

## **Study on efficacy of different *Agrobacterium tumefaciens* strains in genetic transformation of microalga *Chlamydomonas reinhardtii***

**Pratheesh P. T.\*<sup>1</sup>, Shonima G. M.<sup>1</sup>, Jiji Thomas<sup>1</sup>, Abraham C. I.<sup>1</sup> and Muraleedhara Kurup G.<sup>2</sup>**

<sup>1</sup>*School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala 686560*

<sup>2</sup>*Department of Biochemistry, University of Kerala, Kerala 695581*

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### **ABSTRACT**

The objective of this study was to determine the efficacy of three commonly used *Agrobacterium* strains in genetic transformation of the microalga *Chlamydomonas reinhardtii*. Three different strains of *Agrobacterium tumefaciens* (EHA 101, EHA 105, LBA 4404) and pCAMBIA 1304 binary vector hosting the genes coding for GUS ( $\beta$ -glucuronidase) and hpt (hygromycin phosphotransferase) were used for the present study. Transformation processes using *Agrobacterium* were evaluated without the addition of acetosyringone or by wounding the cells. Colonies resistant to hygromycin at 10 mg/l were selected. GUS gene expression in transformed cells was detected with GUS histochemical assay. PCR and RT-PCR analysis confirmed the integration and expression of hpt (610 bp) and GUS (640 bp) genes in transgenic *Chlamydomonas reinhardtii*. *Agrobacterium* strain EHA 105 was found to be the most efficient with a higher rate of transformation frequency.

**Key words:** *Agrobacterium tumefaciens*, transformation, *Chlamydomonas reinhardtii*.

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### **INTRODUCTION**

Transgenesis in microalgae is a complex and fast-growing technology. Different methods have been developed for the nuclear as well as chloroplast transformation of microalgae. Genetic transformation of *Chlamydomonas reinhardtii* was reported as early as 1982. The nuclear transformations are mainly achieved by electroporation [1], agitation with glass beads [2] or silicon carbide whisker [3]. These transformation protocols mainly use cell wall less strains of microalgae, except for particle bombardment. The direct gene delivery methods have disadvantages like insertion of multiple copies of transgenes and a high degree of rearrangement at the site of insertion. *Agrobacterium tumefaciens* mediated transformation has been reported to overcome the major limitations of these transformation procedures, providing stable integration at lower copy number, potentially leading to fewer problems with transgene co-suppression and instability [4]. *Agrobacterium* mediated genetic transformation of microalgae was first reported in *Chlamydomonas reinhardtii* [5]. This technique was also found to be successful in fresh water green alga *Haematococcus pluvialis* [6] and in marine green alga *Dunaliella bardawil* [7]. A study on efficacy of different strains of *Agrobacterium* in the genetic transformation of microalgae has not been reported till date.

*Agrobacterium tumefaciens* has been an invaluable system in studying the fundamental biology of host pathogen interaction and plant biotechnology due to its unique ability to transfer DNA into the plant genome. The process of T-DNA transfer is initiated by the induction of bacterial virulence (*vir*) genes by the phenolic compounds produced and released by the wounded plant cells through a signal transduction system [8]. It has been shown that majority of the dicotyledonous plants produce such phenolic compounds and they have been routinely transformed by *Agrobacterium* [9]. Monocotyledons, which generally do not produce *vir*-inducing compounds, could be transformed by the exogenous addition of such molecules like acetosyringone (AS) [10]. The ease of the procedure, the transfer of relatively large segment of DNA (up to 150 kb) with little rearrangements, preferential insertion of T-

DNA into potentially transcribed regions and the integration of mostly single copy of the transgene(s) into plant chromosomes have made this steadfast method for genetic transformation in dicotyledonous plants [11]. Interestingly, *Agrobacterium* gene transfer has also been extended to fungi [12] and human (HeLa) cells [13]. Research related effective utilization of *Agrobacterium* mediated transformation technique in microalgae is still in its infancy. An attempt has been made to find out the transformation efficiency of commonly used strains of *Agrobacterium tumefaciens* in the microalga *Chlamydomonas reinhardtii*. The study described here may be useful in future research related to *Agrobacterium* mediated transformation in various other algal species.

## MATERIALS AND METHODS

### *Chlamydomonas reinhardtii* strain and culture conditions:

Culture of *Chlamydomonas reinhardtii* CC-125 (mt<sup>+</sup>) was obtained from Chlamydomonas centre, Australia. The culture was grown in Tris Acetate Phosphate (TAP) media [14]. The pH was adjusted to 7 in liquid TAP media and 1.5% agar w/v was added in case of cells grown on solid media. Culture was grown at 24±1°C under light period (16:8h) with mild shaking (80 rpm).

### Sensitivity test of *Chlamydomonas reinhardtii* to hygromycin:

The sensitivity of *Chlamydomonas reinhardtii* CC-125 (mt<sup>+</sup>) to hygromycin was tested by culturing the algal cells in TAP medium containing different concentrations of hygromycin (1, 2, 3, 4, and 5 mg/l). The optical density was measured at regular intervals (600 nm) to assay growth. The hygromycin tolerance of *Chlamydomonas* was also tested at different concentrations in TAP agar medium (2, 4, 6, 8 and 10 mg/l).

### Plasmid construct and Bacterial strains:

Binary vector pCAMBIA1304 was used for the study (M/S CAMBIA, Australia), which harbors *hpt* (hygromycin phosphotransferase) as marker gene and GUS ( $\beta$ -glucuronidase):GFP fusion reporter gene system driven by CaMV 35S promoter (Fig. -1). pCAMBIA 1304 is a widely used vector for transformation studies in microalgae. *Escherichia coli* strain DH5 $\alpha$  was used for the maintenance and multiplication of the plasmids. Different *Agrobacterium tumefaciens* strains used for the study were LBA 4404 (A *vir* helper; harbors disarmed Ti plasmid pAL4404, a T-DNA deletion derivative of pTiAch5, octopine type, Rm<sup>r</sup> Sm<sup>r</sup>) [15], EHA 105 (A *vir* helper, L,L-succinamopine type, harbors T-DNA deletion derivative of pTiBo542, a supervirulent type Ti plasmid, Rm<sup>r</sup>) [16], and EHA 101 (harbors T-DNA deletion derivative of pTiBo542, Km<sup>s</sup>) [17]. Plasmid was introduced into *A. tumefaciens* strains through freeze thaw method [18].

### Transformation of *Agrobacterium*:

For transformation, 1  $\mu$ g plasmid was added to competent cells of *Agrobacterium* and mixed gently. The mixture was kept in ice for 30 min, then frozen at liquid nitrogen and immediately thawed at 37°C for 15 min. LB medium containing 20  $\mu$ g/ml rifampicin and 1  $\mu$ g/ml kanamycin was added to the mixture and incubated at 28°C for 3 hrs with 220 rpm. The cells were plated on LB medium containing 20  $\mu$ g/ml rifampicin and 50  $\mu$ g/ml kanamycin and incubated at 28°C overnight. Colony PCR of the transformants was done to confirm transformation using *hpt* specific (HF, 5'-CGATTGCGTCGCATCGAC-3'; and HR, 5'-GTGCACAGGGTGTACAG-3') and GUS specific primers (GF, 5'-TAGAGATAACCTTCACCCGG-3'; and GR, 5'-CGCGAAAACCTGTGGAATTGA-3'). The PCR reaction was carried out in a total volume of 20  $\mu$ l containing 100  $\mu$ M dNTPs, 250 nM of each primer, 1.5 mM MgCl<sub>2</sub> and 0.5 U *Taq* DNA polymerase (M/S Bangalore Genei, India) with colony as the template (M/S Bio-rad, USA). The PCR profile was as follows: 94°C for 10 min followed by 36 cycles of amplification (94°C for 1 min, 55°C for 1 min, 72°C for 1min) and final extension for 10 min at 72°C. The PCR products were analysed on 1.5% agarose gel.

### *Agrobacterium* mediated transformation of *Chlamydomonas*:

Transformation of *C. reinhardtii* was done following Kumar and co-workers [19]. Single colony of *Chlamydomonas* was inoculated into TAP medium and grown to log phase under illumination. The cells were then plated on to solid TAP medium in 90 mm petriplates and incubated in light for 2 days to allow a lawn of cells to be formed. Overnight *Agrobacterium* culture (A600 = 0.5) was raised in liquid LB medium containing appropriate antibiotics (20 mg/l rifampicin and 50 mg/l kanamycin). The cells were pelleted and resuspended in 200  $\mu$ l TAP broth. The bacterial suspension was spread to the thin layer of *Chlamydomonas* culture growing on agar plate. Plates were incubated for 2 days at 25°C (Co-cultivation). After two days, cells were harvested and washed twice with liquid TAP medium containing 500 mg/l cefotaxime via. resuspension by mild vortexing and centrifugation at 100 $\times$ g for 2 min. The washed *Chlamydomonas* cells were plated on solid TAP agar plates containing 10 mg/l hygromycin and 500 mg/l cefotaxime.

**GUS histochemical analysis:**

The hygromycin resistant colonies were analyzed for GUS activity by staining with X-gluc (5-bromo-4-chloro-3-indoyl  $\beta$ -D-glucuronide) [20]. Transgenic *Chlamydomonas* colonies were collected and resuspended in X-gluc solution and inoculated at 37°C overnight. After incubation, cells were pelleted, bleached with ethanol and analyzed under microscope.

**Isolation of Genomic DNA from *C. reinhardtii*:**

Genomic DNA was isolated using modified CTAB method [21]. Cells from 100ml culture ( $1 \times 10^6$  cells/ml) was pelleted and resuspended in 0.5 ml of CTAB buffer (1% Sucrose, 0.8 M NaCl, 0.022 M EDTA pH 8, 0.22 M Tris-HCl pH 7.8, 0.8% CTAB, 0.14 M mannitol) and incubated at 65°C for 1 hr. An equal volume of Phenol:Chloroform:Isoamylalcohol (25:24:1) was immediately added, mixed by inversion and again incubated at room temperature for 10 min with occasional inversion. Samples were centrifuged for 5 min at 12000 rpm in a microfuge to separate the phase. The upper aqueous phase collected after centrifugation was treated with RNase A (M/S Genei, Bangalore) and incubated at 37°C for 1 h. This was followed by extraction with equal volume of Chloroform:Isoamylalcohol (24:1) as mentioned above. The aqueous layer was transferred to new tube and equal volume of chloroform was added. The upper aqueous phase collected after centrifugation was transferred to a fresh tube and 0.6 volumes of isopropanol was added for precipitation of DNA. Precipitated DNA was pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. DNA pellet was washed with 70% ethanol, air dried and suspended in dH<sub>2</sub>O.

**PCR analysis of transgenic *Chlamydomonas* lines:**

PCR analysis for the confirmation of gene integration was performed using genomic DNA isolated from the transformed cells of *Chlamydomonas*. PCR was performed with *hpt* and GUS specific primers as mentioned earlier using 50 ng DNA as template. The PCR profile was as follows: 94°C for 3 min followed by 36 cycles of amplification (94°C for 30 sec, 55°C for 1 min, 72°C for 1min) and final extension for 10 min at 72°C. The PCR products were analysed on 1.5% agarose gel.

**RNA isolation:**

Method used for RNA isolation is essentially the same as that described in the *Chlamydomonas* Source book. Cell pellets were resuspended in 2 ml of extraction buffer (50 mM Tris-HCl pH 8.0, 0.3 M NaCl, 5 mM EDTA and 2% w/v SDS). Proteinase K was added to a final concentration of 40  $\mu$ g/ml and incubated on an orbital shaker at 250 rpm at room temperature for 20 min. To extract total RNA, an equal volume of phenol:chloroform (1:1) (phenol chloroform equilibrated to pH 4.3) was added, shaken vigorously, and centrifuged at 2000 rpm for 5 min the bottom layers, containing proteins and cellular debris, and the DNA-containing cloudy interface were discarded. The upper aqueous phase, containing the RNA, was re-extracted with phenol:chloroform until there was no cloudy interface (approx. seven rounds of extraction). The RNA was precipitated in 2 volumes of 100% ethanol at -20°C overnight and recovered by centrifugation at 8500 rpm at 4°C for 15 min. Pellets were washed with 70% ethanol, centrifuged for a further 10 min and dried. Pellets were resuspended in RNase-free water and stored at -80°C. The quality and concentration of the isolated RNA was checked on 0.8% agarose gel. To remove contaminating genomic DNA, 1  $\mu$ g RNA was subsequently incubated with RNase-free DNase (2U/ $\mu$ g) (M/S NEB, England) for 15 min at 37°C. The quantity of RNA obtained was determined spectrophotometrically.

**cDNA synthesis:**

Synthesis cDNA was carried out using ImpromII<sup>TM</sup> reverse transcriptase kit for cDNA synthesis (M/S Promega, USA) following the manufacturer's instructions. First strand cDNA synthesis was performed by reverse transcription with 1 $\mu$ g of total RNA using oligo-(dT) 12-18-mer primer and ImpromII RT enzyme. 1  $\mu$ g total RNA (DNase treated) was mixed with 1 $\mu$ l oligo (dT) (0.5  $\mu$ g) 12-18-mer primer. The mixture was heated to 70°C for 10 min and quick-chilled on ice. The content of the tube was collected by brief centrifugation and added the following: 1X First Strand Buffer (containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl,) 3 mM MgCl<sub>2</sub>, 1U RNasin and 500  $\mu$ M dNTP Mix. The content of the tube was mixed by gentle vortex and briefly centrifuged to collect the content to the bottom. After that 1  $\mu$ l ImpromII<sup>TM</sup> RT enzyme was added, mixed gently and incubated at 25°C for 5 min for primer annealing. The tube was then incubated at 37°C for 1 h for extension. After extension, samples were heated for 15 min at 70°C to terminate RT and chilled on ice. 1  $\mu$ l (2 units) of *E. coli* RNase H was added to the RT mix and incubated at 37°C for 20 min to remove RNA complementary to the cDNA.

**RT-PCR analysis:**

Expression of *hpt* and GUS genes in transgenic *Chlamydomonas* was confirmed through RT-PCR analysis. 2  $\mu$ l of the total cDNA synthesised from transgenic *Chlamydomonas* cells served as the template in the PCR amplification. The PCR components and profile were same as mentioned earlier.

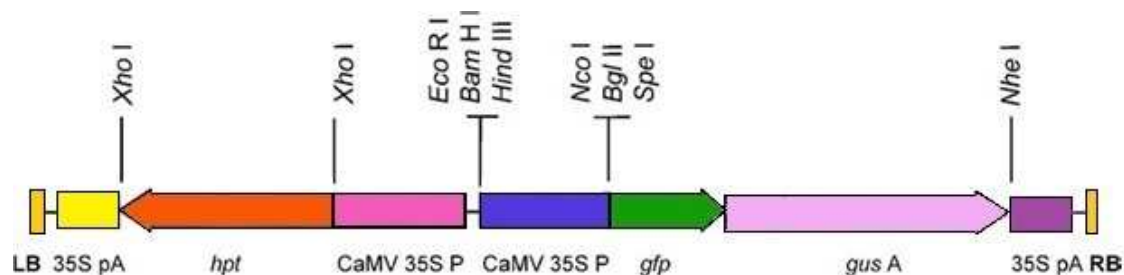


Fig – 1. Schematic representation of the T-DNA region of pCambia 1304.

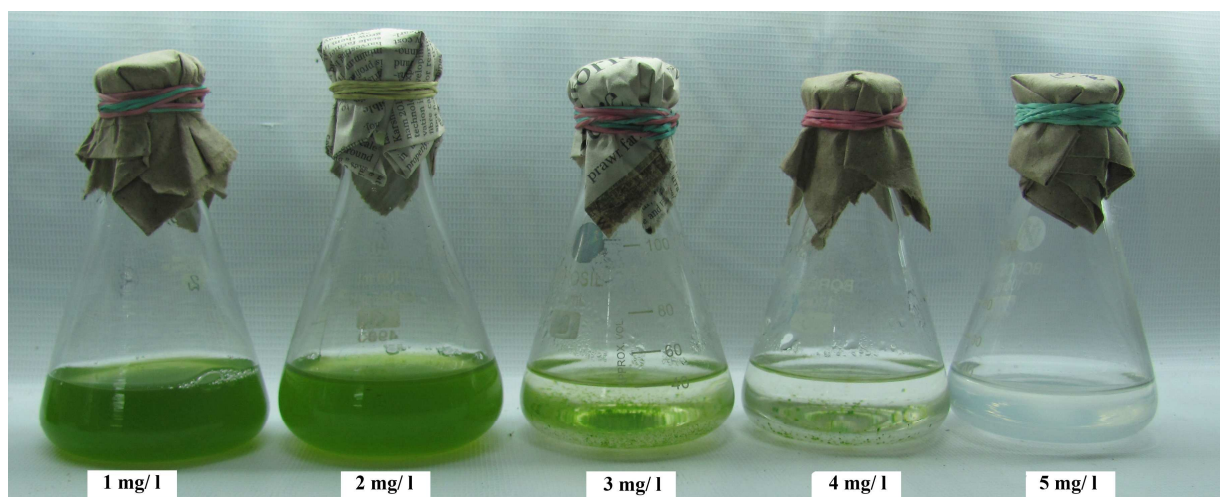


Fig – 2. Hygromycin antibiotic sensitivity test of *Chlamydomonas reinhardtii*

*Chlamydomonas reinhardtii* strain showing hygromycin antibiotic resistance at different concentrations (1, 2, 3, 4 and 5 mg/l) in TAP medium.

Table-1 Transformation frequencies of different strains of *Agrobacterium tumefaciens* in *Chlamydomonas reinhardtii* without acetosyringone

Treatment	No. of cells plated ( $\times 10^6$ )	Number of hygromycin colonies observed	Transformation frequency (per $10^6$ cells $\pm$ SD)
Cocultivation CC-125 in TAP with EHA-105	1.70	14	13.82 $\pm$ 2.8
Cocultivation CC-125 in TAP with EHA-101	1.82	6	5.43 $\pm$ 1.3
Cocultivation CC-125 in TAP with LBA-4404	1.67	7	6.12 $\pm$ 1.4

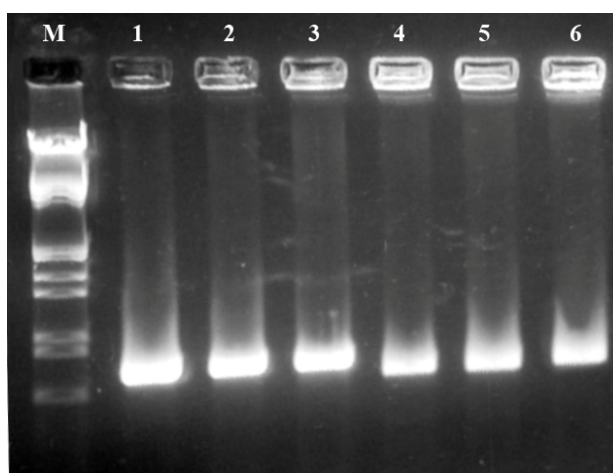


Fig - 3. Colony PCR analysis of transformed *Agrobacterium* strains

M- Molecular weight marker ( $\lambda$ - DNA EcoR I, Hind III double digest).

Lane- 1, 2 and 3- 640 bp amplicon of GUS gene from EHA-105, EHA-101 and LBA-4404 respectively.

Lane- 4, 5, and 6- 610 bp amplicon of hpt gene from EHA-105, EHA-101 and LBA-4404 respectively.



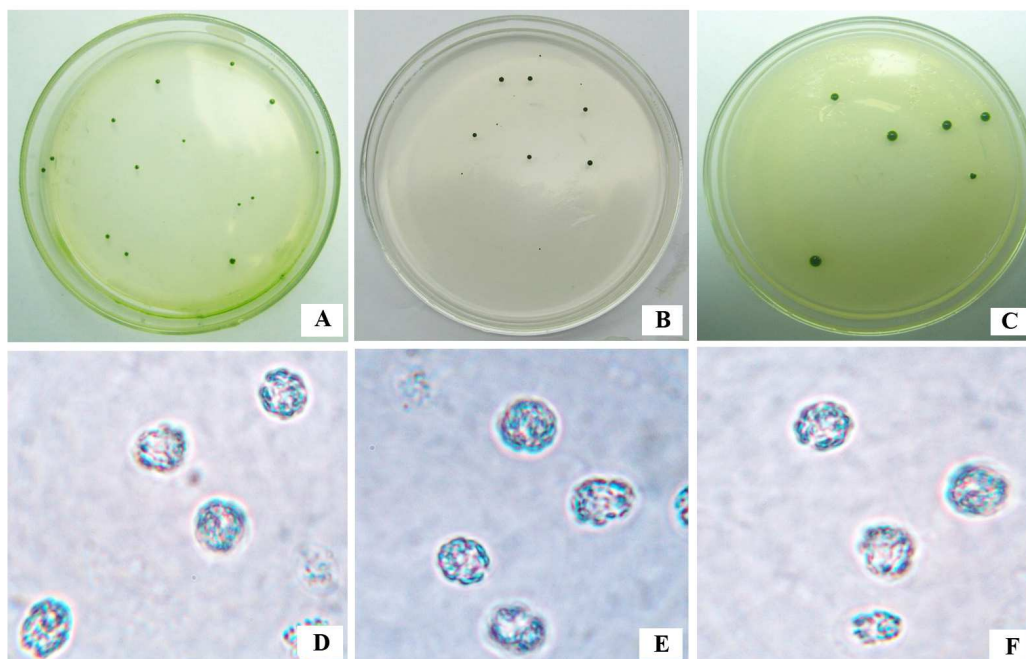


Fig – 4. Screening of Hyg<sup>r</sup> transformed *Chlamydomonas* cells and detection of GUS expression in transgenic *C. reinhardtii* strains

- A. Transformed colonies of *C. reinhardtii* with EHA-105.  
 B. Transformed colonies of *C. reinhardtii* with EHA-101.  
 C. Transformed colonies of *C. reinhardtii* with LBA-4404.  
 D. EHA-105 transformed *C. reinhardtii* GUS expression.  
 E. EHA-101 transformed *C. reinhardtii* GUS expression.  
 F. LBA-4404 transformed *C. reinhardtii* GUS expression.

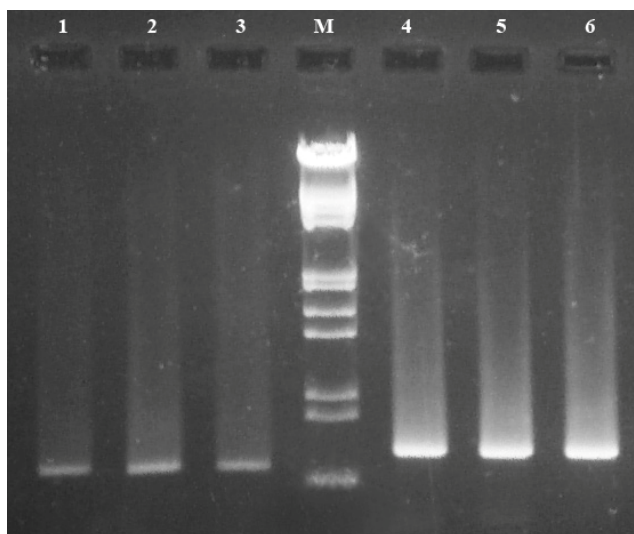


Fig - 5. PCR analysis of genomic DNA isolated from transgenic *C. reinhardtii*  
 Lane -1,2 and 3- 610 bp hpt gene amplicon from transgenic *C. reinhardtii* transformed with EHA-105, EHA-101 and LBA-4404 respectively.  
 M. Molecular weight marker ( $\lambda$ - DNA EcoR I, Hind III double digest).  
 Lane - 4, 5 and 6- 640 bp GUS gene amplicon from transgenic *C. reinhardtii* transformed with EHA-105, EHA-101 and LBA-4404 respectively.

## RESULTS

### Culture conditions:

*Chlamydomonas reinhardtii* was successfully grown in TAP broth as well as on TAP agar at 25°C in 16:8 hr photoperiod. Optimum density for transformation study was observed in 48 hr culture.

### Effect of hygromycin concentration:

The minimum concentration of hygromycin required to inhibit the growth of un-transformed *Chlamydomonas* was tested. Untransformed *Chlamydomonas* was inoculated in TAP medium containing different concentrations of

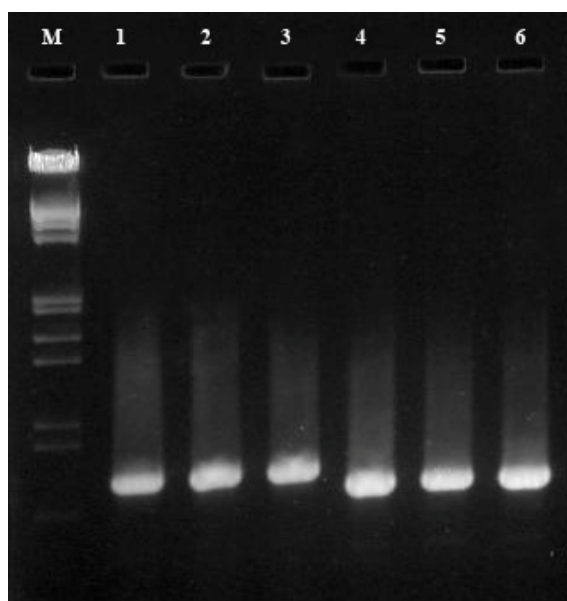
hygromycin. A concentration of 5 mg/l completely blocked regeneration of untransformed algal cells in TAP liquid media (Fig. 2) and 10 mg/l concentration of hygromycin in case of TAP agar media. So these hygromycin concentrations were selected for the screening of transgenic *Chlamydomonas* cells.

#### **Agrobacterium Transformation:**

The three *Agrobacterium* strains under study were successfully transformed with pCAMBIA 1304 through freeze thaw method. Molecular analysis of transformation was done by colony PCR using *hpt* and *GUS* specific primers. Agarose gel electrophoresis of PCR products showed 610 bp amplicon of *hpt* and 640 bp amplicon of *GUS* genes in the three *Agrobacterium* strains (Fig. 3) and confirmed the presence of pCAMBIA 1304 in them.

#### **Transformation of Chlamydomonas:**

*Chlamydomonas reinhardtii* was transformed with three popular strains of *Agrobacterium* to identify the efficient one among them. LBA 4404, EHA 101 and EHA 105 harbouring pCAMBIA 1304 were co-cultivated with *Chlamydomonas* for 2 days. The co-cultivated cells were then grown in TAP selection media containing 10 mg/l hygromycin and 500 mg/l cefotaxime. Transgenic colonies were observed after 5 days incubation in selection media. EHA 105 strain showed the highest frequency of transformation (Table -1).



**Fig - 6. RT-PCR analysis of *hpt* and *GUS* gene expression in transgenic *C. reinhardtii* lines**

*M*- Molecular weight marker ( $\lambda$ - DNA *EcoR* I, *Hind* III double digest)

Lane - 1, 2 and 3- 640 bp amplicon of *GUS* cDNA from transgenic *C. reinhardtii* transformed with EHA-105, EHA-101 and LBA-4404 respectively.

Lane - 4, 5 and 6- 610 bp amplicon of *hpt* cDNA from transgenic *C. reinhardtii* transformed with EHA-105, EHA-101 and LBA-4404 respectively.

#### **GUS histochemical analysis:**

There was high expression of *GUS* gene in transformed cells. The transformants were analyzed for the presence of *GUS* activity. *GUS* positive transgenic *Chlamydomonas* cells showed strong indigo blue colour after incubation with X-gluc (Fig. 4). Most of the cells were completely stained, while some were partly stained. The difference in staining was seen in cells with partially digested cell walls. *GUS* positive colonies were routinely sub-cultured in TAP agar and after 4 weeks subculturing, genomic DNA was isolated.

#### **PCR confirmation of transformation:**

Integration of transgenes into the genome of *Chlamydomonas* was evaluated using PCR analysis. Genomic DNA was isolated from the three different transgenic lines of *Chlamydomonas*, infected with LBA 4404, EHA 101 and EHA 105. PCR analysis showed successful amplification of *hpt* (610 bp) and *GUS* (640 bp) genes from transgenic cells and this confirmed the successful integration of hygromycin and  $\beta$ -glucuronidase genes into the genome of *Chlamydomonas reinhardtii* (Fig. 5).

#### **RT-PCR for gene expression analysis:**

Total RNA was isolated from the three different cell lines of *Chlamydomonas* and cDNA was synthesised. The first strand cDNA derived from the transgenic lines were used as template for PCR. RT-PCR confirmed the presence of *hpt* and *GUS* gene transcripts in all the transgenic lines of *C. reinhardtii* (Fig. 6).

## DISCUSSION

Microalgae have a very high potential in a large number of production technologies [22], as model organism *C. reinhardtii* offer limitless opportunities in this regard. The present work was focused on studying the efficiency on newly developed *Agrobacterium* mediated transformation in *C. reinhardtii*. The TAP medium as used by Kumar and co-workers [5] for the *Agrobacterium* mediated transformation of *Chlamydomonas* was determined to be suitable for the present study. The TAP medium differs from the other media commonly used for *Chlamydomonas* growth in that it contains acetic acid as the carbon source and ammonium chloride as the source of nitrogen, allowing for the simultaneous growth of both algae and bacteria. The *Chlamydomonas* cells were able to tolerate hygromycin concentrations up to 4 mg/l in TAP liquid medium and at 5 mg/l no growth was observed. In case of TAP agar medium 10 mg/l hygromycin completely prevents *Chlamydomonas* growth. This higher tolerance in TAP agar medium may be due to differences in membrane permeability among cells grown in the two media. Higher inoculum cell densities allowed greater tolerance to hygromycin in liquid media, which may be due to quicker degradation of hygromycin to tolerable levels by higher inoculum load than by lower inoculum levels [6].

The use of acetosyringone has been reported to be effective in increasing transformation efficiency by activating the vir genes of the *Agrobacterium*. But *Chlamydomonas* has the capacity to secrete phenolic compounds into the medium [23]. It may be presumed that these compounds may activate the vir genes. Kumar and co-workers [5] reported significantly higher transformation frequency in *C. reinhardtii* while using acetosyringone than without acetosyringone [19]. Similar results of transformation without the use of acetosyringone were also reported in *Haematococcus pluvialis* [6]. In our work, we were able to obtain higher frequency of transformants with *Agrobacterium* strain EHA 105 (13-15 colonies per  $10^6$  cells plated) in the absence of acetosyringone and without wounding the cells. The presence of a super virulent plasmid is expected to be the reason for high frequency of transformation exhibited by this strain.

The *Agrobacterium* mediated transformed *Chlamydomonas* cells were able to sustain hygromycin resistant-phenotype for subsequent generations even when they were maintained in non selection media. Kumar and co-workers [5] reported that *C. reinhardtii* transformed with *Agrobacterium* retained the hygromycin-resistant phenotype for as long as 18 months, even when cells were maintained in hygromycin-free medium. Kathiresan and co-workers [19] reported that the *Agrobacterium* mediated transformed microalga *Haematococcus pluvialis* transformants were stable for more than 2.5 years. The *Agrobacterium* strain EHA 105 has proved to be an efficient tool genetic transformation in plants [24]. The use of *Agrobacterium* for genetic transformation in microalgae is a promising technique in transgenic algal research. The results obtained in this work substantiate that, different strains of *Agrobacterium* can be used for effective genetic transformation of *Chlamydomonas* and EHA 105 is one of the commonly available effective strain for the purpose.

## CONCLUSION

But several challenges still need to be addressed in terms of developing efficient transformation techniques, improving yield and product quality. The present study was focused on improving the transformation technique. All the three different strains of *Agrobacterium* used in this study were capable of inducing nuclear transformation in *C. reinhardtii* in the absence of acetosyringone. *Agrobacterium* strain EHA 105 showed the highest transformation frequency compared to the other strains evaluated in the current investigation. The *Agrobacterium* strain EHA 105 can be preferred over other *Agrobacterium* strains in future genetic transformation experiments in *C. reinhardtii* and other similar microalgal species due to its high efficiency. *Agrobacterium* mediated transformation method for microalga would pave the way for manipulation of many important pathways relevant to the food, pharmaceutical, and nutraceutical industries.

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