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Molecular Mechanism of Plant Growth Promotion and Induced Systemic Resistance to Tobacco Mosaic Virus by *Bacillus* spp.

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***Bacillus* spp., as a type of plant growth-promoting rhizobacteria (PGPR), were studied with regards promoting plant growth and inducing plant systemic resistance. The results of greenhouse experiments with tobacco plants demonstrated that treatment with the *Bacillus* spp. significantly enhanced the plant height and fresh weight, while clearly lowering the disease severity rating of the tobacco mosaic virus (TMV) at 28 days post-inoculation (dpi). The TMV accumulation in the young non-inoculated leaves was remarkably lower for all the plants treated with the *Bacillus* spp. An RT–PCR analysis of the signaling regulatory genes *Coi1* and *NPR1*, and defense genes *PR-1a* and *PR-1b*, in the tobacco treated with the *Bacillus* spp. revealed an association with enhancing the systemic resistance of tobacco to TMV. A further analysis of two expansin genes that regulate plant cell growth, *NtEXP2* and *NtEXP6*, also verified a concomitant growth promotion in the roots and leaves of the tobacco responding to the *Bacillus* spp.**

Keywords: *Bacillus* spp, PGPR, induced systemic resistance, tobacco mosaic virus, growth promotion

The introduction of plant growth-promoting rhizobacteria (PGPR) into soil or the rhizosphere to promote plant growth and elicit plant defenses has been proposed for increasing crop yields and the biological control of fungal, bacterial, and viral threats [14, 15, 32, 35]. Several PGPR-based products have recently become commercially available, and most of these products contain strains of *Bacillus* spp., which also offer solutions to formulation problems due to their heat- and desiccation-resistant spores [7].

Bacillus spp. have been found to promote plant growth and elicit ISR (induced systemic resistance) [8, 15]. The promotion of plant growth is a beneficial effect of PGPR, which increases seed emergence, plant weight, and yield [13, 14, 26]. The mechanisms involved in PGPR-mediated plant growth promotion include the bacterial synthesis of plant hormones, such as indole-3-acetic acid (IAA), cytokinin, and gibberellin; the breakdown of plant-produced ethylene by the bacterial production of 1-amino cyclopropane-1-carboxylate deaminase [8]; and an increased uptake of available minerals, nitrogen, and phosphorus in the soil [5, 9]. Furthermore, bacteria that produce volatiles, such as 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, have been shown to trigger plant growth enhancement and ISR in *Arabidopsis* [26].

Induced resistance is a state of enhanced defensive capacity in a plant stimulated by *elicitors* of *biotic* and *non-biotic* origins. This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants, and even insect herbivores [32]. The two most clearly defined forms of induced resistance are SAR (systemic acquired resistance) and ISR, which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved, as previously demonstrated in model plant systems [24]. Krause *et al.* [15] reported that *Bacillus* spp. can elicit ISR against the foliar disease caused by *Xanthomonas campestris* pv. *armoraciae*, whereas Zehnder *et al.* [35] conducted a greenhouse screen of *Bacillus* spp. for their potential to elicit ISR against the cucumber mosaic virus (CMV) in tomatoes. In addition, *Bacillus* spp. have been tested in field trials for their capacity to reduce the incidence and severity of the tomato mottle virus (ToMoV) that is transmitted by whiteflies [21].

Some interactions between plants and PGPR are able to elicit the expression of certain pathogenesis-related (PR) genes, suggesting that the systemic resistance induced by PGPR is identical with pathogen-induced SAR [33]. SAR signaling in a plant is dependent on salicylic acid (SA) and

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the regulatory protein NPR1 [31]. The enhanced defensive capacity characteristic of SAR is always associated with the accumulation of pathogenesis-related (PR) proteins [10]. However, there is currently no consensus on whether PGPR elicit ISR followed by *PR* gene expression, and most studies have been related to *Pseudomonas* spp. For example, Pieterse *et al.* [25] proposed that *P. fluorescens* WCS417r mediates ISR without the activation of PR protein genes, whereas Saravanakumara *et al.* [28] reported that the protection of tea against *Exobasidium vexans* induced by a bacterial suspension of *P. fluorescens* Pf1 was followed by the induction of chitinase, β-1,3-glucanase, polyphenol oxidase, and peroxidase. Furthermore, only a few studies have discussed whether *Bacillus* spp. elicit ISR followed by *PR* gene expression. For example, Park and Kloepper [22] reported that *Bacillus* spp. induce the *PR-1a* gene in tobacco, and that the induction of the *PR-1a* gene and PGPR-mediated induced systemic disease resistance are linked events. Meanwhile, the *NPR1* gene has been found to encode a protein that controls the expression of various sets of defense genes [1, 25], and although SAR and ISR are different, both pathways require *NPR1* induction [6, 25]. Moreover, the *Coi1* gene is the central regulatory component of the jasmonic acid (JA) signaling pathway and is required for plant fertility and defense responses [34]. Besides, pathogenic microorganism-induced SAR is independent of the JA signaling pathway, whereas PGPR-mediated ISR is dependent on JA [25].

Accordingly, whereas most previous research on PGPR as elicitors of growth promotion or ISR has focused on *Pseudomonas* spp., this study provides evidence of *Bacillus* spp. eliciting ISR to TMV in tobacco plants and the participation of the *PR*, *NPR1*, and *Coi1* genes in ISR, along with the promotion of plant growth and the involvement of two expansin genes, *NtEXP2* and *NtEXP6*.

MATERIALS AND METHODS

Plant Growth-Promoting Rhizobacteria Strains and Media

The *Bacillus* spp. used as PGPR strains in this study were *Bacillus subtilis* G1 and B3, and *B. amyloliquefaciens* FZB24 and FZB42. In addition, a Landy medium [17] was used to grow these PGPR strains.

Evaluation of Effect of PGPR on Plant Growth

To evaluate the effects of the PGPR on tobacco root growth under different environments, experiments were carried out in square Petri dishes and a greenhouse.

Square petri dish experiment. The tobacco (*Nicotiana tabacum* cv. NC89) seeds were first soaked in PGPR suspensions with a final concentration of 1×10^9 CFU per cm^3 for 12 h, and then disinfested in a 1.5% (w/v) solution of sodium hypochlorite for 10 min, washed three times with sterilized distilled water, spread out evenly on an agar medium in a square Petri dish, and finally incubated in an illuminating incubator (200 μE m^{-2} s^{-1} at 25°C) with a 16-h-day and 8-h-night cycle.

Greenhouse experiment. The PGPR strain suspensions (1×10^9 CFU/per cm^3) were mixed with peaty soil (Fertile Soil Inc., Herlongjiang Province, China) at a ratio of 1:50 (v/v). The tobacco seeds were surface sterilized, and then germinated in the amended peaty soil and incubated in a greenhouse at 25±2°C for four weeks post germination. Thereafter, each seedling was transplanted to a one-liter pot with the peaty soil and maintained in the greenhouse as described previously. Five weeks after the transplanting, the plants in the early five- to six-leaf stage were root drenched with the PGPR strain suspensions. The stem height was measured from the soil line to the shoot apex, and the fresh weight above the ground tissue determined 28 days post inoculation (dpi) with TMV.

Evaluation of Effect of PGPR on Disease Severity of Tobacco Mosaic Virus

The TMV was maintained in the tobacco (*Nicotiana tobacum*) cv. NC89 by mechanical passage in a temperature-controlled greenhouse. The TMV inoculum used throughout the experiments consisted of systemically infected NC89 tissue ground in a 50 mM potassium phosphate buffer (pH 7.0) and 10 mM sodium sulfite (1 g tissue: 10 ml buffer). All the inoculation materials were chilled at 4°C prior to the inoculation and maintained on ice during the inoculation. Prior to the inoculation, the first three leaves on each plant were lightly dusted with carborundum. To minimize the influence of an individual tobacco plant on the results, 15 plants were used for each treatment with 3 replications.

The disease severity was evaluated by visual observation and a rating scale of 0 to 4, in which 0=no symptoms observed; 1=light mottling and a few thin yellow veins; 2=mottling and vein clearing unevenly distributed on the leaf; 3=mottling, leaf distortion, and stunting; and 4=severe mottling, leaf curling, and stunting. Mock-inoculated plants were used as a control for each treatment.

Western Blot Analysis

A Western blot analysis was performed as described previously [27]. Briefly, the proteins were separated by 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore) and probed with a primary antibody TMV polyclonal antibody, and finally visualized using diaminobenzidine (DAB) (Beijing Biosynthesis Biotechnology Co., Ltd, China) after binding with a secondary antibody goat anti–rabbit IgG-HRP.

Enzyme-Linked Immunosorbent Assays (ELISAs)

ELISA analyses were carried out to measure the amount of the virus in leaf samples. Each sample was ground in a 50 mM carbonate buffer using a motorized leaf squeezer, and then loaded onto ELISA 96-well plates, coated with plastic wrap, and incubated at 4°C overnight. After washing with a washing buffer (0.5% Tween 20 in PBS), the plates were then blocked with 3% nonfat dry milk in PBS at 37°C for 1 h, and washed five times. Next, the primary antibody rabbit anti-TMV immunoglobulin (Ig) (1.0 μg/ml) (Agdia Inc., Indiana, U.S.A.) was incubated in the 96-well plates at 37°C for 1 h, and after washing the plates six times, the secondary antibody goat anti-rabbit IgG–HRP (1/10,000) was added to each well, followed by incubation at 37°C for 1 h. Finally, after washing seven times, 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide (Amersco) were used as the substrate for developing at 37°C for 15 min, and the reactions stopped by the addition of H_2SO_4. The plates were read using a Multiskan Ascent microplate photometer (Thermo, U.S.A.).

Table 1. PCR and RT–PCR primers used in this study.

Name	Sequence of primers (5'-3')
Coi1 sense primer	GGATTGACTGATTTGGCGAAGG
Coi1 anti primer	TCCCTCACTGGCTACAACTCGT
NPR1 sense primer	GATGTGTGTGTTTGTGTGGACAACGAGT
NPR1 anti primer	CCATCGGATGTCAGATCAGAAGGTCTAG
PR-1a sense primer	GTGTAGAACCTTTGACCTGGGA
PR-1a anti primer	TTCGCCTCTATAATTACCTGGA
PR-1b sense primer	CATGCCCAAAACTCTCAACAAG
PR-1b anti primer	TAGCACATCCAACACGAACCGA
NtEXP2 sense primer	GGCCAACATTGGCATTTTAG
NtEXP2 anti primer	GGGTTGGCCATTGAGATATG
NtEXP6 sense primer	CTCAATGGTGTCATGCTGGA
NtEXP6 anti primer	GCCGCTTCAGCTCTTCTACA
EF-1α sense primer	AGACCACCAAGTACTACTGCAC
EF-1α anti primer	CCACCAATCTTGTACACATCC

Reverse Transcriptional–Polymerase Chain Reaction (RT–PCR) Analysis

Using tobacco plants that had been subjected to two seed treatments with PGPR strain G1 prior to germination, followed by root drenching during the early five- to six-leaf stage, the total RNA was isolated from leaf tissue 12, 24, 48, 72, and 120 h post treatment (hpt) and from untreated controls. The total RNA was isolated using the TRIzol reagent (Invitrogen Biotechnology Co., Carlsbad, CA, U.S.A.) according to the manufacturer's recommendations, and the detection of gene expression was carried out according to methods described elsewhere [23]. The primers for the targeted genes as well as the control gene (*EF1α*) are listed in Table 1. The RT–PCR products were resolved on an agarose gel to determine the expression level of the target gene.

Data Analysis

The data were subjected to an analysis of variance using SPSS software (SPSS Inc., Illinois, U.S.A.). When a significant F test was obtained at $p=0.05$, the separation of the treatment means was accomplished by Fisher's protected LSD.

Results

Promotion of Tobacco Growth

To evaluate the effect of the PGPR strains on the promotion of tobacco growth, two strains of *B. subtilis* (strains G1 and B3) and two strains of *B. amyloliquefaciens* (strains FZB24 and FZB42) were used in this study, where strains FZB24 and FZB42, isolated from plant-pathogen-infested soil, were previously proven to promote plant growth [16].

As shown in Fig. 1, when applying the PGPR strains (1×10^9 CFU/cm^3) to just the tobacco seeds, the ensuing root growth was significantly enhanced when compared with that of the untreated control at 20 dpt. Treatment with strains G1 and FZB42 resulted in a similar increase in root growth of 40%, whereas strains B3 and FZB24 increased the root growth by 27.8% and 19.8%, respectively. When the PGPR strains were applied as a seed treatment, followed by root drenching at the early five- to six-leaf stage, the

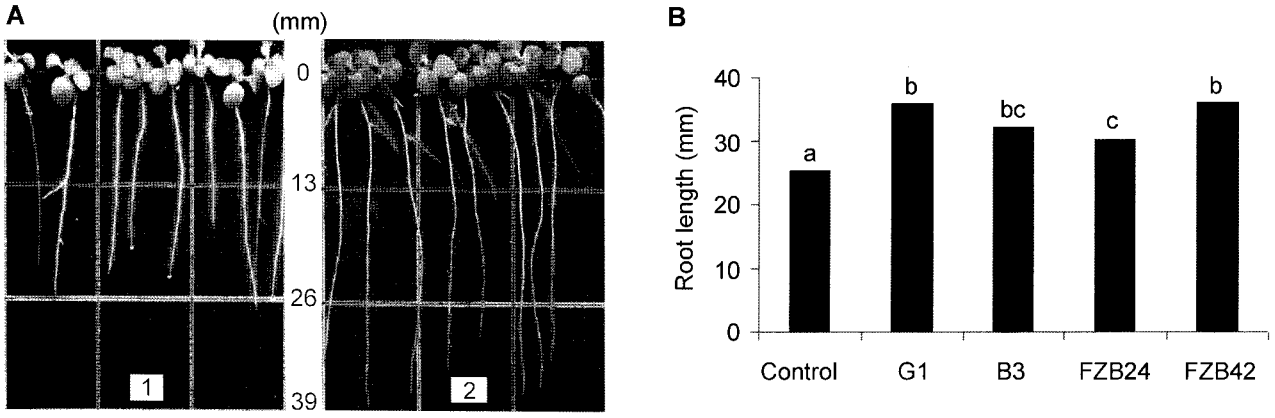

Fig. 1. Effect of PGPR strains on tobacco root growth on agar medium.
A. Growth of tobacco roots. The plants shown are representative of those grown from seeds soaked in a 1×10^9 CFU/cm^3 G1 strain cell suspension for 12 h prior to being sown on the agar medium (2) or the control (1). **B.** Quantification of root growth. Statistical comparisons were made among the treatments for each root length. The different letters indicate significant differences using Fisher's LSD test at $P=0.05$. The photographs in A were taken at 20 dpt. In B, the root length was measured at 20 dpt. This experiment was repeated three times.

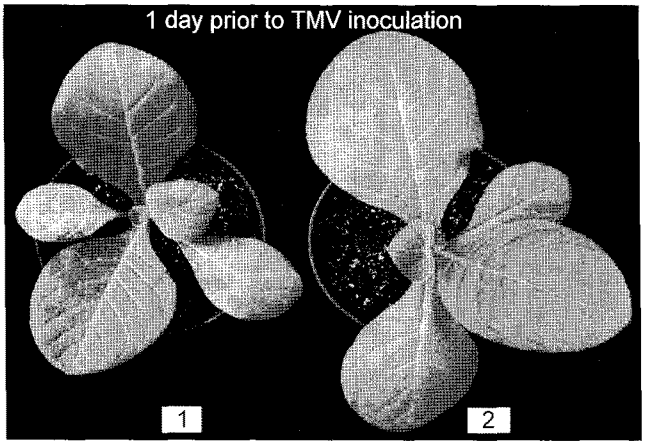

Fig. 2. Effect of *B. subtilis* G1 on tobacco plant growth in greenhouse.
1, control; 2, treated with G1 strain. The photographs were taken 1 day prior to inoculation with the tobacco mosaic virus during the early five- to six-leaf stage. This experiment was repeated three times.

plant growth rates during the initial two weeks were similar for the different strain treatments and untreated control; however, differences became obvious as the time interval increased. At the early five- to six-leaf stage, the untreated control was much smaller than the plants treated with the PGPR strains (Fig. 2).

Two growth parameters, the plant height and plant fresh weight of the aboveground tissue, were evaluated at the end of the experiment (28 dpi) to determine whether the *Bacillus* spp. strains promoted tobacco growth. When the plants were not subjected to TMV inoculation (Figs. 3A and 3B), the plants treated with the PGPR strains G1 and FZB42 were clearly taller than the untreated control plants by 24.1% and 14.2%, respectively, whereas treatment with B3 and FZB24 did not significantly enhance the plant height (Fig. 3A). The PGPR treatments also had a positive effect on the plant fresh weight, as the fresh weights of the plants treated with G1, B3, FZB24, and FZB42 were 86.3%, 38.0%, 17.5%, and 45.1% higher, respectively (Fig. 3B). As such, the G1 strain was the most effective as regards increasing the height and fresh weight of the plants. Meanwhile, when the plants were challenge-inoculated with TMV, the two growth parameters also showed differences between the PGPR treatments and the control (Figs. 3C

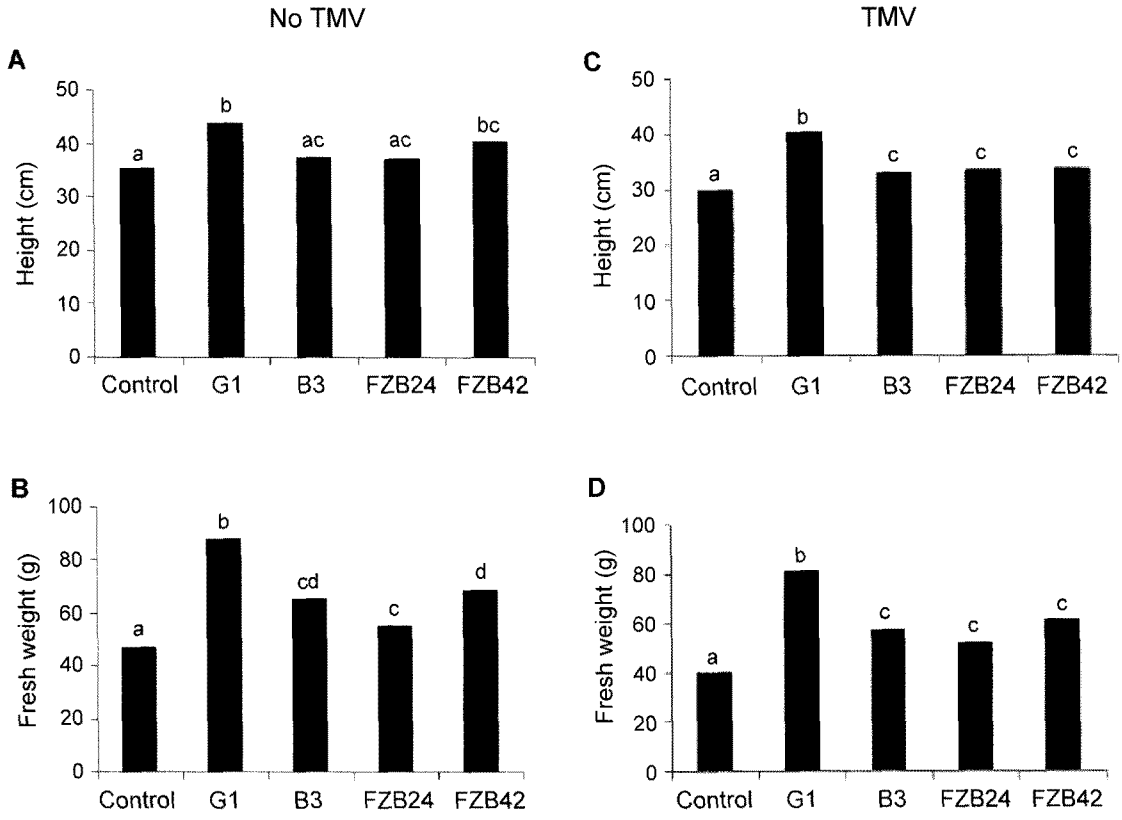

Fig. 3. Mean plant growth characteristics determined for mock-inoculated (no tobacco mosaic virus [TMV]; **A** and **B**) and TMV-inoculated plants **C** and **D**.
For **A** and **C**, the growth characteristic was the stem height (measured from the soil line to the shoot apex), which was determined at 28 dpi. For **B** and **D**, the growth characteristic was the fresh weight of the aboveground tissue, which was determined at 28 dpi. The treatments are listed on the x axis and described in the experimental procedures. Statistical comparisons among the control and PGPR strain treatments were made within a single growth characteristic and the TMV-inoculated or mock-inoculated plants. The different letters indicate significant differences using Fisher's LSD test at p=0.05.

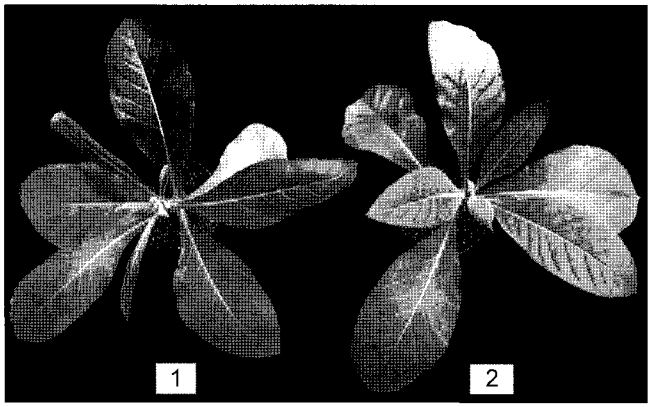

Fig. 4. Induced system resistance against tobacco mosaic virus (TMV) by *Bacillus subtilis* G1 in greenhouse.
1: Control; 2: treatment with G1 strain. The photograph was taken at 28 dpi.

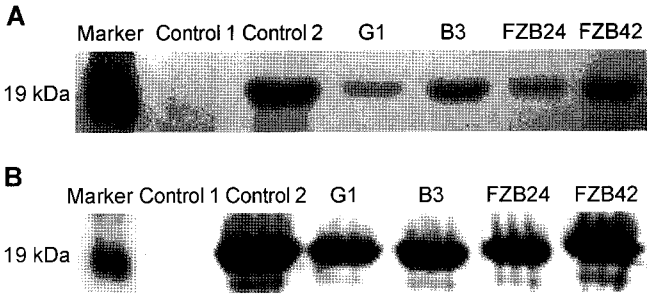

Fig. 5. Detection of tobacco mosaic virus coat protein in tobacco treated with different PGPR strains at 28 dpi.
A. SDS–PAGE analysis. **B.** Western blot analysis. Control 1: tobacco without TMV inoculation. Control 2: tobacco inoculated with TMV, and treated with water. G1, B3, FZB24, and FZB42 represent tobacco treated with these strains and inoculated with TMV.

and 3D). When compared with the untreated control, the treated plants were 35.7%, 11.1%, 12.8%, and 13.5% taller in response to the G1, B3, FZB24, and FZB42 strains, respectively (Fig. 3C). Similarly, the mean plant fresh weight for the aboveground tissue was significantly greater for all the PGPR treatments when compared with the control, at 102.2%, 44.1.0%, 33.2%, and 53.1% in response to the G1, B3, FZB24, and FZB42 strains, respectively (Fig 3D). Again, the G1 strain was the most effective in enhancing the height and weight of the plants infected with TMV.

Induced Systematic Resistance in Tobacco Against TMV

The plant symptoms and virus accumulation were used as the key parameters to evaluate the severity of the viral disease. To analyze the PGPR-elicited ISR against TMV, the severity of the TMV disease was rated based on the tobacco phenotype and amount of TMV accumulated in response to the PGPR strain treatments.

Observation of the plant symptoms indicated that the initial signs of vein clearing and mosaic occurred in the control at 7 dpi, whereas the plants treated with the PGPR strains were symptomless at that time. The occurrence of mosaic in the PGPR-treated plants was delayed by 7 days when compared with that in the control. All the PGPR treatments reduced the disease severity based on the symptoms at 28 dpi (Fig. 4 and Table 2). Furthermore, the effect of treatment with G1 and FZB24 was greater.

The amount of accumulated TMV was determined by detecting the viral coat protein through a Western blot and ELISA using an anti-TMV coat protein antibody.

The TMV accumulation was measured in the young, non-inoculated leaves at 28 dpi. The TMV coat protein accumulation in the tobacco plants exhibited a major band at around 20 kDa in the SDS–PAGE analysis. The amount of this protein was significantly lower in the PGPR-treated plants than in the control (Fig. 5A). The results of a Western blot analysis also confirmed that the protein accumulated in the tobacco plants was the TMV coat protein (Fig. 5B). Among all the PGPR treatments, the plants treated with G1 and FZB24 contained a lower amount of the TMV coat protein than the plants treated with B3 and FZB42. For a quantitative measurement of the amount of the TMV coat protein in the tobacco plants, an ELISA analysis was performed, and the mean ELISA value for the samples collected from the control was clearly higher than that for

Table 2. Response of tobacco treated with different PGPR strains to tobacco mosaic virus (TMV).

Treatments[a]	TMV disease severity[b]	TMV detection by ELISA (450 nm)[c]
Control	3.25a[d]	1.180a
G1	1.92b	0.817b
B3	2.24c	0.878c
FZB24	2.00b	0.826b
FZB42	2.08bc	0.925c

[a]PGPR were applied as seed treatment at time of planting and as root drenches 1 day prior to the TMV inoculation.
[b]Numbers represent the mean disease severity rating at 28 dpi using a 0–4 scale, as described in the experimental procedures.
[c]TMV was detected by an ELISA analysis of the non-inoculated leaves at 28 dpi. Samples were considered positive for TMV infection when the mean ELISA value was greater than the mean plus three standard deviations for comparable healthy control samples (OD_{450}=0.073).
[d]Statistical comparisons are among the treatments within a single column. The different letters indicate significant differences using Fisher's LSD test at p=0.05.

any of the PGPR-treated plants (Table 2). The TMV accumulation in the plants treated with G1 and FZB24 was significantly lower than that for the plants treated with B3 and FZB42. Thus, when taken together, the above results indicated that all the PGPR treatments reduced the severity of the TMV disease based on the plant symptoms and virus accumulation.

Expression of Growth-Related Genes

To explore the molecular mechanism of the noticeable influence of *B. subtilis* G1 as a PGPR strain in the growth promotion of the roots, height, and fresh weight, as shown in Figs. 1 and 3, the present study investigated two effectors, EXP2 and EXP6, that are tobacco expansins encoded by *NtEXP2* and *NtEXP6*, and which function to loosen cell walls and promote cell division and extension, thereby promoting plant growth mediated by ethylene [2]. The results of a RT–PCR analysis revealed that in the case of the untreated control and 12 h post-treatment (hpt), only lower basal signals were detected with no visible effects. However, treatment with the G1 strain elicited a significant expression of *NtEXP2* and *NtEXP6* from 24 to 72 hpt, which then gradually diminished (Fig. 6A), suggesting that the *B. subtilis* G1 treatment induced the expression of *NtEXP2* and *NtEXP6*. Thus, the PGPR strains may have promoted growth by enhancing the expression of these two genes.

Expression of Defense-Related Genes

Plants utilize a broad range of defense mechanisms to prevent invasion by pathogens, and SAR and ISR are two different defense mechanisms induced by pathogens or non-pathogens. Although *PR* gene expression has been suggested as a marker of SAR, there is currently no consensus as to whether PGPR induce ISR accompanied by *PR* gene expression. Thus, to test whether *Bacillus* spp. induce ISR following *PR* gene and signaling regulatory gene expression, this study analyzed the tobacco plants for the presence of several defense-related genes, including the *PR-1a* and *PR-1b* genes, which are most consistently used as an indicator of SAR [3, 4], the *NPR1* gene, a regulator in the expression of various sets of defense genes [19], and the *Coi1* gene, which positively regulates the JA-dependent ISR pathway [34]. Therefore, the total RNA was isolated from the tobacco leaf tissue at various time intervals following seed and root drench treatment with the G1 strain, and the mRNA accumulation of the defense genes examined based on an RT–PCR analysis. The RT–PCR products were further confirmed by sequencing. The RT–PCR results for the defense genes are shown in Fig. 6B. The time courses of *PR-1a*, *PR-1b*, and *Coi1* showed parallel tendencies. For the untreated control and even 12 hpt, no band or a very low constitutive expression was observed for *PR-1a*, *PR-1b*, and *Coi1*, whereas obvious bands of *NPR1* appeared during this period. Interestingly, the expression level of all the defense genes was highest at 24 or 48 hpt, and then gradually decreased. Thus, all the above results indicated that treatment with *B. subtilis* G1 had an effect on inducing the expression of the *PR* genes and other plant defense-related genes involved in the ISR pathways.

DISCUSSION

Several studies have already reported on the utility of *Bacillus* PGPR species for promoting plant growth [14, 26]. The

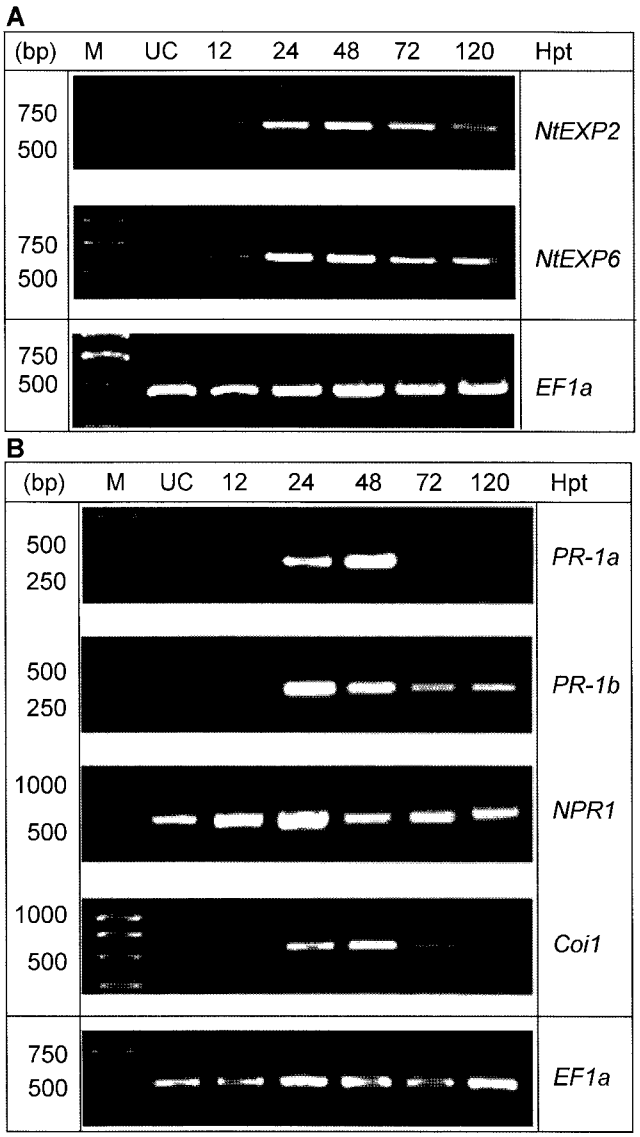

Fig. 6. RT–PCR analysis of expression of expansin genes (**A**) and defense genes (**B**) in leaves of tobacco colonized by *B. subtilis* G1 strain.

UC: untreated control. 12, 24, 48, 72, and 120 hpt represent the time after root drenching when the total RNA was isolated from the leaf tissue. The constitutively expressed *EF1a* gene was used as the standard to verify uniform gene amplification.

mechanisms of PGPR eliciting plant growth promotion include the bacterial synthesis of plant hormones and growth-promoting volatiles, as well as an increased uptake availability of phosphorus, nitrogen, or minerals in the soil [5, 8, 9, 26]. In this study, the greenhouse experimental results revealed that *B. subtilis* PGPR strains G1 and B3, and *B. amyloliquefaciens* PGPR strains FZB24 and FZB42, significantly enhanced the growth of tobacco. *B. amyloliquefaciens* FZB24 and FZB42 have previously been reported to produce indole-3-acetic acid (IAA), which is responsible for promoting plant growth [8]. It is also worth noting that, in the case of the square Petri dish experiments, where the tobacco seeds were soaked in a suspension of *Bacillus* spp. and then disinfested in a solution of sodium hypochlorite, the concentration of growth hormones, including IAA, produced by the *Bacillus* spp. during this short time was not enough to enhance the tobacco root growth. Idris *et al.* [8] also reported that the concentration of IAA produced by FZB24 and FZB42 during a 72 h period was insufficient to promote plant growth. Therefore, these results suggest that IAA was not responsible for the promoted tobacco growth in the square Petri dish. However, an RT–PCR analysis demonstrated that two expansin genes, *NtEXP2* and *NtEXP6*, induced by the *B. subtilis* G1 strain, contributed to enhancing the growth of the tobacco leaves through loosening the cell walls and promoting cell division [2]. Nonetheless, the mechanisms used by *Bacillus* spp. to stimulate plant growth remain elusive. Thus, studies on expansin gene expression during the early stage of interaction with *Bacillus* spp. may provide a new avenue for understanding the molecular mechanisms involved in enhancing plant growth and increasing yield.

Reducing disease severity by root colonizing with *Bacillus* strains is already well documented [20, 28], yet most biological control is directly aimed at soilborne plant pathogens through competing for a niche, essential nutrients, and parasitism, or by antibiosis to suppress pathogen growth [16, 29]. However, very little has been reported on the use of *Bacillus* spp. to elicit plant defenses against the TMV disease without direct contact with the pathogen. Thus, the present study provided evidence that *Bacillus* species were able to reduce the disease severity of TMV based on the visible symptoms, and decrease the virus accumulation based on an ELISA analysis (Table 2). The present results are also similar to those previously reported for ToMoV in tomatoes, where *Bacillus* strains were applied as a seed treatment and powder amendment to the planting medium [21], and consistent with the observation that *Bacillus* spp. greatly suppressed the cucumber mosaic cucumovirus in tomatoes [35].

PGPR-mediated induced systemic resistance (ISR) was initially demonstrated using *Pseudomonas* spp. and other Gram-negative bacteria, yet there has been very limited published research on ISR induction by *Bacillus* spp. ISR is normally considered to require the spatial separation of the pathogen and the inducer [12], and the current results also reflected a spatial separation between the TMV and the *Bacillus* spp., as investigation of the colonization after root drenching with the *Bacillus* spp. revealed that the bacteria remained largely localized on the root surface and lower part of the plant stem, and rarely in the leaves infected with TMV (data not shown). As such, this suggests that the reduced disease severity was most likely the consequence of ISR elicited by the PGPR *Bacillus* spp. In addition, Murphy *et al.* [20] found that the growth enhancement of tomatoes by *Bacillus* spp. resulted in disease protection against the cucumber mosaic virus (CMV), whereas Zhang *et al.* [36] reported a significant relationship between plant growth promotion and systemic protection against blue mold elicited by PGPR strain 90–166. Moreover, the current results suggest that the growth enhancement of tobacco by *Bacillus* spp. may also contribute to a reduced severity of TMV.

Specific host metabolic changes can be associated with a protective effect. In tobacco, ISR triggered by non-pathogen PGPR strains is accompanied by the expression of *PR* genes, encoding pathogenesis-related (PR) proteins, such as chitinase and β-1,3-glucanase, which are most consistently used as an indicator of SAR. Consistent with the results of Park and Kloepper [22], the present RT–PCR analysis results for *PR* genes also showed that *Bacillus* spp. induce *PR* gene expression in tobacco. In addition, Maurhofer *et al.* [18] indicated that the protection of tobacco against the tobacco necrosis virus, induced by the PGPR strain *P. fluorescens* CHA0, was associated with the induction of multiple PR proteins, including PR-1a, -1b, and -1c. Schneider and Ullrich [30] similarly reported that the protection of tobacco against *P. syringae* pv. *tabaci*, induced by culture filtrates of a *P. fluorescens* strain, was associated with the induction of chitinase, β-1,3-glucanase, peroxidase, and lysozyme. Meanwhile, in *Arabidopsis*, Pieterse *et al.* [25] proposed that *P. fluorescens* mediated ISR without any activation of PR protein genes. The specific genus or species used in these studies may explain this distinction.

The elevation of defense-related gene transcriptions has been assumed as molecular evidence of whether or not resistance is induced [11, 22]. Whereas *NPR1* is known to regulate SAR and ISR resistance pathways [19], *Coi1* positively regulates the JA-dependent ISR pathway [34]. In this study, the induced augmented expression of *NPR1* and *Coi1* was consistent with an increased resistance in the processed tobacco, indicating that the use of PGPR plays a role in inducing plant defenses, thereby providing additional persuasive evidence that disease protection by PGPR is based on plant-mediated ISR.

In conclusion, treatment with *Bacillus* spp. was shown to produce changes in the plant physiology and gene

expression, indicating that *Bacillus* spp. may cause a series of physiological and biochemical changes leading to increased resistance to pathogens or the stimulation of plant growth. Additionally, the present results indicated that the application of PGPR led to ISR towards viral infection and enhanced plant growth.

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REFERENCES

1. Cao, H., J. Glazebrook, J. D. Clarke, S. Volko, and X. Dong. 1997. The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88:** 57–63.
2. Choi, D., Y. Lee, H. T. Cho, and H. Kende. 2003. Regulation of expansin gene expression affects growth and development in transgenic rice plants. *Plant Cell* **15:** 1386–1398.
3. Clarke, J. D., N. Aarts, B. J. Feys, X. Dong, and J. E. Parker. 2000. Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in *Arabidopsis*. *Plant Cell* **12:** 2175–2190.
4. Dangl, J. L. and J. D. Jones. 2001. Plant pathogens and integrated defense responses to infection. *Nature* **411:** 826–833.
5. Dobbelaere, S., J. Vanderleyden, and Y. Okon. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Crit. Rev. Plant Sci.* **22:** 107–149.
6. Dong, X. 1998. SA, JA, ethylene, and disease resistance in plants. *Curr. Opin. Plant Biol.* **1:** 316–323.
7. Emmert, E. A. B. and J. Handelsman. 1999. Biocontrol of plant disease: A Gram-positive perspective. *FEMS Microbiol. Lett.* **171:** 1–9.
8. Idris, E. E. S., H. Bochow, H. Ross, and R. Borriss. 2004. Use of *Bacillus subtilis* as biocontrol agent. Phytohormone-like action of culture filtrates prepared from plant growth-promoting *Bacillus amyloliquefaciens* FZB24, FZB42, FZB45 and *Bacillus subtilis* FZB37. *J. Plant Dis. Prot.* **111:** 583–597.
9. Idris, E. E. S., O. Makarewicz, A. Farouk, K. Rosner, R. Greiner, H. Bochow, T. Richter, and R. Borriss. 2002. Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. *Microbiology* **148:** 2097–2109.
10. Kessmann, H., T. Staub, J. Ligon, M. Oostendorp, and J. Ryals. 1994. Activation of systemic acquired disease resistance in plants. *Eur. J. Plant Pathol.* **100:** 359–369.
11. Kim, S. T., S. G. Kim, D. H. Hwang, S. Y. Kang, H. J. Kim, B. H. Lee, J. J. Lee, and K. Y. Kang. 2004. Proteomic analysis of pathogen-responsive proteins from rice leaves induced by rice blast fungus, *Magnaporthe grisea*. *Proteomics* **4:** 3569–3578.
12. Kloepper, J. W., C. M. Ryu, and S. Zhang. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94:** 1259–1266.
13. Kloepper, J. W., F. M. Scher, M. Laliberte, and B. Tipping. 1986. Emergence-promoting bacteria: Description and implications for agriculture, pp. 155–164. *In* T. R. Swinburne (ed.). Iron, Siderphores and Plant Diseases. Plenum, New York.
14. Kloepper, J. W., M. S. Reddy, D. S. Kenney, C. Vavrina, N. Kokalis-Burelle, and N. Martinez-Ochoa. 2004. Theory and applications of rhizobacteria for transplant production and yield enhancement. Proc. XXVI IHC-Transplant Production and Stand Establishment. S. Nicola, J. Nowak, and C. S. Vavrina (eds.). *Acta Hortic.* **631:** 219–229.
15. Krause, M. S., T. J. J. De Ceuster, S. M. Tiquia, F. C. Michel, Jr. L. V. Madden, and H. A. J. Hoitink. 2003. Isolation and characterization of rhizobacteria from composts that suppress the severity of bacterial leaf spot of radish. *Phytopathology* **93:** 1292–1300.
16. Krebs, B., B. Hoding, S. M. Kubart, A. Workie, H. Junge, G. Schmiedeknecht, R. Grosch, H. Bochow, and M. Hevesi. 1998. Use of *Bacillus subtilis* as biocontrol agent. 1. Activities and characterization of *Bacillus subtilis* strains. *J. Plant Dis. Prot.* **105:** 181–197.
17. Landy, M., G. H. Warren, S. B. Roseman, and L. G. Colio. 1948. Bacillomycin, an antibiotic from *Bacillus subtilis* active against pathogenic fungi. *Proc. Soc. Exp. Biol. Med.* **67:** 539–541.
18. Maurhofer, M., C. Hase, P. Meuwly, J. P. Metraux, and G. Defago. 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: Influence of the *gacA* gene and of pyoverdine production. *Phytopathology* **84:** 139–146.
19. Mou, Z., W. Fan, and X. Dong. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113:** 815–826.
20. Murphy, J. F., M. S. Reddy, C. M. Ryu, J. W. Kloepper, and R. Li. 2003. Rhizobacteria-mediated growth promotion of tomato leads to protection against cucumber mosaic virus. *Phytopathology* **93:** 1301–1307.
21. Murphy, J. F., G. W. Zehnder, D. J. Schuster, E. J. Sikora, J. E. Polston, and J. W. Kloepper. 2000. Plant growth-promoting rhizobacterial mediated protection in tomato against tomato mottle virus. *Plant Dis.* **84:** 779–784.
22. Park, K. S. and J. W. Kloepper. 2000. Activation of PR-1a promoter by rhizobacteria that induce systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. *Biol. Control* **18:** 2–9.
23. Peng, J. L., H. S. Dong, H. P. Dong, T. P. Delaney, B. M. Bonasera, and S. V. Beer. 2003. Harpin-elicited hypersensitive cell death and pathogen resistance requires the *NDR1* and *EDS1* genes. *Physiol. Mol. Plant Pathol.* **62:** 317–326.
24. Pieterse, C. M. J., S. C. M. van Wees, E. Hoffland, J. A. van Pelt, and L. C. van Loon. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* **8:** 1225–1237.
25. Pieterse, C. M. J., S. C. M. van Wees, J. A. van Pelt, M. L. R. Knoester, H. Gerrits, P. J. Weisbeek, and L. C. van Loon. 1998.

A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* **10:** 1571–1580.

26. Ryu, C. M., M. A. Farag, C. H. Hu, M. S. Reddy, H. X. Wei, P. W. Pare, and J. W. Kloepper. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* **100:** 4927–4932.
27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
28. Saravanakumara, D., V. Charles, N. Kumarb, and R. Samiyappan. 2007. PGPR-induced defense responses in the tea plant against blister blight disease. *Crop Prot.* **26:** 556–565.
29. Schmiedeknecht, G., H. Bochow, and H. Junge. 1998. Use of *Bacillus subtilis* as biocontrol agent. II. Biological control of potato disease. *J. Plant Dis. Prot.* **105:** 376–386.
30. Schneider, S. and W. R. Ullrich. 1991. Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. *Physiol. Mol. Plant Pathol.* **45:** 291–301.
31. Sticher, L., B. Mauch-Mani, and J. P. Metraux. 1997. Systemic acquired resistance. *Annu. Rev. Phytopathol.* **35:** 235–270.
32. Van Loon, L. C., P. A. H. M. Bakker, and M. J. Pieterse. 1998. Systemic induced resistance by rhizosphere bacteria. *Annu. Rev. Phytopathol.* **36:** 453–483.
33. Wang, Y., Y. Ohara, H. Nakayashiki, Y. Tosa, and S. Mayama. 2005. Microarray analysis of the gene expression profile induced by the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5 in *Arabidopsis*. *Mol. Plant Microbe Interact.* **18:** 385–396.
34. Xie, D. X., B. F. Feys, S. James, M. Nieto-Rostro, and J. G. Turner. 1998. *Coi1*: An *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science* **280:** 1091–1094.
35. Zehnder, G. W., C. Yao, J. F. Murphy, E. R. Sikora, and J. W. Kloepper. 2000. Induction of resistance in tomato against cucumber mosaic cucumovirus by plant growth-promoting rhizobacteria. *Biocontrol* **45:** 127–137.
36. Zhang, S., M. S. Reddy, and J. W. Kloepper. 2004. Tobacco growth enhancement and blue mold disease protection by rhizobacteria: Relationship between plant growth promotion and systemic disease protection by PGPR strain 90–166. *Plant Soil* **262:** 277–288.