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The role of salicylic acid in induced systemic resistance elicited by plant growth-promoting rhizobacteria against blue mold of tobacco

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Abstract

Investigations were conducted to determine the role of salicylic acid (SA) in induced systemic (ISR) resistance against blue mold disease of tobacco elicited by plant growth-promoting rhizobacteria (PGPR). SA did not inhibit germination of sporangia or development of germ tubes of *Peronospora tabacina*, the blue mold pathogen, in vitro. Of three PGPR strains tested, none produced detectable levels of SA in aqueous Murashige and Skoog (MS) medium, and only *Serratia marcescens* strain 90-166 produced SA in tryptic soy broth (TSB). In a microtiter plate system, levels of endogenous free SA in tobacco (*Nicotiana tabacum*) seedlings treated with three PGPR strains significantly increased during the first week after PGPR treatment. In the second week, however, levels of SA were significantly lower than those in nontreated tobacco seedlings. When plants treated with *Bacillus pumilus* strain SE34 were challenged with *P. tabacina*, levels of SA increased markedly 1 day after challenge, compared to the nonbacterized and challenged control. However, a similar increase in SA 1 day after pathogen challenge did not occur in plants treated with PGPR strains 90-166 or *Pseudomonas fluorescens* strain 89B-61. These observations indicate that SA accumulation in tobacco plants may play a role in ISR against tobacco blue mold by PGPR. Disease assays conducted in the microtiter plates showed that the tested PGPR strains significantly reduced disease severity of blue mold in both Xanthi-nc and transgenic NahG tobacco, indicating that systemically induced resistance in tobacco to blue mold by PGPR may be SA-independent.

Keywords: Biological control; Induced systemic resistance; Plant growth-promoting rhizobacteria; Salicylic acid; *Peronospora tabacina*

1. Introduction

Over the past decade, specific strains of plant growth-promoting rhizobacteria (PGPR) have been shown to induce systemic resistance against a broad spectrum of pathogens such as fungi (van Peer et al., 1991; Wei et al., 1991), bacteria (Alström, 1991; Press et al., 1997), and viruses (Murphy et al., 2000; Raupach et al., 1996). PGPR-elicited induced systemic resistance (ISR) is phenotypically similar to pathogen-induced systemic acquired resistance (SAR) (Sticher et al., 1997; van Loon et al., 1998). Both SAR and ISR elicited by PGPR render noninfected parts of previously induced plants more resistant to infection by pathogens and are effective against a broad spectrum of root and foliar pathogens.

Many studies have indicated that salicylic acid (SA) plays an important role in plant defense response against pathogen attack and is essential for the development of SAR (Ryals et al., 1996; Yalpani and Raskin, 1993). White (1979) reported that exogenously applied SA in tobacco (*Nicotiana tabacum* L.) induced resistance against *Tobacco mosaic virus* (TMV). Ward et al. (1991) found that this increased resistance was correlated with accumulation of pathogenesis-related (PR) proteins, which are generally considered to be markers of SAR. A 20-fold increase in the level of endogenous SA after TMV infection was observed in the development of

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hypersensitive response (HR) and SAR in tobacco (Malamy et al., 1990), and this systemic increase occurred in only Xanthi-nc tobacco that carries the *N* resistance gene to TMV. NahG tobacco plants that carry a bacterial *nahG* gene encoding salicylate hydroxylase, which degrades SA into catechol, do not develop SAR by TMV (Gaffney et al., 1993). *Arabidopsis* plants carrying *nahG* gene show higher susceptibility to various pathogens than wild-type plants (Delaney et al., 1994). Leeman et al. (1996) found that SA produced by selected *Pseudomonas fluorescens* (Trevisan) Migula strains is involved in the induction of systemic resistance against Fusarium wilt in radish. Results from a study by De Meyer and Höfte (1997) suggest that SA produced by *Pseudomonas aeruginosa* (Schroeter) Migula strain 7NSK2 is necessary for ISR against *Botrytis cinerea* Pers.:Fr in bean. Chen et al. (1999) reported an increase in SA levels in cucumber roots after treatment with two PGPR strains. Hence, SA is involved in ISR that is triggered by pathogens and by some PGPR.

Although the experiments described above indicate that SA may act as a primary signal in ISR, other studies suggest that SA may not be the translocated signal responsible for induction of ISR. In a reciprocal grafting experiment (Vernooij et al., 1994), SA did not serve as a systemic signal in ISR against TMV since ISR was not induced in the *NahG* scion after inoculation of rootstock leaves with TMV, regardless of which tobacco line was used as the rootstock. In cucumber, increased SA levels and resistance to *Pseudomonas syringae* (van Hall) Ash et al. pv. lachrymans were detected in non-inoculated leaves although the inoculated leaf was removed as early as 4–6 h after inoculation. However, SA was not detected in the phloem sap from the inoculated leaf (Rasmussen et al., 1991). Chen et al. (1996) demonstrated that *Bacillus cereus* Frankland and Frankland strain UW85, which suppressed damping-off of tobacco seedlings caused by *Pythium* spp., did not induce PR-1a expression; whereas SA induced accumulation of PR-1a. Press et al. (1997) showed that SA-negative mutants of an ISR inducing PGPR strain *Serratia marcescens* Bizio 90-166 had the same ISR activity against *P. syringae* (van Hall) Ash et al. pv. *tabaci* in tobacco as did the wild-type strain. Moreover, exogenously applied SA did not induce resistance against take-all caused by *Gaeumannomyces graminis* var. *tritici* J. Walker in wheat (Seah et al., 1996) and root rot by *Pythium aphanidermatum* (Edson) Fitzp. in cucumber (Chen et al., 1999).

Studies of the PGPR strain *Pseudomonas fluorescens* WCS417r, which induces systemic resistance against certain pathogens in several plants, have provided evidence that SA is not an intermediate in ISR. The evidence has shown that WCS417r induces resistance on *Arabidopsis* expressing the bacterial *nahG* gene, and unlike the case with SAR, PR proteins are not activated in plants showing ISR triggered by WCS417r (Hoffland et al., 1996; Pieterse et al., 1998; van Wees et al., 1997). Pieterse et al. (1998) reported that systemic resistance induced by WCS417r requires responsiveness to ethylene and jasmonate. Thus, they have proposed that a distinct pathway from SAR is involved in PGPR-elicited ISR without activation of PR protein genes (Pieterse et al., 1998). Currently, very few reports explain the mechanisms of systemic resistance induced by other rhizobacteria, thus making comparisons to the model of Pieterse et al. difficult. In contrast to the model, Maurhofer et al. (1994) found that induced resistance by PGPR strain *P. fluorescens* strain CHA0 in tobacco resulted in accumulation of eight known PR proteins. Park and Kloepper (2000) reported that induction of PR-1a promoter activity occurs during ISR in tobacco by some PGPR strains, using a reporter gene system for PR-1a in tobacco. The objective of this study was to determine if ISR in tobacco against blue mold disease induced by three diverse PGPR operates in a SA-independent pathway.

2. Materials and methods

2.1. PGPR strains and preparation

PGPR strains *Serratia marcescens* 90-166, *Bacillus pumilus* Meyer and Gottheil SE34, and *Pseudomonas fluorescens* 89B-61 were tested for SA in these assays. The PGPR strains used and the references in which they have been reported are 90-166 (Kloepper et al., 1996; Liu et al., 1995a,b,c; Press et al., 1997; Raupach et al., 1996; Wei et al., 1996; Zehnder et al., 1997a,b), SE34 (Benhamou et al., 1996; Jetiyanon, 1997; Ji et al., 1997; Yao et al., 1997), and 89B-61 (Ji et al., 1997; Kloepper et al., 1996; Wei et al., 1996; Zehnder et al., 1997b). These PGPR strains have elicited ISR activity in tobacco against blue mold disease both in a microtiter plate system and in the greenhouse (Zhang et al., 1999, 2002). For long-term storage, bacterial strains were maintained at −80 °C in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) supplemented with 20% glycerol.

Bacteria were taken from −80 °C, streaked onto tryptic soy agar (TSA), and incubated at 28 °C for 24 h. Bacterial cells were removed from plates into sterile distilled water to a final density of 10^9 colony-forming units (CFU) per milliliter.

2.2. Preparation of P. tabacina inoculum

Isolate No. 79 of *P. tabacina* Adam was provided by W.C. Nesmith, University of Kentucky. Inoculum was obtained from freshly sporulating infected leaves of tobacco (*Nicotiana tabacum* L.) cv. Ky14 on 6- to 8-week-old plants at 7 or 8 days after inoculation. Sporangia were gently brushed into a small quantity of distilled

water, washed three times with distilled water on a 0.45 μm nylon filter, and then resuspended in sterile distilled water to make the sporangial suspension at a final density of 4×10^5 sporangia per milliliter.

2.3. Test for effect of SA in vitro on P. tabacina sporangia germination and development of germ tubes

The basic method for testing germination of *P. tabacina* sporangia described by Shephered (1962) was used. A suspension (0.01 ml) containing 10^3 sporangia was placed on a block of 2% Difco bacto agar ($15 \times 15 \times 3$ mm) resting on a microscope slide in a petri dish with a moistened filter paper on the base. Water or solutions of SA in the amounts of 10 μl were immediately added to the sporangial suspension on the block, which was then incubated for 5 h at 18 °C. One drop of formalin was then added to the suspension followed by 10 μl of trypan blue solution (0.03%). The percentage of germination was determined by counting the number of sporangia that had a recognizable germ tube. One hundred sporangia were examined on each block. In the same experiment, the length of germ tubes was determined with the aid of a microscope ocular micrometer scale calibrated in microns (1 division = 2.0 μm). The lengths of 20 randomly selected germ tubes were measured in each experimental treatment that was on each block.

The experiment consisted of 12 treatments: SA at 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500, and 1000 μg/ml and water control. Treatments were arranged in a randomized complete block with six replications. The experiment was repeated twice. Data from each test were analyzed by ANOVA using JMP software (SAS Institute, Cary, NC). The significance of effect of SA in vitro on germination of *P. tabacina* and development of the germ tubes was determined by the magnitude of the F values ($P = 0.05$). Treatment means were separated by Fisher's protected least significant difference (LSD).

2.4. Extraction and quantification of SA produced by PGPR in culture

Each bacterial strain was inoculated into 10 ml of TSB or Murashige and Skoog (MS; Gibco BRL) liquid medium using a single colony from a TSA plate on which the strain was grown for 24 h at 28 °C. After a 72-h incubation on a shaker at 150 rpm, SA was extracted from bacterial cultures using the technique described by Meyer et al. (1992). The culture was centrifuged at 2800*g* for 20 min at 4 °C. The resulting supernatant was acidified to pH 2.0 with 1 N HCl, filtered through 0.22 μm nylon membrane under a vacuum, partitioned twice with 2 ml of $CHCl_3$, and dried under a nitrogen stream. The sample was resuspended in 1 ml of 23% methanol (v/v) in 20 mM sodium acetate buffer (pH 5.0) and stored at −80 °C for further analysis.

Samples were analyzed with a Delta Pak C18 column (300 Å, 5 μ, 3.9 × 150 mm, Waters) connected to a high performance liquid chromatograph (HPLC). The column was isocratically eluted with 23% methanol in 20 mM sodium acetate buffer (pH 5.0) at a flow rate of 0.5 ml/min. Absorbance at 280 nm was monitored with a photodiode assay (PDA 996, Waters). Fluorescence was recorded with a scanning fluorescence detector (Waters 474). The excitation wavelength remained at 305 nm and the emission wavelength at 407 nm. Ten μl of each sample were injected into the column with a pressure of 21.1 kg/cm^2 (300 psi). The SA peak was separated from others. Ultra-pure water was used for the dilution and the preparation of the solutions. The detection limit was 2.8 ng of SA per milliliter from TSB based on an extraction efficiency of 18% and 2.1 ng of SA per milliliter from MS based on the extraction efficiency of 24%, to which a known quantity of SA was added. Each treatment was replicated four times. Noninoculated TSB or MS was used as controls. This experiment was repeated once.

2.5. Cultivation of tobacco seedlings, PGPR induction treatment, and challenge with P. tabacina

Tobacco seeds (cv. Xanthi-nc) provided by J. Ryals (Novartis Agricultural Biotechnology Research Unit, Research Triangle Park, NC) were used in this assay. Seeds were surface-disinfested with 70% ethanol for 1–2 min, then with 1% sodium hypochlorite for 30 min, and rinsed in sterile distilled water three times. The seeds were placed on MS agar (0.8%) with addition of 3% sucrose in multiwell plates (12-well, Falcon), 1 ml in each well. Tobacco seeds were grown in a growth chamber at 25 °C with a 14 h light and 10 h dark regime. Three weeks after seeding, a PGPR bacterial suspension (10^9 CFU/ml) in the amount of 10 μl was placed on the medium at the base of each stem of a tobacco seedling. Three PGPR strains (90-166, SE34, and 89B-61) were used in these assays. Water treatment was the non-treated control. Treatments were arranged as a randomized complete block with four replications. The tobacco seedlings treated with PGPR were grouped into two sets for the assays: one set of tobacco seedlings was challenged with *P. tabacina* (10^5 sporangia/ml) at 7 days after PGPR treatment, and the other set of seedlings were not challenged. In both assays, plant samples were collected for extraction and detection of endogenous SA at 0, 1, 3, 5, 7, 8, and 10 days after PGPR treatment.

2.6. Extraction and quantification of endogenous-free SA in PGPR-treated tobacco seedlings

SA was extracted from tobacco seedlings using the following technique. One gram of tobacco leaves was homogenized with liquid nitrogen. The ground tissue was mixed with 2.0 ml of 90% methanol, sonicated for

20 min, and centrifuged at 2800*g* for 20 min. The pellet was extracted with 2.0 ml of 100% methanol. The supernatant from two extractions was combined and dried under a nitrogen stream. The resulting residue was resuspended in 4.0 ml of 5% trichloroacetic acid (TCA) and centrifuged at 2800*g* for 20 min. The supernatant was partitioned twice with an extraction solution (ethyl acetate:cyclopentane:isopropanol = 100:99:1), and the top phase was dried under a nitrogen stream. The residues were resuspended in 1 ml of 23% methanol in 20 mM sodium acetate buffer, and solutions were filtered with 0.22 μm nylon membrane. Samples were stored at −80 °C and prepared for HPLC analysis.

The HPLC analysis was conducted using the same method as described above. The detection limit was 10.2 ng of SA from per gram of fresh tobacco leaves based on an extraction efficiency of 49%, which was determined by infiltrating leaf tissues with standard SA (Sigma). The experiment was repeated once. Data from each test were analyzed by ANOVA using JMP software, and significant effects of treatments were determined using LSD at $P = 0.05$.

2.7. Effect of PGPR on blue mold disease in Xanthi-nc and transgenic NahG tobacco

Tobacco seeds (Xanthi-nc and NahG) were planted on MS agar in the microtiter plates as described above. Tobacco seedlings were inoculated with PGPR by pipetting 10 μl of bacterial suspensions (10^9 CFU/ml) onto the medium at the base of the stems three weeks after planting. A week after PGPR treatment, tobacco seedlings were challenged with *P. tabacina*. A pathogen suspension of 2 μl (10^5 sporangia/ml) was placed on one leaf per plant. Three PGPR strains (90-166, SE34, and 89B-61) were used in this test. SA at 1.0 mM was used as a chemical control and a treatment with water as a nontreated control. Treatments were arranged as randomized complete blocks with six replications and 12 seedlings per replication. The experiment was repeated once. Blue mold disease was rated 1–2 weeks after pathogen challenge by counting the number of leaves per tobacco seedling with symptoms. Data were analyzed by ANOVA using JMP software, and significant treatment effects were determined by LSD at $P = 0.05$.

3. Results

3.1. Effect of SA in vitro on P. tabacina sporangia germination and development of germ tubes

SA was tested for in vitro effects on germination of sporangia of *P. tabacina* at 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500, and 1000 μg/ml. Water was used as a nontreated control. Data from a representative test are shown in Figs. 1 and 2. Except at 10 μg/ml, all other SA treatments did not significantly inhibit germination of sporangia of *P. tabacina* compared to the nontreated control (Fig. 1). Similarly, there was no inhibitory effect of SA on the length of germ tubes (Fig. 2). In contrast, SA at 0.05, 50, 100, and 500 μg/ml significantly increased the length of germ tubes compared to the nontreated control.

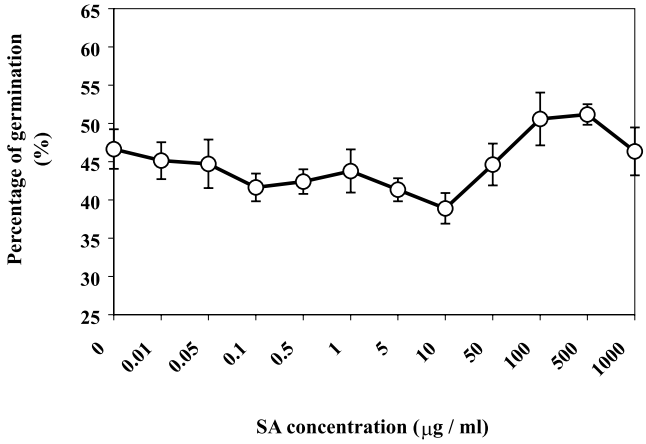

Fig. 1. In vitro effect of SA at different concentrations on germination of sporangia of *P. tabacina*. A suspension containing 10^3 sporangia of *P. tabacina* was placed on a block of 2% Difco bacto agar resting on a microscope slide in a petri dish with a moistened filter paper on the base. SA solutions or water was immediately added to the sporangial suspension on the block, which was then incubated for 5 h at 18 °C. One drop of formalin was then added to the suspension followed by trypan blue solution (0.03%). The percentage of germination was determined by counting the number of sporangia that had a recognizable germ tube. One hundred sporangia were examined on each block as one replication. The experiment was repeated twice. Data were analyzed by ANOVA using JMP software, and significant treatment effects were determined by LSD at $P = 0.05$. Data are from a representative test. Each value is the mean ± SE of six replications.

3.2. Detection and quantification of SA produced by PGPR in culture

After incubation for 72 h, the concentration of bacterial PGPR in TSB (~10^{10} CFU/ml) was much higher than that in MS (~ 10^8 CFU/ml). Of the strains tested, only *S. marcescens* 90-166 produced SA in TSB (38.6 ng /ml culture). However, no SA was detected from MS cultures of all three PGPR strains.

3.3. Time course of endogenous-free SA in PGPR-treated tobacco seedlings

Results from representative tests for endogenous-free SA in PGPR-treated tobacco seedlings without and with pathogen challenge are shown in Figs. 3 and 4, respectively. During the first week after PGPR treatment, levels of free SA significantly increased in tobacco seedlings treated with three PGPR strains compared to

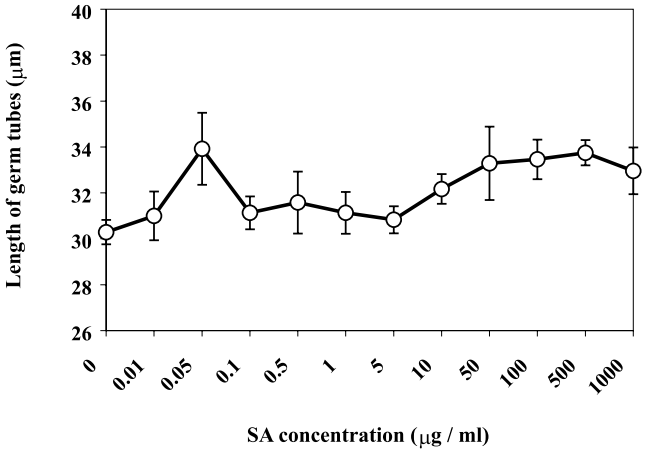

Fig. 2. In vitro effect of SA at different concentrations on development of germ tubes of sporangia of *P. tabacina*. A suspension containing 10^3 sporangia of *P. tabacina* was placed on a block of 2% Difco bacto agar resting on a microscope slide in a petri dish with a moistened filter paper on the base. SA solutions or water was immediately added to the sporangial suspension on the block, which was then incubated for 5 h at 18 °C. One drop of formalin was then added to the suspension followed by trypan blue solution (0.03%). Lengths of germ tubes were measured under a microscope. Twenty germ tubes were randomly selected and measured in each experimental treatment that was on each block. The experiment was repeated twice. Data were analyzed by ANOVA using JMP software, and significant treatment effects were determined by LSD at $P = 0.05$. Data are from a representative test. Each value is the mean ± SE of six replications.

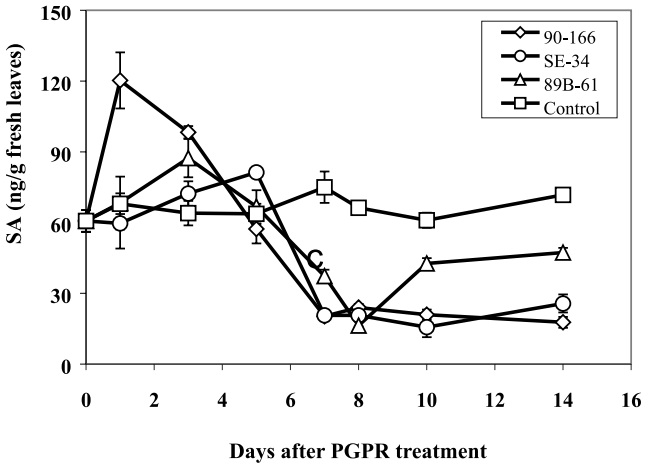

Fig. 3. Time course of levels of endogenous SA in tobacco leaves after PGPR treatment without challenge with *P. tabacina*. Tobacco seedlings were grown in a microtiter plate system under growth-chamber conditions. Three weeks after seeding, PGPR treatments were applied by placing 10 μl of PGPR bacterial suspension on the medium at the base of the stem of a tobacco seedling. Three PGPR strains (90-166, SE34, and 89B-61) were used in these assays. Water treatment was the nontreated control. Treatments were arranged as a randomized complete block with four replications. Plant samples were collected at different days after PGPR treatment. SA was separated by HPLC and measured as described in Section 2. The experiment was repeated once. Data were analyzed by ANOVA using JMP software and significant treatment effects were determined by LSD at $P = 0.05$. Data are from a representative test. Each value is the mean ± SE of four replications.

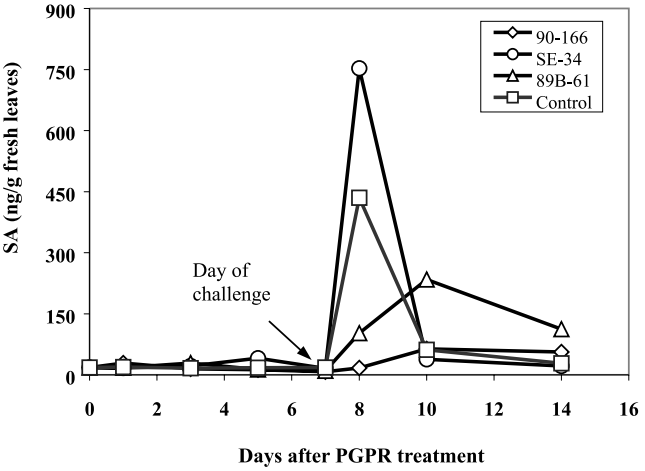

Fig. 4. Levels of endogenous SA in tobacco leaves treated with PGPR and challenged with pathogen 7 days after PGPR treatment. Tobacco seedlings were grown in a microtiter plate system under growth-chamber conditions. Three weeks after seeding, PGPR treatments were applied by placing 10 μl of PGPR bacterial suspension on the medium at the base of the stem of a tobacco seedling. Three PGPR strains (90-166, SE34, and 89B-61) were used in these assays. Water treatment was the nontreated control. One week after PGPR treatment, tobacco seedlings were challenged by dropping 2 μl of *P. tabacina* suspension on a top leaf. Two top leaves were challenged per seedling. Treatments were arranged as a randomized complete block with 4 replications. Plant samples were collected at different days after PGPR treatment. SA was separated by HPLC and measured as described in Section 2. The arrow indicates the time of challenge with *P. tabacina*. The experiment was repeated once. Data were analyzed by ANOVA using JMP software and significant treatment effects were determined by LSD at $P = 0.05$. Data are from a representative test. Each value is the mean ± SE of four replications.

the nontreated control (Fig. 3). SA levels in 90-166-treated tobacco leaves were significantly higher than in the control at 1 and 3 days after PGPR treatment. These levels were a 1.5- to 2-fold increase. Treatments with 89B-61 at 3 days and SE34 at 5 days significantly increased free SA in tobacco seedlings. In the second week after PGPR treatment, however, SA levels in the PGPR treatments were significantly lower than in the nontreated control.

In another assay in which the pathogen challenge was done at 7 days after PGPR treatment, the trend of SA levels over time after pathogen challenge in PGPR-treated tobacco seedlings was different (Fig. 4). One day after challenge, SA levels in the control tobacco seedlings dramatically increased 25-fold, and in SE34-treated seedlings, the levels increased 45-fold, compared to those before challenge. However, in tobacco seedlings treated with 90-166 and 89B-61, SA levels increased only 2- and 10-fold, respectively. Subsequently, the SA levels in SE34-treated tobacco seedlings sharply decreased; but in 90-166 and 89B-61 treatments, SA levels gradually increased up to 3 days after challenge. At this time, the SA levels in 89B-61-treated plants were significantly higher than in control plants. At 7 days after challenge,

levels of SA in 89B-61 and 90-166 treatments were significantly higher than in the control.

3.4. Effect of PGPR on disease severity of blue mold in Xanthi-nc and NahG Tobacco

All three PGPR strains significantly reduced disease severity of blue mold on Xanthi-nc tobacco in microtiter plate system (Table 1). Of these PGPR strains, 90-166 yielded the best protection against blue mold. The other strains (SE34 and 89B-61) had statistically equivalent capacity to protect tobacco plants from *P. tabacina* in the microtiter system.

Significant disease reduction was observed from transgenic NahG tobacco seedlings after they were treated with the same PGPR strains (Table 2), indicating that ISR by these PGPR is SA-independent. SA, which was degraded into catechol by salicylate hydroxylase encoded by the transgenic gene *nahG* in tobacco plants transferred from *Pseudomonas putida*, did not protect tobacco plants from blue mold disease.

4. Discussion

The results from the tests of PGPR on NahG tobacco indicate that ISR elicited by our tested PGPR strains against *P. tabacina* may operate in a SA-independent pathway. In addition, no significant increase in the expression of PR-1a and β-1, 3-glucanase genes was found in the tobacco seedlings treated with 90-166, SE-34, and 89B-61 compared to the nontreated tobacco seedlings (data not shown), indicating that ISR elicited by our tested PGPR strains is not associated with pathogenesis-related (PR) gene activation. These results are in agreement with Pieterse et al. (1996). Pieterse et al. (1998) also reported that ISR in *Arabidopsis* elicited by rhizobacteria is dependent upon ethylene and jasmonic acid pathways. We also examined the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and proteinase inhibitor genes in tobacco seedlings treated with PGPR strains 90-166, SE34, and 89B-61 with ISR activity against *P. tabacina*. No significant increase in the expression of these genes was observed (data not shown), indicating that systemic resistance elicited by these three PGPR is ethylene and jasmonate independent, which is distinct from the pathway proposed by Pieterse et al. (1998). Taken together, our results suggest that ISR elicited by our select PGPR strains against blue mold disease in tobacco may be associated with activation of novel defense pathways.

SA plays a critical role in the classical SAR in induced cucumber and tobacco plants (Malamy et al., 1990; Métraux et al., 1990; Yalpani et al., 1991). Most previous studies on the role of SA have been focused on the interactions between plants and virulent or avirulent pathogens (Conti et al., 1996; Gaffney et al., 1993; Yalpani et al., 1991). Some studies have been done in which SA was detected from the phloem sap of tobacco and cucumber expressing SAR (Métraux et al., 1990;

Table 1
Reduction of disease severity of blue mold in Xanthi-nc tobacco by PGPR in microtiter plates[a]

Treatment	Number of symptomatic leaves per seedling[b]	
	Trial I	Trial II
90-166	0.14 ± 0.02 c	0.18 ± 0.03 d
SE34	0.33 ± 0.03 c	0.55 ± 0.05 bc
89B-61	0.84 ± 0.06 b	0.78 ± 0.07 b
SA (1.0 mM)	0.28 ± 0.03 c	0.32 ± 0.02 cd
Water control	1.89 ± 0.21 a	1.24 ± 0.18 a

[a] Tobacco seeds were planted on MS agar (Murashige and Skoog salt and vitamin mixture, Gibco BRL) amended with 3% sucrose in multiwell (12-well, Falcon) plates after being disinfested with 70% ethanol and 1% sodium hypochlorite. Three weeks after planting, 10 μl of PGPR bacterial suspensions (10^8 CFU/ml), salicylic acid (1.0 mM), or water was pipetted onto the medium at the base of the stem. One week after PGPR induction, tobacco seedlings were challenged with *P. tabacina* by pipetting 2 μl of inoculum (10^5 sporangia/ml) on a leaf. Two fully developed top leaves from each seedling were inoculated. Treatments were arranged in a randomized complete block with five replications and 12 seedlings per replication.

[b] One to two weeks after pathogen challenge, blue mold disease was rated by counting number of symptomatic leaves per seedling. Each value is the mean ± SE of five replications per treatment. Data were analyzed by ANOVA using JMP software and significant treatment effects were determined by LSD at $P = 0.05$. Different letters within a column indicate significant differences among means.

Table 2
Effect of PGPR on blue mold disease in transgenic NahG tobacco in microtiter plates[a]

Treatment	Number of symptomatic leaves per seedling[b]	
	Trial I	Trial II
90-166	0.02 ± 0.002 b	0.02 ± 0.001 b
SE34	0 b	0.02 ± 0.003 b
89B-61	0 b	0.29 ± 0.03 b
SA (1.0 mM)	1.12 ± 0.15 a	2.71 ± 0.34 a
Water control	1.50 ± 0.27 a	3.38 ± 0.42 a

[a] Tobacco seeds were planted on MS agar (Murashige and Skoog salt and vitamin mixture, Gibco BRL) amended with 3% sucrose in multiwell (12-well, Falcon) plates after being disinfested with 70% ethanol and 1% sodium hypochlorite. Three weeks after planting, 10 μl of PGPR bacterial suspensions (10^8 CFU/ml), salicylic acid (1.0 mM), or water was pipetted onto the medium at the base of the stem. One week after PGPR induction, tobacco seedlings were challenged with *P. tabacina* by pipetting 2 μl of inoculum (10^5 sporangia/ml) on a leaf. Two fully developed top leaves from each seedling were inoculated. Treatments were arranged in a randomized complete block with five replications and 12 seedlings per replication.

[b] One to two weeks after pathogen challenge, blue mold disease was rated by counting number of symptomatic leaves per seedling. Each value is the mean ± SE of five replications per treatment. Data were analyzed by ANOVA using JMP software and significant treatment effects were determined by LSD at $P = 0.05$. Different letters within a column indicate significant differences among means.

Rasmussen et al., 1991; Yalpani et al., 1991). However, little work has been done on detecting SA from plants treated with PGPR.

Chen et al. (1999) reported that the capacity of PGPR strains to produce SA was not correlated with elicitation of ISR. In the study, two selected PGPR strains, *Pseudomonas corrugata* Müller strain 13 and *Pseudomonas aureofaciens* strain 63-28, induced the same level of resistance against Pythium root rot of cucumber although they varied in SA production in culture. Supporting this conclusion was a study by Press et al. (1997) in which SA-negative mutants of *Serratia marcescens* strain 90-166 retained the capacity to elicit ISR in cucumber against anthracnose caused by *Colletotrichum orbiculare* (Berk. & Mont.) Arx. Our results with strains 90-166 and SE34 also support the conclusion that PGPR production of SA is not a determinant of ISR capacity. PGPR strain 90-166 is the only one that produced SA in TSB culture, but SE34 induced resistance against blue mold of tobacco at levels statistically equivalent to 90-166 in the greenhouse tests (Zhang et al., 2002). No SA was detected from any PGPR cultures in MS. In the microtiter plate system with MS agar, disease reductions of blue mold by different PGPR were variable. Strain 90-166 showed the highest ISR activity, SE34 showed less effect compared to 90-166, and 89B-61 was the PGPR strain that reduced disease severity the least (Table 1).

Some studies indicate that SA levels have increased in plants induced by pathogen attacks without subsequent challenges with pathogens. Malamy et al. (1990) found that infection of resistant tobacco cultivars with TMV resulted in a 20- to 50-fold increase in levels of endogenous SA in TMV-infected leaves compared to noninoculated leaves. In cucumber, a dramatic rise in SA levels appeared in phloem exudates of the leaves inoculated with *Tobacco necrosis virus*, *Colletotrichum orbiculare*, or *Pseudomonas syringae* pv. *syringae* van Hall (Métraux et al., 1990; Rasmussen et al., 1991). This increase of SA in the phloem preceded appearance of ISR. Increases of SA levels have also been reported in *Arabidopsis thaliana* after infection with *Turnip crinkle virus* or *P. syringae* and in tobacco infected with *Tobacco necrosis virus*, *P. syringae*, *P. tabacina*, and *Erwinia carotovora* (Jones) Hooland (Palva et al., 1994; Silverman et al., 1993; Uknes et al., 1993).

Regarding ISR elicited by PGPR, little work has been conducted to detect the levels of SA from plant tissues, especially after pathogen challenge. Chen et al. (1999), working with a split-root system, detected endogenous SA from cucumber root tissues and reported that PGPR could stimulate cucumber plants to accumulate SA in roots. The plant itself was considered to have produced the SA detected in bacterized cucumber roots. Some SA could be transferred to the induced side from the bacterized side. Our results indicate that accumulation of endogenous SA increased in tobacco leaves during the first week after PGPR treatment (Fig. 3). The SA may be produced in plants in the interaction between PGPR and tobacco plants since no SA was detected in any MS cultures of three PGPR, while a certain amount of SA was found in tobacco seedlings treated with PGPR. The level of SA in tobacco seedlings was about 0.06 ng per gram of fresh plant tissue if the population of PGPR in tobacco was 10^6 CFU/ml as determined in a preliminary assay (unpublished data). However, the increase in SA levels occurred at different times for different treatments with PGPR strains. This discrepancy may reflect the interactions between the tobacco plant and individual PGPR strain.

Increased SA level in a plant may inhibit activity of catalase and ascorbic peroxidase (Chen et al., 1993; Durner and Klessig, 1995), which then leads to increased levels of H_2O_2. The elevated H_2O_2 levels activate PR gene expression (Chen et al., 1993) and increase the rate of polymerization of phenolic compounds into lignin-like substances (Agrios, 1997). This coincides with our finding of necrosis in PGPR-treated tobacco leaves (data not shown), in which treatment with strain 90-166 resulted in the strongest response.

An increase in SA levels in tobacco seedlings during the first week after PGPR treatment (Figs. 3 and 4) may result in elevated H_2O_2 levels, leading to activation of defense responses like polymerization of phenolic compounds into lignin-like substances and making the plants more resistant to pathogen attack. However, no increased expression of PR-1a and β-1,3-glucanase genes was observed in our study. One explanation may be that SA levels induced by PGPR treatments in tobacco plants were not high enough to activate the expression of PR protein genes. In the second week after PGPR induction, SA levels in plants significantly decreased compared to the first week. One of the reasons that the level of disease protection against blue mold of tobacco by PGPR strains decreased may be the prolonged time interval between PGPR treatment and pathogen challenge (Zhang et al., 2002).

When plants are pretreated with a SAR or ISR elicitor, such as a necrotizing pathogen or a synthetic chemical inducer, the systemically protected leaves react more rapidly and more effectively to a subsequent challenge with a virulent pathogen. This phenomenon is known as conditioning or sensitizing (Sticher et al., 1997). The biochemical and physiological changes occurring in a sensitized plant usually become apparent only at the moment of a challenge. At that time, there appears to be a shift toward a greater sensitivity not only to pathogens but also to biotic and abiotic elicitors. Change of SA levels is caused by either pathogen challenge or PGPR treatment. Our results showed that patterns of amounts of endogenous SA in PGPR treated tobacco leaves after challenge with *P. tabacina* were

different. *Peronospora tabacina* challenge alone increased SA accumulation by 25-fold one day after challenge compared to SA accumulation preceding that challenge. At that time, SA accumulation by different PGPR treatments was different depending on the strain. The SA level in SE34-treated plants was significantly higher than that in 90-166- and 89B-61-treated plants, indicating that sensitization of tobacco plants by PGPR was dependent on different strains.

No in vitro inhibition effect of SA was found on germination of *P. tabacina* sporangia and development of germ tubes. Exogenously applied SA on tobacco plants significantly reduced blue mold disease both in greenhouse assays and in the microtiter plate assays (Zhang et al., 1999, 2002). However, three PGPR substantially reduced blue mold disease both in Xanthi-nc and NahG tobacco, indicating that PGPR-elicited ISR is independent of SA. Taken together, therefore, SA may not be a primary signal, but it did accumulate when tobacco plants were induced by PGPR. SA accumulation in tobacco plants may play a role in ISR against tobacco blue mold by PGPR, or it may be a coincidental event.

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