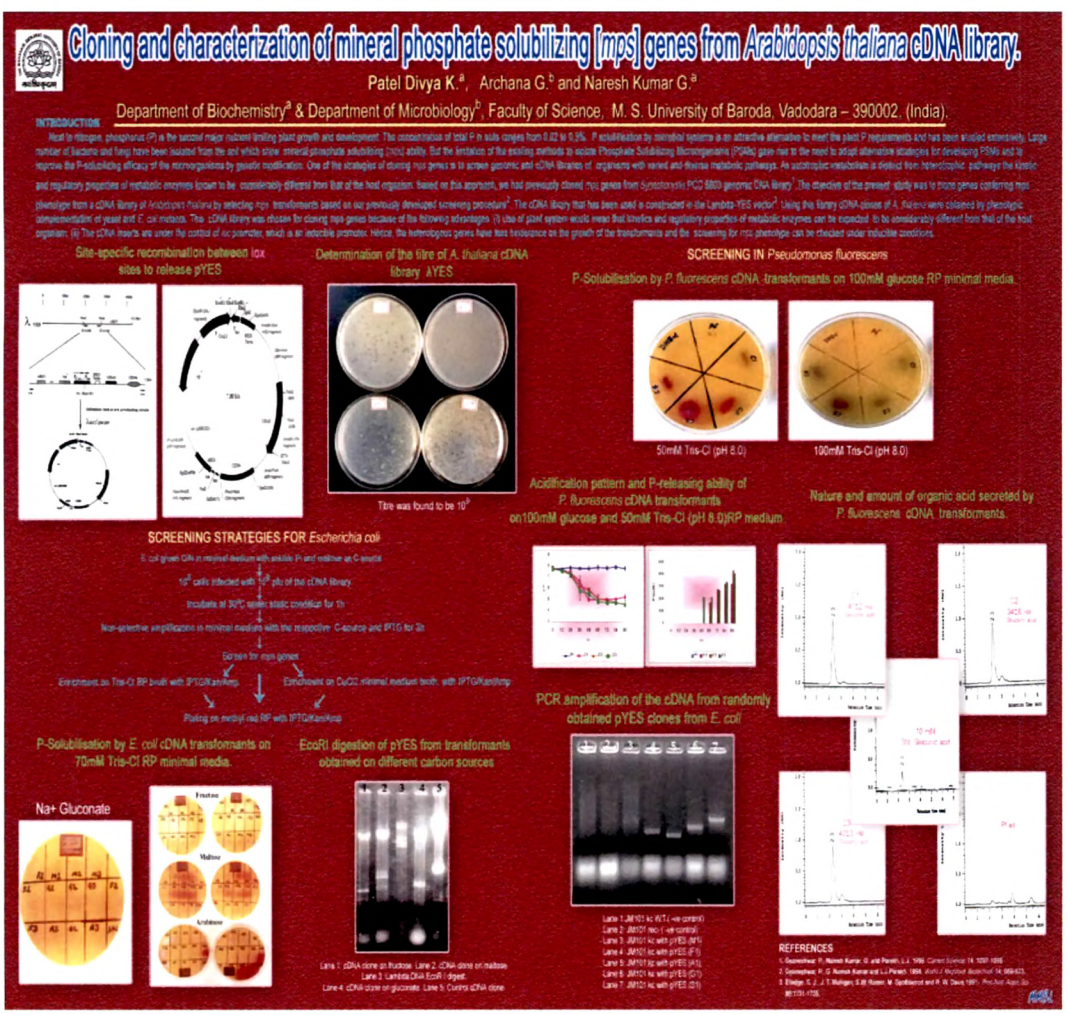
CHAPTER 4

Screening of *Arabidopsis thaliana* cDNA Library for Mineral Phosphate Solubilizing (mps) Ability



4.1: INTRODUCTION

P solubilization by microorganisms is an attractive alternative to meet the plant P requirements and has been studied extensively. Many bacteria and fungi have been isolated from the soil with efficient P solubilizing ability under laboratory conditions (Cunningham and kuiack, 1992; Vassilev *et al.*, 1996; Sharma *et al.*, 2005). The mechanism of P solubilization by these PSMs mainly involves secretion of organic acids like gluconic, 2-ketogluconic, citric, oxalic, acetic, succinic, tartaric and other TCA cycle intermediates or proton extrusion (Bolan *et al.*, 1994; Goldstein 1995; Sharma *et al.*, 2005). However, the biochemical and molecular mechanism for the acid secretion by PSMs are not yet fully characterized (Goldstein *et al.*, 1993).

It is paradoxical that although PSMs are abundant in soils and in the rhizosphere of most plants, phosphorus remains one of the major limiting nutrient for plant growth (Kucey *et al.*, 1989). One of the important pre-requisite for a PSM to be effective in the field conditions is its capability to produce strong organic acids in sufficient amounts to release P from mineral phosphates in soil. About 10 mM citric or oxalic acid was shown to be required to drop the pH of alkaline vertisol soil to 4.0 and release approximately 1 mM of soil Pi, whereas around 50 mM gluconic or 20 mM tartaric or 100 mM lactic acid was required to bring about the same drop in pH while the amount of Pi released was ~ 0.6 mM. However, organic acids secreted by the PSMs were found to be 20 to 50 times less than that required to solubilize P from alkaline soil. The high buffering capacity of the alkaline vertisol soils is responsible for this limitation of PSMs (Gyaneshwar *et al.*, 1998a).

Another prime requirement is the ability of the PSM to colonize the roots of a wide variety of plants along with its ability to show P solubilization from different soil types under various environmental conditions (Halder and Chakrabarty, 1993). Some of the biotic and abiotic factors such as predation, competition, nutrients supplied by the roots and the pH of the soil, clay minerals content, water tension, presence of toxic chemicals etc are also known to affect the P solubilizing ability of the PSMs drastically. In addition, the limitation of the conventional screening methods is that it allows for the selection of only 1% of the culturable population of microbes. All these factors along with several others, pose severe drawback for a PSM to work efficiently under the actual

field conditions. As a result, even though there are several reports of P solubilizing microbes under lab conditions belonging to different genera in the recent years, field trials with PSM inoculations showed variable effects on plant growth and crop yields. *Penicillium billai*, which secretes 10 mM each of citric and oxalic acids (Cunningham and kuiack, 1992) is the only PSM shown to be effective in releasing P in the field conditions with calcareous Chernozemic soil which had low available P (Asea *et al.*, 1988).

There is no single PSM which can probably be effective under all soil and environmental conditions where plants experience P deficiency. The limitation of the existing methods to isolate versatile PSMs gave rise to the need to adopt alternative strategies by genetic modification for developing PSMs and improve the P solubilizing efficacy of the microorganisms. Metabolic engineering of bacteria would allow to incorporate mps phenotype (organic acid secreting ability) into any of the existing native rhizobacteria.

Thus, genetic transfer of any isolated gene involved in mps to induce or improve phosphate-dissolving capacity in PGPR strains, would be an interesting approach. An attempt to incorporate mps ability in *E. coli*, using the metabolic engineering approach, was carried out in our lab too. Genes responsible for conferring mps ability were cloned from *Synechocystis* PCC 6803, a unicellular cyanobacterium, which by itself does not show any mps ability. *E. 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 insoluble P. The transformants were isolated using mannitol as the C source under buffered medium conditions. The mps phenotype was found to be plasmid associated as shown by transformation of *E. coli* strain with the isolated plasmids. The transformants also showed DCP solubilization with glucose and glycerol as C source under buffered conditions (Gyaneshwar *et al.*, 1998b). However, the transformants were not able to solubilize RP under buffered conditions. Moreover the mps genes were expressed under their natural promoters as it was a genomic DNA library. Much better mps phenotype could be achieved if the genes are expressed from strong artificial promoters. Thus, use of cDNA library from heterologous systems

expressed from inducible stronger promoters may be suitable for giving stronger mps phenotype.

With this background the main objective of the present study was to clone genes conferring mps phenotype from a cDNA library of *Arabidopsis thaliana* by selecting mps transformants based on the screening procedure on buffered minimal medium with RP as the sole P source that has been developed in the lab. One of the advantages the library will provide is the heterologous gene overexpression using appropriate promoters in different host bacteria, particularly those having high propensity to colonize the rhizosphere of important plants.

This cDNA library was chosen because of the advantages that it would offer, such as:

(i) Use of plant system would mean that kinetics and regulatory properties of metabolic enzymes can be expected to be considerably different from that of the host organism. This is of particular importance since plant tissues show autotrophy and heterotrophy in a tissue specific manner as a consequence of significant contributions from the precursor organellae ancestors. Hence, this could give rise to the necessary modification in the metabolism to allow enhanced deregulated acid production.

(ii) The cDNA inserts are under the control of *lac* promoter which is an inducible promoter. Hence, the heterologous genes have less hinderance on the growth of the transformants and the screening for mps phenotype can be checked under inducible conditions. Interestingly, *lac* is a strong promoter for *Pseudomonas* and *Rhizobium* sp., thus facilitating the transfer of the mps phenotype into these rhizobacteria.

(iii) The cDNA library is constructed in the Lambda-YES vector. This vector is particularly convenient since it is a phasmid allowing the ease of phage infection and facility for conversion to plasmid form.

4.2: MATERIALS AND METHODS

Table 4.1: Bacterial strains used for the study

STRAINS	GENOTYPE
E. coli BL21	B F- *dcm*, *omp*T, *hsd*S (rB-mB-), *gal*
E. coli BL21 -λkc	BL21 lysogenised with λkc
E. coli DH5α	*sup*E44, Δ*lac*U169 (Φ80 *lac*Z ΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, elA1.
E. coli DH5α -λkc	DH5α lysogenised with λkc
E. coli JM101	*sup*E, *thi*-1Δ (*lac-pro*AB) [F' *tra*D36 *pro*AB *lac*IqZ ΔM15]
E. coli JM101 -λkc	JM101 lysogenised with λkc
E. coli LE392	*Sup*E44, *sup*F54, *hsd*R514, *gal*K2, *gal*T22, *met*B, *trp* R55, *lac*Y1
E. coli LE392 - λkc	LE392 lysogenised with λkc
P. fluorescens ATCC 13525 (Pf)	Wild Type
P. fluorescens (C1,C2,C3,C4)	*P. fluorescens* ATCC 13525 (Pf) transformed with plasmid cDNA library (pYES)

4.2.1: *Arabidopsis thaliana* cDNA Library

The *Arabidopsis thaliana* cDNA library was obtained from Dr. Stephen J. Elledge, Baylor College of Medicine, Houston (Elledge *et al.*, 1991). The library consists of cDNA prepared from the above ground parts of Arabidopsis plants which varied in size (from those which had just opened their primary leaves to those which had bolted and were flowering). The library was constructed in the λYES vector. It is a multifunctional vector capable of replicating as a lambda phage lysogen or a plasmid in *E. coli* and as a centromere plasmid in yeast. The cDNAs were inserted non-directionally into an *Xho*I site and flanked by *Eco*RI sites of the λYES vector. In one direction they

can be expressed from the *E. coli lac* promoter and in the other direction from the yeast *Gal*1 promoter (**Fig. 4.1**). The selectable markers are ampicillin resistance in *E. coli* and Ura-3 for yeast. The plasmid part of the vector can be automatically looped out of the lambda phage by site specific recombinase using Cre protein (for Causes REcombination), a trans acting function and the loxP site (for Locus of crossing over [x]) in the vector, a 24 bp sequence. The plasmid portion of λYES is flanked by direct repeats of lox sites (**Fig. 4.2**).

Fig 4.1: Restriction map of pYES

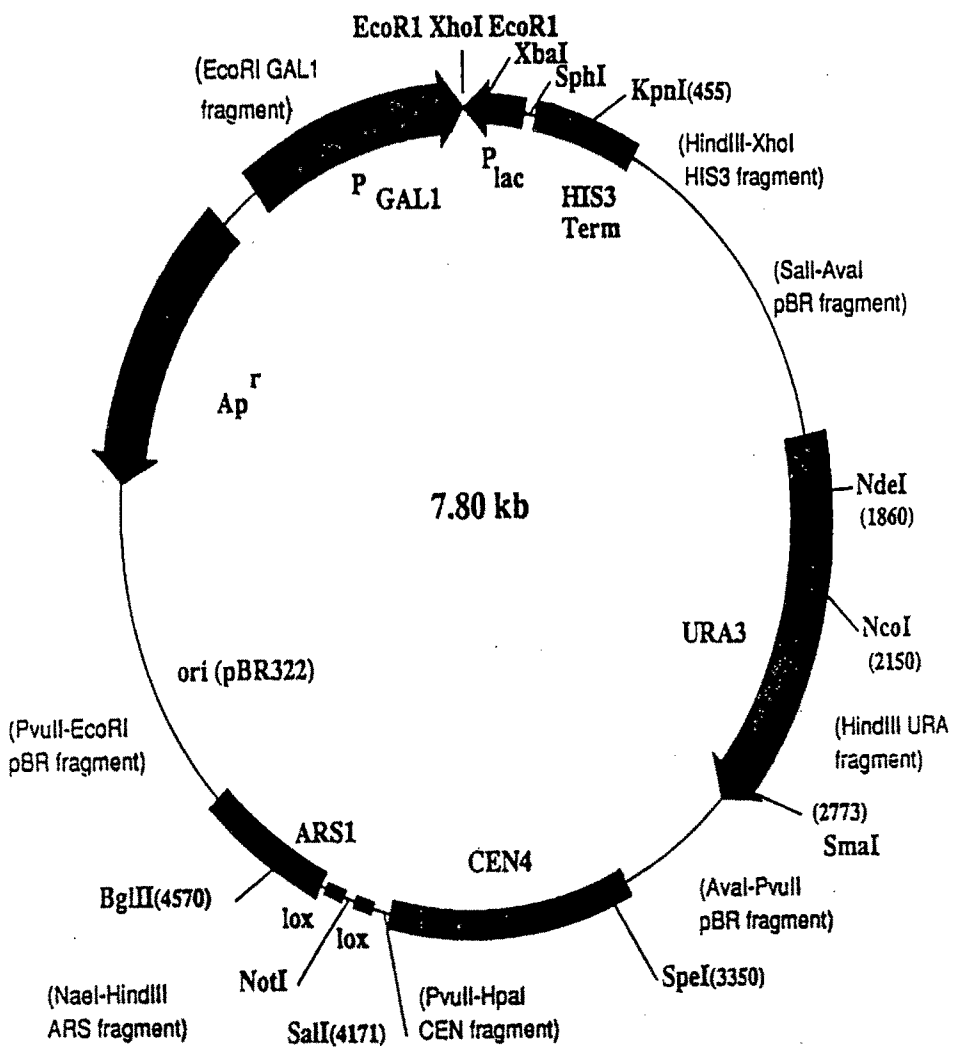
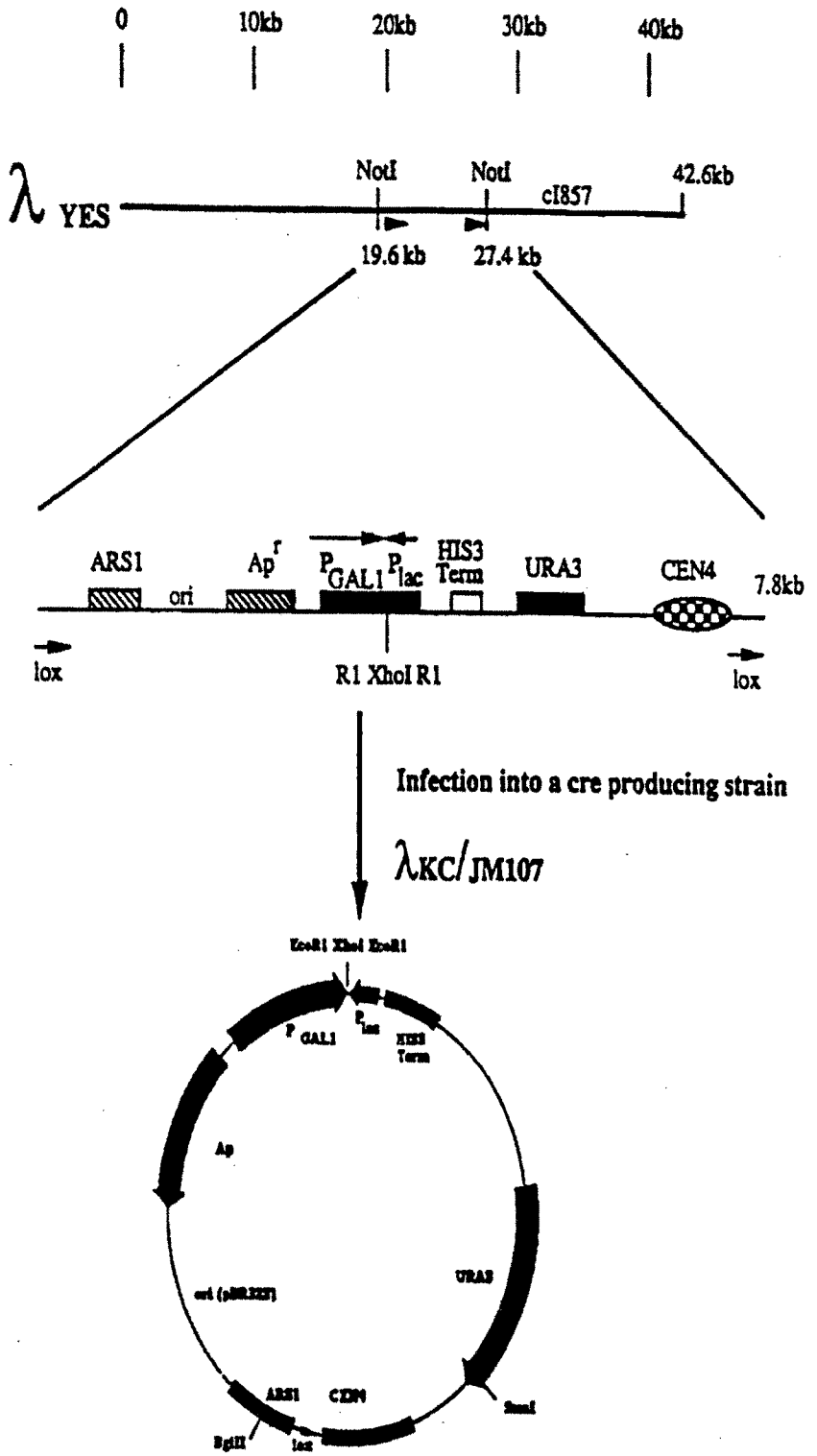


Fig 4.2: Site specific recombination between lox sites to release pYES



4.2.2: Preparation Of Plating Bacteria For Phage Infection

50 mL of Luria broth was supplemented with 0.2% maltose in a 250 mL flask and inoculated with a single bacterial colony. After the culture grew overnight at 37°C with shaking at 250 cycles/min, the cells were spun down at 4000 g for 5 min at room temperature and supernatant was discarded. The cell pellet was resuspended in 20 mL of 0.01M $MgSO_4$, transferred to a 100 mL sterile flask, incubated for 1h at 37°C with shaking and then stored at 4°C till further use.

4.2.3: Determination Of Titre Of *A. thaliana* cDNA Library

E. coli strain LE392 was used as the host for determination of the cDNA library titre. Serial dilutions of the phage library was made using lambda dilution buffer.

Composition of Lambda dilution buffer (1L):

NaCl	5.8g
1M Tris Cl (pH 7.5)	50.0 mL
$MgSO_4$ $7H_2O$	2.0g
2% Gelatin	5.0 mL
D/W	945.0 mL

An aliquot of the appropriate dilution was used to infect *E. coli* strain LE392. After allowing adsorption of the phage to the host bacterium for 30min. at 30°C under static conditions, the infection mixture was added to 3mL of soft agar and overlayed on LA plates. The plates were incubated at 30°C overnight and the number of plaques was counted.

4.2.4: Preparation Of Lambda Stocks From Plaques For Library Amplification

For the amplification, 1 mL of host *E. coli* strain LE392 (i.e. approx. 10^8) was infected with 1uL of the original λYES phage cDNA library of known titre (i.e. approx. 10^7) to maintain MOI of 0.1. After infection the culture was overlayed with the help of top agar on a LA/amp plate such that the entire surface of the plate was filled with plaques. Next day, to the plates containing the plaques, 5 mL of suspension medium (SM) was added and the plates were stored at 4°C for several hours with intermittent gentle shaking. The SM was harvested with the help of a pipette from the plate and stored in a microfuge tube. Additional 5 mL of SM was added to the same plates and

stored for 15 min. in a tilted position to allow all the fluid to drain into one area. This SM was also pooled along with the previous harvest. To this recovered lysate 0.1 mL of chloroform was added, vortexed very briefly and centrifuged at 4000g for 10 min. at 4°C. The supernatant was recovered and a drop of chloroform was added along with 7% v/v of DMSO which is particularly useful for long term storage of lambda lysate at -20°C. An aliquote of the lysate was used for the phage titre determination.

Composition of suspension medium (SM) (1L)

NaCl	5.8 g
$MgSO_4$. $7H_2O$	2.0 g
1 M Tris Cl (pH 7.5)	50 mL
2% Gelatin solution	5 mL

4.2.5: Construction Of λkc Lysogen

E. coli was grown overnight in LB such that the cell density reaches approximately 1-2 x 10^8/ mL. 0.5 mL of these overnight grown cells were then placed in a sterile microfuge tube along with 0.05 mL of the λkc lysate (considering the multiplicity of infection [MOI] to be 0.1) and 0.1 M $MgCl_2$ and incubated at 30°C under static conditions without shaking for 1h to allow phage adsorption and subsequent expression of the kanamycin resistance gene. Cells were selected by growth on LA/kanamycin plates at 30°C.

4.2.6: Infection Of *E. coli* With *A. thaliana* λYES cDNA Library

Overnight grown *E. coli* cells were used for preparing the plating bacteria as described above. To this host culture, the phage library was added at an appropriate MOI and incubated under static condition at 30°C for 30 min. to allow the phage to adsorb to the host bacteria. The infection mixture was then allowed to grow non-selectively in the presence of IPTG in either LB for 1h or in complete minimal medium for 2-3 h after which the transformants were screened on selective plates. The infection efficiency was determined by performing a viable count on LA/amycin-ampicillin plates.

4.2.7: Screening *A. thaliana* cDNA Library In *E. coli*

4.2.7.1: Determining The Minimal Tris Cl (pH 8.0) Buffering Conditions In *E. coli* For Screening The cDNA Library

The carbon (C) sources selected for screening the library were Na-gluconate, Na-acetate, glucose, lactose, fructose, xylose, arabinose, maltose, glycerol and sucrose. They were used at a concentration of 100 mM throughout the study.

To determine the minimum buffering conditions that do not show acidification on minimal medium plates containing methyl red with Rock Phosphate (RP) as the sole Pi source by the *E. coli* λkc lysogen. The culture was spotted on the respective C sources with buffer concentrations ranging from 0 to 100mM of Tris Cl (pH-8.0). For this the *E. coli* λkc strain with self ligated pYES backbone was used.

4.2.7.2: Screening of *A. thaliana* cDNA Library in *E. coli* For mps Genes

The *E. coli* λkc-pYES infection mixture was spread on the RP plates with kanamycin/ampicillin. IPTG was used in the medium to induce the expression of the inserted genes. The plates were monitored for the growth and change in colour of methyl red from yellow to red which indicates acidification. *E. coli* λkc transformed with Self ligated pYES backbone was used as a control wherever necessary.

4.2.8: Screening *A. thaliana* cDNA Library In *Pseudomonas fluorescens*

4.2.8.1: Preparation And Extraction Of *A. thaliana* Plasmid (pYES) cDNA Library For Screening In *P. fluorescens*

E. coli lysogenized with the phage λkc was infected with the λYES library and the infection mixture was allowed to grow in 10 mL of LB with kanamycin and ampicillin for 2-3 h. The 10 mL broth was then transferred to a larger amount of LB and next day pYES plasmid was extracted by standard alkali lysis method. Infection efficiency was determined by plating an appropriate aliquote of the serially diluted infection mixture on LA kanamycin/Ampicillin plate.

4.2.8.2: Screening Of *A. thaliana* Plasmid (pYES) cDNA Library In *P. fluorescens* For mps Genes

The pYES plasmid library of *A. thaliana* was used to transform *P. fluorescens*. The transforments were plated on the RP containing minimal medium plates with methyl red and respective C source in a similar manner as described for *E. coli*. The buffering conditions for screening in *P. fluorescens* were maintained to 100 mM Tris Cl (pH 8.0) along with 100 mM of the C source.

4.2.8.3: Characterization Of mps Ability Of *P. fluorescens* pYES cDNA Transformants

P solubilizing ability of the isolate was determined using buffered minimal medium with Senegal RP as source of insoluble P or 10 mM KH_2PO_4 as soluble P source and 100mM of the specified C source under broth conditions. The concentration of Tris Cl (pH 8.0) was varied according to requirement to either 50 mM or 100 mM (Gyaneshwar *et al.*, 1998a). Aliquots of the medium supernatant were withdrawn at 24h intervals. Absorbance at 600nm and pH drop were used as parameters for the growth pattern and acidification, respectively. Culture supernatant when the pH dropped below 5.0 was used for identification and quantification of organic acids and for estimating soluble Pi. Results are Mean ± S.D. of three independent experiments performed in duplicates.

4.2.9: Analytical Methods

Organic acids were analysed by LaChrom Merck Hitachi High Performance Liquid Chromatography (HPLC). The culture supernatant was filtered through 0.22μ nylon filter. The organic acids were separated using RP-18 column. The mobile phase consisted of 0.1% phosphoric acid with a flow rate of 1ml/min. Acids were detected by absorbance at 210nm. The organic acids were identified and quantitated by comparing the retention times and peak areas with standard acids.

Phosphate estimations were done according to Ames method using KH_2PO_4 as standard (Ames, 1964). Molecular biology techniques followed throughout the work was according to Sambrook and Russel (2001).

4.2.10: PCR Amplification Of cDNA Inserts From The pYES Clones

Amplification of cDNA inserts from the pYES transformants was carried out using colony PCR using "Techne" thermal cycler from Progene. Primers corresponding to the *lac* and *gal* promoters which flank the cDNA inserts in the pYES vector were designed and ordered from Sigma Chemical Co. Forward primer corresponding to the *lac* promoter was 5' TGT GGA ATT GTG AGC GG 3'(17bp) and the reverse primer corresponding to the *gal* Promoter was 5' ACT TTA ACG TCA AGG AG 3'(17bp). Colony PCR was programmed at an anneling temperature of 50°C for 30 sec. and elongation was carried out at 72°C for 90 sec for 30 cycles. The assay system was set as described below.

Assay system:

DNA sample (genomic or plasmid)	colony
Forward primer (10 μM stock)	1.5μL
Reverse primer (10 μM stock)	1.5μL
dNTPs (2.5mM stock)	3.0μL
Taq DNA polymerase buffer	5.0μL
Taq DNA polymerase	0.5μL
D D/W	make up volume to 50μL

4.3: RESULTS

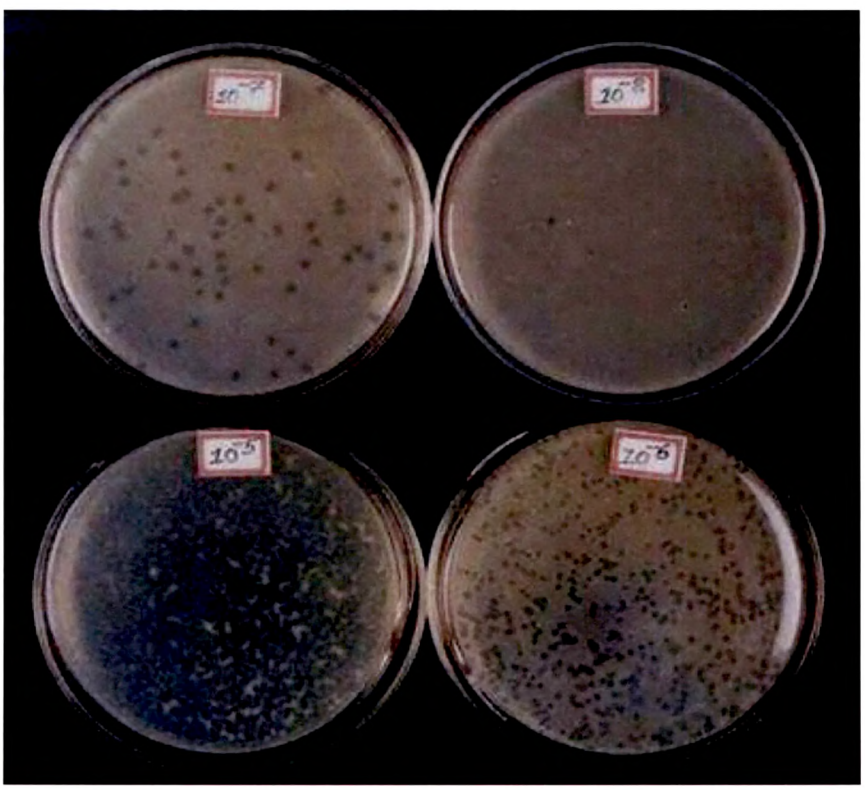
4.3.1: Determination Of Titre Of *A. thaliana* Phage (λYES) cDNA Library

It was necessary to determine the titre of the original λYES cDNA library before proceeding with the further experiments. *E. coli* strain LE392 has been recommended for phage studies and hence this strain was used for carrying out all the lambda phage work including titre determination. The titre of the λYES cDNA library was found to be approximately 10^9 pfu/mL **(Fig. 4.3).**

Since the titre of the original λYES cDNA library was determined to be 10^9 pfu/mL, which is not recommendable for screening of any genes. Hence, the library was amplified by infection of *E. coli* strain LE392 with original λYES cDNA library. After amplification the titre of the lysate containing the amplified library was found to be

$5.21x10^{10}$. Thus a 10 fold increase in the titre of the λYES cDNA library was achieved along with the increase in the volume of the library to 10 mL.

Fig. 4.3: Determination of the titre of *A. thaliana* λYES cDNA library



4.3.2: Construction Of *E. coli* λkc Lysogen And Its Confirmation

The λYES is a phasmid vector, so it has the advantage of being infected with efficiency which is higher than transformation. After infection it can be very conveniently and easily converted into plasmid form which allows the ease of isolating the gene of interest as compared to the vector remaining in the lysogenic form. However, to take benefit of this advantage the *E. coli* strain has to be first lysogenised with another lambda phage namely λkc. This lambda phage contains the *cre* recombinase gene which facilitates the plasmid part from the λYES vector to be looped out after its infection into the host *E. coli* strain by site specific recombination. Also the λkc contains the kanamycin resistance gene as selection marker. Thus the first step in the screening of the λYES cDNA library was the construction of the *E. coli* host with the λkc lysogenised

into it. For this the *E. coli* strains were infected with the λkc lysate at appropriate MOI under static conditions and then selected for kanamycin resistance.

Kanamycin resistant *E. coli* so obtained were confirmed for lysogeny by checking for the induction of the λ prophage and for immunity to superinfection. The lysogens were subsequently infected by the λYES phage library and excision of the pYES was monitored by plasmid isolation and presence of the cDNA insert was confirmed by restriction enzyme digestion. This approach was used to convert part of the phage library to plasmid library. The efficiency of library infection was monitored by performing a viable count on LA Ampicillin/kanamycin plate for each round of infection before plating on the respective screening medium, in order to ensure the minimum 10^5 transformants/mL.

4.3.3: Determining The Minimal Tris Cl (pH 8.0) Buffering Conditions In *E. coli* For Screening The cDNA Library

The C sources selected for the screening were glucose, Na-gluconate, Na-acetate, lactose, fructose, xylose, arabinose, maltose, glycerol and sucrose.

It is known that *E. coli* was inherently able to produce small amounts of acid because of which it was able to show red coloration on RP containing minimal medium with methyl red. Hence, it was necessary to determine the minimum Tris Cl (pH 8.0) buffering concentration which the untransformed *E. coli* could tolerate for growth without any acidification so that it could be used for screening of mps genes. For this, different *E. coli* strains like BL21, DH5α and JM101 were lysogenised with λkc and then transformed with self ligated pYES backbone. This *E. coli* transformants were then spotted on methyl red containing plates with RP, which varied in their Tris Cl buffering concentrations ranging from 0 to 100 (in multiples of 10) for a particular C source and then monitored for reddening of the plates. The concentration which allowed for just the growth without acidification was determined as tabulated and selected for screening the cDNA library.

Since different C sources were selected for screening the library and the acidification varied with the type of C source used, The experiment was repeated for all

the selected C sources and the minimum Tris Cl (pH 8.0) was decided for each of the C sources **(Table 4.2).**

Table 4.2: Carbon sources and concentrations of Tris Cl (pH 8.0) buffering selected for screening on *E. coli*

Carbon source (100mM)	Tris-Cl (pH 8.0) (mM)
Glucose	100
Na^+ Gluconate	70
Na^+ Acetate	50
Lactose	90
Fructose	70
Xylose	70
Arabinose	70
Maltose	70
Glycerol	40
Sucrose	10

4.3.4: Infection Of *E. coli* With *A. thaliana* λYES cDNA Library

The λYES cDNA library was infected into *E. coli* λkc which aids in bringing about site specific recombination at a 24 bp lox sites that flank the plasmid portion of the λYES library thus allowing efficient conversion of the phage vector into plasmid form. The efficiency of infection was determined to be about 10^5 cfu/mL from the viable count of the transformants on LA kanamycin/ampicillin plates.

The transformants were further confirmed by extracting the pYES plasmid from the culture and restriction digestion with *Eco*R1 which flanks the cDNA inserts and hence results in the release of cDNA inserts of varied sizes from the pYES backbone **(Fig. 4.4).**

4.3.5: Screening Of *A. thaliana* Phage (λYES) cDNA Library In *E. coli* For mps Genes

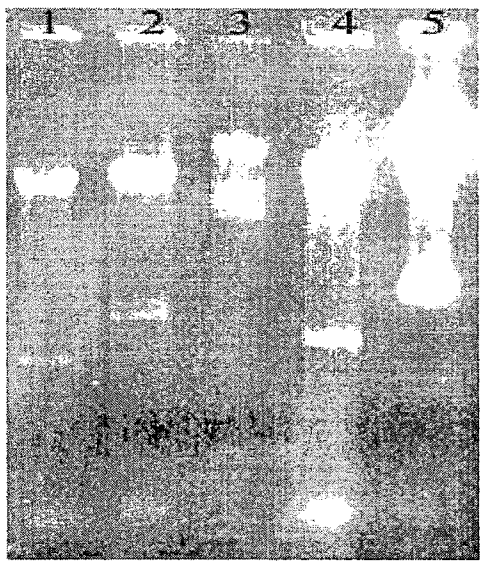
Three *E. coli* strains (BL21, DH5α and JM101) were selected for screening the *A. thaliana* cDNA library. These strains were used to different extents during the course of screening.

Fig. 4.4: Confirmation of conversion of λYES cDNA library of *A. thaliana* into pYES

Lane 1: Undigested pYES a
Lane 2: pYES a digested with *Eco*RI
Lane 3: pYES b digested with *Eco*RI
Lane 4: pYES c digested with *Eco*RI
Lane 5: lambda *Hind*III/*Eco*RI
double digest marker

The C sources used for screening of acid producing clones were glucose, Na-gluconate, Na-acetate, lactose, fructose, xylose, arabinose, maltose, glycerol and sucrose. The above C sources are utilised by *E.coli* using different metabolic pathways and hence we could expect obtaining different sets of clones on different C source giving rise to the different acid production. Different strategies were employed during the course of screening. Firstly, transformants were plated directly on buffered RP minimal medium containing methyl red as indicator for acid production, with the predetermined concentration of Tris Cl (pH 8.0) on each C source. This strategy was also used to screen the plasmid library in *P. fluorescens* as described below. After infecting the phage cDNA library into *E. coli* λkc lysogens, the infection mixture was spread on the RP containing screening medium. Using this strategy, clones able to grow on fructose, maltose, arabinose and Na-gluconate with 70mM Tris Cl buffering were obtained. The plasmids obtained from the clones was digested with *Eco*RI which flanks the insert to release the cDNA **(Fig. 4.5).** It was seen that the cDNA insert size was different in case of all the different C sources indicating obtainment of different cDNA clones.

Fig. 4.5: *Eco*RI digestion of pYES from cDNA transformants obtained on different carbon sources



Lane 1: cDNA clone on fructose
Lane 2: cDNA clone on maltose
Lane 3: Lambda DNA *Eco*RI digest
Lane 4: cDNA clone on gluconate
Lane 5: cDNA clone on arabinose

The plasmid isolated from these clones was used to retransform *E. coli.* Three independent colonies were picked up randomly from each of the sub transformants and used for further spotting (F1, F2, F3 for clones on fructose, M1, M2, M3 for clones on maltose and so on for the clones on arabinose and gluconate) **(Fig. 4.6).** All the clones were spotted for acid production on 70 mM Tris Cl buffered minimal methyl red plates containing the original C source from which they were isolated. In addition, they were also grown on the rest of the three C sources to check if they displayed acid production on more than one C source. However, the red zone indicating acid production was not found to be consistent. However, broth experiments were performed to reconfirm and characterize the P solubilizing ability of the cDNA inserts on each of the four C source. No drop in pH was observed which was further confirmed by HPLC profile of the supernatant that was similar to the control containing only the self ligated pYES backbone. Thus, despite several rounds of screening using all the above mentioned C sources no positive clones were obtained using this strategy.

Hence, a second screening strategy was employed by subjecting the infection mixture to liquid enrichment on unbuffered minimal medium with RP on the respective C source before spreading on the selective screening medium under buffered conditions. In this case pH drop was observed in the screening medium with xylose and arabinose as C sources. However, when the cells from the enriched broth were spread on the solid

medium with the same C source under buffered conditions, no colonies appeared. The failure to grow on the buffered medium could be either due to the low amount of acid produced which is insufficient to solubilize P and sustain growth under buffered conditions or *E. coli* could not survive due to drop in pH. HPLC analysis of the supernatant of enriched broth was done to determine any difference in the acid secretion profile A new additional peak with a retention time of 3.5 min was present compared to the self ligated pYES backbone used as control This indicates that some new organic acid was being produced by the clones. Also, the peak at the retention time of 4.20 min was increased. On comparision with the standard organic acids, the acid with the retention time of 3.5 min was found to be pyruvate while the one with 4.2 min was determined to be acetic acid **(Fig. 4.7 a and b).**

Fig. 4.6: P solubilization by *E. coli* cDNA transformants on 70mM Tris Cl buffered RP minimal medium

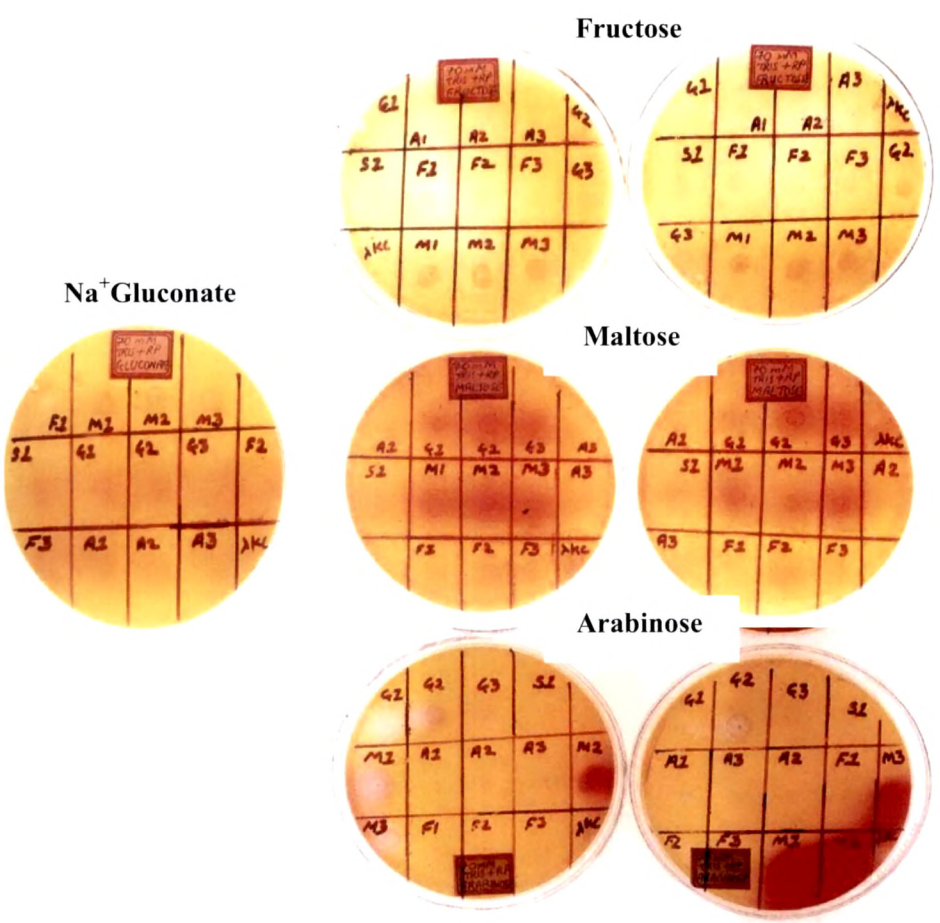


Fig. 4.7 (a): HPLC profile of organic acids secreted by *E. coli* cDNA transformants on xylose as C source

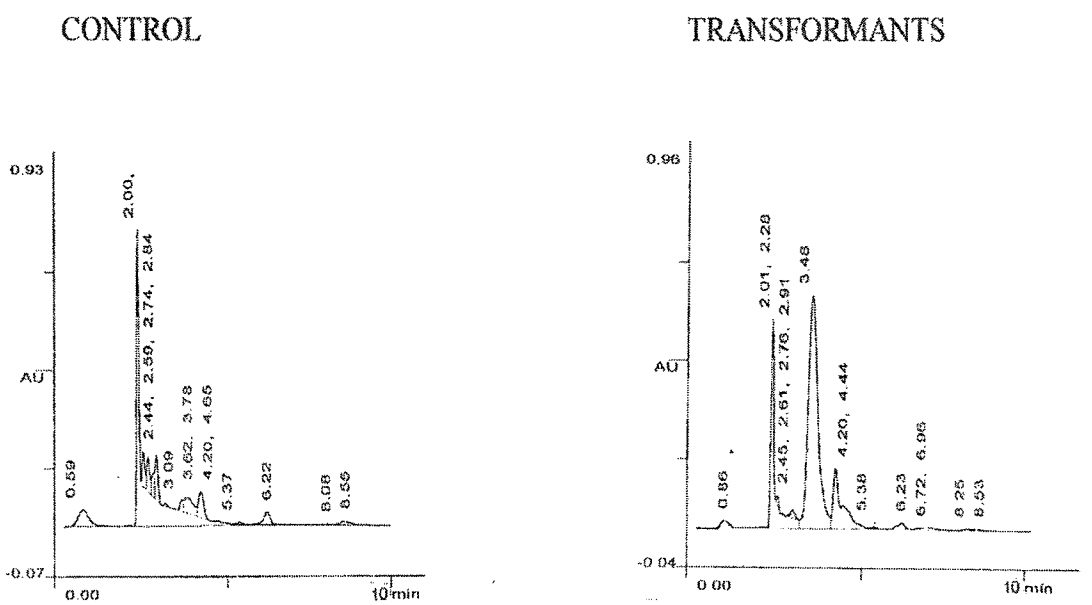
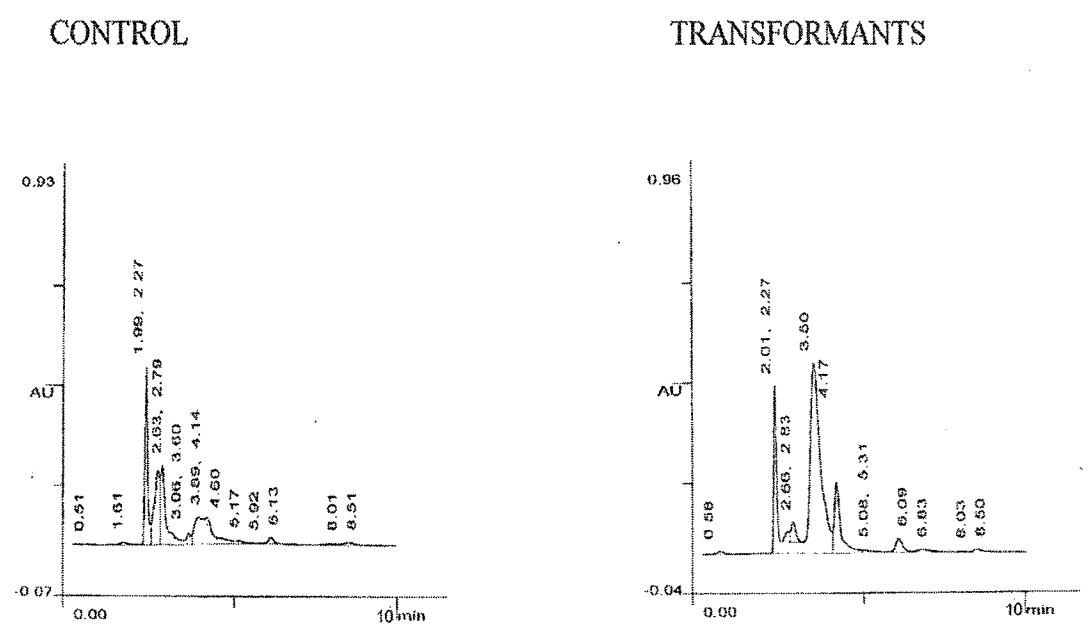


Fig. 4.7 (b): HPLC profile of organic acids secreted by *E. coli* cDNA transformants on arabinose as C source



4.3.6: Preparation Of Plasmid (pYES) From Phage (λYES) cDNA Library For Screening In *P. fluorescens*

The λYES vector is a multifunctional vector capable of replicating as a lambda phage lysogen as well as a plasmid in *E. coli*. The plasmid part of the vector can be excised out of the lambda phage by *cre* mediated site specific recombination. Moreover, the lambda phage infection is highly specific to *E. coli*. Thus, for screening the library in *P. fluorescens* it is necessary to first convert the phage library into plasmid (pYES) form so that it can be transformed into *P. fluorescens*. Plasmid library was prepared by using the amplified phage cDNA library. The plasmid was visible as a diffused band on agarose gel, as it is expected to comprise of many plasmids with varying sizes. The plasmid library showed transformation efficiency of $2x10^4$ cfu/mL in *P. fluorescens*.

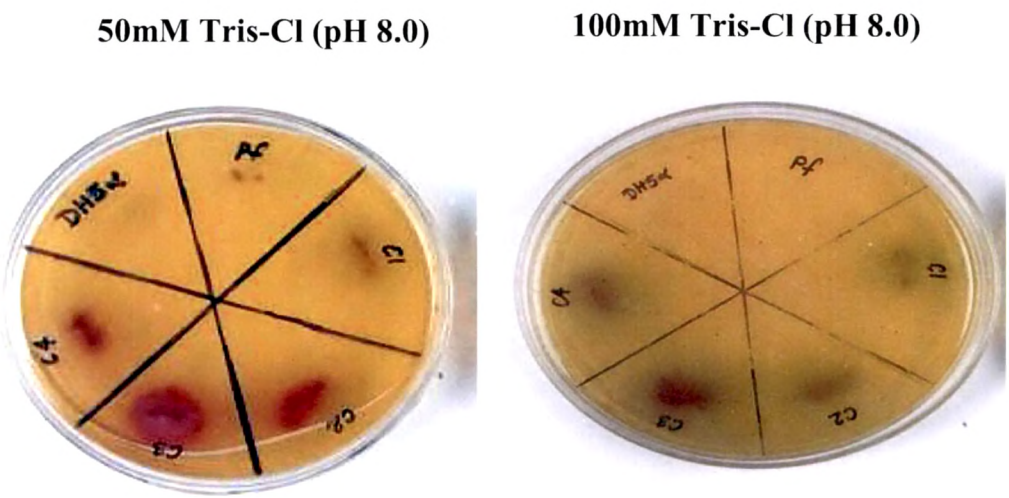
4.3.7: Screening Of *A. thaliana* Plasmid (pYES) cDNA Library In *P. fluorescens* For mps Genes

The plasmid cDNA library was used for screening of mps genes in *P. fluorescens*. Glucose was selected as the C source for the screening. pYES was transformed into *P. fluorescens* and spread on the selective minimal medium plate with RP as the sole Pi source. After 24 h of incubation a lawn of tiny colonies appeared and the entire plate turned pink. On further incubation for 5 days few colonies among the lawn began to emerge which grew bigger than the others turning the medium around them red in colour. These colonies were subcultured onto 100mM Tris Cl buffered RP plates several times to reconfirm acid production and finally 4 (C1, C2, C3and C4) out of 13 *P. fluorescens* cDNA transformants were selected for further characterization on the basis of consistent acid producing phenotype on the methyl red plates.

4.3.8: Characterization Of mps Ability Of *P. fluorescens* pYES cDNA Transformants

The ability of *P.fluorescens* transformants C1, C2, C3 and C4 to solubilise RP were monitored on100mM as well as 50mM Tris-Cl (pH 8.0) with untransformed *P. fluorescens* as control **(Fig. 4.8).** It was observed that at lower buffering (50 mM) the red zone of acidification was much better as compared to that at 100 mM whereas untransformed *P. fluorescens* as well as DH5α which were used as controls failed to grow.

Fig. 4.8: P solubilization by *P. fluorescens* cDNA transformants on RP minimal medium with glucose



In order to characterize the P solubilizing efficacy of the cDNA transformants elaborately, growth, acidification and P releasing ability were monitored in the liquid medium with 100 mM Tris Cl (pH 8.0) buffering. It was seen that the untransformed *P. fluorescens* could grow upto 10^8 cfu/mL but failed to lower the pH, whereas C1, C2 and C3 could grow upto 10^8 cfu/mL and also decreased the pH of the medium from about 8.0 to 4.5 which is an indication of acidification. This decrease in pH was observed after 48h. of incubation. The amount of P solubilized in the supernatant was also determined. It was found that only after the pH drops below 6.5 is the P release detectable. untransformed *P. fluorescens* failed completely to show any P release whereas in the case of cDNA transformants the amount of P released gradually increased over the days with a maximum on the 4^{th} day i.e. after 96 hrs of growth. However, C1 showed the least amount of P release and at 96 hrs only. The amount of P released averaged to around 400µM in case of all the transformants **(Fig. 4.9 a and b).**

To investigate whether the P solubilizing phenotype was dependent on the presence of insoluble P, acidification was also checked with 10 mM KH_2PO_4 as soluble Pi source using 100 mM Tris Cl (pH 8.0) buffer. The transformants could grow and

acidify the medium in the presence of KH_2PO_4 suggesting that the P releasing ability was not induced by phosphate starvation **(Fig. 4.10).**

Fig. 4.9: (a) Acidification and (b) P releasing ability of *P. fluorescens* cDNA transformants on glucose with 100 mM Tris Cl (pH 8.0)

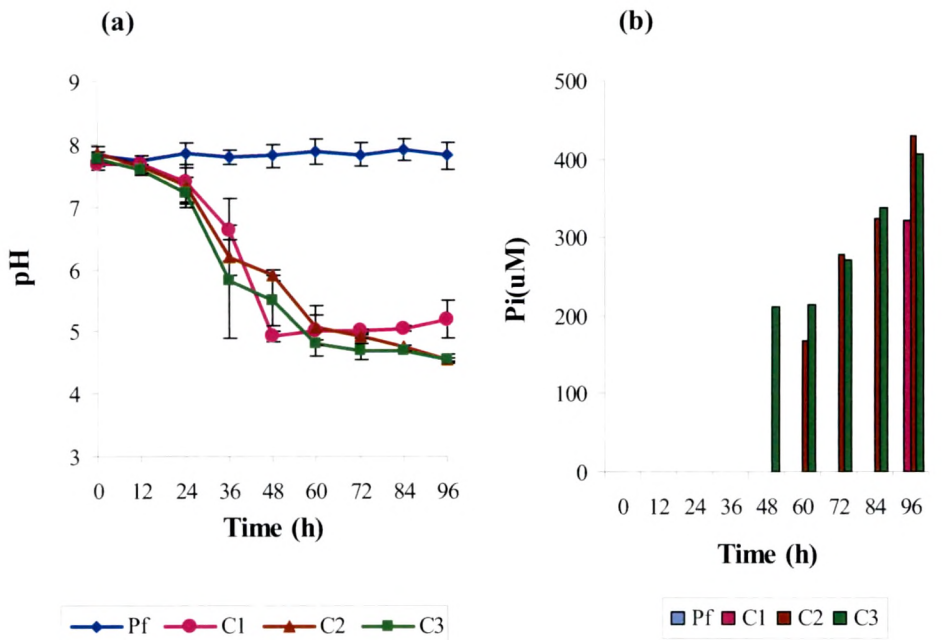
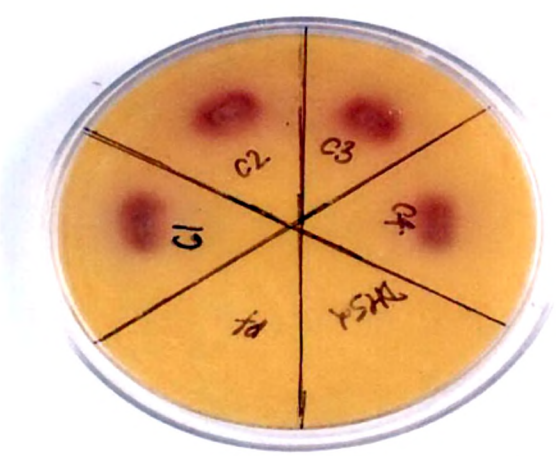
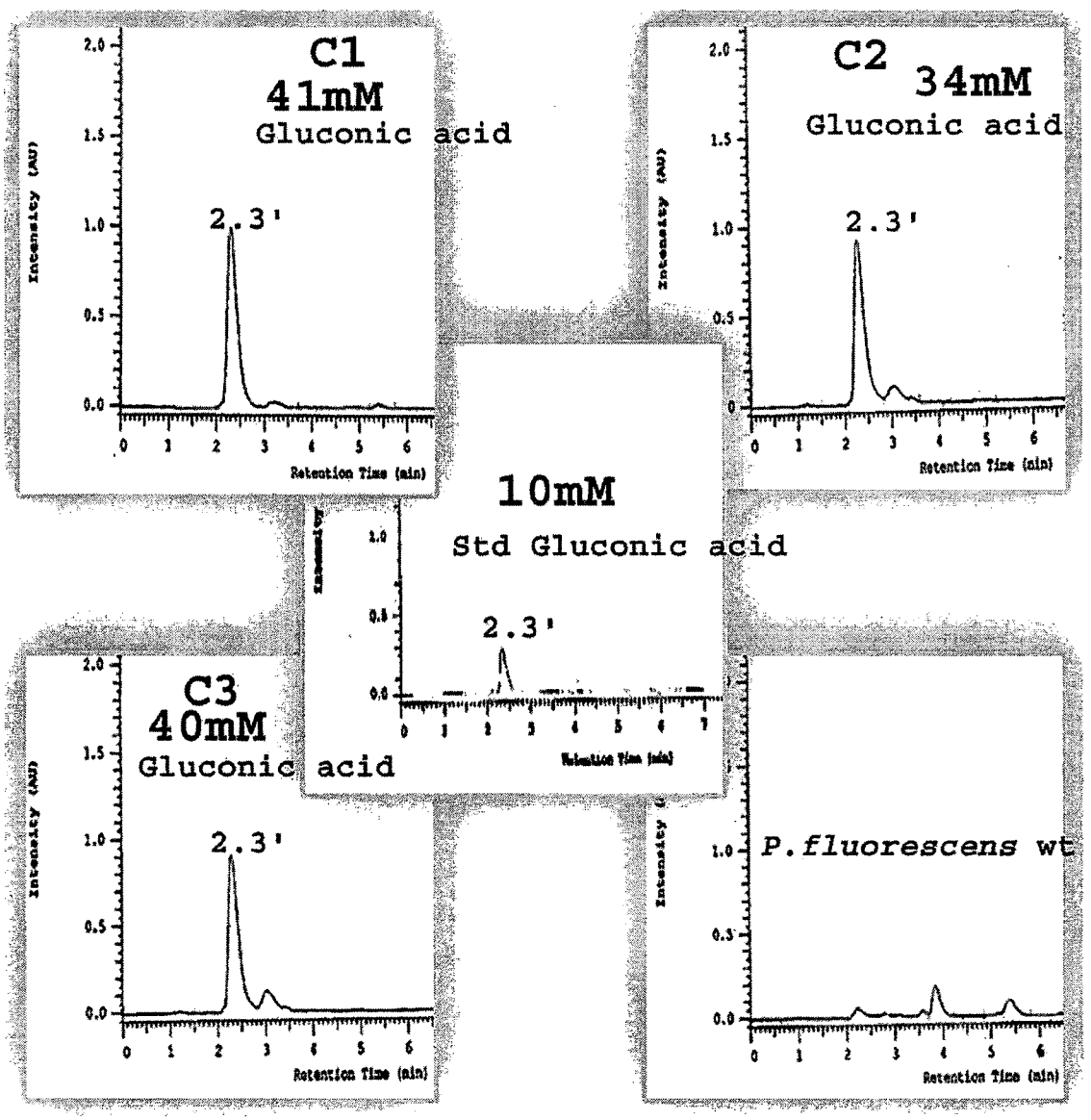


Fig. 4.10: Acidification by *P. fluorescens* cDNA transformants on glucose with 10mM KH_2PO_4 as soluble Pi source with 100 mM Tris Cl (pH 8.0) buffered media



HPLC analysis of the medium supernatant was done to determine the type of organic acid produced by the cDNA transformants and to quantitate it **(Fig. 4.11).** The transformants were found to secrete gluconic acid as determined by comparision with the retention times of standard organic acids whereas the untransformed *P. fluorescens* failed to show any acid production. Both C1 and C3 secreted around 40 mM gluconic acid while the clone C2 could secrete about 34mM.

Fig. 4.11: Characterization of nature and amount of organic acid secreted by *P. fluorescens* cDNA transformants on glucose with 100 mM Tris Cl (pH 8.0)



In order to further characterise the P solubilising ability of the transformants, they were also checked for growth and acid production on C sources other than glucose such as sucrose, lactose, xylose, glycerol and fructose with 50 mM of buffering. On these C sources, the transformants could grow but failed to show any red zone of acidification on the RP plates except for that on xylose **(Table 4.3).** This indicates that the transformants were not able to utilize these C sources for organic acid production and thereby P solubilization. In presence of xylose as C source, however, the transformants were able to show good growth and acidification when the buffering was 50 mM in contrast to the untransformed *P. fluorescens* **(Fig. 4.12).**

Table 4.3: Growth and acid secretion by *P. fluorescens* cDNA transformants on different C sources

Carbon source	Pf	C1	C2	C3	C4
Glucose	++/-	++/++	++/++	++/++	++/++
Sucrose	++/-	++/-	++/-	++/-	++/-
Lactose	+/-	+/-	+/-	+/-	+/-
Xylose	++/-	++/+++	++/+++	++/+++	++/+++
Glycerol	+/-	+/-	+/-	+/-	+/-
Fructose	++/-	++/-	++/-	++/-	++/-

Growth/Acid production

The pYES plasmid is a shuttle vector capable of autonomous replication in *E. coli* and yeast. Since it has the *E. coli* ori (origin of replication), it is not capable of self replication in *P. fluorescens.* Thus, C1, C2, C3 and C4 transformants could be a result of genomic integration of the pYES which served as a suicide vector for *P. fluorescens.* This was confirmed by failure to retrive the plasmid pYES from the cDNA transformants. To further confirm the presence of pYES integration in the genome of the *P. fluorescens* cDNA transformants, primers were designed complementary to the *lac*

and *gal* promoters which flank the cDNA in the pYES. PCR amplification of the cDNA was first attempted from the randomly obtained pYES clones from *E. coli* to standardize the PCR reaction **(Fig. 4.13).** However, no amplification was obtained from the *P. fluorescens* pYES cDNA genomic integrants C1, C2, C3, and C4 with the above designed primers despite several attempts.

Fig. 4.12: P solubilization by *P. fluorescens* cDNA transformants on 100mM xylose and 50 mM Tris Cl pH (8.0) buffered RP minimal medium

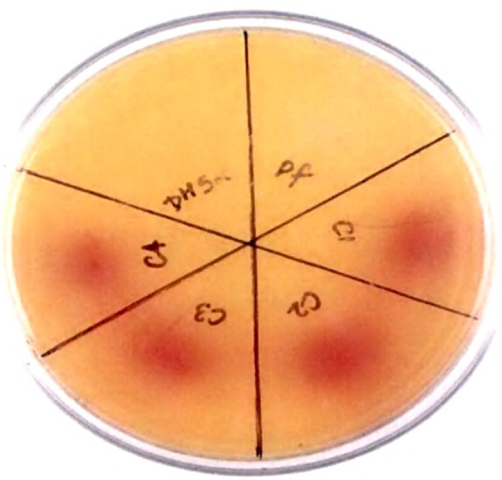
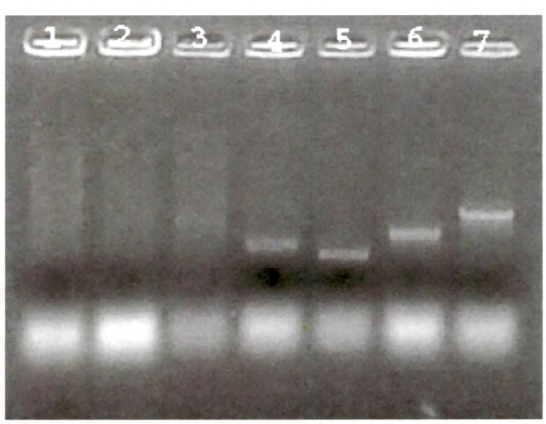


Fig. 4.13: PCR amplification of the cDNA from randomly obtained pYES clones from *E. coli*

Lane 1:JM101λkc (-ve control)
Lane 2: JM101 rec$^-$ (-ve control)
Lane 3: JM101λkc with pYES (M1)
Lane 4: JM101λkc with pYES (F1)
Lane 5: JM101λkc with pYES (A1)
Lane 6: JM101λkc with pYES (G1)
Lane 7: JM101λkc with pYES (S1)



4.4: DISCUSSION

The screening of *A. thaliana* plasmid cDNA library resulted in the selection of *P. fluorescens* transformants which could solubilize RP in the presence of glucose on 100 mM Tris Cl (pH 8.0) buffered minimal medium. However, screening of the phage cDNA library in *E. coli* failed to generate any clones with consistent P solubilizing ability. This success and failure can be attributed to the differences in the nature and amount of organic acid production by the two microorganisms. Although many mono- and di-carboxylic acids have been implicated in the P solubilizing ability of bacteria, gluconic and 2-ketogluconic acid have been responsible for most efficient mps phenotype (Hoon *et al.*, 2003; Sharma *et al.*, 2005). Moreover, incorporation of genes associated with gluconic acid secretion have been shown to confer P solubilizing ability to non PSMs such as *E. coli*, *Pseudomonas* sp. PSS and *Burkholderia cepacia* IS-16 (Liu *et al.*, 1992; Kim *et al.*, 1998a; Rodriguez *et al.*, 2000).

The pYES library screening in *P. fluorescens* also resulted in the gluconic acid production. However screening the cDNA library for mps genes in E. coli, resulted in production of pyruvic acid and increased the amount of acetic acid production in contrast to the reported gluconic acid production Differences in the metabolism between the two microorganisms could account for this phenotype. In the case of *E. coli*, which belongs to the Enterobacteriaceae family, Embden-Meyerhof- Parnas (EMP) or glycolysis is the central pathway utilized for sugar metabolism whereas for *P. fluorescens* belonging to the Pseudomonadaceae family, Entner-Doudoroff (ED) is the main pathway involved in the sugar utilization (Conway, 1992). In addition to the phosphorylative pathway, in the case of *P. fluorescens* glucose can also be utilized by direct periplasmic oxidation to gluconic acid via membrane bound enzyme glucose dehydrogenase (Goldstein, 1995).

E. coli K-12 derivatives however, cannot produce gluconic acid as it contains only the apo GDH but is unable to produce PQQ (Matsushita *et al.*, 1997), the cofactor of GDH essential for the formation of holoenzyme. This inability of some *E. coli* strains is due to the presence of cryptic pqq synthase genes (Liu *et al.*, 1992; Kim *et al.*, 1998a). Thus, genetic manipulation of *E. coli* for mps phenotype so far has resulted in cloning of one of the genes from the pqq synthase gene cluster that could complement cryptic pqq synthase genes or involved in PQQ transport, thus allowing gluconic acid production and

thereby solubilize P. For example, a putative PQQ synthase gene was cloned from *Deinococcus radiodurans* KR1 in *E. coli* under the control of an inducible promoter and the expression of this gene was shown to complement the mps activity in recombinant *E. coli* (Khairnar *et al.*, 2003). The absence of gluconic acid producing transformants in *E. coli* could be attributed to the selection of the plant cDNA library. *A. thaliana* genome has been completely sequenced and annoted. A search in the genome showed that it lacks the pqq synthase gene cluster and hence it was not possible to compliment the cryptic pqq synthase genes in *E. coli.*

The extracellular secretion of puruvic acid was observed in *E .coli* carrying acetate kinase (*ack*A) and phosphotransacetylase (*pta*) mutations as a result of redistribution of the metabolic flux from acetyl-CoA branch (Yang *et al.*, 1999 a, b). Acetate pathway mutants (*pta, ack, rpo*S and *pta ack*) have been shown to accumulate some pyruvate under aerobic conditions (Diaz-Ricci *et al.*, 1991; Contiero *et al.*, 2000). However, which gene overexpression is responsible for pyruvic acid production could not be determined because the *E. coli* transformants displayed instability and inconsistency in their phenotype.The failure to grow on the buffered medium could be either due to the weak nature and low amount of pyruvic and acetic acid produced, as compared to gluconic acid, which is insufficient to solubilize P and sustain growth under buffered conditions or the inability of *E. coli* to survive due to drop in pH. Thus completely different acid secretion profile was seen in case of *E. coli*, a success that could be achieved only due to the randomness of the cloning strategy.

All the four *P. fluorescens* cDNA transformants C1, C2, C3 and C4 showed similar P solubilizing and gluconic acid producing properties, but the genetic basis for this phenotype could not be determined as the cDNA was integrated into the genome. The phenotype could be due to inactivation of the gene where the cDNA was integrated leading to unregulated expression of the direct oxidative pathway genes giving rise to increased gluconic acid production. An attempt to amplify the integrated cDNA by PCR, however failed. It could be attributed to the way in which the integration of the pYES might have taken place in the genome of *P. fluorescens* as explained schematically in the **Fig. 4.14.** If the integration has occurred as a result of random illegitimate recombination event, the cDNA could be amplified and retrieved by the primers designed against the *lac* and *gal* promoters. However, if the pYES has integrated due to

homologous recombination with the cDNA it is not possible to retrieve the cDNA with the primers designed against the above mentioned regions.

Fig. 4.14: Schematic representation of integration of pYES in the genome of *P. fluorescens* and its effect on PCR amplification of the cDNA