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Activation of PR-1a Promoter by Rhizobacteria That Induce Systemic Resistance in Tobacco against *Pseudomonas syringae* pv. *tabaci*

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Investigations were conducted to determine whether induction of PR-1a gene promoter was correlated with systemic resistance, induced by rhizobacteria, against wildfire disease of tobacco. Ten different strains of plant growth-promoting rhizobacteria (PGPR), including the species *Bacillus pumilus, Serratia marcescens, Pseudomonas fluorescens, P. putida, Curtobacterium flaccumfaciens,* and *Bukholderia gladioli,* were tested. Induction of PR-1a gene activity was assessed using transgenic tobacco plants expressing the *β*-glucuronidase (GUS) gene fused to the PR-1a gene promoter. In a microtiter plate assay, GUS activity was significantly enhanced, compared to that of water-treated controls by salicylic acid (SA), four PGPR strains with known induced systemic resistance activity, and one endophytic bacterium previously shown to lack induced systemic resistance activity in cucumber. No enhanced GUS activity was noted with three control bacteria (two plant-associated strains and *Escherichia coli* strain HB-101). In a separate assay, infiltration of greenhouse-grown tobacco leaves with these same bacteria resulted in significant increases in GUS activity compared to that of the water-treated control, for all strains which induced GUS activity in the microtiter plate assay. The two plant-associated bacterial controls did not affect GUS activity. Treatment of tobacco with SA and all bacterial strains which enhanced GUS activity in the microtiter and leaf assays led to reduced symptoms of wildfire disease caused by *Pseudomonas syringae* pv. *tabaci* in the greenhouse, whereas none of the three control bacterial strains significantly affected disease. These results support the conclusion that induction of PR-1a promoter activity and PGPR-mediated induced systemic disease resistance are linked events for the PGPR strains studied.

Key Words: **systemic acquired resistance (SAR); pathogenesis-related proteins; rhizobacteria; immunization; biological control.**

INTRODUCTION

One strategy for the development of alternatives to broad-spectrum fungicides and bactericides for controlling plant diseases is the activation of host plant defenses by application of various agents which induce systemic disease resistance. Systemic plant resistance induced by infection with a necrotizing pathogen has been termed "systemic acquired resistance (SAR)" and involves a salicylic acid (SA)-mediated pathway of defense reactions in the plant (reviewed in Ryals *et al.*, 1996). During activation of SAR, induced plants show an early increase in exogenous salicylic acid (Métraux *et al.*, 1990) and activation of pathogenesis-related (PR) protein genes (reviewed in Hunt and Ryals, 1996; Uknes *et al.*, 1993; van Loon and van Strien, 1999). With tobacco, PR-1a is the PR protein that is most consistently used as an indicator of SAR, as it accumulates in high levels after pathogen challenge (van Loon and van Strien, 1999).

A similar approach to inducing systemic protection against pathogens is to use plant growth-promoting rhizobacteria (PGPR) instead of necrotizing pathogens or chemicals as the inducing agent, a process that has been termed "induced systemic resistance (ISR)." In recent years, several research groups working on various plant/pathogen systems have reported that certain strains of PGPR can also act as inducers for ISR (Wei *et al.*, 1991; van Peer *et al.*, 1991; Alström, 1991; Zhou and Paultitz, 1994). Induction of systemic resistance by seed or soil treatment with PGPR protects plants against various pathogens, including fungi (Hofland *et al.*, 1996; Liu *et al.*, 1995b,c; van Loon *et al.*, 1988; van Wees *et al.*, 1997; van Peer *et al.*, 1991; Wei *et al.*, 1991; Zhou and Paulitz, 1994), bacteria (Alström, 1991; Hofland *et al.*, 1996; Liu *et al.*, 1995a; van Wees *et al.*, 1997), and viruses (Mauhofer *et al.*, 1994; Raupach *et al.*, 1996), under both greenhouse and field conditions (Wei *et al.*, 1996; Raupach *et al.*, 1997; Raupach and Kloepper, 1998), resembling the classical SAR.

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A series of studies on one PGPR strain, *Pseudomonas fluorescens* (Trevisan) Migula strain WCS417r, which induces systemic protection in several plants, has presented evidence that the physiological response of the plant during induction is different from that of classical SAR (reviewed in van Loon *et al.*, 1998). In contrast to the case with SAR, PR proteins are not activated by plants showing protection mediated by WCS417r (Hoffland *et al.*, 1996; Pieterse *et al.*, 1998; van Loon and van Strien, 1999; van Wees *et al.*, 1997), and SA is not an intermediate in the induced systemic protection since the bacterium induces resistance in plants expressing the bacterial salicylate hydroxylase (*nahG*) gene, whereas SAR is not active on such plants. Another distinguishing feature is that systemic resistance induced by WCS417r requires responsiveness to jasmonate and ethylene, whereas classical SAR does not (Pieterse *et al.*, 1998). Based on these differences, Pieterse *et al.* (1998) proposed that rhizobacterial-mediated ISR operates by a pathway distinct from that of SAR, without activation of PR protein genes in ISR. Because this model is based primarily upon studies with the single strain WCS417r, it remains to be tested whether most PGPR with induced resistance activity fit the model of ISR or that of SAR. To date, only a very few reports considering the mechanism of induced resistance by other rhizobacterial strains have been published; so, comparisons to the model of Pieterse *et al.* (1998) are difficult. In contrast to this model, Maurhofer *et al.* (1994) reported that induced resistance by PGPR strain *P. fluorescens* CHA0 in tobacco leads to accumulation of eight known PR proteins. In support of the model, Press *et al.* (1997) reported that induced resistance by PGPR strain *Serratia marcescens* Bizio 90-166 occurred in NahG tobacco plants. The objective of this study was to determine whether any of a collection of diverse PGPR strains with ISR activity on tobacco activate the PR-1a promoter, using a reporter gene system for PR-1a in tobacco.

MATERIALS AND METHODS

Sources and culture of bacteria and tobacco. Bacteria used in the experiments described below included 10 PGPR strains previously demonstrated to induce systemic resistance and 4 control strains. All of the selected PGPR strains led to significant reduction in foliar disease following application as seed treatments on cucumber, and some have also been reported to induce disease resistance in tomato against foliar diseases. The PGPR strains used and the references in which they have been reported are *Bacillus pumilus* Meyer and Gottheil strains T-4 (Raupach *et al.*, 1997), SE-34 (Jetiyanon, 1997; Benhamou *et al.*, 1996; Yao *et al.*, 1997; Ji *et al.*, 1997), SE-49 (Jetiyanon, 1997; Jetiyanon *et al.*, 1997), SE-76 (Jetiyanon, 1997), and INR-7 (Kloepper *et al.*, 1996; Wei *et al.*, 1996; Zehnder *et al.*, 1997a,b); *S. marcescens* strain 90-166 (Liu *et al.*, 1995a,b,c; Raupach *et al.*, 1996; Kloepper *et al.*, 1996; Wei *et al.*, 1996; Zehnder *et al.*, 1997a,b; Press *et al.*, 1997); *P. fluorescens* strain 89B-27 (Liu *et al.*, 1995a,b,c; Raupach *et al.*, 1996); *P. putida* (Trevisan) Miguala strain 89B-61 (Kloepper *et al.*, 1996; Wei *et al.*, 1996; Zehnder *et al.*, 1997b; Ji *et al.*, 1997); *Curtobacterium flaccumfaciens* (Hedges) Collins and Jones strain INR-5 (Kloepper *et al.*, 1996; Wei *et al.*, 1996; Zehnder *et al.*, 1997a,b); and *Burkholderia gladioli* (Severini) Yabuuchi *et al.* strain IN-26 (Ji *et al.*, 1997). Bacterial strains used as controls included *Enterobacter asburiae* Brenner *et al.* strain JM-22, which is a model endophytic strain used in several previous studies (Musson *et al.*, 1995; Quadt-Hallmann and Kloepper, 1996; Quadt-Hallmann *et al.*, 1997) but which does not induce resistance in cucumber (Wei *et al.*, unpublished); *Escherichia coli* (Migula) Castellani and Chalmers strain HB-101; *Clavibacter michiganensis* (Smith) Davis *et al.* strain TE5, originally isolated from stems of field-grown cucumber and lacking the ISR activity in cucumber (Wei, unpublished); and *Paenibacillus macerans* Schardinger strain S8G6, which was selected in another study for biological control of *Pythium ultimum* Trow on cotton (Kloepper, unpublished).

All bacterial strains were maintained at −80°C in tryptic soy broth (TSB) amended with 20% glycerol. Inoculum for treatment of tobacco was prepared by streaking strains from −80°C onto tryptic soy agar (TSA) plates, incubating plates at 28°C for 24 to 30 h, and scraping bacterial cells off plates in 0.02 M K_2HPO_4 buffer, pH 6.8, to yield 10^9–10^{10} colony-forming units (cfu)/ml.

Seeds of *Nicotiana tabacum* L. cv. Xanthi-nc, which was genetically engineered with a GUS reporter gene fused to the PR-1a promoter (Uknes *et al.*, 1993), were provided by J. Ryals (Novartis Agricultural Biotechnology Research Unit, Research Triangle Park, NC). Seeds were grown in the greenhouse in soilless potting medium for seed increase. For testing activation of PR-1a in the seedling growth chamber assay, seeds were surface-disinfected by mixing for 3 min in 20% household bleach, followed by 3 min in 75% MeOH, and rinsing three times in sterile distilled water. Two seeds were placed, along with 0.1 ml of aqueous Murashige and Skoog (MS) medium without hormones, into each well of a sterile 24-well microtiter plate. Plates were covered and incubated at 28°C with 12 h light.

Activation of PR-1a promoter in a microtiter plate assay. To test large numbers of bacteria, an assay was developed by growing seeds in microtiter plates and treating seeds with SA, which induces SAR and β-glucuronidase (GUS) activity in the transgenic plants (Uknes *et al.*, 1993). GUS activity was measured in leaflets of tobacco plants using a fluorometric assay

described by Jefferson (1987). The assay conditions were further optimized for various plant-growing environments by timing the capacity of bacteria to activate the PR-1a promoter of the transgenic tobacco. According to preliminary results, 7 days after placing the tobacco seeds into MS medium in microtiter plates as described above, various treatments were applied. Treatments consisted of 20 μl of prepared bacterial suspensions each, indicated above, or the same volume of sterile water as a negative control, or 0.5 mM SA as a positive control.

GUS activity was determined at various sampling times (see methods of individual experiments). Twenty milligrams of plant tissue from each replication of each treatment was removed from the microtiter plates and ground in an Eppendorf tube with 300 μl of GUS extraction buffer (Jefferson, 1987). Extracts were centrifuged twice at 8000*g* for 5 min at 4°C, and 20 μl of the resulting supernatant was incubated with 200 μl of 2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) at 37°C for 1 h. The reaction was terminated by adding 960 μl of 0.2 M sodium carbonate solution, and the fluorescence was measured with a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Background fluorescence was determined by carrying out the reaction in the absence of MUG, and this value was subtracted from each sample. As a calibration standard, 4-methylumbelliferone (MU) was used (Wilson *et al.*, 1992), and, therefore, GUS activity was expressed as nM of MU/10 mg of sample/h.

Experiment 1 was conducted to determine the optimum time after bacterial treatment to test for activation of the PR-1a promoter by measuring enhanced GUS activity. Treatments included bacterial strains T4, SE-49, TE-5, and IN-26 along with SA (0.5 mM) and sterile water. Strain TE-5 was the negative bacterial control since it lacked ISR activity in previous trials on cucumber, whereas all other strains expressed ISR activity previously. Four replications of each treatment were used, and GUS activity was determined at 3, 5, 7, 10, 12, 14, 16, and 20 days after treatment with bacteria. Data were analyzed with ANOVA in SAS JMP software (SAS Institute, Cary, NC). Significant differences in treatment means on each sample date were determined using LSD at $P = 0.05$.

In Experiment 2, a larger number of strains was evaluated for potential activation of GUS activity under the same experimental conditions as described for Experiment 1, except that sampling was done at 7, 10, and 12 days after treatment. In addition to the same six treatments used in the Experiment 1, the following seven additional bacteria were tested: INR-7, 89B-61, 90-166, JM-22, 89B-27, SE-34, and SE-76. All of the new strains, except JM-22, were previously shown to induce systemic disease protection in cucumber. Experiment 2 was designed as a randomized complete block with three replications of each treatment, and the whole experiment was repeated twice. After confirming homogeneity of variances between the trials, the data from both trials were combined and analyzed as in Experiment 1.

In Experiment 3, strains S8G6 and HB101, which have never been demonstrated to induce resistance, were used as additional negative control strains to test the possibility that enhanced GUS activity was a general rather than a strain-specific phenomenon. In addition to these new strains, the previous two negative control strains used in Experiments 1 and 2, TE-5 and JM-22, were also tested along with four PGPR strains previously shown to exhibit ISR on cucumber (strains T-4, 89B-61, 90-166, and IN-26). Additional controls included water treatment and treatment with SA. The experimental design was a randomized complete block with four replications per treatment per sample time. Samples were taken at 3, 7, 10, and 13 days after bacterial treatment.

Activation of PR-1a promoter in tobacco plants grown in the greenhouse. Three greenhouse trials were conducted to determine whether the bacteria that induced the PR-1a promoter in the microtiter plate assay also induced PR-1a promoter activity in tobacco plants. All three trials were randomized complete blocks with 10 treatments, each replicated four times, with each replication consisting of one plant. Treatments included bacterial strains T-4, JM-22, 89B-61, 90-166, IN-26, TE-5, S8G, and HB-101, as well as a water control and a SA control.

In Trial 1, 6 weeks after planting in the greenhouse, which represented the sixth-leaf stage, leaves were infiltrated on the lower side with 0.1 ml of a 10^8 cfu/ml bacterial suspension. Controls were infiltrated with the same volume of sterile water or 0.5 mM SA. At 4 or 5 days after infiltration, three leaf discs were removed from the leaf area immediately adjacent to the infiltrated zone of each replication with a No. 3 cork borer, and they were ground and processed as described above for the microtiter plate assay for GUS activity. GUS activity values were analyzed for significant differences in each of the three trials, and data from the three trials were not combined due to heterogeneity of variances.

In Trial 2, PGPR were applied to tobacco seedlings germinated on MS agar by pipetting 10 μl of a 10^8 cfu/ml bacterial suspension over each seedling. Seedlings were then immediately planted into soilless mix in pots in the greenhouse. Twenty days later, leaves were sampled and processed for GUS activity as in Trial 1.

In Trial 3, PGPR were applied as root drenches to 6-week-old plants by adding 0.1 ml of a 10^8 cfu/ml bacterial suspension to each plant. Leaves were sampled for GUS activity 10 days after treatment with PGPR.

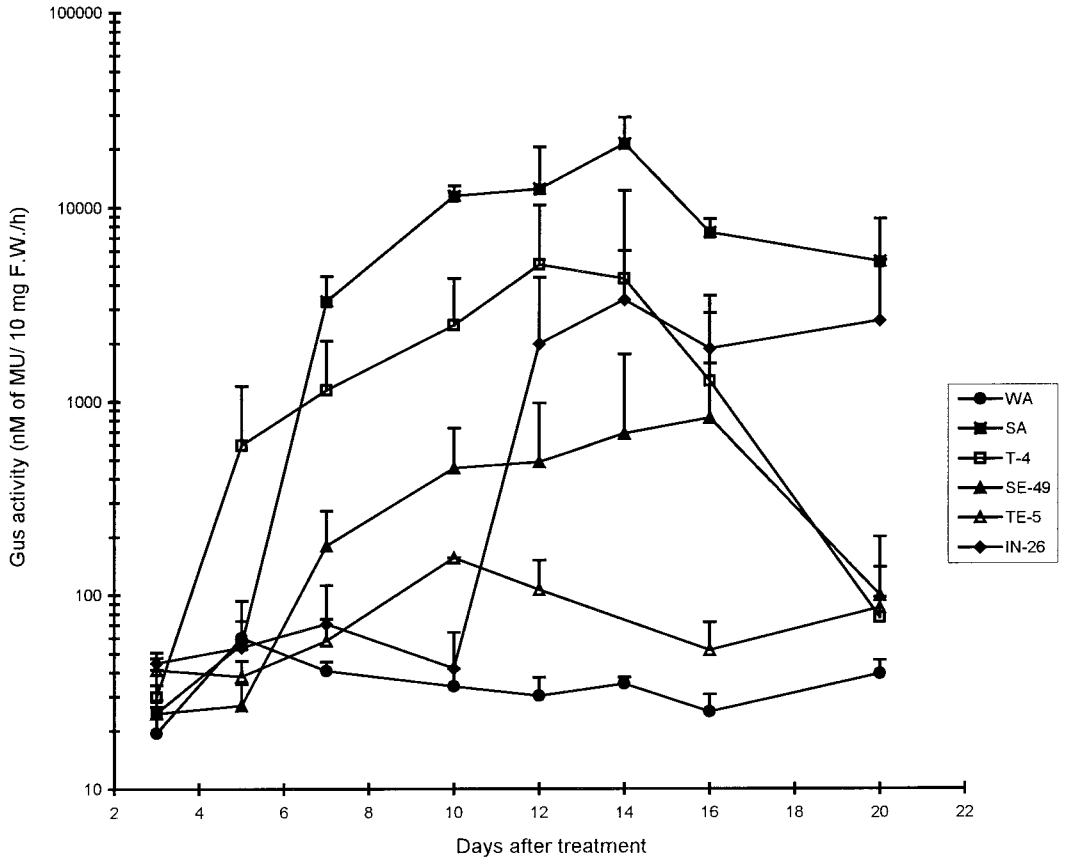

FIG. 1. PGPR-mediated induction of β-glucuronidase activity as a reporter for PR-1a promoter induction in microtiter plate assay, Experiment 1. Bacterial suspensions (20 μl of 10^8 cfu/ml) were applied to two 7-day-old tobacco seedlings per well of a 24-well microtiter plate. Four replicate wells were tested at each sample time for GUS activity, as described under Materials and Methods. Treatment legend: WA, water control; SA, 0.5 mM salicylic acid control; T-4 and SE-49, *Bacillus pumilus;* TE-5, *Clavibacter michiganensis;* IN-26, *Burkholderia gladioli.* Vertical bars indicate standard error of the mean. MU, 4-methylumbelliferone; F.W., fresh weight.

Protection against Pseudomonas syringae pv. tabaci. Transgenic tobacco plants were inoculated with eight selected bacterial strains by drenching bacterial suspensions (10^8 cfu/ml) into the soil of 6-week-old tobacco plants. Controls included a soil drench with water and foliar spray of entire plants to run-off with 0.5 mM SA, since SA did not induce resistance as did soil drench. All treatments were replicated five times. Ten days after induction, entire plants were sprayed with a suspension of 10^8 cfu/ml of the pathogen *Pseudomonas syringae* (van Hall) Ash *et al.* pv. *tabaci* in 0.02% Silwet L-77 (Union Carbide, Tarrytown, NY) to run-off, and they were then placed in a humidity chamber (100% relative humidity) for 24 h prior to placing on a greenhouse bench. Disease symptoms were recorded by determining the percentage of leaf surface with symptoms 10 days after challenge. On each replicate plant, symptoms were recorded on two leaves, and the mean value per plant was analyzed with ANOVA using SAS JMP software. The experiment was conducted twice. After confirming homogeneity of variances with Bartlett's test, combined data were analyzed with ANOVA and significant differences in treatment means were determined using LSD at $P = 0.05$.

RESULTS

Activation of PR-1a promoter in a microtiter plate assay. In Experiment 1, GUS activity, indicating activation of the PR-1a promoter, was significantly enhanced compared to the background activity in the water control with all treatments except the bacterial strain TE-5 (Fig. 1). The minimum magnitude of the increase, relative to the control, was threefold for all of the bacterial strains. Maximum enhancement of GUS activity was induced by SA. Among the tested bacterial strains, T-4 induced the highest overall level of GUS activity, and this was statistically equivalent to that of the SA treatment at 12 days after treatment. The time interval between the treatment with bacteria and the detection of a statistically significant enhancement of GUS activity, compared to the water treatment, varied among the strains from a low of 5 days with strain T-4 to a high of 12 days with strain IN-26.

TABLE 1

PGPR-Mediated Induction of β-Glucuronidase (GUS) Activity as a Reporter for PR-1a Promoter Induction, Microtitre Plate Assay, Experiment 2[a]

Treatment	GUS activity (nM MU[b]/10 mg fresh weight/h) 7 DAT[c]	10 DAT	12 DAT
Water control	21	21	105
Salicylic acid	22,843*	23,622*	53,088*
T-4	16	7,799	3,892
JM-22	1,245	33,050*	29,523*
89B-61	7,814	35,145*	22,793*
90-166	215	12,162*	34,543*
IN-26	713	14,507*	25,388*
TE-5	24	69	268
SE-34	20	102	1,002
SE-49	28	228	900
89B-27	52	40	9,466
SE-76	10	28	240
INR-7	23	926	2,233
$LSD_{0.05}$	10,827	10,875	12,464

[a] The experiment was a randomized complete block with three replications of each treatment. Data shown are pooled from two trials after confirming homogeneity of variances between the two trials.

[b] MU, 4-methylumbelliferone.

[c] DAT, days after treatment.

* Indicates significant increase in GUS activity compared to that of the water control at $P = 0.05$.

Based on the results from Experiment 1, sampling times of 3, 7, 10, and 12 days after treatment with bacteria were used for testing activation of PR-1a by a larger number of bacterial strains in Experiment 2. Significant increases in GUS activity relative to the water-treated control were observed at one or more sampling times with SA and four of the bacteria (Table 1). The same bacterial strain used as negative control (strain TE-5) in Experiment 1 also failed to increase GUS activity in Experiment 2. In contrast, strain JM-22, which was included in the Experiment 2 as another negative control strain significantly increased GUS activity at 10 and 12 days after treatment.

In Experiment 3, the two additional control strains, S8G6 and *E. coli* strain HB-101, failed to induce significant changes in GUS activity relative to that of the water control (Table 2). Strain TE-5, which was another negative control strain tested in Experiments 1 and 2, also did not induce GUS activity in Experiment 3, whereas the fourth negative control strain, JM-22, significantly increased GUS activity in Experiment 3 as it did in Experiment 2. Two of the PGPR strains with known ISR activity, strains 89B-61 and IN-26, also significantly increased GUS activity at one or more sampling times.

Activation of PR-1a promoter: plant assay in greenhouse. Bacterial strains that previously were demonstrated to activate the PR-1a promoter, as determined by GUS activity assay in the microtiter plate assay (T-4, JM-22, 89B-61, 90-166, and IN-26), significantly induced GUS activity, relative to that of the water control, when infiltrated into tobacco leaves in two of the three trials (Table 3). SA significantly induced GUS activity in all three trials. Two of the negative control bacterial strains (S8G6 and TE-5) significantly increased GUS activity in only one of the three trials. Background GUS activity of *E. coli* strain HB-101, which produces glucuronidase (Wilson *et al.*, 1992), was detected in all three trials.

GUS activity in tobacco leaves was significantly enhanced, relative to that of the water-treated control, by four of the PGPR strains applied to tobacco seedlings and by two of the PGPR strains applied as root drenches (Table 4).

Protection against Pseudomonas syringae pv. tabaci. Treatment with all bacterial strains that previously demonstrated enhanced GUS activity in the microtiter plate assay resulted in statistically significant reductions in severity of angular leaf spot symptoms compared to that of the water control (Table 5). In contrast, the three bacterial strains that did not enhance GUS activity in the microtiter plate assay, TE-5, S8G6, and HB-101, generally had no significant effect on symptoms. The SA control also significantly reduced symptom severity.

TABLE 2

PGPR-Mediated Induction of β-Glucuronidase (GUS) Activity as a Reporter for PR-1a Promoter Induction, Microtitre Plate Assay, Experiment 3[a]

Treatment	GUS activity (nM MU[b]/10 mg fresh weight/h) 3 DAT[c]	7 DAT	10 DAT	13 DAT
Water control	106	146	228	356
Salicylic acid	591*	2,022*	2,152	5,952*
T-4	112	370	610	2,647
JM-22	440*	453	12,100*	2,990
89B-61	124	1,052	3,165	3,602
90-166	112	744	2,214	—
IN-26	124	1,185	2,145	4,595*
TE-5	121	238	632	—
S8G6	81	372	—	—
HB-101	123	439	460	
$LSD_{0.05}$	231	1127	3119	3114

[a] The experimental design was a randomized complete block with four replications per treatment per sample time.

[b] MU, 4-methylumbelliferone.

[c] DAT, days after treatment.

* Indicates significant increase in GUS activity compared to that of the water control at $P = 0.05$.

TABLE 3

Induction of β-Glucuronidase (GUS) Activity in Tobacco Leaves Infiltrated with PGPR in the Greenhouse[a]

Treatment	GUS activity (nM MU[b]/10 mg fresh weight/h) Trial 1	Trial 2	Trial 3
Water control	54	370	1,050
Salicylic acid	1,840*	33,460*	8,610*
T-4	31	1,024*	4,985*
JM-22	24	14,368*	12,195*
89B-61	55	23,862*	10,532*
90-166	192	21,170*	10,270*
IN-26	38	18,938*	7,750*
TE-5	82	16,792*	3,807
S8G6	62	10,570*	1,067
HB-101	1,902*	23,460*	7,860*
$LSD_{0.05}$	713	6,514	3,685

[a] Leaves of Xanthi-nc tobacco plants at the sixth-leaf stage were infiltrated on the lower side with 0.1 ml of a 10^8 cfu/ml bacterial suspension; controls were infiltrated with the same volume of sterile water or 0.5 mM salicylic acid. At 4 days after infiltration in Trial 1 and 5 days after infiltration in Trials 2 and 3, three leaf discs were removed from the leaf area immediately adjacent to the infiltrated zone of each plant and processed for analysis of GUS activity. Each trial was a randomized complete block with four replications of single plants per treatment.

[b] MU, 4-methylumbelliferone.

* Indicates significant increase in GUS activity compared to that of the water control at $P = 0.05$.

DISCUSSION

The results reported here indicate that there is a strong correlation between the capacity of PGPR strains to induce the PR-1a gene promoter in transgenic Xanthi-nc tobacco and the capacity to induce systemic disease resistance. This correlation was noted for a group of bacterial strains that were previously selected for induced systemic resistance activity in cucumber. Strains T-4, 89B-61, 90-166, and IN-26, with known ISR activity in cucumber, all significantly enhanced GUS activity, compared to that of the water control, in the assay for PR-1a promoter activation in one or more of the three experiments in the microtiter plate assay (Fig. 1, Tables 1 and 2), as well as in two of the three trials with leaf infiltration into 6-week-old plants (Table 3). All of these strains induced significant protection against wildfire disease caused by *P. syringae* pv. *tabaci* in the greenhouse (Table 4), whereas none of the control strains had any effect. The strain *E. asburae* JM-22 was originally selected as a negative control because it colonized the endorhiza without inducing systemic resistance in cucumber in preliminary experiments (unpublished). As reported in this paper, JM-22 induced GUS activity in both the microtiter plate assay (Tables 1 and 2) and the leaf-infiltration tests (Table 3). It also induced systemic protection against wildfire disease, which further supports the conclusion that disease protection and PR-1a promoter activation are linked. Also supporting this hypothesis is that none of the three control strains, which did not induce disease protection, *C. michiganensis* strain TE-5, *P. macerans* strain S8G6, and *E. coli* strain HB 101, promoted GUS activity significantly in the microtiter plate assay (Table 2).

The transgenic tobacco system used here with the GUS reporter gene has been used for studying regulation or induction of PR-1 genes (Beilmann *et al.*, 1992; Grüner and Pfitzner, 1994; Ohshima *et al.*, 1990; Uknes *et al.*, 1993; van de Rhee *et al.*, 1990). Precisely how much increased GUS activity, following application of an inducing agent, constitutes induction of the PR-1 promoter was different in each of these studies, and in none was "induction" based on a statistically significant increase in GUS activity compared to that of the control. Two studies (Ohshima *et al.*, 1990; van de Rhee *et al.*, 1990) set an arbitrary level of increase over the control of 50% (Ohshima *et al.*, 1990) or 5-fold (van de Rhee *et al.*, 1990) to be scored as "induced." Uknes *et al.* (1993) discussed the difficulty of comparing conclusions regarding induction of PR-1 in different studies using the GUS reporter system because of the

TABLE 4

PGPR-Mediated Induction of β-Glucuronidase (GUS) Activity in Tobacco Leaves in Greenhouse Trials

Treatment	GUS activity (nM MU[a]/10 mg fresh weight/h) PGPR applied to seedlings[b]	PGPR applied as root drench[c]
Water control	352	298
Salicylic acid	6,542*	6,194*
T-4	1,832	21,390*
JM-22	3,986*	2,322*
89B-61	3,587*	1,081
90-166	4,198*	542
IN-26	2,952*	943
TE-5	661	565
S8G6	715	251
HB-101	318	280
$LSD_{0.05}$	2,522	1,732

[a] MU, 4-methylumbelliferone.

[b] Tobacco seedling germinated on MS agar were inoculated with 20 μl of a 10^8 cfu/ml suspension of PGPR and then planted into soilless potting media. Three leaf discs per plant were sampled at 20 days after PGPR treatment for analysis of GUS activity. Values shown are means of four replications.

[c] PGPR were applied to 6-week-old tobacco plants by drenching 100 μl of a 10^8 cfu/ml suspension of PGPR into each pot. Three leaf discs per plant were sampled at 10 days after PGPR treatment for analysis of GUS activity. Values shown are means of four replications.

* Indicates significant increase in GUS activity compared to that of the water control at $P = 0.05$.

TABLE 5

Protection against *Pseudomonas syringae* pv. *tabaci* on Xanthi-nc Tobacco[a]

Treatment	Disease severity (% leaf surface with lesions)[b]		
	Trial 1	Trial 2	Combined data
Water control	15	33	24
Salicylic acid	4*	16*	10*
T-4	4*	11*	8*
JM-22	0.4*	6*	3*
89B-61	2*	6*	4*
90-166	0.1*	15*	7*
IN-26	13	11*	12*
TE-5	15	20*	18
S8G6	17	25	21
HB-101	18	25	22
$LSD_{0.05}$	6.2	8.4	6.6

[a] The experiment was a randomized complete block with five replications of single plants per treatment. Data are combined from two trials after confirming homogeneity of variances with Bartlett's test. Bacteria and water control treatments were applied as root drenches of 6-week-old tobacco plants. Salicylic acid was applied as a foliar spray. Ten days after treatment, plants were challenge-inoculated with the pathogen by foliar spray of bacterial suspension.

[b] Disease severity was measured 10 days after pathogen inoculation by recording the percentage of leaf area covered with lesions on each of two leaves per replication.

* Indicates significant reduction in disease severity compared to that of the water control at $P = 0.05$.

lack of standardization of how much enhancement of GUS activity is needed to indicate induction. In the study by Uknes *et al.* (1993) with transgenic tobacco, a mean increase in GUS activity of 13-fold, compared to that of the control, was induced by TMV, whereas SA induced an average 5-fold increase of GUS activity, but it was not stated what minimum increase in GUS activity was deemed to represent "induction" of PR-1. Due to this lack of agreement, we chose the conservative approach of using statistical significance at $P = 0.05$ as the cut-off for indicating induction.

The conclusion from this study, that induction of PR-1a promoter activity and induction of systemic disease resistance are linked events, disagrees with the model for PGPR-mediated ISR proposed by Pieterse *et al.* (1998) based on extensive studies with the PGPR strain WCS417r, which proposes that PGPR-mediated ISR does not lead to PR protein accumulation and is not salicylate dependent. Previous work with one of the strains used in our study, *S. marcescens* 90-166 (Press *et al.*, 1997), indicated that induction of systemic protection of tobacco against *P. syringae* pv. *tabaci* was not dependent on the salicylate pathway, since protection occurred in *NahG*-tobacco plants, which agrees with the model of Pieterse *et al.* (1998). However, the finding here that this PGPR strain induced the PR-1a promoter does not agree with the model of Pieterse *et al.* (1998). This apparent contradiction may be explained by the fact that strain 90-166 produced salicylate, which may result in activation of the PR-1a promoter in the GUS reporter system. Other indications that some PGPR or rhizosphere bacteria may induce PR proteins, as SAR, have been reported. Maurhofer *et al.* (1994) indicated that induced protection of tobacco against tobacco necrosis virus by PGPR strain *P. fluorescens* CHA0 was associated with the induction of multiple PR proteins, including PR-1a, 1-b, and 1-c. Schneider and Ullrich (1991) similarly reported that protection of tobacco against *P. syringae* pv. *tabaci*, induced by culture filtrates of a *P. fluorescens* strain, was associated with induction of chitinase, β-1,3-glucanase, peroxidase, and lysozyme. Hence, as suggested by Maurhofer *et al.* (1998), systemic resistance by bacteria appears to involve multiple mechanisms, and further work should be done with the ISR-active strains used in this study to determine how they relate to mechanistic models for ISR and SAR.

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