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Molecular Basis of Endophytic *Bacillus megaterium*-induced Growth Promotion in *Arabidopsis thaliana*: Revelation by Microarray-based Gene Expression Analysis

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Abstract Black pepper root endophytic *Bacillus megaterium* BP17 (BmBP17) displayed excellent antagonistic activity against diverse plant pathogens. BmBP17 endophytically colonized plantlets of *Arabidopsis* with significant growth promotion as exemplified by increased root and shoot length. To elucidate the molecular basis of growth promotion, microarray-based gene expression profiling was performed on interactome of *Arabidopsis*-BmBP17. A total of 150 genes were found differentially expressed which represented 80 up-regulated and 70 down-regulated plant genes. Key up-regulated *Arabidopsis* genes were (i) *NIR1, AMT1–5, TIP2–3,* and *SULTR1–2* participating in the transport of nutrients through transmembranes; (ii) *SHV3, MMP, RLP44, PROPEP4, AGL42, SCPL30, ANAC010*, and *KNAT7* involved in cell organization, biogenesis, and transcription; (iii) *SRO5, ANNAT7*, and *DDF1* associated with abiotic stress tolerance like salt stress and water deprivation; and (iv) *MYB7, MYB4, MYB49, WRR4, ATHCHIB*, and *ATOSM34* involved in defense against biotic stress. Strikingly, most of the genes participating in growth and development were up-regulated in diverse plant tissues right from root to seed. The up-regulation of nutrient mobilization and uptake-related genes could be attributed to plant growth promotion. The regulation of endogenous population of BmBP17 could be due to the activation of biotic stress defense-associated genes. The bacterial colonization triggered down-regulation of genes coding for transcription factors of ethylene-responsive genes such as *ERF5, ERF71, ERF104, ERF105, TEM1*, and *RAP2.6* and salicylic acid and jasmonic acid-responsive gene such as *BAP1, SIB1, BT4, MKK9*, and *PLA2A*. Our study showed that the plant growth promotion as observed in *Arabidopsis thaliana* Col 0 could be attributed to the up-regulation of nutrient uptake-associated genes and down-regulation of genes coding for transcription factors of ethylene-responsive genes.

Keywords *Arabidopsis* · *Bacillus megaterium* · Differential gene expression · Endophytic colonization · Growth promotion · Real-time PCR

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Introduction

Plant endophytic bacteria colonize the plant endosphere for their nutritional and spatial requirements and in turn contribute to plant growth and development as well as plant disease suppression. Bacteria-mediated plant growth promotion can be attributed to a number of interlinked metabolic events such as nutrient mobilization and acquisition, phyto-hormonal regulation, and modulation of plant developmental processes. For instance, poorly soluble inorganic nutrients that are rate limiting for growth can be made available through organic acids and other metabolites of bacterial origin (Bashan and others 1989; Kapulnik

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1996; Vessey 2003). A number of root endophytes have been shown to fix atmospheric nitrogen in rhizosphere (Dobbelaere and others 2003). Among the plant-associated *Bacillus* species, *Bacillus megaterium* is one of the most adapted spore-forming bacterium found in diverse habitats including the plant interior as an endophyte (Vary 1994; Aravind and others 2009; Salgaonkar and others 2013; Munjal and others 2016). Much like its adaptive behavior, the use of *B. megaterium* is highly versatile. To cite a few, *B. megaterium* is an excellent producer of vitamin B_{12}, oxetanocin—an antiviral chemical and penicillin amidase besides its use as a host to express high-quality foreign proteins in biotechnological applications (Vary 1994; Morita and others 1999). In agriculture, *B. megaterium* is known for its ability to promote plant growth as well as its antagonistic activity against plant pathogens (Aravind and others 2009; Kaymak and others 2008; Munjal and others 2016). *Bacillus*-mediated growth promotion is largely attributed to air-borne volatiles such as acetoin, 2-pentylfuran, and increased mineral availability (Han and others 2006; Supanjani and others 2006; Lopez-Bucio and others 2007; Zou and others 2010). It is further presumed that bacterial interaction with plants triggers modifications in root morphogenesis possibly by phytohormone stimulation and signaling (Kapulnik and others 1985; Dubeikovsky and others 1993; Pattern and Glick 1996; Xie and others 1996). *Bacillus megaterium* BP17 (BmBP17) is a black pepper-associated root endophyte that displayed characteristic volatile-mediated antagonism against diverse plant pathogens (Aravind and others 2010, 2012; Munjal and others 2016). Interestingly, BmBP17 has been reported as a broad host range endophyte in ginger and the model plant, *Arabidopsis* (Munjal and others 2016). The broad host range of this endophyte has been reported by several other workers (McInroy and Kloepper 1995; Vendan and others 2010; Chen and others 2014). BmBP17 was found to promote plant growth during its endophytic interaction with *Arabidopsis thaliana* (Munjal and others 2016). Although the bacterial traits responsible for growth promotion are partly deciphered, the specific mechanisms of plant growth promotion and signaling pathways modulating growth promotion by *B. megaterium* are not conclusively understood.

During the last decade, transcription microarray and RNA seq-based transcriptome technologies have been applied to improve our understanding of plant–microbe interactions (Maleck and others 2000; Schenk and others 2000). To elucidate molecular mechanisms of plant growth promotion triggered by BmBP17, we have performed a microarray-based gene expression analysis on *A. thaliana* Col 0 using Affymetrix Genechip probe arrays. Here, we report that a single inoculation of *A. thaliana* Col 0 seeds with the endophytic strain of BmBP17 during germination exerted positive phenotypic effects on plant growth and development. The bacterium not only multiplied endogenously but also significantly promoted the growth of plantlets as a consequence of altered expression of a number of plant genes associated with growth and development.

Materials and Methods

Bacterial Strain and Culture Conditions

Unless stated otherwise *Bacillus megaterium* BP17 (BmBP17) was routinely cultured in Luria–Bertani broth (LBB, g L^{-1}: Tryptone 10, Yeast Extract 5, NaCl 10; pH 7.2) at 28 °C for 24 h in a rotary shaker at 200 rpm (Lab Companion, Daihan Scientific, South Korea). Bacterial cells were pelleted after 24 h at 6000 rpm for 5 min and washed pellets were used for inoculating the *A. thaliana* plants after resuspending in sterile distilled water. A spontaneous rifamycin-resistant BmBP17 developed in the laboratory was genetically tagged with pMUTIN-gfp conferring erythromycin resistance and inducible GFP gene. The dual antibiotic resistance enabled us to precisely track the endogenous cells of BmBP17 (Munjal and others 2016).

Effect of *Bacillus megaterium* BP17 on Growth of *Arabidopsis thaliana*

Plant growth conditions Seeds of *A. thaliana* Col-0 surface disinfected using 70 % ethanol and 1 % NaOCl were placed on half-strength Murashige and Skoog medium (Murashige and Skoog 1962), and 10 µl of varying decimal dilutions of BmBP17 cell suspension such as 10^9, 10^8, and 10^7 cells mL^{-1} were seed inoculated. Five seeds were arranged in line on the surface of the medium and incubated for 48 h at 4 °C. Subsequently the plates were transferred to a climate chamber preset at 22/20 °C (day/night) temperature, 24 h light period, and 40 % relative humidity. Sampling was done on 21 dpi and phenotypic changes such as root and shoot length were recorded. Also, the endogenous population size of BmBP17 was estimated using the plate count method as described below. Another set of plantlets were aseptically harvested, transferred to microfuge tubes, and preserved at −80 °C for gene expression analysis.

Bacterial Inoculation and Tissue Localization

Estimation of endophytic BmBP17 The endogenous population size of the BmBP17 in bacterized *Arabidopsis* was estimated on rifamycin and erythromycin amended LBA (LBA, g L^{-1}: Tryptone 10, Yeast Extract 5, NaCl 10, Agar 15; pH 7.2). Briefly, the whole plantlet of *A. thaliana* was

harvested on 21 dpi, surface disinfected using sodium hypochlorite (0.5 %), amended with Tween 20 (0.01 %) for 20 min and ethyl alcohol (70 %) for 1 min followed by rinsing with sterile distilled water 2–3 times. Each sample was ground aseptically with 2 mL of phosphate buffered saline [PBS, g L^{-1} NaCl 8; KCl 0.2; Na_2HPO_4 1.44; KH_2PO_4 0.24; pH-7.4] was decimally diluted up to 10^{-6}. From this, one mL was pour plated in LBA amended with rifamycin (50 µg mL^{-1}) + erythromycin (1 µg mL^{-1}) and incubated at 28 °C for 48 h. Colonies were counted and expressed as CFU per gram of fresh tissue.

Bio-PCR assay to detect endogenous BmBP17 For detection of endogenous BmBP17 in *Arabidopsis* plants, bio-PCR was performed (Munjal and others 2016). Briefly, surface sterilized plantlets were ground with 1.0 mL of distilled water and the homogenates were vortexed for five seconds and 500 µl was inoculated into LB broth amended with rifampicin (50 µg mL^{-1}) and erythromycin (1 µg mL^{-1}). The broth was incubated at 37 °C for 48 h and two µl of this broth was used as a template for PCR using BmBP17 specific primers cbiD_F: 5′TTCCGT CCCCTACTTCCTTT3′ and cbiD_R: GCCGCTTTGCT ACATTTCAT3′ that is known to yield 862 bp amplicon.

Statistical Analysis

Analysis of variance (ANOVA) of root length, shoot length, and population size data was done using the online statistical software OPSTSAT (hau.ernet.in/about/op-stat.php) and appropriate normalized data sets were used for statistical analysis. Means were compared using critical difference (CD) at P = 0.05.

Gene Expression Analysis Using Microarray

RNA extraction, cDNA preparation, and microarray analysis Three-week-old seedlings that emerged on MS medium from seeds treated with BmBP17 were carefully detached from the agar plate, rinsed with sterile distilled water, and frozen in liquid nitrogen. Plants were macerated in a sterile mortar pestle and RNA was extracted using the Qiagen RNAeasy kit as per the manufacturer's protocol. RNA quality and quantity were checked spectrophotometrically (Eppendorf Biophotometer, Germany). The quality of RNA was further checked in agarose gel electrophoresis. Two hundred nanograms of total RNA were converted to cDNA using a cDNA synthesis kit from Affymetrix. IVT labeling was done for the synthesized cDNA strand followed by quantification, purification, and fragmentation of aRNA using a GeneChip® 3′ IVT Express Kit following its protocol. Fragmented aRNA was hybridized on Affymetrix ATH1 Genechip probe arrays representing approximately 22,500 genes. The standard wash and double-stain protocols were applied using an Affymetrix GeneChip Fluidics Station 450 as per instrument protocol. The arrays were scanned on an Affymetrix GeneChip scanner 3000. Raw data files generated through the Affymetrix Gene chip instrument were analyzed using Agilent's Gene Spring software (Gene Spring 12.6 version).

Data analysis The 22,500 probes on *A. thaliana* ATH1 Gene chip were used to compare across samples. The data were analyzed by one-way analysis of variance (ANOVA) using a *T* test with $P \leq 0.05$. Differentially expressed genes were identified by outlier detection of a contaminated bivariate distribution. To determine the differentially expressed candidate genes from these outliers, the fold-change values were calculated providing up- and down-regulated genes across the samples as compared to mock. The biological significance and functional classification of differentially expressed genes were performed using the Gene Ontology program. Annotation and gene ontology data were retrieved from The Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org/index.jsp).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Representative Genes

For validation of microarray data, real-time quantitative PCR expression analysis was performed. Total RNA from plantlets was isolated using the RNAeasy Mini Kit—Qiagen in accordance with the manufacturer's protocol. Isolated total RNA was treated with DNAse I (Takara Bio, Shiga, Japan) to avoid contamination with genomic DNA. RNA was reverse transcribed using an ImProm-II™ Reverse Transcription System in accordance with the manufacturer's instructions; 500 ng of RNA was mixed with 4 µl of ImProm-II™ 5X Reaction Buffer, 4.8 µl MgCl2, 1 µl of dNTPs 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor, 1 µl of ImProm-II™ Reverse Transcriptase, and incubated at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. The cDNA obtained was used as a template for real-time quantitative PCR. PCR amplification was performed using a 2× SYBR Mix (Roche diagnostics), 1 µM each of gene-specific forward and reverse primers, and 15–20 nanogram of cDNA template. Gene-specific RT-PCR primers were designed using primer 3 plus software and synthesized (IDT, USA). The primers used for the quantitative PCR are listed in Supplementary Table 3. The genes were amplified using a Light cycler 96 PCR machine (Roche diagnostics, Switzerland). For normalizing expression levels of a constitutively expressed gene, protein phosphatase 2A (PP2A) (At1g13320) was used as a reference gene (Hong and others 2010). The thermal profile used consisted of an initial denaturation step at 95 °C for

5 min, followed by 40 cycles of 95 °C for 10 s, annealing for 15 s (annealing temperature given in Table S3), and 72 °C for 15 s followed by one cycle of 95 °C for 10 s, AT + 5 °C for 60 s, 97 °C for 1 s, and a final cooling of 37 °C for 30 s. Agarose gel electrophoresis of the qRT-PCR products was performed to confirm that the individual qRT-PCR products corresponded to a single homogeneous cDNA fragment of expected size.

Results

Effect of BmBP17 on Growth of *Arabidopsis thaliana*

The effect of seeds inoculated with *B. megaterium* on plant growth and development was investigated using the model plant, *A. thaliana* (Ecotype: Col-0). Seeds inoculated with BmBP17 yielded plantlets with significantly higher root and shoot length than mock (Table S1, Figs. 1, 2). Seeds bacterized with high bacterial titer displayed significantly longer root and shoots as compared to low titer inoculations as well as mock.

Endophytic Colonization of BmBP17 in *Arabidopsis thaliana*

Dual antibiotic resistance makers of genetically transformed BmBP17 enabled us to precisely quantify the endogenous population of bacterium in bacterized plantlets. BmBP17 endophytically colonized the plantlets of *Arabidopsis* wherein plantlets treated with varying dilutions of bacterial cells recorded a more or less identical endogenous bacterial population size. The log CFU of 5–6 was recorded for plantlets from seeds bacterized with 10^{7-9} bacterial cells (Table S2, Fig. 2). Bio-PCR with BmBP17 specific primers yielded a BmBP17 specific amplicon that further confirmed its endogenous presence in plants (data not shown).

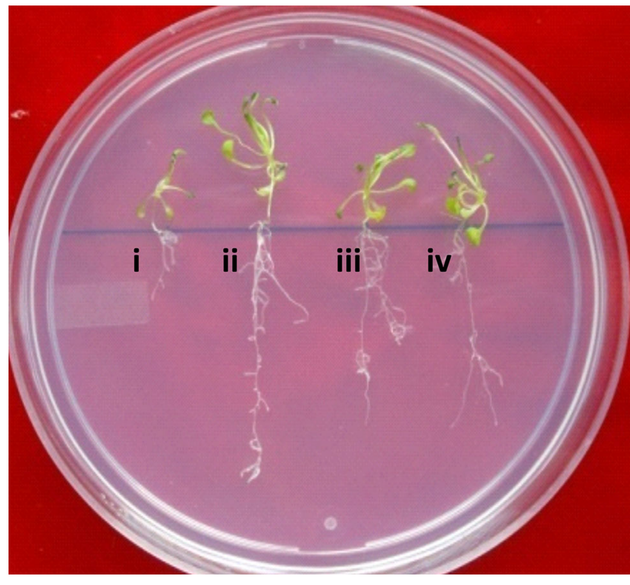


Fig. 1 Changes observed in phenotype (increased root and shoot length) of 21-day-old *Arabidopsis thaliana* plants due to inoculation of *Bacillus megaterium* BP17 on *Arabidopsis* seeds. *i* Control plants; *ii* plants treated with 10^9 cfu of BmBP17; *iii* plants treated with 10^8 cfu of BmBP17; *iv* plants treated with 10^7 cfu of BmBP17. Experiment was performed twice with five replicates

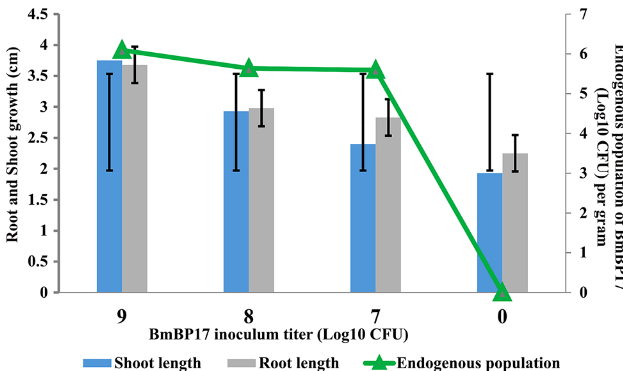
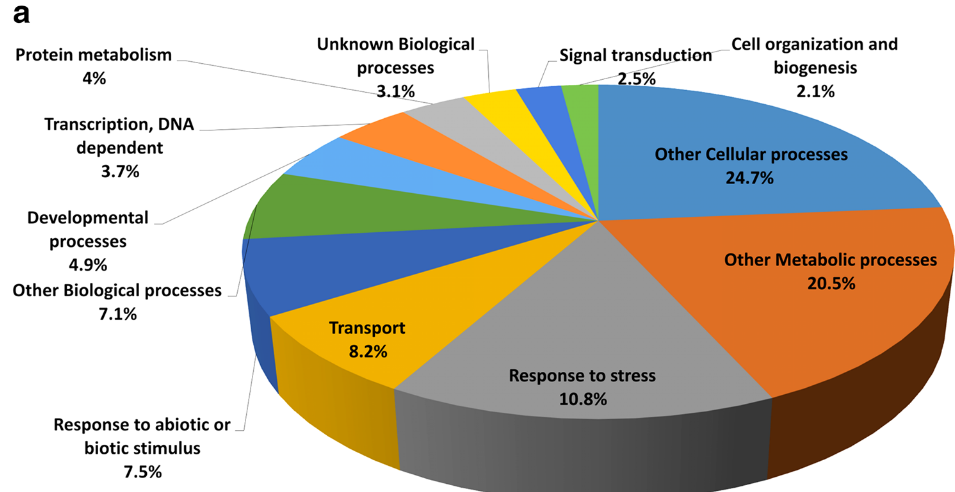


Fig. 2 Root and shoot length of 21-day-old *Arabidopsis thaliana* Col 0 colonized by BmBP17 and endogenous population size of BmBP17 in *Arabidopsis thaliana* Col 0. Experiment was performed twice with five replicates. *Error bars* indicate mean values with SEM

Gene Expression Analysis Using Microarray

Microarray-based gene expression profiling provided a global view of transcript modification during the *A. thaliana*–BmBP17 interaction. A total of 150 genes were differentially expressed with fold change of at least 1.5 in 21-day-old endophyte-treated *Arabidopsis*. Among them 80 were up-regulated genes and 70 represented down-regulated plant genes. A complete list of up-regulated/down-regulated genes is furnished in Supplementary Tables 4 and 5. Functional categorization of differentially expressed genes based on their involvement in various processes is shown in Fig. 3a, b.

Arabidopsis genes up-regulated by BmBP17 Plant genes participating in cellular and metabolic processes (45.2 %) followed by stress-responsive genes and genes expressed due to biotic and abiotic stimuli (18.3 %) were significantly up-regulated upon endophytic colonization (Fig. 3a). Endophytic colonization of *Arabidopsis* by BmBP17 up-regulated the number of genes participating in nutrient mobilization and uptake (*NIR1*, AMT1–*5*, *TIP2–3,* and *SULTR1–2*); growth and elongation of shoot and root (SHV3, *MMP, RLP44, PROPEP4, AGL42, SCPL30, ANAC010*, and *KNAT*); abiotic stress tolerance (*SRO5*, *ANNAt7*, and *DDF1*); and defense genes against biotic stress (*MYB7, MYB4, MYB49, WRR4, ATHCHIB*, and *ATOSM34*) (Tables 1, 2).



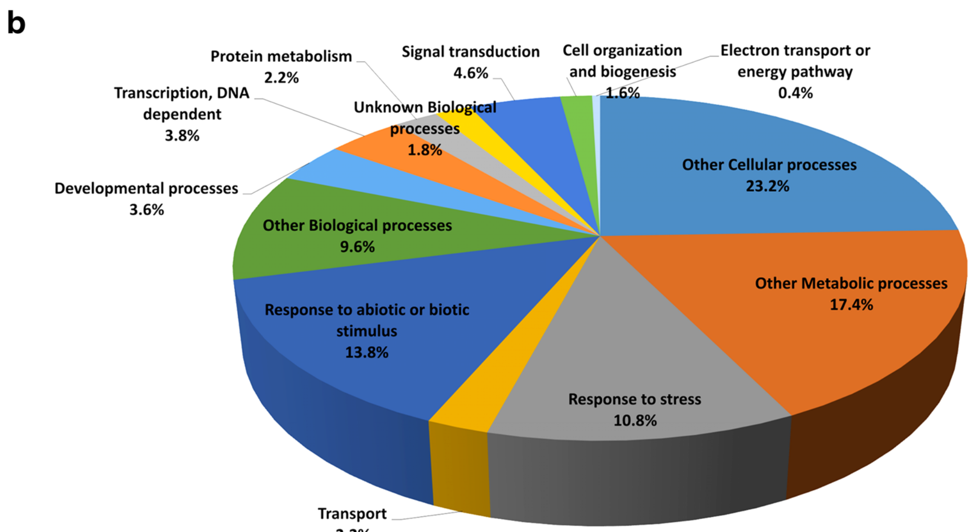


Fig. 3 Functional classification of *Arabidopsis thaliana* genes based on their annotations in terms of GO (Gene Ontology) molecular component **a** Up-regulated **b** Down-regulated by endophytic *Bacillus megaterium* BP17

Arabidopsis genes down-regulated by BmBP17 Among the down-regulated genes were those associated with cellular and metabolic processes in plants (40.6 %). Nearly 24 % genes participating in stress responses and expressed upon biotic and abiotic stimuli were down-regulated (Fig. 3b). Down-regulated genes in *A. thaliana* Col-0 upon endophytic colonization by BmBP17 are furnished in Supplementary Table 5. Significantly, BmBP17 colonization triggered down-regulation of a number of genes coding for transcription factors of ethylene-responsive genes such as *ERF5, ERF71, ERF104, ERF105, TEM1*, and *RAP2.6;* and genes participating in response to salicylic acid and jasmonic acid such as *BAP1, SIB1, BT4, MKK9*, and *PLA2A* (Table 3). With the help of the Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org/index.jsp), up-regulated and down-regulated genes were retrieved and classified based on their expression and function in different tissues of *A. thaliana* (Table 4).

Quantitative RT-PCR validation To verify the Gene Chip results, a quantitative RT-PCR analysis was performed for selected up- and down-regulated genes identified in the microarray analysis. The relative visible intensity of PCR amplicons clearly indicated the elevated expression level of up-regulated genes as compared to mock-inoculated plants. Similarly for down-regulated genes the visible intensity was less than mock. RT-PCR results on randomly selected genes further authenticated the gene expression data obtained from microarray analysis (Table 5).

Discussion

Plant-associated endophytic bacteria are known to modulate plant growth, development, and defense against plant pathogenic microorganisms. Endophytic *B. megaterium* BP17 isolated from black pepper root is reportedly an

Table 1 List of 'up-regulated defense'—associated genes upon colonization by plant endophytic *Bacillus megaterium* BP17 in 21-day-old *Arabidopsis thaliana* Col 0

Gene name/ other name	AGI number	Remarks	Reference
1. MYB7	At2g16720	AtMYB7 is a repressor of flavonol biosynthesis. Expression of MYB7 is induced by salt stress	Fornale and others (2013)
2. MYB4	At4g38620	AtMYB4, regulates the accumulation of the UV-protectant compound sinapoylmalate by repressing the transcription of the gene encoding the phenylpropanoid enzyme cinnamate 4-hydroxylase. AtMYB4 is thus a key regulator of phenylpropanoid pathway gene expression	Hemm and others (2001)
3. MYB49	At5g54230	MYB proteins are a superfamily of transcription factors that play regulatory roles in developmental processes and defense responses in plants	Eulgem (2005), Rushton and Somssich (1998)
4. WRR4	At1g56510	Encodes a TIR-NB-LRR protein that confers broad-spectrum white rust resistance in *Arabidopsis thaliana* to four physiological races of *Albugo candida*	Borhan and others (2008), Rushton and Somssich (1998)
5. ATHCHIB	At3g12500	Encodes a basic chitinase involved in ethylene/jasmonic acid-mediated signaling pathway	Stintzi and others (1993)
6. ATOSM34	At4g11650	Osmotin like protein form a subclass of thau-1 proteins also known as PR-5 proteins. They are involved in defense response to fungus	Capelli and others (1997)

antagonist of several plant pathogens (Aravind and others 2010; Munjal and others 2016). The antagonism inflicted by BmBP17 is attributed to antimicrobial volatile organic compounds that belong to the pyrazine class of chemicals (Munjal and others 2016). Apart from its antagonistic activity, the bacterium endogenously colonized not only black pepper but also the model plant, *Arabidopsis,* at 6 log units of colonies per gram of plant tissue (Munjal and others 2016). The bacterium, thus, behaved as a broad-spectrum plant endophytic bacterium. The endophytic bacterial population size did not increase with increasing bacterial titer used in seed bacterization, which clearly indicated a regulated endogenous multiplication and proliferation. Regulated endogenous multiplication of plant endophytic *Pseudomonas putida* BP25 in *Arabidopsis* was recently reported (Sheoran and others 2016), which prompted us to speculate that the endophytic regulation of bacterial multiplication is an essential trait for being an endophyte. Interestingly, colonization of *Arabidopsis* plantlets by BmBP17 triggered beneficial phenotypic changes as exemplified by enhanced root and shoot growth. These results clearly indicated that BmBP17 is a typical plant growth-promoting endophytic bacterium in plants. Plant growth enhancement by a few other strains of *B. megaterium* was reported previously by Lopez-Bucio and others (2007), who showed that growth promotion is independent of auxin and ethylene signaling. To support this observation, Ortiz-Castro and others (2008) reported a possible role of cytokinin signaling in plant growth promotion by *B. megaterium*. Here, microarray-based gene expression profiling was employed to delineate the endogenous interaction of BmBP17 with *Arabidopsis* as we did in the case of the *Arabidopsis thaliana–Pseudomonas putida* BP25 endophyte system (Sheoran and others 2016).

BmBP17 endophytic colonization up-regulates the expression of genes participating in nutrient uptake Expression of 80 plant genes was up-regulated upon endophytic colonization in *Arabidopsis* plants. The growth promotion observed in BmBP17-colonized plants could be attributed to the expression of a number of 'Growth and Development'—associated genes in all plant parts. Nutrient uptake-associated genes such as *NIR1*, *AMT1–5*, *TIP2–3,* and *SULTR1–2* were up-regulated upon bacterial colonization. *NIR1* expressed in plant parts such as carpel, cauline leaf, cotyledon, flower, guard cell, hypocotyl, plant embryo, pollen, root, seed, and stem of *Arabidopsis* is involved in nitrite assimilation and high NIR activity is considered to confer high tolerance for NO_2 (Yoneyama and others 1979; Shimazaki and others 1992). Takahashi and others (2001) showed that NIR is a controlling enzyme in NO_2 assimilation and overexpression of the NIR gene and consequent biosynthesis of NIR enzyme would improve the ability of plants to assimilate NO_2. *AMT 1–5* is involved in ammonium transport and is reportedly expressed in the roots of *Arabidopsis*. *AMT1–1* genes from tomato and *Arabidopsis* conferred high-affinity NH_4^+ uptake to yeast mutants defective in NH_4^+ transport (Ninnemann and others 1994; Lauter and others 1996). Similarly *TIP2–3* is a tonoplast intrinsic protein, which is known to be expressed in roots of *Arabidopsis*, transports ammonium (NH3) and methyl ammonium across the tonoplast membrane, and is involved in diurnal regulation

Table 2 List of up-regulated genes involved in growth, development and stress tolerance upon colonization by plant endophytic *Bacillus megaterium* BP17 in 21-day-old *Arabidopsis thaliana* Col 0

Gene name/ other name	AGI number	Remarks	Reference
1. NIR1	At2g15620	NIR catalyzes the six electron reduction of nitrite to ammonium in the second step of nitrate assimilation pathway	Takahashi and others (2001)
2. AMT1.5	At3g24300	Encodes ammonium transporter which is involved in ammonium uptake from the soil	Loque and others (2006)
3. SULTR1;2	At1g78000	Encodes a sulfate transporter involved in transmembrane sulfate transport	Nakashita and others (2003)
4. SHV3	At4g26690	Glycerophosphoryl diester phosphodiesterase-like protein involved in cell wall cellulose accumulation and pectin linking. Impacts root hair, trichome, and epidermal cell development	Hayashi and others (2008)
5. SRO5	At5g62520	P5CDH and SRO5 proteins are key components of a regulatory loop controlling ROS production and stress response	Borsani and others (2005)
6. DDF1	At1g12610	Encodes a member of the DREB subfamily A-1 of ERF/AP2 transcription factor family (DDF1). The protein contains one AP2 domain. Overexpression of this gene results in increased tolerance to high levels of stress	Kang and others (2011)
7. ANNAT7	At5g10230	Annexins play a role in the Golgi-mediated secretion of newly synthesized plasma membrane and wall materials in plant cells	Postupolska and others (2014)
8. PROPEP4	At5g09980	PROPEP genes are endogenous amplifiers of endogenous immune response initiated by PAMPS	Huffaker and Ryan (2007)
9. SCPL30	At4g15100	Serine carboxypeptidase-like proteins (SCPLs) comprise a large family of protein hydrolyzing enzymes that play roles in multiple cellular processes	Liu and others (2008)
10. RLP44	At3g49750	RLP44 is required for normal growth and stress responses and connects with the BR signaling pathway, presumably through a direct interaction with the regulatory receptor-like kinase BAK1	Wolf and others (2014)
11. TIP2–3	At5g47450	Tonoplast intrinsic protein, transports ammonium (NH3) and methylammonium across the tonoplast membrane, gene expression shows diurnal regulation and is up-regulated by ammonium (NH3)	Loque and others (2004)
12. KNAT7	At1g62990	Encodes a homeodomain transcription factor of the Knotted family. Involved in secondary cell wall biosynthesis. Mutants have moderately irregular xylem development. Expression of this gene is up-regulated by SND1 and MYB4	Li and others (2012)
13. MMP	At1g70170	Mutation of the Matrix Metalloproteinase At2-*MMP* inhibits growth and causes late flowering and early senescence in *Arabidopsis*	Golldack and others (2001)
14. AGL42	At5g62165	Encodes a MADS box transcription factor. Promotes flowering in the shoot apical and axillary meristems	Fornell and others (2011)

(Loque and others 2006). *SULTR1–2* is involved in sulfate uptake and plants utilize sulfate for the synthesis of various organic compounds through a complex metabolic network (Leustek and Saito 1999; Leustek and others 2000; Grossman and Takahashi 2001). Nakashita and others (2003) reported that the *SULTR1–2* transporter is expressed in *Arabidopsis* roots where it functions as a major component of the initial sulfate uptake system. It is also expressed in other tissues such as cauline leaf, cotyledon, flower, guard cell, hypocotyl, and pollen. Sulfur deficiency causes retarded and chlorotic growth of plants and significantly reduces crop productivity.

BmBP17 endophytic colonization up-regulates the expression of genes participating in growth and morphogenesis Genes involved in root and associated structural development were up-regulated due to BmBP17 colonization. *SHV3* is involved in root hair cell differentiation, root hair elongation, and cell wall organization (Hayashi and others 2008). Another up-regulated gene, *MMP,* is reportedly expressed in root, flower, and pollen and is involved in developmental vegetative growth, multicellular organismal development, and more importantly the negative regulation of leaf senescence and proteolysis (Golldack and others 2001). *RLP44* is essential for normal growth of plants (Wolf and others 2014). *AGL42* is expressed in all plant tissues like leaf, flower, guard cell, root, seed, stem, embryo, and encodes a MADS box transcription factor that promotes flowering in the shoot apical and axillary meristems (Fornell and others 2011). *SCPL* is expressed in flower and root and codes for serine carboxypeptidase-like proteins that play roles in multiple cellular processes. SCPL is involved in the regulation of

Table 3 Significantly down-regulated genes in 21-day-old *Arabidopsis* upon endophytic colonization *by Bacillus megaterium* BP17

Sl no	Gene symbol/gene name	AGI number	Remarks	Reference
1.	PLA2A	At2g26560	Patatin-like protein 2 (PLP2), a pathogen-induced patatin-like lipid acyl hydrolase, promotes cell death and negatively affects *Arabidopsis* resistance to the fungus *Botrytis cinerea*	La camera and others (2005, 2009)
2.	GSTU3	At2g29470	Plant glutathione transferases (GSTs) are induced by diverse biotic and abiotic stimuli, and are important for protecting plants against oxidative damage	Sappl and others (2009)
3.	BAP1	At3g61190	Repression of BAP1 leads to enhanced disease resistance to virulent pathogens largely through an R-gene SNC1	Yang and others (2006)
4.	PHOSPHOGLYCERATE MUTASE FAMILY PROTEIN	At3g60420	Phosphoglycerate mutase plays critical roles in stomatal movement, vegetative growth, and pollen production in *Arabidopsis thaliana*	Zhao and others (2011)
5.	UGT74E2	At1g05680	Member of uridine diphosphate (UDP)-glycosyltransferase (UGT). UGT mutant shows enhanced resistance to *Pseudomonas syringae* pv. tomato infection in *Arabidopsis*	Park and others (2011)
6.	PLANT INVERTASE/ PECTIN METHYLESTERASE INHIBITOR SUPERFAMILY PROTEIN	At3g47380	PMEI might indirectly play a role in plant defense against pathogen attack by inhibiting the endogenous PME and maintaining the pectin in a highly methylated form	Juge (2006)
7.	RAP2.6	At1g43160	Overexpression of RAP2.6 in *Arabidopsis* results in a dwarf phenotype with extensive secondary branching and small siliques	Zhu and others (2010)
8.	DREB19	At2g38340	DREB19 exhibit tissue specific expression, and participates in plant developmental processes as well as biotic and/or abiotic stress signaling	Krishnaswamy and others (2011)
9.	ERF105	At5g51190	Ethylene response factors (ERFs) constitute the largest family of transcription factors in *Arabidopsis.* Many ERFs have been implicated in plant defense responses	Meng and others (2013)
10.	UGT73C1	At2g36750	UGT73C1 belongs to a family of plant glycosyltransferases involved in stress response	Meurinne and others (2005)
11.	MAPKKK19	At5g67080	MKK9 acts as a negative regulator of the abiotic stress response	Alzviy and Morris (2007)
12.	CYP94B1	At5g63450	Overexpression of CYP94B1 Results in JA-deficient Phenotypes	Koo and others (2014)

defense responses against pathogen infection and oxidative stress. Plants overexpressing SCPL show an increased tolerance to oxidative stress (Liu and others 2008). *KNAT7* encodes a homeodomain transcription factor of the Knotted family which is expressed in leaf, flower, root, stem, pollen. It is also involved in secondary cell wall biosynthesis and xylem development (Li and others 2012).

BmBP17 endophytic colonization up-regulates the expression of genes participating in abiotic stress tolerance BmBP17 colonization up-regulated many stress tolerance genes like *SRO5*, *ANNAT7*, and *DDF1,* which are reported to govern tolerance to abiotic stresses like salt and water deprivation. *SRO5* proteins are components of a regulatory loop controlling ROS production and stress response and are expressed in leaf, flower, root, seed, embryo, guard cell, and pollen (Borsani and others 2005). *ANNAt* (Annexins) are a multigenic–multifunctional family of Ca^{2+}-dependent membrane-binding proteins expressed in flower, root, vascular leaf, and serve as components of Ca^{2+} signaling pathways (Mortimer and others 2008). Annexin is up-regulated in *Medicago sativa* and *Arabidopsis* in response to osmotic stress, abscisic acid (ABA), and drought (Kovacs and others 1998; Kreps and others 2002; Postupolska and others 2014). *DDF1* is expressed in flower, guard cell, root, and leaf and enhances tolerance to cold, drought, and heat stresses in *Arabidopsis thaliana* (Kang and others 2011).

BmBP17 endophytic colonization up-regulates the expression of genes involved in biotic stress defense Defense-related genes such as *MYB7, MYB4, MYB49, WRR4, ATHCHIB*, and *ATOSM34* were also up-regulated in *Arabidopsis*. *MYB* proteins are a large family of plant transcription factors linked to biotic and abiotic stress responses (Yang and Klessig 1996; Singh and others 2002). *MYB7* and *MYB49* are expressed in leaf, flower, embryo, root, seed, and guard cell. *MYB7* negatively regulates ABA-mediated inhibition of seed germination, which is governed by *ABI5* in *Arabidopsis* seeds (Kim and others 2014). *MYB4* along with WRKY transcription factors are

Table 4 Classification of up-regulated and down-regulated genes based on their expression in different parts of *Arabidopsis thaliana* Col 0

Plant part	Gene name[a]					
	Up-regulated			Down-regulated		
	Growth and development	Stress tolerance	Defense	Growth and development	Stress tolerance	Defense
Seed	NIR1,SHV3,RLP44, PROPEP4, AGL42	SRO5	MB7, MYB4	TEM1, BAP1	MKK9	SIB1, BT4,
Hypocotyl	NIR1, SULTR1-2	–	–	–	–	–
Stem	NIR1, SHV3, AGL42, ANAC010, KNAT7	–	WRR4, ATHCHIB, ATOSM34	ERF5, ERF71, ERF104, TEM1		SIB1, PLA2A
Cauline leaf	NIR1, SULTR1-2	–	–	–	–	–
Vascular leaf	–	ANNAT7	–	–	–	–
Leaf	SHV3, RLP44, PROPEP4, AGL42, ANAC010, KNAT7	SRO5, DDF1	MYB7, MYB4, WRR4, ATHCHIB	ERF5, ERF71, ERF104, TEM1, RAP2.6	MKK9	BAP1, SIB1, BT4, PLA2A
Root	NIR1, AMT1-3, SULTR1-2, SHV3, MMP, RLP44, PROPEP4, AGL42, SCPL30, TIP2-3, ANAC010, KNAT7	SRO5, ANNAT7, DDF1	MYB7, MYB4, MYB49, ATHCHIB, ATOSM34	ERF5, ERF71, ERF104, TEM1, RAP2.6	MKK9	BAP1, SIB1, BT4, PLA2A
Guard cell	NIR1, SULTR1-2, SHV3, PROPEP4, AGL42	SRO5, DDF1	MYB7, MYB4	–	–	–
Flower	NIR1, SULTR1-2, SHV3, MMP, RLP44,PROPEP4, AGL42, SCPL30, ANAC010, KNAT7,	SRO5, ANNAT7, DDF1	MYB7, MYB4, WRR4	ERF5, ERF71, ERF104,TEM1, RAP2.6	MKK9	BAP1, SIB1, BT4
Pollen	NIR1, SULTR1-2, SHV3,MMP, KNAT7	SRO5	ATHCHIB, ATOSM34	ERF5, ERF71, ERF104, TEM1	MKK9	BAP1, SIB1, BT4, PLA2A
Embryo	NIR1, RLP44, PROPEP4, AGL42	SRO5	MYB7, MYB4, WRR4	ERF5, ERF71, ERF104,TEM1	MKK9	BAP1, SIB1, BT4
Cotyledon	NIR1, SULTR1-2	–	–	–	–	–

[a] Expression data was retrieved from The Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org/index.jsp)

known to provide resistance in plants against biotic stresses (Datta and others 2013). *MYB49,* which is expressed in roots, plays regulatory roles in the developmental processes and defense responses in plants (Eulgem 2005; Rushton and Somssich 1998). *WRR4* encodes a cytoplasmic TIR-NB-LRR receptor-like protein in leaf, stem, flower, embryo, and shoot of *A. thaliana* and confers disease resistance against *Albugo candida. ATHCHIB,* which is expressed in root, leaf, stem, and pollen is a well-known pathogenesis-related protein (PR3) produced in response to a variety of biotic stresses (Van Loon 1985). *OSM* gene, which is expressed in root, stem, and pollen, is known to provide resistance to tobacco against fungal diseases (Liu and others 1994; Zhu and others 1996). Plant endophytic *P. putida* BP25 was recently reported to induce salicylic acid-mediated plant defense in *Arabidopsis* with concomitant regulation of the endogenous bacterial population (Sheoran and others 2016). Nearly identical endogenous regulation of BmBP17 in *Arabidopsis* could be attributed to the up-regulation of defense-related genes. Together with the report of Sheoran and others (2016), it could be confirmed that up-regulation of plant defense gene expression is one of the regulatory mechanisms used by endophytic microorganism to cohabitate as an endophyte.

BmBP17 endophytic colonization down-regulates the expression of genes associated with ethylene signaling A total of 70 genes were down-regulated in *Arabidopsis* upon endophytic colonization. Some transcription factors of ethylene-responsive genes such as *ERF5, ERF71, ERF104, ERF105, TEM1*, and *RAP2.6* were down-regulated in *Arabidopsis* colonized by BmBP17, which is consistent with the observation of Verhagen and others (2004) who postulated that the onset of ISR is associated with a reduction in ethylene signaling. *ERF5, ERF71*, *ERF104,*

Table 5 Reverse transcription-polymerase chain reaction analysis of mRNA expression of randomly selected genes based on the microarray results

Control	Treated	Gene	Product size (bp)	Regulation	Fold change	Identity
		PP2A	168	Reference	Reference	Protein phosphatase 2
		NIR1	197	UP	2.0	NITRITE REDUCTASE 1
		AMT1;3	185	UP	2.0	AMMONIUM TRANSPORTER 1;3
		ANNAT7	190	UP	2.5	Encodes a calcium-binding protein annexin
		RAP2.6	190	DOWN	2.3	Encodes a member of the ERF (ethylene response factor) subfamily
		ERF5	198	DOWN	1.6	ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR-5

ERF105, and *TEM1* are expressed in pollen, leaf, flower, stem, embryo, and root. *RAP2.6* is expressed in root, flower, and leaf.

BmBP17 endophytic colonization down-regulates the expression of genes associated with salicylic acid and jasmonic acid signaling The down-regulated genes also included some defense-related genes participating in salicylic acid and jasmonic acid signaling *BAP1, SIB1, BT4, MKK9*, and *PLA2A*. BmBP17 might have suppressed some defense-related genes to facilitate its early endophytic colonization. A population size of 5–6 Log CFU per gram of tissue was recorded for BmBP17 in *Arabidopsis*. Bacterial colonization mediated by repression of defense genes was reported in the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5, and *P. putida* BP25 (Wang and others 2005; Sheoran and others 2016). *BAP1* is expressed in embryo, seed, root, leaf, flower. and pollen and its repression leads to enhanced disease resistance to virulent pathogens largely through an R-gene *SNC1* (Yang and others 2006). The MAPK cascade involving MKK9-MPK6 is shown to play an important role in regulating leaf senescence in *Arabidopsis* (Zhou and others 2009). *MKK9* is expressed in embryo, seed, root, leaf, flower, and pollen of *Arabidopsis*. It has been shown that a mutation in *MKK9* results in enhanced seedling stress tolerance, suggesting that *MKK9* may act as a negative regulator of abiotic stress responses (Alzwiy and Morris 2007).

Other species of *Bacillus,* especially *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, are known to promote plant growth by air-borne volatiles such as 2, 3-butanediol and acetoin through cytokinin signaling (Ryu and others 2003). Growth promotion by volatiles of *B. subtilis* GB03 is also attributed to auxin and regulation of cell wall loosening enzymes for enabling cell expansion (Zhang and workers 2007). Another mechanism of growth promotion by *B. subtilis* is up-regulated photosynthetic capacity in *Arabidopsis* via adjustment of glucose/ABA sensing *in planta* (Zhang and others 2008). Transcripts of genes encoding chloroplast proteins associated with photosynthesis were up-regulated and genes for chlorophyll a/b binding protein and two RuBisCO subunit binding proteins were also up-regulated with GB03 exposure (Zhang and others 2008). However, physical interaction of these two *Bacillus* species within the endosphere of the plant is not known. BmBP17 promoted *Arabidopsis* growth upon its

characteristic endophytic multiplication in the intercellular spaces. A comparative differential gene expression profile of *Arabidopsis* triggered by *B. subtilis* and *B. megaterium* is furnished in Supplementary Table 6. It appears that the mechanism of plant growth promotion triggered by endophytic BmBP17 is completely different from that of *B. subtilis*. The differences in altered gene expressions observed in BmBP17-colonized plants could be due to its endogenous physical interaction, where a number of diverse bacterial components or elicitors are likely to interact with plant cell receptors and trigger downstream cellular responses. Significant up-regulation of several genes participating in nutrient mobilization and transport, growth and development, and down-regulation of ethylene signaling associated genes; SA and JA signaling genes were also found among bacterium-altered genes.

Conclusion

Our study showed that the endophytic *B. megaterium*-mediated plant growth promotion as observed in the model plant, *A. thaliana* Col 0 could be attributed to selective up-regulation of genes participating in nutrient transport and uptake, plant growth, and development as well as down-regulation of transcription factors involved in phytohormone signaling especially ethylene. Regulation of the endogenous population size of *B. megaterium* BP17 in *Arabidopsis* could be attributed to up-regulation of defense-related genes.

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