Biocompounds

www.bic.reapress.com

Biocompd. Vol. 1, No. 1 (2024) 63–73.

Paper Type: Original Article

A New Trend for the Highly Efficient Transformation of the Microalga Chlorella Vulgaris by Agrobacterium Tumefaciens

Somayeh Asghari*

Department of Environmental Health Engineering, Faculty of Health, Health Sciences Research Center, Mazandaran University of Medical Sciences, Sari, Iran; som.asghari28@yahoo.com.

Citation:

Received: 27 September 2023 *Revised: 09 November 2023* *Accepted: 10 February 2024*	Asghari, S. (2024). A new trend for the highly efficient transformation of the microalga Chlorella Vulgaris by Agrobacterium tumefaciens. *Biocompounds, 1*(1), 63-73.

Abstract

The microscopic green microalga Chlorella vulgaris is an important member of the photosynthetic microorganisms, producing a wide range of natural, high-value compounds. Recently, C. Vulgaris has gained great attention as a green bioreactor for the production of valuable biologicals ranging from therapeutic proteins to biofuels. Because of the lack of robust, efficient and low-cost transformation techniques, the significant potential of this expression system is often limited. Agrobacterium-mediated genetic transformation, one of the most efficient methods, can be an ideal solution for microalgae genetic engineering. The transformation of C. Vulgaris was evaluated using A. tumifaciens strain EHA101, carrying the pCAMBIA1304 binary vector. Integration and expression of mgfp:uidA and hptII genes in the transformed cells were determined by Polymerase Chain Reaction (PCR) and Reverse Transcription Polymerase Chain Reaction (RT-PCR), FACS flow cytometry, and β-Glucuronidase (GUS) activity assays. PCR data confirmed the successful integration of the mgfp and hptII genes into the C. Vulgaris genome. RT-PCR analysis showed that the transcripts of the genes were present in the transcriptome of the transformants. The expression of the mgfp and uidA genes was successfully evaluated through the FL-1 channel in the facs flow cytometry and GUS activity assays. The strain EHA101 of A. tumifaciens could be used as a more ideal platform for the transformation of Chlorella in order to achieve high-efficiency production of valuable recombinant proteins.

Keywords: Agrobacterium-mediated transformation, Chlorella vulgaris, Gene expression, Microalgae, Recombinant protein.

1 | Introduction

The planktonic unicellular green microalga Chlorella Vulgaris has noticeable potential to produce vital compounds such as amino acids, proteins, lipids, carbohydrates, pigments, vitamins, and minerals [1]. These compounds are highly required in the human food, animal feed, aquaculture, and pharmaceutical industries [2], [3]. Besides its use as a food source or dietary supplement, Chlorella has significant potential as a recombinant protein expression system to revolutionize biotechnology due to its many advantages. The

Corresponding Author: som.asghari28@yahoo.com

https://doi.org/10.48313/bic.vi.74

growth rate of Chlorella is very high, and it can be inexpensively, easily, and rapidly cultured on a large scale. Furthermore, Chlorella is considered Generally Recognized As Safe (GRAS) for humans by the U.S. Food and Drug Administration (FDA), and its recombinant proteins could be used without costly purification steps [4], [5]. Importantly, as a eukaryotic microalga, Chlorella is capable to perform the post-translational modifications on the expressed proteins [6]. In fact, Chlorella is exploited as a promising platform for the large-scale production of foreign proteins owing to its inherent characteristics.

Up to now, the different types of genetic transformation methods such as particle bombardment (biolistic) [7], electroporation [8], agitation in the presence of glass beads and Polyethylene Glycol (PEG) [9], and Agrobacterium-mediated genetic transformation [10], [11] have been used to deliver the specific genetic sequences into the nuclear genome of Chlorella. In this context, Agrobacterium-mediated transformation is considered a more ideal system for Deoxyribonucleic Acid (DNA) delivery. Relatively high rate of transformation, stable integration of DNA pieces with minimal rearrangement, introduction of lower transgene copy number into the host chromosomes, the transfer of relatively large fragments of DNA, the integration patterns of more predictable transgenes, and its requirement for simple and inexpensive equipment [10], [12] are the major advantages of Agrobacterium-based transformation.

Agrobacterium tumefaciens, the gram-negative soil pathogen, induces crown gall tumors in many plants. Indeed, this bacterium can transfer the Transfer-DNA (T-DNA) fragment from its Ti plasmid (tumour-inducing plasmid) to the host genome and cause the disease [13], [14]. Accordingly, modified Agrobacterium strains are generated with alterations in the Ti plasmid by replacing the genes that induce tumor formation with any gene of interest. More recently, Agrobacterium-mediated transformation has been reported in several non-plant organisms such as fungi, HeLa cells, and microalgae [15–17]. To date, A. tumefaciens-mediated transformations have been successfully reported for a few species of microalgae such as Chlamydomonas reinhardtii, Dunaliella pseudosalina, and Spirulina platensis [4], [5], [18].

Chlorella is presented as a robust candidate bioreactor for the production of different types of pharmaceutical and industrial biologics [7], [9], [19], [20]. However, there is little information about the ability of various A. tumifaciens strains to transform C. Vulgaris. Therefore, the purpose of this study was to evaluate the efficacy of strain EHA101 of A. tumifaciens in the transformation of the green microalga C. Vulgaris. Our data showed that the strain of A. tumifaciens can efficiently and stably transfer the mgfp, uidA, and hptII genes into the genome of C. Vulgaris.

2 | Materials and Methods

2.1 | Microalgae and Culture Conditions

C. Vulgaris was supplied by the Culture Collection of Algae from Bushehr Shrimp Research Institute, Iran. The axenic culture of the microalga was maintained in BG11medium $NaNO_3$ (1.5 g/L), K_2HPO_4 (4.0 g/L), $MgSO_4.7H_2O$ (7.5 g/L), $CaCl_2.2H_2O$ (3.6 g/L), $C_6H_8O_7$ (0.6 g/L), $(NH_4)_5[Fe(C_6H_4O_7)_2$ (0. 6 g/L), $EDTANa_2$ (0.1 g/L), Na_2CO_3 (2.0 g/L), H_3BO_3 (2.86 g/L), $MnCl_2.4H_2O$ (1.81 g/L), $ZnSO_4.7H_2O$ (0.22 g/L), $Na_2MoO_4.2H_2O$ (0.39 g/L), $CuSO_4.5H_2O$ (0.08 g/L), $Co(NO_3)$ $2.6H_2O$ (0.05 g/L) [21]. Subsequently, the alga was incubated at 25 °C under a 16:8 h light: dark cycle with daily manual agitation.

2.2 | Bacterial Strain and Vector Construct

To transform C. Vulgaris cells, strain EHA101 (Nopaline type, pTiBo542DT-DNA plasmid) of A. tumefaciens was obtained from the Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari, Iran. The binary vector pCAMBIA1304 was used to transform the strain EHA101 by the freeze-thaw method [22]. This vector contains the hygromycin phosphotransferase (hptII) gene as an antibiotic marker gene for the selection of transformed cells, as well as the mgfp:gus fusion as a reporter gene. Both genes were under the control of the constitutive CaMV35S (Cauliflower Mosaic Virus) promoter. In addition, the plasmid carries the nptII gene (kanamycin-resistance gene) as a selection marker for the selection of the transformed

bacteria (*Fig. 1*). The strain DH5α of Escherichia coli was employed for the maintenance and propagation of the plasmid construct. Both strains were kept on solid LB medium containing 50 mg/L kanamycin.

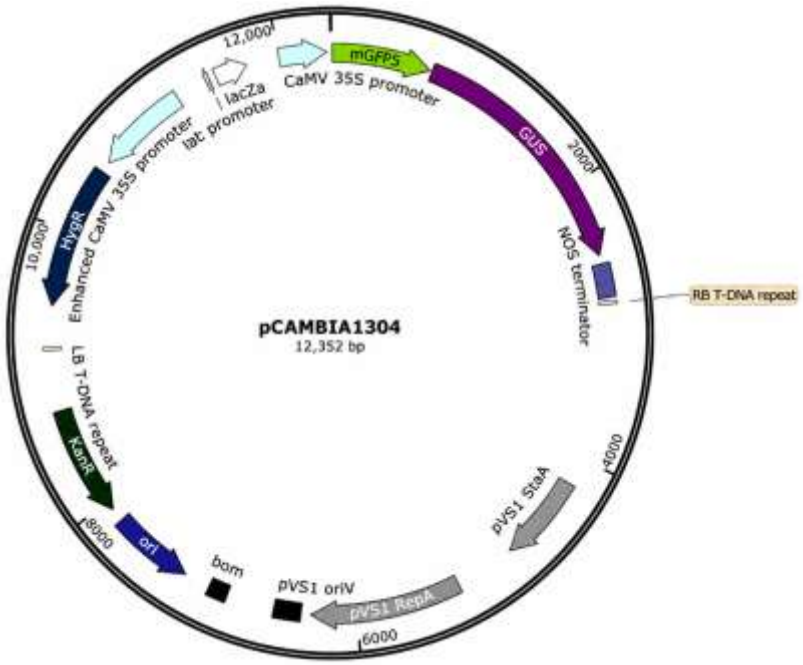


Fig. 1. The schematic map and some features of the pCAMBIA1304 plasmid.

2.3 | Sensitivity of C. Vulgaris to Hygromycin

Approximately 1.5×10^6 cells per mL of C. Vulgaris (OD_{600}: 0.6) were cultivated on the solid BG11 medium containing various concentrations of hygromycin (10–50 mg/L). The BG11 agar plates without antibiotic were used as the control sample. These experiments were done in three biological replicates. The agar plates were incubated under standard growth conditions for C. Vulgaris for two weeks.

2.4 | Agrobacterium-Mediated Transformation Procedures

Transformation procedures of C. vulgaris were performed using the method proposed by Kumar [18] with some modifications. About 150 µL of algal suspension in the exponential growth phase (OD_{600} = 0.6) was transferred to the BG11 agar plates and incubated under standard growth conditions for 5 days to form an algal lawn. The EHA101 strain of Agrobacterium was grown in LB solid medium containing appropriate antibiotics (25 mg/L rifampicin and 50 mg/L kanamycin) at 28˚C for 48 h. Subsequently, a single colony of EHA101 was inoculated into liquid LB medium supplemented with the mentioned antibiotics and then incubated at 28˚C overnight at 200 rpm in the dark (OD_{600}= 1.0). The cells were collected by centrifugation at 5000 rpm for 5 min. After discarding the supernatant, the bacterial pellets were resuspended in liquid BG11medium. Subsequently, two hundred microlitres of the bacterial suspension were spread over the thin algal lawn formed on BG-11 solid medium. After 3 days of incubation at 25˚C, cells were collected by centrifugation at 1000 rpm for 2 min and washed twice with BG-11 medium containing 500 mg/L cefotaxime to remove the bacterial residues. Finally, the Chlorella cells were spread onto BG11 agar plates containing 50 mg/L hygromycin and 500 mg/L cefotaxime. The individual transformed colonies that appeared in the selection medium after 2 weeks were added to the liquid BG11 medium and were used for downstream process analyses [5].

2.5 | Analysis of Transfer-Deoxyribonucleic Acid Integration

The algal samples in the late log phase of growth were centrifuged at 3000 rpm for 5 min, and then genomic DNA was extracted by Dawson et al. [7]. Subsequently, Polymerase Chain Reaction (PCR) analyses were used to confirm the integration of mgfp and hptII genes and the absence of the nptII gene in the C. Vulgaris

genome using specific primers (*Table 1*) [5]. Primers for PCR were designed by the Oligo version 7 software and synthesized by Takapo Zist Company, Tehran, Iran. The cycling conditions comprised 5 min strand separation at 94°C, 2 min annealing at 53°C, and 1 min extension at 72°C for 32 successive cycles, followed by a final extension step at 72°C for 5 min.

Table 1. The sequence of primers used.

Primers	Forward Sequence	Reverse Sequence
hptII	5'-GCGTCGGTTTCCACTATCG-3'	5'-ATGAAAAAGCCTGAACTCAC-3'
mgfp	5'-ATGGTAGATCTGACTAGTAAAGG-3'	5'-TCATCCATGCCATGTGTAATC-3'
nptII	5'-CCA ATC AGG CTT GAT CCC CAG-3'	5'-GAT ACG GAA GGA ATG TCT CCT GC-3'

2.6 | Ribonucleic Acid Extraction and Reverse Transcription Polymerase Chain Reaction Analysis

Total Ribonucleic Acid (RNA) was extracted from the transgenic and non-transgenic lines of Chlorella using TRIzol® reagent according to the manufacturer's instructions. After extraction of total RNA, the quality and concentration of the isolated RNA were analyzed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Subsequently, cDNA synthesis was performed using total extracted RNA, reverse transcriptase, and random hexamer primers according to the manufacturer's protocol (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, USA). The expression of hptII and mgfp genes in transgenic Chlorella cells was checked through Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis. About 2 µl of the total synthesized cDNA was used as the template in PCR amplification. The PCR profile was the same as the described "PCR analysis" [5].

2.7 | β-Glucuronidase Activity Experiment

The activity of β-Glucuronidase (GUS), encoded by the uidA gene, was evaluated by using the 5-Bromo-4-chloro-3-indolyl beta-D-glucuronide (Sigma-Aldrich, St. Louis, Missouri, USA) as its substrate. To eliminate chlorophyll, the microalgae cells were kept in 95% ethanol for 8 hours. Further, the microalgal cells were collected by centrifugation, washed, and resuspended in 450 µL of GUS assay buffer. These reactions were performed for ~24 hours at 37 °C [4].

2.8 | Flow Cytometry Analysis

The transformed and untransformed microalgal cells were centrifuged at 3000 rpm for 3 min and washed twice with Phosphate-Buffered Saline (PBS) buffer. Afterward, the emission of the mGFP gene was detected in the FL1 channel and the Fluorescein Isothiocyanate (FITC) spectrum range by a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) [4].

3 | Results

3.1 | Achievement of Transformed Cells

Our data showed that 50 µg/mL of hygromycin could inhibit the growth and proliferation of the C. Vulgaris cells and, accordingly, such a concentration of the antibiotic was used for the selection of the transformants (*Fig. 2*). After about seven days, the hygromycin-resistant colonies were apparent in the solidified BG-11 medium. Our microscopic observations showed that the EHA101 strain of A. tumefaciens could transform the microalgae cells with high efficacy (*Fig. 3*).

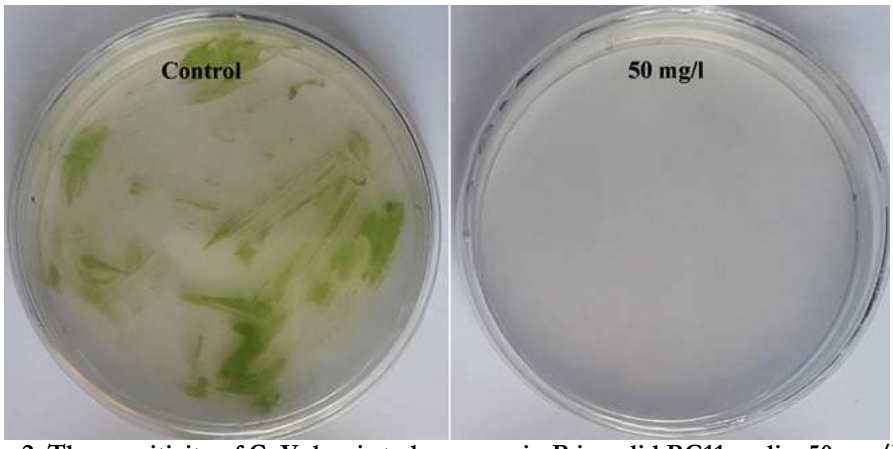


Fig. 2. The sensitivity of C. Vulgaris to hygromycin B in solid BG11media. 50 mg/L of hygromycin could be used for the selection of transformed microalgae cells.

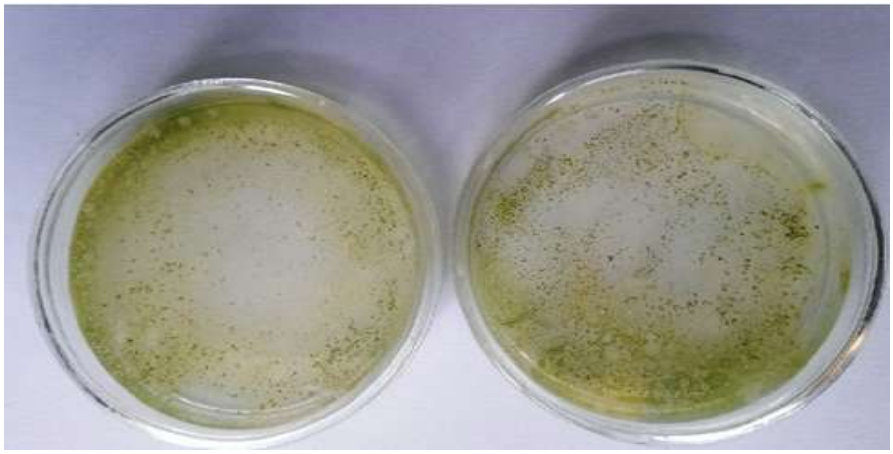
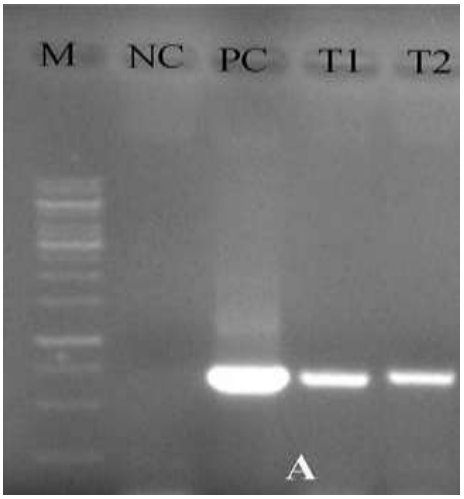


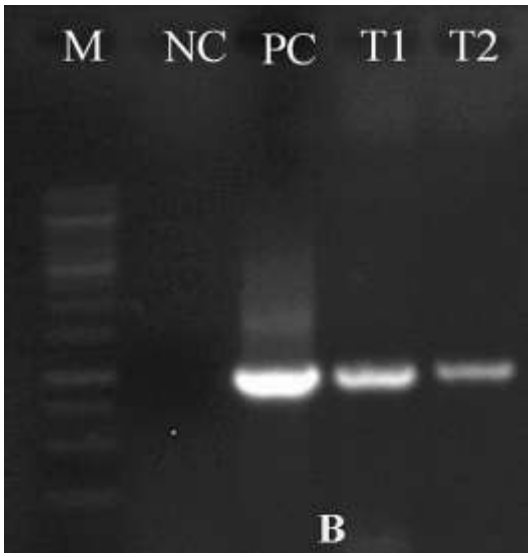
Fig. 3. Hygromycin-resistant colonies that appeared within about seven days on the selection medium.

3.2|Integration of the Transfer-Deoxyribonucleic Acid into the C. Vulgaris Genome

Hygromycin-resistant colonies of Chlorella were selected randomly and grown in the liquid BG11 medium. PCR analyses from the genomic DNA of the transformed and untransformed Chlorella cell lines using the specific primer pairs for the mgfp and hptII genes, respectively, verified the presence of ~ 680 bp and ~ 1.0 kb amplicons in the transgenic cells. In comparison, untransformed cells did not show any PCR band (*Fig. 4*).



a.

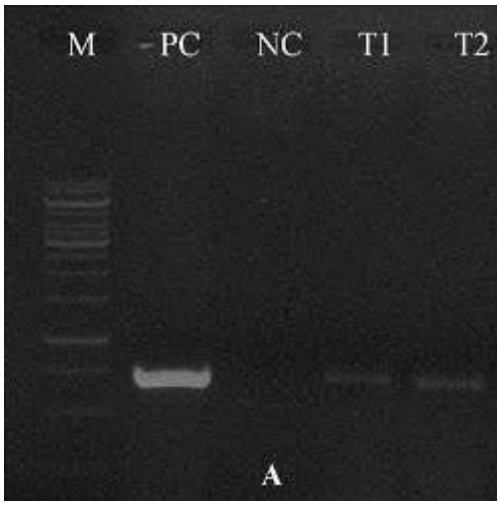


b.

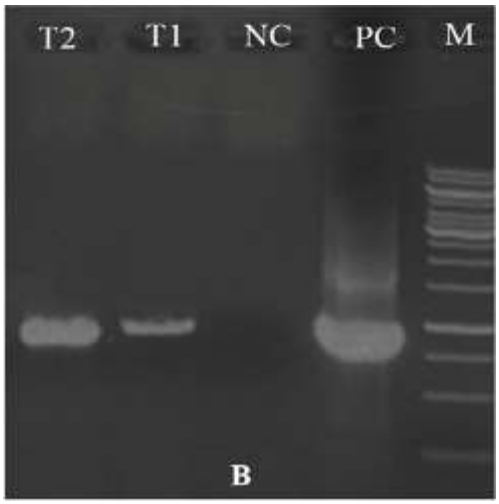
Fig. 4. DNA analyses of the transformed C. Vulgaris colonies: a. PCR amplification using mgfp-specific primers, and b. PCR amplification using hptII-specific primers. M: 1-kb DNA ladder; PC: pCAMBIA1304 plasmid used as the positive control; NC: untransformed microalgal cells used as the negative control; T1 and T2: transformed cells.

3.3 | Identification of the Transfer-Deoxyribonucleic Acid Transcripts in the C. Vulgaris Transcriptome

RT-PCR reaction was performed to determine the presence of mgfp and hptII transcripts in the Chlorella transgenic cells. The cDNA transcripts encoding hptII and mgfp genes were amplified by PCR using the specific oligonucleotide primers. As shown in *Fig. 5*, a band of ~ 680 bp (mgfp) and ~ 1.0 kb (hptII) was observed in all transgenic cell lines.



a.



b.

Fig. 5. Transcriptome analysis of the transformed C. Vulgaris colonies: a. RT-PCR-amplified ~680 bp amplicon of the mgfp gene from transgenic Chlorella colonies, and b. RT-PCR-amplified ~1.0 Kb amplicon of the hptII gene from the transgenic Chlorella lines. M: 1kb ladder; PC: pCAMBIA1304 plasmid is used as positive control; NC: untransformed microalga cells are used as negative control; T1, T2: transformed cells.

3.4 | Gene Expression Analyses

3.4.1 | Gus activity assay

Regarding the presence of the uidA gene in the T-DNA part of the pCAMBIA1304 plasmid, the biological activity of the GUS enzyme was confirmed using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide as its substrate. Our data revealed that the transformed Chlorella cells showed GUS activity, while the untransformed colonies were colorless (*Fig. 6*).

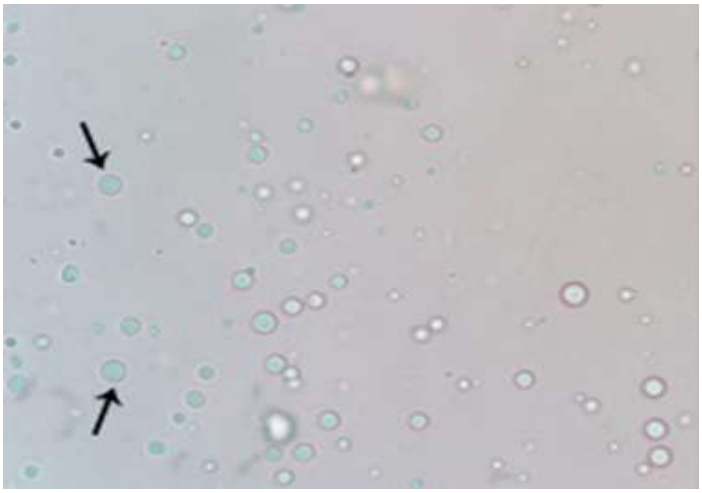


Fig. 6. Evaluating the biological activity of GUS by GUS assay. Histochemical microscopic analyses showed that the GUS enzyme is produced in the transformed Chlorella cells (arrows), while untransformed colonies remain colorless.

3.4.2 | Green fluorescent protein detection assessment

The successful expression of the Green Fluorescent Protein (GFP) gene was identified through the FL1 channel and FITC spectrum range of flow cytometry in the transformants (*Fig. 7*). To eliminate the autofluorescence of chlorophyll, the microalgae were resuspended in absolute ethanol and acetone (9:1).

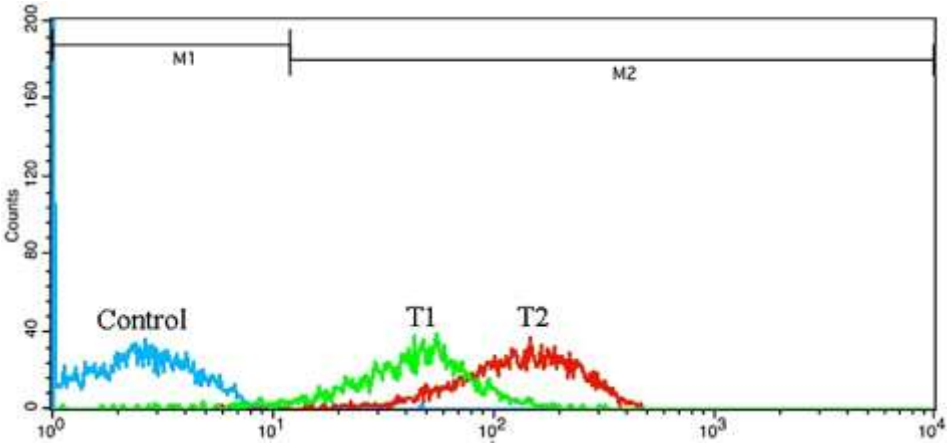


Fig. 7. Detection of GFP protein through flow cytometry analysis. Regarding the excitation (395 nm) and emission (475 nm) wavelengths of GFP, the presence of the expressed protein is identified with a flow cytometry instrument in the transformed Chlorella colonies. T1, T2: transformed cells.

3.4.3 | Evaluating the A. tumefaciens contamination in the transformants

In order to evaluate the possible contamination of Agrobacterium, a PCR reaction was done by specific primers of nptII gene, which is located outside the T-DNA part of the pCAMBIA1304 plasmid. The analysis showed no amplicon for this gene in the transformed cell lines of C. Vulgaris, while a single corresponding band (~600 bp) was obtained on the electrophoresis gel in the positive control (i.e., pCAMBIA1304 plasmid), which confirmed the absence of A. tumefaciens in the transformants (*Fig. 8*).

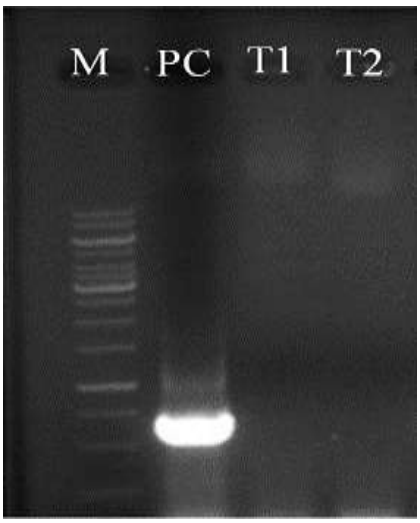


Fig. 8. Screening for A. tumefaciens contamination in transformants. PCR analysis of the transformants using nptII gene-specific primers; M: 1kb ladder; PC: purified pCAMBIA1304 plasmid was used as a positive control; T1 and T2: transformed cells.

4 | Discussion

To date, several approaches have been employed for the transformation of different species of the eukaryotic microalgae, including particle bombardment, electroporation, and agitation with glass beads with some inherent advantages and disadvantages for each of them [10], [11], [23]. Among these methods, glass bead

agitation and electroporation methods required the cell wall-lacking microalgae for significant transformation, while particle bombardment could be done with intact microalgae [5], [24]. Additionally, the high mortality rate of cells after exposure to electric pulses with high voltage is considered one of the major limitations of the electroporation method [24]. The main drawback of the particle bombardment technique is the high cost of transformation compared to other methods as well [23], [24]. Because of such difficulties, Agrobacterium-mediated genetic transformation is introduced as a more suitable platform with high transformation and expression efficacy to transform the microalgae [5], [25]. Accordingly, the successful transformation of Chlorella and some other microalgae using A. tumefaciens strain LBA4404 has been previously reported [10], [11], [18], [26–28]. Also, the efficacy of different strains of A. tumefaciens, including LBA4404, EHA101, and EHA105, was evaluated in the genetic transformation of the microalga Chlamydomonas reinhardtii. The results showed that strain EHA105 of Agrobacterium was more competent, with a higher transformation frequency compared to the two mentioned strains [29]. Furthermore, the strains LBA4404 and EHA105 of A. tumefaciens harboring the pCAMBIA 2301 vector were employed for the transformation of the marine microalga Schizochytrium.29 Integration and expression of egfp and gus genes were verified by PCR reaction and GUS activity assays. According to these findings, the LBA4404 strain showed higher transformation efficiency to transform the host cells [28]. Similarly, to evaluate the efficiency of the A. tumefaciens approach in the transformation of microalgae for producing recombinant proteins, the integration of different genes into the genome of microalgae species was examined after transformation by different strains of A. tumefaciens. For instance, C. reinhardtii was transformed by the strain LBA4404 of A. tumefaciens carrying pCAMBIA1304. The stable integration and the expression of T-DNA in the hygromycin resistant colonies is confirmed by histochemical, microscopical and molecular analyses. In addition, Agrobacterium-based protocol efficiency, which was used in the mentioned study, was 50 times more than the glass beads method [30]. Furthermore, the successful integration and expression of uidA, hptII, and gfp genes was reported in the genome of Haematococcus pluvialis using EHA101 strain of Agrobacterium [26]. Recently, the potential of three strains of A. tumefaciens (i.e., EHA101, GV3301 and GV3850) were evaluated for the stable transformation of D. pseudosalina, in which the different molecular and proteomic analyses showed the GV3301 strain had a higher potential to efficient transform the microalgal cells [5]. Further analyses confirmed the GV3301 strain could be utilized for the stable transformation of the blue-green microalga Spirulina platensis with high efficacy [4]. Accordingly, the development of a robust transformation method in these algae along with the stability of the integrated transgene may allow the manipulation of many important pathways for the commercial synthesis of heterologous proteins [26], [27]. However, there are no reports available for the transformation of C. Vulgaris by A. tumefaciens strain EHA101. Our results demonstrated the successful transfer of T-DNA into the C. vulgaris genome using the Agrobacterium strain EHA101. Moreover, our data confirmed the successful incorporation of the T-DNA fragment of the plasmid into the genome of transformed C. vulgaris via the EHA101 strain of Agrobacterium, as well as the successful expression of the mgfp and hptII genes. Hence, it could be concluded that the Agrobacterium-based protocol seems to be a more ideal method for DNA delivery because of the simplicity of gene transfer, the high frequency of the transformation, the precision of T-DNA integration, and so on.

5 | Conclusion

In the present work, we successfully present an efficient and simple transformation procedure for the stable transformation of the microalga C. Vulgaris by A. tumefaciens strain EHA101. According to our findings, the successful integration of the plasmid was verified by PCR amplification of the mgfp and hptII genes. In addition, the expression of these genes in the transformed microalgal strain was confirmed by RT-PCR, GUS activity, and flow cytometry analyses. Since C. Vulgaris can produce valuable molecules ranging from high-quality recombinant proteins to biofuels, handling different strains of Agrobacterium for the transformation of C. Vulgaris, as well as other similar microalgal species for biotechnological applications, is highly necessary.

Authors' Contributions

The author was responsible for all stages of the research and manuscript preparation and approved the final version.

Data Availability

All data are included in the text.

Funding

This research was not supported by any specific grant from funding bodies in the public, commercial, or not-for-profit sectors.

Conflict of Interest

There are no competing interests to declare.

Consent for Publication

The author confirms consent for the publication of this work

Ethics Approval and Consent to Participate

This article does not contain any studies with human participants performed by the author.

References

[1] Yang, B., Liu, J., Jiang, Y., & Chen, F. (2016). Chlorella species as hosts for genetic engineering and expression of heterologous proteins: Progress, challenge and perspective. *Biotechnology journal*, *11*(10), 1244–1261. https://doi.org/10.1002/biot.201500617

[2] Kalhor, A. X., Movafeghi, A., Mohammadi-Nassab, A. D., Abedi, E., & Bahrami, A. (2017). Potential of the green alga Chlorella vulgaris for biodegradation of crude oil hydrocarbons. *Marine pollution bulletin*, *123*(1–2), 286–290. https://doi.org/10.1016/j.marpolbul.2017.08.045

[3] Gong, Y., Hu, H., Gao, Y., Xu, X., & Gao, H. (2011). Microalgae as platforms for production of recombinant proteins and valuable compounds: Progress and prospects. *Journal of industrial microbiology and biotechnology*, *38*(12), 1879–1890. https://doi.org/10.1007/s10295-011-1032-6

[4] Dehghani, J., Adibkia, K., Movafeghi, A., Barzegari, A., Pourseif, M. M., Maleki Kakelar, H., ... & Omidi, Y. (2018). Stable transformation of Spirulina (Arthrospira) platensis: A promising microalga for production of edible vaccines. *Applied microbiology and biotechnology*, *102*(21), 9267-9278. https://doi.org/10.1007/s00253-018-9296-7

[5] Dehghani, J., Movafeghi, A., Barzegari, A., & Barar, J. (2017). Efficient and stable transformation of Dunaliella pseudosalina by 3 strains of Agrobacterium tumefaciens. *BioImpacts: Bi*, *7*(4), 247. https://doi.org/10.15171/bi.2017.29

[6] Rasala, B. A., Muto, M., Lee, P. A., Jager, M., Cardoso, R. M., Behnke, C. A., ... & Mayfield, S. P. (2010). Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of Chlamydomonas reinhardtii. *Plant biotechnology journal*, *8*(6), 719-733. https://doi.org/10.1111/j.1467-7652.2010.00503.x

[7] Dawson, H. N., Burlingame, R., & Cannons, A. C. (1997). Stable transformation of Chlorella: Rescue of nitrate reductase-deficient mutants with the nitrate reductase gene. *Current microbiology*, *35*(6), 356–362. https://doi.org/10.1007/s002849900268

[8] Wang, C., Wang, Y., Su, Q., & Gao, X. (2007). Transient expression of the GUS gene in a unicellular marine green alga, Chlorella sp. MACC/C95, via electroporation. *Biotechnology and bioprocess engineering*, *12*(2), 180–183. https://doi.org/10.1007/BF03028646

[9] Kim, D. H., Kim, Y. T., Cho, J. J., Bae, J. H., Hur, S. B., Hwang, I., & Choi, T. J. (2002). Stable integration and functional expression of flounder growth hormone gene in transformed microalga, Chlorella ellipsoidea. *Marine biotechnology*, *4*(1), 63-73. https://doi.org/10.1007/s1012601-0070-x

[10] Cha, T. S., Yee, W., & Aziz, A. (2012). Assessment of factors affecting Agrobacterium-mediated genetic transformation of the unicellular green alga, Chlorella vulgaris. *World journal of microbiology and biotechnology*, *28*(4), 1771–1779. https://doi.org/10.1007/s11274-011-0991-0

[11] Sanitha, M., Radha, S., Fatima, A. A., Devi, S. G., & Ramya, M. (2014). Agrobacterium-mediated transformation of three freshwater microalgal strains. *Polish journal of microbiology*, *63*(4), 382–387. https://doi.org/10.33073/pjm-2014-052

[12] Cha, T. S., Chen, C. F., Yee, W., Aziz, A., & Loh, S. H. (2011). Cinnamic acid, coumarin and vanillin: Alternative phenolic compounds for efficient Agrobacterium-mediated transformation of the unicellular green alga, Nannochloropsis sp. *Journal of microbiological methods*, *84*(3), 430-434. https://doi.org/10.1016/j.mimet.2011.01.005

[13] Gelvin, S. B. (2000). Agrobacterium and plant genes involved in T-DNA transfer and integration. *Annual review of plant biology*, *51*(1), 223–256. https://doi.org/10.1146/annurev.arplant.51.1.223

[14] Hamilton, C. M., Frary, A., Lewis, C., & Tanksley, S. D. (1996). Stable transfer of intact high molecular weight DNA into plant chromosomes. *Proceedings of the national academy of sciences*, *93*(18), 9975–9979. https://doi.org/10.1073/pnas.93.18.9975

[15] Bundock, P., den Dulk-Ras, A., Beijersbergen, A., & Hooykaas, P. (1995). Trans-kingdom T-DNA transfer from Agrobacterium tumefaciens to Saccharomyces cerevisiae. *The embo journal*, *14*(13), 3206–3214. https://doi.org/10.1002/j.1460-2075.1995.tb07323.x

[16] De Groot, M. J. A., Bundock, P., Hooykaas, P. J. J., & Beijersbergen, A. G. M. (1998). Agrobacterium tumefaciens-mediated transformation of filamentous fungi. *Nature biotechnology*, *16*(9), 839–842. https://doi.org/10.1038/nbt0998-839

[17] Kunik, T., Tzfira, T., Kapulnik, Y., Gafni, Y., Dingwall, C., & Citovsky, V. (2001). Genetic transformation of HeLa cells by Agrobacterium. *Proceedings of the national academy of sciences*, *98*(4), 1871–1876. https://doi.org/10.1073/pnas.98.4.1871

[18] Kumar, S. V., Misquitta, R. W., Reddy, V. S., Rao, B. J., & Rajam, M. V. (2004). Genetic transformation of the green alga—Chlamydomonas reinhardtii by Agrobacterium tumefaciens. *Plant science*, *166*(3), 731–738. https://doi.org/10.1016/j.plantsci.2003.11.012

[19] Chen, Y., Wang, Y., Sun, Y., Zhang, L., & Li, W. (2001). Highly efficient expression of rabbit neutrophil peptide-1 gene in Chlorella ellipsoidea cells. *Current genetics*, *39*(5), 365–370. https://doi.org/10.1007/s002940100205

[20] Koo, J., Park, D., & Kim, H. (2013). Expression of bovine lactoferrin N-lobe by the green alga, Chlorella vulgaris. *Algae*, *28*(4), 379–387. https://doi.org/10.4490/algae.2013.28.4.379

[21] Stanier, R. Y., Kunisawa, R., Mandel, M., & Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological reviews*, *35*(2), 171–205. https://doi.org/10.1128/br.35.2.171-205.1971

[22] Holsters, M., De Waele, D., Depicker, A., Messens, E., Van Montagu, M., & Schell, J. (1978). Transfection and transformation of Agrobacterium tumefaciens. *Molecular and general genetics (MGG)*, *163*(2), 181–187. https://doi.org/10.1007/BF00267408

[23] Coll, J. M. (2006). Methodologies for transferring DNA into eukaryotic microalgae: A review. *Spanish journal of agricultural research*, *4*(4), 316–330. https://doi.org/10.5424/sjar/2006044-209

[24] Luo, D., & Saltzman, W. M. (2000). Synthetic DNA delivery systems. *Nature biotechnology*, *18*(1), 33–37. https://doi.org/10.1038/71889

[25] Gelvin, S. B. (2003). Agrobacterium-mediated plant transformation: The biology behind the "gene-jockeying" tool. *Microbiology and molecular biology reviews*, *67*(1), 16–37. https://doi.org/10.1128/mmbr.67.1.16-37.2003

[26] Kathiresan, S., Chandrashekar, A., Ravishankar, G. A., & Sarada, R. (2009). Agrobacterium-mediated transformation in the green alga Haematococcus pluvialis (Chlorophyceae, Volvocales) 1. *Journal of phycology*, *45*(3), 642–649. https://doi.org/10.1111/j.1529-8817.2009.00688.x

[27] Anila, N., Chandrashekar, A., Ravishankar, G. A., & Sarada, R. (2011). Establishment of Agrobacterium tumefaciens-mediated genetic transformation in Dunaliella bardawil. *European journal of phycology*, *46*(1), 36–44. https://doi.org/10.1080/09670262.2010.550386

[28] Cheng, R., Ma, R., Li, K., Rong, H., Lin, X., Wang, Z., ... & Ma, Y. (2012). Agrobacterium tumefaciens mediated transformation of marine microalgae Schizochytrium. *Microbiological research*, *167*(3), 179-186. https://doi.org/10.1016/j.micres.2011.05.003

[29] Pratheesh, P. T., Vineetha, M., & Kurup, G. M. (2014). An efficient protocol for the Agrobacterium-mediated genetic transformation of microalga Chlamydomonas reinhardtii. *Molecular biotechnology*, *56*(6), 507–515. https://doi.org/10.1007/s12033-013-9720-2

[30] Rajam, M. V, & Kumar, S. V. (2006). Green alga (Chlamydomonas reinhardtii). In *Agrobacterium protocols* (pp. 421–433). Springer. https://doi.org/10.1385/1-59745-131-2:421