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Effect of Transgenic Rhizobacteria Overexpressing *Citrobacter braakii appA* on Phytate-P Availability to Mung Bean Plants

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Rhizosphere microorganisms possessing phytase activity are considered important for rendering phytate-phosphorus (P) available to plants. In the present study, the *Citrobacter braakii* phytase gene (*appA*) was overexpressed in rhizobacteria possessing plant growth promoting (PGP) traits, for increasing their potential as bioinoculants. AppA was cloned under the *lac* promoter in the broad-host-range expression vector pBBR1MCS-2. Transformation of the recombinant construct pCBappA resulted in high constitutive phytase activity in all of the eight rhizobacterial strains belonging to genera *Pantoea*, *Citrobacter*, *Enterobacter*, *Pseudomonas* (two strains), *Rhizobium* (two strains), and *Ensifer* that were studied. Transgenic rhizobacterial strains were found to display varying levels of phytase activity, ranging from 10-folds to 538-folds higher than the corresponding control strains. The transgenic derivative of *Pseudomonas fluorescens* CHA0, a well-characterized plant growth promoting rhizobacterium, showed the highest expression of phytase (~8 U/mg) activity in crude extracts. Although all transformants showed high phytase activity, rhizobacteria having the ability to secrete organic acid showed significantly higher release of P from Ca-phytate in buffered minimal media. AppA overexpressing rhizobacteria showed increased P content, and dry weight (shoot) or shoot/ root ratio of mung bean (*Vigna radiata*) plants, to different extents, when grown in semisolid agar (SSA) medium containing Na-phytate or Ca-phytate as the P sources. This is the first report of the overexpression of phytase in rhizobacterial strains and its exploitation for plant growth enhancement.

Keywords: *appA* overexpression, phytate-P availability, phytase, plant growth promotion, transgenic rhizobacteria

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Organic phosphorus (Po) in soils generally accounts for 29–65% of total soil phosphorus (P), depending upon the nature of the soil [38]. *myo*-Inositol hexakisphosphate (phytic acid) or its salts are a predominant form of Po [38]. Specialized enzymes known as phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are required for releasing P from phytates, since general phosphatases are not efficient at phytate hydrolysis [9]. Phytases are widely distributed in plants, microorganisms, and animals [40], and hydrolyze phytate in a stepwise manner leading to release of P and formation of lower phosphate derivatives of inositol phosphates. Based on their amino acid sequences, three-dimensional structure, and active-site amino acids, phytases are classified into three major types as histidine acid phosphatases (HAPs), purple acid phosphatases (PAPs), and β-propellor phytases (BPPs) [22].

Most plants are unable to acquire their P requirement from phytate [11, 29] since their root systems do not secrete phytases. Phytase overexpression in transgenic plants has been an important strategy to increase phytate-P availability to plants [18, 31]. Another significant approach is to employ rhizosphere phytase-producing microorganisms to help plants acquire phytate-P for growth [13, 26, 29]. The existence of rhizosphere microorganisms in a P-deficient soil is equally effective as extracellular phytase secretion by transgenic plants [5].

Several rhizosphere isolates belonging to the genera *Pantoea*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Burkholderia*, *Pseudomonas*, and *Bacillus* have been shown to possess phytase enzymes that may be cell bound (periplasmic) or secreted extracellularly [19, 26, 31, 39]. Despite the existence of phytase-positive soil organisms representing 30–50% of total soil microbial flora [8, 30] and ranging 3–19% in the rhizosphere of different plants [26], phytates remain the most recalcitrant pool of Po in soils [24]. The apparent inability of microorganisms to hydrolyze soil phytates has been mainly attributed to the lack of availability

of phytates in the solution phase [6]. Phytates can easily complex with soil cations to give rise to insoluble complexes such as Al-, Fe-, or Ca-phytates [38], or may adsorb to soil mineral components [7]; such type of phytate complexes cannot be hydrolyzed by any of the three major types of phytases [37]. In addition, the expression of phytase in native bacterial strains is regulated by nutrient availability or growth phase [9], and its expression in the rhizosphere has not been studied yet.

Low-molecular-weight organic acids have been reported to solubilize mineral complexes of phytates and render them susceptible for enzymatic hydrolysis [26, 37]. Many rhizosphere bacteria are known to secrete different organic acids [10, 26, 32]. Organic-acid-producing (OAP) and phytate-mineralizing (PM) rhizobacteria make phytate-P available to plants [26, 39]. The beneficial effect of such bacteria could be further enhanced by increasing phytase production and rendering it free from the regulatory controls that may be a detriment to achieving high phytase levels in rhizosphere.

Considering these important points, this work was aimed towards the constitutive heterologous overexpression of the *Citrobacter braakii appA* gene encoding the HAP-type of phytase [15] in different rhizosphere bacteria possessing various plant-beneficial traits. The phytase encoded by the *appA* gene of *C. braakii* possesses high specific activity and is resistant to proteases [14]. The bacterial strains selected as hosts had one or more of the following plant-beneficial traits: secretion of low-molecular-weight organic acids, the ability to fix nitrogen, the ability to colonize plant roots, and the ability to secrete antifungal metabolites or plant growth promoting (PGP) substances. The plant growth promoting abilities of phytase-overexpressing transformants with respect to phytate-P availability to plants were studied *in vitro* in semisolid agar (SSA) medium using mung bean plants grown in the presence of Na-phytate or Ca-phytate as the sole source of P.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

Table 1 shows the bacterial strains and plasmids used in this study and lists important plant beneficial traits that the native strains possess. All bacteria were routinely grown in Luria–Bertani broth (LB), except for the rhizobial strains, which were grown in Ashby's mannitol medium. Broad-host-range plasmid pBBR1MCS-2, known to be stably maintained in various gram-negative bacteria even in the absence of selection [16], was used for the construction of the *appA*-bearing plasmid pCBappA. Plasmid pB-phyF, carrying a genomic fragment of *C. braakii* harboring the *appA* gene (a kind gift from Dr. Han-Woo Kim, Biotechnology Research Center, NFRDI, Busan, Korea), was used to PCR amplify the *appA* gene for clone construction. pBBR1MCS-2 and pCBappA bearing stains were grown in media containing 50 μg/ml kanamycin. pB-phyF-bearing *E. coli* DH5α was grown in medium containing 100 μg/ml ampicillin.

Construction of pCBappA

The *appA* gene was amplified from pB-phyF by PCR using specific primers (Forward: 5'-GG**GGTACC**GAAAAGGTGGTGCTGG-3' and reverse: 5'-GG**GGATCC**TTATTCCGTAACTGCA-3'). The underlined sequence in the forward primer indicates a ribosomal binding site (RBS), and the bold letters in the forward and reverse primers indicate the *Kpn*I and B*am*HI restriction sites, respectively. The PCR product carrying the *Kpn*I and *Bam*HIsites was ligated into pBBR1MCS-2 at the *Eco*RV site to yield pCBappA. Plasmid pCBappA was transferred into various rhizobacterial strains possessing one or more PGP traits (Table 1) by $CaCl_2$-mediated transformation, except for the rhizobial strains, where conjugation using *E. coli* S17-1 was carried out. For every strain, a control was constructed that carried the empty vector pBBR1MCS-2.

Phytase and Phosphatase Activity Measurements in pCBappA Transformants

The *appA* expression was monitored by phytase assays. Bacterial transformants were grown in 100 ml of growth media at 30°C until they reached the stationary phase. Culture was centrifuged at 13,000 ×*g* for 10 min and washed three times with 30 ml of sterile 0.85% NaCl. The bacterial pellet was resuspended in 5 ml of 0.85% NaCl and subjected to sonication for 3.33 min at 9.9 s on/off pulses at 28% amplitude using a Sonics vibracell ultrasonicator. The lysate was centrifuged at 13,000 ×*g* for 15 min at 4°C, and the supernatant obtained was used for determining phytase activities. Phytase activity was determined at pH 4.5, as described by Patel *et al.* [26], where Na-phytate was used as the substrate. Since AppA also shows phosphatase activity [14], the acid phosphatase activity was determined from the extracts by the method previously described by Patel *et al.* [26], where *p*-nitrophenyl phosphate (pNPP) was used as the substrate.

Hydrolysis of Ca-phytate by pCBappA Transformants

Ca-phytate hydrolysis by the transformants was studied in minimal medium containing 100 mM glucose, 100 mM Tris-Cl (pH 8.0), 50 mM potassium nitrate (KNO_3), 8.9 mM NaCl, 2 mM magnesium sulfate ($MgSO_4 \cdot 7H_2O$), and 100 μM calcium chloride ($CaCl_2$). Ca-phytate (0.3%) was used as the sole source of P and added separately after autoclaving. After inoculating the bacterial cultures, the pH of the medium and the P released in solution were measured in aliquots sampled at regular time intervals. P was estimated by a modified ammonium molybdate method [12].

Plant Inoculation Studies

Short-term plant inoculation experiments were performed using mung bean plants (*Vigna radiata* Var. Guj 4) grown in glass tubes containing 25 ml of semisolid agar (SSA) medium, containing one tenth diluted Murashige and Skoog medium [23]. KH_2PO_4 was replaced with 1 mM Na-phytate (filter sterilized and added separately) or 1 mM Ca-phytate (autoclaved separately and added) and the medium was solidified by 0.8% agar. The pH of the medium was adjusted to 8.0 prior to autoclaving, and methyl red (0.03%) was added as the pH indicator dye to observe the change in pH during the growth of plants. Mung bean seeds were surface sterilized with 0.1% $HgCl_2$, washed thoroughly, and germinated in sterile distilled water.

Table 1. Characteristics of bacterial strains and plasmids used in this study.

Bacterial strains or plasmids	Description	Reference
E. coli strains		
DH5α	Used for maintaining plasmids	[33]
S17-1	Used for conjugative transfer of plasmid; *recA pro hsdR* (RP4-2 Tc::Mu Km::Tn*7*)	[36]
Other bacterial strains		
Citrobacter sp. DHRSS	Wild type; OAP; PSB, PM laboratory isolate	[25, 26]
DHRSS (pBBR1MCS-2)	*Citrobacter* sp. DHRSS vector control	This study
DHRSS (pCBappA)	*Citrobacter* sp. DHRSS *appA* transformant	This study
Enterobacter asburiae PSI3	Wild type; OAP; PSB, PM laboratory isolate	[10, 25, 26, 34, 35]
EaPSI3 (pBBR1MCS-2)	*E. asburiae* PSI3 vector control	This study
EaPSI3 (pCBappA)	*E. asburiae* PSI3 *appA* transformant	This study
Pantoea sp. PP1	Wild type; OAP; PSB, PM laboratory isolate	[26]
PP1 (pBBR1MCS-2)	*Pantoea* sp. PP1 vector control	This study
PP1 (pCBappA)	*Pantoea* sp. PP1 *appA* transformant	This study
Pseudomonas putida KT2440	Root colonizer, siderophore-producing	[21]
PpKT2440 (pBBR1MCS-2)	*P. putida* KT2440 vector control	This study
PpKT2440 (pCBappA)	*P. putida* KT2440 *appA* transformant	This study
Pseudomonas fluorescens CHA0	Antifungal compounds- and siderophore-producing	[3]
PfCHA0 (pBBR1MCS-2)	*P. fluorescens* CHA0 vector control	This study
PfCHA0 (pCBappA)	*P. fluorescens* CHA0 *appA* transformant	This study
Ensifer meliloti (ATCC No. 9930)	Wild type, N_2-fixing	[41]
Em (pBBR1MCS-2)	*E. meliloti* vector control	This study
Em (pCBappA)	*E. meliloti appA* transformant	This study
Rhizobium sp. ST-1	Wild type; N_2-fixing	[27]
ST-1 (pBBR1MCS-2)	*Rhizobium* sp. ST-1 vector control	This study
ST-1 (pCBappA)	*Rhizobium* sp. ST-1 *appA* transformant	This study
Rhizobium sp. IC-3109	Wild type; N_2-fixing	[27]
IC-3109 (pBBR1MCS-2)	*Rhizobium* sp. IC-3109 vector control	This study
IC-3109 (pCBappA)	*Rhizobium* sp. IC-3109 *appA* transformant	This study
Plasmids		
pB-phyF	Ap^r, plasmid carrying genomic fragment of *C. braakii* harboring *appA*	[15]
pBBR1MCS-2	Km^r, expression vector	[16]
pCBappA	Km^r, pBBR1MCS-2 harboring ~1.3 kb *C. braakii appA* under *lac* promoter	This study

OAP: Organic-acid-producing; PM: phytate-mineralizing; PSB: Phosphate-solubilizing bacterium; Km^r: kanamycin resistant; Ap^r: ampicillin.

Bacterial suspensions were inoculated onto the surface-sterilized seedlings to obtain ~10^4 or ~10^6 CFU, respectively, for *Pseudomonas* and non-*Pseudomonas* strains, per seedling. Inoculated seedlings were planted in growth media such that each tube contained two plants. Each treatment was replicated in 5 tubes (10 plantlets for each treatment). Control treatments consisted of uninoculated plants. Plants were grown under natural daylight at ambient temperature for 15 days, after which they were gently uprooted; the roots and shoots were separated and dried at 65°C to constant weight, and the dry weight was measured. Dried shoots were ashed at 550°C for 16 h and the ashes were dissolved in 3 ml of 5.6 M HNO_3. The P content of the acid extracts was determined by using a modified ammonium molybdate method [12].

Data Analysis and Statistical Treatments

Data values are given as the means of 3–5 independent experiments (exact number of replicates mentioned in the legends to Figures and Tables) along with standard deviation (SD). Significant differences in the means were established using general analysis of variance (ANOVA) and the treatment means compared by LSD (SigmaStat 3.5).

Results

Construction of pCBappA

PCR amplification of the *appA* gene from pB-phyF, using the specific primers, showed the expected ~1.3 kb DNA band. After ligating into pBBR1MCS-2, the recombinant construct pCBappA was subjected to *appA*-specific PCR amplification, where the expected ~1.3 kb *appA* amplicon was obtained. RE digestion with *Eco*RI and *Hin*dIII conformed to the expected band pattern (Fig. 1). The orientation of *appA* in the recombinant plasmids was checked by digestion

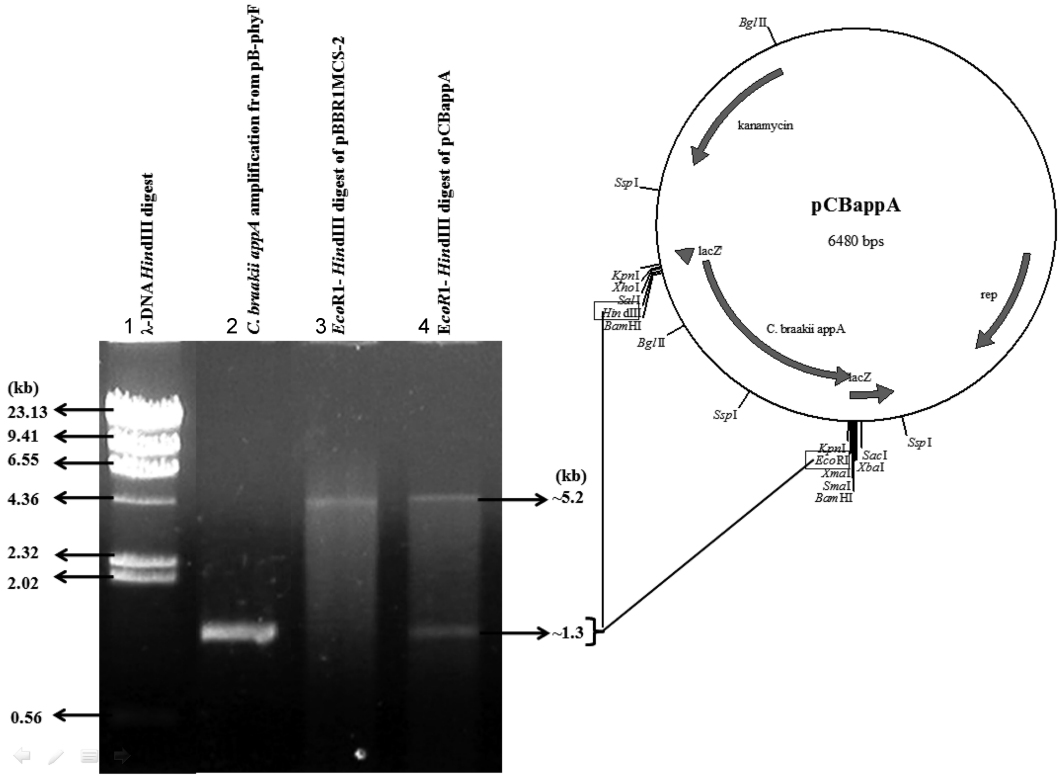

Fig. 1. Physical map of pCBappA and agarose gel electrophoresis showing the presence of *appA* by PCR and the release of *appA* from pCBappA by the *Eco*RI–*Hin*dIII digest.
Lane: 1, λ-DNA *Hin*dIII digest; 2, *C. braakii appA* amplification from pB-phyF; 3, *Eco*RI–*Hin*dIII digest of pBBR1MCS-2; 4, *Eco*RI–*Hin*dIII digest of pCBappA.

with *Kpn*I and *Bam*HI since both sites are present on the vector as well as the ends of the amplicon, and the construct with correct orientation with respect to the *lac* promoter was sequenced using the M13 sequencing primer. The insert showed a 100% match with *C. braakii appA* (Accession No. AY471611 [15]), and this construct was termed pCBappA. After transferring the plasmid into different rhizobacteria, the presence of pCBappA in them was confirmed by PCR using *C. braakii appA*-specific primers (data not shown).

Phytase Activity in pCBappA Transformants

All transformants bearing pCBappA showed higher phytase activity as compared with the corresponding vector control strains bearing pBBR1MCS-2 (Fig. 2A). The specific phytase activity of crude cell lysates ranged form ~0.5 (*Pantoea* sp. PP1) to ~8.0 (*P. fluorescens* CHA0) U/mg protein, and the fold increase ranged from approximately 10-fold (*Pantoea* sp. PP1, *Citrobacter* sp. DHRSS), to 171-fold (*P. putida* KT2440), and highest increase was found in the case of *P. fluorescens* CHA0, which showed a 538-fold increase in specific phytase activity as compared with the corresponding vector control. Rhizobial strains do not show detectable basal phytase activity, hence the fold increase cannot be estimated. As compared with the *C. braakii* native strain (0.27 U/mg) [14], the crude phytase activity of transformants was about ~2 times (in *Pantoea* sp. PP1) to 30 times (in *P. fluorescens* CHA0) higher. In agreement with the phytase activity measurements, the reported ~47 kDa protein band of *C. braakii* of AppA [14] was observed to be overexpressed in crude cell lysates of *P. fluorescens* CHA0 (pCBappA) on SDS–PAGE gel, but overexpression could not be discerned in SDS–PAGE of crude extracts of the other strains (data not shown), although phytase activity was higher than that corresponding to the vector control for all the strains. The phytase activities of pCBappA transformants of *P. fluorescens* CHA0, *P. putida* KT2440, and *E. meliloti* were higher than that of *E. coli* BL21 (DE3) expressing the *phyM* of *P. syringae* MOK1 (2.514 U/mg) [1].

Since the phytase enzyme encoded by the *C. braakii appA* also has general phosphatase activity, it was of interest to check this activity in the transformants (Fig. 2B). As seen, the phosphatase activities of the strains showed an increase by 5- to 38-folds as compared with the corresponding vector controls; however, the vector transformant strains of rhizobia did not exhibit the native acid phosphatase activity.

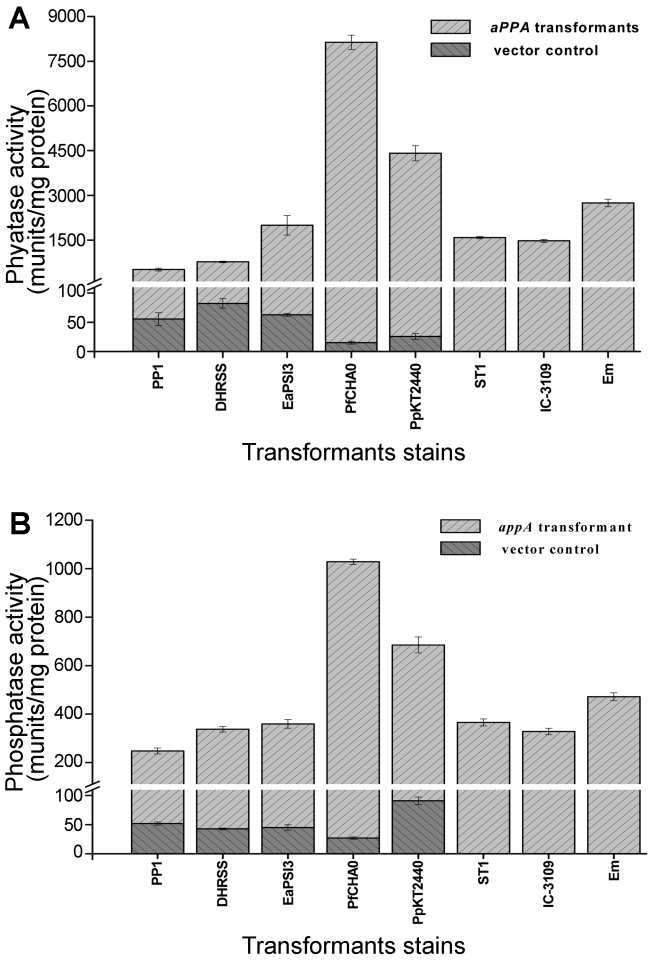

Fig. 2. Phytase (**A**) and phosphatase (**B**) activities of *appA*-expressing transformants (pCBappA) and vector controls (pBBR1MCS-2) of various transformants strains.

Hydrolysis of Ca-Phytate by pCBappA Transformants

Sparingly soluble Ca-phytate is a predominant form of phytate present in alkaline soils [38]. The rhizobacterial transformants expressing *appA* were tested for their ability to hydrolyze Ca-phytate in minimal medium containing 100 mM Tris-Cl (pH 8) buffer. The *appA*-expressing transformants of *Pantoea* sp. PP1, *Citrobacter* sp. DHRSS, *E. asburiae* PSI3, and *P. putida* KT2440 showed significant P release from Ca-phytate than their respective vector control strains (Fig. 3A), although both pCBappA transformants and vector control strains brought about a similar drop in the pH of the medium (Fig. 3B). Strains *Pantoea* sp. PP1 [26], *Citrobacter* sp. DHRSS [25], and *E. asburiae* PSI3 [10, 35] have been previously reported to produce organic acids such as gluconic and acetic acids, which aid in solubilization of sparingly soluble Ca-phytate, thus rendering it more susceptible to enzymatic attack by cell-associated phytase [26]. *P. fluorescens* CHA0 (pCBappA) (which showed the highest phytase activity; Fig. 2A) was not able to drop the medium pH below 5.6 (Fig. 3D), and P release in this case was significantly lower (Fig. 3C) than other transformant strains (Fig. 3A), although the *appA* transformant showed higher than the vector control. Rhizobial transformants neither showed significant Ca-phytate hydrolysis (Fig. 3C) nor lowered the medium pH (Fig. 3D) as compared with their corresponding vector control.

Effects of Transformants Overexpressing *C. braakii appA* on Plant Growth

Plant growth was monitored in short-term experiments as dry weight of plants, dry shoot/root ratio, and P content of plants. When grown in the presence of Na-phytate, plants inoculated with *appA*-expressing transformants showed significant increases in dry shoot weight [*Pantoea* sp. PP1, *E. asburiae* PSI-3, and *Ensifer meliloti* (previously known as *Sinorhizobium meliloti*)], in dry shoot/ root ratio (all except *E. asburiae* PSI-3 and *Rhizobium* sp. IC-3109), and in dry shoot P content (all except *Citrobacter* sp. DHRSS and *E. asburiae* PSI-3) as compared with the uninoculated plants (Table 2). However, as compared with plants inoculated with vector control strains, the *appA*-expressing transformant showed significant increases in dry shoot weight (*P. putida* KT2440 and *E. meliloti*), in dry shoot/ root ratio (*P. fluorescens* CHA0 and *P. putida* KT2440) and in dry shoot P content in all strains (except *Citrobacter* sp. DHRSS and *E. asburiae* PSI-3) (Table 2).

Whether the plant growth promotion by phytase-overexpressing microorganisms is also seen with poorly soluble phytates such as Ca-phytate is not well studied. Therefore, plant inoculation experiments were also carried out using Ca-phytate, where all pCBappA transformants except rhizobial strains (as these were not able to hydrolyze Ca-phytate) were used. In these experiments, all transformants showed significant increases in dry shoot weight (except *Citrobacter* sp. DHRSS and *E. asburiae* PSI-3), in dry shoot/root ratio (except *Pantoea* sp. PP1 and *Citrobacter* sp. DHRSS), and in dry shoot P content of plants (Table 3), as compared with uninoculated control plants. However, as compared with plants inoculated with vector control strains, significant increases by *appA*-expressing strains were observed in dry shoots (by *Pantoea* sp. PP1 and *P. putida* KT2440), in dry shoot/root ratio (by *Pantoea* sp. PP1 and *P. fluorescens* CHA0), and in shoot P content in all strains expressing *appA* (except *Citrobacter* sp. DHRSS) (Table 3).

Discussion

This work deals with the overexpression of the *appA* gene-encoding HAP-type phytase from *C. braakii* in eight plant-associated strains, including members of *Citrobacter*, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Rhizobium*, and

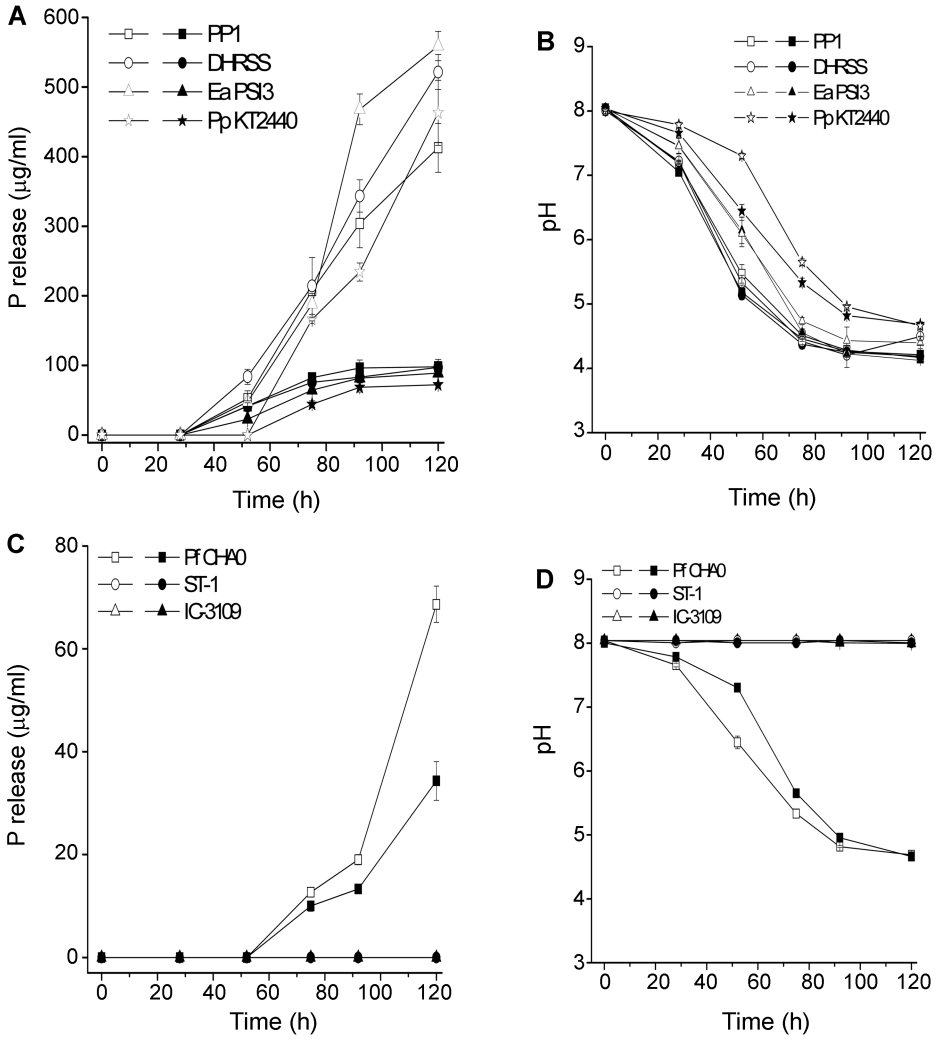

Fig. 3. The P released and pH profile of transformants during growth on minimal media containing Ca-phytate.
(**A**) P released and (**B**) pH profile of *Pantoea* sp. PP1, *Citrobacter* sp. DHRSS, *E. asburiae* PSI3, and *P. putida* KT2440 transformants; (**C**) P released and (**D**) pH profile of *P fluorescens* CHA0 and rhizobial transformants (ST-1 and IC-3109). Open symbols indicate pCBappA transformants and closed symbols are vector controls with pBBR1MCS-2.

Ensifer. The hosts used for heterologous expression of *appA* included two widely studied model strains, the plant growth promoting rhizobacterium (PGPR) strain *P. fluorescens* CHA0 [2, 3] and the metabolically versatile root-colonizing strain *P. putida* KT2440 [21, 28]. *P. fluorescens* CHA0 produces various antifungal metabolites as well as siderophores, and *P. putida* KT2440 is a siderophore-producing strain. *E. asburiae* PSI3 [10, 35], *Citrobacter* sp. DHRSS [25], *Rhizobium* sp. ST-1 [4, 27] and *Pantoea* sp. PP1 [26] represent well-characterized plant-associated isolates from this laboratory with different plant-beneficial traits such as P solubilization and N fixation. It is interesting to note that all native strains possessed varying levels of phytase activity, with *Citrobacter* sp. DHRSS having the highest levels and rhizobial strains displaying no detectable activity (Fig. 2A). This is the first report of phytase activity in the model strain *P. putida* KT2004 and the well-characterized *P. fluorescens* CHA0, which have never been earlier reported for their phytase activities. Although the genome of *P. putida* KT2440 is sequenced, phytase was not annotated in the genome sequence; the genome sequence does not have an *appA* homolog. Upon transformation with pCBappA, the phytase activity was not proportional to the basal levels of the native strains, and *P. fluorescens* CHA0 showed the highest activity, about seven times higher than *Citrobacter* sp. DHRSS, in spite of the gene being obtained from the latter genus.

The AppA enzyme of *C. braakii* is highly specific for phytate but also displays activity as a general phosphatase [14]. In the transformants, both the activities were high under uninduced conditions, in agreement with the observation that the *lac* promoter is a strong, constitutively

Table 2. Dry weight and shoot P content of mung bean plants grown in SSA medium containing Na-phytate as a sole source of P, inoculated with vector control and *appA* transformants of different bacterial strains.

Bacterial strains	Parameters					
	Dry shoot weight (mg)		Dry shoot/root ratio of plants (mg)		Shoot P concentration of plants (mg/g dry wt)	
	Vector control	*appA* transformant	Vector control	*appA* transformant	Vector control	*appA* transformant
Uninoculated control	17.63		4.56		41.88	
PP1	19.73 ns	20.66 * NS	5.70 *	5.78 * NS	42.30 ns	45.05 * †
DHRSS	18.62 ns	19.62 ns NS	5.56 *	6.06 ** NS	44.51 *	43.05 ns NS
EaPSI-3	19.33 ns	21.25 * NS	5.15 ns	5.39 ns NS	41.97 ns	43.42 ns NS
Pf CHA0	18.42 ns	19.11 ns NS	5.69 *	6.77 ** †	40.88 ns	46.36 ** ††
PpKT2440	17.10 ns	20.54 ns †	5.13 ns	6.22 ** †	39.59 **	48.69 ** ††
ST1	19.25 ns	18.50 ns NS	5.46 *	5.97 ** NS	38.03 **	49.15 ** ††
IC-3109	19.66 ns	19.80 ns NS	4.44 ns	4.82 ns NS	41.39 ns	46.91 ** ††
Em	16.87 ns	21.10 * ††	4.67 ns	5.41 * NS	43.92 ns	50.22 ** ††

Plants were grown (2 plants/tube, 7 replicates) for 15 days, and the data are means of individual experiments (for dry weight and shoot/root ratio of plants, n=10, and for shoot P content, n=4). Means followed by ns/NS do not significantly differ (Fisher LSD test, $p<0.05$). ns: data comparison with uninoculated control; NS: data comparison with corresponding vector control; *: significant in comparison with uninoculated control; †: significant in comparison with the corresponding vector control (single sign: $p<0.05$; double sign: $p<0.001$).

expressed promoter in gram-negative bacteria such as rhizobia and pseudomonads [17]. The varying levels of expression in transformants may reflect the difference in the expression of the *lac* promoter in the different species [17]. The Phytase activities of pCBappA-bearing transformants were 2 (*Pantoea* sp. PP1) to 8 (*P. fluorescens* CHA0) folds higher than their phosphatase activity, and this could be because of the inherent phosphatase activity of the strains. As nonspecific phosphohydrolases play an important role in the final stages of P release from phytate [38], the higher phosphatase activity of AppA may be beneficial in field conditions to make available more P from lower P derivatives of phytates and also from other Po present in soil.

It has been shown that in the case of phytase-exuding transgenic plants, the assimilation of phytate-P is limited by the availability of phytate in solution [20]. *appA*-overexpressing transgenic transformants such as rhizobial strains, which did not possess inherent organic acid-producing ability, were not able to hydrolyze Ca-phytate, most likely since they were unable to render the phytate ion in the soluble phase, which is a prerequisite for its hydrolysis by the HAP type of phytase [26]. However, *P. fluorescens* CHA0 (pCBappA) released up to ~70 μg/ml P,

Table 3. Dry weight and shoot P content of mung bean plants grown in SSA medium containing Ca-phytate as a sole source of P, inoculated with vector control and *appA* transformants of selected bacterial strains.

Bacterial strains	Parameters					
	Dry shoot weight (mg)		Dry shoot/root ratio of plants (mg)		Shoot P concentration of plants (mg/g dry wt)	
	Vector control	*appA* transformant	Vector control	*appA* transformant	Vector control	*appA* transformant
Uninoculated control	14.60		4.73		38.58	
PP1	14.50 ns	17.28 * †	3.81ns	4.88 ns †	42.79 **	45.82 ** †
DHRSS	16.00 ns	15.15 ns NS	4.56 ns	5.12 ns NS	46.91 **	46.62 ** NS
Ea PSI-3	13.70 ns	14.57 ns NS	4.98 ns	5.48 * NS	42.84 **	46.62 ** †
Pf CHA0	16.92 *	18.00 * NS	5.70 *	8.37 ** ††	40.47 **	45.64 ** ††
Pp KT2440	14.00 ns	16.76 * †	5.73 *	6.36 ** NS	36.80 ns	47.70 ** ††

Plants were grown (2 plants/tube, 7 replicates) for 15 days, and the data are the means of individual experiments (for dry weight and shoot/root ratio of plants, n=10, and for shoot P content, n=4). Means followed by ns/NS do not significantly differ (Fisher LSD test, $p<0.05$). ns: data comparison with uninoculated control; NS: data comparison with corresponding vector control; *: significant in comparison with uninoculated control; †: significant in comparison with the corresponding vector control (single sign: $p<0.05$; double sign: $p<0.001$).

which was higher than that released by the vector control strain (Fig. 3C), in spite of the strain bringing about only a modest drop in pH (~5.6) (Fig. 3D).

A beneficial effect of phytase-producing bacterial isolates on plant growth, when phytate is supplied as the sole P source, has been previously reported [26, 29, 31]. Recently, George *et al.* [5] demonstrated that the presence of rhizosphere microorganisms can overcome the need of extracellular phytase secretion by transgenic plants in a P-deficient soil. The present work describing the transgenic overexpression of HAP-phytase in plant-associated beneficial bacteria demonstrates its usefulness for enhancing their plant growth promoting characteristics. The practical application of the engineered strains in natural conditions will require genomic integration of the *appA* gene, because the maintenance of plasmids, at least under certain conditions, may affect the metabolism of transformants [34].

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