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Transgenic expression of glucose dehydrogenase in *Azotobacter vinelandii* enhances mineral phosphate solubilization and growth of sorghum seedlings

Burla Sashidhar and Appa Rao Podile*
Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad-500 046, Andhra Pradesh, India.

Summary

The enzyme quinoprotein glucose dehydrogenase (GDH) catalyses the oxidation of glucose to gluconic acid by direct oxidation in the periplasmic space of several Gram-negative bacteria. Acidification of the external environment with the release of gluconic acid contributes to the solubilization of the inorganic phosphate by biofertilizer strains of the phosphate-solubilizing bacteria. Glucose dehydrogenase (*gcd*) gene from *Escherichia coli*, and *Azotobacter*-specific glutamine synthetase (*gln*A) and phosphate transport system (*pts*) promoters were isolated using sequence-specific primers in a PCR-based approach. *Escherichia coli gcd*, cloned under the control of *gln*A and *pts* promoters, was mobilized into *Azotobacter vinelandii* AvOP and expressed. Sorghum seeds were bacterized with the transgenic azotobacters and raised in earthen pots in green house. The transgenic azotobacters, expressing *E. coli gcd*, showed improved biofertilizer potential in terms of mineral phosphate solubilization and plant growth-promoting activity with a small reduction in nitrogen fixation ability.

Introduction

Ever increasing demand for expensive chemical fertilizers to supplement macronutrients like nitrogen and phosphorous to the crop plants and their excessive use in cropping systems contributes to imbalance in the soil ecology in more than one way. Free-living plant growth-promoting rhizobacteria (PGPR) present in the soil enhance the growth and protect the plants in many different ways such as nitrogen fixation, mineral phosphate solubilization (MPS), sequestration of iron by secretion of siderophores, production of phytohormones and release of volatiles (Podile and Kishore, 2006). Phosphorous is one of the essential macronutrients, next only to nitrogen, required for plant growth and productivity. The total content of phosphorous in many soils is normal, but its availability to plants is meager (Goldstein, 1986). Phosphorous often tends to form complexes with metal ions such as Mg^{2+}, Ca^{2+} and Fe^{2+} etc., in the soil and crop plants are deprived of essential phosphorous to attain maximum productivity even in the presence of added phosphatic fertilizers. *Sorghum bicolor* L. (Sorghum) is an important food crop in Africa, Central America and South Asia, more particularly in the semi-arid tropics. It is the fifth most important cereal crop grown in the world and India is the second greatest producer of sorghum worldwide (Anonymous, 1995).

Membrane-bound glucose dehydrogenase (GDH) in Gram-negative bacteria plays an important role in MPS via direct oxidation (DO) pathway, catalysing oxidation of D-glucose to D-gluconate (Goldstein, 1995). Glucose dehydrogenase requires pyrroloquinoline quinone (PQQ) as a cofactor, and has binding sites for Mg^{2+} (*in vitro*), Ca^{2+} (*in vivo*), ubiquinone, as well as for substrate glucose (Hommes *et al*., 1984; Matsushita *et al*., 1997). *Escherichia coli* is unable to synthesize PQQ; therefore, GDH occurs as an apoenzyme, but is readily reconstituted by incubation with PQQ and metal ions (Matsushita *et al*., 1997). The location of the active domain of the enzyme towards the periplasmic site facilitates release of the oxidized product gluconate into the external medium, which is further oxidized to 2-keto gluconate by gluconate dehydrogenase. Gluconate and 2-keto gluconate are the strongest known organic acids produced by the Gram-negative bacteria that are associated with MPS (Goldstein, 1995) and facilitate release of soluble inorganic phosphate.

Azotobacters are free-living aerobic, soil-dwelling organisms with a unique array of metabolic capabilities, in addition to reduction of atmospheric nitrogen into ammonium compounds. Different strains of *Azotobacter* are capable of producing compounds such as alginates, poly-β-hydroxybutyrates, siderophores, pigments and plant hormones (Kennedy and Toukdarian, 1987), while MPS ability of these azotobacters is less known. To enhance/improve the biofertilizer potential of nitrogen-fixing

Received 14 April, 2008; accepted 13 April, 2009. *For correspondence. E-mail arpsl@uohyd.ernet.in; podilerao@gmail.com; Tel. (+91) 40 2313 4503; Fax. (+91) 40 2301 0120.

Azotobacter, we have adopted a transgenic approach in which the membrane-bound GDH of *E. coli* was expressed under *Azotobacter*-specific glutamine synthetase (*gln*A) and phosphate transport system (*pts*) promoters to understand the expression of MPS phenotype simultaneously under nitrogen-fixing and phosphate-limiting conditions respectively. Here we report and discuss the transgenic expression *gcd*-enhanced MPS activity of transgenic *Azotobacter*, and also the increased growth of pot-grown sorghum plants bacterized with transgenic strains.

Results

Construction of E. coli *gcd under the control of* Azotobacter*-specific* glnA *and* pts *promoters*

Azotobacter-specific glutamine synthetase (*gln*A) and phosphate transport system (*pts*) gene promoters were isolated using primers designed upstream to the respective genes and cloned in pUC18 as HindIII and BamHI fragments. Similarly, 2.4 kb glucose dehydrogenase (*gcd*) gene from *E. coli* was amplified, and cloned into pUC18 as BamHI and EcoRI fragment to derive pUCGDE. The *E. coli gcd* was excised from pUCGDE, cloned under *Azotobacter*-specific *gln*A and *pts* promoters resulted in the plasmid pGDEGS1 and pGDEPS1, respectively in *E. coli* PP2148. The constructs, i.e. promoters with *E. coli gcd*, were separately excised using HindIII and EcoRI, and religated into broad-host-range vector pMMB206. The resulting clones pMMBEGS1 and pMMBEPS1 were used to transform *Azotobacter vinelandii* AvOP.

Acid production phenotype of E. coli *PP2418 and transgenic* A. vinelandii *AvOP expressing* E. coli gcd

Escherichia coli PP2418 harbouring pGDEGS1 and pGDEPS1, spotted onto MacConkey or NBRIP medium, showed purple colour on MacConkey agar (Fig. 1A) and clear zones of solubilization around the colony in NBRIP medium indicating acid production (Fig. 1D and E), whereas *E. coli* PP2418 harbouring pUCGDE did not show coloration (Fig. 1A). Transgenic *A. vinelandii* strains showed a purple colour on MacConkey agar, without added PQQ, suggesting that *Azotobacter* is capable of producing PQQ, unlike *E. coli*, which requires PQQ for holoenzyme formation (Fig. 1B).

The RNA isolated from both transgenic and wild-type *Azotobacter*, *E. coli* PP2418 and *E. coli* DH5α was used as template for RT-PCR to detect the expression of *gcd* on agarose gel. The *gcd* expression was observed in transgenic azotobacters under the control of both *gln*A and *pts* promoters, but not in *E. coli* PP2418 (Fig. 1C). *Escherichia coli* DH5α in 1% glucose LB medium expressed higher *gcd*, similar to transgenic *E. coli* PP2418, while the transgenic azotobacters had significantly low *gcd* expression. In azotobacters, the *gcd* expression, driven by *pts* promoter (pMMBEPS1), was high compared with *gln*A promoter (pMMBEGS1). Similarly on semi-solid NBRIP medium, the *E. coli* DH5α and the transgenic *E. coli* PP2418 solubilized tricalcium phosphate (TCP) better than either of the *Azotobacter* transformants (Fig. 1D and E). The HPLC analysis of gluconic acid (Fig. 1F) using culture supernatant of different bacterial strains also confirmed the increase in the gluconic acid content by 1.5-fold (18 mM) (pMMBEGS1) and two-fold (23.4 mM) (pMMBEPS1) in transgenic azotobacters, when compared with the wild type (11.3 mM). While gluconic acid produced from culture supernatants of transformed *E. coli* PP2418 with recombinant *gcd* clones under regulation of *gln*A (pGDEGS1) (83 mM) and *pts* (pGDEPS1) (45.6 mM) promoters was less than the wild-type *E. coli* DH5α (112 mM).

Growth, phosphate solubilization and nitrogen fixation of transgenic A. vinelandii *AvOP*

The transformants pMMB206, pMMBEPS1 and pMMBEGS1 of *A. vinelandii* AvOP had reduced growth in Burk's nitrogen-free medium by about 26% compared with the wild type. Nitrogenase activity of these transformed strains was reduced by about 6% compared with wild type as assessed by acetylene reduction (Fig. 2). The transformants pMMB206, pMMBEPS1 and pMMBEGS1 reduced 4828, 4833 and 4812 μmol acetylene per OD of culture per 24 h, respectively, while the wild-type strain reduced 5148 μmol during the same time. The phosphate solubilization by both these transformants in liquid NBRIP and Burk's media was more than the wild type (Fig. 3). A notable improvement in the release of inorganic phosphate from tricalcium phosphate was visible with transgenic strains early during the incubation and progressively increased at least up to 3 days in NBRIP liquid medium, with or without ammonium salts. The observed improvement sustained up to 6 days for both the transformants in the medium with ammonium salt. However, pMMBEGS1 was unable to sustain the improvement beyond 3 days. The absence of the ammonium salts did not affect the MPS ability of pMMBEPS1 at least up to 6 days.

Plant growth-promoting activity of transgenic A. vinelandii *AvOP*

Sorghum seeds bacterized with transgenic and non-transgenic azotobacters showed difference in the fresh weight of seedlings. The fresh weight and height of the seedlings were significantly higher in seedlings that were

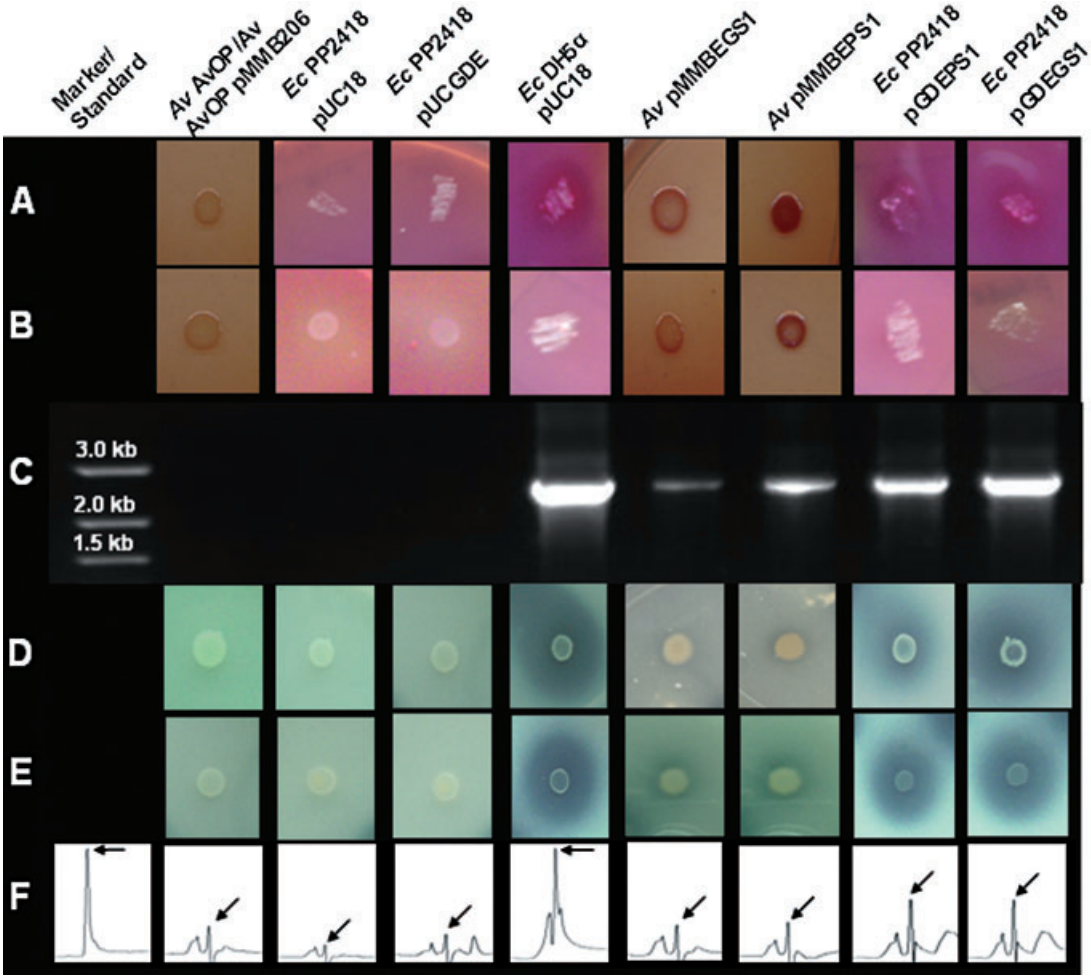

Fig. 1. Transgenic expression of glucose dehydrogenase. Transgenic expression of glucose dehydrogenase (*gcd*) under *Azotobacter*-specific *gln*A and *pts* promoters was assessed in both *Escherichia coli* (*Ec* pGDEGS1 and *Ec* pGDEPS1) and *Azotobacter vinelandii* (*Av* pMMBEGS1 and *Av* pMMBEPS1) in comparison with *A. vinelandii* AvOP, *E. coli* PP2418 pUCGDE, *E. coli* DH5α pUC18. Azotobacters were incubated at 30°C, while *E. coli* strains were incubated at 37°C.
A and B. Bacterial strains spotted on MacConkey agar with and without PQQ respectively and photographed after 4 days of incubation.
C. The total RNA isolated from the log-phase cultures was used as template in an RT-PCR using *gcd*-specific primer and subjected to agarose gel electrophoresis with molecular size marker on the extreme left.
D and E. Bacterial strains spotted on buffered and non-buffered NBRIP medium, containing 5% TCP respectively and photographed after 7 days of incubation.
F. Chromatograms of gluconic acid produced by different bacterial strains using gluconic acid standard on extreme left. Arrows on the chromatogram indicate the gluconic acid peak with retention time between 2.6 and 2.8 min.
Av, *A. vinelandii*; *Ec*, *E. coli*.

inoculated with transgenic *Azotobacter* strains compared with the non-transgenic *Azotobacter*-inoculated seedlings (Fig. 4). Superphosphate with *Azotobacter* significantly increased the height of the seedlings compared with the seedlings inoculated with transgenic *Azotobacter* (Fig. 4B). The cumulative growth-promoting effect of the transgenic strains was comparable to the effect of superphosphate application up to 3 weeks and improved further with prolonged growth period. The height of the seedlings treated with transgenic *Azotobacter* was high compared with the superphosphate-treated seedlings by fourth week. The seedlings treated with transgenic *Azotobacter*, compared with wild type, were more vigorous. However, the combined effect of native *Azotobacter* with the superphosphate was most effective in promoting the growth of sorghum seedlings over 4 weeks.

Discussion

The use of phosphate-solubilizing bacteria as inoculants increases both phosphorus uptake by the plant and crop yield. Azotobacters are routinely used as free-living nitrogen-fixing inoculants in agriculture and horticulture. It would be of interest to develop improved mineral

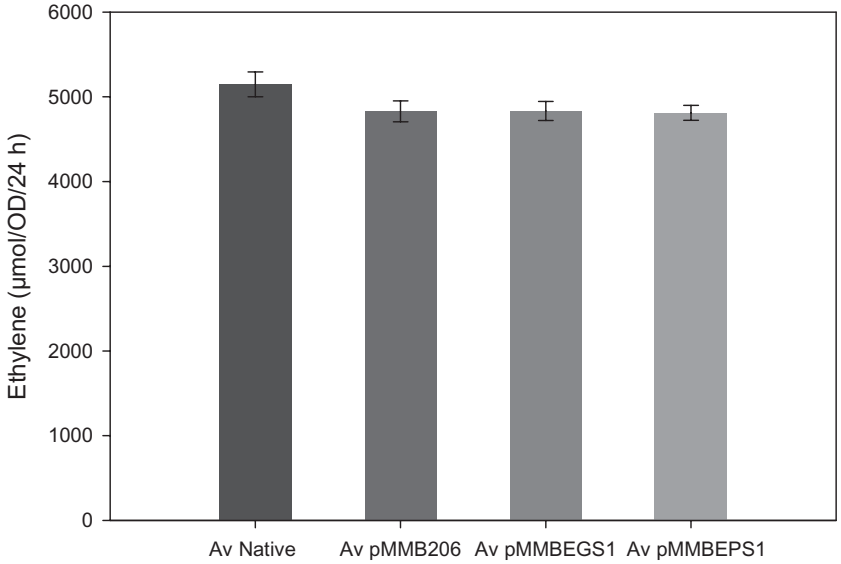

Fig. 2. Nitrogenase activity of azotobacters. Nitrogenase activity of transgenic and wild-type azotobacters was evaluated by acetylene reduction assay. *Azotobacter* strains were grown in Burk's nitrogen-free medium for 48 h. The cells were pelleted, transferred to fresh Burk's nitrogen-free medium tubes with rubber stopper, flushed with argon and replaced with acetylene. Reduction of acetylene to ethylene was estimated after 24 h. The values represent the mean of the triplicates in three independent experiments. The vertical bars on the histogram indicate standard error.

phosphate-solubilizing azotobacter strains that would simultaneously fix atmospheric nitrogen in the soil for dual benefits to the crop plants. The expression of *E. coli gcd* under the regulation of *gln*A and *pts* promoters in *E. coli* PP2418 (*gcd*::*cm* and *pts*$^-$) on MacConkey glucose and NBRIP medium was supplemented with PQQ, since *E. coli* forms only apoenzyme of GDH (Matsushita *et al.*, 1997). Certain mutants of *E. coli* were capable of producing PQQ to form PQQ-GDH holoenzyme without external supplementation (Biville *et al.*, 1991). *Azotobacter vinelandii* AvOP harbouring pMMBEGS1 and pMMBEPS1, without supplementation of PQQ, showed pink colouration of the colony, solubilized TCP and also released inorganic phosphate in liquid media more than the wild type, suggesting that the *Azotobacter* was able to synthesize the cofactor PQQ. *Azotobacter vinelandii* AvOP genome sequencing project also revealed the presence of ORFs with homology to the PQQ cofactor biosynthetic genes. The wild-type *Azotobacter* was less efficient in solubilizing mineral phosphate with a single copy of *gcd* in the genome (GenBank Accession No. NZ_AAAU02000003).

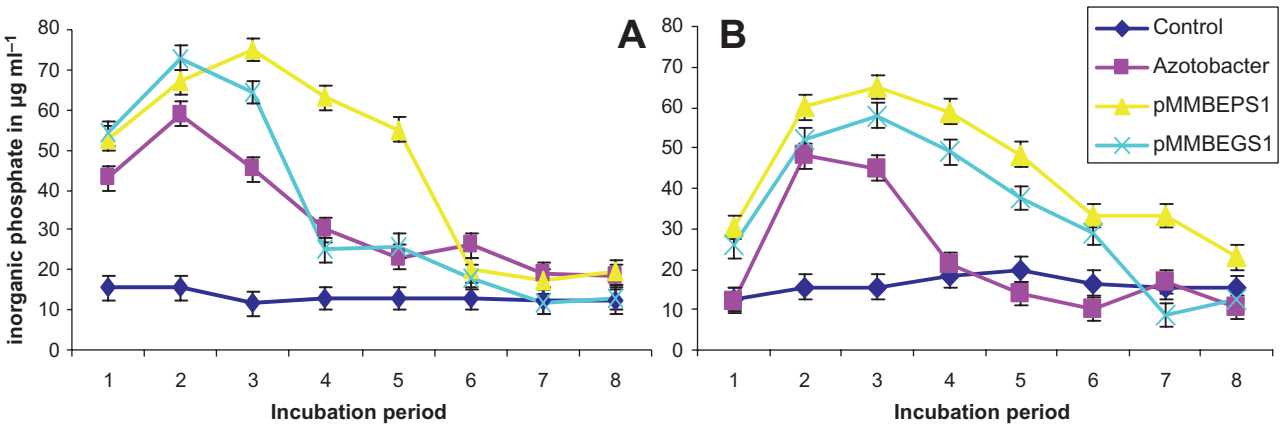

Fig. 3. Inorganic phosphate release by azotobacters in non-buffered NBRIP liquid medium supplemented with/without ammonium sulfate. Transgenic and wild-type *Azotobacter* were separately inoculated in non-buffered NBRIP liquid media. Every day 1.5 ml of samples were with drawn, centrifuged at 13 000 r.p.m. for 5 min, and the supernatant was used for estimation of inorganic phosphate over a period of 8 days. (A) Medium with ammonium sulfate and (B) medium containing sodium molybdate and iron sulfate in place of ammonium sulfate. The values represent the mean of the triplicates in three independent experiments. The vertical bars indicate standard error.

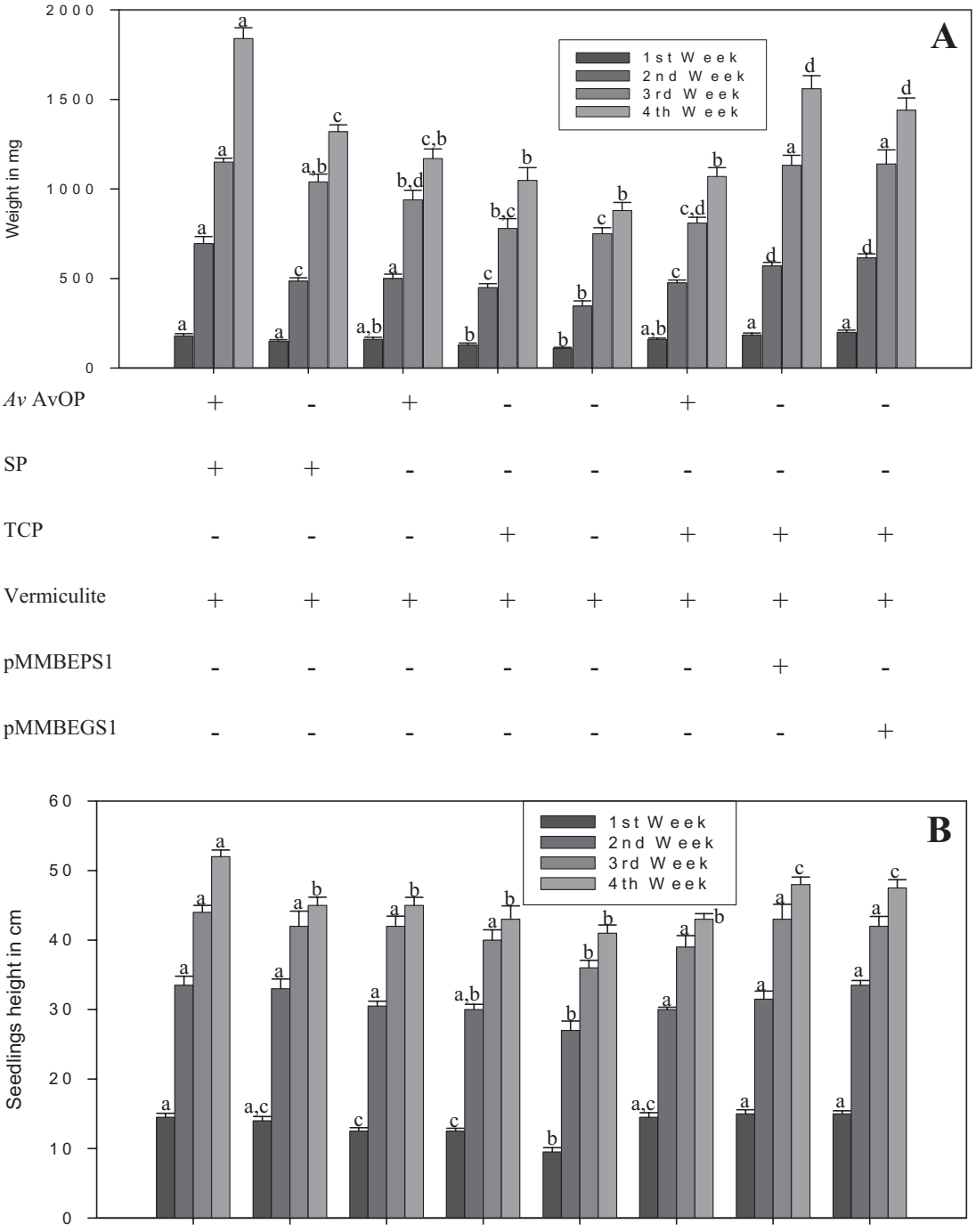

Fig. 4. Plant growth-promoting activity of transgenic *Azotobacter* in bacterized sorghum seedlings. Sorghum seeds bacterized with transgenic and wild-type azotobacters were raised in sterilized vermiculite-filled earthen pots in green house. Treatment consisted of duplicate pots containing five seedlings each and was repeated three times. The data on (A) wet weight and (B) height of the seedlings were collected. Data represent the mean (±SD, $n = 10$) of the three independent experiments. Different letter on each bar at each time point represents values that are statically different ($P < 0.05$) (by all pair-wise multiple comparison procedure Student–Newman–Keuls method and one-way ANOVA test). AvOP, *A. vinelandii* AvOP; SP, super phosphate; TCP, tricalcium phosphate; pMMBEGS1, *E. coli gcd* under *gln*A-*p*1 in pMMB206; pMMBEPS1, *E. coli gcd* under *pts-p*1 in pMMB206.

When the *in vitro* phosphate-solubilizing ability of the transgenic *Azotobacter* was tested on semi-solid medium, the zone of clearance on the non-buffered NBRIP medium was slightly larger compared with the cleared zone in the buffered medium (Fig. 1D and E) suggesting possibly a weak expression of MPS phenotype by the transgenic *Azotobacter*. In liquid buffered NBRIP medium there was no significant release of phosphate by the transgenic azo-

tobacters, as in wild type (data not shown). The bacterial strains were grown in buffered medium to mimic the buffering conditions of the soil. The HPLC analysis of gluconic acid from the culture supernatants of bacterial strains showed an increase in production of gluconic acid by transgenic azotobacters. The transgenic strains solubilized mineral phosphate more efficiently than the wild type. The production of additional gluconic acid by the transgenic azotobacters and *E. coli* PP2418 possibly is involved in DO of glucose as reported earlier (Goldstein, 1986; 1995).

The promoter activity was assayed in terms of MPS phenotype in transgenic azotobacters. The *in silico* promoter prediction showed a high score for *gln*A-*p* compared with *pts-p* (http://www.fruitfly.org/seq_tools/promoter.html). However, *gcd* expression through *gln*A-*p* released less of soluble phosphate into the medium than *pts-p*, probably due to the presence of ammonium sulfate in the NBRIP medium, which may negatively regulate glutamine synthetase expression/activity. Replacement of ammonium sulfate, with salt III of *Azotobacter* Burk's nitrogen-free medium, enhanced the phosphate release (Fig. 3B), supported the possible negative influence of excess of reduced forms of nitrogen on glutamine synthetase. Maximum inorganic phosphate was released by the end of the third day in transgenic azotobacters transformed with pMMBEPS1 but steadily decreased thereafter indicating that the *pts* promoter, in presence of inorganic phosphate in the medium, negatively influenced the expression of *gcd*. It appears, therefore, reduced forms of the nitrogen in case of *gln*A promoter and availability of inorganic phosphate in the medium for *pts* promoter negatively influenced the expression of *gcd*.

The transformation of high-molecular-weight plasmid DNA to *Azospirillum brasilence* Cd affected the morphology and growth rate of the bacterium (Holguin and Glick, 2003). Similar changes were observed when the broad-host-range plasmids pMMB206, pMMBEGS1 and pMMBEPS1 were introduced in *A. vinelandii* AvOP in this study. The transformed cells showed changes in size and morphology from the parent azotobacter cells (Glick *et al.*, 1985; Kennedy and Toukdarian, 1987). Transformed cells were smaller, produced more capsular exopolysaccharide, less ability to fix nitrogen or synthesize siderophores (Kennedy and Toukdarian, 1987) and with the production of the characteristic yellow-green *Azotobacter* sp. pigment (Glick *et al.*, 1985). In contrast there was no change in the morphology and nitrogen fixation ability of *Azospirillum* when broad-host-range vector pMCG 898 was transformed (Vikram *et al.*, 2007). The exopolysaccharide being highly hygroscopic matrix should increase the water availability to the bacteria and showed better survival in the adverse environmental conditions (Holguin and Glick, 2003). The nitrogen fixation ability by the transgenic *Azotobacter* was marginally affected with the acquisition of MPS phenotype. The growth of sorghum seedlings bacterized with transgenic *Azotobacter* steadily increased over a period of 4 weeks, although there was no significant increase in the first 2 weeks. The significant enhancement in growth of sorghum in the third and fourth weeks indicated that these transgenic azotobacters imparted long-term benefits to the plants like other biofertilizers.

In this article, we have reported the enhanced biofertilizer potential of transgenic *A. vinelandii* AvOP. The heterologous expression of *E. coli gcd* under the control of *Azotobacter*-specific glutamine synthetase (*glnA*) and phosphate transport system (*pts*) promoters in transgenic *A. vinelandii* AvOP had significantly improved the MPS ability and the plant growth-promoting activity of the transgenic strains with little compromise in the nitrogen-fixing ability. The marginal effect on the nitrogen-fixing ability could be because of the introduction of wide-host-range plasmid into *Azotobacter* (Kennedy and Toukdarian, 1987) and not because of introduction of additional copy of *gcd*. It was reported that introducing high-molecular-weight plasmid DNA into azotobacters and *A. brasilence* Cd affects the nitrogen-fixing ability (Kennedy and Toukdarian, 1987; Holguin and Glick, 2003).

Experimental procedures

Bacterial strains and growth conditions

The list of the bacterial strains and plasmids used or generated in this study was given in Table 1. *Escherichia coli* was grown in Luria–Bertani (LB) medium at 37°C with or without ampicillin (100 μg ml^{-1}), while *A. vinelandii* AvOP was grown in Burk's nitrogen-free medium (Newton *et al.*, 1953) at 30°C with or without chloramphenicol (30 μg ml^{-1}).

Plasmid isolation and transformation of E. coli *DH5α, PP2418 and* A. vinelandii *AvOP*

Plasmid DNA was routinely prepared by the alkaline lysis and *E. coli* transformation with plasmid DNA by $CaCl_2$-treated cells (Sambrook *et al.*, 1989), while transformation of *A. vinelandii* was carried out as described (Glick *et al.*, 1985).

Cloning of Azotobacter*-specific promoters and* E. coli gcd *gene.* The details of the primers used for the amplification of the promoters and *gcd* sequences are given in Table 2.

*Glutamine synthetase (*gln*A) gene promoter (*gln-p*) and phosphate transport system (*pts*) gene promoter (*pts-p*) of* A. vinelandii *AvOP.* Promoter-specific primers were designed upstream to the *gln*A and *pts* genes (GenBank Accession Nos. M57275 and AAAU03000002) of *A. vinelandii* AvOP. Thirty rounds of PCR were preformed using genomic DNA of *A. vinelandii* AvOP as the template, with PS-Fp-1, PS-Rp-1, GS-Fp-1 and GS-Rp-1 as the primers, each consisting of a

Table 1. List of the bacterial strains/plasmids used or generated in this work.

Strain/plasmid	Purpose/description	Growth conditions	Source/reference
Azotobacter vinelandii AvOP	Promoter isolation and expression host	30°C	Professor C. Kennedy, University of Arizona
Escherichia coli DH5α	*gcd* isolation and cloning host	37°C	Clonetech
E. coli PP2418 (*gcd*::*cm* & *pts*$^-$)	Expression host	37°C, cm (30 μg ml^{-1})	Cleton-Jansen *et al.* (1990)
Ec pUC18 (2.6 kb)	Cloning vector	37°C, amp (100 μg ml^{-1})	Our collection
Ec pUCGS1 (2.83 kb)	*Azotobacter*-specific *gln*A-*p*1 in pUC18	37°C, amp (100 μg ml^{-1})	This work
Ec pUCPS1 (2.78 kb)	*Azotobacter*-specific *pts*-*p*1 in pUC18	37°C, amp (100 μg ml^{-1})	This work
Ec pUCGDE (5.0 kb)	*E. coli gcd* in pUC18	37°C, amp (100 μg ml^{-1})	This work
Ec pGDEGS1 (5.23 kb)	*E. coli gcd* under *gln*A-*p*1 in pUC18	37°C, amp (100 μg ml^{-1})	This work
Ec pGDEPS1 (5.18 kb)	*E. coli gcd* under *pts*-*p*1 in pUC18	37°C, amp (100 μg ml^{-1})	This work
Az pMMB206 (9.31 kb)	Wide-host-range cloning vector	30°C, cm (30 μg ml^{-1})	Morales *et al.* (1991)
Az pMMBEGS1 (11.9 kb)	*E. coli gcd* under *gln*A-*p*1 in pMMB206	30°C, cm (30 μg ml^{-1})	This work
Az pMMBEPS1 (11.8 kb)	*E. coli gcd* under *pts*-*p*1 in pMMB206	30°C, cm (30 μg ml^{-1})	This work

Ec, *E. coli*; *Az*, *A. vinelandii*; cm, chloramphenicol; amp, ampicillin.

denaturation step at 95°C for 1 min, an annealing step at 56°C for 1 min and an elongation step at 72°C for 1 min resulting in *gln-p* (228 bp) and *pts-p* (182 bp). The amplicons *gln-p* (228 bp) and *pts-p* (182 bp) and pUC18 were double digested with HindIII and BamHI, gel purified and ligated using T4 DNA ligase at 16°C for 16 h, and the recombinants were designated as pUCGS1 and pUCPS1 respectively.

*Glucose dehydrogenase (*gcd*) of* E. coli*.* The 2.4 kb glucose dehydrogenase (GenBank Accession No. D12651) of *E. coli* was PCR-amplified using the genomic DNA as template, with GCD-Fp and GCD-Rp as primers and 30 cycles of denaturation step at 95°C for 1 min, an annealing step at 58°C for 1 min and an elongation step at 72°C for 5 min. The amplicon 2.4 kb and pUC18 were double digested with BamHI and EcoRI, gel purified and ligated using T4 DNA ligase at 16°C for 16 h, resulting in pUCGDE. The *E. coli gcd* was later released from pUCGDE, ligated to BamHI and EcoRI double-digested pUCGS1and pUCPS1. The resulting clones were designated as pGDEGS1 and pGDEPS1 respectively.

Cloning and transformation of Azotobacter *with recombinant wide-host-range vector pMMB206*

The *gcd* along with the promoter constructs, released from pGDEGS1 and pGDEPS1, was ligated to HindIII and EcoRI double-digested broad-host-range vector pMMB206 and designated as pMMBEGS1and pMMBEPS1 respectively. The recombinant pMMBEGS1, pMMBEPS1 and native pMMB206 vectors were mobilized into *A. vinelandii* AvOP as described earlier (Glick *et al.*, 1985).

Expression of E. coli gcd *under* Azotobacter*-specific promoters*

RT-PCR. The total RNA from overnight cultures of native and transgenic *Azotobacter* and *E. coli* PP2418 harbouring different clones, grown in LB medium containing 1% glucose with the appropriate antibiotics at 30°C and 37°C respectively, was isolated using Tri-reagent (Sigma). First-strand cDNA was synthesized from total RNA as template, with GCD-Rp primer and one cycle of 50°C for 45 min using cDNA synthesis kit (Promega). The reaction was stopped by heating the reaction mixture at 94°C for 10 min. Two microlitres of the synthesized first-strand cDNA was then used as template and followed the PCR amplification of *E. coli gcd* using gene-specific primers as described in the section *Glucose dehydrogenase (*gcd*) of* E. coli.

Detection of acid secretion both by plate assay and by HPLC. Escherichia coli PP2418 (*gcd*::*cm* and *pts*$^-$) harbouring pUCGDE, pGDEGS1 and pGDEPS1 were spotted onto MacConkey medium containing 200 nM PQQ, 1% glucose and 100 μg ml^{-1} ampicillin. Similarly transformants of *A. vinelandii* with pMMBGDE, pMMBEGS1 and pMMBEPS1 were spotted on MacConkey medium containing 1% glucose,

Table 2. Details of the primers used to isolate the *gcd* of *Escherichia coli* and *Azotobacter*-specific *gln*A and *pts* promoters.

Serial No.	Primer code	Primer sequence[a]	Restriction site	Expected amplicon size
1.	PS-Fp-1	TGCAAGCTTGAGGACCGATTATAGCGG	HindIII	228 bp
2.	PS-Rp-1	CCGCGTGACGGATCCATGAATACAAGATG	BamHI	
3.	GS-Fp-1	ATCAAGCTTCCCAGGCACATAAACAGAGC	HindIII	182 bp
4.	GS-Rp-1	GCGACTTCGGGATCCTGTCCTCCAGGTGG	BamHI	
5.	GCD-Fp	GCGGATCCATGGCAATTAACAATACAGG	BamHI	2.4 kb
6.	GCD-Rp	CCCGAATTCTTACTTCACATCATCCGGCA	EcoRI	

a. Underlined are the restriction sites used for cloning.

30 μg ml^{-1} chloramphenicol, and without PQQ. For the analysis of gluconic acid, bacterial cultures were centrifuged at 8000 r.p.m. for 10 min and the supernatants were filtrated through 0.2 μm filter (Millipore, GTBP). Twenty microlitres of filtrates were injected to HPLC [Shimadzu SDP-10 Avp, isocratic system; Luna 5 μm C18 (2) 100 A column (250 × 4.6 mm)] with methanol water (1:1 v/v) as solvent at a flow rate of 1 ml min^{-1}. The gluconic acid was detected using UV-visible PDA detector at 210 nm. The gluconic acid peaks were analysed using class-VP-Shimadzu software.

Solubilization of tricalcium phosphate. Azotobacters, *E. coli* DH5α and PP2418 were plated on both buffered (with 100 mM Tris HCl, pH 7.0) and non-buffered NBRIP medium (Nautiyal, 1999) containing 5% TCP supplemented with 200 nM PQQ to test their ability to solubilize insoluble form of phosphate.

Acetylene reduction assay

Azotobacter vinelandii AvOP was grown in 20 ml of Burk's nitrogen-free medium for 48 h at 30°C with shaking at 150 r.p.m. Five millilitres of the culture was distributed in to 15 ml tubes, air tightened by rubber stoppers, flushed with argon for 30 s and replaced by equal volume of acetylene, and the tubes were incubated in slanting position without shaking at 30°C for 3, 12 or 24 h. Ethylene formation was analysed in a Shimadzu gas chromatograph using a hydrogen flame ionization detector. The amount of ethylene formed was expressed as μmol per OD of culture over 24 h as described (Sasi Kala *et al*., 1990).

Estimation of released inorganic phosphate

The transformants and non-transformants of *A. vinelandii* AvOP were separately inoculated in 150 ml conical flasks containing 30 ml of NBRIP medium with and without ammonia supplementation, and incubated at 30°C for 1 week at 150 r.p.m. Every day, 1.5 ml of the culture was drawn and centrifuged at 13 000 r.p.m. for 5 min. Inorganic phosphate released into supernatant was estimated as described by Ames (1964).

Seed bacterization and evaluation of growth-promoting activity of transgenic Azotobacter *in green house*

Sorghum seeds were surface sterilized using crude ethanol for 5 s and later soaked twice in 10% sodium hypochlorite with one drop of Tween100 for 5 min. Seeds were then washed five times to remove the detergent with sterile double-distilled water. *Azotobacter vinelandii* AvOP strains were grown in Burk's nitrogen-free plates (supplemented with 30 μg ml^{-1} chloramphenicol in case of transformants) for 48 h, culture was then scraped and suspended in 0.5% carboxy methyl cellulose (CMC) and sorghum seeds were bacterized as described (Kishore *et al*., 2005). Bacterized sorghum seeds were sown in experimental pots (diameter 18 cm and 15 cm deep) containing 2 kg of vermiculite sterilized for 2 h at 130°C, 200 mg of TCP per pot and raised in green house with about 12 h of light and 12 h of dark at 30°C. Control pots containing 500 mg of superphosphate per pot, only vermiculite and wild-type *A. vinelandii* AvOP. Sterile water was provided to the pots at regular intervals to sustain the growth of the seedlings. Height and fresh weight of the seedlings were recorded by uprooting the seedlings, after every 7 days, for 4 weeks. Experiments were repeated three times containing 5 plants in each pot and a total of 10 plants were taken for mean comparison of the data which was subjected to one-way ANOVA using SigmaStat (Systat Software, California, USA).

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