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Molecules and Cells



# Inhibition of Primary Roots and Stimulation of Lateral Root Development in *Arabidopsis thaliana* by the Rhizobacterium *Serratia marcescens* 90-166 Is through Both Auxin-Dependent and -Independent Signaling Pathways

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**The rhizobacterium *Serratia marcescens* strain 90-166 was previously reported to promote plant growth and induce resistance in *Arabidopsis thaliana*. In this study, the influence of strain 90-166 on root development was studied *in vitro*. We observed inhibition of primary root elongation, enhanced lateral root emergence, and early emergence of second order lateral roots after inoculation with strain 90-166 at a certain distance from the root. Using the *DR5*::GUS transgenic *A. thaliana* plant and an auxin transport inhibitor, *N*-1-naphthylphthalamic acid, the altered root development was still elicited by strain 90-166, indicating that this was not a result of changes in plant auxin levels. Intriguingly, indole-3-acetic acid, a major auxin chemical, was only identified just above the detection limit in liquid culture of strain 90-166 using liquid chromatography-mass spectrometry. Focusing on bacterial determinants of the root alterations, we found that primary root elongation was inhibited in seedlings treated with cell supernatant (secreted compounds), while lateral root formation was induced in seedlings treated with lysate supernatant (intracellular compounds). Further study revealed that the alteration of root development elicited by strain 90-166 involved the jasmonate, ethylene, and salicylic acid signaling pathways. Collectively, our results suggest that strain 90-166 can contribute to plant root development via multiple signaling pathways.**

## INTRODUCTION

The capacity of microorganisms to stimulate development of plants has been used for *in vitro* and *in vivo* cultivation of agricultural, industrial, and ornamental plants (Ayyadurai et al., 2006; Kim et al., 2009; Noel et al., 1996; Unno et al., 2005). Plant growth-promoting rhizobacteria (PGPR) that are mainly found in the rhizosphere and rhizoplane, colonize roots and stimulate plant growth. As more plant tissues are analyzed for the presence of bacteria, an increasing number of indole acetic acid (IAA)-producing PGPR are being detected (Dey et al., 2004; Rosenblueth and Martinez-Romero, 2006; Unno et al., 2005). Studies of PGPR such as *Azospirillum* spp. revealed that bacterial IAA biosynthesis contributed to plant root proliferation (Dobbelaere et al., 1999; Tsavkelova et al., 2007), as *Azospirillum* mutants deficient in IAA production did not enhance root development (Dobbelaere et al., 1999). Increased root formation enhances plant mineral uptake and root secretion, which in turn stimulates both bacterial colonization and plant growth. However, a balanced interplay of different factors in bacterial IAA synthesis, rather than IAA production alone, is needed to account for growth promotion, as evidenced in a study of *Pseudomonas putida* CR 12-2 IAA-overproducing mutants (Xie et al., 1996).

In plants, IAA is the main auxin and affects many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity (Taiz and Zeiger, 1998; Teale et al., 2006; Woodward and Bartel, 2005). Thus, when IAA-producing bacterial strains interact with plants they have the potential to influence any of these processes by changing the plant auxin pool both spatially and temporally, leading to diverse changes in plant development and basal plant defense mechanisms (Chung et al., 2008; Spaepen et al., 2007). Altered plant development has been reported for IAA-producing rhizobacteria. Inhibition of plant growth was observed when plants were treated with the IAA-producing strain *P. thivervalensis* at concentrations above $10^6$ colony forming units (cfu)/ml, but not at concentrations below $10^5$ cfu/ml (Persello-Cartieaux et al., 2001).

Previous studies of selected PGPR strains have reported promotion of plant growth and induction of systemic resistance

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against a variety of pathogens for several crop species, including cucumber, tomato and tobacco (Liu et al., 1995; Raupach et al., 1996; Ryu et al., 2003b; Zhang et al., 2002). In addition, we have also reported that selected PGPR strains promoted plant growth and induced systemic resistance in *Arabidopsis thaliana* as a model plant system (Ryu et al., 2003a; 2003b; 2004; 2005). When searching for bacterial components from the PGPR strains capable of promoting plant growth, we discovered that volatile organic compounds (VOCs) from a PGPR strain of *Bacillus subtilis* GB03 (Ryu et al., 2003a) promoted plant growth via an EIN2- and CRE1-dependent cytokinin-signaling pathway. Furthermore, VOCs from strain GB03 promoted plant growth by regulating plant auxin homeostasis (Zhang et al., 2007). We found previously that plant exposure to several distinct PGPR both *in vivo* (in the soil) and *in vitro* (on medium) resulted in promotion of plant growth (Ryu et al., 2005). However, for the Gram-negative PGPR model strain *Serratia marcescens* 90-166, little is known regarding the bacterial components and the mechanisms involved in promotion of plant growth.

In this study, vertically-grown seedlings treated with *S. marcescens* 90-166 *in vitro* were used to study how this PGPR strain inhibited primary root growth and induced lateral root formation. We characterized the alteration of plant root development by strain 90-166, and investigated whether the auxin signaling pathway was involved in the triggered plant root developmental changes by using a *DR5*::GUS transgenic auxin reporter plant and an auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) assay. In addition to auxin, plant defense-related signaling molecules including jasmonic acid, ethylene and salicylic acid were also assessed to determine whether strain 90-166 contributes to the induced alterations in plant root development.

## MATERIALS AND METHODS

### Bacterial culture and inoculum preparation

PGPR strain *S. marcescens* 90-166 originally isolated from cucumber root (Wei et al., 1991) was used in this study. *Escherichia coli* DH5α (Qiagen, USA) was used as a control. The stock cultures amended with 20% glycerol were removed from -80°C storage, streaked onto tryptic soy agar (TSA) plates (Difco Laboratories, USA), and incubated at 30°C for 24 h to check for purity. Single colonies were transferred to a new TSA plate and incubated for two days. For experimental use, fully-grown bacterial colonies were scraped off the plates and re-suspended in sterilized distilled water (SDW). The bacterial suspensions were adjusted to $10^8$ cfu/ml ($OD_{600}$ = 1) based on optical density.

### Plant material and growth conditions

Transgenic *NahG* (salicylic acid degraded to an inactive form, catechol) *A. thaliana* plants were obtained from Dr. Xinnian Dong (Duke University, USA; Cao et al., 1994). Transgenic plants containing the auxin-responsive promoter *DR5*::GUS construct were obtained from Dr. Tom J. Guilfoyle (University of Missouri, USA; Ulmasov et al., 1997). The mutants ethylene-insensitive (*etr1*) (Chang et al., 1993), cytokinin-insensitive (*cre1*) and jasmonic acid-insensitive (*jar1*) were obtained from the Ohio State University Stock Center. All mutant and transgenic lines were derived from the parental *A. thaliana* ecotype Columbia (Col-0), which was obtained from the Ohio State University Stock Center. *A. thaliana* seeds were surface-sterilized with 6% sodium hypochlorite (100% commercial laundry bleach) containing 0.1% Triton X-100, washed four times with SDW, and maintained at 4°C for two days to enhance germination. The seeds were spread in a single line on MS medium with 0.6% plant agar and 1% (w/v) sucrose (Murashige and Skoog, 1962). Plants were grown vertically at 22°C under a 16/8 h light-dark cycle.

### *In situ* assay for detection of bacterial IAA production

The *in situ* assay for IAA was modified from a protocol developed by John M. Bric (Bric et al., 1991). Briefly, 3 μl of cells were resuspended in SDW and incubated on TSA plates at 30°C overnight; colonies were then overlaid with a 0.45 μm pure nitrocellulose membrane (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, USA), and incubation continued for 2 h. The membrane was removed from the plate and Salkowski reagent B in 1.2% 0.5 M $FeCl_3$ and 37% sulfuric acid (Sigma, USA) was applied. The membrane was then saturated in a Petri dish by overlaying on a reagent-saturated filter paper (Whatman No. 2, Whatman Co., USA). The reaction was allowed to proceed for 3 h at room temperature for proper color development, and images were captured by a digital camera (Nikon, Nikon CORP., Japan). The experiment was repeated three times with similar results.

### IAA detection by LC-MS/MS analysis

Liquid chromatography-mass spectrometry (LC-MS) was performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., USA) equipped with an electrospray ionization (ESI) source. High-performance liquid chromatography (HPLC) separation was performed on the Finnigan Surveyor™ Modular HPLC System (Thermo Electron Co., USA), using a Waters XTerra MS C18 (5 μm, 2.1 × 150 mm, Ireland) with a BetaBasic-18 guard column (2.1 × 10 mm, Thermo, USA). Mobile phase A was water and mobile phase B was acetonitrile; both containing 0.4% formic acid. The gradient elution at a flow rate of 0.3 ml $min^{-1}$ was performed as follows: 0-10 min 5-60% B (linear gradient) and 10-15 min 100% B (isocratic). The mass spectra were obtained in the range *m/z* 100-400 in positive mode. Data-dependent tandem mass spectrometry (MS/MS) experiments were controlled by the menu-driven software provided with the Xcalibur system. The experiment was repeated three times with similar results.

### Strain 90-166 treatment *in vitro*

Three-day-old vertically-grown *A. thaliana* Col-0 seedlings were treated with strain 90-166. For each treatment, 50 μl cell suspensions at $10^8$ cfu/ml were incubated on a sterilized filter paper disk (Tokyo Roshi Kaisha Ltd., Japan) and placed approximately 1 cm under the root tips of the seedlings. Control treatments consisted of an equivalent volume of SDW or cell suspension of strain DH5α. Seedlings were collected 7 days after treatment (DAT), and the morphological changes in root development were detected by digital camera (Nikon, Nikon CORP., Japan) and a Zeiss Axiophoto fluorescence microscope (Karl Zeiss, Germany).

In the transport inhibitor assay, two-day-old seedlings were transferred and grown vertically on MS plates containing the auxin inhibitor NPA (5 μM; Sigma, USA). Treatment with strain 90-166 was carried out after one day of additional growth, and the root phenotype was assessed at 7 DAT. The experiment was repeated three times with similar results.

### GUS assay

Histochemical staining for β-glucuronidase (GUS) activity was performed on transgenic *DR5*::GUS plants. Four seedlings treated with strain 90-166 and NPA were collected for GUS staining at 3, 7, and 11 days after drenching with 100 nM NPA

for the control (DMSO) or strain 90-166 in SDW. Whole seedlings were immersed in staining solution (2 mM X-Gluc in *N, N*-dimethylformamide, 100 mM $NaH_2PO_4$, 10 mM $Na_2EDTA$, 0.5 mM K-ferrocyanide, and 0.1% Triton X-100, pH 7.0) followed by incubation at 37°C overnight in the dark. The seedlings were cleared of chlorophyll by treatment with 70% ethanol after staining at room temperature. Stained samples were observed and photographed under a microscope.

**Identification of bacterial determinants of altered root development**

For preparation of bacterial cell lysis, a single colony was picked and incubated in tryptic soy broth at 30°C overnight in a shaking incubator. Following centrifugation, the supernatant was collected and this was termed the supernatant of culture. The bacterial pellet was resuspended in distilled water, and sonicated on ice for 10 min. The lysate supernatant was collected, and the pellet containing the bacterial cell wall components was resuspended in distilled water. A sample of each of the three fractions was streaked onto TSA and incubated at 30°C for 24 h. No colonies grew, confirming no bacterial cell contamination of any fraction. A sample (50 ul) of each fraction was then applied to a paper disk which was located below the *A. thaliana* seedlings. Root growth was assessed at 7 DAT. The experiment was repeated twice with similar results.

**Data analysis**

Data was subjected to analysis of variance using JMP software (SAS Institute Inc., USA). The significance of the PGPR treatment effects was determined by the magnitude of the *F* value at $P = 0.05$. When a significant *F* value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD) at $P = 0.05$. Results of repeated trials of each experiment outlined above were similar. Hence, a representative trial of each experiment is reported in the Results section.

## RESULTS

**Multiple effects of strain 90-166 on plant root development**

During our initial studies, plant growth of seedlings treated with strain 90-166 *in vitro* was promoted at 14 DAT, which was consistent with our previous results (Ryu et al., 2005). However, most seeds did not germinate, or germinated late, when directly inoculated with strain 90-166, and root growth of germinated seedlings was strongly inhibited (data not shown). This could be due to a high concentration of certain compound(s) produced by strain 90-166 during bacterial growth on the seeds.

To avoid direct attachment of strain 90-166 to plants or seeds and to characterize the effects on plant growth after treatment, a new method was developed. Strain 90-166-inoculated paper disks were instead placed under vertically-grown seedlings, and this method was used for further experiments. At 7 DAT, primary root elongation of strain 90-166-treated seedlings was largely inhibited and the inhibition strongly depended upon the distance the paper disk was from the seedlings, while no inhibition was detected in SDW-treated seedlings (Figs. 1A-1D). The inhibition of primary root elongation was maintained as late as 18 DAT (Fig. 1C). To analyze these observations in more detail, seedlings were divided into two groups for quantification of root length: seedlings grown within 2 cm (< 2 cm) and further than 2 cm (> 2 cm) distance from the paper disk. For the < 2 cm group, the primary root length of seedlings treated with strain 90-166 was only 10.2 mm, while those of SDW-treated seedlings reached 39.8 mm. In comparison, for the > 2 cm group, significant inhibition of primary root length was only detected when directly compared with the SDW control seedlings (Fig. 2A).

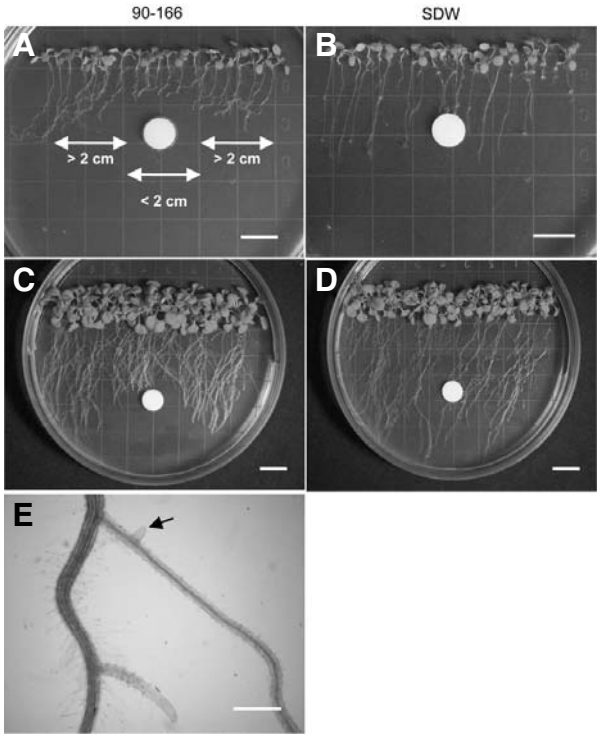


**Fig. 1.** Alteration of root development by strain 90-166 treatment. Seedlings on MS plated were divided into < 2 cm and > 2 cm groups based on distance from the paper disk, as indicated in A. Primary root elongation was inhibited in strain 90-166-treated plants at 7 days after treatment (DAT) (A), compared to sterilized distilled water (SDW) control (B). Second order lateral roots (indicated by arrow) were detected only in strain 90-166-treated plants at 7 DAT (E). Inhibition of primary root elongation was maintained in strain 90-166-treated plants (C) compared to SDW control (D) at 18 DAT. Scale bars: 1 cm in (A, B, C, and D); 100 μm in (E). This experiment was repeated three times with similar results.

Induction of lateral root formation was also detected after strain 90-166 treatment, with 9.2 lateral roots in the < 2 cm group and 5.2 in the > 2 cm group, which was close to or more than double when compared to the SDW controls (Fig. 2B). Surprisingly, specific second order lateral roots, which emerged from the primary lateral roots, were only detected in strain 90-166-treated seedlings (Fig. 1E), and not in SDW controls.

**Detection of IAA production by strain 90-166**

IAA analogs produced from root-associated bacteria such as *Rhizobium leguminosarum* and *P. putida* have been found to affect host root development (Boot et al., 1999; Patten and Glick, 2002). IAA was detected in all three strains tested including a positive control *P. chlororaphis* O6 (data not shown), which has previously been reported to produce IAA (Kang et al., 2006). Fractions of strain 90-166 culture were tested; supernatant of culture, lysate supernatant, and bacterial cell wall components. A weak signal only was detected in the supernatant of culture, and no signal was recorded in any fraction of lysate supernatant or bacterial cell wall components (data not shown), indicating that strain 90-166 can secrete IAA analogs out of the bacterial cells. Unexpectedly, the supernatant of the

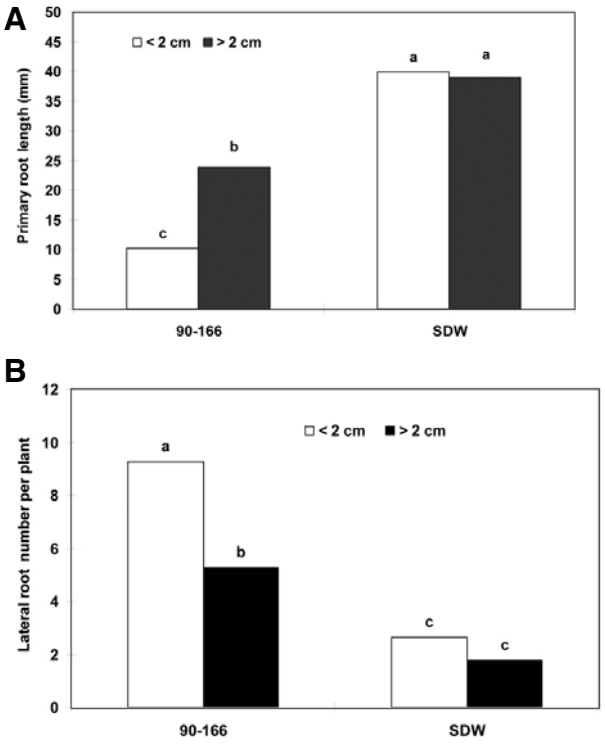


**Fig. 2.** Quantification of primary root inhibition and lateral root induction. (A) Quantification of primary root length (mm) in strain 90-166- or SDW-treated seedlings on MS plates at 7 DAT. (B) Quantification of lateral root numbers per plant in strain 90-166- or SDW-treated seedlings on MS plates at 7 DAT. Different letters indicate significant differences using Fisher's protected LSD test at $P = 0.05$. This experiment was conducted three times, and results of repeated trials of each experiment outlined above were similar. Hence, one representative trial of each experiment is reported here.

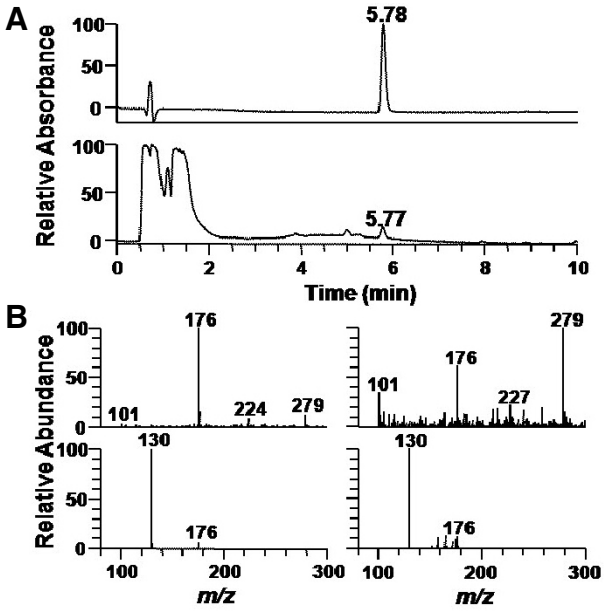


**Fig. 3.** IAA production by strain 90-166 examined using LC-MS/MS analysis. (A) HPLC chromatogram at 280 nm of 100 μM standard IAA (upper row) and 90-166 supernatant (bottom row). (B) Positive ion mass spectra and MS/MS fragmentation patterns of the standard IAA (left column) and 90-166 (right column), designated as 5.78 min and 5.77 min, respectively, showed the same patterns.

*E. coli* DH5α used as a bacterial control clearly produced a positive reaction in our IAA detection system (data not shown). The structure of the compound secreted by strain 90-166 was confirmed by comparison with standard IAA, exhibiting identical retention time, mass spectra, and MS/MS spectra under the same conditions. IAA showed a mass spectrum with a molecular ion ($[M + H]^+$) peak at *m/z* 176 (Fig. 3A), and the mass spectrum and tandem mass spectrum of strain 90-166 sample showed the same pattern as the standard IAA compound (Fig. 3B). Therefore, the peak at 5.77 min for the strain 90-166 supernatant of culture sample was identified as IAA.

**Altered root development by low levels of auxin production from strain 90-166**

IAA analogs produced from strain 90-166 indicated that an auxin-dependent signaling pathway might be involved in 90-166-triggered alterations in root development. To test our hypothesis, plant auxin accumulation was directly examined in the auxin reporter *DR5*::GUS transgenic plants treated with strain 90-166. On the leaves and roots, similar GUS staining levels were detected in strain 90-166- and SDW-treated seedlings at 5 DAT (Fig. 4A). Surprisingly, the GUS staining level was maintained on strain 90-166-treated plants even at 11 DAT, whereas there was no detectable signal remaining in SDW-treated seedlings at this time, indicating that the alteration of plant root development at 7 DAT was not due to changes in the auxin balance in the plant-tissues (Fig. 4B).

The auxin-dependent pathway was tested with seedlings grown on MS plates containing the auxin transport inhibitor NPA (5 μM). Both primary root elongation and lateral root formation were reduced due to the inhibition of auxin transport, consistent with lateral root initiation promoted by auxin transport (Dhooge et al., 2001). Significant inhibition of root growth and initiation of lateral roots were detected in the < 2 cm group of strain 90-16-treated seedlings when compared with those of the > 2 cm group or controls (Fig. 5). This indicated that alteration of root development by 90-166 can be independent of auxin signaling, which is consistent with the results of GUS staining in *DR5*::GUS transgenic plants treated with strain 90-166.

**Multiple compounds from strain 90-166 contribute to alteration of root development**

The distance-dependent alteration of root development described above indicated that some unknown secreted compounds were produced by strain 90-166, and probably diffused through the MS agar and affected plant root development. To assess this, the supernatant from cell cultures and the fractions of cell lysis (lysate supernatant and bacterial cell wall components) were prepared and applied to plants. Interestingly, primary root elongation was only inhibited in seedlings treated with supernatant of cell culture, while lateral root formation was only induced in seedlings treated with lysate supernatant. This indicated that distinct secreted metabolites and intracellular compounds from strain 90-166 may be involved in the inhibition of primary root growth and in the lateral root development, respectively (Fig. 6).

**Screening plant mutant(s) defective for hormones involved in alteration of root development by strain 90-166**

To search for mechanisms other than auxin signaling by which

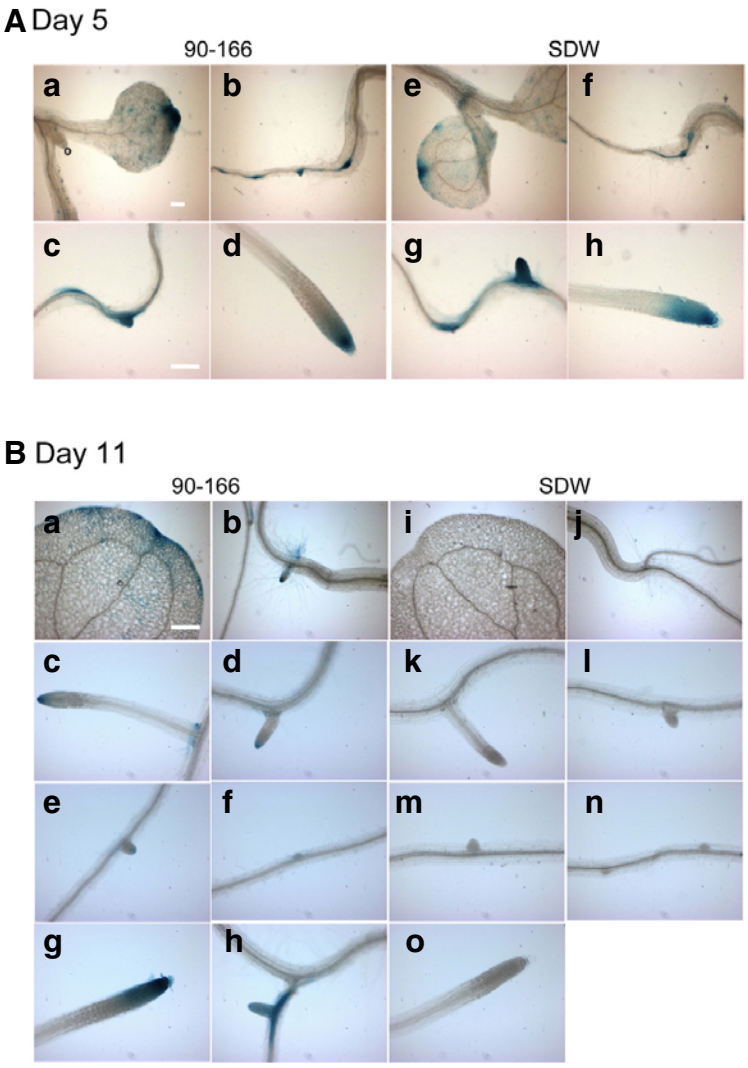


**Fig. 4.** (A) Day 5; at 5 days after treatment (DAT), the expression of *DR5*::GUS induced by strain 90-166 was not different to that induced by sterile distilled water (SDW). The panels show GUS staining in *DR5*::GUS transgenic plants following strain 90-166 (a-d) or SDW (e-h) treatment. Differential GUS staining was shown in the cotyledon (a, e), root code (b, f), lateral roots (c, g), and primary root tip (d, h). Three replicates were performed with a total seedling population of 20; representative images are shown. Scale bar in a and c : 100 μm. The first group (photos a, b, e, and f) and second group (photos c, d, g, and h) are at the same magnitude. (B) Day 11; the expression of *DR5*::GUS induced by strain 90-166 was maintained through the late stages of plant growth. The panels show GUS staining in *DR5*::GUS transgenic plants following strain 90-166 (a-h) or SDW (i-o) treatment at 11 DAT. Differential GUS staining was shown in the cotyledon (a, i), root code (b, j), and lateral roots at different stages (c-f, k-n), primary root tip (g, o), and second order lateral root (h). Three replicates were performed with a total seedling population of 20; representative images are shown. Scale bar in a: 100 μm.

strain 90-166 may alter root development, strain 90-166 treatment was tested in a series of *A. thaliana* mutants defective in specific plant hormone signaling pathways. Specific second order lateral roots were observed for wild-type Col-0 plants treated with strain 90-166 at 7 DAT, while significant reduction of second order lateral roots was observed in *etr1*, *jar1,* and *NahG*, but not *cre1* mutants (Table 1). This indicates that the ethylene, jasmonic acid, and salicylic acid-signaling pathways contributed to the induction of second order lateral root growth. In addition, instead of observing recovery of primary root elongation as expected, enhanced inhibition was observed in several of the mutant lines tested (Table 1).

## DISCUSSION

In this study, we developed and employed an *in vitro* method to study bacterial effects on root development in *A. thaliana*. Using this method, we found that a Gram-negative model PGPR strain 90-166 is able to affect plant root development, including inhibition of primary root elongation and induction of lateral root formation. However, the plant auxin signaling pathway may be only partially involved in the alteration of root development; unknown bacterial determinants from strain 90-166 other than IAA may also contribute to the alteration of root development.

Initial studies showed that *A. thaliana* seeds inoculated with a strain 90-166 cell suspension exhibit severely inhibited or delayed germination and altered root development, instead of the promotion of plant growth seen when plants were exposed to *in vivo* or *in vitro* treatment, as reported in our previous study (Ryu et al., 2005). The difference in these results may indicate the different colonization level for strain 90-166 on the *A. thaliana* seeds and roots, as PGPR strains have been reported to grow slowly on MS medium. Thus, the effects on plant development by strain 90-166 are probably dependent on the concentration of certain compounds produced by strain 90-166, because the bacterium promoted plant growth at low concentrations during indirect treatment but inhibited seed germination and plant root growth at high concentrations during direct seed treatment. Inhibition of plant growth was found only in a few plants grown next to the bacterial source after treatment *in vitro*, which is consistent with our hypothesis of a dose-dependent mechanism.

Inoculation with strain 90-166 affected the root system in wild-type Col-0 plants, including inhibition of primary root growth and enhancement of root branching (Figs. 1 and 2). The most significant influence on root architecture was exhibited in the < 2 cm group of strain 90-166-treated seedlings; while primary

**Table 1.** Root development response *of Arabidopsis thaliana* mutants on MS plates following treatment with strain 90-166

| Plant | Description | Second order lateral roots[a] | Primary root elongation[b] |
|---|---|---|---|
| Col-0 | Wild-type | +++ | + |
| *etr1* | Ethylene-insensitive | + | - |
| *jar1* | Jasmonic acid-insensitive | + | ND |
| *NahG* | Salicylic acid-insensitive | ++ | - |
| *cre1* | Cytokinin-insensitive | +++ | + |

Symbols indicate differences in second order lateral roots and root length in Col-0 wild-type and mutants in Col-0 background after strain 90-166 treatment in *A. thaliana* seedlings on MS plates, relative to sterilized distilled water (SDW) or DH5α (control) treatment.
[a] +, ++, and +++ indicate average number of second order lateral roots per seedling at 7 DAT (+ = < 1; ++ = 1 – 2; +++ = > 2; ND = not detected).
[b] (-, +, +++) indicate primary root length at 15 DAT, wild-type seedlings with (+) and without (+++) strain 90-166 treatment were established as standard; (-) indicates enhanced inhibition of root elongation with strain 90-166 treatment; ND, not detected. The experiments were repeated three times with similar results.

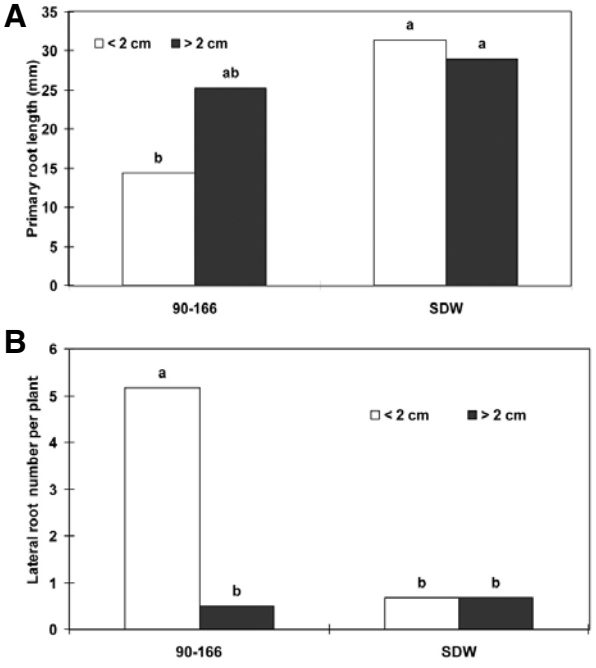


**Fig. 5.** Auxin signaling in strain 90-166-altered root development, identified by the *N*-1-naphthylphthalamic acid (NPA) assay. (A) Quantification of primary root length (mm) in strain 90-166- or SDW-treated seedlings on MS plates containing 5 μM NPA at 7 DAT. (B) Quantification of lateral root number per plant in strain 90-166- and SDW-treated seedlings on MS plates containing 5 μM NPA at 7 DAT. Different letters indicate significant differences using Fisher's protected LSD test at $P = 0.05$. This experiment was conducted three times, and results of repeated trials of each experiment were similar. Hence, one representative trial of each experiment is reported here.

root growth was slightly inhibited by the supernatant of cell culture, and lateral roots were slightly induced by the supernatant of cell lysate (Fig. 6). These results indicate that continued production of compounds by strain 90-166 is necessary for full alteration of root development; the distinct components produced were probably contributing to the inhibition of primary root growth and induction of lateral root branching, respectively. The development of early branching of roots was reported to directly contribute to water uptake and facilitate the extraction of nutrients essential for robust plant growth and development (Spaepen et al., 2007). It will be interesting to check whether strain 90-166 triggers plant growth by promoting direct stimulation of root branching in the soil. Primary root growth inhibition is indicated to be caused by a reduction in cell elongation and by a reduction of cell proliferation in the root meristem, and both primary and lateral root elongation was inhibited by the soil bacterium *B. megaterium* (Lopez-Bucio et al., 2007). A different phenotype, with normal elongation of lateral roots but inhibited growth of primary roots has been identified in our study, suggesting the involvement of different compounds that act through separate signaling pathways.

Auxin is known to be required at several developmental stages to facilitate lateral root formation (Himanen et al., 2002; Torrey, 1950). Furthermore, specifically endogenous overproduction of auxin results in an increase in the number of lateral roots (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995). It has also been reported that IAA-producing bacteria can stimulate or inhibit root growth depending on bacterial concentrations (Persello-Cartieux et al., 2001). Our results showed, somewhat unexpectedly, that DH5α was able to produce IAA analogs and partially alter root development (data not shown). Rescued alteration of root development by use of NPA on DH5α-treated plants indicates that the auxin signaling pathway and IAA analogs may be involved. Unlike the case for DH5α, NPA was not able to rescue alteration of root development triggered by strain 90-166, but did reduce the level of inhibition of primary root elongation and the lateral root number (Fig. 5), indicating that other signaling pathways combined with auxin signaling contribute to the root system architecture. Recently, increased growth of lateral roots was reported to be mediated by auxin redistribution rather than auxin accumulation caused by *B. megaterium* (Lopez-Bucio et al., 2007). However, similar levels and patterns of GUS staining in strain 90-166- and SDW-treated *DR5*::GUS seedlings suggest that induction of lateral roots was not caused by auxin accumulation or redistribution (Fig. 4A). Nevertheless, auxin may be slightly induced or indirectly affected by strain 90-166 treatment, as plant auxin levels are maintained throughout the relatively late stages of root development, and after root alteration is completed. The mechanism for maintaining auxin in the plant tissues as a result of the presence of strain 90-166 is not clear. However, maintaining auxin in plants may directly contribute to promotion of plant growth by stimulating additional initiation of lateral root development. It is worth noting that *E. coli* DH5α produced detectable levels (data now shown) of IAA, suggesting a need to examine why a human gut bacterium secretes a plant growth hormone.

Specific second order lateral roots are initiated in most seedlings in the < 2 cm group following strain 90-166 treatment, but not in the control or the > 2 cm group, and this is probably due to the high concentration of compounds arising from strain 90-166. Ethylene, in crosstalk with auxin, has recently been re-

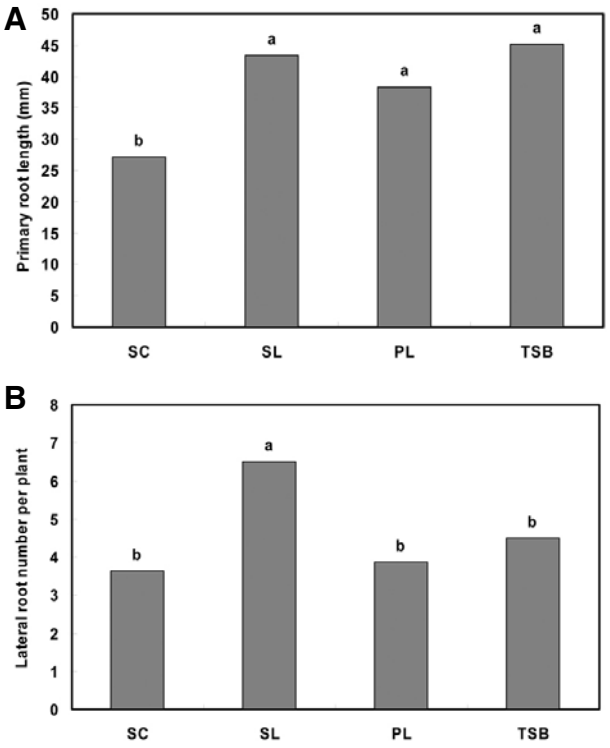


**Fig. 6.** Different compounds were produced by strain 90-166 for root growth inhibition and lateral root induction, respectively. (A) Quantification of primary root length (mm) in strain 90-166 fraction-treated seedlings on MS plates at 7 DAT. (B) Quantification of lateral root number per plant in strain 90-166 fraction-treated seedlings on MS plates at 7 DAT. Fractions of cell culture and cell lysis: SC, supernatant of cell culture; SL, supernatant of cell lysate; PL, pellet of cell lysate; TSB, tryptic soy broth. Different letters indicate significant differences using Fisher's protected LSD test at $P = 0.05$. This experiment was conducted three times, and results of repeated trials of each experiment were similar. Hence, one representative trial of each experiment is reported here.

ported to regulate lateral root formation in a dose-dependent manner (Ivanchenko et al., 2008; Negi et al., 2008). The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) promotes initiation of primordial lateral roots at low concentrations, but inhibits pericycle cells to initiate new primordial lateral roots at high concentrations; interestingly, specific second order lateral roots are observed at high ACC concentrations (Ivanchenko et al., 2008). Few or no second order lateral roots are detected in *jar1*, *etr1* and *NahG* mutants inoculated with strain 90-166, indicating that rather than ethylene signaling, complex hormone signaling pathways are involved in this process. However, neither inhibition of primary root growth nor enhanced lateral root formation was recovered in any of the mutant plants tested. Thus, mechanisms other than the plant hormone signaling pathways tested here may be responsible for the altered root development; alternatively, multiple phytohormone signaling pathways rather than a single pathway may contribute to the observed effects on root development.

Recently, three reports showing similar results to ours were published on the alteration of root architecture induced by microbes or their metabolites. A bacterial antibiotic, 2,4-diacetylphloroglucinol (DAPG) produced by the biological control agent *P. fluorescens* inhibited primary root growth and stimulated lateral root production in tomato seedlings (Brazelton et al., 2008). Using auxin-resistant *diageotropica* tomato mutant seedlings and auxin-inducible *GH3 promoter-luciferase reporter*-harboring tobacco plants, the authors concluded that DAPG-mediated changes in root architecture were relayed by an auxin-dependent signaling pathway. Similarly, inoculation with a plant symbiotic fungus *Trichoderma virens* resulted in characteristic auxin-related effects such as promotion of plant growth and stimulation of lateral root development (Contreras-Cornejo et al., 2009). In addition, the effect of *T. virens* on root growth was decreased in the auxin transport or signaling *A. thaliana* mutants *aux1, big, eir1* and *axr1*, indicating that auxin-dependent signaling plays a critical role in the stimulation of lateral root growth by *T. virens*. In contrast to these previous two reports, strain 90-166 promoted lateral root growth even on the NPA-containing plates (Fig. 5) suggesting the existence of auxin-independent or weakly auxin-dependent signaling. Intriguingly, similarly to our results, bacterial production of *N*-acylhomoserine lactones (AHLs), bacterial signaling molecules conferring quorum sensing, affected alteration of primary root and lateral root formation in an auxin-independent manner (Ortíz-Castro et al., 2008; von Rad et al., 2008). Among five different AHLs tested, pharmaceutical application of *N*-decanoly-homoserine lactone (C10-HL) on *A. thaliana* seedlings greatly affected root development. Moreover, using auxin mutant plants *aux1-7, doc1,* and *axr2,* differentiation of root architecture induced by C10-HL was confirmed to be through auxin-independent signaling (Ortíz-Castro et al., 2008). Through use of an indicater strain, we found that strain 90-166 produced AHL molecules (data not shown). Further studies with different PGPR mutant strains including mutants defective in AHL production and in alteration of root morphology, and other plant mutant lines, may broaden our understanding of the mechanisms underlying PGPR-induced alterations in plant root development.

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