No Role for Bacterially Produced Salicylic Acid in Rhizobacterial Induction of Systemic Resistance in *Arabidopsis*

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ABSTRACT

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The role of bacterially produced salicylic acid (SA) in the induction of systemic resistance in plants by rhizobacteria is far from clear. The strong SA producer *Pseudomonas fluorescens* WCS374r induces resistance in radish but not in *Arabidopsis thaliana*, whereas application of SA leads to induction of resistance in both plant species. In this study, we compared *P. fluorescens* WCS374r with three other SA-producing fluorescent *Pseudomonas* strains, *P. fluorescens* WCS417r and CHA0r, and *P. aeruginosa* 7NSK2 for their abilities to produce SA under different growth conditions and to induce systemic resistance in *A. thaliana* against bacterial speck, caused by *P. syringae* pv. *tomato*. All strains produced SA in vitro, varying from 5 fg cell^{-1} for WCS417r to >25 fg cell^{-1} for WCS374r. Addition of 200 μM $FeCl_3$ to standard succinate medium abolished SA production in all strains. Whereas the incubation temperature did not affect SA production by WCS417r and 7NSK2, strains WCS374r and CHA0r produced more SA when grown at 33 instead of 28°C. WCS417r, CHA0r, and 7NSK2 induced systemic resistance apparently associated with their ability to produce SA, but WCS374r did not. Conversely, a mutant of 7NSK2 unable to produce SA still triggered induced systemic resistance (ISR). The possible involvement of SA in the induction of resistance was evaluated using SA-nonaccumulating transgenic NahG plants. Strains WCS417r, CHA0r, and 7NSK2 induced resistance in NahG *Arabidopsis*. Also, WCS374r, when grown at 33 or 36°C, triggered ISR in these plants, but not in ethylene-insensitive *ein2* or in non-plant pathogenesis-related protein-expressing *npr1* mutant plants, irrespective of the growth temperature of the bacteria. These results demonstrate that, whereas WCS374r can be manipulated to trigger ISR in *Arabidopsis*, SA is not the primary determinant for the induction of systemic resistance against bacterial speck disease by this bacterium. Also, for the other SA-producing strains used in this study, bacterial determinants other than SA must be responsible for inducing resistance.

Additional keywords: root colonization.

Several nonpathogenic, root-colonizing bacteria, notably of the genus *Pseudomonas*, are able to suppress various diseases by competition, antibiosis, or lytic enzymes directed against soilborne pathogens, or by induction of systemic resistance in the plant, extending protection to foliar pathogens (38). The induced systemic resistance (ISR) is phenotypically similar to pathogen-induced systemic acquired resistance (SAR) in that it confers an enhanced defensive capacity against diseases caused by fungi, bacteria, viruses, and nematodes. SAR is associated with the accumulation of plant pathogenesis-related proteins (PRs), some of which have been demonstrated to possess antimicrobial properties (37). Some ISR-eliciting rhizobacteria have been shown to likewise induce PRs in protected tissues, but effective ISR without accumulation of PRs or activation of *PR* genes also has been demonstrated (12,19,26).

The central signaling compound in the induction of PRs in plants is salicylic acid (SA) (33). Levels of endogenously produced SA increase both locally and systemically in incompatible plant–pathogen interactions and, to a lesser extent, in necrotizing compatible interactions, and this increase is both necessary and sufficient for SAR to develop and *PR* genes to be activated (22, 42). Many bacteria that have been described to elicit ISR produce SA in vitro under iron-limiting conditions, when the compound is likely to serve as an iron-scavenging siderophore (43). When produced in the rhizosphere, SA might elicit both ISR and PRs, and this type of ISR would be SA-dependent. However, some rhizobacteria that do not produce SA but do elicit ISR must act through an SA-independent mechanism (27,28). These contrasting observations suggest that rhizobacteria may have more than one mechanism of eliciting ISR, making the significance of SA produced by rhizobacteria in the elicitation of ISR enigmatic.

There are several observations that support a role for SA in the elicitation of ISR by specific rhizobacterial strains. For instance, *Pseudomonas fluorescens* CHA0 has the ability to produce SA (24), induces PRs in tobacco, and elicits ISR against *Tobacco necrosis virus* (TNV) (19). Expression of the SA-biosynthetic genes of *P. aeruginosa* PAO1 in the non-SA-producer *P. fluorescens* P3 made this strain an elicitor of ISR in tobacco against TNV (20), demonstrating that bacterially produced SA at the root surface can trigger ISR. *P. corrugata* strain 13 and *P. aureofaciens* strain 63-28 induced systemic resistance against *Pythium aphanidermatum* in cucumber roots, associated with their ability to produce SA in culture. However, exogenously applied SA did not elicit ISR to cucumber root rot caused by *P. aphanidermatum* (5). *Pseudomonas aeruginosa* strain 7NSK2 has the ability to produce SA and elicits ISR against *Tobacco mosaic virus* (TMV) in tobacco (7) and against *Botrytis cinerea* or *Colletotrichum lindemuthianum* in bean (3,9). By using bacterial mutants with altered SA production capacities, as well as tobacco *NahG* transformants in which SA-dependent responses are abolished as a result of SA-hydroxylase-mediated conversion of SA to catechol, it was proven that elicitation of ISR in tobacco was SA dependent (7). However, in induced tobacco plants, the marker PR protein PR-1 was not

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detectable. This latter discrepancy could be explained by observations that tiny amounts of SA that are insufficient for inducing PRs already suffice to elicit ISR (8).

In contrast, several other reports describe that bacterial SA production and elicitation of ISR are not related. ISR elicited in tobacco against *P. syringae* pv. *tabaci* by the SA-producing strain *Serratia marcescens* 90-166 was maintained in NahG plants, suggesting that ISR by this strain is SA independent. Indeed, SA-nonproducing mutants of this biocontrol strain induced resistance to the wildfire disease in tobacco to the same levels as wild-type bacteria, and likewise against anthracnose, caused by *C. orbiculare*, in cucumber (32). In strain 90-166, production of a catechol siderophore plays a key role in triggering ISR (31). A comparative analysis of strains *P. putida* WCS358, *P. fluorescens* WCS374, and *P. fluorescens* WCS417 indicated that both WCS358 and WCS417 elicited ISR in *Arabidopsis* against several fungi and bacteria, whereas WCS374 did not (35,41). None of the strains induced PRs under these conditions. Nevertheless, WCS374 and WCS417 produced 50 and 10 μg of SA ml^{-1} of standard succinate medium, respectively, whereas WCS358 never was found to produce SA under iron-limited conditions (18,21). Thus, the non-SA-producer WCS358 did elicit ISR, whereas the strong SA-producer WCS374 did not (29). Pseudobactin, lipopolysaccharides, and flagella of WCS358 all can trigger ISR in *A. thaliana* (2,25). In contrast, upon root colonization of radish, the ability of the three strains to elicit ISR against *Fusarium oxysporum* did correlate with their capacity to produce SA (i.e., both WCS374 and WCS417 elicited ISR, whereas WCS358 did not). Moreover, the level of resistance induced by the former two strains was higher when rhizosphere conditions were optimized for bacterial SA production by lowering iron availability (18).

The observation that, in *Arabidopsis*, the strong SA-producer WCS374 did not elicit ISR was unexpected because, upon treatment of *Arabidopsis* with SA, systemic resistance is induced concomitant with the activation of PR genes (40). WCS374 colonizes Arabidopsis roots to levels similar to those of the strains that do elicit ISR. Thus, it seems that our standard bioassay conditions are not conducive to the production of SA by WCS374 in the Arabidopsis rhizosphere. To investigate the extent to which SA produced by WCS374 could contribute to ISR in *Arabidopsis*, bacteria were grown under varying conditions of iron availability and temperature, because both factors have been described to affect the production of factors implicated in ISR (9,18). For instance, in bean, ISR elicited by *P. aeruginosa* 7NSK2 was evident only when the bacterium was grown under conditions of low iron availability (9). Effects of WCS374 were compared with those of other SA-producing strains WCS417, CHA0, and 7NSK2 and to the non-SA-producing strain WCS358. To analyze whether ISR could be SA dependent, *Arabidopsis NahG* transformants were used in which SA-dependent ISR is abolished (6). The ethylene-insensitive mutant *ein2* does express SA-dependent induced resistance, but SA-independent ISR, as elicited by WCS417, is lost (30). Therefore, this mutant was included to further assess a possible role for SA.

MATERIALS AND METHODS

Bacterial cultures. Bacterial strains used are listed in Table 1. All fluorescent *Pseudomonas* strains were grown on King's medium B (KB) agar plates (15) with or without 200 μM $FeCl_3$ at 28 or 33°C for 20 to 24 h. Strain WCS374r additionally was grown at 31 and 36°C for 24 and 48 h, respectively. Bacterial cells were collected in sterile 10 mM $MgSO_4$, washed twice by centrifugation at 7,600 × *g* for 10 min, and resuspended in 10 mM $MgSO_4$. Densities of the bacterial suspensions were adjusted based on their optical density at 660 nm.

The pathogen *P. syringae* pv. *tomato* DC3000 (44) was cultured overnight in liquid KB medium at 28°C and shaking at 240 rpm. The suspension was washed twice with 10 mM $MgSO_4$ by centrifugation at 7,600 × g for 10 min.

Analysis of in vitro production of SA by fluorescent *Pseudomonas* spp. Strains were cultured in 25 ml of liquid standard succinate medium (SSM) (23) for 24 h at 28°C and 240 rpm. Then, 50 μl was transferred into a 100-ml flask containing 25 ml of SSM (three flasks per treatment) with or without 200 μM $FeCl_3$, and incubated for 48 h at the desired temperature and 240 rpm. After determining the bacterial population density at 660 nm, the culture was centrifuged at 12,000 × g for 10 min, and the supernatant acidified to pH 1.5 to 2 with 2 M HCl. SA in the acidified supernatant was determined according to Meyer et al. (24), and expressed as fg per cell.

Plant cultivation and inoculation. Seed of wild-type *A. thaliana* accession Col-0, transgenic NahG plants, and mutants *ein2* (16,30) and *npr1* (4), all in the Col-0 background, were germinated in quartz sand, supplemented with 100 ml of half-strength Hoagland nutrient solution (11) containing 10 μM Fe-ethylenediamine di-o-hydroxyphenylacetic acid (FeEDDHA; Syngenta, Basel, Switzerland) per kilogram of sand. Two-week-old seedlings were transplanted into pots containing 100 g of a potting soil/sand mixture (12:5 vol/vol, autoclaved twice for 1 h on alternate days), supplemented with 5 ml of 10 mM $MgSO_4$ containing fluorescent *Pseudomonas* spp. to a final density of 5×10^7 CFU g^{-1} soil, or an equal volume of sterile 10 mM $MgSO_4$ as a control. Seedlings were grown for another 3 weeks in a climate chamber with an 8-h day and 16-h night cycle at 24 and 20°C, respectively, and at 70% relative humidity. Half-strength Hoagland solution without FeEDDHA was applied to the plants once a week, and tap water was given when needed.

When 5 weeks old, 25 plants per treatment were challenge inoculated with *P. syringae* pv. *tomato*. The rosette leaves of Col-0 wild-type and *npr1* and *ein2* mutant plants were dipped into a *P. syringae* pv. *tomato* suspension containing 2.5×10^7 CFU ml^{-1} and 0.01% (vol/vol) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands). Because of the enhanced susceptibility of the NahG transformant to *P. syringae* pv. *tomato* (29), NahG plants were inoculated similarly with 2.5×10^6 CFU ml^{-1}. The inoculated seedlings were kept at 100% relative humidity for disease to develop. After 3 or 4 days, the percentage of leaves per plant showing chlorotic or necrotic lesions was determined.

TABLE 1. *Pseudomonas* strains used in this study

Strain	Relevant characteristics[a]	Reference
Pseudomonas aeruginosa 7NSK2	Wild type; Pch^+, Pvd^+, SA^+; amp^r, chl^r	6
KMPCH	Chemical mutant of MPFM1; Pch^-, Pvd^-, SA^+; amp^r, chl^r, Km^r	6
MPFM1	*Tn*5 mutant of 7NSK2; Pch^+, Pvd^-, SA^+; amp^r, chl^r, Km^r	6
MPFM1-569	*pch*A replacement mutant of MPFM1; Pch^-, Pvd^-, SA^-; amp^r, chl^r, Km^r	6
P. fluorescens CHA0r	Isolated from tobacco rhizosphere; HCN^+, Phl^+, Plt^+, Pvd^+, SA^+; amp^r, chl^r, rif^r	18,34
P. fluorescens WCS374r	Isolated from potato rhizosphere; SA^+, amp^r, chl^r, rif^r	10
P. fluorescens WCS417r	Isolated from wheat rhizosphere; SA^+, amp^r, chl^r, rif^r	17
P. putida WCS358r	Isolated from potato rhizosphere; SA^-, amp^r, chl^r, rif^r	10
P. syringae pv. *tomato* DC3000	Causal agent of bacterial speck; rif^r	44

[a] Abbreviations: HCN = hydrogen cyanide, Pch = pyochelin, Phl = 2,4-diacetylphloroglucinol, Plt = pyoluteorin, Pvd = pyoverdin; SA = salicylic acid; amp^r, chl^r, Km^r, and rif^r = resistant to ampicillin, chloramphenicol, kanamycin, and rifampin, respectively.

Rhizosphere populations of fluorescent *Pseudomonas* spp. At the end of each experiment, roots of five plants per treatment were harvested separately and shaken vigorously for 1 min in 10 vol (g vol^{-1}) of 10 mM $MgSO_4$ with 0.5 g of glass beads (0.6 mm in diameter). Aliquots of 100 µl at proper dilutions were pipetted into 24-well tissue culture plates and 400 µl of KB agar supplemented with ampicillin (40 µg ml^{-1}), cycloheximide (100 µg ml^{-1}), and chloramphenicol (13 µg ml^{-1}) (KB+), at 45 to 50°C, was added to each well. For strains WCS358r, WCS374r, WCS417r, and CHA0r, KB+ agar was supplemented with rifampin (150 µg ml^{-1}). Plates were incubated at 28°C for 24 to 36 h. *P. aeruginosa* 7NSK2 was grown in KB+ only and incubated at 37°C for 16 to 20 h. For mutants KMPCH, MPFM1, and MPFM1-569, KB+ was supplemented with kanamycin (200 µg ml^{-1}). After incubation, the colonies that had developed in each well were counted with the aid of a light microscope and the number of CFU per gram of fresh root was calculated.

Data analysis. Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased (29). The percentages of diseased leaves were statistically analyzed using one-way analysis of variance (ANOVA), followed by Fisher's least significant differences test ($P \leq 0.05$), using SPSS software (SPSS for Windows, version 8.0).

All experiments were performed at least twice and yielded comparable results.

TABLE 2. Salicylic acid production by fluorescent *Pseudomonas* spp. growing at different temperatures in liquid standard succinate medium without iron[a]

Strain	28°C	31°C	33°C
WCS374r	18.8 ± 0.46	27.2 ± 0.87	26.0 ± 0.61
WCS417r	4.5 ± 0.17	nd	5.4 ± 0.47
CHA0r	6.0 ± 0.55	nd	13.7 ± 0.09
7NSK2	8.4 ± 0.20	nd	8.2 ± 0.48
WCS358r	0	nd	nd

[a] Salicylic acid production is expressed as fg $cell^{-1}$ (means ± standard error); nd = not determined.

RESULTS

SA production in vitro by *Pseudomonas* spp. To compare the capacity of the fluorescent *Pseudomonas* strains to produce SA at different temperatures, WCS374r, WCS417r, CHA0r, and 7NSK2 were grown in liquid SSM medium. Strain WCS374r hardly grew in liquid culture at 36°C and analysis of SA production at this temperature was not possible. Of all strains, WCS374r produced the highest level of SA, amounting to ≈19 fg $cell^{-1}$ at 28°C and increasing to >25 fg $cell^{-1}$ at 31 and 33°C (Table 2). Temperature had no significant effect on SA production by WCS417r and 7NSK2, but CHA0r produced more than double the amount of SA at 33 compared with 28°C. As previously observed (18,21), strain WCS358 did not produce SA.

Supplementing the medium with 200 µM $FeCl_3$ completely suppressed SA production in all producer strains (data not shown), confirming that SA production by the bacteria is iron regulated.

Effects of iron availability and temperature during bacterial growth on induction of systemic resistance. As shown previously (41), strain WCS374r, when grown at 28°C on iron-limited KB agar plates, did not induce resistance, whereas strains WCS417r, CHA0r, and 7NSK2, as well as the non-SA-producing strain WCS358, all induced significant systemic resistance in

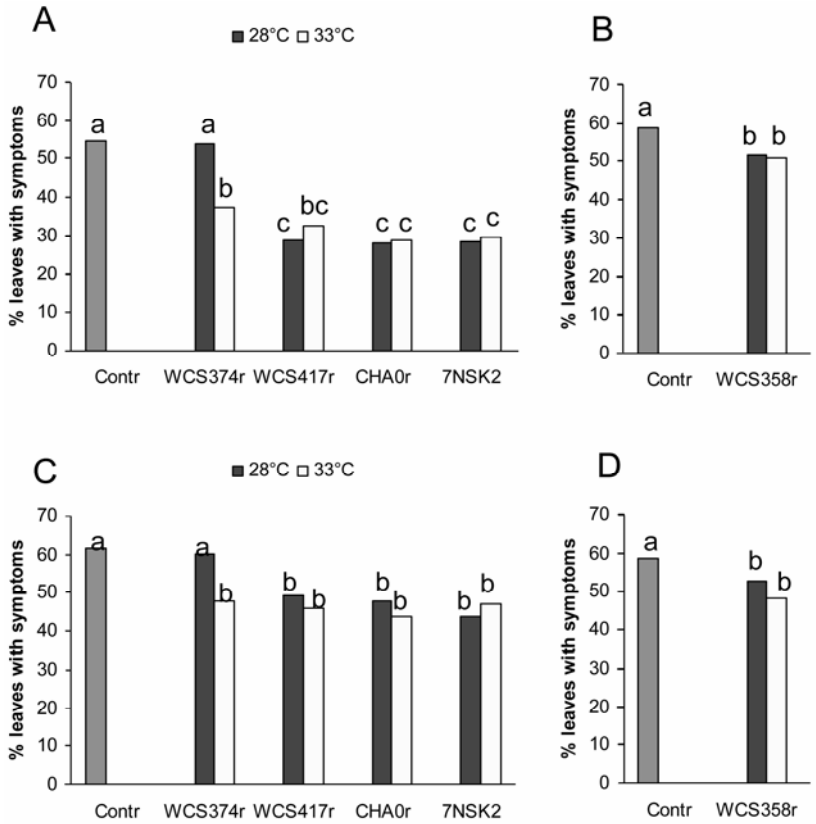

Fig. 1. Induction of systemic resistance in *Arabidopsis* by fluorescent *Pseudomonas* strains grown on **A and B,** King's medium B (KB) agar plates or **C and D,** KB agar plates supplemented with 200 µM $FeCl_3$ at 28 or 33ºC. Two-week-old seedlings were transplanted into soil that was mixed with either 10 mM $MgSO_4$ (Contr = control) or 5×10^7 bacterial cells g^{-1} of soil in 10 mM $MgSO_4$. Plants were grown for another 3 weeks and challenge inoculated by dipping the rosette leaves in a suspension of *Pseudomonas syringae* pv. *tomato* (2.5×10^7 CFU ml^{-1}). Disease severity is expressed as the mean percentage of leaves with necrotic water-soaked symptoms per plant. For each frame, different letters indicate statistically significant differences between treatments (Fischer's least significant difference test; $P \leq 0.05$).

Arabidopsis (Fig. 1A and B). Addition of iron to the growth medium did not alter the abilities of the strains to induce resistance (Fig. 1C and D). Increasing the temperature of growth from 28 to 33°C had no effect on the abilities of strains WCS417, CHA0, 7NSK2, and WCS358 to induce resistance in either the absence (Fig. 1A and B) or the presence (Fig. 1C and D) of iron. However, WCS374 became ISR-inducing when grown at 33°C in both the absence (Fig. 1A) or the presence (Fig. 1C) of iron. Population densities of the introduced strains in the rhizosphere of *Arabidopsis* varied between 6.5 and 7.0 log CFU g^{-1} of fresh root. Incubation temperature and iron availability did not significantly influence the population densities in the rhizosphere (data not shown). Thus, the inability of strain WCS374r to induce resistance when grown at 28°C did not result from insufficient rhizosphere populations. Moreover, because SA production in this strain is iron regulated, the resistance induced at 33°C was unlikely to be the exclusive result of bacterially produced SA.

Effects of iron-regulated factors of strain 7NSK2 on elicitation of ISR. The availability of specific mutants of strain 7NSK2 allowed further investigation of the possible involvement of SA and the iron-regulated siderophores pyoverdin and pyochelin in the elicitation of ISR by this strain. Mutants MPFM1, deficient in the production of pyoverdin, KMPCH, lacking both pyoverdin and pyochelin, and MPFM1-569, impaired in synthesis of both siderophores as well as SA, all reduced the percentage of diseased leaves to comparable extents (Fig. 2). These results demonstrate that pyoverdin, pyochelin, or SA are not necessary for the induction of ISR by 7NSK2 in *Arabidopsis*.

Signaling pathways in the induction of systemic resistance in *Arabidopsis*. To further test for an involvement of SA in the induction of systemic resistance by strains WCS417r, CHA0, and 7NSK2, the experiments were repeated using SA-non-accumulating NahG plants. As expected, strain 7NSK2 elicited significant ISR (Fig. 3), similar to that in wild-type plants (Fig. 1). Also, strains WCS417r and CHA0r induced systemic resistance in this transformant, although to a significantly lesser extent (Fig. 3). Nevertheless, it can be concluded that significant ISR is triggered by these strains independent of SA signaling.

Because induction of systemic resistance by strain WCS374r was temperature dependent (Fig. 1), its inducing activity was investigated in more detail by growing the bacterium at different temperatures and testing for ISR in wild-type, NahG-transformed, and *ein2* and *npr1* mutant plants. The ability of WCS374r to induce resistance increased gradually when the growth temperature of the bacteria was increased from 28 to 36°C (Fig. 4A). Essentially similar results were obtained in NahG plants (Fig. 4B), supporting the notion that SA produced by WCS374r grown at higher temperatures was not the main factor to induce resistance. Similar tests in ethylene-insensitive *ein2* plants demonstrated that, independent of its growth temperature, WCS374r did not induce resistance (Fig. 4C). These results clearly indicate that elicitation of ISR by WCS374r in *Arabidopsis* requires sensitivity to ethylene. Likewise, no significant resistance was induced in the *npr1* mutant when WCS374 was grown at either 28 or 33°C (data not shown). Thus, resistance induced by WCS374r also is dependent on NPR1. The lack of resistance induction in *ein2* or *npr1* plants was not due to poor root colonization, because the population densities of WCS374r in the rhizosphere of the wild-type, transgenic, and mutant plants did not differ significantly in any of these assays (data not shown).

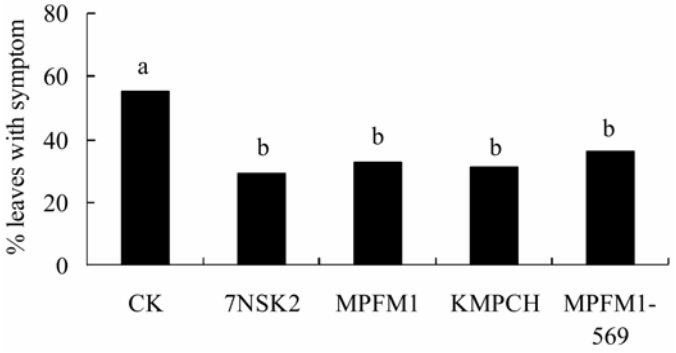

Fig. 2. Induction of systemic resistance in *Arabidopsis* by *Pseudomonas aeruginosa* strain 7NSK2 and its mutants defective in biosynthesis of pyoverdin (MPFM1), pyoverdin and pyochelin (KMPCH), and pyoverdin, pyochelin and SA (MPFM1-569). Two-week-old seedlings were transplanted into soil that was mixed with either 10 mM $MgSO_4$ (Contr = control) or 5×10^7 bacterial cells g^{-1} of soil in 10 mM $MgSO_4$. Plants were grown for another 3 weeks and challenge inoculated by dipping the rosette leaves in a suspension of *Pseudomonas syringae* pv. *tomato* (2.5×10^7 CFU ml^{-1}). Disease severity is expressed as the mean percentage of leaves with necrotic water-soaked symptoms per plant. For each frame, different letters indicate statistically significant differences between treatments (Fischer's least significant difference test; $P \leq 0.05$).

DISCUSSION

Our study focused on understanding further the contribution of the SAR-signaling compound SA in the elicitation of ISR in *Arabidopsis* by several *Pseudomonas* strains. To study a possible role of bacterially produced SA in the induction of systemic resistance in vivo, the bacteria were grown at different temperatures and iron availabilities before applying them onto the plant roots. Our assumption was that growing the bacteria at conditions that promote bacterial SA production would increase their eliciting activity.

Four of the five strains used consistently induced systemic resistance against the pathogen *P. syringae* pv. *tomato*. When grown under standard conditions, WCS374 did not elicit ISR. On *Arabidopsis*, WCS374 was the only non-ISR inducing-strain described (41); however, mutants of CHA0 that lack production of 2,4-diacetylphloroglucinol also do not induce systemic resistance in *Arabidopsis* (13). Therefore, the ability of *Pseudomonas* bacteria to induce ISR in this plant species appears to be linked to specific bacterial traits. All five strains studied, with the exception of *P. putida* WCS358, had the capacity to produce SA in vitro under iron-limiting conditions. Because WCS358 does not produce SA (18,21), this strain elicits ISR by an SA-independent mechanism (2,25). Several lines of evidence indicate that this also applies to the other strains, which have the capacity to produce SA. Similar levels of ISR were evident in plants treated with WCS374, WCS417, CHA0, and 7NSK2 grown in the absence or presence

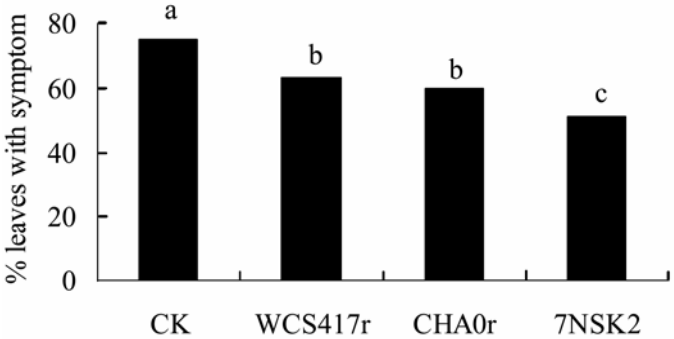

Fig. 3. Induction of systemic resistance in the NahG transformant of *Arabidopsis* by fluorescent *Pseudomonas* strains WCS417r, CHA0r, and 7NSK2. Two-week-old seedlings were transplanted into soil that was mixed with either 10 mM $MgSO_4$ (Contr = control) or 5×10^7 bacterial cells g^{-1} of soil in 10 mM $MgSO_4$. Plants were grown for another 3 weeks and challenge inoculated by dipping the rosette leaves in a suspension of *Pseudomonas syringae* pv. *tomato* (2.5×10^7 CFU ml^{-1}). Disease severity is expressed as the mean percentage of leaves with necrotic water-soaked symptoms per plant. Different letters indicate statistically significant differences between treatments (Fischer's least significant difference test; $P \leq 0.05$).

of iron, conditions that lead to induction and repression, respectively, of the synthesis of SA by these strains. However, it could not be excluded that iron-limiting conditions prevailed in the rhizosphere of treated plants, and that SA production occurred in situ on plant roots irrespective of previous culture conditions.

The resistance-inducing action of SA is abolished in transformed plants carrying the *NahG* gene (6). Testing the strains on NahG plants showed that WCS417, CHA0, and 7NSK2 still induced resistance. That the induction by 7NSK2 was fully SA independent was borne out by the use of bacterial mutants impaired in SA or siderophore production. Like SA, siderophores are produced only under iron-limited conditions. Moreover, SA is an intermediate in the biosynthesis of the pyochelin siderophore. All mutants elicited ISR comparable with the wild type, indicating that neither SA nor the pyoverdin or pyochelin siderophores are necessary for the induction of systemic resistance by 7NSK2 in *Arabidopsis*. Notably, the involvement of SA in ISR elicitation by 7NSK2 appears to be plant species specific. De Meyer and Höfte (9) observed induction of resistance to gray mold in bean only when the bacterium was prepared from iron-limited KB, but not when grown on iron-rich LB medium. Further results obtained by use of the bacterial mutants strongly suggested that SA produced by 7NSK2 is required for the induction of systemic resistance in both bean against *B. cinerea* (9) and tobacco against TMV (7). However, it recently was demonstrated by Audenaert et al. (1) that, whereas ISR elicited by 7NSK2 requires the SA signaling pathway in tomato, the bacterial trigger of ISR is the combination of the antibiotic pyocyanin and the SA-containing pyochelin, rather than SA itself.

The significantly lesser resistance induced by WCS417 and CHA0 in NahG plants compared with wild-type plants is difficult to interpret, but might point to a small contribution of SA on the level of resistance attained. However, the strongly increased SA production of CHA0r grown in vitro at 33°C had no additional effect on its ability to elicit ISR, suggesting that SA does not contribute to the induction of systemic resistance by this strain in *Arabidopsis*. In contrast, in tobacco, induction of systemic resistance by CHA0 against TNV seems to be dependent on accumulation of SA (20). CHA0 can secrete a number of antimicrobial compounds, including 2,4-diacetylphloroglucinol (14). Recently, it was shown that this antibiotic can act as a determinant of ISR elicitation by this strain and that this induction occurs independently of SA (13). As for strain WCS417, Pieterse et al. (29,30) previously established that WCS417r was as active in triggering ISR in *Arabidopsis* NahG as in wild-type plants, and that the signaling pathway in the plant leading to ISR was different from SAR and required sensitivity to ethylene.

Of the strains examined, WCS374 had by far the largest capacity to produce SA. Yet, when cultivated at 28°C, it did not elicit ISR in *Arabidopsis*. Nevertheless, increasing the temperature of cultivation induced in this strain an ability to trigger ISR, associated with a 40% increase in SA production in vitro. However, SA did not seem to be involved, because the same ability was evident when the bacterium was cultured in the presence of 200 μM $FeCl_3$, when no SA was produced, or when bioassays were conducted on NahG plants, in which SA-dependent ISR is abolished. When incubated at 28 or 31°C, WCS374r did not trigger ISR, but this strain reduced the percentage of diseased leaves consistently in both wild-type and NahG plants when the incubation temperature was raised to 33 or 36°C. WCS374r produced comparably high amounts of SA in vitro when grown at 31 or 33°C. If SA was involved, one might have expected that, when grown at either of these incubation temperatures, it would have triggered ISR to similar levels. However, the difference in the level of ISR attained was significant. Thus, it has to be concluded that the increased disease resistance observed upon application of WCS374r grown at higher temperatures was not due to SA. This conclusion is further supported by the results obtained

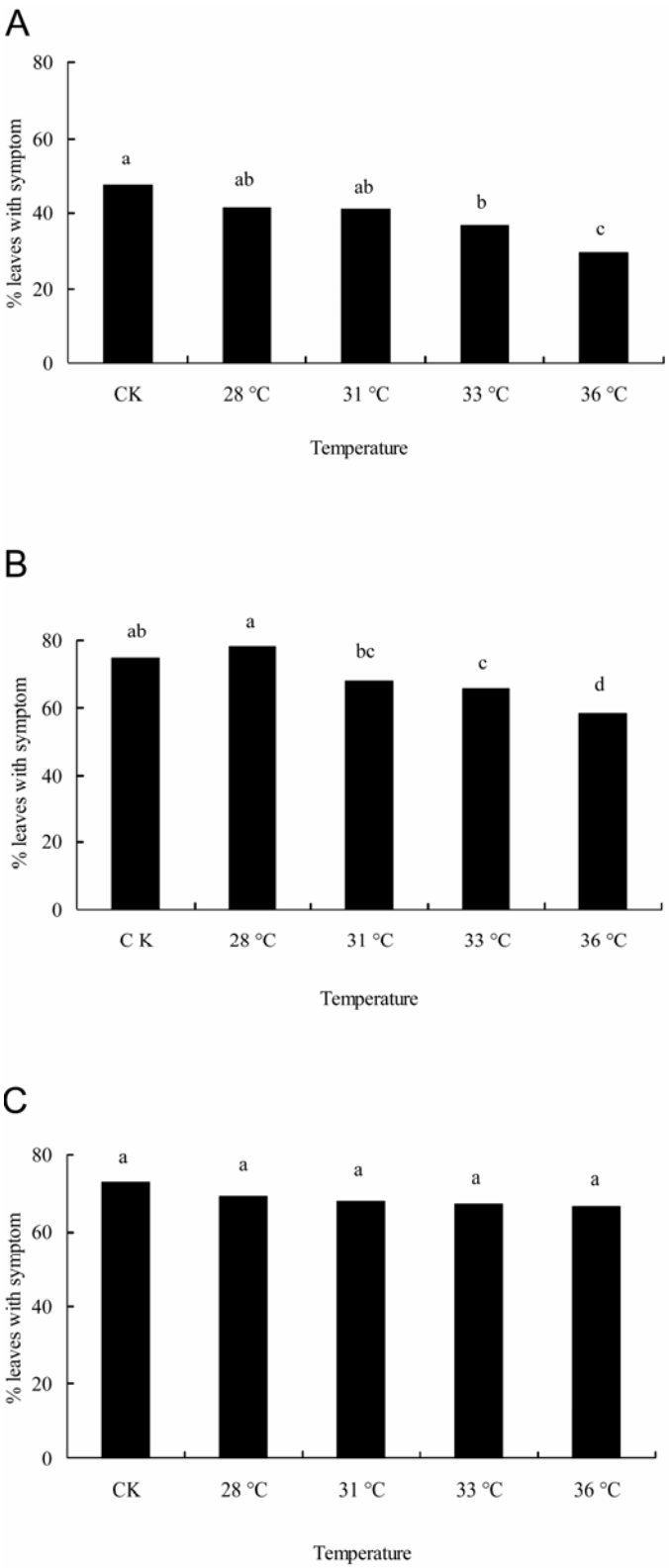

Fig. 4. Effect of *Pseudomonas fluorescens* strain WCS374r grown on King's medium B agar plates at different temperatures on induction of systemic resistance in *Arabidopsis* **A,** wild-type, **B,** transgenic NahG, and **C,** mutant *ein2* plants. Two-week-old seedlings were transplanted into soil that was mixed with either 10 mM $MgSO_4$ (Contr = control) or 5×10^7 bacterial cells g^{-1} of soil in 10 mM $MgSO_4$. Plants were grown for another 3 weeks and challenge inoculated by dipping the rosette leaves in a suspension of *Pseudomonas syringae* pv. *tomato* (2.5×10^7 CFU ml^{-1}). Disease severity is expressed as the mean percentage of leaves with necrotic water-soaked symptoms per plant. For each frame, different letters indicate statistically significant differences between treatments (Fischer's least significant difference test; $P \leq 0.05$).

with the ethylene-insensitive mutant *ein2*. In this mutant, application of SA or avirulent pathogens triggers SAR (40). However, WCS374r did not induce systemic resistance in *ein2*, irrespective of the incubation temperature. Also, in mutant *npr1* plants, WCS374r was not effective. Thus, it appears that WCS374r grown at 33°C triggers the same pathway as WCS417r. The incubation temperature at which the bacterial inoculum was produced may have affected other traits in WCS374, such as the lipopolysaccharides or the flagella, which have both been implicated in WCS358-mediated ISR in *Arabidopsis* (2,25).

Whereas, in *P. aeruginosa* 7NSK2, production of SA is linked to the synthesis of pyochelin (9), in *P. fluorescens* WCS374 it is associated with the production of the SA-containing siderophore pseudomonine (21). The synthesis of pseudomonine is regulated by iron availability; however, in addition, this siderophore is produced at elevated temperatures, whereas the production of the fluorescent siderophore pseudobactin is shut down at these temperatures (21). Because siderophores also have been implicated in the induction of systemic resistance (2,39), effects of iron availability and temperature on the in vitro SA production by specific resistance-inducing fluorescent *Pseudomonas* strains may mediate elicitation of ISR in vivo through synthesis of specific siderophores. This hypothesis is supported by the iron dependency of ISR induced by CHA0 against TNV and *Thielaviopsis basicola* (18,36) in tobacco, and by *S. marcescens* 90-166 against *C. orbiculare* and *P. syringae* pv. *tabaci* in cucumber and tobacco, respectively (32). Although *S. marcescens* was capable of producing SA in vitro, SA was not the primary determinant of ISR elicited by this strain. Our results confirm those of Iavicoli et al (13) that, in *Arabidopsis*, CHA0 does not elicit ISR through an SA-dependent mechanism. Taking into account that SA does appear to be involved in systemic resistance induced by specific strains on some plant species, our findings clearly demonstrate that plant species can differ in their reaction to resistance-inducing rhizobacteria by activating different signaling pathways leading to ISR.

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