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RESEARCH ARTICLE

Physiological and proteomic analysis of plant growth enhancement by the rhizobacteria *Bacillus* sp. JS

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Abstract In this study, the effects of the plant growth-promoting rhizobacterium (PGPR), *Bacillus* sp. JS on the growth of tobacco (*Nicotiana tabacum* 'Xanthi') and lettuce (*Lactuca sativa* 'Crispa'), were evaluated by comparing various growth parameters between plants treated with the bacterium and those exposed to water or nutrient broth as control. In both tobacco and lettuce, fresh weight and length of shoots were increased upon exposure to *Bacillus* sp. JS. To explain the overall de novo expression of plant proteins by bacterial volatiles, two-dimensional gel electrophoresis was performed on samples from PGPR-treated tobacco plants. Our results showed that chlorophyll a/b binding proteins were significantly up-regulated, and total chlorophyll content was also increased. Our findings indicate the potential benefits of using *Bacillus* sp. JS as a growth-promoting factor in agricultural practice, and highlight the need for further research to explore these benefits.

Keywords 2-DE analysis · *Bacillus* sp. JS · Chlorophyll a/b binding protein · Chlorophyll content · Plant growth-promoting rhizobacteria

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Introduction

Plant growth may be influenced by a number of abiotic and biotic factors, including some soil microorganisms. The soil and root-colonizing bacteria that promote plant growth are called plant growth-promoting rhizobacteria (PGPR). Since the term PGPR was first used by Kloepper and coworkers (Kloepper and Schroth 1978), numerous studies have addressed PGPR-driven improvements in plant growth. PGPR are thought to enhance plant growth by (1) increasing the nutrient availability to plants, (2) producing plant growth regulators, and (3) enabling plants to withstand biotic and abiotic stresses.

Leguminous plants obtain their nitrogen (N) requirements through N-fixation by rhizobia, such as *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium*, which reside in their root nodules (Vessey 2003). However, non-leguminous crops depend on atmospheric nitrogen that is fixed and made available by PGPR (Kennedy and Tchan 1992). Soil fertility, including the available soil nitrogen, is one of the most important factors affecting plant growth, development, and crop yield. *Azospirillum* in maize (Garcia de Salamone et al. 1996), rice (Malik et al. 1997), and wheat (Boddey et al. 1986), *Burkholderia* sp. in rice (Baldani et al. 2000), *Gluconacetobacter diazotrophicus* in sugarcane (Boddey et al. 2001; Sevilla et al. 2001), and *Herbaspirillum* sp. in sorghum and rice (James et al. 1997, 2002) are documented PGPR species that improve the growth of crop plants through nitrogen fixation. PGPR may, therefore, be considered as biofertilizers in that they are microorganisms, which colonize the rhizosphere and promote plant growth (Vessey 2003).

By playing the role of an elicitor and by triggering the induced systemic resistance (Ryu et al. 2004), PGPR may enable plants to endure biotic stresses (Raj et al. 2003; Guo

et al. 2004). Drought/salt stress is a common challenge in crop production (Hu and Schmidhalter 2005). Some studies have shown that PGPR confer tolerance to stress, particularly to abiotic stresses, such as drought, salt stress, and extreme temperatures (Yang et al. 2009). For example, Mayak et al. (2004) reported that *Achromobacter piechaudii* ARV8 increased the fresh and dry weights of both tomato (*Lycopersicum esculentum* Mill 'F144') and pepper (*Capsicum annuum* L. 'Maor') seedlings under transient water stress. The mechanism suggested for this positive effect was that the production of ACC deaminase by PGPR causes the degradation of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), which in turn, prevents the formation of ethylene (Khan et al. 2009); high ethylene concentrations are known to inhibit plant growth and development (Davies 2004). In addition, wheat (*Triticum aestivum*) inoculated with *Azospirillum brasilense* Sp245 showed enhanced lateral root growth, root hair development, higher water content, and greater grain yield than did the non-inoculated plants under water stress; this effect could be the result of nitric oxide production by *Azospirillum brasilense* Sp245 (Creus et al. 2004, 2005).

The repeated physical and chemical treatment of soil is known to reduce its fertility and is environmentally unsustainable. In contrast, the use of PGPR that confer growth benefits to plants may be a more sustainable approach over the long term. Among the PGPR studied in recent years, *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a were reported to promote *Arabidopsis* growth via the production of bacterial volatile 2,3-butanediol (Ryu et al. 2003). Moreover, *Bacillus megaterium* XTBG34 promoted the growth in *Arabidopsis* by emitting 2-pentylfuran (Zou et al. 2010).

A noble bacterial strain was characterized and designated as *Bacillus* sp. JS in previous research (Song et al. 2012). The aim of this study was to determine the growth-promoting effects of the volatiles of *Bacillus* sp. JS on plants and to elucidate the mechanistic underpinnings of these effects.

Materials and methods

Bacterial culture

Bacillus sp. JS, *Escherichia coli* DH5α, *Pseudomonas syringae* pv. *tomato, Xanthomonas campestris* pv. *vesicatoria, B. megaterium, P. putida, B. cereus* BS101, and *B. cereus* BS107 were streaked on nutrient agar medium (NA, Difro, Detroit, USA) and cultured at 28 °C.

A single bacterial colony was transferred from NA to 30 mL of nutrient broth (NB, Difro, USA) and grown in a reciprocating shaker (110 rpm) at 28 °C for 14 h. The bacterial concentration was adjusted to 1×10^7 colony-forming units (CFU)/mL for the *in vitro* test, and to 1×10^8 CFU/mL for the field test.

In vitro plant growth promotion

Seeds of *Nicotiana tabacum* 'Xanthi' were sterilized by treating with 70% ethanol for 30 s, 1% sodium hypochlorite for 2 min, and were finally rinsed four times with sterile distilled water. A divided Petri dish test was performed in which ten tobacco seeds were placed on one side of the divided Petri dish (100 × 15 mm) containing half strength Murashige–Skoog's (MS) medium with 1.5% sucrose. After 7 days, 50 µL of *Bacillus* sp. JS suspension was added onto the side of the divided Petri dish with no tobacco seeds. In the vertical plate test, seeds were placed on the upper side of a square plate (120 × 120 mm) containing half strength MS medium with 1.5% sucrose. After 7 days, 50 µL each of *Bacillus* sp. JS, *E. coli* DH5α, *P. syringae* pv. *tomato, X. campestris* pv. *vesicatoria, B. megaterium, P. putida, B. cereus* BS101, and *B. cereus* BS107 suspensions were spread on the lower side of the plate. The square plate was kept vertically and incubated under 16-h light/8-h dark conditions, at 26 °C. For growth evaluation, 30 seedlings were randomly collected from three or four plates at 5 and 10 DAT (days after treatment) for the vertical test and after 8 days for the divided plate test. The data were calculated by analysis of variance (ANOVA), with means separated by Tukey's HSD test at $p < 0.01$.

Growth response of lettuce

Lettuce seeds were sown in 50-plug trays and grown for 10 days. The 10-day-old lettuce seedlings were then placed in a top-open container (with a 5-cm gap from the bottom) containing 250 mL of the bacterial culture diluted with 750 mL dH_2O. The bacterial suspension was changed every 5 days. The containers with one-half strength NB, instead of bacterial suspension, were used as controls. Fifty lettuce plants were randomly selected from three plug trays. The fresh weight of shoots was measured at 25 and 35 DAT (i.e., 35- and 45-days-old shoots). These shoot-weight data were compared using the independent-samples *t* test at $p < 0.01$.

Determination of total chlorophyll content

Seven tobacco seedlings were grown on half-strength MS media containing 1.5% sucrose on one-half of the divided plates. These plates were exposed to 16-h light/8-h dark cycles at 26 °C for 7 days, after which, 50 µL of *Bacillus* sp. JS suspension (at a concentration of 1×10^7 CFU/mL) was dropped onto the other side of the divided Petri dish. Deionized water was used, instead of the bacterial suspension, as a control. Seven days after bacterial inoculation, 0.5 g

tissue from 20 tobacco seedlings was ground in liquid nitrogen using a mortar and pestle. Thereafter, 50 mL of acetone was added to the sample, and the mixture was centrifuged at 1500× *g* for 5 min. The absorbance of the supernatant was measured at 645 and 663 nm using a DU 730 UV–Vis spectrophotometer (Beckman, U.S.A.). The total chlorophyll content of the sample was calculated based on the method of Arnon et al. (1949) using the formula:

$$\text{Chlorophyll concentration (mg g}^{-1}\text{ FW)} = (20.29 \times A_{645}) + (8.02 \times A_{663}) \times \text{volume of acetone (mL)/fresh weight (mg)}$$

where A_{645} and A_{663} are the absorbance of the sample at 645 and 663 nm, respectively.

Protein extraction and 2-DE analyses

To determine the change in the expression of proteins in the seedlings caused by the volatiles of *Bacillus* sp. JS, two-dimensional gel electrophoresis (2-DE) was performed with three biological replicates. Tobacco seeds were placed on one side of the divided Petri dishes (100 × 15 mm) containing half strength MS medium with 1.5% sucrose. After 7 days, 50 µL of *Bacillus* sp. JS suspension was added to the side of the divided Petri dish with no tobacco seeds. The inoculated plates were incubated under 16-h light/8-h dark conditions at 26 °C. Nutrient broth was used as a control. The seedling samples were collected 96 h after *Bacillus* sp. JS treatment, and were stored at − 80 °C. The protein was extracted using Mg/NP-40 buffer, consisting of 0.5 M Tris–HCl (pH 8.3), 2% v/v Nonidet P-40 (NP-40), 20 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% w/v polyvinyl polypyrrolidone, following the method described by Kim et al. (2004), and was subsequently fractionated with PEG 4000. The isoelectric focusing (IEF) gel mixture consisted of 4.5% w/v acrylamide solution, 9.5 M urea, 2% v/v NP-40, and 2.5% v/v pharmalytes (pH 3–10:5–8:4–6.5 = 1:3.5:2.5; Amersham Biosciences, CA, USA). Each 150-µg protein sample was loaded onto an IEF gel (18-cm tube gel). In the second dimension, SDS–PAGE was conducted following the method of Laemmli (1970), using 12% polyacrylamide gels. The 2-DE gels were CBB-stained in 0.2% w/v Coomassie R-250, 50% v/v methanol, and 10% v/v glacial acetic acid followed by destaining in 10% v/v glycerol and 50% v/v methanol. PDQuest software (Version 6.2.1; Bio-Rad, CA, USA), recommended by the supplier, was used for the analysis of gel image. The intensities of the induced protein spots from the samples were recorded as digital images, using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer; Bio-Rad).

The spots were picked from the gel, washed with 50% v/v acetonitrile in 0.1 M NH_4HCO_3, and vacuum-dried. The gel fragments were reduced for 45 min at 55 °C in a solution of 10 mM DTT in 0.1M NH_4HCO_3. After cooling, the DTT solution was immediately replaced with a solution of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. After washing in a solution of 50% acetonitrile in 0.1M NH_4HCO_3, the dried gel pieces were made to swell in a minimum volume of 10 mL digestion buffer containing 25 mM NH_4HCO_3 and 12.5 ng/µL trypsin (sequencing grade; Promega, Madison, USA), and incubated at 37 °C overnight. The trypsin-digested peptides were extracted following the method of Kim et al. (2003).

All the samples were finally analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). The parent ion masses were measured in the reflectron/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000%, a guide wire voltage of 0.010%, and a delay time of 150 ns. A two-point internal standard for calibration was used with des-Arg1-Bradykinin (m/z 904.4681) and angiotensin 1 (m/z 1296.6853). The peptides were selected in the mass range of 500–3000 Da. For data processing, the software package PerSeptive-Grams was used. The database searches were performed using Protein Prospector (http://prospector.ucsf.edu).

Results

In vitro plant growth promotion

The results of the divided plate experiment, in which the effect of *Bacillus* sp. JS on tobacco was investigated, showed approximately 90% increase in plant growth when tobacco seeds were treated with the PGPR for 8 days over that in the seeds treated with the control (Fig. 1). The divided plate has a central partition through which only volatile materials can move. Therefore, any effect of the bacteria on the plant seedlings would imply that effects are mediated by the volatiles. This positive effect was likely related to the volatile chemicals emitted by *Bacillus* sp. JS.

The assessment of growth performance in the vertical square plates showed that when compared to the various microorganisms used by us, *Bacillus* sp. JS had greater overall positive effects on the plant growth (Fig. 2). In contrast to the *B. megaterium* and *B. cereus* BS101 treatments where significantly lower plant growth was observed than in the control, the *Bacillus* sp. JS-treated plants showed significant growth promotion effects after 10 days of treatment (Fig. 2). The treatment with *Pseudomonas putida* also induced root growth but it had no effect on the fresh weight of shoots as compared to that in the control treatment.

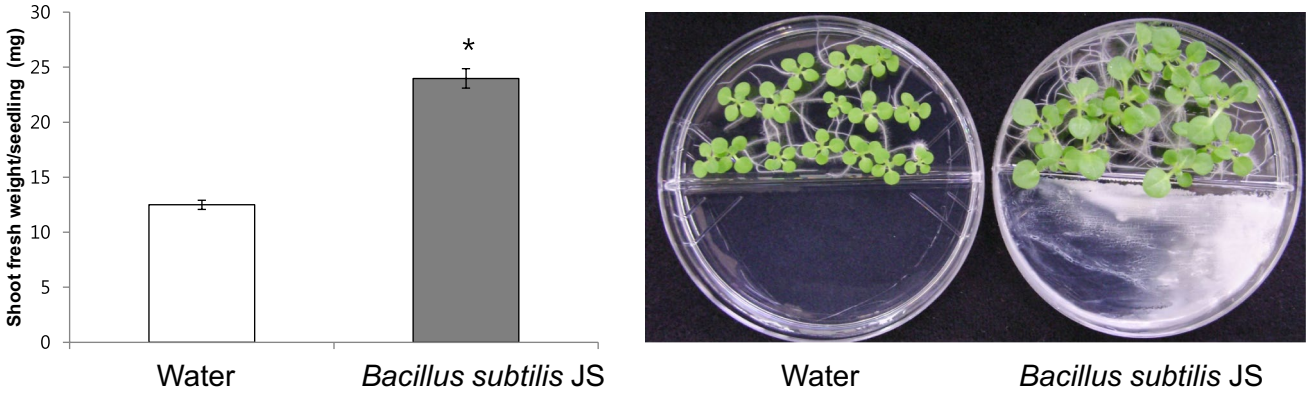

Fig. 1 Growth promotion in tobacco upon exposure to the volatiles of *Bacillus* sp. JS. The asterisk indicates a significant difference based on independent samples *t* test at $p < 0.01$. Data represent the average of three replicates of eight seedlings

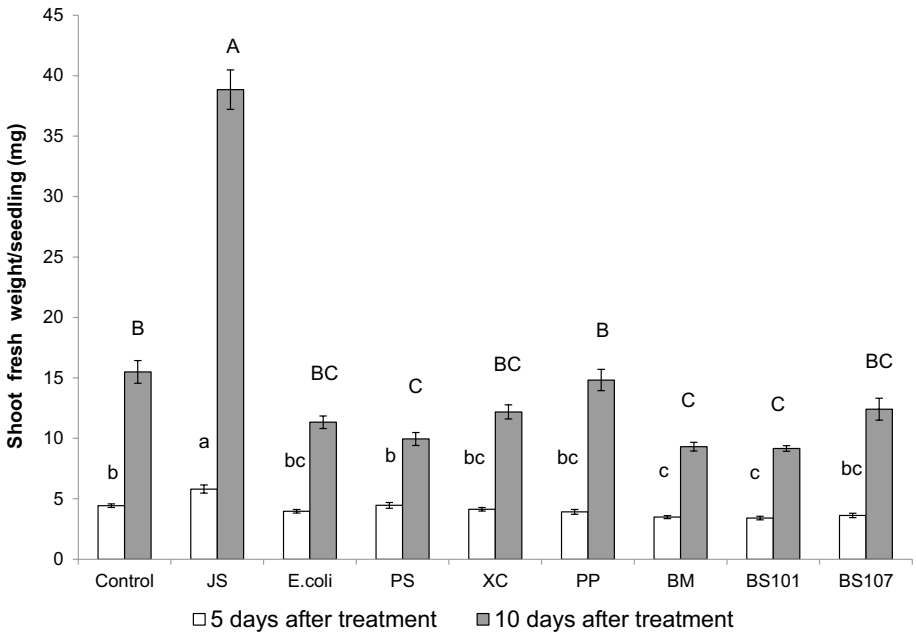

Fig. 2 Growth promotion of tobacco seedlings in response to *Bacillus* sp. JS treatment. Control: water, JS: *Bacillus* sp. JS, *E. coli: Escherichia coli* DH5α, PS: *Pseudomonas syringae* pv. *tomato* DC3000, XC: *Xanthomonas campestris* pv. *vesicatoria*, PP: *Pseudomonas putida*, BM: *Bacillus megaterium*, BS101: *Bacillus cereus* strains BS101, BS107: *Bacillus cereus* strains BS107. Different letters of the alphabet denote significant difference between the factors (according to Tukey's HSD at $p < 0.01$). Each data point represents the mean ± standard error

Growth enhancement in lettuce

To evaluate the growth promoting effect of the volatiles of *Bacillus* sp. JS in field, lettuce seedlings in open top plug tray were employed. The results showed that the shoot weight of lettuce plants treated with *Bacillus* sp. JS was significantly higher (by approximately 78% at 25 DAT by 31% at 35 DAT) than that of plants treated with the control (Fig. 3).

Alteration of protein and chloroplast density in *Bacillus* sp. JS treated samples

In the 2-DE gel analysis, we detected nine differential spots (Fig. 4), among which spot number 5, corresponding to hairpin binding protein 1, showed decreased intensity (Table 1). The protein group with increased expression consisted of elongation factors TuA and TuB, chloroplast ferredoxin-NADP reductase, chloroplast chlorophyll A-B binding protein 40, chloroplast chlorophyll a/b binding protein cab-BO3-1, mannose-6-phosphate isomerase class

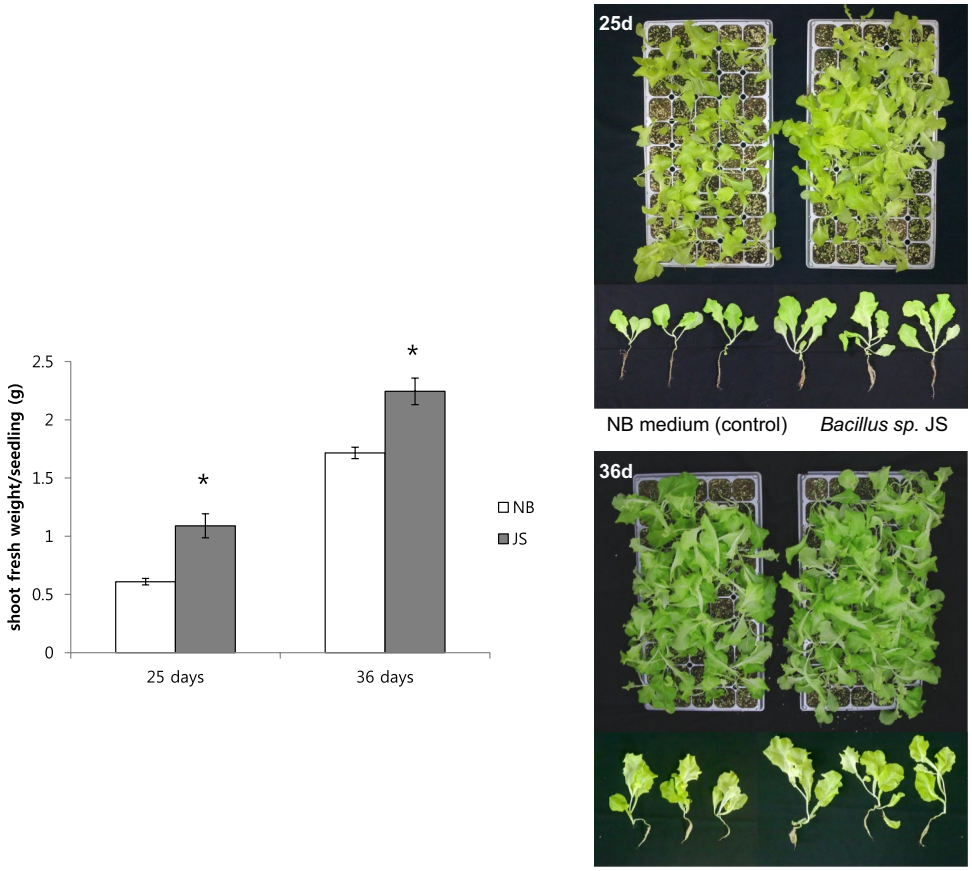

Fig. 3 Quantification of growth promotion in lettuce induced by exposure to volatiles of *Bacillus* sp. JS in the open-plug tray field trial. *Bacillus* sp. JS suspension was administered at the bottom and lettuce were kept afloat. Each data point represents the mean ± standard error, and asterisks denote significant differences based on the independent samples *t* test ($p < 0.01$)

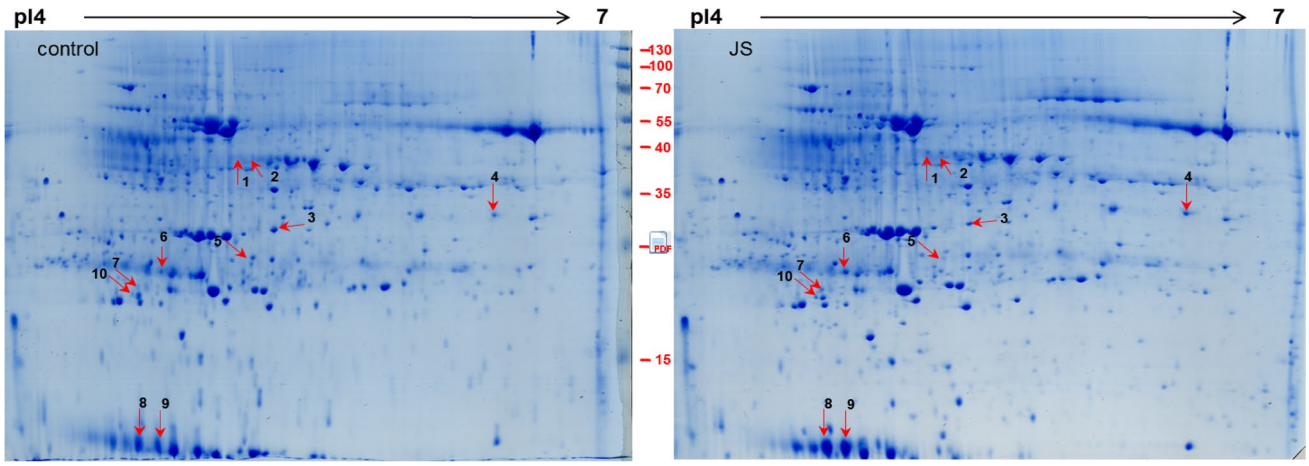

Fig. 4 Two-dimensional gel electrophoretic (2-DE) analysis of samples from tobacco plants treated with bacterial volatiles. C means the representative 2-DE images of control, JS represent the same for tobacco plants treated with bacteria

I, and small chain of ribulose-bisphosphate carboxylase. The chlorophyll a/b binding protein in particular, which is known to increase during development and with light exposure, was increased by about two-fold (Table 1).

Discussion

The genome sequencing of *Bacillus* sp. JS was recently completed (Song et al. 2012). The sequence data of *Bacillus* sp. JS revealed that the *Bacillus* sp. JS genome consists of a single circular chromosome of 4,120,406 bp with 43.9% GC content, 4240 protein-coding sequences, 10 rRNA

Table 1 Details of differentially expressed proteins identified after bacterial volatile exposure of tobacco plants

No.[a]	Protein	Accession number[b]	Nominal mass (Mr)	pI value	SC[c] (%)	Fold change[d]
1	Elongation factor TuA, chloroplastic	Q40450	52,152	6.34	32	+2.50
2	Elongation factor TuB, chloroplastic	Q43364	52,769	5.95	28	+2.03
3	Chloroplast ferredoxin-NADP + reductase	A4UTS5	40,392	8.57	28	+1.39
4	Harpin binding protein 1	Q5QJB2	30,208	8.8	43	−1.56
5	Chloroplast chlorophyll A–B binding protein 40	Q677D0	22,651	9.75	21	+1.52
6	Mannose-6-phosphate isomerase, class I	F1T7R5	39,300	6.9	24	+3.53
7	Ribulosebisphosphate carboxylase small chain	Q84QE5	20,496	7.57	48	+1.10
8	Ribulosebisphosphate carboxylase small chain, chloroplastic	P69250	20,526	7.6	62	+1.36
9	Chloroplast chlorophyll a/b binding protein cab-BO3-1	B7SAW4	28,790	5.25	30	+2.08

[a]Numbers correspond to the 2-DE gels shown in Fig. 4

[b]Accession number from EMBL database

[c]Sequence coverage

[d]'+' up-regulation; '−' down-regulation after *Bacillus* sp. JS volatiles exposure

operons, and 8669 tRNAs. In addition, the *Bacillus* sp. JS genome was found to be similar to those of *B. subtilis* 168 and BSn5 genomes, with a 70 ANIb value of 95.31% (Song et al. 2012). The PGPR bacterium used in the subsequent experiments in this study was designated as *Bacillus* sp. JS.

The genome sequence has been deposited in GenBank under accession no. CP003492. This study revealed that the volatiles of *Bacillus* sp. JS enhance the growth in several plants, including tobacco, lettuce, and *Arabidopsis*. We further aimed to determine the mechanisms of plant growth promotion by *Bacillus* sp. JS. In the divided plate experiment, JS remarkably increased the fresh weight of the shoots of tobacco seedlings. This observation suggested that the plant growth promoting effect of *Bacillus* sp. JS is due to the volatiles produced by *Bacillus* sp. JS. The number of lateral root and root hairs and the primary root length of tobacco seedlings were also increased by the treatment with *Bacillus* sp. JS (data not shown). Among the mechanisms that might explain these effects is the production of plant hormones by the PGPR, which stimulate cell division and elongation (Davies 2004).

To understand the mechanism of growth promotion by *Bacillus* sp. JS volatiles, 2-DE analyses were carried out. Eight proteins were up-regulated and one was down-regulated upon treatment with *Bacillus* sp. JS volatiles. The photosynthesis pathway-related proteins, such as chloroplast chlorophyll A–B binding protein 40 and chloroplast chlorophyll a/b binding protein cab-BO3-1 were up-regulated. The increased chlorophyll A–B binding protein was demonstrated to correlate with the up-regulation of its mRNA in an earlier SSH analysis (Kim et al. 2015). The level of mRNA has been shown to parallel the accumulation of chlorophyll in *Pinus palustris* seedlings (Peer et al. 1996). The comparison of chlorophyll content in tobacco seedlings treated with *Bacillus* sp. JS volatiles and those that were not-treated revealed an approximately 60% increase in the chlorophyll content in the PGPR-treated samples (Fig. 5). The positive effect of *Bacillus* sp. JS volatiles on the chlorophyll content, reported here, supports the notion that one of the pathways by which PGPR exerts growth-promoting effects is the increase of chlorophyll content in plants.

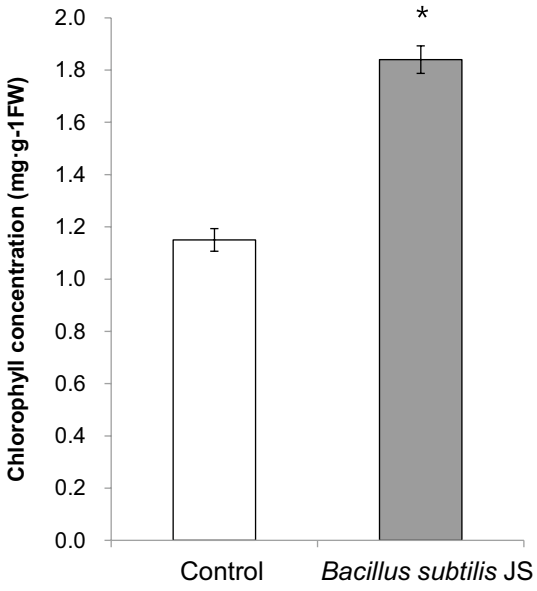

Fig. 5 Chlorophyll concentration in tobacco plants treated with bacterial volatiles. The representative samples were taken 14 days after the bacterial inoculation. The data points show mean ± standard error, and asterisk denotes significant differences based on the independent samples *t* test ($p < 0.01$)

The PGPR-induced growth enhancement seen in lettuce seedlings, as evidenced by the increase in fresh weight of

the shoots of seedlings, highlights the potential of *Bacillus* sp. JS application in increasing the yields of lettuce in commercial cultivation. PGPR have previously been shown to exert beneficial effects on plant development; for example, the application of OSU-142 and M3 stimulated the yield and quality parameters of sugar beet, barley (Cakmakci et al. 2001), raspberry (Orhan et al. 2006), and apple (Aslantas et al. 2007) in the field, via direct or indirect mechanisms. However, ours is the first report of growth promotion by PGPR in lettuce. PGPR-mediated increase in the availability of nutrients in the rhizosphere has been proposed as the mechanism by which PGPR enhance the crop yield and increase the fruit size (Bar-Ness et al. 1992; Richardson 2001). However, because in the present study, plants were adequately supplied with all the nutrients, the notion that the observed positive growth effects may be the consequence of hormone production gets credence (Gutierrez-Manero et al. 2001). The growth-promoting effects of phytohormones, produced or induced by PGPR, are thought to alter the assimilation–partitioning patterns in plants, and therefore, alter the growth and the fructification process (Cakmakci et al. 2001; Orhan et al. 2006; Aslantas et al. 2007). Our results suggest that *Bacillus* sp. JS has the potential to increase the yield of lettuce. However, additional field experiments are required to verify the consistency of these positive effects in the conventional production systems.

We report a novel finding with regard to PGPR and their role in agriculture, i.e., the potential usefulness of *Bacillus* sp. JS in increasing the crop yield in species, such as tobacco and lettuce. We assume that the volatiles of *Bacillus* sp. JS act as elicitors and activate the chlorophyll synthesis in plant growth promotion. The application of *Bacillus* sp. JS can not only enhance the plant growth in agriculturally important species but can also make agriculture environmentally safe and sustainable, thereby contributing to the redressal of problems related with the use of agrochemicals.

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Compliance with ethical standards

Conflict of interest Ji Seong Kim declares that he has no conflict of interest. Jeong Eun Lee declares that he has no conflict of interest. Hualin Nie declares that he has no conflict of interest. Yong Jae Lee declares that he has no conflict of interest. Sun Tae Kim declares that he has no conflict of interest. Sun-Hyung Kim declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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