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Systemic Resistance and Expression of the Pathogenesis-Related Genes Mediated by the Plant Growth-Promoting Rhizobacterium *Bacillus amyloliquefaciens* EXTN-1 Against Anthracnose Disease in Cucumber

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Plants have the ability to acquire an enhanced level of resistance to pathogen attack after being exposed to specific biotic stimuli. To obtain plant growth-promoting rhizobacteria inducing resistance against cucumber anthracnose by *Colletotrichum orbiculare*, more than 800 strains of rhizobacteria were screened in the greenhouse. Among these strains, *Bacillus amyloliquefaciens* solate EXTN-1 showed significant disease control efficacy on the plants. Induction of pathogenesis-related (*PR-1a*) gene expression by EXTN-1 was assessed using tobacco plants transformed with *PR-1a::β-glucuronidase* (*GUS*) construct. GUS activities of tobacco treated with EXTN-1 and salicylic acid-treated transgenic tobacco were significantly higher than those of tobacco plants with other treatments. Gene expression analyses indicated that EXTN-1 induces the accumulation of defense-related genes of tobacco. The results showed that some defense genes are expressed by the treatment with EXTN-1 suggesting the similar resistance mechanism by salicylic acid.

KEYWORDS: Biological control, Induced systemic resistance (ISR), Pathogenesis-related proteins, Rhizobacteria

Systemic resistance is the phenomenon that a plant exhibits an increased level of resistance to pathogen infection after appropriate stimulation. Since Ross (1961a, b) described this resistance in the virus-infected plants, many researches have demonstrated that induction of systemic resistance can be a potential mechanism for control of wide range of diseases (Hammerschmidt and Kuc, 1995). Systemic resistance is comprised with systemic acquired resistance (SAR) and induced systemic resistance (ISR). The former is induced by a predisposing infection with necrotizing pathogens (Kuc, 1982), treatment of chemicals including benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Friedrich *et al.*, 1996; Gorlach *et al.*, 1996) and methyl-2,6-dichloroisonicotinic acid (DCINA) (Delaney, 1997), and certain elicitors (Binet *et al.*, 2001). ISR-mediated plant protection is induced by colonization of the rhizosphere with plant growth-promoting rhizobacteria (PGPR). Seed coating or soil drenching with PGPR protected plants against various pathogens including fungi, bacteria, and viruses (Alstrom, 1991; Hoffland *et al.*, 1996; Liu *et al.*, 1995a, b, c; van Peer *et al.*, 1991; van Wee *et al.*, 1997; Wei *et al.*, 1996) in greenhouses and field conditions.

Extensive studies on the biological roles and mechanisms of plant ISR by PGPR have been performed (Jetiyanon, 1997; Raupach *et al.*, 1996; Yao *et al.*, 1997). Many of them suggested that rhizobacteria-mediated ISR is independent of salicylic acid accumulation and pathogenesis-related (PR) gene transcript accumulations. Pieterse *et al.* (1996) supported above paradigm by demonstrating that a biocontrol bacterial strain, *Pseudomonas fluorescens* WCS417r, induces the systemic resistance in the Arabidopsis mutant NahG, the gain-of-function mutant of bacterial salicylate hydroxylase (*nahG*) gene (Gaffney *et al.*, 1993) and no expression of *PR-1* in the biocontrol bacteria-treated wild type Col-0. Similar phenomena were observed frequently in tobacco or other plants. Press *et al.* (1997) also described that induced resistance by PGPR strain 90-166 triggered in NahG tobacco plants.

On the other hand, some reports have indicated the PGPR-mediated transcript accumulation of PR genes. Another PGPR strain *P. fluorescens* CHA0, which conferred systemic resistance on tobacco against *tobacco mosaic virus*, provoked the accumulation of eight tobacco PR proteins (Maurhofer *et al.*, 1994). In addition, mutation of *NPR1* also resulted in the non-expression of systemic resistance in the Arabidopsis against *P. syringae* DC3000 (Pieterse *et al.*, 1998). These results suggested the participation or dependency of salicylic acid-mediated defense pathway in the rhizobacteria-mediated systemic resistance.

Recently, induction of *PR-1a* gene activity by PGPR was assessed using transgenic tobacco plants expressing β-glucuronidase (GUS) gene fused to the *PR-1a* promoter (Park and Kloepper, 2000). Infiltration of several selected PGPR into transgenic tobacco leaves resulted in the activation of GUS expressions and reduction of wildfire disease casued by *P. syringae* pv. *tabaci* (Park and Kloepper, 2000). These results indicated that expression of *PR-1a* is induced by the PGPR and this defense pathway may be effective in the practical plant disease control. The objectives of this study were to select rhizobacteria induc-

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ing systemic resistance to cucumber anthracnose and to investigate the defense pathway activated by selected PGPR.

Materials and Methods

Sources of bacteria and plants. Bacteria used in the experiments were EXTN-1, PGPR strains inducing systemic resistance, positive (89B-61), and negative (TE-5 and HB-101) control strains. Strain 89B-61 was included for comparison in this study that was previously reported (Park and Kloepper, 2000) to protect tobacco wildfire disease. Bacterial strains used as negative controls were *Escherichia coli* strain HB-101 and *Clavibacter michiganensis* strain TE-5. This strain TE-5 is originally isolated from stems of field-grown cucumber. Both strains are known to be lack of the ISR activity in tobacco and cucumber (Wei, unpublished). All bacterial strains were maintained at -80°C in tryptic soy broth (TSB) amended with 20% glycerol. PGPR was prepared by streaking strains onto tryptic soy agar (TSA), then incubated them at 28°C for 24 to 30 h, and bacterial cells were harvested by scrapping plates in 0.02 M K_2HPO_4 buffer (pH 6.8) to yield 10^9-10^{10} colony forming units (cfu)/m*l*.

Seeds of *Nicotiana tabacum* 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, U.S.A.). For testing activation of *PR-1a*, seeds were surface-sterilized by dipping in 1% NaOCl for 3 min., 75% methanol for 3 min., and then rinsed 3 times with sterile distilled water. Two seeds were placed in each well of 24-well cell culture plates containing 1 m*l* Murashige and Skoog (MS) medium. Plates were incubated at 28°C with 12 hour light.

Protection against *Colletotrichum orbiculare*. Cucumber plants (*Cucumis sativus* L. cv. Eunsung, 2 leaf stage) were treated with four selected bacterial strains by soil drench of bacterial suspensions (10^8 cfu/m*l*). Controls included a soil drench with water (negative) and leaf spray of entire plants until run-off with 0.5 mM salicylic acid (positive), since salicylic acid did not induce resistance by soil drenching. All treatments were conducted with 5 replicates. Seven days after induction, entire plants were spray-inoculated with a conidial suspension (1.0×10^5 conidia/m*l*) of cucumber anthracnose pathogen *C. orbiculare* in 0.02% Silwet L-77 (Union Carbide, Tarrytown, NY, U.S.A.) until run-off and then placed in a humidity chamber (100% relative humidity) for 24 hours. After that, all plants were transferred in a greenhouse at 20~28°C. Disease symptoms were recorded 7 days after inoculation by estimation of the percentage of symptomatic leaf areas. Symptoms were recorded on 2nd leaves of each replicate, and the mean value per plant was analyzed with ANOVA using SAS JMP software (SAS Institute, 1995). The experiments were conducted two times. After confirming homogeneity of variances with Bartlett's test, combined data were analyzed with ANOVA and significant differences in treatment means were detected with LSD at $P = 0.05$.

Activation of *PR1-a* promoter. GUS activity was measured in leaflets of tiny tobacco plants by using a flurometric assay described by Jefferson (1987) and Park and Kloepper (2000). The assay conditions were further optimized for various growth environments and other factors including the timing of bacterial treatments. Treatments included bacterial strains EXTN-1, 89B-61 (positive control), and TE-5 (negative control) along with 0.5 mM salicylic acid (positive control) and sterile water (negative control). Strain 89B-61 previously reported as the PR-1a inducer, whereas strain TE-5 did not induced the expression of *PR-1a* (Park and Kloepper, 2000). Four replications of each treatment were used, and GUS activity was determined 7 days after bacterial treatments.

Ten mg of plant tissue from each replication of all treatments were removed from the cell culture plates and ground in an Eppendorf tube with 20 m*l* of extraction buffer (Jefferson, 1987). Extracts were centrifuged twice at 8,000 × g for 5 min. at 4°C, and 20 μl of the resulting supernatant were incubated with 20 m*l* of 2 mM 4-methylumbeliferyl-β-D-glucuronide (MUG) at 37°C for an hour. 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, U.S.A.). GUS activity was expressed as nM of MU/10mg of sample/hour.

Data were analyzed with ANOVA in SAS JMP software (SAS Institute, 1995). Significant differences in treatment means on each sample date were determined using LSD at $P = 0.05$.

Total RNA isolation and RNA blot hybridization analyses. Leaves of rhizobacteria-drenched tobacco plants were harvested 0, 3, 18, and 36 hours after treatments and stored at -80°C until use. All samplings were performed in the growth chamber with 16 h light/8 h dark regime at 25°C, 80% relative humidity.

Total RNA was extracted from tobacco plant powder using the lithium chloride-precipitation methods as described by Davis and Ausubel (1989). For hybridization analyses, 15 μg of total RNA was separated electrophoretically in denaturing formaldehyde-agarose gels (8% formaldehyde, 0.5 × MOPS, 1.5% agarose) and blotted into Hybond-N^+ membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) by capillary transfer, as described by Sambrook *et al.* (1989). Equal sample loading was confirmed by ethidium bromide staining of the rRNA in the gel.

RNA gel blots were hybridized and washed as described previously (Kim *et al.*, 2001) and exposed to an X-ray film (AGFA-Gevaert N. V., Germany). DNA probes were labeled with (α-^{32}P) dCTP by random primer labeling (Boeringer-Mannheim, Germany). Probes were prepared by polymerase chain reaction (PCR) with M13 primer (5'-GGAAACAGCTATGACCATG-3') and reverse primer (5'-GTAAAACGACGGCCAG-3'). Probe DNAs used in Northern blot hybridization analyses were PCR products of PR protein gene (*PR-1a*), phenylalanine ammonia-lyase (*PAL*) gene, ascorbate peroxidase (*APX*) gene, and 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*; 1,031 bp) gene (Kang *et al.*, 1998; Park *et al.*, 1999).

Results

Selection of plant growth-promoting rhizobacteria. Among more than 800 strains of rhizobacteria recovered from various rhizosphere, a systemic resistance-inducing strain, EXTN-1 was selected on the basis of disease inhibition in the greenhouse tests (Fig. 1). EXTN-1 was identified as *Bacillus amyloliquefaciens* by MIDI Shorlock system.

Protection against *C. orbiculare*. In greenhouse test, treatment with strains EXTN-1 and 89B-61 resulted in significant reductions against anthracnose disease compared to the water treatment (Table 1 and Fig. 1). In contrast, the bacterial strains TE-5 and HB101, which were used as the negative control, had no significant effect on disease control. Salicylic acid as a positive control also significantly reduced disease severity (Table 1).

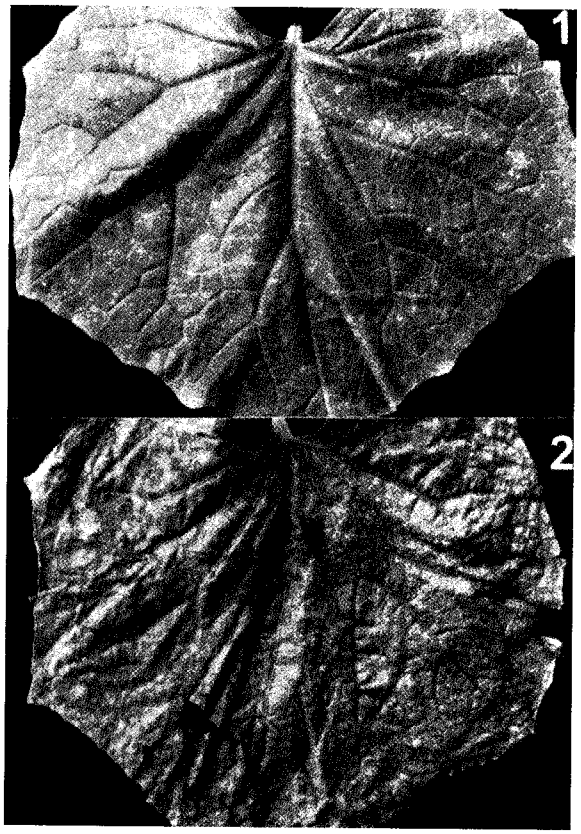

Fig. 1. Protection of cucumber plants against *Colletotrichum orbiculare* by *Bacillus amyloliquefaciens* strain EXTN-1. 1. EXTN-1 treatment, 2. Control.

Table 1. Protection against anthracnose disease caused by *Colletotrichum orbiculare* on cucumber plants by the treatment with PGPR strains and salicyclic acid

Treatments[b]	Disease severity (% of lesion area)[a]		
	Trial 1	Trial 2	Combined data
Water control	41.0[c]	46.0	43.5
Salicylic acid (0.5 mM)	2.3*	0.2*	1.3*
TE-5	25.0	50.5	37.7
HB-101	23.3	44.4	33.9
89B-61	19.6	6.0*	12.8
EXTN-1	4.3*	6.8*	5.6*
$LSD_{0.05}$	11.1	11.9	11.5

[a]Disease severity was measured 7 days after pathogen inoculation by recording the percentage of leaf area covered with lesions on each of two leaves per replication.
[b]Drenching with *E. coli* strain HB101 and TE-5 were negative controls because both bacterial strains did not induce systemic resistance. Application with salicylic acid and 89B-61 were positive controls because both induced systemic resistance on plant (see Materials and Methods).
[c]The experiment was carried out a randomized complete block with twelve replications of single plant per treatment. Data are combined from two trials after confirming homogeneity of variances with Bartlett's test. The treatments were applied as root drenches with bacteria and spraying with salicylic acid of 3-week-old cucumber plants seven days before challenge inoculation with the pathogen.
*Indicates significant reduction in disease severity compared to that of the water control at $P = 0.05$.

Activation of *PR-1a* promoter. GUS assay indicated

Table 2. Induction of β-glucuronidase (GUS) activity in tobacco transformed with *PR-1a::GUS* construct

Treatments[b]	GUS activity (nM MU[c]/10 mg fresh weight/hour)
Water control	1,050
Salicylic acid (0.5 mM)	8,610*
TE-5	3,870
EXTN-1	7,890*
89B-61	10,270*
$LSD_{0.05}$	3,685

[a]The experimental design was a randomized complete block with four replications per treatment in the microtitre plate.
[b]Drenching with TE-5 was negative control because the bacterial strain did not induce systemic resistance. Application with salicylic acid and 89B-61 were positive controls because both induced *PR-1a* transcript accumulation (see Materials and Methods).
[c]MU, 4-methylumbeliferone.
*Indicates significant increase in GUS activity compared to that of the water control at $P = 0.05$.

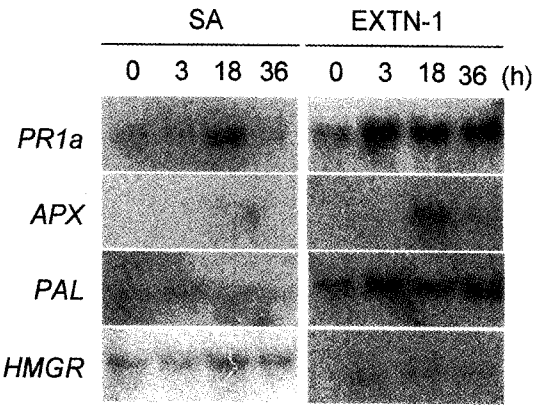

Fig. 2. RNA blot analysis of the expression of the induced systemic resistance (ISR) response gene *PR-1a* in Xanti nc of tobacco plants. ISR was induced in tobacco plant by dipping the plants in 1 mM salicylic acid and culture of plant growth-promoting rhizobacteria strain EXTN-1. *PR-1a*, *APX*, *PAL*, and *HMGR* genes were used as the probes.

that PR-1a accumulation was significantly enhanced in the EXTN-1- and 0.5 mM salicylic acid-treated tobacco compared to those of water and TE-5 treated one (Table 2). Among the tested bacterial strains, 89B-61 induced the highest overall level of GUS activity 7 days after treatment (Table 2).

Expression of defense-related genes. Northern blot analyses were carried out to investigate effect of EXTN-1 on the expressions of defense-related genes including *PR-1a* (Fig. 2). In the salicylic acid-treated tobacco, strong expression was observed 18 hours after treatment and disappeared 36 hours after treatment. Transcript accumulations of *APX*, *PAL*, and *HMGR* were not clearly induced by the salicylic acid treatment. On the other hand, high degree of expression of *PR-1a* and *PAL* was appeared within 3 hours in the EXTN-1-treated tobacco leaves. Although *APX* was not expressed 3 hours after treatment, it was also expressed 18 hours after treatment. Moreover, expression degree of *PR-1a*, *APX*, and *PAL* of EXTN-1-treated tobacco was much more higher than those of salicylic acid-treated one. The results of gene expression showed that the EXTN-1 activated the defense genes which are important for the resistance expression. Accumulation of HMGR transcript was not observed in the EXTN-1-treated tobacco leaves.

Discussion

In greenhouse screening tests, seven promising strains were selected that significantly reduced cucumber anthracnose. The most promising strain EXTN-1 showed significant disease control efficacy against anthracnose of cucumber plant. The strain also induced *PR-1a* expression as shown in GUS assay. EXTN-1 infiltration resulted in the typical hypersensitive reaction (HR). Furthermore, Northern blot hybridization analyses evidently exhibited that EXTN-1 induced *PR-1a* expression.

The most important result presented in this study was the activation of *PR-1a* by EXTN-1 treatment through soil drenching. Induction of the *PR-1a* expression is one of the unique features of disease resistance in the incompatible interaction (Davis and Ausubel, 1989). The expression of ascorbate peroxidase (*APX*) and phenylalanine ammonia-lyase (*PAL*) gene is also known to be unique in defense responses. These results might be interpreted as follows; the first, ascorbate peroxidase and phenylalanine ammonia-lyase may play a role in the expression of EXTN-1-mediated systemic resistance. Cross-talk between (among) different defense pathways might be possible during the expression of EXTN-1-mediated systemic resistance and expression of *PR-1a*. Maurhofer *et al.* (1994) indicated that induced protection of tobacco necrosis virus by PGPR strain *P. fluorescens* CHAO was associated with the induction of multiple PR proteins. Schneider and Ullrich (1991) similarly reported that protection of tobacco against *P. syringae* pv. *tabaci*, inducing resistance by culture filtrates of a *P. fluorescense* strain, was associated with induction of chitinase, β-1,3-glucanase, peroxidase, and lysozyme. Hence, our results suggest that the induced systemic resistance by PGPR strain EXTN-1 activate *PR-1a* promoter and some defense gene at microtiter assay and that Northern blot analyses, which indicates ISR, could be linked events for PGPR. More analyses using *PDF1* and additional data about the activation of systemic resistance in the mutant lines will be needed for the explanation of EXTN-1-mediated resistance expression.

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