

Cloning of mineral phosphate solubilizing genes from *Synechocystis* PCC 6803

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Genes responsible for conferring mineral phosphate solubilizing (mps) ability were cloned from *Synechocystis* PCC 6803, a unicellular cyanobacterium, which by itself does not show mps ability. *Escherichia coli* was transformed with the genomic DNA library of the *Synechocystis* PCC 6803 and two mps genes were selected based on the ability of the transformants to show zone of clearance on solid medium containing dicalcium phosphate (DCP) as the sole source of phosphorus (P). The mps phenotype was found to be plasmid-associated as shown by transformation of *E. coli* strain with isolated plasmids. These transformants also solubilized rock phosphate.

MANY soil microorganisms have the ability to solubilize mineral phosphates in pure culture conditions by the secretion of organic acids, viz. lactic, succinic, oxalic, citric, gluconic, etc. These phosphate-solubilizing microorganisms (PSMs) are believed to play an important role in supplementing P to plants, thus allowing the sustainable use of phosphatic fertilizers¹⁻³. Besides the conventional approach of screening natural isolates having mineral phosphate solubilizing (mps) phenotype, engineering of non-mps organisms to become mps has been achieved by the cloning and expression of mps genes from natural P-solubilizing isolates. The mineral phosphate solubilizing genes, so far, have been cloned only from gluconic acid secreting, P-solubilizing bacteria like *Erwinia herbicola* and *Pseudomonas cepacia* by construction of genomic DNA library of these bacteria and screening for the *E. coli* transformants for mps phenotype. The mps genes from *E. herbicola* were found to encode for pyrroloquinoline quinone (PQQ) synthase⁴ while the *P. cepacia* gene encodes either a PQQ transporter or a regulatory protein involved in PQQ biosynthesis⁵. *E. coli* cannot produce gluconic acid as it cannot synthesize PQQ, the cofactor for the apo-glucose dehydrogenase enzyme that *E. coli* normally possess⁶. The cloned genes apparently allowed *E. coli* strains to produce gluconic acid and thus solubilize P.

In this paper, we have addressed the question whether mps phenotype can be conferred on an organism by the

cloning of mps genes and overexpression of mps genes from another non-mps organism, that is metabolically distinct from the host of organism. Our rationale is that overexpression of genes involved in biosynthesis/regulation of organic acid production from a different organism may lead to metabolic deregulation resulting in enhanced acid production. To test this postulate, we selected the genomic DNA library of wild-type *Synechocystis* PCC 6803 (a photoautotrophic, unicellular, and non-nitrogen fixing cyanobacterium) which does not possess P-solubilizing ability, for transforming *E. coli*, a heterotroph. Although nitrogen-fixing bacteria are known to solubilize tricalcium phosphate (TCP) and rock phosphate, they do not acidify the medium^{7,8}. It is known that nonnitrogen-fixing cyanobacterium, *Anacystis nidulans* (*Synechococcus* 7942), does not solubilize tricalcium phosphate⁷.

E. coli is known to solubilize DCP (ref. 9). In order to determine the maximum level of acid production and DCP solubilization by *E. coli*, a minimal medium containing 100 mM mannitol as carbon source and 10 mM NH₄Cl as nitrogen source was used. DCP was precipitated *in situ*⁹. The medium was buffered with different concentrations of Tris buffer pH 8.0 and the zone of clearance on DCP was measured. *E. coli* DH5 α was able to solubilize DCP on media buffered with 30 mM Tris buffer pH 8.0 or with lower buffering capacity but not with 40 mM buffer (Table 1).

E. coli DH5 α was transformed with the genomic DNA library of *Synechocystis* PCC 6803 by the calcium chloride method¹⁰. The genomic DNA library was made in plasmid Bluescript SK with an average insert size of 3 kb (ref. 11). A total of 10⁴ transformants were screened for the mps phenotype on solidified DCP containing medium with 100 mM mannitol, 10 mM NH₄Cl and 40 mM Tris buffer pH 8.0. Ampicillin was added at 100 μ g/ml. Two independent mps transformants were isolated based on their ability to show a zone of clearance (Table 2). The purified plasmid isolated from these mps transformants was able to transform *E. coli* DH5 α , conferring the mps phenotype, thereby suggesting this property to be plasmid-borne. The subsequent experiments were done with the secondary *E. coli* transformants.

Table 1. Effect of buffering of media on DCP solubilization by *E. coli* strain DH5 α

Concentration of tris buffer pH 8.0 (in mM)	0	10	20	30	40
Zone of clearance (in cm)	1.5	1.0	0.7	0.3	UD

UD, Undetectable.

Medium contained 100 mM mannitol and 10 mM NH₄Cl as C and N sources, respectively. The zone size was determined after 72 h of growth and the values are mean of three independent experiments.

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The plasmids from the transformants were isolated and restriction endonuclease digestion showed that both MPS1 and MPS2 clones contained an insert of about 3 kb in size (Figure 1). The MPS1 and MPS2 clones contained genomic fragments which are distinct as seen from the fact that the MPS 1 insert had single sites for *HincII*, *Sall* and *XhoI*, whereas the MPS 2 insert contained single sites for *XbaI*, *ClaI*, *SacI*, *EcoRV* and *HindIII*. The transformants could also solubilize DCP with glucose as well as glycerol as the carbon source at buffer concentrations 50 and 20 mM, respectively. The

Table 2. DCP solubilization by *mps*⁺ transformants of *E. coli* strain DH5 α

Plasmid	Zone of clearance (cm)			
	12 h	24 h	48 h	72 h
Bluescript SK	UD	UD	UD	UD
MPS 1	UD	0.2	0.4	0.5
MPS 2	UD	0.3	0.5	0.5

Media contained 40 mM tris buffer pH 8.0, 100 mM mannitol, 10 mM NH₄Cl and 100 μ g/ml ampicillin. The values are mean of three independent experiments.

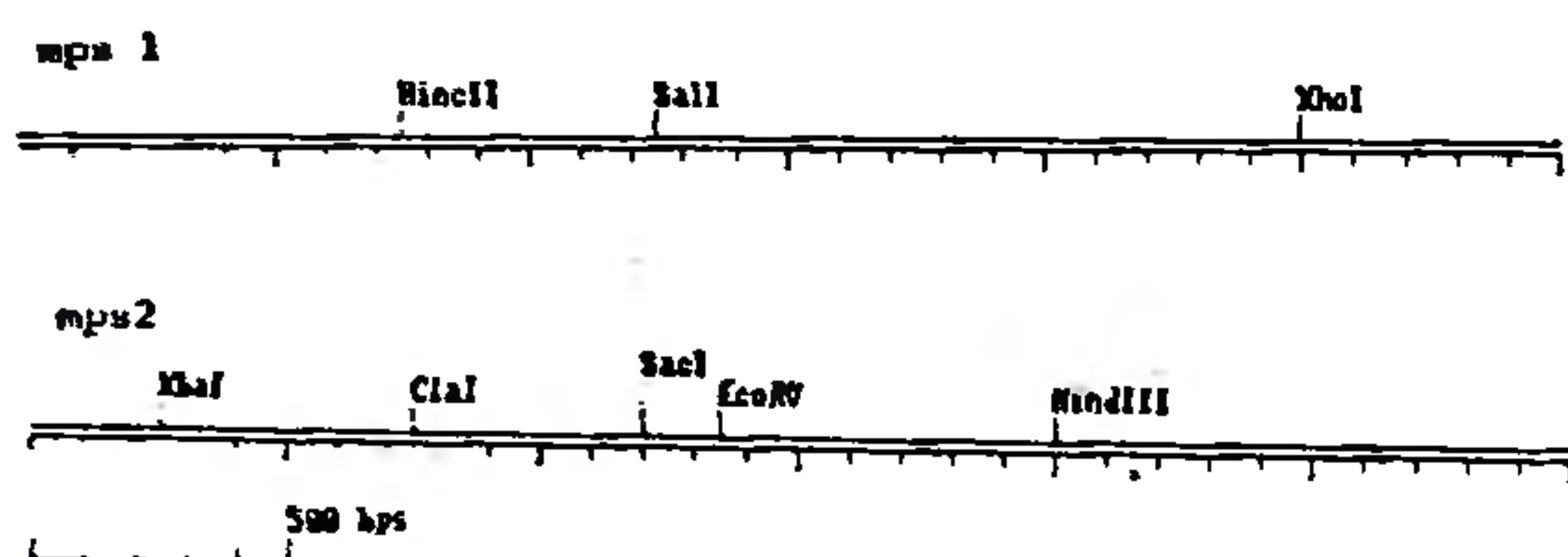


Figure 1. Restriction maps of the genomic inserts of MPS 1 and MPS 2 clones.

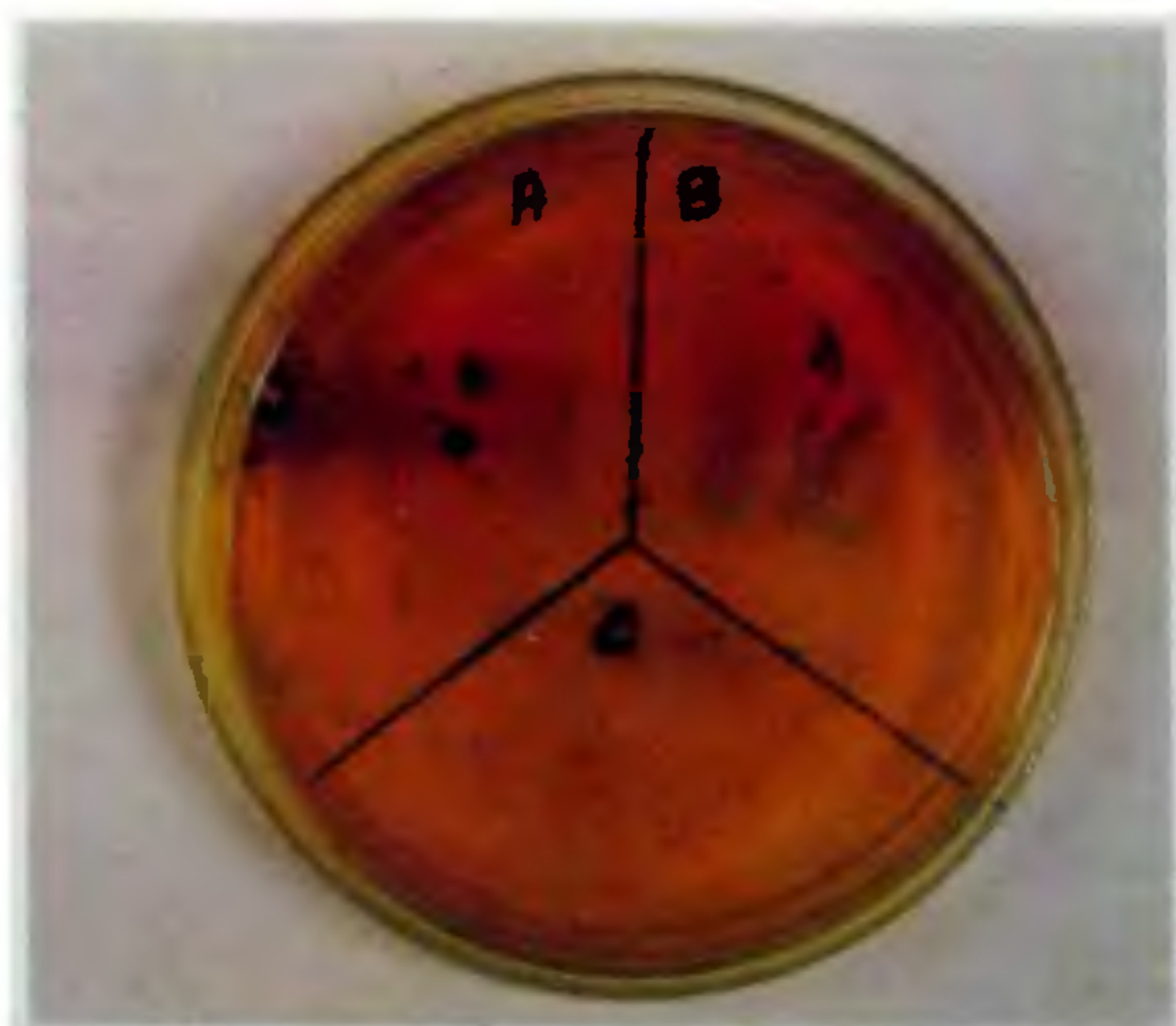


Figure 2. RP solubilization by *E. coli* containing *mps* plasmids 1 and 2. A, *mps* plasmid 1; B, *mps* plasmid 2 and C, Bluescript SK. Minimal media plates with 40 mM tris pH 8.0, 50 mM glucol, 0.1% (wt/vol) RP and 0.01% (wt/vol) methyl red.

Table 3. RP solubilization by *mps*⁺ transformants of *E. coli* strain DH5 α on different carbon sources

Plasmid	Time					
	12 h		24 h		48 h	
	pH	P (mM)	pH	P (mM)	pH	P (mM)
Glucose						
Bluescript SK	7.0	0.1	6.0	0.15	5.5	0.25
MPS 1	6.0	0.2	5.0	0.6	4.8	0.7
MPS 2	6.5	0.1	5.5	0.5	4.5	0.8
Mannitol						
Bluescript SK	7.0	0.1	7.0	0.1	6.5	0.2
MPS 1	6.5	0.2	5.0	0.6	5.0	0.6
MPS 2	6.0	0.25	4.8	0.7	4.8	0.65
Glycerol						
Bluescript SK	8.0	UD	7.5	0.05	7.5	0.05
MPS 1	7.0	0.1	6.0	0.25	5.0	0.65
MPS 2	6.5	0.25	5.0	0.7	4.8	0.6

The medium contained carbon sources (glucose, mannitol and glycerol) at 50 mM and 0.1% (wt/vol) RP. Concentration of tris buffer pH 8.0 was 40 mM with glucose and mannitol, and 10 mM with glycerol.

Phosphate estimation was carried out by ascorbate method¹².

mps transformants were also tested for P release from 'Senegal' rock phosphate (RP). For this, minimal medium containing RP 1% (wt/vol) as sole P and methyl red indicator dye was used at 0.01% (wt/vol). Both the transformants could grow and acidify in the buffered media plates, as seen from the red zone around the colonies (Figure 2). The recombinant transformants were also tested for their ability to release P from RP in liquid medium. It was seen that both the transformants released about 3-fold higher P on glucose and mannitol and about 10-fold higher in glycerol compared to the *E. coli* strains harbouring the vector alone (Table 3), indicating that the genomic insert was responsible for the P-solubilizing ability of the transformants.

The above experiments show that *mps* genes are not limited to PSMs. Overexpression and/or expression of genes in a metabolically distinct organisms from a non-PSM has been shown here to confer *mps* ability to the host organism. This strategy could also be employed with cDNA libraries constructed under stronger promoters to screen for genes conferring *mps* phenotype to plant growth promoting rhizobacteria like *Pseudomonas* and *Rhizobium*.

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Shoot bud regeneration from leaf explants of a medicinal plant: *Enicostemma axillare*

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Adventitious shoot bud regeneration was achieved from leaf explants excised from a medicinal plant *Enicostemma axillare* (Lam.) Raynal cultured on Murashige and Skoog (MS) medium. MS medium supplemented with 8.9 μ M N6-benzyladenine (BA) was found to be more efficient for adventitious shoot bud regeneration than MS medium containing a combination of 8.9 μ M BA and 0.54 μ M NAA. Adventitious shoot buds were induced directly on the margin and mid vein of the leaf explant. The shoot buds multiplied in media containing 4.4 μ M BA. Hormone-free MS medium was used for further growth and development. Roots were developed in media containing 0.054–0.54 μ M NAA. Results suggest that *Enicostemma axillare* can be mass propagated within a short time for the production of microbe-free plants for the extraction of drugs.

TRADITIONAL medicine based on herbal remedies has always played a key role in the health system of many countries. An estimated three quarters of prescription drugs are derived from plants^{1,2}, and were discovered because of their prior use in indigenous medicine and related purposes. Several thousands of medicinal plants

are disappearing from the earth due to neglect and human activities.

Enicostemma axillare (Lam.) Raynal belonging to the family Gentianaceae is a species known for its medicinal properties. It is used as a bitter tonic, stomachic, laxative, blood purifier and as a drug for curing dropsy and malaria³. It is also used in rheumatism, abdominal ulcers, hernia, swellings, itches and insect poisoning⁴. It contains ophelic acid. *In vitro* propagation of this useful medicinal plant species could provide a means of disease-free healthy clones for the extraction of pure drugs. The results pertaining to *in vitro* shoot regeneration for mass propagation of this species is presented in this paper.

Top shoot cuttings of *Enicostemma axillare* were collected from healthy disease-free plants growing in Nagamali Hills, Madurai, India, and washed in soap water prior to surface sterilization. The fully-expanded leaves were excised and surface-sterilized with 20% commercial chlorox solution containing 1.05% sodium hypochlorite and a drop of tween 20 for 15 min. After washing in sterile distilled water, the leaves were dipped in 0.1% mercuric chloride solution for 3 min followed by thorough rinsing in sterile distilled water three times. Finally the plant materials were dipped in 70% ethanol for one second and rinsed in sterile distilled water. Surface-sterilized leaves were cut into 5 mm pieces and used as explants. These explants were placed on the MS medium⁵ containing different concentrations of BA in 25 \times 150 mm culture tubes. The pH of the media was adjusted to 5.6 prior to autoclaving. All the cultures were incubated under 1000 lux light intensity provided by white fluorescent lamps for 16 h photo period at 25 \pm 1°C. For each treatment, 40 replicates were made and the experiment was repeated twice. The explants were subcultured once in 15 days. After 45 days the shoot buds were transferred to hormone-free media for shoot growth and elongation. The isolated plantlets were planted on MS media containing 0.054–0.54 μ M NAA for rooting. Rooted plantlets were washed and planted on autoclaved soil mix containing sand, peat moss and humus (1:1:1) for acclimatization.

Leaf explants enlarged after 2 weeks of incubation in media with 8.9 μ M BA and 0.54 μ M NAA, produced light green callus along the cut surfaces mainly along the midrib region and at the petiolar base. After 30 days in culture, several globular shoot bud initials were observed on the surface of the callus. This kind of globular shoot bud initials also developed directly on the midrib of the explants (Figure 1a) in media containing 8.9 μ M BA alone. After 20 days, the globular buds proliferated in the same media. These shoot buds developed into plantlets (Figure 1b) when transferred to hormone-free media or media containing low concentration of BA (0.44 μ M). Each one of the isolated shoot buds when