



## Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*

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### Abstract

Plant growth-promoting rhizobacteria (PGPR) colonize plant roots and exert beneficial effects on plant health and development. We are investigating the mechanisms by which PGPR elicit plant growth promotion from the viewpoint of signal transduction pathways within plants. We report here our first study to determine if well-characterized PGPR strains, which previously demonstrated growth promotion of various other plants, also enhance plant growth in *Arabidopsis thaliana*. Eight different PGPR strains, including *Bacillus subtilis* GB03, *B. amyloliquefaciens* IN937a, *B. pumilus* SE-34, *B. pumilus* T4, *B. pasteurii* C9, *Paenibacillus polymyxa* E681, *Pseudomonas fluorescens* 89B-61, and *Serratia marcescens* 90-166, were evaluated for elicitation of growth promotion of wild-type and mutant *Arabidopsis* *in vitro* and *in vivo*. *In vitro* testing on MS medium indicated that all eight PGPR strains increased foliar fresh weight of *Arabidopsis* at distances of 2, 4, and 6 cm from the site of bacterial inoculation. Among the eight strains, IN937a and GB03 inhibited growth of *Arabidopsis* plants when the bacteria were inoculated 2 cm from the plants, while they significantly increased plant growth when inoculated 6 cm from the plants, suggesting that a bacterial metabolite that diffused into the agar accounted for growth promotion with this strain. *In vivo*, eight PGPR strains promoted foliar fresh weight under greenhouse conditions 4 weeks after sowing. To define signal transduction pathways associated with growth promotion elicited by PGPR, various plant-hormone mutants of *Arabidopsis* were evaluated *in vitro* and *in vivo*. Elicitation of growth promotion by PGPR strains *in vitro* involved signaling of brassinosteroid, IAA, salicylic acid, and gibberellins. *In vivo* testing indicated that ethylene signaling was involved in growth promotion. Results suggest that elicitation of growth promotion by PGPR in *Arabidopsis* is associated with several different signal transduction pathways and that such signaling may be different for plants grown *in vitro* vs. *in vivo*.

### Introduction

Free-living, root-colonizing bacteria (rhizobacteria) have been studied for the past century as possible inoculants for increasing plant productivity (Kloepper, 1992). Several mechanisms have been postulated to explain how plant growth-promoting rhizobacteria (PGPR) stimulate plant growth. These mechanisms are broadly categorized as direct or indirect (Glick, 1995). Direct mechanisms elicit growth promotion by bacterial determinants, including production of

plant hormones such as IAA, GA, and cytokinin, and phosphate solubilization (Glick, 1995; Idriss et al., 2002). PGPR also promote plant growth by suppressing growth of plant pathogens and deleterious rhizosphere microorganisms, thus freeing the plant from growth limitations that would have resulted because of the presence of these microorganisms (Kloepper, 1992; Schippers et al., 1987). These indirect mechanisms, such as suppression of harmful microorganisms and induced systemic resistance (ISR), are normally recognized as having a role in biological control (Kloepper, 1992; Dobbelaere et al., 2003). Studies

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of mechanisms for biological control by PGPR have concentrated on how PGPR antagonize pathogens (Whipps, 2001; Antoun and Kloepper, 2001).

Most studies on mechanisms for plant growth promotion by PGPR have focused on bacterial traits without examining the host plant's physiological responses (Bloemberg and Lugtenberg 2001). *Arabidopsis thaliana* has often been chosen as a model system for understanding plant-microbe interactions because of its rapid growth rate and its extensive and well-characterized genetic background (O'Callaghan et al., 2001; Mantelin and Touraine 2004). The availability of many mutants, which impair specific steps in signal transduction pathways, makes *Arabidopsis* particularly suited for the present study. Mutants are available in hormone-response pathways, including auxin, gibberellin, cytokinin, abscisic acid, ethylene, and brassinosteroid pathways, as well as several other potential signaling pathways of plant growth promotion (McCourt, 1999). *Arabidopsis* mutants have been used to investigate the relationship between PGPR-induced signal pathways in plants and induced systemic resistance (ISR) (van Loon et al., 1998; Ryu et al., 2003a, 2004; Kloepper et al., 2004a; Kloepper and Ryu, 2004b) but have not yet been used for studies on plant growth promotion.

The objectives of this study were to determine if eight different PGPR strains, which were previously shown to elicit plant growth promotion on various crops under greenhouse and field conditions, could promote growth of *Arabidopsis thaliana* and to gain an understanding of the plant's signal transduction pathways through the use of various mutant lines of *A. thaliana*.

## Materials and methods

### *PGPR and Arabidopsis used in the experiment*

Eight different PGPR strains, *Bacillus subtilis* GB03, *B. amyloliquefaciens* IN937a, *B. pumilus* SE34, *B. pumilus* T4, *B. pasteurii* C9, *Paenibacillus polymyxa* E681, *Pseudomonas fluorescens* 89B61, and *Serratia marcescens* 90-166, were evaluated *in vitro* and *in vivo* on *A. thaliana* ecotypes Columbia-0 (Col-0) for elicitation of growth promotion. These strains have been shown to elicit plant growth promotion and ISR on several different hosts (reviewed in Kloepper et al., 2004a; Kloepper and Ryu, 2004b). For experimental use, all bacteria were streaked onto tryptic soy

agar (TSA) plates (Difco Laboratories, Detroit, MI) and incubated at 28 °C in the absence of light for 24 h. PGPR cells were harvested from TSA plates in sterile distilled water (SDW) to yield 10<sup>9</sup> colony-forming units (CFU)/mL as determined by optical density. For long-term storage bacterial cultures were maintained at -80 °C in tryptic soy broth that contained 20% glycerol.

All mutant and transgenic lines were derived from parental *A. thaliana* ecotypes Col-0 or Wassilewskija (WS), which were obtained from the Ohio State University Stock Center (Columbus, OH). Mutant lines included the following: transgenic NahG (encodes salicylate hydroxylase and degrades salicylic acid [SA]) and mutants *npr1* (lacks systemic acquired resistance [SAR] and production of SA [Cao et al., 1997]), *cpr1* (exhibits SAR and constitutive production of SA [Bowling et al., 1994] X. Dong, Duke U, Durham, NC), *ein2.5* (ethylene-insensitive [Alonso et al., 1999] J.R. Ecker, U of Penn, PA), *cbb1* (insensitive to brassinosteroid [Kauschmann et al., 1996] C. Müssig, Max-Planck-Institute Golm, Germany), *coi1* (insensitive to jasmonic acid [Xie et al., 1998] J. G. Turner, University of East Anglia, Norwich, UK), *gai2* (insensitive to gibberellic acid [Peng et al., 1997] N. P. Harberd, John Innes Centre, Norwich, UK), and *eir1-3* (insensitive to auxin [Luschnig et al., 1998]). The background of *gai2* and *eir1-3* is WS and that of the last of mutant is Col-0.

### *In vitro experiments of growth promotion on Arabidopsis by PGPR*

For *in vitro* testing, *Arabidopsis* seeds were surface-sterilized with 5% sodium hypochlorite (100% commercial laundry bleach) containing 0.1% triton X-100, rinsed three times with sterile water, and kept at 4 °C for 2 days to enhance germination. The seeds were then suspended in 0.4% low-melting-point agarose and seeded 2, 4, and 6 cm from a bacterial culture. Different distances were used because we suspected that diffusible bacterial metabolites might account for plant growth promotion and that the effect of these on plant growth would be concentration-dependent. Plants and bacteria were grown on sterile MS media (Murashige and Skoog, 1962) containing 1.5% sucrose solidified with 1% agar. The medium was adjusted to pH 5.7 prior to autoclaving at 15 psi for 25 min.

One day prior to conducting the plant experiments, we prepared the bacterial strains as described

above. Plastic Petri-dishes (100 × 15 mm) were prepared with MS solid media, and three-day-old germinated *A. thaliana* seedlings (6–10 seedlings/plate) were transferred to the plates at distances of 2, 4, and 6 cm from site of bacterial inoculation. Twenty  $\mu\text{L}$  of a given bacterial strain or SDW was applied dropwise onto the edge of each plate. The plates were then sealed with parafilm.

Following inoculation, the plates were incubated in a growth chamber supplied with 12 h of light and 12 h of dark each day. Effects on plant growth were assessed by obtaining foliar fresh weight and measuring total leaf surface area (TLSA) with an integrated digital video image analysis system (AGVISION system; AgImage Plus Version 1.08, Decagon Devices, Inc. Pullman, WA, and Panasonic CCTV camera MODEL WV-BL200, Pullman, WA). This experiment was designed as a randomized complete block (RCB) with 12 replications and one plant per replication. The experiment was repeated three times and data from all three trials were combined after confirming homogeneity of variances with Bartlett's test.

#### *Comparison between transplanting and direct seeding methods on effect of PGPR on growth of A. thaliana in vivo experiments*

To evaluate growth promotion of Arabidopsis by PGPR strains *in vivo*, we tested Arabidopsis growth under different conditions and at different ages of plant after germination. After preliminary experiments, we optimized two methods to test growth promotion by PGPR strains: 'direct seeding' and 'transplanting' methods. For the direct seeding method, 1000 mL of  $10^9$  cfu/mL PGPR suspension were added to 10 L of the potting media (Speedling Mix, Speedling Inc, Sun City, FL), and the seeds were directly seeded on the peat-based soilless potting media containing PGPR strains in plastic pots. The pots were placed in the greenhouse with natural light at 22–25 °C and 70–80% relative humidity. Plants were fertilized weekly with soluble Peter's light (20:10:20). For the transplanting method, 3-week-old Arabidopsis seedlings, grown in soilless media without bacterial treatment, were transplanted into PGPR-treated soil. The final concentration of PGPR was  $10^7$ – $10^8$  cfu/g soil. A water-treated control was included. TLSA and foliar fresh weight of plant were measured seven weeks after seeding. This experiment was designed as a randomized complete block (RCB) with 10 replications and one plant per replication. The experiment was con-

ducted twice. Significant treatment effects in the two trials were similar, so representative data from one trial are presented in the results.

#### *In vivo experiments of growth promotion on hormonal mutant lines of Arabidopsis by PGPR*

To define signal transduction pathways associated with growth promotion elicited by PGPR strains, we used Arabidopsis signaling mutants as previously described. For this experiment we used direct seeding methods. For screening mutant lines of Arabidopsis, the seeds of wild type and mutant lines of Arabidopsis were seeded in plastic trays (60 × 20 cm) containing peat-based soilless potting media. The trays were placed in the greenhouse under conditions described above. PGPR were mixed into the potting media at  $10^8$  cfu/g before seeding. A water-treated control was included. The seeds from 0.4% low melting agarose were seeded in a row with 12 Arabidopsis lines in the tray containing PGPR mixed soilless potting media. Seeding 15–20 seeds per each Arabidopsis line resulted in a stand of 10–12 seedlings. TLSA and foliar fresh weight were measured seven weeks after seeding. No symptoms of disease were present on any plants in the *in vitro* or *in vivo* assays. This experiment was designed as a randomized complete block (RCB) with three replications (trays) and 12 plants per tray. The experiment was repeated twice. Significant treatment effects in the two trials were similar, so representative data from one trial are presented in the results.

#### *Statistical analysis*

Data were subjected to analysis of variance using JMP software version 4.04 (SAS Institute Inc., Cary, NC). The effect of PGPR treatments was considered significant according to the magnitude of the *F* value ( $P = 0.05$ ). When a significant *F* test was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD).

## **Results**

#### *Effect of PGPR distance from bacterial inoculation on growth of A. thaliana on MS medium*

When Arabidopsis plants were grown *in vitro* with the eight PGPR strains, significant increases in total leaf surface area and foliar fresh weight of Arabidopsis over the control were observed with all strains at

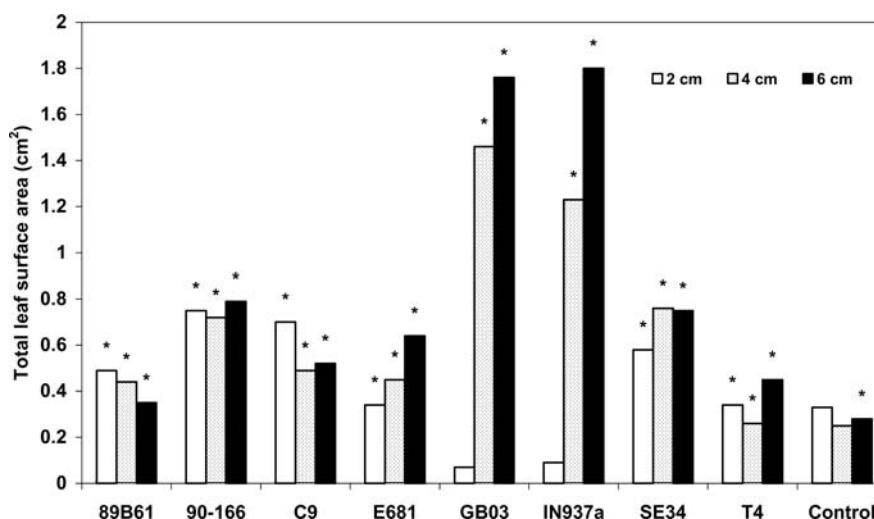


Figure 1. Effect of plant growth promoting rhizobacteria at different distances from the point of bacterial inoculation on growth of *A. thaliana* Col-0 *in vitro* on MS medium. <sup>a</sup>Bars represent means of 12 replications per treatment, with one seedling per replication.

<sup>b</sup>PGPR were inoculated at the center on MS medium. The 3-4 seeds of different lines of *A. thaliana* were placed at distances of 2, 4, and 6 cm from PGPR inoculation. PGPR were inoculated at the time of seeding.

\*Indicates significant difference from water control using Fisher's LSD test at  $P = 0.05$ .

The experiment was conducted three times. Data from the three trials were combined after confirming homogeneity of variances with Bartlett's test.

specific distances from bacterial inoculation site (Figure 1). The result of foliar fresh weight followed the same trends as those of the total leaf surface area (data not shown). Thus, we present total leaf surface area as representative data. However, the magnitude of growth promotion varied among the strains and among their distances from the bacterial inoculation site. For example, growth promotion with strain T4 was less than with other strains and was only significant when plants were located 6 cm from the bacterial inoculation site. Other strains that elicited large growth-promoting effects (e.g., GB03 and IN937a) also tended to cause maximum growth increases as distance from the bacterial inoculation site increased. PGPR strains GB03 and IN937a inhibited growth of *Arabidopsis* plants *in vitro* when the bacterium was inoculated 2.0 cm from the plants (Figure 1) but demonstrated large growth promotion at distances of 4 and 6 cm.

#### Comparison between transplanting and direct seeding methods on PGPR effect on growth of *A. thaliana*

With the direct seeding method, all PGPR strains except strain 89B61 enhanced TLSA and foliar fresh weight seven weeks after seeding compared to the control (Table 1). Strain 89B61 increased TLSA but not foliar fresh weight compared to the control. With the transplanting method, all PGPR strains except strain

T4 increased TLSA and foliar fresh weight compared to the control (Table 1).

#### *In vitro* effect of PGPR on growth of hormone mutants of *A. thaliana*

Elicitation of plant growth promotion by PGPR on *Arabidopsis* hormonal mutants varied among mutants in the *in vitro* assay. We present results of foliar fresh weight of *Arabidopsis* at 6 cm from the bacterial inoculation point because plant responses at 2 and 4 cm varied considerably (data not shown). Strain SE34 increased foliar fresh weight on two of the nine *Arabidopsis* lines. Strains E681, 90-166, T4, and C9 enhanced growth of four of the nine mutant lines compared to the appropriate wild-type. Strains 89B61 and IN937a increased plant growth of four mutant lines (Table 2). Strain GB03 did not elicit growth promotion on *cbbl*, *NahG*, and *npr1*, which are insensitive to brassinosteroids and salicylic acid. Strains SE34, 90-166, T4, IN937a, and C9 failed to enhance foliar fresh weight of WS (a wild-type for *eir1* and *gai2* mutants) (Table 2). Growth promotion of ethylene insensitive mutant *ein2* was elicited by PGPR strains 89B61, IN937a, GB03, T4, and C9. Growth promotion of *cbbl* and *NahG* lines was not elicited by any PGPR strains. Growth promotion of jasmonic acid insensitive mutant *coi1* was elicited by PGPR strains E681,

Table 1. Effect of PGPR on growth of *A. thaliana* directly seeded or transplanted into soilless growth media under greenhouse condition seven weeks after seeding

| Treatment          | Growth promotion by PGPR <sup>1</sup> |                            |                          |               |
|--------------------|---------------------------------------|----------------------------|--------------------------|---------------|
|                    | Total leaf area (cm <sup>2</sup> )    |                            | Foliar fresh weight (mg) |               |
|                    | Direct seeding                        | Transplanting <sup>2</sup> | Direct seeding           | Transplanting |
| 89B61              | 68.4*                                 | 61.6*                      | 18.5                     | 47.4*         |
| 90-166             | 77.3*                                 | 77.2*                      | 20.6*                    | 70.1*         |
| C9                 | 96.3*                                 | 64.4*                      | 26.0*                    | 50.4*         |
| E681               | 77.2*                                 | 44.6                       | 24.3*                    | 23.3          |
| GB03               | 102.7*                                | 66.9*                      | 29.3*                    | 50.7*         |
| IN937a             | 75.0*                                 | 54.8*                      | 21.5*                    | 33.9          |
| SE34               | 112.3*                                | 58.5*                      | 32.1*                    | 40.5*         |
| T4                 | 110.5*                                | 49.5                       | 31.5*                    | 27.6          |
| Control            | 43.1                                  | 37.6                       | 15.2                     | 20.0          |
| LSD ( $P = 0.05$ ) | 20.7                                  | 14.36                      | 5.06                     | 1.79          |

<sup>1</sup>The plant parameters were measured 7 weeks after seeding in the greenhouse. Numbers represent mean of 12 replications per treatment, one seedling per replication.

<sup>2</sup>PGPR were mixed in soilless medium in the 10 X10 cm square pots and 10–15 cold treated seeds of *A. thaliana* ecotype Col-0 were seeded evenly in the pots. For transplanting study, 4 two-week old seedlings were transplanted in the PGPR amended soil.

\*Indicates significant difference from water control using Fisher's LSD test at  $P = 0.05$ .

The experiment was conducted twice. Significant treatment effects were identical in the two trials. Data shown are from one trial.

Table 2. Effect of plant growth-promoting rhizobacteria on different hormone mutants of *Arabidopsis in vitro*<sup>1</sup>

| Treatment <sup>3</sup> | Fresh weight (mg) of each line at 6 cm from PGPR inoculation <sup>2</sup> |               |             |             |             |             |       |             |               |
|------------------------|---|---------------|-------------|-------------|-------------|-------------|-------|-------------|---------------|
|                        | Col-0 <sup>4</sup>  | <i>ein2.5</i> | <i>cbb1</i> | <i>coil</i> | <i>npr1</i> | <i>nahG</i> | WS    | <i>gai2</i> | <i>eir1-3</i> |
| 89B61                  | 25.8*   | 71.8*         | 14.5        | 20.3        | 4.3         | 43.3        | 18.8* | 24.6        | 21.6*         |
| 90-166                 | 28.3*   | 29.3          | 22.7        | 32.3*       | 45.3*       | 37.8        | 13.5  | 25.5        | 17.0          |
| C9                     | 18.7*   | 60.6*         | 9.0         | 66.5*       | 10.3        | 33.8        | 15.2  | 23.5        | 18.3          |
| E681                   | 19.3*   | 17.0          | 27.8        | 34.0*       | 25.0        | 37.0        | 31.0* | 16.5        | 16.0          |
| GB03                   | 45.0*   | 65.3*         | 32.3        | 58.0*       | 11.0        | 32.3        | 20.7* | 52.8*       | 23.5*         |
| IN937a                 | 71.0*   | 95.3*         | 33.2        | 23.8        | 44.0*       | 46.6        | 14.0  | 0.0         | 10.5          |
| SE34                   | 24.4*   | 36.8          | 15.8        | 15.0        | 41.0*       | 21.3        | 9.5   | 30.4        | 11.0          |
| T4                     | 37.0*   | 83.8*         | 32.0        | 22.8        | 18.5        | 34.7        | 16.2  | 42.7        | 29.3*         |
| Control                | 8.3   | 19.5          | 33.0        | 10.8        | 17.3        | 36.2        | 13.8  | 35.0        | 15.5          |
| LSD ( $P = 0.05$ )     | 8.7   | 28.6          | 6.4         | 15.8        | 13.7        | 8.3         | 3.7   | 10.0        | 5.7           |

<sup>1</sup>The assay was conducted on MS medium. Data are representative from one of two trials with similar results.

<sup>2</sup>Numbers represent means of 12 replications per treatment, one seedling per replication.

<sup>3</sup>PGPR were inoculated at the center of MS medium on Petri dish. The 3-4 seeds per plate of different lines of *A. thaliana* were placed at distances of 2, 4, and 6 cm from the PGPR inoculation point. PGPR were inoculated at the time of seeding.

<sup>4</sup>Col-0 and WS = wild type ecotypes; *gai2* = GA insensitive; *eir1-3* = auxin insensitive; *cbb1* = brassinosteroid insensitive; *ein2.5* = ethylene insensitive; *coil* = jasmonic acid insensitive; *npr1* = non-expressed PR proteins; *nahG* = *nahG* (SA degrading) transgenic on Col-background.

\*Indicates significant differences from nontreated control with Fisher's LSD test at  $P = 0.05$ .

90-166, GB03, and C9. Strains SE34, 90-166, and IN937a promoted growth of *npr1* mutant, which lacks production of SA and PR proteins. Growth of auxin insensitive *eir1-3* was promoted by strains 89B61, GB03, and T4. Only strain GB03 elicited growth promotion on GA mutant *gai2* (Table 2).

#### In vivo effect of PGPR on growth of hormone mutants of *A. thaliana*

Growth promotion of the *Arabidopsis* hormonal mutants *in vivo* varied depending on PGPR strains (Table 3). Strain GB03 increased foliar fresh weight compared to the control in all mutant lines and wild

Table 3. Growth promotion of hormone mutants of *A. thaliana* by plant growth-promoting rhizobacteria mixed into the soilless medium under greenhouse conditions 3 weeks after seeding<sup>1</sup>

| Treatment <sup>3</sup> | Foliar fresh weight (mg) <sup>2</sup> |               |             |             |             |             |         |             |               |
|------------------------|---------------------------------------|---------------|-------------|-------------|-------------|-------------|---------|-------------|---------------|
|                        | Col-0 <sup>4</sup>                    | <i>ein2.5</i> | <i>cbb1</i> | <i>coi1</i> | <i>npr1</i> | <i>nahG</i> | WS      | <i>gai2</i> | <i>eir1-3</i> |
| 89B61                  | 81.50                                 | 29.30         | 80.11       | 32.13       | 41.27       | 92.70       | 84.63   | 44.00       | 51.80         |
| 90-166                 | 51.70                                 | 26.00         | 70.30       | 71.10       | 56.60       | 68.56       | 70.10   | 29.60       | 56.50         |
| C9                     | 206.60*                               | 43.80         | 232.70*     | 175.50*     | 89.67       | 233.80*     | 286.30* | 154.80*     | 305.63*       |
| E681                   | 218.29*                               | 55.67         | 301.25*     | 182.50*     | 222.67*     | 220.75*     | 345.78* | 174.25*     | 244.00*       |
| GB03                   | 268.50*                               | 34.75         | 266.70*     | 218.50*     | 197.00*     | 349.00*     | 318.10* | 155.71*     | 230.40*       |
| IN937a                 | 274.70*                               | 105.50*       | 172.14*     | 140.90*     | 160.10*     | 134.80*     | 295.60* | 152.40*     | 140.70*       |
| SE34                   | 235.13* <sup>4</sup>                  | 51.13         | 234.44*     | 95.88*      | 180.00*     | 234.33*     | 303.89* | 172.67*     | 185.10*       |
| T4                     | 140.00*                               | 56.44         | 311.20*     | 135.70*     | 150.80*     | 112.60*     | 247.40* | 141.50*     | 240.70*       |
| Control                | 50.29                                 | 32.63         | 61.60       | 29.90       | 51.00       | 39.43       | 72.10   | 30.40       | 19.80         |
| LSD ( $P=0.05$ )       | 58.62                                 | 29.01         | 63.59       | 51.10       | 44.78       | 56.06       | 71.62   | 43.97       | 67.19         |

<sup>1</sup>The assay was conducted on peat based soilless medium. Data are representative from one of two trials with similar results.

<sup>2</sup> Mean of 12 replications, one seedling per replication.

<sup>3</sup> PGPR were mixed with soilless media at the time of seeding at  $10^8$  cfu/ g soil. The 10–20 seeds of different lines of *A. thaliana* were seeded in rows in the  $60 \times 20$  cm tray containing PGPR-mixed soil.

<sup>4</sup> Col-0 and WS = wild type ecotypes; *gai2* = GA insensitive; *eir1-3* = auxin insensitive; *cbb1* = brassinosteroid insensitive; *ein2.5* = ethylene insensitive; *coi1* = jasmonic acid insensitive; *npr1* = non-expressed PR proteins; *nahG* = *nahG* (SA degrading) transgenic on Col-background.

\*Indicates significant different from nontreated control with Fisher's LSD test at  $P = 0.05$ .

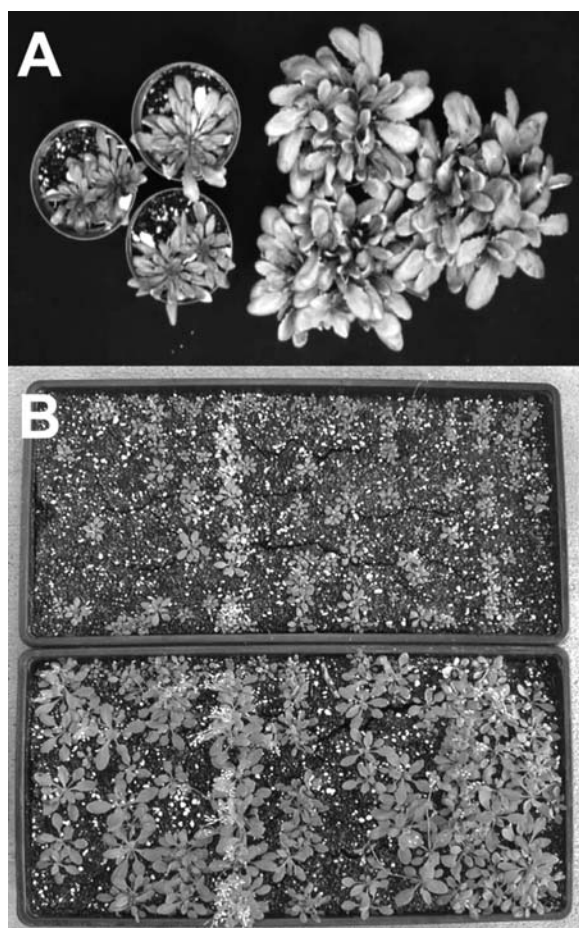
types except ethylene insensitive *ein2.5* mutant. Treatment with strains E681, SE34, T4, and GB03 resulted in enhancement of foliar fresh weight of every Arabidopsis line except *ein2.5*. Strains 89B61 and 90-166, which elicited growth promotion of some Arabidopsis lines *in vitro*, did not affect growth of any Arabidopsis lines. PGPR strain IN937a elicited growth promotion in all Arabidopsis lines tested. Growth promotion of all Arabidopsis lines except *ein2* and *npr1* mutants was elicited by all PGPR strains except 90-166 and 89B61. PGPR strain IN937a was the only strain to elicit growth promotion of ethylene insensitive mutant *ein2*. Growth promotion of *npr1* mutant, which lacks production of SA and PR proteins, was elicited by PGPR strains SE34, E681, IN937a, GB03, and T4 (Table 3).

## Discussion

Some PGPR strains previously shown to promote growth on other crops enhanced the growth of Arabidopsis seedlings both *in vitro* and *in vivo*. In the *in vitro* tests, all PGPR strains directly enhanced plant growth, depending on their distance from seeds. Our finding that the magnitude of growth promotion was dependent on the PGPR strain's distance from the plant suggests that a diffusible bacterial

metabolite caused plant growth promotion. Bacterial metabolites, such as hormones, would be expected to diffuse into the agar at decreasing concentrations at increased distances from the bacteria. For example, some *Pseudomonas* strains isolated from Arabidopsis affect the root system in a way that suggests the effects to be mediated by auxin. IAA-producing bacteria stimulate or inhibit root growth depending on bacterial concentrations (Persello-Cartieaux et al., 2003).

Alternatively, the PGPR could have mineralized nutrients, making them more available to plants. In one study, some bacilli PGPR strains promoted growth of maize seedlings through production of extracellular phytase, which degrades phytate (myo-inositol hexakisphosphate) under the conditions of limited phosphate availability. The dilution of culture filtrates obtained from the defective phytase strain did not enhance plant growth, but culture filtrates from wild type did (Idriss et al., 2002). However, because our plants were grown in a rich medium containing sucrose and excess nitrogen and other nutrients, bacterial metabolites seem to be the more plausible explanation. The strains that were the most promotive *in vitro*, e.g. GB03 and IN937a, were almost ineffective in the *in vivo* assays. Further, the majority of PGPR strains that were most effective at promoting plant growth *in vitro* were only mildly effective *in vivo*. Hence, different mechanisms of growth promotion likely occur *in vitro* and *in vivo*.



**Figure 2.** Visual illustration of growth promotion of *Arabidopsis* by PGPR in the greenhouse. A. PGPR were mixed into soilless medium at  $10^7$ – $10^8$  cfu/g soil, placed into 4-inch pots, and then seeded with *A. thaliana* ecotype Col-0 at time of planting. B. PGPR were mixed into soilless medium at  $10^7$ – $10^8$  cfu/g soil, placed into 60 cm  $\times$  30 cm tray, and then seeded with mutant lines of *A. thaliana* at time of planting.

*In vitro* tests reflect completely different responses than those seen in the greenhouse because, under the gnotobiotic conditions of *in vitro* tests, mechanisms of growth promotion that involve interactions with other microorganisms cannot be functional. Hence, in the *in vitro* tests, we can speculate that growth promotion effects involve only direct mechanisms such as production of plant growth regulators by PGPR, while growth promotion in *in vivo* tests may be related to both direct and indirect mechanisms. In addition, environmental processes, such as fluctuations of temperature, light intensity at day and night, and watering, which were not present in the *in vitro* test were present *in vivo* (in the greenhouse). Thus, different operational

mechanisms for growth promotion likely account for the differing strain-specific responses in the two tests. This point deserves further investigation as it may shed light on the kinds of *in vitro* screening that could be done to facilitate optimal greenhouse and field performance, while at the same time assisting to elucidate physiological mechanisms by which PGPR promote plant growth.

Results from our test with hormone mutant lines of *Arabidopsis* further indicate the lack of concordance between *in vitro* and the *in vivo* assays. Strain 90-166, for example, enhanced plant growth of *cbb1* and *npr1* in the *in vitro* assay but did not affect plant growth in the *in vivo* assay. Given the difference between *in vitro* and *in vivo* results, we believe that a meaningful understanding of signal pathways related to growth promotion comes from examining results with the *Arabidopsis* mutants *in vivo*. Among the eight PGPR strains tested, GB03 and IN937a promoted growth on the largest number of hormone mutant plants *in vivo*. The results of the *in vivo* experiment with strain GB03 and IN937a agree with our previous data, which showed that bacterial volatiles elicited growth promotion (Ryu et al., 2003). Growth promotion of *ein2* mutant was elicited by strain IN937a but not by strain GB03 (Table 3, Ryu et al., 2003). Ethylene insensitive *ein2* mutant is an ethylene and cytokinin double mutant (Alonso et al., 1999). To identify in detail the signaling pathways of growth promotion elicited by strain GB03 and IN937a, we need a single mutant of ethylene and cytokinin such as *etr1* or *cre1*.

From our signal transduction study using plant hormonal mutants of *Arabidopsis*, growth promotion induced by PGPR strains tested involved brassinosteroid, SA, and GA signaling *in vitro* and ethylene signaling *in vivo*. Some rhizobacteria have been reported to produce SA, GA, and ethylene (Press et al., 1997; Gutierrez-Manero et al., 2001; Berner et al., 1999). To our knowledge, there is no report of bacteria that produce brassinosteroid. The approach of isolating brassinosteroid-producing bacteria as PGPR strains should be considered because brassinosteroid acts positively in plant development (Nemhauser and Chory, 2004). *In vivo* screening suggested that ethylene might play an important role in growth promotion. Previously, several papers have reported that some PGPR strains reduce ethylene levels in plant tissues by producing ACC deaminase, which is an enzyme that degrades endogenous ethylene (Glick et al., 1998). The results reported here will be used to further inves-

tigate mechanisms of growth promotion *in vivo* and *in vitro* by specific PGPR strains.

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