Omat AR film. The DNA probes were labeled with  $\alpha$ -<sup>32</sup>P-dCTP by random primer labeling (Feinberg and Vogelstein 1983) with a Ready-To-Go DNA Labeling Kit (Pharmacia Biotech, Roosendaal, the Netherlands). PR-1, PR-2, and PR-5 probes originated from *Arabidopsis* PR-1, PR-2, and PR-5 cDNA clones, respectively (Uknes et al. 1992).

### In vitro antagonism assay.

To test antibiotic activity by WCS417r, WCS374r, and WCS358r, the bacterial strains were spotted at three positions on KB and rhizosphere medium (Buyer et al. 1989) agar plates supplemented with 200 µM  $\text{FeCl}_3$  (Duijff et al. 1993). After incubation at 28°C for 2 days, a suspension of *P. syringae* pv. *tomato* ( $1 \times 10^7$  CFU/ml) or *F. oxysporum* f. sp. *raphani* ( $5 \times 10^6$  conidia/ml) in 10 mM  $\text{MgSO}_4$  was sprayed evenly onto the plates. After an additional incubation for 2 days at 28°C for *P. syringae* pv. *tomato* or at 24°C for *F. oxysporum* f. sp. *raphani*, plates were inspected for the occurrence of zones of inhibited growth of *P. syringae* pv. *tomato* or *F. oxysporum* f. sp. *raphani* around the colonies of the biocontrol bacteria.

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