

***Agrobacterium*-Mediated Transformation of Three Freshwater Microalgal Strains**

MARY SANITHA, SUDHAKAR RADHA, ANWAR ALIYA FATIMA, SELVARAJU GAYATHRI DEVI
and MOHANDASS RAMYA*

Department of Genetic Engineering, School of Bioengineering, SRM University, Kattankulathur,
Kancheepuram District, Tamil Nadu, India

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Abstract

Microalgal transformation has gained interest in recent years. *Agrobacterium*-mediated transformation remains as the most efficient method for the development of transgenic plants and microalgae due to its wide host range, inexpensive procedure and transfer of large segments of DNA. In the present study, three different microalgal species were isolated from freshwater environment and identified based on the morphological characteristics and ITS-2 region amplification. *Agrobacterium*-mediated transformation was successful for the isolates *Chlorella* sp., *Ankistrodesmus* sp and *Scenedesmus bajacalifornicus*. Gene integration and expression was confirmed by PCR amplification of *hptII* and GUS histochemical assay. *A. tumefaciens* contamination was checked by amplification of *npt II* gene (kanamycin resistant) which lies outside the T-border. Based on GUS assay, transformation efficiencies were found to be 12.25% for *Chlorella* sp. 2.96% for *Scenedesmus bajacalifornicus* and 3.5% for *Ankistrodesmus* sp.

Key words: *Agrobacterium tumefaciens*, GUS intron, LBA4404, transgenic microalgae

Introduction

Microalgae have emerged as an important system for various biotechnological applications such as production of biofuel, vitamins, carotenoids, enzymes, pharmaceuticals and recombinant proteins (Barrow and Shahidi, 2008; Liam and Philip, 2010; Potvin and Zhang, 2010). Freshwater microalgal species are advantageous for to its high biomass and lipid production over marine and waste water microalgae (Mata *et al.*, 2010). Freshwater species like *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina* and *Spirulina maxima* (cyanobacteria) are used as nutritional supplements in humans and as feed additives in animals (Pulz and Gross, 2004).

In the current scenario genetic improvement of microalgae is required to enhance the microalgal potential for biotechnological applications. For the purpose of genetic improvement suitable transformation methods are required. Algae can be genetically transformed by using *Agrobacterium* (Rajam and Kumar, 2006), by direct transformation using glass beads or silicon carbide whiskers (Kindle, 1990; Wang *et al.*, 1995), by particle bombardment (Koop *et al.*, 2007) and by electroporation (Ladygin, 2003; Niu *et al.*, 2011).

In recent years considerable progress has been made in *Agrobacterium*-mediated transfer of gene to microalgal species. *Agrobacterium* mediated transformation has significant advantage over other methods, in producing higher transformation efficiency, stable integration of DNA fragments reducing the frequency of rearrangements, introduction of fewer copies of the transgene into the host genome. *Agrobacterium tumefaciens* can be used to transfer large sized genes (Hallmann, 2007; Kilians *et al.*, 2011).

Agrobacterium-mediated genetic transformation was successful in a variety of freshwater algal strains such as *Chlamydomonas reinhardtii* (Kumar *et al.*, 2004), *Haematococcus pluvialis* (Kathiresan and Sarada, 2009), *Chlorella vulgaris* (Cha *et al.*, 2012) and marine microalgal strains *Parachlorella kessleri* (Rathod *et al.*, 2013), *Schizochytrium* (Cheng *et al.*, 2012), *Nannochloropsis* sp. (Cha *et al.*, 2011) and *Dunaliella bardawil* (Anila *et al.*, 2011).

Genetic transformation using *Agrobacterium* has been successful only for a few species amongst the freshwater microalgal strains (Kumar *et al.*, 2004; Cha *et al.*, 2012; Kathiresan and Sarada, 2009). Fresh water microalgae being economically important, methodology development for genetic transformation studies will be

* Corresponding author: M. Ramya, Department of Genetic Engineering, School of Bioengineering, SRM University, Kattankulathur, Kancheepuram District, Tamil Nadu, India; e-mail: ramya.mohandass@gmail.com

useful for product improvement or value addition. In this study, fresh water microalgal isolates *Chlorella* sp., *Ankistrodesmus* sp. and *Scenedesmus bajacalifornicus* were genetically transformed using *Agrobacterium tumefaciens* harboring vector pCAMBIA 1301. Stable gene integration was studied using the GUS (β -glucuronidase) and hygromycin markers in transformed algal species.

Experimental

Materials and Methods

Medium and antibiotics used. BG 11 medium (pH 7 ± 0.05) was used for culturing of all the algal strains. The macronutrients of the medium consists of NaNO_3 (1.5 g l^{-1}); K_2HPO_4 (40 mg l^{-1}); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (75 mg l^{-1}); $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (36 mg l^{-1}); Na_2CO_3 (20 mg l^{-1}); Na_2EDTA (1 mg l^{-1}); Citric acid (6 mg l^{-1}) and $\text{C}_6\text{H}_8\text{FeNO}_7$ (6 mg l^{-1}) and the micronutrients (mg l^{-1}) were H_3BO_3 (28.6); MnCl_2 (18); ZnSO_4 (2.2); CuSO_4 (7); $\text{Co}(\text{NO}_3)_2$ (4.9) and Na_2MoO_4 (39) (Stanier et al., 1971). A binary vector pCAMBIA 1301, which has a bacterial *hpt* and a GUS intron each under the CaMV35S promoter was used in this study (Rathod et al., 2013). The vector pCAMBIA 1301 was maintained in *Agrobacterium tumefaciens* LBA4404 which was gifted by MSSRF (Chennai, India). Kanamycin ($50 \mu\text{g ml}^{-1}$) was used as the bacterial selection and hygromycin ($25 \mu\text{g ml}^{-1}$) was the algal selection marker.

Isolation and identification of microalgal strains.

Water samples were collected from various freshwater lakes and ponds from different parts of Tamil Nadu, India. Water samples (10 ml) were inoculated into 100ml of sterilized BG-11 broth and incubated at $25 \pm 1^\circ\text{C}$ temperature, under light intensity $100 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ with 16:8 h light and dark cycles. The algal samples were subjected for purification using serial dilution followed by plating in BG11 medium. The individual algal colonies were inoculated into liquid BG-11 media and incubated for further growth. The morphology of the isolates was checked under light microscope (Hund Wizard, Germany). *Chlamydomonas reinhardtii* (CC-124) obtained from *Chlamydomonas* Genetics Centre, Duke University, Minnesota was used as a positive control (Kumar et al., 2004). All the strains were maintained on solid and liquid BG-11 (pH 7 ± 0.05) in plant growth chamber under the above growth conditions.

Molecular identification of microalgae. Colony PCR protocol was followed for all the species as per previous study (Radha et al., 2013). The internal transcribed spacer (ITS-2) region of each isolate was amplified using universal primer (Hall et al., 2010). The

primer sequence for ITS-2 region includes forward 5'-ATGCGATACTTGGTGTGAAT-3' and reverse 5'-GACGCTTCTCCAGACTACAAT-3'. The PCR reaction components for 50 μl reaction mix includes 1 μl of DNA template, 1U of *Taq* DNA polymerase (Bangalore Genei, India), 5 μl of 10X PCR buffer, 1 μl dNTP mix (10 mM each nucleotide), 1 μl of primer (5 pico moles of each, forward and reverse) (Bioserve, India). The conditions for PCR used were initial denaturation at 95°C for 5min, annealing at 55°C for 30 seconds, extension at 72°C for 1 min for 35 cycles of ITS-2 region. The PCR reaction was carried out using Thermal Cycler (Applied Biosystems, USA). The PCR products were examined on 1% (w/v) agarose gel with the 100 bp DNA ladder. The amplified products were purified using EZ-10 column purification kit (Biobasic Inc., USA) and subjected to sequencing using Automated DNA Sequencer AB1 3130 XL (Genetic Analyser, Applied Biosystems, USA). The obtained sequence was analyzed using BLASTN (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and submitted to GenBank. The obtained sequences were aligned using CLUSTAL W and subjected to phylogenetic tree construction using MEGA4 software (Tamura and Dudley, 2007). The unrelated species *Uronema heteromarinum* (JN885101.1) has been chosen as an out group to build a phylogenetic tree.

Sensitivity of microalgal strains towards antibiotic hygromycin. The algal strains were serially diluted and plated (100 μl) on BG-11 plates containing the antibiotic hygromycin ($25 \mu\text{g ml}^{-1}$) and the plates were incubated at $25 \pm 1^\circ\text{C}$ for a week to observe the growth (Rathod et al., 2013).

Transformation of microalgal strains by Agrobacterium co-cultivation. Transformation was done by co-cultivating the microalgal strains with *A. tumefaciens* LBA4404 (Kumar et al., 2004). The algal cultures were plated onto the solid BG-11 medium and were incubated in light to allow a lawn of cells to be formed. *A. tumefaciens* LBA4404 culture in liquid

Table I
Sensitivity of isolated freshwater microalgal strains with Hygromycin ($25 \mu\text{g ml}^{-1}$)

No	Microalgal strains used in the study	Hygromycin ($25 \mu\text{g ml}^{-1}$)
1	<i>C. proboscideum</i> . SRM01	Sensitive
2	<i>Chlorella</i> sp. SRM02	Sensitive
3	<i>Desmodesmus</i> sp. SRM03	Sensitive
4	<i>Scenedesmus</i> sp. SRM04	Sensitive
5	<i>S. obliquus</i> SRM05	Sensitive
6	<i>C. sorokiniana</i> SRM06	Resistant
7	<i>Micractinium</i> sp. SRM07	Sensitive
8	<i>S. bajacalifornicus</i> SRM08	Sensitive
9	<i>Ankistrodesmus</i> sp. SRM 09	Sensitive

YEP (A600=0.5 OD) was plated onto the thin-layer of algal cultures grown on BG-11 plate. Following co-cultivation for 48 h, cells were harvested, washed twice with liquid BG-11 medium containing 500 $\mu\text{g ml}^{-1}$ cefotaxime by centrifugation at 10,000 rpm for 2 min, resuspended in liquid BG-11 media and plated on to solid BG-11 containing 25 $\mu\text{g ml}^{-1}$ hygromycin and 500 $\mu\text{g ml}^{-1}$ cefotaxime. Transformed colonies appeared in a week; and transformed individual colonies were maintained on the same medium containing hygromycin (25 $\mu\text{g ml}^{-1}$) and cefotaxime (500 $\mu\text{g ml}^{-1}$).

Selection of transformed algal colonies by colony PCR. After co-cultivation, transformed colonies were selected and inoculated into BG-11 broth containing hygromycin (25 $\mu\text{g ml}^{-1}$). Further these colonies were used for colony PCR using primers for GUS intron (190 bp), CaMV35S promoter (800 bp) and *npt II* gene (800 bp). Amplification of ITS-2 region was used as positive control. The following primer sequences were used in the study. GUS intron F 5'AGTAAA TTTCTAG TTTTCTCC3' GUS intron R 5'TGTAA CTATCATCATCATC3', CaMV35SF 5'GTCGACGCTT GCCAACATGGTGGGA3' CaMV35SR 5'TCTAGAAGA GATAGATTTGTAGAGAAG3', ITS F 5'AGGAGAA-GTCGTAACAAGGT 3', ITS R 5'TCCTCCGCTTATT-GATATGC3' and *npt II* F 5'TCAGAAGAAGTTCGT-CAAGAAG 3', *npt II* R 5'ATGGGGATTGAACAAGATG 3' (Subramanyam *et al.*, 2011). The presence of *Agrobacterium tumefaciens* contamination was checked using PCR amplification of *npt II* gene fragment (800 bp). This gene confers resistance to kanamycin. Amplification of *npt II* in plasmid pCAMBIA 1301 was used as positive control.

Histochemical assay. Transformed algal cultures (1 ml of each) were harvested by centrifugation at 6500 rpm and washed with sterile distilled water and 0.1 M Na_3PO_4 buffer (pH 7.0). The algal cell pellet was resuspended in 500 μl GUS histochemical assay buffer

and incubated overnight at 37°C. Following GUS assay, chlorophyll was cleared by incubation for 1–2 h in 70% (v/v) ethanol and cells were resuspended in 40% (v/v) glycerol (Cha *et al.*, 2011). Cells were counted in a haemocytometer chamber under a light microscope (Hund wizard, Germany). Cells spotted as blue were recorded as transformed. Transformation efficiency was calculated as the number of GUS positive cells/total number of cells counted ($\times 100\%$). All the experiments were repeated thrice and the mean value was taken for the analysis.

Results and Discussion

Isolation of microalgae. Microalgae are present in diverse environments such as aquatic, terrestrial, freshwater, brackish water, seawater and wastewater (Richmond, 2004). It is estimated that more than 100,000 species exist (Lin *et al.*, 2011). In this study, three different microalgal species were isolated from freshwater samples and morphological identification of the isolates was carried out using microscopic observation (Hund wizard, Germany). Based on the morphological features like cell shape and size the isolated strains were identified as *Scenedesmus* (4 cells are joined together and lying parallel to each other), *Ankistrodesmus* (cells are curved with tapered ends, size is about 20–30 μm in diameter) and *Chlorella* (spherical in shape, size 2–20 μm in diameter) (Fig. 1).

Molecular identification of microalgal strains. Morphologically different microalgal colonies were obtained upon serial dilution and plating. These colonies were subjected to molecular identification using ITS-2 as a marker. ITS-2 amplified products of the isolated microalgal strains were gel purified and sequenced. The sequences were analyzed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The Genbank

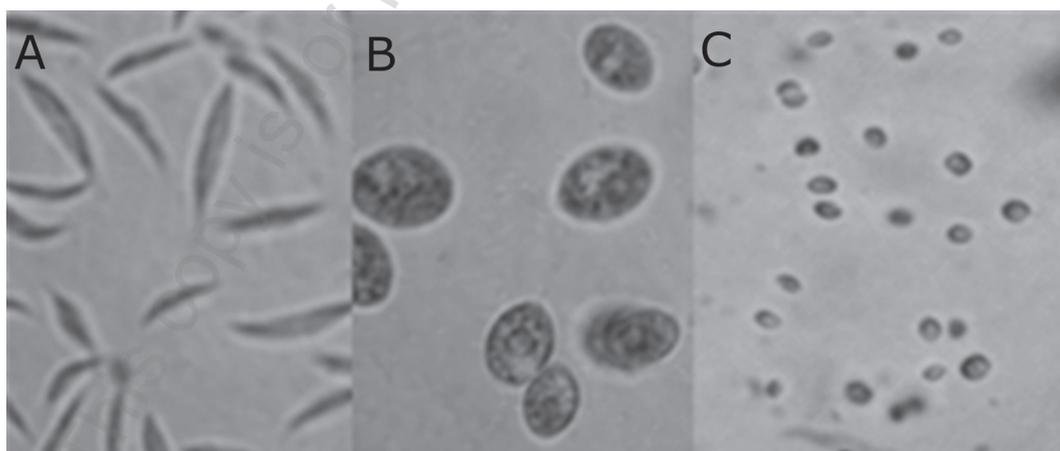


Fig.1. Microscopic (40X) observation of three freshwater isolates (a) *Ankistrodesmus* sp. (b) *S. bajacalifornicus* and (c) *Chlorella* sp.

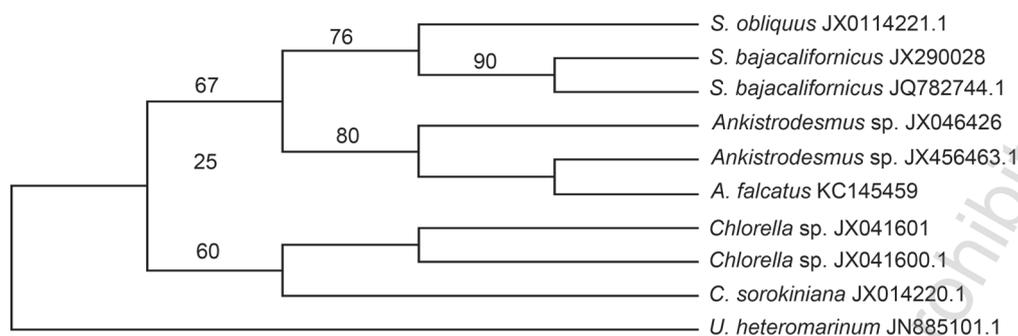


Fig. 2. Phylogenetic analysis results of *Chlorella* sp., *S. bajacalifornicus* and *Ankistrodesmus* sp.

accession number and percentage similarity values of the isolates were as follows: *Chlorella* sp. JX041601 (100%), *Scenedesmus bajacalifornicus* JQ782744 (95%) and *Ankistrodesmus* sp. JX456463 (88%).

Phylogenetic tree is used to determine the evolutionary relationship between various species (Penny *et al.*, 1992). Molecular based phylogenetic techniques have been employed as an important tool in algal taxonomic studies (Brodie and Lewis, 2007). ITS-2 sequence has been widely used in species level identification of green algae (O'Kelly *et al.*, 2010). In a study carried out by Ratha *et al.*, (2012), phylogenetic tree was constructed using ITS-2 molecular marker for microalgal biodiversity analysis. The percentage similarity values were obtained for all the isolated species after pair wise alignment of the ITS-2 sequence. Phylogenetic tree shows that, the isolated species were closely related to the genus of *Scenedesmus*, *Chlorella* and *Ankistrodesmus* sp (Fig. 2).

Antibiotic sensitivity of microalgal strains. Microalgal strains isolated from fresh water samples were tested for sensitivity against hygromycin before transformation analysis. When the algal strains were plated on BG-11 plates containing hygromycin ($25 \mu\text{g ml}^{-1}$), no growth was observed for the strains *Ankistrodesmus* sp. *Chlorella* sp and *S. bajacalifornicus* at the end of 12 days. This shows that these strains were sensitive to hygromycin; hence they were used for further transformation study with pCAMBIA 1301 which has hygromycin as selection marker.

Transformation of microalgal strains. *Agrobacterium tumifaciens* containing plasmid pCAMBIA

1301 was co-cultivated with algal strains and a mat of cells were formed. After 72 h, the cells were harvested, serially diluted and plated on solid BG-11 containing hygromycin ($25 \mu\text{g ml}^{-1}$) and cefotaxime ($500 \mu\text{g ml}^{-1}$). After 9 days, colonies were observed on the plate. Cefotaxime inhibits bacterial cell wall synthesis and thereby prevents the growth of the bacteria (Da silva *et al.*, 2001). After co-cultivation, growth was observed for all the three isolates *Ankistrodesmus* sp., *Chlorella* sp. and *S. bajacalifornicus*. Among the isolated species *Chlorella* sp. were studied earlier for *Agrobacterium*-mediated transformation (Cha *et al.*, 2012). So far, there are no reports available for the other two strains *Ankistrodesmus* sp. and *S. bajacalifornicus* used in this study.

Selection of transformed colonies. CaMV35S promoter drives the reporter genes *Uida* (β -glucuronidase-GUS) and selection marker *hptII* (Hygromycin phosphotransferase). Amplification for GUS intron and CaMV35S promoter regions in the transformed algal species indicates that the plasmid had been integrated into the microalgal strains through *A. tumifaciens*. The expected sizes of the amplified GUS intron and CaMV35S promoter regions are 190 and 800 bp, respectively. The predicted PCR products were obtained in *Ankistrodesmus* sp., *Chlorella* sp., *S. bajacalifornicus* and positive control *C. reinhardtii* CC-124 after transformation (data not shown).

Agrobacterium tumifaciens contamination in the transformed colonies was analyzed by PCR amplification of the *npt II* gene fragment. This kanamycin resistance gene (*npt II*) is commonly used as a bacte-

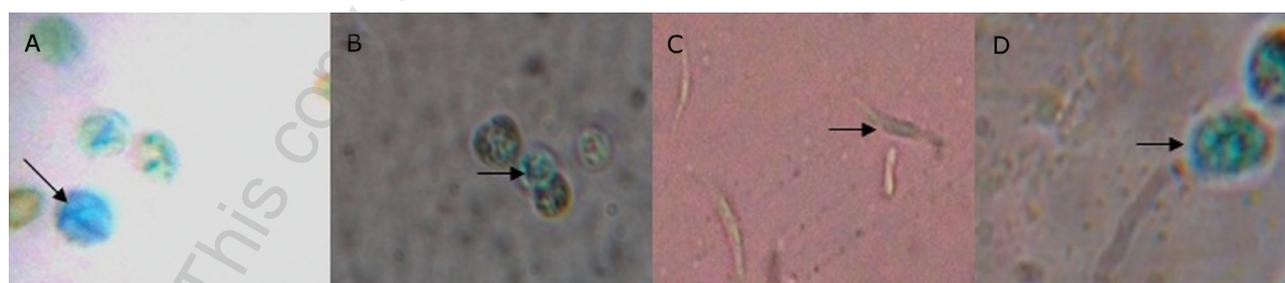


Fig. 3. Microscopic observation (100X) of GUS-positive transformed cells. Arrow indicates the blue coloured cell demonstrating the transfer of pCAMBIA into (a) *S. bajacalifornicus* (b) *Chlorella* sp. (c) *Ankistrodesmus* sp., and (d) *C. reinhardtii* CC-124

rial selection marker. Therefore, if there was any bacterial contamination, amplification would have been obtained at 800 bp. No amplification for *npt II* gene for all transformed microalgal strains was detected, which indicates that there is no *A. tumefaciens* contamination in the transformed colonies.

GUS histochemical assay. GUS histochemical assay was performed to check the expression of GUS in transformed microalgal cells. This assay was performed after hygromycin screening for all the isolated species. GUS gene (β -glucuronidase) commonly utilized as a “marker gene” for plant genetic transformation. β -glucuronidase in the cells reacts with the substrate (X-gluc) 5-bromo-4-chloro-3-indolyl glucouronide. This reaction makes the cell to appear in blue colour (Jefferson *et al.*, 1987). Figure 3 (a, b, c and d), shows the microscopic view of the transformed *S. bajacalifornicus*, *Chlorella* sp., *Ankistrodesmus* sp. and *C. reinhardtii* CC-124. The blue coloured cells were recorded as transformed cells whereas the non transformed cells showed no blue color. Based on the GUS assay, the transformation efficiency of three microalgal species were found to be, *Chlorella* sp. (12.25%), *S. bajacalifornicus* (2.96%) and *Ankistrodesmus* sp. (3.5%).

In conclusion, *Agrobacterium*-mediated gene transfer is a commonly used technique for plants and it is a less expensive and easy method. Recent studies suggested that, *Agrobacterium*-mediated gene transfer is possible for microalgal species. The current study will provide a platform for further development in biofuel production and other applications of microalgae. In the present study, three freshwater microalgal species *Ankistrodesmus* sp., *Chlorella* sp. and *S. bajacalifornicus* were isolated from freshwater samples and subjected for *A. tumefaciens* mediated transformation, using pCAMBIA 1301. The plasmid integration was detected by PCR amplification for GUS intron gene and CaMV35S promoter region. GUS histochemical assay was performed to check the expression of GUS gene in transformed microalgal strains to determine the transformation efficiencies. This technique has to be further exploited for the genetic improvement of many other microalgal species.

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