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Bacillus megaterium Strain XTBG34 Promotes Plant Growth by Producing 2-Pentylfuran

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Several chemical changes in soil are associated with plant growth-promoting rhizobacteria. An endospore-forming bacterium, strain XTBG34, was isolated from a Xishuangbanna Tropical Botanical Garden soil sample and identified as *Bacillus megaterium*. The strain's volatiles had remarkable plant growth promotion activity in *Arabidopsis thaliana* plants; after 15 days treatment, the fresh weight of plants inoculated with XTBG34 was almost 2-fold compared with those inoculated with DH5α. Head space volatile compounds produced by XTBG34, trapped with headspace solid phase microextraction and identified by gas chromatography–mass spectrometry, included aldehydes, alkanes, ketones and aroma components. Of the 11 compounds assayed for plant growth promotion activity in divided Petri plates, only 2-pentylfuran increased plant growth. We have therefore identified a new plant growth promotion volatile of *B. megaterium* XTBG34, which deserves further study in the mechanisms of interaction between plant growth-promoting rhizobacteria and plants.

***Keywords*:** 2-pentylfuran, *A. thaliana*, *B. megaterium*, gas chromatography-mass spectrometry (GC/MS), plant growth-promoting rhizobacteria (PGPR), solid phase microextraction (SPME)

Plant growth-promoting rhizobacteria (PGPR) can stimulate plant growth in association with the root systems of plants. This phenomenon occurs in most natural soils. PGPR have been applied to a wide range of agricultural species to improve growth, e.g. to increase seedling emergence, plant weight, crop yield, and disease control (Kloepper *et al*., 1980, 1991; Cakmakci *et al*., 2006). For instance, canola seedling emergence increases by 10-40% when seeds are coated with PGPR before planting (Kloepper *et al*., 1991); plant weight of tuber-treated potatoes increases by 80% on average by midseason (Kloepper and Schroth, 1981). Leaf, root, and sugar yield of sugar beet also significantly increase after PGPR inoculation (Cakmakci *et al*., 2006). The use of PGPR as a replacement for chemical soil fertilizers is becoming more and more popular in organic and sustainable agriculture.

PGPR can promote plant growth by producing antibiotics or siderophores that prevent the deleterious effects of plant pathogenic microorganisms (Leong, 1986; Sivan and Chet, 1992), and secreting various phytohormones (Loper and Schroth, 1986; MacDonald *et al*., 1986; Xie *et al*., 1996; Timmusk *et al*., 1999). PGPR can also produce substances that increase mineral, phosphorus, and nitrogen availability in the soil (Lin *et al*., 1983; De Freitas *et al*., 1997; Rabouille *et al*., 2006). PGPR 1-aminocyclopropane-1-carboxylate deaminase can modulate plant growth and development (Safronova *et al*., 2006), and break down plant-produced ethylene (Glick, 1999). Pyrroloquinoline quinine from *Pseudomonas fluorescens* B16 improves plant growth by scavenging reactive oxygen species and hydrogen peroxide (Choi *et al*., 2008), and the volatile components 2,3-butanediol and acetoin are released exclusively from two bacterial strains that trigger growth promotion of *Arabidopsis thaliana* (Ryu *et al*., 2003). Although PGPR plant growth promotion compounds have been identified by a range of methods, their relatively high volatility and low molecular weights means that they cannot be easily identified or traced during plant growth promotion.

In this study, we describe the isolation of a *Bacillus megaterium* strain that has a remarkable ability to promote plant growth in *Arabidopsis*. The strain's volatiles have considerable plant-growth promotion activity. We identified volatile organic compounds from the XTBG34 strain, characterized the volatiles that promote plant growth, and classified and identified XTBG34. Our results suggest that 2-pentylfuran plays an important role in the plant growth promotion activity of this bacterial strain.

Materials and Methods

Soil sampling

Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (21°54'N, 101°38'E), Yunnan, Province of China, was chosen for sampling. Soil samples (~200 g) were taken randomly from the top layer (2-15 cm) of soil. Samples were passed through a 2 mm sieve and stored at 4°C until use.

Plant material

A. thaliana ecotype Columbia seeds were surface-sterilized (soaked

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for 2 min in 70% ethanol followed by 15 min in 1% sodium hypochlorite), rinsed three times with sterile distilled water, and placed on Petri plates containing half-strength Murashige and Skoog salt (½ MS) medium containing 0.6% agar and 1.5% sucrose adjusted to pH 5.8. Seeds were vernalized for 3 days at 4°C in the dark and then placed in a greenhouse set to a 12-h light/12-h dark cycle under 40 W fluorescent lights (100 μE/m^2/sec). The temperature was maintained at 22°C with a relative humidity of 50-60%. Germinated seedlings were transferred to plates after 2 days for subsequent experiments.

Isolation and screening of soil bacteria

Bacteria were isolated from soil samples by the dilution plating technique on ¼ MS agar medium to simulate the nutrient-poor conditions in soil. Isolates were selected and purified, maintained in Luria-Bertani broth (LBB) containing 20% glycerol and stored at -80°C. Isolates with significant plant growth promoting activity were screened according to the modified method described in Ryu *et al*. (2003). One day before plant experiments, bacterial strains were cultured on tryptic soy agar plates as described above and scraped into sterile distilled water. The liquid suspension culture was diluted with water to yield 10^9 CFU/ml based on optical density and serial dilutions with plate counts. A small glass Petri plate (6 cm diameter) was placed inside a larger glass Petri plate (12 cm diameter) (Fig. 1) prepared with MS solid medium, and 2-day-old germinated *Arabidopsis* seedlings (14 seedlings per plate) were transferred to the larger part of the plates. Treated plants were inoculated with 20 μl of a given bacterial strain or sterile distilled water applied dropwise onto the center of the small Petri plate. Plates were sealed with parafilm and arranged in a completely randomized design, and placed the greenhouse under the conditions mentioned above. *Escherichia coli* strain DH5α, which lacks plant growth promotion activity, was used as control. After incubation for 15 days at 28°C, the fresh weight per plant was measured.

Strain identification

Strain morphology and motility of bacteria were investigated by microscopic examination. Gram reaction, oxidative/fermentative glucose utilization, presence of catalase and oxidase, temperature sensitivity, and the assimilation of 49 different carbon sources were studied using the API kit (bioMérieux, France) according to the manufacturer's protocol. Bacterial 16S rRNA gene was amplified and sequenced by PCR using the combination of universal primer 1492r (5′-GGTTACC TTGTTACG ACTT-3′) and bacterial primer 27f (5′-AGAGTTTGAT CCTGGCTCAG-3′) (Lane, 1991); and bacterial recA gene was amplified and sequenced by forward primer (5′-CAACAGCAGGGC GGACAGGC-3′) and reverse primer (5′-TGTTCACGCACTTGTCC CGCA-3′). The resulting 16S rRNA gene and recA gene sequences were compared in a BLAST search to those in the National Library of Medicine (Bethesda, USA) database (Altschul *et al*., 1997). Phylogenetic analysis was performed using the MEGA software packages (Version 4.0) (Kumar *et al.*, 2008) after multiple alignments of data by CLUSTAL X (Thompson *et al.*, 1997), with gaps treated as missing data. The relationships among sequences were analyzed using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by analyzing 1,000 randomized data sets (Felsenstein, 1985).

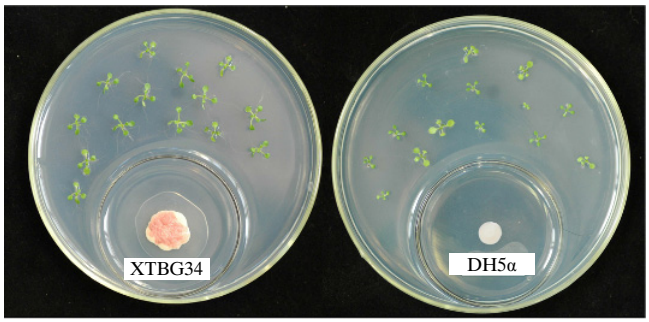

Fig. 1. Representative examples of 7-day-old *A. thaliana* seedlings grown on plates with airborne exposure to *B. megaterium* XTBG34 and *E. coli* DH5α bacterial strains. The small plates were prepared as gnotobiotic systems so that the inoculated bacteria were the only microorganisms present.

Identification of volatiles of the XTBG34

Volatiles were extracted from fresh bacterial cultures in ½ MS medium by solid phase microextraction (SPME) methods (Augusto and Valente, 2002; Wady *et al*., 2003) using a 75 μm CAR/PDMS SPME fiber (Supelco, USA). The fiber was conditioned at 250°C for 15 min in the hot injector, and exposed in culture vials at 28°C for 3 days. After extraction, the SPME fiber was directly inserted into the front inlet of a gas chromatograph (GC, HP 6890A) connected to a mass spectrometer (MS, HP 5973) (Agilent Technologies, USA) and desorbed at 250°C for 2 min. The temperatures of the transfer line and ion trap were 250 and 300°C, respectively. The temperature of the oven was 50°C for 5 min, 50-180°C at a rate of 10°C/min, 180-250°C at 10°C/min, and then held at 250°C for 5 min. Standard Wiley27 and NBS75 K MS-libraries were used to identify volatiles. Samples were tested three times.

Plant growth promotion activities of commercial volatiles

Commercial volatiles that were produced by XTBG34 (2-pentylfuran, 2,3-dimethyl-butanedinitrile, 1-ethenyl-4-methoxy-benzene, 3,5-dimethoxy-toluene, hexadecane, 2,6,10-trimethyl-dodecaneand propylbenzene) were purchased from Sigma-Aldrich (USA). Their plant growth promotion activities were assessed using modified methods described by Ryu *et al*. (2003). Two-day old germinated *Arabidopsis* seedlings were transferred to one part of a two-compartment Petri dish. One milliliter of volatile was diluted separately in alcohol or CH_2Cl_2, and 20 μl of the resulting suspension, or solvent alcohol alone, was applied to a sterile paper disk (d=1 cm) (Whatman) on the other part of the two-compartment Petri dish. For each candidate volatile, 1 mg, 100 μg, 10 μg, 1 μg, and 0.1 μg doses were tested. Three independent biological replicates were used for each PGPR test. For each replicate, there were at least three repeats for different treatment, the fresh weight of individual *Arabidopsis* plant was tested and recorded, and more than 20 individual *Arabidopsis* plants were included in each experiment. In totally, there were at least 60 samples for performing statistical analysis.

Data analysis and statistics

Data were analyzed using analysis of variance (ANOVA), and means were compared by the test of least significant difference (LSD) at $P=0.05$ using SPSS 11.0 for Windows (SPSS Inc., USA).

Results

Identification of strain XTBG34

Fifty-three isolates differing in colony morphology in MS medium were evaluated in terms of their volatiles to improve

the growth of *Arabidopsis*. Among these, strain XTBG34 secreted aromatic compounds with significant plant growth promotion activity, and was further analyzed (Fig. 1).

Strain XTBG34 had the general characteristics of the genus *Bacillus*. The isolate was mobile, Gram-positive, rod-shaped with endospores, aerobic, formed catalase, and oxidase, and its biochemical characteristics (data not shown) strongly suggested that this strain was a member of the *Bacillus* family. Computer analysis using APILAB software identified the strain as *B. megaterium* with 99.8% identity. When the 16S rRNA gene sequence of XTBG34 (1,420 bp) was compared to the NCBI database by BLAST, the closest relatives of XTBG34 were *B. megaterium* strain KSC_SF2d [DQ870698] and A10-2 [AB244298] (99.9% identity). In addition, the *recA* gene sequence of XTBG34 (724 bp) was compared to the NCBI database, the closest relatives of XTBG34 were *B. megaterium* QM B1551 [CP001983] (99.5% identity) and DSM319 [CP001982] (98.5% identity). Neighbor-joining dendrograms were generated using the 16S rRNA and recA gene sequences (Figs. 2A and B), respectively, and the representative sequences of *Bacillus* were download from GenBank. All these results supported that the strain XTBG34

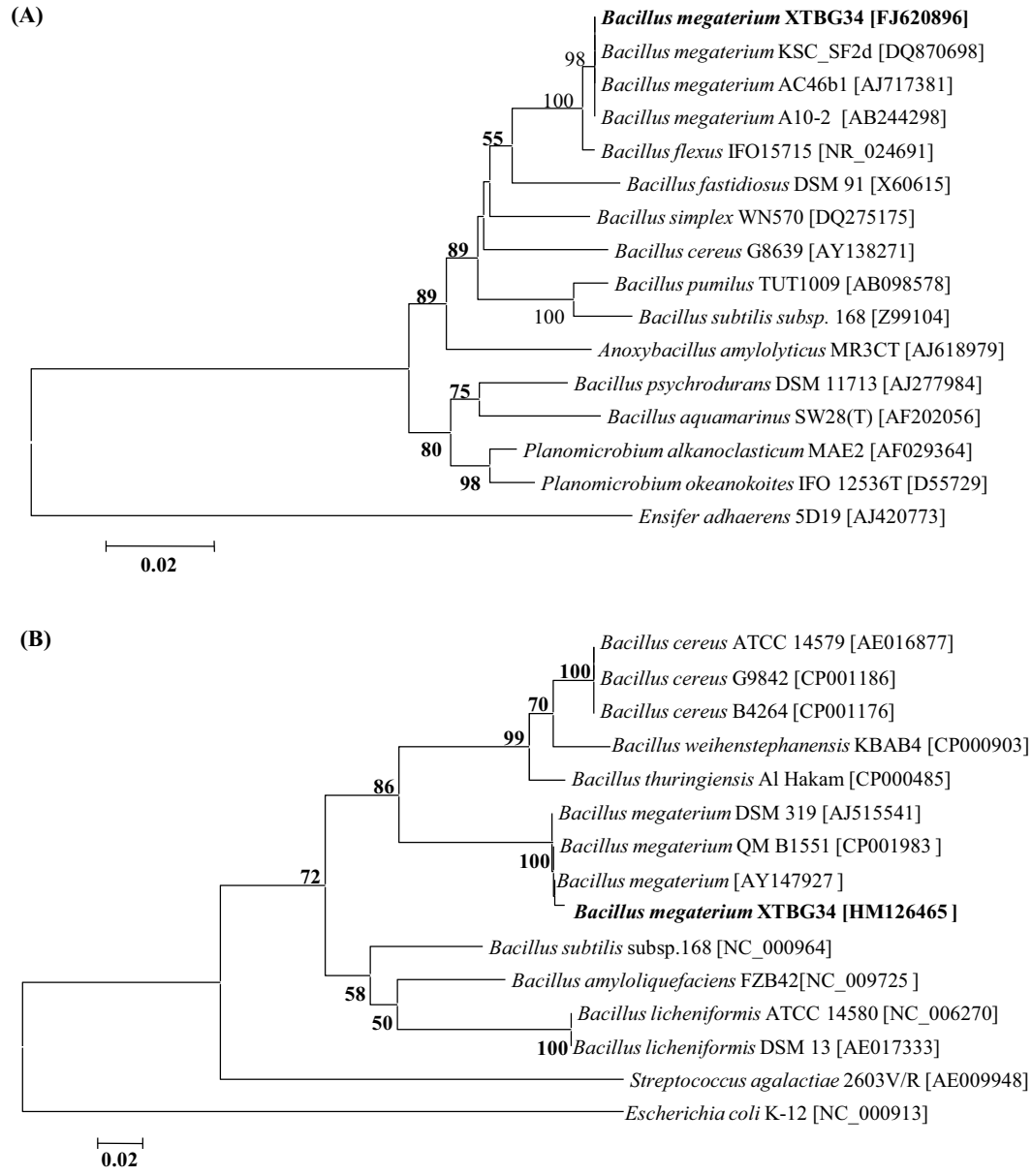

Fig. 2. Neighbor-joining phylogenetic trees based on 16S rRNA and recA gene sequences. (A) Phylogenetic tree based on 16S rRNA gene sequences, *Ensifer adhaerens* (AJ420773) was used as outgroup. (B) Phylogenetic tree based on recA gene sequences, *E. coli* (NC_000913) was used as outgroup. The strain XTBG34 was shown in bold. Bootstrap values (n=1,000 replicates) of ≥50% are reported as percentages, the scale bar represents the number of changes per nucleotide position, and the accession numbers are given at the end of each sequence. The reference sequences of *Bacillus* were downloaded from GenBank.

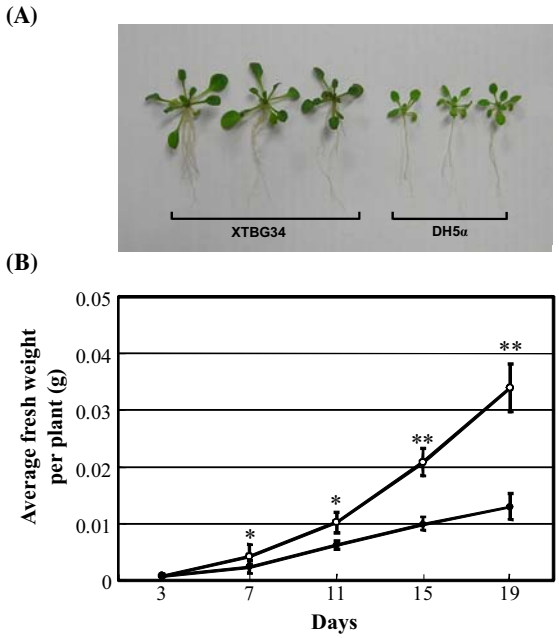

Fig. 3. Plant growth promotion of *A. thaliana* by *B. megaterium* strain XTBG34. (A) Representative examples of 19-day-old *Arabidopsis thaliana* seedlings after airborne exposure to strain XTBG34 or DH5α. (B) Growth was assessed by measuring the fresh weight per plant, bars indicate standard deviations of three independent biological samples, at least 60 individual plants were counted. White circles represent treatment with strain XTBG34; black circles represent treatment with DH5α control. Differences between the treatment and control are significant at the $0.01<P<0.05$ (*) or $P<0.01$ (**) levels.

was a member of the *B. megaterium*.

The 16S rRNA gene and *recA* gene sequence determined for strain XTBG34 were submitted to the GenBank database under the accession number FJ620896 and HM126465, rexpectively.

Plant growth promotion of the XTBG34 strain

Growth promotion in plants activated by volatile chemicals released from PGPR was tested in the laboratory using divided Petri plates (referred to as I plates). I plates contained a center partition, so that only airborne signals could be transmitted between bacteria and the plant seedlings. Inoculation with XTBG34 significantly promoted growth of *Arabidopsis* compared with the DH5α controls (Fig. 1A). Plant fresh weight was measured every 4 days up to 19 days after inoculation with XTBG34 or DH5α. A significant promotion effect of XTBG34 was observed from day 7 ($P<0.05$), and the average fresh weight of plants treated with XTBG34 was about 1.7-fold higher than plants treated with DH5α. The difference between the two treatments increased with time, reaching 2.6-fold on day 19 (Fig. 3B).

Identification and confirmation of growth promotion volatiles of XTBG34

SPME-GC/MS was used to detect volatile components in XTBG34 and DH5α (Fig. 4). In triplicate experiments, 27-32 peaks were observed in total ion current chromatograms of volatile detection in XTBG34, whereas 28-30 peaks were observed in DH5α. Database searching yielded 29 organic compounds, which included a range of aldehydes, alkanes, alcohols, organic acids, ketones, and aroma components.

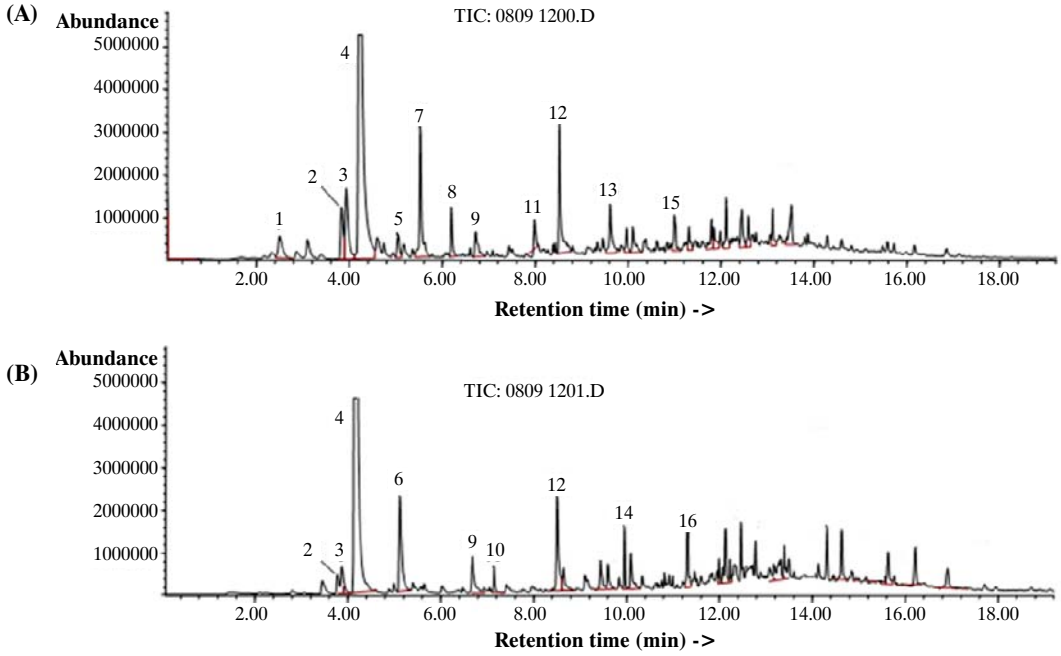

Fig. 4. A representative example of SPME total ion current chromatograms showing volatiles identified from strain XTBG34 and DH5α cultures. (A) Strain XTBG34, which had plant growth promotion activity in *Arabidopsis thaliana*; (B) strain DH5α, which did not have plant growth promotion activity in *A. thaliana*. Key: (1) 2,3-dimethyl-butanedinitrile, (2) ethylbenzene, (3) 1,3-dimethyl-benzene, (4) styrene, (5) propyl-benzene, (6) benzaldehyde, (7) 2-pentylfuran, (8) 2,4-dimethyl-2,3-heptadien-5-yne, (9) 1-phenyl-ethanone, (10) nonanal, (11) 1-ethenyl-4-methoxy-benzene, (12) naphthalene, (13) 3,5-dimethoxy-toluene, (14) tridecane, (15) 2,6,10-trimethyl-dodecane, (16) tetradecane.

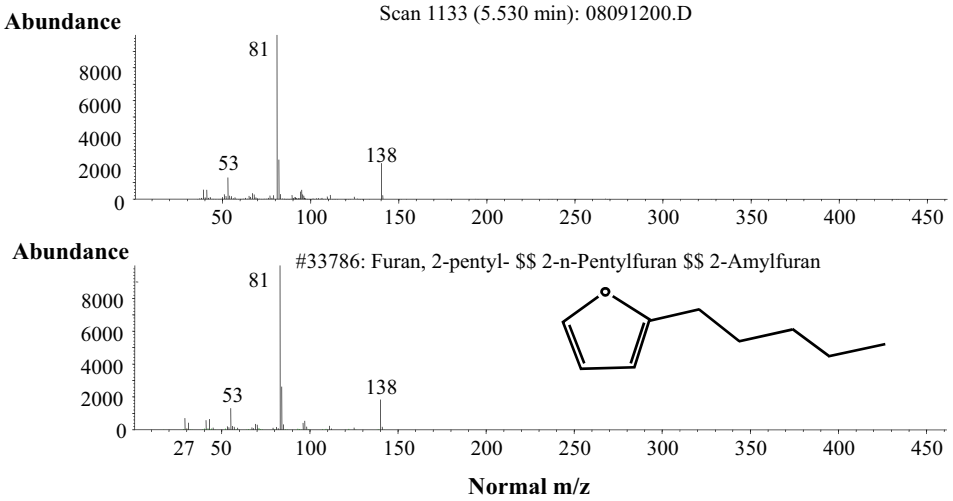

Fig. 5. GC/MS data analysis of 2-pentylfuran.

Organic volatile compounds with >85% quality similarity in database searches were chosen as candidates for plant growth promotion activity assays in divided plates (Fig. 5).

The volatiles 2,3-dimethyl-butanedinitrile, 2-pentylfuran, hexadecane, propyl-benzene, 2,4-dimethyl-2,3-heptadien-5-yne, 3,5-dimethoxy-toluene, 1-ethenyl-4-methoxy-benzene, and 2,6,10-trimethyl-dodecane were found in XTBG34 cultures, but not in DH5α cultures. The volatiles benzaldehyde, tridecane, and tetradecane were found in DH5α cultures, but not in XTBG34 cultures (Fig. 4).

The volatiles produced by XTBG34 are likely to play essential roles in plant growth promotion. To test their functions, the commercial compounds of 2,3-dimethyl-butanedinitrile, 2-pentylfuran, hexadecane, propyl-benzene, 3,5-dimethoxy-toluene, 1-ethenyl-4-methoxy-benzene, and 2,6,10-trimethyl-dodecane were tested for their plant growth promotion activity in *Arabidopsis* plants. 2-Pentylfuran significantly increased plant growth; the degree of growth promotion varied depending on the dose of 2-pentylfuran (Fig. 6). Plant growth promotion was observed at a dose of 0.1 μg, achieved a maximum value at 10 μg, and then decreased as dose increased.

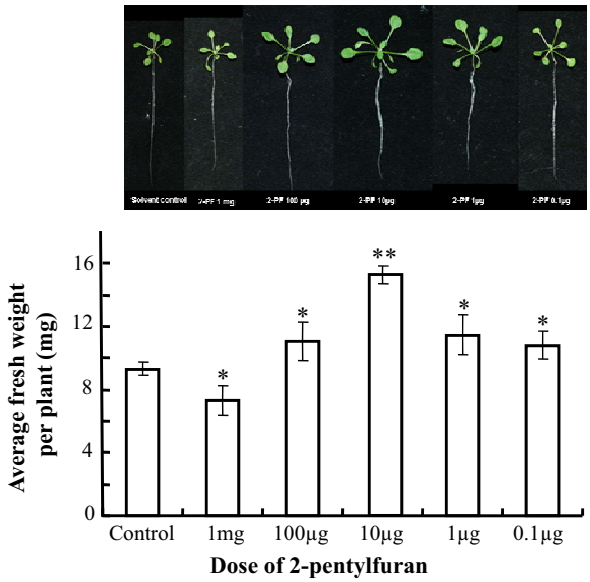

Fig. 6. Representative examples of 15-day-old *A. thaliana* seedlings grown on plates with different doses of synthetic 2-pentylfuran. Growth was assessed by measuring the fresh weight per plant, bars indicate standard deviations of three independent biological samples, and at least 60 individual plants were counted. Differences between the treatment and control are significant at the $0.01<P<0.05$ (*) or $P<0.01$ (**) levels.

Plant growth promotion effects of 2-pentylfuran

The fresh weight of *Arabidopsis* plants was measured every 4 days up to 19 days after treatment with 10 μg 2-pentylfuran or solvent alcohol. A significant promotive effect of 2-pentylfuran on plant growth was observed on day 7 ($P<0.05$), and the average fresh weight of plants treated with 2-

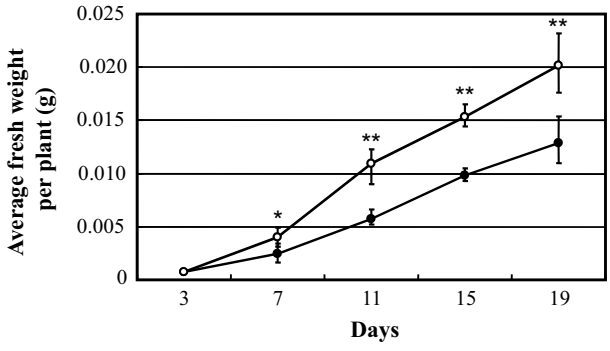

Fig. 7. Effect of 10 μg 2-pentylfuran on plant growth promotion in *A. thaliana*. White circles indicate treatments with 2-pentylfuran (10 μg); black circles indicate solvent control. Growth was assessed by measuring the fresh weight per plant, bars indicate standard deviations of three independent biological samples, and at least 60 individual plants were counted. White circles represent treatment with 2-pentylfuran (10 μg); black circles represent treatment with solvent control. Differences between the treatment and control are significant at the $0.01<P<0.05$ (*) or $P<0.01$ (**) levels.

pentylfuran was about 1.6-fold higher than control plants treated with solvent alcohol. The maximum difference between the two groups was reached on day 11, when the average fresh weight of plants treated with 2-pentylfuran was about 1.9-fold higher than control plants (Fig. 7). Compared to plants inoculated with XTBG34, the difference in fresh weight between 2-pentylfuran and solvent alcohol-treated plants was less than the difference in fresh weight between XTBG34 and DH5α-inoculated plants, and did not increase with time. The variance in fresh weight between 2-pentylfuran and solvent alcohol-treated plants first increased and then decreased.

Discussion

PGPR were first defined by Kloepper and Schroth (1978). They include soil bacteria that colonize plant roots following inoculation onto seed, and improve plant growth.

To be an effective PGPR, an organism must be able to colonize the rhizosphere or plant surface in nutrient-deficient soils at population densities sufficient to produce a beneficial effect. Therefore, we chose nutrient-poor ¼ MS medium when isolating PGPR. *Bacillus* species are typical inhabitants of the rhizosphere, and they are also commonly present on the surface of plants. *B. megaterium* can be detected in the olive phylloplane at all times during the year (Ercolani, 1991) and can infect maize roots through cracks formed at lateral root junctions (Liu *et al*., 2006). *B. megaterium* strain B153-2-2 can easily colonize soybean seedlings, roots, and hypocotyls (Liu and Sinclair, 1993). In experiments in our laboratory (data not shown), the XTBG34 strain colonized soil and the surface of *Arabidopsis* for a long time.

Today, PGPR are increasingly used as inoculants for biocontrol, biofertilization, and phytostimulation. Therefore, identifying plant growth promotion compounds and their mechanisms has been a hot spot for research. After reviewing much research on PGPR, Li and Wilhelm (2004) suggested that they modulate plant growth via their own components or secondary metabolites, which directly or indirectly affect the regulatory networks of plant growth and development. The interactions between plants and PGPRs are generally based on intense, precise exchanges of information between the plant and the bacteria. Understanding this type of communication on a molecular level is one of the most fascinating challenges at the borderline between microbiology, chemistry, and molecular genetics (Li and Wilhelm, 2004). Our work suggests that the volatile 2-pentylfuran of XTBG34 plays an important role in the growth promotion of *Arabidopsis*, and is worthy of future attention.

A growing body of reports suggest that *B. megaterium* can improve plant growth by increasing the available phosphorus. For example, using P-solubilizing *B. megaterium* RC07 as biofertilizer improves the growth and quality of sugar beet in the greenhouse (Cakmakci *et al*., 2006), and De Freitas *et al*. (1997) identified that this isolate of *B. megaterium* was an effective phosphate-solubilizing PGPR. Until now, there have been no reports about volatile signals from *B. megaterium* that can trigger plant growth promotion. Chemical and plant growth studies of *B. subtilis* GB03 and *B. amyloliquefaciens* IN937 revealed that the volatile components 2,3-butanediol and acetoin could improve plant growth promotion in *Arabidopsis* (Ryu *et al*., 2003). However, Farag *et al*. (2006) showed that 2-pentylfuran was also released from both *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a at very low levels, so this compound did not attract much attention.

Based on our chemical and biochemical experiments, we suggest that 2-pentylfuran is an essential bacterial component responsible for airborne chemical signaling triggering growth promotion in *Arabidopsis*. Comparative analysis of volatile profiles of growth-promoting and non-growth-promoting strains showed that release of 2-pentylfuran was distinct from other volatiles; this component was detected exclusively in strain XTBG34, which triggered plant growth promotion by emission of volatiles (Fig. 4). Subsequent testing of commercially available 2-pentylfuran established that exogenous application of this volatile component resulted in a dose-dependent stimulation of plant growth (Fig. 6). However, the other major GC peaks associated with bacterial volatiles from growth-promoting or non-growth-promoting strains did not show the same activity. In terms of biologically relevant concentrations, the initial concentrations of the 1 mg, 100 μg, 10 μg, 1 μg, and 0.1 μg doses were about 1690, 169, 16.9, 1.69, and 0.169 μg/L, respectively, according to the volume of our divided Petri plates. When the initial concentration is 1690 μg/L, the chemical property of 2-pentylfuran is more significant than its biological properties, so the compound shows plant-growth inhibition (Fig. 6A).

Plant growth responses were variable and dependent on the dose of 2-pentylfuran (Fig. 6), similar to results observed for 2,3-butanediol and acetoin (Ryu *et al*., 2003). During XTBG34 plant growth promotion, 2-pentylfuran was released continuously from this strain, so that the difference in growth promotion between XTBG34 and DH5α increased gradually (Fig. 3). However, during 2-pentylfuran plant growth promotion, the compound was added to the plate only once, and its concentration decreased gradually with time; therefore, the difference in growth promotion between 2-pentylfuran and the solvent control reduced gradually (Fig. 7), confirming that 2-pentylfuran plant growth promotion was dose-dependent.

Our study demonstrates that the isolate XTBG34 can promote plant growth by producing 2-pentylfuran. 2-furan-2-yl-(1,3)dioxolane considerably promoted the growth of *Triticum aestivum,* with an increase of 227% in root number, 234% in root length, and 295% in shoot dry mass (Tian *et al*., 2007). 2-amino-furan (patent numbers EP4931-A2, EP4931-A, BR7902392-A, DE2817449-A, DK7901612-A, JP54140725-A, US4240820-A, EP4931-B, and DE2960554-G) can regulate the growth of plants. Based on their chemical structures, these plant growth-regulating compounds are categorized into furan derivatives. Therefore, the furan nucleus may play an essential role in plant growth promotion.

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