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ORIGINAL PAPER

Contribution of indole-3-acetic acid in the plant growth promotion by the rhizospheric strain *Bacillus amyloliquefaciens* SQR9

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Abstract *Bacillus amyloliquefaciens* SQR9, isolated from the rhizosphere of cucumber, can control *Fusarium* wilt of cucumber and directly stimulate plant growth. To evaluate its potential agricultural use, the plant growth promotion of *B. amyloliquefaciens* SQR9 was evaluated, and the relative mechanisms, especially the production of the phytohormone indole-3-acetic acid (IAA), were investigated. The related plant-growth-promoting factors were genetically and chemically analyzed, and a mutant library was constructed for selecting strains with different IAA production. *B. amyloliquefaciens* SQR9 showed a growth-promoting activity in greenhouse experiments. Plant-growth-promoting factors like extracellular phytase, volatile components including acetoin, 2,3-butanediol, and phytohormone IAA were detected in *B. amyloliquefaciens* SQR9 cultures grown under laboratory conditions. Three IAA production mutant strains showed variation in plant-growth-promoting effect. IAA production in *B. amyloliquefaciens* SQR9 was related to its plant-growth-promoting effect, but IAA alone could not account for the overall observed plant-growth-promoting effect. The promoted plant growth by the rhizospheric strain *B. amyloliquefaciens* SQR9 can be attributed to multiple factors, including production of phytohormones, volatile compounds, and extracellular enzymes. Therefore, the strain *B. amyloliquefaciens* SQR9 may be used as a plant-growth-promoting agent to increase crop yield.

Keywords *Bacillus amyloliquefaciens* SQR9 · Plant-growth-promoting factors · Indole-3-acetic acid · Greenhouse experiment

Introduction

Plant-growth-promoting rhizobacteria (PGPR) are a wide range of microorganisms, colonizing the root surface and enhancing seed emergence, plant biomass, and crop yield (Kloepper et al. 1989; Frommel et al. 1991; Babalola 2010). PGPR can directly stimulate plant growth through various mechanisms, including production of phytohormones (Bottini et al. 2004; Chaiharn and Lumyong 2011; Kochar et al. 2011), increasing the availability of plant nutrients in the rhizosphere (Idriss et al. 2002; Adesemoye et al. 2008; Palacios et al. 2014), suppressing ethylene production by root cells (Penrose and Glick 2001; Madhaiyan et al. 2006), and emitting plant-growth-promoting volatile compounds (Ryu et al. 2003). Today, most of the commercial PGPR are *Bacillus* strains because they form heat- and desiccation-resistant endospores, which facilitate their survival during production and storage period (Emmert and Handelsman 1999). The use of *Bacillus* strains as a bioorganic fertilizer also depends on the production of various antibiotics (Liu et al. 2013; Wang et al. 2013). It is very likely that plant-growth-promoting effect of rhizosphere bacilli results from the complex combined action of several factors mentioned above, though little is known about the basic molecular mechanisms responsible for this beneficial action.

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Low-molecular-weight C6-volatile compounds such as methyl jasmonate, jasmonate, and terpenes, have been identified as signal molecules for plant growth (Farmer 2001; Farag and Paré 2002). Airborne chemicals released by specific PGPR can regulate auxin homeostasis are implicated in cell expansion, trigger growth promotion, and induce systemic resistance (ISR) in *Arabidopsis thaliana* seedlings (Ping and Boland 2004; Ryu et al. 2004; Farag et al. 2006; Zhang et al. 2007). Studies on *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a revealed that 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol could be the volatile chemical signals enhancing the growth of *A. thaliana* (Ryu et al. 2003, 2004).

Improving P nutrition is achievable by releasing P from insoluble inorganic form such as polyphosphates and/or phytate (myo-inositol hexakiphosphate), which accounts for 20–50 % of the total organic P in soil (Richardson et al. 2001). The possible role of microbial phytase produced by PGPR in supporting plant growth has been investigated in *B. amyloliquefaciens* FZB45, which can promote maize growth under P limiting condition (Idriss et al. 2002).

Auxin (indole-3-acetic acid (IAA)), which can promote plant growth (Idris et al. 2007; Spaepen et al. 2007), was the first hormone identified in plants. This phytohormone can change cell wall in plants, increase cell permeability with swelling of exposed plant cells, and induce the production of another hormone, ethylene (Woodward and Bartel 2005; Teale et al. 2006). IAA can be produced by 80 % of rhizosphere bacteria (Idris et al. 2004). Studies using IAA-deficient bacterial mutants showed that microbial IAA production increases lateral root length and number as well as root hair density (Barbieri and Galli 1993). By increasing the physical size of the root system, plant mineral uptake, and root exudation, it also increases bacterial colonization on plant roots. *B. amyloliquefaciens* FZB42 IAA mutant strains were less efficient in promoting plant growth, indicating that IAA and plant growth promotion are functionally related in the bacterial strain (Idris et al. 2007). However, the relative importance of IAA in the plant-growth-promoting effect of *Bacillus* is not well understood.

B. amyloliquefaciens SQR9 isolated from cucumber rhizosphere showed an efficient root colonization (Cao et al. 2011; Qiu et al. 2012). The antifungal antibiotic, bacillomycin D, is involved in the biofilm formation and root colonization by *B. amyloliquefaciens* SQR9 (Xu et al. 2013), whereas the global transcription regulator AbrB inhibited its root colonization (Weng et al. 2013). *B. amyloliquefaciens* SQR9 was an IAA production strain, and we hypothesized that IAA produced by SQR9 was important for its plant-growth-promoting activity. To determine the effects of IAA production by *B. amyloliquefaciens* SQR9 on plant growth promotion, three IAA mutant strains were selected from a random insertion mutant library, and changes in plant-growth-promoting effect of these mutants were monitored and compared to the effects of the wild-type strain in greenhouse experiments.

Materials and methods

Strains and growth conditions

B. amyloliquefaciens SQR9 (China General Microbiology Culture Collection Center, CGMCC accession No. 5808), green fluorescent protein (GFP)-labeled *B. amyloliquefaciens* SQR9 (SQR9-*gfp*) (Cao et al. 2011), and *Escherichia coli* DH5α (DH5α) were grown at 37 °C in Luria-Bertani (LB) medium, when necessary with the appropriate antibiotics (kanamycin at 10 μg ml^{-1} and erythromycin at 3 μg ml^{-1}), solidified with 1.5 % agar. Strains were stored at −80 °C in LB containing 30 % glycerol. The production of phytase was determined by fermentating *B. amyloliquefaciens* SQR9 in 500-ml Erlenmeyer flasks with 100 ml of artificial sea water (ASW) medium (Idriss et al. 2002) in a rotary shaker at 37 °C, at 200 r min^{-1}. Both acetoin and 2,3-butanediol production were determined by incubating *B. amyloliquefaciens* SQR9 cells in the LB medium containing glucose (1 % [*w/v*] final concentration) at 37 °C, at 90 r min^{-1}. For IAA production, *B. amyloliquefaciens* SQR9 was cultured in liquid Landy medium (Landy et al. 1948) with or without L-tryptophan (3 mM) at 25 °C at 90 r min^{-1} shaking in the dark.

Growth-promoting assays

Cucumber (Jinchun 4) seeds were surface-sterilized (2-min, 70 % ethanol soaking followed by a 5-min, 10 % sodium hypochlorite soaking) and rinsed (four times) with sterile distilled water, and then grown in the nursery cups. They were transplanted to pots filled with 400 g soil-less growth media (Klasmann-Deilmann Base Substrate, Recipe-No. 422, blended with sterile vermiculite, 1:1) at two true leaves age. The experiment includes four treatments: CK1, plants were treated with 5 ml of inactivated (moist heat sterilized for 20 min) *B. amyloliquefaciens* SQR9 suspensions (10^8 cells ml^{-1}); CK2, plants treated with 10 ml of inactivated *B. amyloliquefaciens* SQR9 suspensions (10^8 cells ml^{-1}); T1, plants treated with 5 ml *B. amyloliquefaciens* SQR9 suspensions (10^8 cells ml^{-1}); and T2, plants treated with 10 ml *B. amyloliquefaciens* SQR9 suspensions (10^8 cells ml^{-1}). Suspensions of *B. amyloliquefaciens* SQR9 were prepared by shaking cells for 6 h in the liquid LB medium, and then cells were collected by centrifugation for 10 min at 8000×*g* and suspended in sterile distilled water (washed twice by sterile distilled water). Each treatment was replicated 30 times, and the experimental plan included three blocks in a completely randomized design (10 plants for each block). All treatments were incubated in a greenhouse at 70 % humidity,

with 27±2 °C at day and 22±2 °C at night, natural light. Plants were irrigated regularly during the growing period, and pots were fertilized with 1 % (*w/w*) commercial fertilizer (alkali-hydrolyzed N, 6.27 %; available P, 4.71 %; available K 10.01 %). Ten randomly selected plants of each treatment were harvested after 55 days of transplanting, and plant height, root length and surface area, and shoot dry weight were measured. Data were analyzed using JMP software (SAS Institute Inc., Cary, NC).

Seeds of *A. thaliana* (ecotypes Columbia) were surface-sterilized as described above for cucumber seeds and then inoculated with *B. amyloliquefaciens* SQR9. Seeds were placed on Petri dishes containing half-strength modified Murashige and Skoog (MS) medium with 1.5 % (*w/v*) sucrose. The seeds were vernalized for 2 days at 4 °C in the dark. Then, the seedlings were placed in a growth chamber (14-h-light/10-h-dark cycles under 40-W fluorescent lights; the temperature was maintained at 25±1 °C with a 50–60 % relative humidity). After 3 days, germinated seedlings (6–10 seedlings per plate) were transferred to one side of a divided Petri dish prepared with modified MS solid medium; then, 20 μl of *B. amyloliquefaciens* SQR9 (10^8 cells ml^{-1}) in phosphorous buffer (PBS buffer, pH 7.0) and *E. coli* DH5α suspensions (10^8 cells ml^{-1}) in PBS buffer (pH 7.0) (control) or sterilized PBS (control) were spotted onto the other side. The Petri dishes were sealed with parafilm. Co-cultivation was performed for 14 days in a growth chamber as described above.

Investigation of plant-growth-promoting factors

The phytase activity of *B. amyloliquefaciens* SQR9 was measured as described by Shimizu (1992). Detection and quantification of acetoin and 2,3-butanediol produced by *B. amyloliquefaciens* SQR9 were performed by the method of Nicholson (2008). HPLC and ELISA analysis were used for determining IAA produced by *B. amyloliquefaciens* SQR9. After incubation for 65 h, the bacterial abundance of each culture was measured at 600 nm, and the cells of *B. amyloliquefaciens* SQR9 and mutant strains were separated by centrifugation (5000×*g*, 20 min, 4 °C). Next, 50 μl culture supernatant of each strain were subjected to ELISA analysis with the IAA ELISA KIT (Cloud-Clone Corp., USA), and the IAA content quantified. Supernatants were adjusted to pH 2.5 with 1.0 M HCl and extracted three times with ethyl acetate (1:3, *v/v*). The organic solvents were vacuum-dried at 37 °C and then dissolved in 3 ml methanol. The extracted samples were filtered through a 0.45-μm membrane before HPLC (1200 series, Agilent, USA) detection at 220 nm using a UV detector. Mobile phase was methanol 0.1 % acetic acid (60/40) at a flow rate of 0.4 ml min^{-1} for 20 min. IAA was determined and quantified by integrating the areas of peaks with the help of standard samples (supplementary material).

Transposon mutagenesis

A shuttle plasmid pMarA (Breton et al. 2006), which contained a *mariner*-based transposon, was used to construct a random insertion library for screening mutant strains with different IAA production. The pMarA plasmids (kindly supplied by Plant Protection College, Nanjing Agricultural University) were transformed into competent cells of *B. amyloliquefaciens* SQR9 (Cao et al. 2011) selecting for Kan^r and Em^r at 30 °C. Plasmid DNA was then extracted from the transformants, subjected to *Kpn*I digestion, and *HimarI* transposon fragments were amplified by PCR primers (oAtnpFwd and oAtnpRev, Table 1) to verify if these clones contained the original intact plasmid. A representative plasmid-containing colony was cultured overnight in liquid LB at 30 °C, then diluted (10^{-4}) and plated on LB agar plates containing kanamycin and grown at 50 °C to select transposants.

Screening of IAA production variation mutants

Bacteria were cultivated for 12 h in Landy medium without L-tryptophan, and then a 20-μl aliquot was transferred into 5 ml of fresh Landy medium supplemented with 3 mM L-tryptophan. After 48 h (at 25 °C), the abundance of bacteria cells was measured at 600 nm; then, cells were separated from culture medium by centrifugation (5000×*g*, 20 min). One hundred microliters of the supernatant were mixed with 100 μl Salkowski's reagent (0.5 M $FeCl_3$ 35 % $HClO_4$,

Table 1 Oligonucleotides used in this study

Primer and name	Sequence (5′-3′)[a]	Reference
oAtnpFwd	cccggtcaaTGGAGCAATTCGGACGATTGACAAGC	Breton et al. (2006)
oAtnpRev	cccggtcaaGTCGACGCAGATTCCGGTCTAACAAAG	Breton et al. (2006)
oIPCR1	GCTTGTAAATTCTATCATAATTG	Breton et al. (2006)
oIPCR2	AGGGAATCATTTGAAGGTTGG	Breton et al. (2006)
oIPCR3	GCATTTAATACTAGCGACGCC	Breton et al. (2006)

[a] Italic nucleotides indicate sites in the primer sequences corresponding to the attack of the restriction enzymes

2:100) in a 96-well plate. After 30 min at room temperature in the dark, the absorbance was measured at 535 nm. The concentration of IAA in the culture was determined by a standard IAA (Sigma) curve. IAA levels produced by SQR9 and its mutants were calculated by the OD_{535}/OD_{600} ratio. IAA production of mutants was also quantified by HPLC analysis and ELISA analysis as described above with the growth curve being determined by OD_{600}. Measurements were replicated three times.

The transposon insertion sites were analyzed by inverse-PCR (IPCR) as reported by Breton et al. (2006). The oIPCR1 and oIPCR2 (Table 1) primers, which face outward from the transposon sequence, were used in IPCR, whose products were purified by the quick PCR purification kit and sequenced after amplification by the oIPCR3 primer (Table 1).

Investigation of phytase, acetoin, and 2,3-butanediol produced by the mutant strains

SQR9 strains were cultured in the ASW and LB (containing 1 % glucose [*w/v*]) media for phytase, and acetoin and 2,3-butanediol productions, respectively. The detection methods were described above and shown in details in the supplementary material; the abundance of bacteria cells was continuously measured at 600 nm.

Colonization assay of *B. amyloliquefaciens* SQR9 strains

To study root colonization, green fluorescent protein (GFP)-labeled *B. amyloliquefaciens* SQR9 (Cao et al. 2011) was used instead of SQR9. Cultures of the SQR9 strains were grown in 50 ml LB media with kanamycin at 37 °C until reaching the stationary phase. The cells were washed twice in sterile PBS buffer (pH 7.0) and resuspended in PBS buffer (OD_{600}=0.5) prior to use. Axenic-prepared cucumber seedlings were incubated in bacterial suspensions for 1 h at room temperature. Then, the seedlings were transplanted to containers with 200 ml of sterile 1/2 MS medium. The plants were grown in a growth chamber at 27 °C with a 16-h-light regimen. Each treatment was replicated 10 times. After 5 days, bacteria were extracted from 0.2-g roots randomly selected from each treatment by briefly rinsing the roots in sterile water; then, roots were homogenized in 1.8 ml PBS buffer using a mortar and pestle. The homogenates were serially diluted and plated onto LB plates containing kanamycin. SQR9 strains on cucumber seedling roots were determined.

Greenhouse experiment of *B. amyloliquefaciens* SQR9 and its derived strains

Pot experiments were performed for comparing the plant growth promotion effect of the wild-type *B. amyloliquefaciens* SQR9 and its strains with different IAA production (SQR9-IAA1, SQR9-IAA2, and SQR9-IAA3). Cucumber (Jinchun 4) seeds were surface-sterilized as described above. Seedlings were treated with 10-ml suspensions (10^8 CFU ml^{-1}) of SQR9 strains, equal amounts of sterile distilled water served as control. Suspensions of SQR9 strains were prepared by shaking cells for 6 h in liquid LB media, and cells were collected by 10-min centrifugation at 5000×*g*; then, cells were suspended in sterile distilled water (washed twice by sterile distilled water). Each treatment was replicated 30 times and the experimental plan included three blocks in a completely randomized design (10 plants for each block). Pots were incubated in a greenhouse at 70 % humidity with a natural light and 27±2 °C at day and 22±2 °C at night. Plants were irrigated regularly during the growing period. Ten randomly selected plants of each treatment were harvested after 55 days. Plant height and shoot dry weight were measured. Data were analyzed using JMP software (SAS Institute Inc., Cary, NC).

Results

B. amyloliquefaciens SQR9 significantly stimulated growth of cucumber and *A. thaliana*

Equal amounts of inactivated *B. amyloliquefaciens* SQR9 cells were used as control (CK). Obvious differences among CK replicates were not observed, and this revealed that the presence of bacterial cells is not a factor of plant growth promotion (Table 2). When 5-ml *B. amyloliquefaciens* SQR9 suspensions (10^8 cells ml^{-1}) were applied to each pot (T1 treatment), the cucumber yield, shoot height, root length, and root surface area increased by 60.1, 45.7, 29.3, and 30.7 %, respectively (Table 2). When 10-ml cell suspensions (10^8 cells ml^{-1}) were applied (T2 treatment), increases amounted to 90.0, 71.6, 56.3, and 65.6 %, respectively (Table 2).

The effects of volatile chemicals produced by *B. amyloliquefaciens* SQR9 on plant growth were tested with divided Petri dishes so that only airborne signals could be transmitted between bacteria and the plant seedlings. After 14 days of incubation, *B. amyloliquefaciens* SQR9 significantly stimulated *A. thaliana* growth compared with the PBS and *E. coli* DH5α controls (Fig. 1a).

B. amyloliquefaciens SQR9 production of potentially plant-growth-promoting factors

Acetoin production by *B. amyloliquefaciens* SQR9 was low during exponential growth, increased during stationary phase, and peaked at 60 h after inoculation, reaching a concentration of 15.87 mM (Fig. 1b). Thin-layer chromatography (TLC) analysis showed that *B. amyloliquefaciens* SQR9 could

Table 2 Effect of *B. amyloliquefaciens* SQR9 on growth of cucumber transplant plugs 55 days after seeding under greenhouse conditions (n=10)

Treatment	Dry weight (g)	Height (cm)	Root length (cm)	Root surface area (cm^2)
CK1	2.44±0.24c	80.41±5.61c	957.44±43.24c	326.05±17.17c
CK2	2.57±0.16c	81.88±3.74c	1000.71±27.76c	326.12±12.23c
T1	4.01±0.28b	118.24±5.65b	1266.36±40.14b	426.18±12.61b
T2	4.76±0.19a	139.27±7.13a	1529.99±25.86a	539.90±11.45a

Different letters indicate significantly differences at P<0.05, least significant difference. CK1, seedlings inoculated with 5-ml inactivated bacterial suspensions (10^8 CFU ml^{-1}); CK2, seedlings inoculated with 10-ml inactivated bacterial suspensions (10^8 CFU ml^{-1}); T1, seedlings inoculated with 5-ml bacterial suspensions (10^8 CFU ml^{-1}); T2, seedlings inoculated with 10-ml bacterial suspensions (10^8 CFU ml^{-1})

produce 2,3-butanediol (Fig. 1c), which peaked at 22 h after inoculation (Fig. 1b). *B. amyloliquefaciens* SQR9 was incubated in phosphate-free ASW, and phytase activity increased markedly during the late exponential growth and peaked at 100 h after inoculation (0.28 U ml^{-1}) (Fig. 2).

B. amyloliquefaciens SQR9 cultured in Landy medium with or without L-tryptophan (3 mM) showed positive color reaction by using Salkowski reagent, indicating the presence of an IAA-like substance in the supernatant. Both HPLC and ELISA analysis of supernatants confirmed the production of IAA by *B. amyloliquefaciens* SQR9 (Fig. S1) and the produced IAA reached a concentration of 9.46 mg l^{-1} when L-tryptophan was added to Landy medium (Fig. 3), which was almost 3.6-fold compared to the no-L-tryptophan treatment (data not shown). This observation indicated that IAA production in *B. amyloliquefaciens* SQR9 is tryptophan dependent.

Screening of SQR9 strains differing in the IAA production

Above results showed that SQR9 is an efficient plant-growth-promoting strain producing IAA. To evaluate the contribution of IAA production to plant growth promotion, a random transposon mutant library of SQR9 was constructed to select different IAA production strains. About 3000 mutants were initially screened by colorimetric assays. Three mutants were selected, one mutant (designated as SQR9-IAA1) was almost completely deficient in IAA production, another mutant (designated as SQR9-IAA2) showed decreased IAA production, and the third mutant (designated as SQR9-IAA3) showed increased IAA production. In mutant SQR9-IAA1, SQR9-IAA2, and SQR9-IAA3 strains, the TnYLB-1 transposon was inserted at *qoxB*, *ylmD*, and *yngF* genes, respectively.

IAA production and bacterial growth were measured in culture supernatants after 65 h (Fig. 4). Both SQR9-IAA2

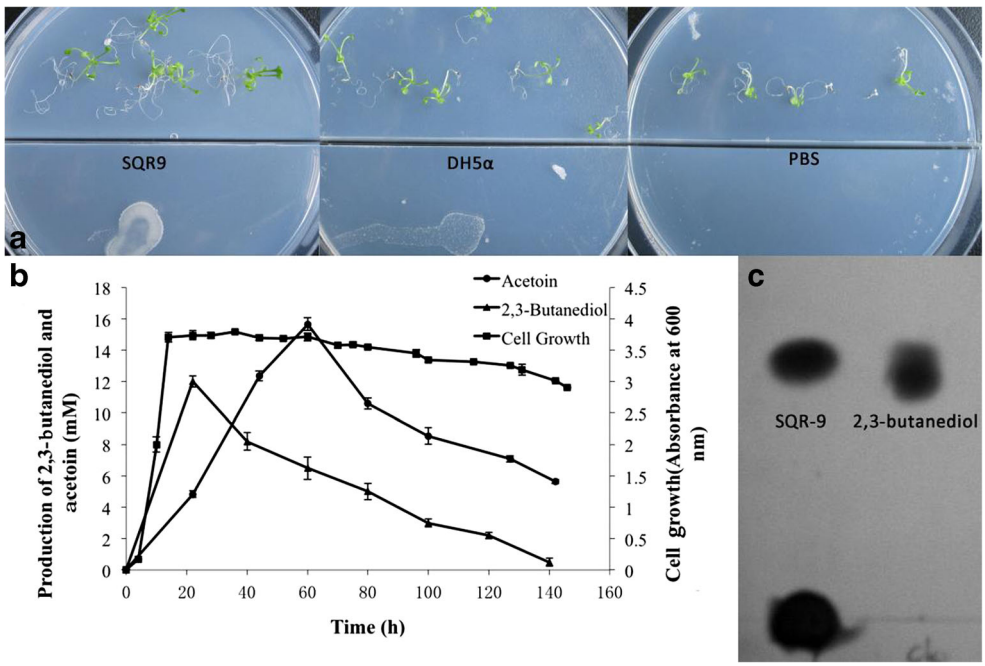

Fig. 1 Acetoin and 2,3-butanediol produced by *B. amyloliquefaciens* SQR9. **a** Growth promotion of *A. thaliana* after exposure to airborne compounds released from *B. amyloliquefaciens* SQR9 compared with a nongrowth-promoting *E. coli* strain DH5α and PBS treatments. **b** The profile of acetoin and 2,3-butanediol productions and growth curve of *B. amyloliquefaciens* SQR9. *Bars* represent standard deviations of three biological replicates. **c** TLC assay of 2,3-butanediol in culture filtrates of *B. amyloliquefaciens* SQR9 in a TLC plate after spraying with modified Seebach solution and heat treatment

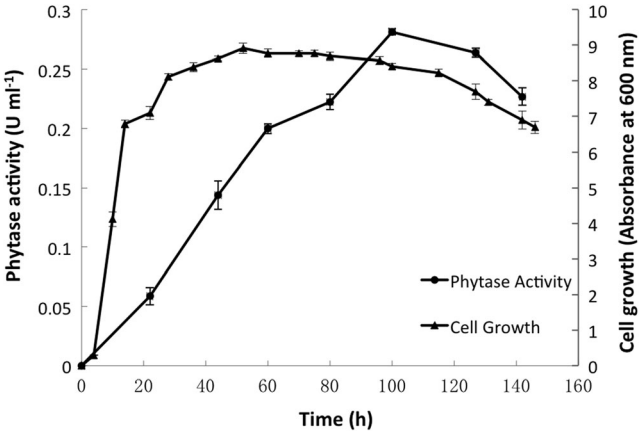

Fig. 2 Phytase activity during the growth of *B. amyloliquefaciens* SQR9 cultivated in ASW media for 6 days. The enzyme activity was assayed in Tris–HCl (pH 7.0) buffer with 10 mM $CaCl_2$ at 50 °C for 30 min. Cell growth was measured at 600 nm

and SQR9-IAA3 strains exhibited almost identical growth curves with the wild-type strain, while SQR9-IAA1 strain grew slower than the wild-type strain. Less than 10 % of the IAA produced by the wild type was detected in the culture supernatant of SQR9-IAA1 strain, and IAA production in SQR9-IAA2 strain was nearly 50 % of the wild-type strain, while in SQR9-IAA3 strain, the IAA production was nearly 240 % compared with that of the wild-type strain (Fig. 4).

Phytase, acetoin, and 2,3-butanediol produced by IAA production variation mutant strains

As these mutants could have effects unrelated to IAA synthesis, we investigated their abilities to produce phytase, acetoin, and 2,3-butanediol. The three mutants showed no difference in growth curve in ASW medium and LB medium containing glucose. In addition, phytase, acetoin, and 2,3-butanediol produced by the three mutants were not affected by the transposon mutagenesis (Fig. 5).

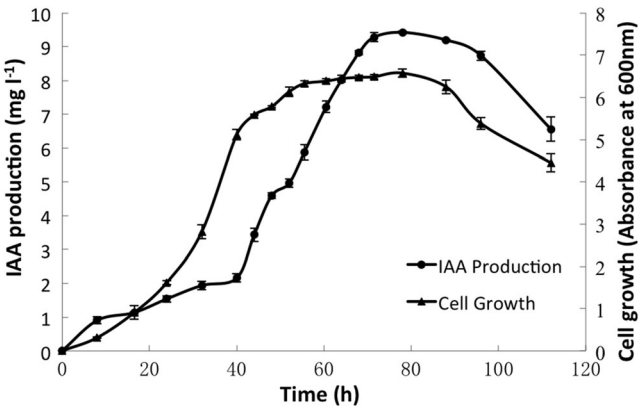

Fig. 3 Time curve of IAA synthesized by *B. amyloliquefaciens* SQR9 grown in Landy medium with 3 mM tryptophan at 25 °C and under limited aeration. Quantification of the phytohormone was conducted by HPLC. *Bars* represent standard deviations of three biological replicates

Root colonization

The population size attained by the mutant strains on the surface 5 days after inoculation was similar to that attained by the SQR9-*gfp* (about 10^6 CFU g^{-1} root) suggesting that the reduced and increased IAA production in SQR9 strains did not affect the ability to colonize cucumber seedling roots (Fig. 6).

Effect of SQR9 and IAA production on cucumber growth

All bacterial treatments showed different plant-growth-promoting effects compared with the non-treated control. Strain SQR9-IAA1, nearly deficient in IAA production, decreased the biomass weight and shoot height by 24 and 18.9 % compared to the wild-type strain (Fig. 7). SQR9-IAA3 strain, which produced approximately 240 % IAA of the wild-type strain, also significantly ($P<0.05$) promoted cucumber biomass over the other strains. Although SQR9-IAA2 strain produced about 50 % IAA of the wild-type strain, it showed no reduction in plant growth promotion compared with the wild-type strain (Fig. 7).

Discussion

B. amyloliquefaciens SQR9 is a rhizobacterium isolated from the rhizosphere of cucumber (Cao et al. 2011). We have demonstrated that *B. amyloliquefaciens* SQR9 can directly promote plant growth by various mechanisms. The prerequisite of disease suppression and plant growth promotion is the efficient colonization of plant roots by the PGPR strain (Qiu et al. 2014). Indeed *B. amyloliquefaciens* SQR9 is a root colonizer since it can maintain the population at 10^6 CFU g^{-1} root after 23 days of inoculation (Cao et al. 2011).

Genomic analysis of SQR9 (NCBI accession no. CP006890) revealed several genes involved in the plant-growth-promoting effects. A whole set of genes *alsRSD* (V529_35850, V529_35860, and V529_35870) responsible for acetoin synthesis were identified. In this cluster, *alsS* gene potentially encodes the enzyme α-acetolactate synthase, which convert pyruvate to α-acetolactate; *alsD* gene encodes α-acetolactate decarboxylase catalyzing the conversion of α-acetolactate to acetoin; *alsR* gene, which encodes a positive transcriptional regulator of *alsSD*, is located downstream of *alsSD* (Renna et al. 1993). *YwrO* was found upstream of *alsRSD* gene cluster, which encodes a putative NAD (P)-dependent oxidoreductase in *Bacillus* (Renna et al. 1993). However, the *bdhA* gene of *B. subtilis* (Nicholson 2008), which encodes 2,3-butanediol dehydrogenase responsible for the conversation of acetoin to 2,3-butanediol, was not found in

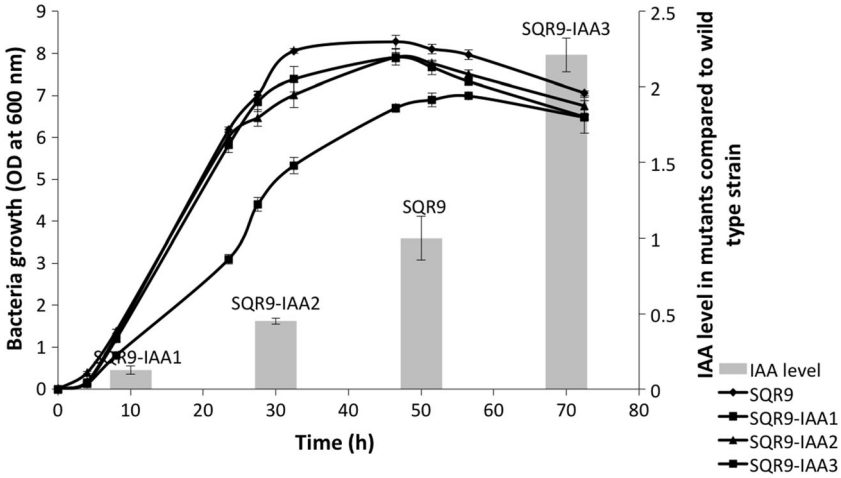

Fig. 4 Growth curve and IAA production (column diagram) of *B. amyloliquefaciens* SQR9 strains grown in Landy medium supplemented with tryptophan. IAA production was quantified at stationary phase (65 h) and the IAA concentration produced by SQR9 was defined as 1. *Bars* represent standard deviations of three biological replicates

B. amyloliquefaciens SQR9 genome. The *phy* gene (V529_21880), which encodes phytase in *Bacillus* spp. (Kerovuo et al. 1998; Kim et al. 1998; Idriss et al. 2002), was also identified in *B. amyloliquefaciens* SQR9. Moreover, acetoin, 2,3-butanediol and phytase activity were detected in the culture supernatant of *B. amyloliquefaciens* SQR9. The volatiles acetoin and 2,3-butanediol released by *Bacillus* strains can enhance plant growth (Ryu et al. 2003). Also, extracellular bacteria phytase released by *B. amyloliquefaciens* FZB strains can contribute to the plant-growth-promoting activity under P limiting condition (Idriss et al. 2002; Makarewicz et al. 2006).

Tryptophan has been postulated as a main precursor for IAA synthesis in bacteria (Spaepen et al. 2007; Duca et al. 2013); we also showed that the primary route of IAA biosynthesis in *B. amyloliquefaciens* SQR9 depends on the presence of this amino acid since lower IAA concentration was detected in the supernatant without tryptophan supply. Genes with high homology to *ysnE* (putative IAA transacetylase) and *yhcX* (putative nitrilase) in *B. amyloliquefaciens* FZB42 (Idris et al. 2007) were found in the genome of *B. amyloliquefaciens* SQR9 (V529_38080 and V529_08860), and these two genes were suggested to be involved in tryptophan-dependent IAA synthesis pathways in *B. amyloliquefaciens* FZB42. Three strains showing variation in IAA production were obtained by construction of a mutant library with plasmid pMarA. The transposon was inserted into an ORF coding for quinol oxidase polypeptide I (V529_38120) in strain SQR9-IAA1. The menaquinol oxidase (aa_3-600) catalyzes oxidation of quinols with release of energy during vegetative growth; the relation between growth rate and *qox* expression reflects the need of energy of the cell (Santana et al. 1992). In glucose minimal medium, the *qoxB* mutant strain grows slower than the *B. subtilis* wild-type strain (Santana et al. 1992), as also found by us with the Landy medium containing 20 g l^{-1} glucose and 1 g l^{-1} yeast extract. We suggest that the quinol oxidase was

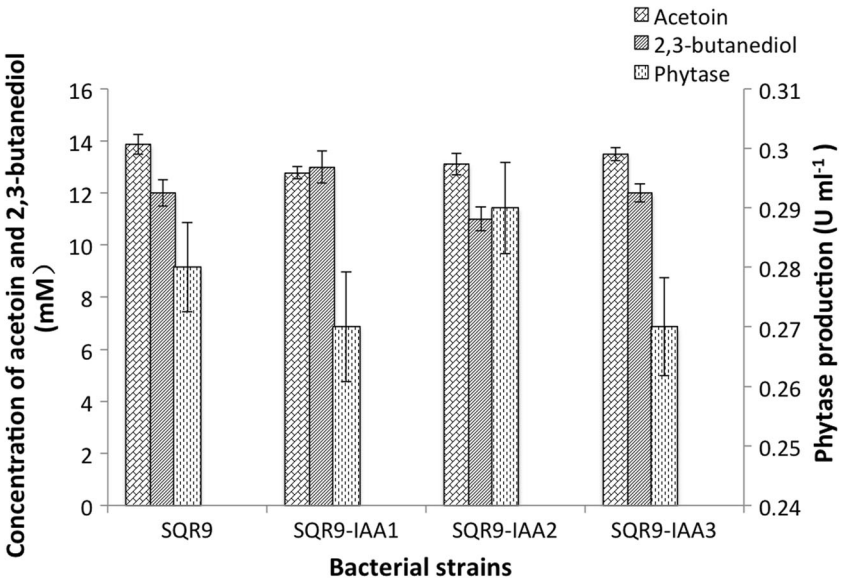

Fig. 5 Strains were cultured in the ASW medium and LB medium containing glucose (1 % [*w/v*] for phytase, acetoin, and 2,3-butanediol production). *Bars* represent standard deviations of three biological replicates

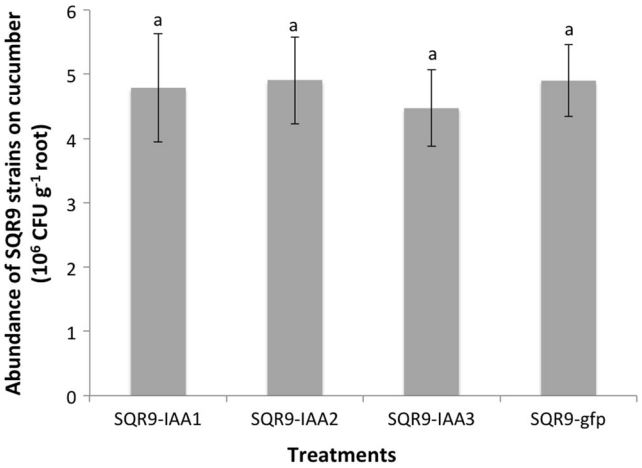

Fig. 6 Abundance of *B. amyloliquefaciens* SQR9 strains colonizing cucumber seedling roots. *Bars* represent standard deviations of three biological replicates

involved in the electron transport chains of tryptophan metabolism, since the IAA biosynthesis contains oxidation-reduction reactions like indole-3-acetaldehyde dehydrogenase converting indole-3-acetaldehyde to IAA. In strain SQR9-IAA2, the transposon was inserted into *ylmD* ORF (V529_14750), encoding the multi-copper polyphenol oxidoreductase laccase, which may be involved in cytochrom c maturation system (Kuras et al. 2007). In strain SQR9-IAA3, the transposon was inserted into *yngF* ORF (V529_18040), which is located in *yng* operon and is expected to be required for degradation of fatty acids to acetyl-CoA (Koburger et al. 2005). 3-Methylbutyryl-CoA, produced by

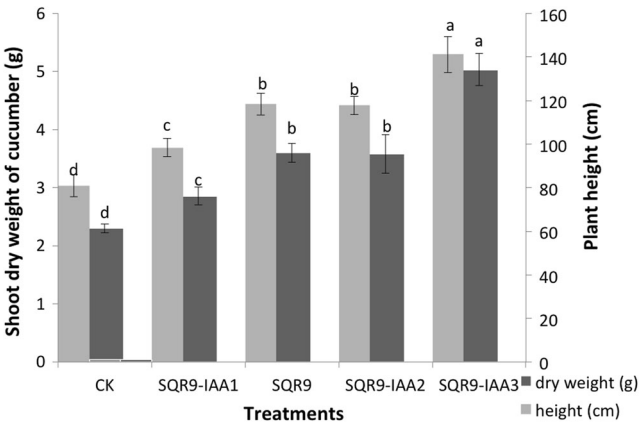

Fig. 7 Effect of *B. amyloliquefaciens* SQR9 strains on growth of cucumber transplant plugs. *Different letters* indicate significantly differences at $P<0.05$, least significant difference. CK, seedlings inoculated with 10 ml sterile distilled water; SQR9-IAA1, seedlings inoculated with 10-ml cell suspensions of SQR9-IAA1 strain (10^8 CFU ml^{-1}); SQR9, seedlings inoculated with 10-ml cell suspensions of the wild-type SQR9 (10^8 CFU ml^{-1}); SQR9-IAA2, seedlings inoculated with10-ml cell suspensions of SQR9-IAA2 strain (10^8 CFU ml^{-1}); SQR9-IAA3, seedlings inoculated with 10-ml cell suspensions of SQR9-IAA3 strain (10^8 CFU ml^{-1}). Plants were grown in greenhouse for 55 days. *Bars* represent standard deviations of three biological replicates

the oxidation of fatty acids, can be decarboxylated to acetyl-CoA and acetoacetyl-CoA by enzyme encoded by *yng* operon; this intermediate can also be produced by the degradation of branched chain amino acids (Kanehisa et al. 2002). However, the role of *qoxB*, *ylmD* and *yngF* in tryptophan metabolism and IAA synthesis in *B. amyloliquefaciens* SQR9 remains to be illustrated.

Greenhouse experiment of SQR9 strains showed that IAA production was directly related to its plant-growth-promoting effect. However, the lowest IAA production mutant SQR9-IAA1 still promoted growth of cucumber compared to the control, and SQR9-IAA2 strain, with decreased synthesis of IAA, showed similar plant growth promotion effect of the wild-type SQR9 strain. The reduced or enhanced growth promoting effects were not attributed to the root colonization since all mutants showed similar root colonization ability to the SQR9-*gfp* strain. This confirms the report by Patten and Glick (2002), who showed that the *Pseudomonas putida* GR12-2 IAA mutant strain was able to colonize canola roots as the wild-type strain, while IAA mutant strain of *Erwinia herbicola* 299R was less competitive than the wild-type strain for root colonization (Brandl and Lindow 1997). Plant-growth-promoting factors like phytase, acetoin, and 2,3-butanediol produced by the three IAA production variation mutants were not affected by the transposon mutagenesis. These results suggest that IAA production is involved in the plant-growth-promoting effect but IAA biosynthesis alone cannot account for the overall effects of *B. amyloliquefaciens* SQR9. In our study, seedlings were grown in sterile soil, and this avoided the effects of soil native microflora. PGPR can interact with other microbes in soil, especially mycorrhizal fungi, to give a net growth response (Meyer and Linderman 1986). Another review by Bashan et al. (2004) on *Azospirillum* indicated the potential role of multiple factors in plant growth enhancement including co-inoculation of *Azospirillum* with other microbial species.

In conclusion, our results indicated that in *B. amyloliquefaciens* SQR9, IAA production alone cannot account for the overall observed effects, since its stimulation of plant growth can be attributed to multiple factors, including generation of phytohormone, production of volatile compounds, and release of extracellular enzymes. Our study not only has improved our understanding of the plant growth promotion mechanisms but also has provided some indications for the isolation of plant-growth-promoting rhizosphere bacteria for agricultural application.

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