Molecular cloning and expression of TB antigen protein in microalga 
Chlamydomonas reinhardtii

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Microalgae based therapeutics has had great success over the last few years. Although biotechnological processes based on transgenic microalgae are still in its infancy, researchers and companies are considering their high potential as bioreactors for drug development. In the present study, an efficient and reproducible protocol for Agrobacterium tumefaciens-mediated genetic transformation and regeneration of microalga Chlamydomonas reinhardtii with esxH gene of Mycobacterium tuberculosis H37Rv under the control of CaMV 35S promoter has been developed. C. reinhardtii strain CC-125 was transformed with A. tumefaciens strain LBA 4404, harbouring the binary vector pCAMBIA 1304 containing the sequence coding for hygromycin phosphotransferase (hpt) as the selectable marker gene, β-glucuronidase (GUS) as the reporter gene and the sequence encoding 10 kDa T-cell antigen (esxH) of M. tuberculosis. The transformation event was confirmed by PCR amplification with hpt, GUS and esxH gene-specific primers. Expression of esxH gene in transgenic Chlamydomonas was confirmed through RT-PCR. In future, this microalgal expression system can be used to meet the ever growing need for therapeutic proteins by the pharmaceutical industries.

Keywords: Agrobacterium tumefaciens, Chlamydomonas reinhardtii, esxH gene, expression system, TB antigen

Introduction

Chlamydomonas reinhardtii is a unicellular eukaryotic green alga that has been used as a powerful model system for understanding several biological processes in eukaryotes at the molecular level¹. One of the recent technological advances that helped to establish C. reinhardtii as a model photosynthetic organism has been the development of a transformation system for the stable introduction of DNA into the nuclear genome². Different methods have been developed for the nuclear transformation of Chlamydomonas, which include particle bombardment³,⁴, electroporation⁵, agitation with glass beads⁶ or silicon carbide whiskers⁷. Agrobacterium-mediated genetic transformation of C. reinhardtii was reported by Kumar et al⁸, which is a simple, stable and economical transformation system.

Chlamydomonas as an expression system has several advantages, which include reduced length of time required from the initial transformation event to having usable quantity of a protein, ease of scaling up process and growth in containment area, reducing any concern about environmental contamination⁹,¹⁰. The algal systems have the ability to perform post-transcriptional and post-translational modifications effectively¹¹. Moreover, because algae do not have the pollen, there are no potential for the introduction of transgenes into food crops, as potentially could occur in higher plants by gene flow (via pollen) to surrounding plants¹². Additional benefits include the ability to produce secreted proteins and the availability of a wide variety of well characterized chloroplast and nuclear promoter elements¹³,¹⁴. Besides the fact that most green algae are “generally regarded as safe” (GRAS), they can be cultivated in full containment reducing any concern about environmental contamination. These characteristics make the algal systems an attractive approach for the expression of recombinant proteins¹⁵.

Despite the development in health sciences, Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), remains a major health threat. Each year, ~8 million new TB cases are reported and 2 million individuals die of TB¹⁶. Moreover, it is estimated that one third of the population is latently infected with M. tuberculosis, of which ~10% will develop active disease during lifetime¹⁷. The

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immunonodominant T-cell antigen expressed by *esxH* gene is of great importance for the development of an efficient vaccine against TB\(^{18,19}\). Even though different transgenic proteins have been expressed in microalgae\(^{20}\), expression of therapeutic proteins via *Agrobacterium*-mediated transformation is not reported till date. Present study describes *A. tumefaciens*-mediated genetic transformation of *C. reinhardtii* with the gene encoding T-cell antigen of *M. tuberculosis* under the control of CaMV 35S promoter. The gene coding for T-cell antigen protein (*esxH*) was selected for the genetic transformation with the objective of developing transgenic microalga, which can be used for the large scale production of anti-tuberculosis vaccine.

**Materials and Methods**

**Alga and Culture Conditions**

*C. reinhardtii* strain CC-125 was obtained from Chlamydomonas Centre, Australia. The cells were raised in Tris acetate phosphate (TAP) medium (pH 7) at 24±1°C under light/dark (16/8 h) period with mild shaking (80 rpm)\(^{21}\). The cells were then plated on to TAP agar (1.5% w/v) medium in 90 mm Petri plates and incubated in light for 2 d to allow a lawn of cells to be formed and these were used for *Agrobacterium* co-culture.

**Plasmid Construct and Agrobacterium Strains**

The binary vector pCAMBIA-1304 (M/s CAMBIA, Australia), which harbors *hpt* (hygromycin phosphotransferase) as marker gene, GUS (β-glucuronidase) as reporter gene and *M. tuberculosis* *esxH* gene driven by CaMV 35S promoter was used for the study. Genomic DNA from *M. tuberculosis* was isolated using *M. tuberculosis* (MTB) Genomic DNA Isolation Kit (M/s PREMAS Biotech Pvt. Ltd. India). The 291 bp *esxH* gene was obtained by PCR amplification of *M. tuberculosis* genomic DNA using primer sequences corresponding to the previously published sequence of *esxH* gene\(^{22}\). The *esxH* gene was ligated to the binary vector pCAMBIA 1304 to develop a functional vector for transformation experiments (Fig. 1). The binary vector was transferred to *Agrobacterium* strain LBA 4404 through freeze thaw method.

*Agrobacterium* was inoculated into LB broth containing rifampicin (30 µg/mL) and kanamycin (50 µg/mL) and incubated at 28°C in a rotary shaker at 220 rpm. The actively growing bacteria were collected by centrifugation at 5000 rpm for 5 min. The pellet was resuspended in TAP medium containing 100 µM acetosyringone. The bacterial density of the culture was adjusted to 5×10\(^8\) cells/mL\(^{23}\). The bacterial suspension was spread to the thin layer of Chlamydomonas culture growing on agar plate. Plates were incubated for 2 d at 25°C (co-cultivation). After 2 d, cells were harvested and washed twice with liquid TAP medium containing 500 mg/L cefotaxime via resuspension by mild vortexing and centrifugation at 100 × g for 2 min. The washed Chlamydomonas cells were plated on solid TAP agar plates containing 500 mg/L cefotaxime to eliminate the overgrowth of *Agrobacterium* and 10 mg/L hygromycin for the selection of transformed cells\(^{24}\). Four subcultures were made at 2 wk intervals for the elimination of escapes.

**Detection of Reporter Gene Expression**

The hygromycin resistant colonies were analyzed for the expression of GUS gene. Transgenic Chlamydomonas colonies were collected and resuspended in X-gluc solution and incubated at 37°C overnight. After incubation time, cells were pelleted, bleached with ethanol and analyzed under microscope\(^{25}\).

**DNA Isolation**

DNA was extracted from transgenic as well as control Chlamydomonas cells following modified CTAB method\(^{26}\). Cells from 100 mL culture (1×10\(^6\) cells/mL) was pelleted and resuspended in 0.5 mL of CTAB buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8, 2% CTAB) and incubated at 65°C for 30 min. An equal volume of phenol, chloroform and isoamylalcohol (25:24:1) was immediately added, mixed by inversion and centrifuged for 5 min at 14000 rpm. The aqueous layer was transferred to a new tube, equal volume of chloroform was added and centrifuged for 5 min at 14000 rpm. The upper layer was collected and DNA was precipitated by the addition of 0.6 volumes of isopropanol. Precipitated DNA was spooled out, washed with 70% ethanol, air dried and resuspended in water.

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Fig. 1—Modified pCAMBIA 1304 vector showing *hpt*, *esxH*, GFP and GUS genes inside the T-DNA borders.
PCR Amplification

PCR amplification was done for the confirmation of transformation (M/s Eppendorf, Germany). The detection of hpt gene was done using 5′-AGCTGCGCCGATGGTTTCTACAA-3′ forward and 5′-ATCGCCTCGCCTCCAGTCAATG-3′ reverse primers, flanking hpt coding region of the plasmid vector. The transgene (exsH) was amplified with exsH cDNA specific primers 5′-TATGGGCCGGATATGCCGGCA-3′ forward and 5′-CGAAGCCGCCAAATGGGGCG-3′ reverse and GUS gene amplification was done using GUS specific 5′-CTGTAGAAACCCCCACACG-3′ forward and 5′-TGCAGCGCTACCTAAGGCCG-3′ reverse primers (Sigma-Aldrich, Bangalore). The condition for PCR reaction was: initial denaturation at 94°C for 4 min, followed by 36 cycles of 94°C for 1 min, 55°C for 1min and 72°C for 1min. The final extension was followed by 36 cycles of 94°C for 1 min, 55°C for 1min and 72°C for 1min. The condition for PCR reaction was: initial denaturation at 94°C for 4 min, followed by 36 cycles of 94°C for 1 min, 55°C for 1min and 72°C for 1min. The final extension was followed by 36 cycles of 94°C for 1 min, 55°C for 1min and 72°C for 1min.

DNA Isolation

RNA Isolation

Method used for RNA isolation was essentially the same as described in the Chlamydomonas Sourcebook27. 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 µg/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. Both 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 using a vacuum desiccator for 10-15 min. 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, the RNA preparation (500 ng/µL) was subsequently incubated with RNase-free DNase (2 U/µg) (M/s NEB, England) for 15 min at 37°C. The quantity of RNA obtained was determined spectrophotometrically.

cDNA Synthesis

Synthesis of cDNA was carried out using ImpromII™ reverse transcriptase kit (M/s Promega, USA) following the manufacturer’s instructions. First strand cDNA synthesis was performed by reverse transcription with 1 µg of total RNA using oligo-(dT) 12-18-mer primer and ImpromII RT enzyme. 1 µg total RNA was mixed with 1 µL oligo (dT) (0.5 µg) 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: 1× first strand buffer [containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl], 3 mM MgCl₂, 1 U RNasin and 500 µM 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 µL ImpromII™ 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 µL (2 units) of Escherichia coli RNase H was added to the RT mix and incubated at 37°C for 20 min to remove RNA complementary to the cDNA.

Expression of exsH Gene in Transgenic Chlamydomonas

Expression of exsH gene in transgenic Chlamydomonas was confirmed through RT-PCR synthesis. Total cDNA served as the template in the PCR amplification of exsH cDNA using specific primers. PCR amplification was performed. The reaction was carried out in 20 µL reaction volume, which was composed of 1 buffer, 50 mM KCl, 1.5 mM MgCl₂, 100 mM dNTPs, 0.5 U of Taq DNA polymerase, 2 µL of the first strand cDNA mixture and 250 nM of each primer (exsH gene specific primers mentioned earlier).

Results and Discussion

The current vaccine against TB, introduced over 80 years ago, is the live attenuated bacterium M. bovis bacillus Calmette-Guerin (BCG), designed as a prophylactic vaccine for pre-infection administration. BCG is known to protect young children against
severe forms of TB. However, it does not efficiently and consistently protect adults against the most prevalent form of the disease, nor does BCG offer protection from establishment of latent TB and subsequent reactivation\textsuperscript{28,29}. Thus an important criterion in seeking protective antigens should be that they induce T-cell mediated immunity. For many years, great efforts have been made to develop a new vaccine against tuberculosis. 10 kDa antigen, which is shared by \textit{M. tuberculosis} and \textit{M. bovis} BCG, stimulates T-cell mediated immunity\textsuperscript{30} and is recognized by sera from tuberculosis patients.\textsuperscript{31} The gene encoding low mol wt (10 kDa) antigen protein has been cloned and characterized by Baird \textit{et al}\textsuperscript{31}. Earlier, the 10 kDa antigen protein was expressed in \textit{E. coli} expression system\textsuperscript{32,33}. However, there are distinct disadvantages in producing transgenic protein in \textit{E. coli} as it may be contaminated by endotoxin, which causes an adverse reaction in patients\textsuperscript{34}. In the present study, a more efficient microalgal expression system was used for the expression of 10 kDa antigen.

\textit{C. reinhardtii} strain CC-125 was cultured in TAP medium and was used for transformation procedure (Fig. 2a). The \textit{esxH} gene was successfully cloned into the binary vector pCAMBIA 1304 and the functional vector was introduced to \textit{Agrobacterium}. \textit{Chlamydomonas} was co-cultivated with \textit{Agrobacterium} (Fig. 2b), harvested after 2 d and plated onto the selection medium with cefotaxime (500 mg/L) and hygromycin (10 mg/L). Transformed colonies appeared after 1 wk of selection (Fig. 2c). The trasformants were analyzed for the presence of GUS activity. GUS positive transgenic \textit{Chlamydomonas} cells showed strong indigo blue colour after incubation with X-gluc (Fig. 2d). GUS positive colonies were routinely sub-cultured in TAP agar and after 4 wk sub-culture, genomic DNA was isolated. The total DNA isolated from transgenic as well as control \textit{Chlamydomonas} colonies was employed to examine the presence of transgene by PCR analysis. Specific primers for \textit{hpt}, GUS and \textit{esxH} genes were used for the PCR analysis to amplify the DNA fragments of 500 bp \textit{hpt} gene, 700 bp GUS gene (Fig. 3a) and 230 bp \textit{esxH} gene (Fig. 3b) from \textit{Chlamydomonas} transformants; while control DNA did not show any amplification in all the three cases. PCR amplification of transgenes from the genomic DNA confirmed the transformation status of \textit{Chlamydomonas}.

To confirm the expression of \textit{esxH} gene in transgenic \textit{Chlamydomonas}, RT-PCR analysis was done. The isolated cDNA was used as the template for RT-PCR. Using specific primers, a 230 bp fragment of \textit{esxH} cDNA was successfully amplified at 55°C annealing temperature (Fig. 4). The preliminary results presented here are promising, but it would be useful to elucidate the expression pattern of \textit{esxH} gene in \textit{Chlamydomonas}, which will provide the basis for the large scale expression of the T-cell antigen protein in \textit{Chlamydomonas}.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{C. reinhardtii under pure culture, co-cultivation, screening and GUS expression: a. Untransformed \textit{C. reinhardtii} on TAP agar plate; b. \textit{C. reinhardtii} and \textit{Agrobacterium} co-cultivation; c. Transgenic \textit{C. reinhardtii} cells on hygromycin selection medium; d. Microscopic view of \textit{C. reinhardtii} showing GUS expression.}
\end{figure}

\begin{figure}[h]
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\caption{a. \textit{hpt} and GUS genes [M. Mol wt marker (\lambda-DNA, EcoRI, HindIII double digest); \textit{hpt}, 500 bp amplicon of \textit{hpt} gene from transgenic DNA; C, Control; GUS, 700 bp amplicon of GUS gene from transgenic DNA; C, Control]; b. \textit{esxH} gene [\textit{esxH}, 230 bp amplicon of \textit{esxH} gene from transgenic DNA; M, Mol wt marker (50 bp ladder); C, control].}
\end{figure}
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References