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A volatile producing Bacillus subtilis strain from the rhizosphere of Haloxylon ammodendron promotes plant root development

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Abstract

Aims

The colonization of plant growth-promoting rhizobacteria (PGPR) along plant roots in turn facilitates their ability to promote plant growth and health. In this study, we found that *Bacillus subtilis* strain WM13-24 from the rhizosphere of *Haloxylon ammodendron* was able to promote the growth of both Arabidopsis and its host plant. Furthermore, we found that volatile compounds (VCs) from strain WM13-24 could promote plant growth by stimulating lateral root formation and root hair growth. However, the molecular mechanism underlying WM13-24-stimulated root development is still unknown.

Methods

In this study, a series of Arabidopsis mutants defective in specific plant hormone signaling pathways were used as materials to preliminarily study the effect of volatile compounds (VCs) released by strain WM13-24 on plant root development through genetic and pharmacological methods. The volatile compounds of strain WM13-24 were identified by solid-phase microextraction gas chromatography-mass spectrometry (SPME-GC-MS).

Results

The results showed that auxin signaling and transport played a crucial role in WM13-24-stimulated changes of root architectures in Arabidopsis. SPME-GC-MS analysis revealed that WM13-24 produces 2, 3-butanediol and benzyl alcohol and the growth responses of plants to the two VCs were concentration dependent.

Conclusions

All these results suggested that VCs emitted from *Bacillus subtilis* strain WM13-24 from the rhizosphere of *Haloxylon ammodendron* improves root development depending on auxin signaling.

Introduction

Terrestrial plants harbor a highly complex microbiota community in soil closely adhering to their rhizosphere. The rhizosphere provides a niche for interactions between plant roots and microbes (Bulgarelli et al. 2013; Sasse et al. 2018; Qu et al. 2020). Root-associated soil bacteria or fungi can strongly influence plant fitness (Vílchez et al. 2020). Some beneficial bacteria or fungi living in the rhizosphere are referred to as plant growth-promoting rhizobacteria or fungi (PGPR or PGPF), which promote plant growth by inducing the adaptive development of vigorous root systems and enhancing the capacity of the host plants to acquire nutrients and water from the soil (Verbon et al. 2016). The

production of bioactive metabolites in microbes is strain-dependent and these metabolites include volatile (Ryu et al. 2003; Camarena-Pozos et al. 2019; Garnica-Vergara et al. 2016; Syed-Ab-Rahman et al. 2019; Cordovez et al. 2018; Li et al. 2021) and nonvolatile substances (Wang et al. 2020; Sun et al. 2020; Inaji et al. 2020). The microbial volatile organic compounds (VOCs) database contains thousands of logged VOCs from a wide range of microbes, as well as proposed metabolomic pathways (https://bioinformatics.charite.de/mvoc/) (Lemfack et al. 2018). Microbial volatile compounds diffuse freely into the environment through aqueous solutions and permeate through the atmosphere, this means volatile compounds have important roles as signals in intra-kingdom and inter-kingdom communication at low concentrations and over long distances (Syed-Ab-Rahman et al. 2019; Bailly and Weisskopf 2017; Schulz-Bohm et al. 2017; Piechulla et al. 2017; Ryu et al. 2003). These volatile compounds of low molecular weight (< 300 Da) belong to a broad range of chemical classes such as aldehydes, ketones, alkyls, alcohols, esters, alkynes, acids, sulfur and nitrogen-containing compounds (Kai et al. 2009; Xu et al. 2015; Garbeva et al. 2020). Plants either take up VOCs as nutrient sources or perceive them as infochemicals (Meldau et al. 2013; Bailly et al. 2014; Matsui 2016; Aziz et al. 2016; Zhou et al. 2016; Morcillo et al. 2020).

Roots play important roles in the growth and development for plants (Lv et al. 2021). The root system of dicots is formed by one embryonically formed primary root and post-embryonically developed lateral roots (LRs) of different orders (Du and Scheres 2018; Jia et al. 2021). Lateral roots (LR) formation involves priming, initiation, patterning and emergence (Malamy and Benfey 1997; Péret et al. 2009). It is well known that many PGPR and PGPF strains caused alterations in the root system architecture of plants by promoting the formation of lateral root and root hair and resulted in enhanced plant growth (Sun et al. 2020; Zamioudis et al. 2013; Garnica-Vergara et al. 2016; Li et al. 2021). PGPR modified root system architecture and the structure of root tissues mainly through interfering with the homeostasis and signaling of endogenous plant hormones, such as auxin, cytokinin, gibberellin, ethylene (ETH), brassinosteroid, jasmonic acid (JA) and abscisic acid (ABA) (Osmont et al. 2007; Sharifi and Ryu 2018). Therefore, root system growth are complex processes that are modulated by a variety of phytohormones and signaling molecules (Van de Poel et al. 2015). Auxin in plant fulfils multiple roles throughout LR development (Péret et al. 2009; Du and Scheres 2018). Auxin distribution is regulated mainly by auxin transport proteins, including members of the AUXIN 1/Like AUXIN (AUX1/LAX) family of influx carriers and PIN-FORMED (PIN) proteins of efflux carriers (Adamowski and Friml 2015; Li et al. 2020; Chen et al. 2015).

The increased root development leads to an increased root surface that could improve plant nutrition and thus would be a key factor for plant growth promotion by PGPR. *Bacillus* sp. represent one of the most abundant genera of the root microbiome. Exposure of Arabidopsis seedlings to VCs emitted by *Bacillus* sp. increased root branching and biomass production (Ryu et al. 2003; López-Bucio et al. 2007; Hossain et al. 2019; Li et al. 2021). Thus, VCs-dependent plant growth and development is apparently under control of complex mechanisms, although its signaling components have remained largely unidentified. In this study, a volatile producing *Bacillus subtilis* strain from the rhizosphere of *Haloxylon ammodendron* was used to investigate its growth promotion effect on both Arabidopsis and its host plant. Then, the

physiological and molecular mechanism underlying root development effect was probed and we investigated how this strain induced Arabidopsis mutants defective in phytohormone signaling. We found that *Bacillus subtilis* WM13-24 was able to promote the growth of both Arabidopsis and its host plant. *Bacillus subtilis* WM13-24 inoculation altered the root architecture of Arabidopsis by increasing the number of lateral roots and elongating root hairs. Auxin signaling and transport participated in WM13-24 induced plant root development.

Materials And Methods

Suspension culture of the bacterial strains

Bacillus amyloliquefaciens GB03 (Ryu et al. 2003), *Bacillus subtilis* WM13-24 and *Escherichia coli* strain DH5α were cultured on LB medium (composition in gram per liter: tryptone 10; yeast extract 5; NaCl 10, pH = 7) without light for 24 h at 28°C or 37°C (DH5α). Cells of PGPR strain WM13-24 were harvested from LB medium in distilled water to yield 10^9 (OD = 600) colony forming units (CFU) mL^{-1}, as determined by optical density and serial dilutions.

Plant materials and growth conditions

For the *in vitro* assays, *Arabidopsis thaliana* ecotype Col-0 was used as the wild type to compare with the mutants, including *axr1* (Estelle and Somerville 1987), *axr2* (Timpte et al. 1994), *axr4* (Hobbie and Estelle 1995), *eir1* (Roman et al. 1995), *cre1* (Inoue et al. 2001), *etr1* (Hua and Meyerowitz 1998), *gai* (Stotz et al. 2011), *cbb1* (Kauschmann et al. 1996), *coi1-1* (Xie et al. 1998), and *NahG* (Lawton et al. 1995) transgenic plants were used in this study.

The *arf7-1* mutant and *DR5::GUS* transgenic plant line were presented by Dr. Hong-Ju Yin at Lanzhou University, China. The mutants of *arf19-1*, *arf7arf19*, *ein3-1* and *jar* were presented by Prof. Lai-Sheng Meng in Jiangsu Normal University, Xuzhou, China. The mutant of *aux1-7* was presented by Dr. Hai-Qing Liu at Lanzhou University, China. The *PIN1::PIN1:GFP*, *PIN2::PIN2:GFP*, *PIN4::PIN4:GFP* and *PIN7::PIN7:GFP* transgenic plant lines were presented by Prof. Chuan-You Li in Chinese Academy of Sciences, China.

Seeds were surface-sterilized with 75% (v/v) ethanol for 3 min and 1% sodium hypochlorite (v/v) for 5 min, washed eight times with sterile water. Seeds were vernalized for 3 days at 4°C in darkness. Petri plates were prepared by adding 1/2 Murashige and Skoog (MS) agar medium (1/2 MS salts, 1% sucrose, and 0.75% agar, pH 5.7). Unless otherwise indicated Arabidopsis plants were cultured in Petri dishes containing sucrose-free solid MS. In the divided petri plates (I-plates), bacterial suspension was applied drop wise in one of the compartments and Arabidopsis seeds were sown in the other compartment.

To investigate the effects of volatile compounds (benzyl alcohol) in Arabidopsis, the 1.5 mL short thread vial (Beijing Labgic Technology Co., Ltd) contained benzyl alcohol with concentrations of 50, 100, 200,

500, 750, 5000 μM, mixture compounds of benzyl alcohol and 2,3-butanediol with concentrations of 50 + 50, 50 + 100, 50 + 200, 100 + 50, 100 + 100, 100 + 200 μM, and solvent controls contained water. The vial was placed in one of the compartment and five Arabidopsis thaliana seeds were grown in the other compartment. The petri dishes were transferred and positioned vertically in a growth chamber under a long-day photoperiod (16 h of light, with light intensity of 100 μmol m^{-2} s^{-1}) at 22 ± 1°C.

For pot experiment, seeds of *Haloxylon ammodendron* were collected from wild plants in Alxa League Right Banner, Inner Mongolia Autonomous Region, China (Lü et al. 2019). The disinfected seeds were sown in plastic pots (5 × 5 × 5 cm; 10 seedling/pot) containing heat-sterilized vermiculite (*H. ammodendron*) and half strength Hoagland nutrient solution for growth (Gao et al. 2020). When plants reached four weeks-old, they were divided into two groups, inoculation bacterial suspension group and VCs treatment group. Each pot of *H. ammodendron* was inoculated with 5 mL bacterial suspension culture of WM13-24 or water as control. For VCs treatment experiment, six pots of *H. ammodendron* were transplanted to sealed containers (8 L, 6 pots). The container contained half strength Hoagland nutrient solution and 1.5 mL vials (100 μM benzyl alcohol, 500 μM 2,3-butanediol, both 100 μM benzyl alcohol and 500 μM 2,3-butanediol, respectively), which were added every two days. The plant growth promotion parameters were typically assessed at 14 d after treatment. Plants were grown in greenhouse under 28/23°C (day/night), the photoperiod was 16/8 h (light/dark) and the light intensity was 1200 μmol photons $m^{-2}s^{-1}$ under metal halide and high-pressure sodium lamps, and the relative humidity was about 70%.

Each pot of Arabidopsis was inoculated with bacterial suspension culture of WM13-24 or water as control. The plant growth promotion parameters were typically assessed at 14 d after treatment. Plants were grown as described above.

Quantification of shoot and root growth

For shoot and total fresh weight measurements, seedlings were immediately measured on an analytical balance. The method for measurement leaf chlorophyll content was the same as described by He et al (2018).

Photographs were taken with a digital camera (Cannon EOS 7D, Cannon Inc., Japan). Primary root (PR) length was measured using a ruler. Lateral root (LR) density was calculated with LR number divided by the PR length of each seedling. Total root length was determined using a root scan apparatus (Perfection V700Photo, Seiko Epson Corp., Japan) equipped with WinRHIZO software (Regent Instruments Inc., Canada). The specific procedure was the same as described by Wang et al (2016).

Histochemical staining and microscopy

Wild type Arabidopsis Col-0 and transgenic line *DR5::GUS* were immersed in staining buffer (50 mM $K_3Fe(CN)_6$, 50 mM $K_4Fe(CN)_6$ ž $3H_2O$, 1 M Na_2HPO_4, 1 M NaH_2PO_4, 0.25 M Na_2EDTA, Trifonx-100,

methanol, ddH_2O, 2 mM X-Gluc) and incubated overnight at 37°C. Seedlings were cleared in consecutive washes 7 % (v/v) ethanol. Images were obtained using fluorescence microscope (DM6 B, Leica, Germany).

Microscopy

The number of emerged LR and images of the root hair were obtained using stereomicroscope (M205 A, DMC5400, Leica, Germany). Green fluorescence from roots of *DR5::GFP* was observed under a fluorescence microscope (DM6 B, DFC2000T, Leica, Germany) and *PIN::GFP* transgenic plants were observed under a laser scanning confocal microscope (SP8 SR, Leica, Germany).

RNA isolation and qRT-PCR

Total RNA was isolated using RNAiso Plus Kit (TaKaRa) in accordance with the manufacturer's protocol. Gene expressions in Arabidopsis were analyzed by qRT-PCR with SYBR Green dye (SYBR® Green Real-time PCR Master Mix-Plus, Code No. QPK-212) and performed on an ABI StepOnePlus Real-Time PCR System. *AtActin2* was used as internal control. The relative expression levels of all these genes were gained using the $2^{-\triangle\triangle CT}$ method. The primers used are listed in supporting information Table S1.

Measurement of IAA content

Under control and strain WM13-24 treatment, 12-day-old seedlings root were collected for measurement of IAA content. The IAA content was determined using the method described by He et al (2021).

Analysis of VCs by SPME-GC-MS

The VCs released by WM13-24 were analyzed in petri dishes containing 1/2 MS medium with a solid-phase microextraction (SPME) technique and GC-MS. The compounds were collected for 15 h with gray SPME fiber (Carboxen/DVB/PDMS, 2 cm, 50/30 μm; Agilent, USA) and desorbed at 230 ℃ for 5 min in the injector port of a gas chromatograph (Agilent 7890D, USA), equipped with an MS detector (7000D, USA), and the MassHunter Workstation software for data acquisition and processing.

Agilent DB1701 capillary column (30 m × 0.25 μm × 0.25 μm) was used. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The column was maintained at 35 ℃ for 3 min at the beginning, then was programmed to 180 ℃ at a rate of 3 ℃/min, further to 230 ℃ at 25 ℃/min, and ultimately held for 5 min. The mass fragments were analysed using electron impact ionization at 70 eV. The compounds were identified by comparison with mass spectra from NIST. Only compounds with the match > 700 were taken and compared with the mass spectra of the NIST/EPA/NIH Mass Spectral Library version (NIST14.L). There were four treatments shown as follows: 1/2 MS medium (control), 1/2 MS medium

contains 14 days-old Arabidopsis, co-cultivation of 14 days-old Arabidopsis and bacterium WM13-24 and 1/2 MS medium only contains bacterium WM13-24.

Statistical analysis

Growth and physiological parameters were presented as means with standard errors ($n \geq 3$). All data were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple comparison test or student's t test were used to detect the significant differences among means at a significance level by SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Effect of *Bacillus subtilis* WM13-24 on seedling growth of Arabidopsis and *H. ammodendron* and root development of Arabidopsis

Inoculation with strain WM13-24 was found to promote biomass production of Arabidopsis in *vitro* and in soil. Strain WM13-24 at three concentrations (10^4, 10^8 and 2×10^8 CFU mL^{-1}) significantly increased shoot dry weight (1.2-fold, 1.6-fold and 1.9-fold, respectively), root dry weight (93%, 1.2-fold and 1.5-fold, respectively), leaf chlorophyll *a* (14%, 32% and 43%, respectively) and chlorophyll *b* content (12%, 28% and 39%, respectively) ($P < 0.05$) of Arabidopsis compared to control (Fig. 1A, B, C). Correspondingly, strain WM13-24 at the concentration of 10^4 and 2×10^8 CFU mL^{-1} significantly ($P < 0.05$) increased plant photosynthetic rate by 33% and 51%, respectively (Fig. 1D). In addition, strain WM13-24 significantly ($P < 0.05$) increased total fresh weight of *H. ammodendron* seedlings by 17% and 35% and total dry weight by 30% and 25% 14 and 40 days after inoculation of strain WM13-24 at 10^8 CFU mL^{-1}, respectively, compared to control (Fig. 2).

Then, the effects of WM13-24 on Arabidopsis seedling growth and root development were evaluated in sterile plates (Fig. 3A). Strain WM13-24 significantly increased the number of lateral root by 41% ($P < 0.05$) 14 days after inoculation, compared to control (Fig. 3B). In addition to positive effects on LR formation, WM13-24 had a strong impact on root hair development (Fig. 3C). *B. subtilis* WM13-24 showed the similar growth promotion effect on Arabidopsis compared to *B. amyloliquefaciens* GB03 as shown in Fig. S1.

Screening Arabidopsis-signaling pathway mutants for regulatory control of root development

In order to probe the mechanism by which bacterial volatiles can regulate root development, PGPR strain WM13-24 was tested against a series of Arabidopsis mutants defective in specific regulatory pathways,

and total root length, LR number and LR density were analyzed at 14 days. The mutants included *ein3-1* and *etr1* for ethylene, *cre1* for cytokinin, *gai* for gibberellin acid, *jar* and *coi1* for jasmonic acid, *cbb1* for brassinosteroid, *NahG* for salicylic acid. As showed in Fig. 4, these mutants (*ein3-1*, *etr1*, *cre1*, *gai*, *jar*, *coi1*, *cbb1* and *NahG*) responded to VCs like Col-0 by increasing total root length (1.9-fold, 63%, 30%, 1.2-fold, 38%, 1.6-fold, 1.2-fold and 77%, respectively) (Fig. 4B), LR number (77%, 73%, 28%, 94%, 41%, 43%, 47% and 32%, respectively) (Fig. 4C) and lateral root density (73%, 67%, 29%, 64%, 56%, 35%, 59% and 33%, respectively) ($P < 0.01$) (Fig. 4D) 14 days after inoculation. Although strain WM13-24 also promoted root development in the cytokinin receptor mutant *cre1* to a certain extent, the effect was much lower than that of the wild type Col-0, indicating that cytokinin was involved in the process of root development promoted by WM13-24, and the role of cytokinin in this process needs to be further investigated.

To further address the role of auxin signaling in WM13-24-induced root development, we analyzed the responses of Col-0 and Arabidopsis single or double mutants (*arf7*, *arf19*, *arf7arf19*, *axr1*, *axr2*, *axr4* and *eir1*) defective in auxin signaling to VCs emitted from strain WM13-24 (Fig. 5). The *arf19*, *axr1*, *axr4* and *eir1* mutants, which formed a significantly reduced total root length under control treatment, responded to VCs emitted from strain WM13-24 with increased total root length, but only approximately 45%, 40%, 46% and 58% of that of WM13-24-treated Col-0, respectively (Fig. 5B). However, the double mutant *arf7arf19* did not respond to the VCs without increased total root length (Fig. 5B). The VCs increased LR number of *arf7*, *arf19*, *axr1*, *axr2*, *axr4* and *eir1* mutants by 2-fold, 88%, 25%, 21%, 39% and 11% ($P < 0.01$), respectively, compared to their individual control, however, was not capable of rescuing the LR-defective phenotype of the *arf7arf19* mutant, whose lateral root formation is completely abolished (Fig. 5C).

Effect of *Bacillus subtilis* WM13-24 VCs on auxin response of Arabidopsis

LR formation is tightly correlated with auxin signaling (Fukaki et al. 2007). To test the role of auxin response in WM13-24 VCs-induced LR formation, transgenic plants harbouring *DR5::GUS* and auxin-responsive marker *DR5::GFP* were used. WM13-24 VCs enhanced the expression of the GUS in the entire root, especially in the lateral root primordia, compared with control (Fig. 6A). Similarly, the VCs also increased the green fluorescence level of GFP in the apical meristem and lateral root (Fig. 6D). To probe the stages of LRP that are affected by strain WM13-24, the developmental stage of each LRP on WM13-24-treated roots was classified according to Malamy and Benfey (1997). LRP stages - and - were significantly increased in strain WM13-24 VCs treated seedlings (Fig. 6B). Strain WM13-24 VCs significantly increased the number of total LRP by 180%, compared to control (Fig. 6C). Furthermore, the expression levels of genes related to LR formation were analyzed using qRT-PCR. The expression levels of *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) and *LBD29* were induced significantly by WM13-24 VCs, although those of *ARF7* and *ARF19* were not altered (Fig. 6E).

Therefore, we further measured root IAA level and the expression levels of genes related to IAA biosynthesis, *ASA1* (*ANTHRANILATE SYNTHASE SUBUNIT 1*), *TAA1*, *YUC5*, *YUC8*, *YUC9*, *NIT1* (*NITRILASE*

1) and *NIT2*, and auxin dynamic balance maintenance, *GH3.1* (*GRETCHEN HAGEN 3.1*), *GH3.5* and *GH3.6* (Staswick et al. 2005), using qRT-PCR. WM13-24 VCs enhanced root IAA level by 73% compared with control (Fig. 7A). WM13-24 VCs significantly increased the expression levels of *ASA1*, *YUC5*, *YUC8*, *YUC9*, *NIT1*, *NIT2, GH3.1*, *GH3.5* and *GH3.6* by 25%, 43%, 17%, 28%, 21%, 37%, 27%, 17% and 39%, compared with their individual controls ($P < 0.05$) (Fig. 7B). The major route of IAA biosynthesis is the indole-3-puruvate pathway (Kasahara 2016). To test whether the increase in LR formation was caused by auxin biosynthesis under strain WM13-24 VCs treatment. The involvement of the indole-3-puruvate pathway was evaluated using 0.5 and 1 μM L-kynurenine and 4-phenoxyphenyl boronic acid, which inhibit TAA1/TARs and YUCs (Inaji et al. 2020), respectively (Fig. S2A). In the presence of 0.5 and 1 μM 4-phenoxyphenyl boronic acid, WM13-24 VCs still stimulate LR formation with increased LR number, whereas in the presence of 1 μM L-kynurenine, LR formation was inhibited (Fig. S2B). The results shown that TAA1-mediated auxin biosynthesis may require for strain WM13-24 VCs-promoted LR formation.

Effect of *Bacillus subtilis* WM13-24 VCs on auxin polar transport of Arabidopsis

In order to investigate the role of auxin transport in WM13-24 VCs-induced lateral root formation, the LR phenotype of the auxin influx mutant *aux1-7* was used (Fig. 8A). The LR number of *aux1-7* was less than that of Col-0 under control condition, but WM13-24 VCs could still increase LR number of *aux1-7* by 47% (similarly by 41% for Col-0) ($P < 0.05$) (Fig. 8B). Furtherly, to test the possible role of auxin polar transport in WM13-24 VCs-induced lateral root formation, the auxin efflux inhibitor 2-((1 naphthalenylamino)-carbonyl) benzoic acid (NPA) was supplemented to the plant growth media. In the presence of 1 μM NPA, WM13-24 VCs still stimulate LR formation with increased LR number, whereas in the presence of 5 μM NPA, LR formation was completely inhibited and WM13-24 VCs could not stimulate LR formation (Fig. 8C). Above results suggested auxin efflux was required for WM13-24 VCs-stimulated LR formation. Thus, we analyzed the levels of four auxin efflux carriers, PIN1, PIN2, PIN4 and PIN7, in WM13-24 VCs exposed transgenic lines expressing *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN4::PIN4::GFP* and *PIN7::PIN7::GFP*. As shown in Fig. 9, WM13-24 VCs significantly reduced the green fluorescence level of PIN4 and slightly reduced those of PIN1 and PIN2, whereas significantly induced that of PIN7 compared with their individual controls (Fig. 9A, B). Subsequently, the gene expression levels of above four auxin efflux carriers and auxin influx carriers, AUX1, LAX1, LAX2 and LAX3, were analyzed using qRT-PCR. The expression levels of *PIN1*, *PIN2* and *PIN4* were reduced, whereas *PIN7* was induced significantly by WM13-24 VCs, compared with their individual controls (Fig. 9C), which was consistent with the changes of their green fluorescence levels. The expression levels of *AUX1*, *LAX1* and *LAX2* were significantly reduced by WM13-24 VCs (Fig. 9C).

Analysis of *Bacillus subtilis* WM13-24 VCs and their effects on plant growth promotion

Solid-phase microextraction and GC-MS were used to identify volatile compounds produced by strain WM13-24 alone or WM13-24 in the interaction with Arabidopsis seedlings. A total of 18 kinds of VCs, including alcohols, esters and others, were detected in the headspace of plates with strain WM13-24 compared with control (1/2 MS medium) or Arabidopsis seedling only (Table 1). Among these 18 kinds of VCs, acetoin, 2,3-butanediol and benzyl alcohol were detected in the plates with WM13-24 alone (Fig. S3) and acetoin and 2,3-butanediol in the plates with WM13-24 in the interaction with Arabidopsis seedlings (Fig. S4).

Table 1
VCs produced by strain WM13-24 as measured by SPME-GC-MS

No.	VCs	Retention time (min)	Molecular formula	Match
1	nitroso-methane,	1.381	CH_3NO	875
2	Ethyl acetate	2.166	$C_4H_8O_2$	882
3	Acetic acid ethenyl ester	2.243	$C_4H_6O_2$	923
4	1-butanol	3.516	$C_4H_{10}O$	859
5	Acetic acid	4.206	$C_2H_4O_2$	931
6	α-methyl-benzene ethanol	4.537	$C_9H_{12}O$	778
7	Acetoin	6.456	$C_4H_8O_2$	889
8	[S-(R*, R*)]-2, 3-butanediol	10.160	$C_4H_{10}O_2$	939
9	1-ethyl-2-methyl-benzene	11.956	C_9H_{12}	802
10	2-ethyl-butanoic acid	12.204	$C_5H_{10}O_2$	837
11	α-Methylstyrene	12.398	C_9H_{10}	920
12	1-methyl-4-propyl-benzene	12.962	$C_{10}H_{14}$	890
13	Formic acid, heptyl ester	14.272	$C_8H_{16}O_2$	872
14	1-octanol	18.915	$C_8H_{18}O$	922
15	Benzyl alcohol	20.054	C_7H_8O	935
16	Phenol	20.778	C_6H_6O	886
17	4-(1, 1-dimethylpropyl)-cyclohexanone	22.187	$C_{11}H_{20}O$	714
18	1-nonanol	23.415	$C_9H_{20}O$	946

2,3-butanediol has been reported to promote plant growth (Perez-Flores et al. 2017; Ryu et al. 2003; Li et al. 2021). Therefore, the effect of other compounds (α-methyl-benzeneethanol, 1-nonanol, 4-(1,1-dimethylpropyl)-cyclohexanone and 1-butanol), benzyl alcohol and mixtures of benzyl alcohol and 2, 3-butanediol on plant growth were examined in this study. To determine the growth promotion effects of benzyl alcohol, the compound at various concentrations were applied to Arabidopsis (petri dishes), respectively. The growth promotion effect of benzyl alcohol is dose-dependent (Fig. 10A). Benzyl alcohol

at 50 and 100 μM significantly increased the total fresh weight of Arabidopsis seedlings by 21% and 24%, respectively, however, significantly reduced the total fresh weight by 35% and 59%, respectively, at 750 and 5000 μM (Fig. 10B). Benzyl alcohol at 50, 100, 200 and 500 μM significantly increased LR number by 43%, 68%, 52% and 22%, respectively (Fig. 10C). Benzyl alcohol at all concentrations tested significantly increased LR density (Fig. 10D).

Above result indicated that benzyl alcohol at 50 and 100 μM had the best growth promotion effect. 50 or 100 μM of benzyl alcohol mixed with 2,3-butanediol at 50, 100 or 200 μM were applied to Arabidopsis, respectively (Fig. 11A). Mixture of benzyl alcohol and 2,3-butanediol at 50 + 50, 50 + 100, 50 + 200, 100 + 50, 100 + 100 and 100 + 200 μM significantly increased LR number by 22%, 31%, 29%, 44%, 50% and 50% (Fig. 11C), and lateral root density by 25%, 44%, 23%, 23%, 49% and 51%, respectively ($P < 0.05$) (Fig. 11D). Moreover, other compounds such as α-methyl-benzeneethanol, 1-nonanol and 4-(1,1-dimethylpropyl)-cyclohexanone, detected in strain WM13-24 VCs cannot induce LR formation tested by those pure compounds (Fig. S6, S7). 1-butanol at 100 μM increased the total fresh weight and lateral root density of Arabidopsis seedlings (Fig. S5).

Benzyl alcohol, 2, 3-butanediol and their mixture were also applied to *Bacillus subtilis* WM13-24's host plant *H. ammodendron* to determine their growth promotion effects. Benzyl alcohol at 100 μM, 2,3-butanediol at 500 μM and their mixture at 100 + 500 μM significantly increased the total fresh weight by 13%, 9% and 7%, and total dry weight by 20%, 15% and 10%, respectively ($P < 0.05$) (Fig. S8).

Discussion

PGPR or PGPF colonize the plant rhizosphere and provide a number of beneficial functions for their host and induce plant systematic resistance against biotic and abiotic stresses (Zamioudis 2013; Sun et al. 2020). The contribution of PGPR or PGPF can be exerted through different mechanisms including root system architecture modulation and growth promotion by production of phytohormones such as auxin or indole derivative, small molecules or volatile compounds (Spaepen et al. 2014; Verbon et al. 2016; Sun et al. 2020; Li et al. 2021). In current study, plant root inoculation with *Bacillus subtilis* WM13-24 from the rhizosphere of *Haloxylon ammodendron* in pots significantly promoted the growth of both model plant Arabidopsis and its host plant *Haloxylon ammodendron*. By employing a germ-free experimental system, we demonstrated the possible mechanism of how VCs released from WM13-24 promoted root development using mutants of Arabidopsis with perturbations in hormone signaling.

Auxin signaling and transport are essential for *Bacillus subtilis* WM13-24-stimulated root architecture improvement

Auxin plays essential roles in root system development and root meristem maintenance (Overvoorde et al. 2010; Hu et al. 2021). The auxin distribution in the root tip controls many aspects of root phenotype (Hu et al. 2021). Other phytohormones were shown to regulate LR development through crosstalk with auxin (Ivanchenko et al. 2008; Sharifi et al. 2018). López-Bucio et al (2007) demonstrated that *Bacillus megaterium* promoted plant growth and altered root architecture may involve auxin- and ethylene

independent mechanisms. Arabidopsis root system development upon *Martelella endophytica* YC6887 colonization was dependent on auxin signaling, but independent of ethylene and jasmonic acid signaling (Khan et al. 2016). Indole emitted by *Proteus vulgaris* JBLS202 stimulated the growth of Arabidopsis through an interplay between the auxin, cytokinin, and brassinosteroid pathways (Bhattacharyya et al. 2015). *Bacillus siamensis* YC7012 can promote the growth of Arabidopsis by producing VCs independent of auxin, ethylene, or jasmonic acid signaling pathway (Hossain et al. 2019). Garnica-Vergara et al (2016) reported that the production of 6-pentyl-2H-pyran-2-one by *Trichoderma atroviride* directly influences root architecture via modulation of ethylene signaling and auxin transport (Garnica-Vergara et al. 2016). In this study, WM13-24 altered root architecture by stimulating lateral root formation and root hair development, therefore, water and nutrient acquisitions were increased, leading to improved biomass production. the *arf7arf19* mutant did not form any LRs in response to VCs (Fig. 5C), suggesting that VCs emitted from WM13-24 directly influences auxin response transcription factors AUXIN RESPONSE FACTOR7 (ARF7) and ARF19-mediated auxin signaling pathway in the root of Arabidopsis.

We observed enhanced *DR5::GUS* and *DR5::GFP* blue spots and fluorescence after WM13-24 VCs treatment in the roots, particularly in the apical meristem and LR primordia, indicating that the auxin signaling and auxin distribution were altered by WM13-24 VCs during the LR initiation program (Fig. 6). Auxin accumulation and distribution were regulated by its biosynthesis and transport (Stepanova et al. 2008; Yamada et al. 2009; Sun et al., 2020). Strain WM13-24 VCs treatment significantly increased the expressions of IAA biosynthesis-related genes and IAA content in roots (Fig. 7). Our results indicated that WM13-24 VCs increased the expression of the DR5:GUS and DR5:GFP reporter in root by enhancing auxin biosynthesis.

Polar auxin transport plays important roles to modulate local auxin levels (Blilou et al. 2005; Krecek et al. 2009; Liu et al. 2015; Hu et al. 2021). Treatment with the auxin transport inhibitor NPA affects root growth (Zhang et al. 2007; Zamioudis et al. 2013), and these results suggest that disturbing polar auxin transport changes auxin accumulation in root and subsequently alters root growth (Raya-González et al. 2014). In this study, NPA compromised the ability of WM13-24 to promote LR formation, suggesting that polar auxin transport is involved in WM13-24 VCs-mediated root growth. Consistent with this result, PIN4-GFP was significantly reduced by WM13-24 VCs treatment, whereas PIN1-GFP and PIN2-GFP fluorescence were not significantly changed (Fig. 9). PGPR increased LR formation associated with regulated expression of auxin transporters (Sun et al. 2020; Garnica-Vergara et al. 2016). Therefore, these results indicated that PIN4 at least partly was involved in WM13-24 VCs-induced changes of polar auxin transport in root and root growth. How PIN4 is involved in WM13-24-induced root growth remains to be explored. Taken together, the results demonstrated that the VCs from strain WM13-24 stimulated lateral root formation by increasing DR5:GUS and DR5:GFP reporter expression in root via enhancement of auxin biosynthesis and PIN4-mediated polar auxin transport.

Benzyl alcohol and 2,3- butanediol are major growth promotion-related VCs emitted by strain *Bacillus subtilis* WM13-24

Microbial volatiles are highly diverse and strain-specific (Blom et al. 2011; Lee et al. 2016). The VCs produced by certain PGPR or PGPF can be used for plant-bacterium communication as plant growth promotion triggers. In recent years, a number of VCs that increase fresh weight, root length and the number of lateral roots in plants had been identified, such as acetoin (Ryu et al. 2003; Li et al. 2021); 6-pentyl-2H-pyran-2-one (Garnica-Vergara et al. 2016); dimethyl disulfide, dimethyl trisulfide, acetophenone and 3-hexanone (Cordovez et al. 2018; Groenhagen et al. 2013); ethyl isovalerate, isoamyl acetate, 3-methyl-1-butanol, benzyl alcohol, 2-phenylethyl alcohol and 3-(methylthio)-1-propanol (Camarena-Pozos et al. 2019); 3-methylbutanol, dodecyl aldehyde, isovaleraldehyde, isoamyl propionate, isovaleric acid and 2-heptanone (Syed-Ab-Rahman et al. 2019). 2,3-butanediol and acetoin were already reported to promote plant growth and LR development (Ryu et al. 2003; Li et al. 2021). To understand the possible role of VCs released from WM13-24 in phytostimulation, we monitored VCs production by strain WM13-24 alone or WM13-24 in the interaction with Arabidopsis seedlings. The results showed that benzyl alcohol and the mixture treatment (combination of benzyl alcohol and 2,3-butanediol) increased LR number, especially benzyl alcohol treatment had higher LR numbers than mixture (Fig. 10; Fig. 11). These results indicated that the effects of VCs on plant growth and development cannot be simply explained by the arithmetic addition of compounds, because organisms were evolutionarily attuned to relative concentrations of volatiles than to absolute amounts (Sharifi and Ryu 2018). Li et al (2021) reported that acetoin, a major component of *Bacillus amyloliquefaciens* VCs, is less active in promoting root development compared to VC blends from *B. amyloliquefaciens* (Li et al. 2021). In many cases, exposure of plants to discrete (individual) VCs or VOC mixtures either failed to reproduce or only partially reproduced the effects induced by the complex blends of VCs emitted by PGPR (Cordovez et al. 2017; Groenhagen et al. 2013; Naznin et al. 2013; García-Gómez et al. 2019). Our result showed that mixture (combination of benzyl alcohol and 2,3-butanediol) treatment produced fewer LR numbers compared to inoculation with WM13-24.

Our approach to screen for VCs from WM13-24 involved in growth promotion using Arabidopsis was also effective in host plants *H. ammodendron*. Benzyl alcohol, 2,3-butanediol and their mixture also promote the growth of desert plant *H. ammodendron*. This results suggesting that application of the identified volatiles in the field could speed up growth of desert plants. However, it remains to be elucidated how VCs are perceived by plants and how these signals are interpreted and processed across plant species (Sharifi and Ryu 2018; Camarena-Pozos et al. 2018).

Conclusion

Bacillus subtilis WM13-24 was able to promote the growth of both Arabidopsis and its host plant, *Haloxylon ammodendron*. *Bacillus subtilis* WM13-24 inoculation altered the root architecture of Arabidopsis by increasing the number of lateral roots and elongating root hairs through enhancing auxin accumulation in roots via increased auxin biosynthesis and PIN4-mediated polar auxin transport. Bioactive VCs emitted by WM13-24 mainly include 2, 3-butanediol and benzyl alcohol and they, alone or in combination, promoted growth of both Arabidopsis and host plant *Haloxylon ammodendron*. These VCs could be applied in plant production in arid and semi-arid environments.

Declarations

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Author contributions

Jin-Lin Zhang conceived the project and planned the experiments. Ao-Lei He, Wei Ren, Hui-Ru Li and Ling-Yu Zhao carried out the experiments. Ao-Lei He and Wei Ren analyzed the data. Ao-Lei He and Jin-Lin Zhang wrote the manuscript., Paul W. Paré and Qi Zhao revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

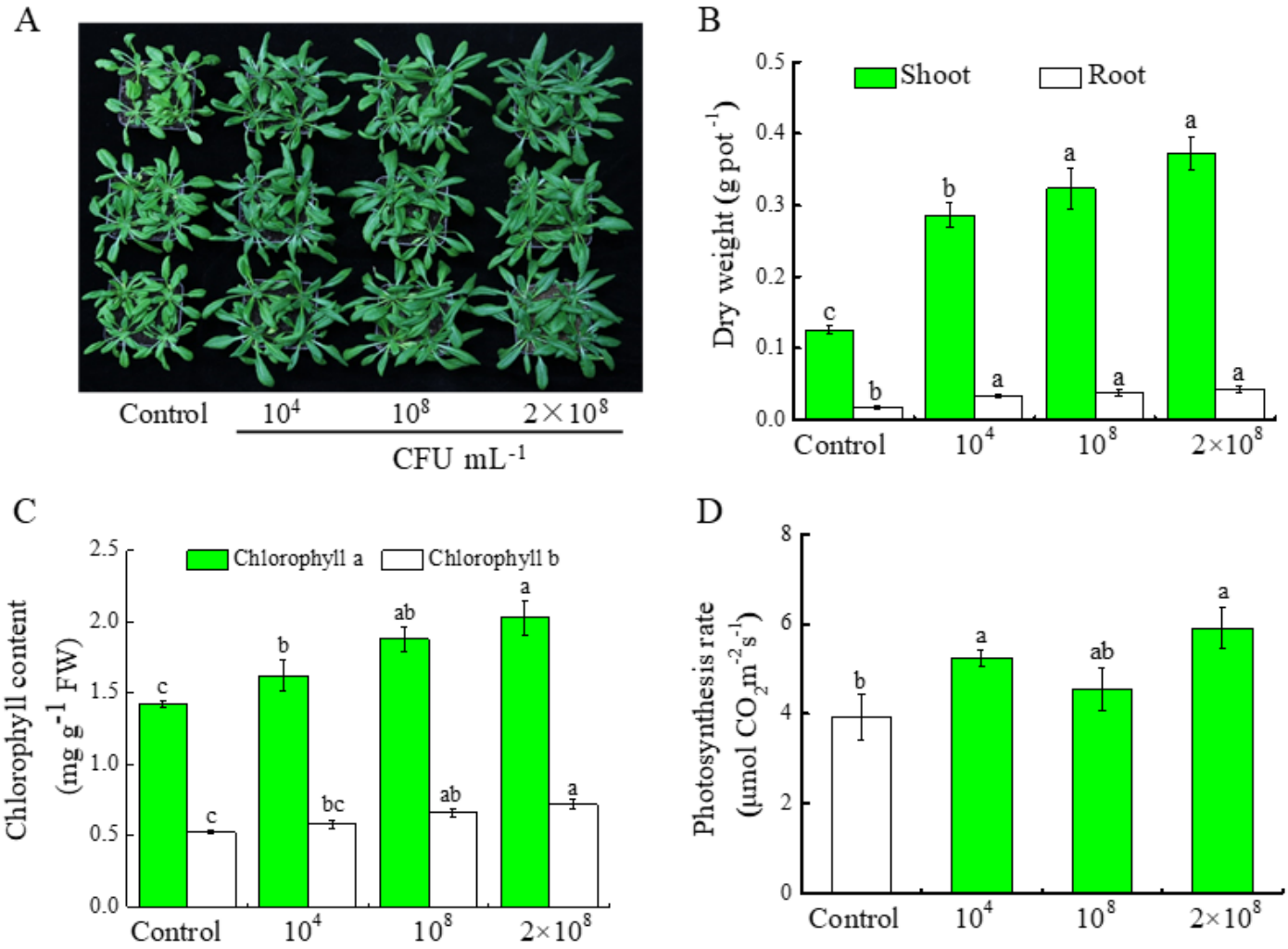


Figure 1

Effects of the inoculation of *Bacillus subtilis* WM13-24 on the growth of Arabidopsis in the pots. Plant growth performance (A). Plant dry weight (B), Chlorophyll contents (C) and photosynthesis rate (D) were measured 14 days after inoculation. Values are means and bars indicated standard errors (SEs) (n = 10). Same colored columns with different letters indicated significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

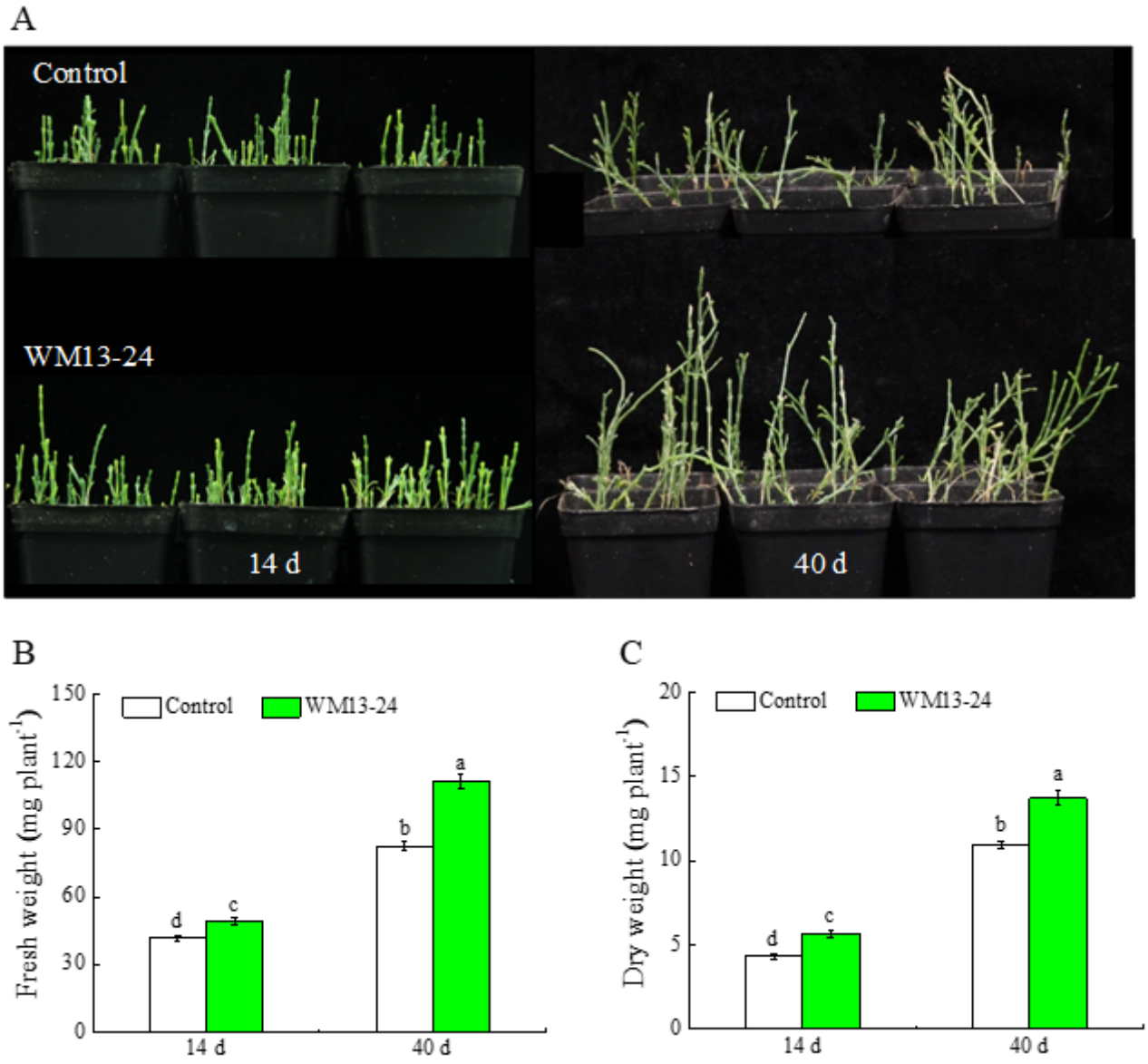


Figure 2

Effects of the inoculation of *Bacillus subtilis* WM13-24 on the growth of *Haloxylon ammodendron* in the pots. Plant fresh weight (B) and dry weight (C) were measured 14 or 40 days after inoculation. Values are means and bars indicated standard errors (SEs) (n= 10). Columns with different letters indicated significant differences at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

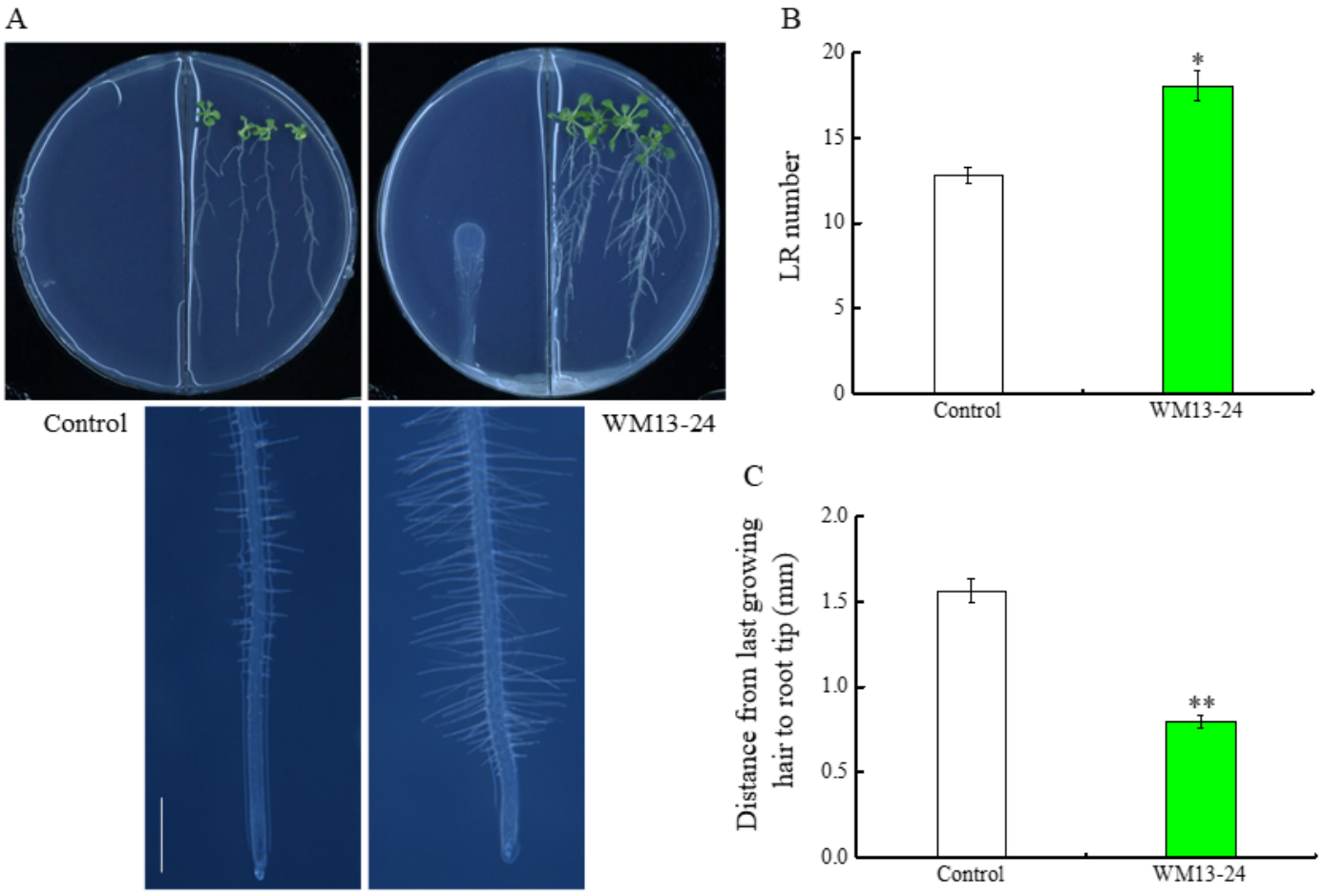


Figure 3

Effects of VCs released from *Bacillus subtilis* WM13-24 on root architecture of Arabidopsis. Lateral root (LR) number (B) and the distance from the last growing root hair to root tip (C) were measured 14 days after inoculation of *Bacillus subtilis* WM13-24 and sowing of the seeds of Arabidopsis. Values are means and bars indicated standard errors (SEs) (n = 10). Asterisks indicated significant differences between WM13-24 treatment and control (P < 0.01).

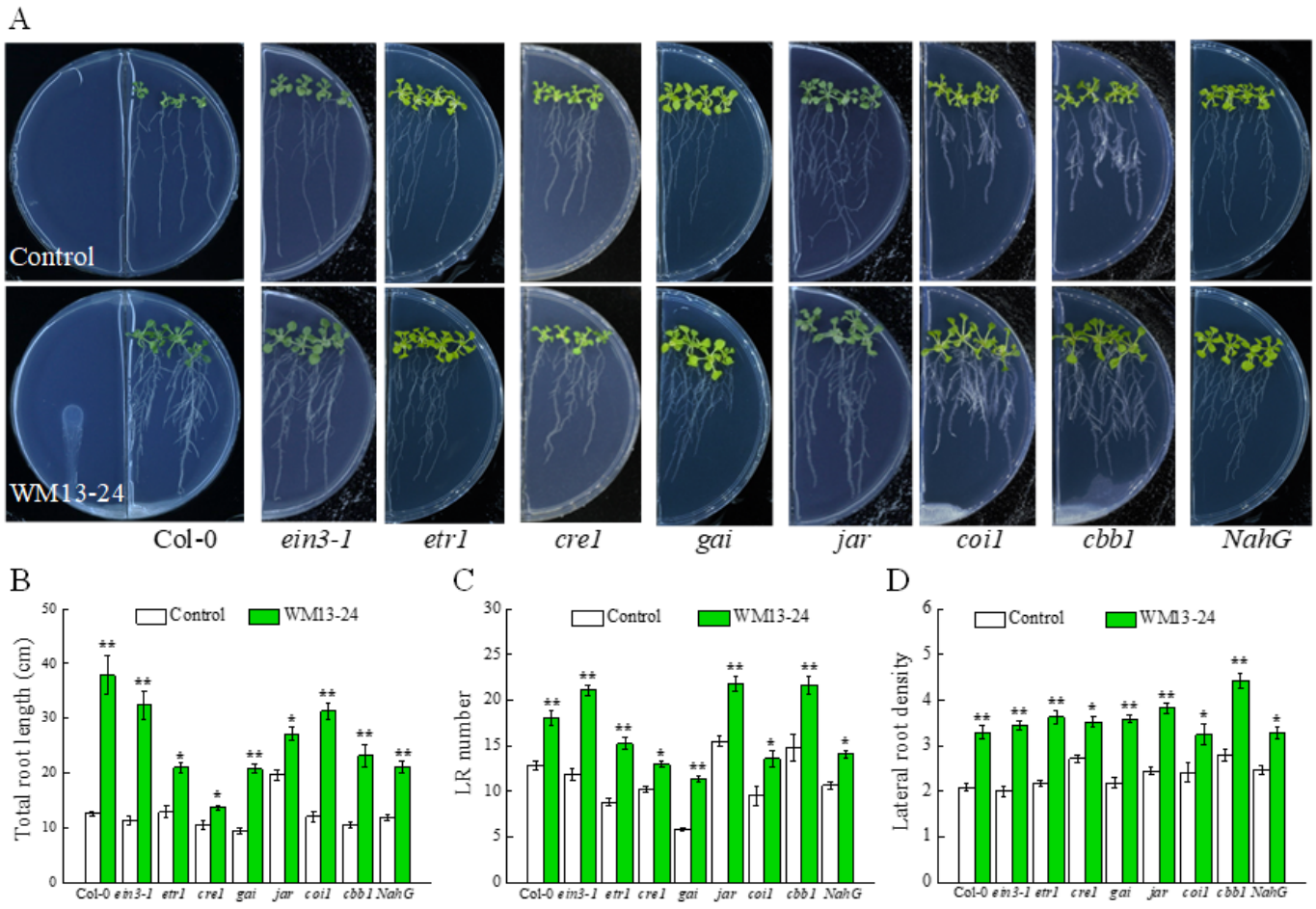


Figure 4

Effects of VCs released from *Bacillus subtilis* WM13-24 on the growth of Arabidopsis mutants (*ein3-1*, *etr1*, *cre1*, *gai*, *jar*, *coi1*, *cbb1* and *NahG*). Plant growth performance (A). Total root length (B), LR number (C) and lateral root density (D) were measured 14 days after inoculation of *Bacillus subtilis* WM13-24 and sowing of the seeds of Arabidopsis. Total root length was observed using a root scan apparatus (Perfection V700Photo, Seiko Epson Corp, Japan) equipped with WinRHIZO software (Regent Instruments Inc., Canada). Values are means and bars indicated standard errors (SEs) (n= 20). Asterisks indicated significant differences between WM13-24 treatment and control (P < 0.01).

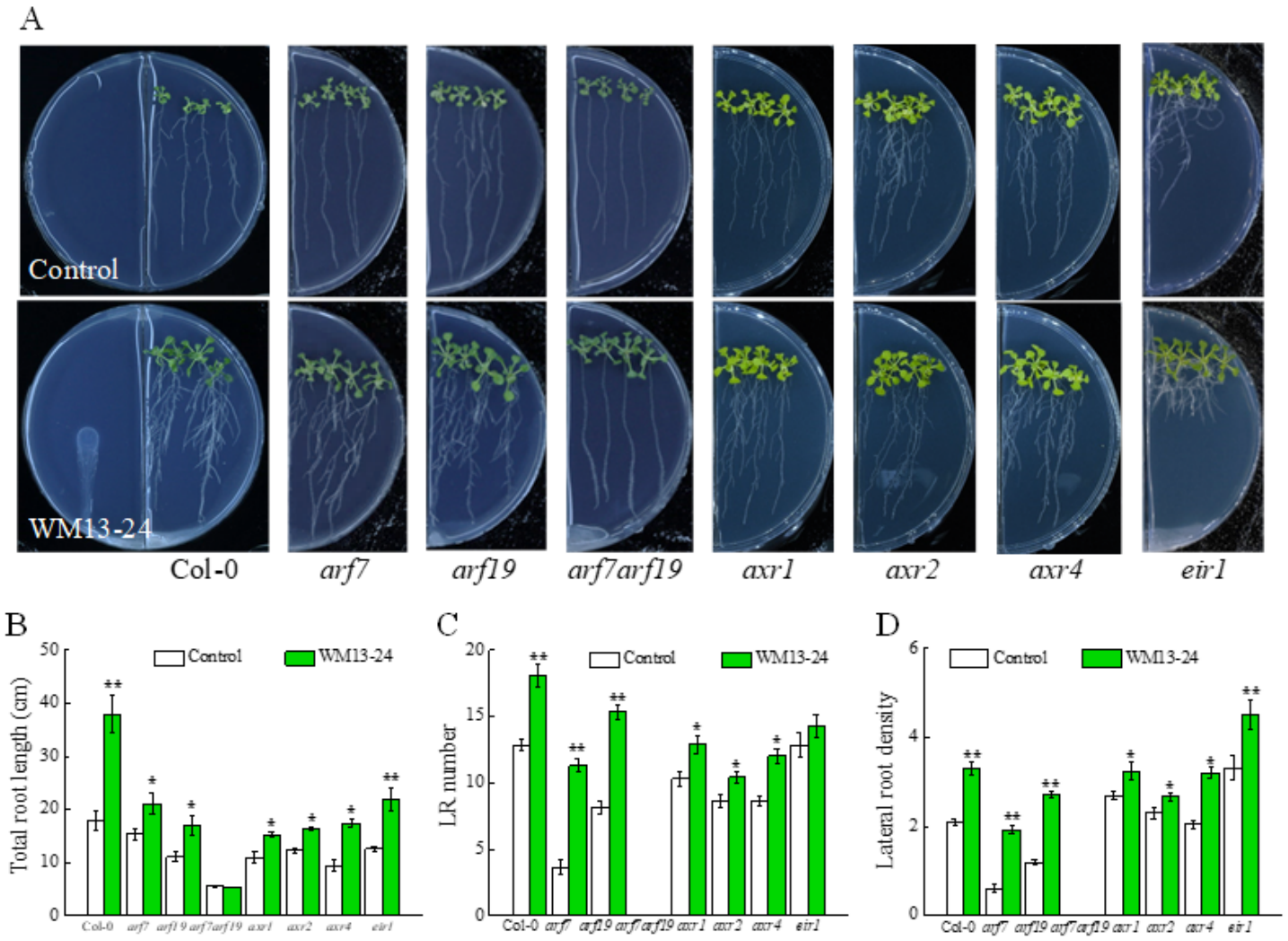


Figure 5

Effects of VCs released from *Bacillus subtilis* WM13-24 on the growth of Arabidopsis mutants (*arf7*, *arf19*, *arf7arf19*, *axr1*, *axr2*, *axr4* and *eir1*). Plant growth performance (A). Total root length (B), LR number (C) and lateral root density (D) were measured 14 days after inoculation of *Bacillus subtilis* WM13-24 and sowing of the seeds of Arabidopsis. Total root length was observed using a root scan apparatus (Perfection V700Photo, Seiko Epson Corp, Japan) equipped with WinRHIZO software (Regent Instruments Inc., Canada). Values are means and bars indicated standard errors (SEs) (n = 20). Asterisks indicated significant differences between WM13-24 treatment and control (P < 0.01).

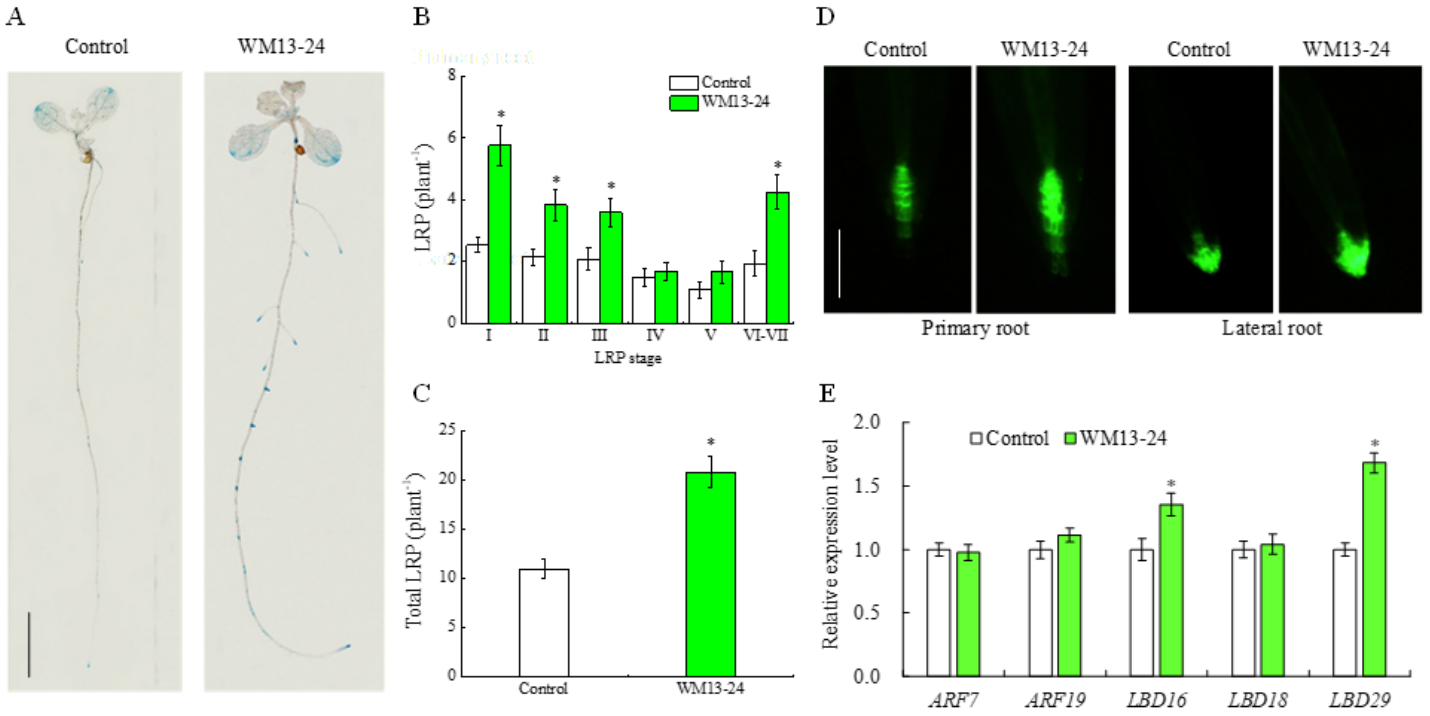


Figure 6

Effects of *Bacillus subtilis* WM13-24 VCs on auxin response in Arabidopsis. Auxin responses monitored using *DR5::GUS* transgenic plants (Scale bar, 50 mm) (A), LRP number at different stages (B), The total number of LRP (C), Auxin responses monitored using *DR5::GFP* transgenic plants (Scale bar, 5 mm) (D), The expressions of auxin signaling and lateral root growth related genes in the roots of Col-0 seedlings (E). *AtActin2* was used as the reference gene. Values are means and bars indicated standard errors (SEs) (n = 3 or 12). "*" indicated significant differences between WM13-24 treatment and control (P < 0.05).

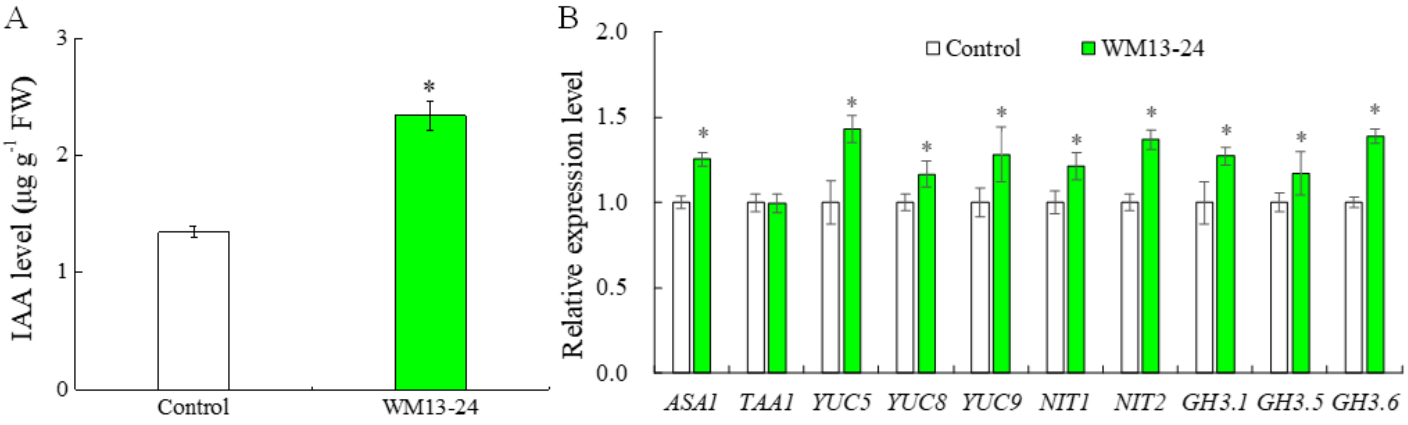


Figure 7

Effects of VCs released from *Bacillus subtilis* WM13-24 on IAA content of Arabidopsis root. IAA content (A), The expression of auxin biosynthesis related genes in the roots of Col-0 seedlings (B). *AtActin2* was used as the reference gene. Values are means and bars indicated standard errors (SEs) (n = 3). "*" indicated significant differences between WM13-24 treatment and control ($P < 0.05$).

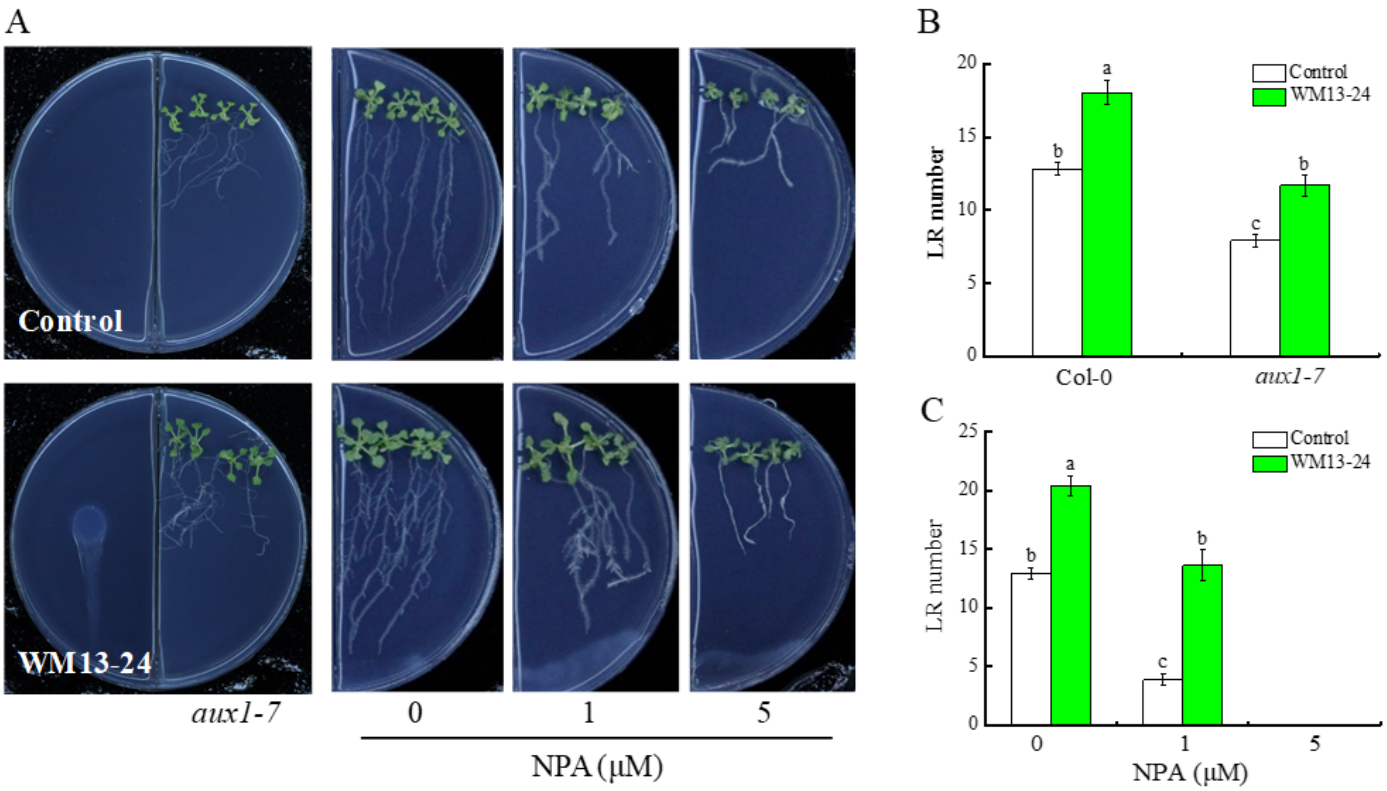


Figure 8

Effects of auxin transport on *Bacillus subtilis* WM13-24 VCs-mediated LR formation in the Arabidopsis. Plant growth performance (A). LR number in the auxin influx mutant *aux1-7* (B) and Arabidopsis wild-type (Col-0) (C) were measured 14 days after inoculation of *Bacillus subtilis* WM13-24 and sowing of the seeds of Arabidopsis. Col-0 seedlings were grown on a medium containing 0, 1 or 5 μM NPA dissolved with DMSO (Dimethyl sulfoxide). Values are means and bars indicated standard errors (SEs) (n = 20). Columns with different letters indicated significant differences at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

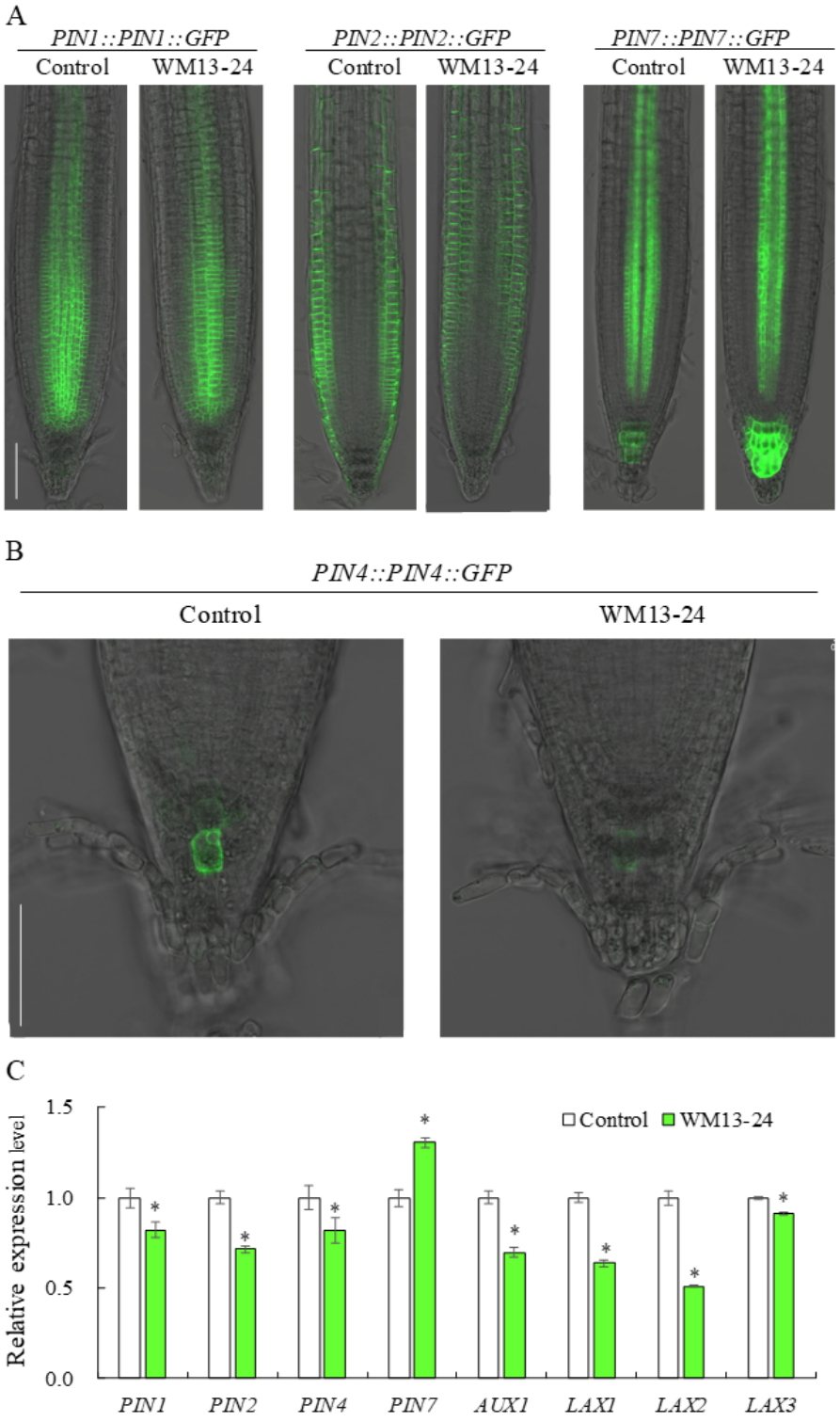


Figure 9

Bacillus subtilis WM13-24 regulated auxin polar transport. Confocal images of Arabidopsis transgenic *PIN1::PIN1::GFP*, *PIN2::PIN2::GFP*, *PIN7::PIN7::GFP* (A) and *PIN4::PIN4::GFP*(B) seedlings of the primary root tips were photographed and the expressions of auxin carrier genes in the roots of Col-0 seedlings (C) were analyzed by qRT-PCR 7 days after inoculation of *Bacillus subtilis* WM13-24 and sowing of the seeds of Arabidopsis. Scale bars represented 75 μm. *AtActin2*was used as the reference gene. Values are

means and bars indicated standard errors (SEs) (n = 3). Asterisks indicated significant differences between WM13-24 treatment and control (P < 0.05).

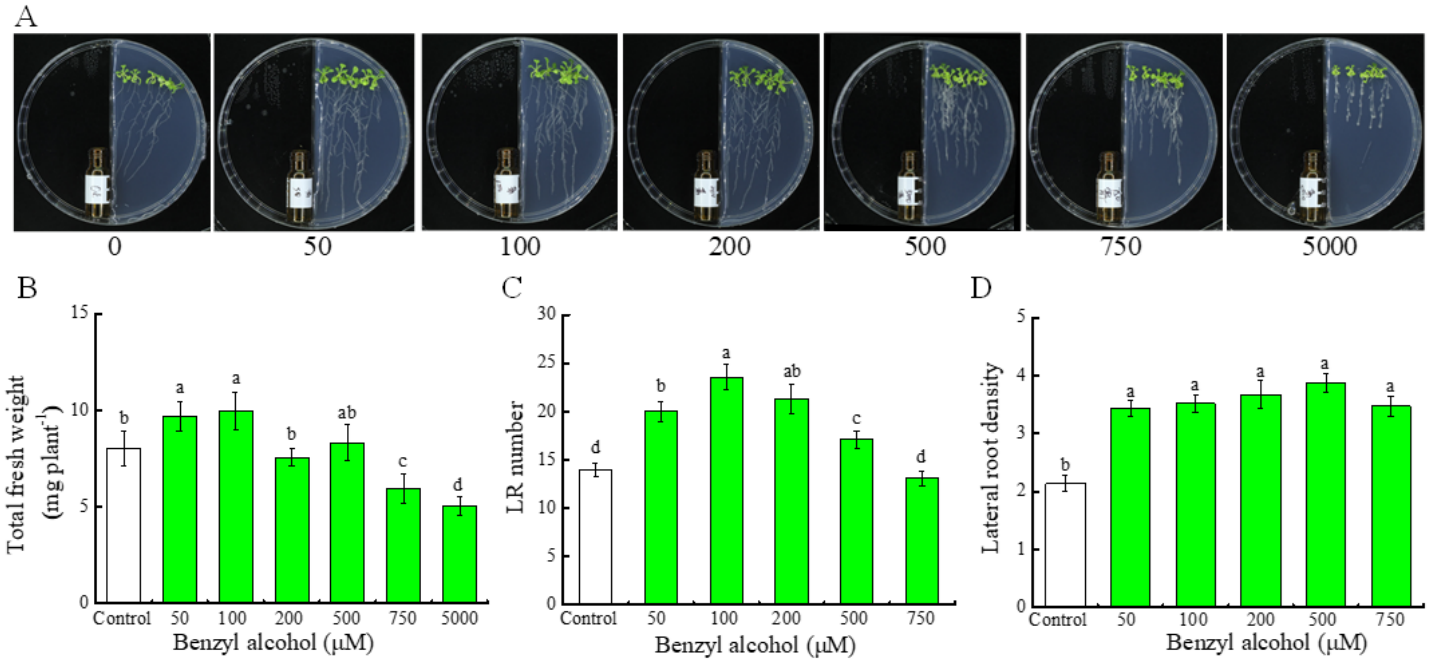


Figure 10

Effects of benzyl alcohol on the growth of Arabidopsis. Plant growth performance (A). Total fresh weight (B), LR number (C) and lateral root density (D) were measured 14 days after exposed to benzyl alcohol at various concentrations. Values are means and bars indicated standard errors (SEs) (n = 20). Same colored columns with different letters indicated significant difference at P < 0.05 (ANOVA and Duncan's multiple comparison test).

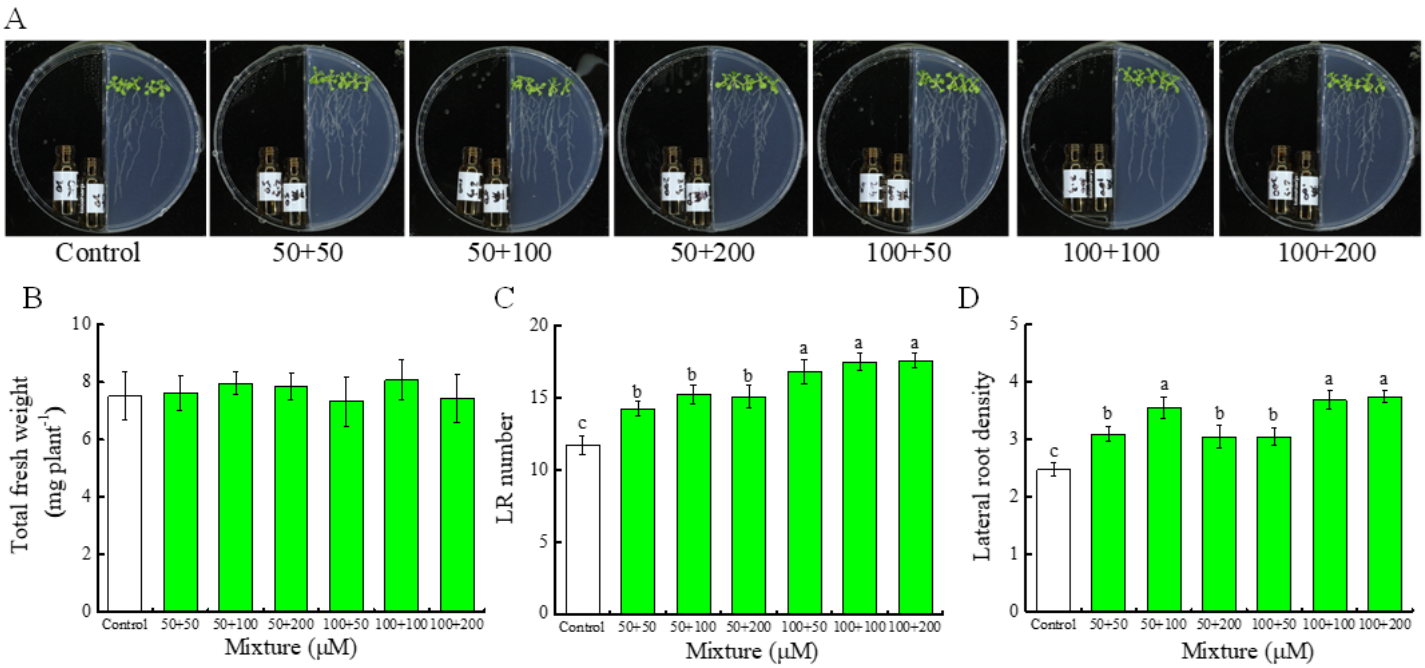


Figure 11

Effects of mixture (combination of 2,3-butanediol and benzyl alcohol) on the growth of Arabidopsis. Plant growth performance (A). Total fresh weight (B), LR number (C) and lateral root density (D) were measured 14 days after exposed to mixture at various concentrations. Values are means and bars indicated standard errors (SEs) (*n*= 20). Same colored columns with different letters indicated significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

Supplementary Files

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- SupplementaryMaterial.docx