Genetic transformation of *Chlorella vulgaris* mediated by HIV-TAT peptide

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Abstract

Scientific interest in microalgal species is growing and, genetic transformation has definitely opened more avenues, in the ongoing research on microphytes. In the present study, we have attempted to transform *Chlorella vulgaris* by mobilizing double-stranded linear Transfer DNA (T-DNA) comprised of *green fluorescent protein (egfp)* gene cassette and *hygromycin phosphotransferase II (hptII)* gene cassette non-covalently bound to TAT peptide, into *C. vulgaris* cells treated with Triton X-100. The transformed *C. vulgaris* cells when examined under fluorescent microscope, exhibited green fluorescence in comparison to the untransformed cells. The transformed cells were further screened, and the surviving colonies were sub-cultured, on BG11 medium fortified with Hygromycin. The surviving colonies were confirmed for the presence of integrated T-DNA by Polymerase Chain Reaction with *egfp* and *hptII* gene-specific primers. This methodology has potential to substitute the existing tedious transformation methodologies and ease the future studies in microalgae.

Keywords *Chlorella vulgaris* · HIV-TAT · Cell-penetrating peptide · Genetic transformation · Microalgae

Introduction

Chlorella is a popularly studied single-celled microalga that thrives both in freshwater and marine environments. It is composed of essential amino acids, bioactive compounds, chlorophyll, fatty acids, high-quality proteins, and minerals, thereby is considered as an excellent food source for rotifers,

fish larvae and humans (Cha et al. 2012). Since, *Chlorella* also has a high lipid and biomass content, it is also a promising source for bio-fuel production (Converti et al. 2009). With an intent to enhance the strain characteristics, to date, several transformation methodologies have been reported for *Chlorella*, such as the microprojectile bombardment technique (Dawson et al. 1997; El-Sheekh 1999), PEG-CaCl₂ method (Kim et al. 2002), Agrobacterium-mediated transformation (Cha et al. 2012), and electroporation (Gomma et al. 2015); however, all these techniques are time-consuming and tedious (Suresh and Kim 2013).

In plants, as an alternative to the tedious conventional methodologies for transgene delivery, a simple approach was established by Ziemienowicz et al. (2012). They demonstrated that a cell-penetrating peptide (CPP) could successfully deliver and integrate a linear dsDNA into the genome of triticales microspores. CPPs are small peptides (< 30 amino acids) that can efficiently transport biological materials (proteins, DNA, RNA, and drug molecules) across cellular membranes (Chuah et al. 2015). HIV-TAT peptide used by Ziemienowicz et al. (2012) is a small (11 aa), extensively studied, popular cell-penetrating peptide (CPP). HIV-TAT peptide can reportedly translocate drug molecules, oligo nucleotides, and proteins in a receptor,

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energy and temperature-independent fashion into mammalian, microalgal and plant cells, with a strong affinity towards the nucleus and no reported cytotoxicity (Suresh and Kim 2013; Zou et al. 2017).

CPPs have been used to deliver biological cargo in algal cells as well. Hyman et al. (2012) demonstrated that octa- and nona-arginine CPPs transferred enzymes and proteins into *Chlamydomonas reinhardtii*. Suresh and Kim (2013) demonstrated that FITC-conjugated CPPs (pVEC, TRA, PEN, and TAT) could successfully translocate into *C. reinhardtii*. Wei et al. (2015) demonstrated that R9 (nona-arginine) successfully transported dsRNA into *Dunaliella salina*. Kang et al. (2017) showed that pVEC could successfully translocate proteins into *C. reinhardtii*, *Nannochloropsis salina*, and *Chlorella vulgaris*. However, to best of our knowledge, CPP-mediated genetic transformation of microalgae has not yet been reported.

Therefore with the aim to establish a simple and economical methodology to genetically transform microalgae, we have treated the *C. vulgaris* cells with a non-ionic surfactant Triton X-100 (TX-100) prior to transfection, to permeabilize the cell. HIV-TAT peptide (11aa) has been used to translocate a linear dsDNA T-DNA with *egfp* expression cassette into the nucleus of *C. vulgaris*. The incorporation and expression of the linear dsDNA in *C. vulgaris* is evaluated by fluorescent microscopy, PCR, and Southern hybridization analysis.

Materials and methods

Chlorella vulgaris culture

The green microalgae *C. vulgaris* was obtained from the 'The Centre for Conservation and Utilization of Blue Green Algae' (CCUBGA), Division of Microbiology, IARI, New Delhi, India. The microalgae were routinely cultured in BG-11 medium (HiMedia, India) and maintained in a culture room at 25 ± 1 °C with a 16 h photoperiod, under a light irradiance of $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ provided by Cool White Fluorescent tubes. The cultures were gently agitated at an interval of 12 h and sub-cultured every 15 days.

Reconstitution of T-DNA in pGreen 0179 vector

The binary vector pBI121:*egfp* was digested with *Hind*III and *Eco*RI restriction enzymes to release an approximately 1.9 kb dsDNA (double-stranded DNA) fragment, composed of CaMV35s promoter::*egfp*::nosA terminator cassette. The purified linear dsDNA cassette was ligated between similar restriction endonuclease sites of the Multiple Cloning Site (MCS) in pGreen0179 vector.

The confirmed pGreen0179 vector harboring *egfp* cassette (pGreen0179:*egfp*) was digested with *Bgl*III restriction enzyme to release a double-stranded linear T-DNA region of ~4.5 kb. The digested mixture was resolved on a 0.8% agarose gel, and the ~4.5 kb double-stranded T-DNA fragment was purified using gel extraction kit (Qiagen, USA). The linear purified T-DNA fragment harboring *hptII* cassette and *egfp* cassette, hereafter termed as *gh*, was used for transformation of *C. vulgaris*. Plasmid isolation and cloning were carried out as previously described by Sambrook and Russell (2001).

Complexing TAT peptide to *gh*

HIV-1 TAT Protein (47–57) was procured from Sigma-Aldrich (USA). The binding activity of TAT peptide to the linear dsDNA *gh* was analyzed by complexing one microgram of purified *gh* with TAT peptide at N/P ratios of 1:0.5, 1:1, 1:2, 1:3, 1:4, and 1:5 (*gh*:TAT). The reaction volumes were made up to 30 μl in a 0.2 ml tube with PBS and incubated at 37 °C for 1 h. The *gh*:TAT mixtures were then subjected to electrophoresis at 60 V for 45 min on a 0.8% agarose gel and stained with ethidium bromide (EtBr). The DNA mobility was documented using UV transilluminator.

Transfection of *C. vulgaris*

Approximately 2×10^5 exponentially growing *C. vulgaris* cells were harvested in individual Eppendorf tubes (2 ml) by centrifuging at 2000 rpm for 5 min and resuspended in 500 μl of fresh BG11 medium. The tubes were then inoculated with *gh*:TAT (1:4) complex prepared as previously described, and *gh* alone individually. One set was maintained without any inoculation and termed as a control.

For treatment involving TX-100, the harvested cells were suspended in different concentrations (0.05%, 0.10%, 0.15%, 0.20%, 0.25%, and 0.30%) of TX-100 (500 μl) and tapped gently for 5 min to keep the cells suspended. The cells were then washed thrice with fresh sterile BG11 medium by centrifugation and finally suspended in 500 μl of fresh BG11 medium supplemented with *gh*:TAT (1:4) complex. The cultures were incubated for different time durations (12 h, 24 h, 36 h, 48 h, 60 h, and 72 h) under optimum growth conditions with intermittent agitation. Consequently, the cultures were washed and suspended in fresh 100 μl of BG11 medium.

Ten microliters of the culture from Eppendorf tubes transfected with *gh*, *gh*:TAT and TX-100 treated before transfection with *gh*:TAT, were spread on glass slides and enclosed with cover-slips before observation under Leica SP5 confocal microscope for fluorescence. The images were captured at 488 nm and 633 nm wavelengths (Watts et al. 2017). The remaining cultures were individually spread on selection plates (BG11 medium + Hygromycin 50 mg l^{-1}).

Selection of the putative transgenics and PCR analysis

The hygromycin-resistant colonies were randomly selected and streaked onto fresh selection plates and sub-cultured every 15 days successively thrice on selection plates to ensure the stability of the transformants.

The genomic DNA was isolated from the resistant and untransformed cultures by SDS-potassium acetate method (Sambrook et al. 1989). PCR was carried out using *egfp* gene-specific primers (FP: 5'-CAAGGGCGAGGAGCT GTT-3' and RP: 5'-CTTGTACAGCTCGTCCATGC-3') that amplify a 709 bp sequence and *hptII* gene-specific primers (FP: 5'-GCGAAGAATCTCGTGCTTTC-3', RP: 5'-GAT GTTGGCGACCTCGTATT-3') that amplifies a 605 bp fragment in a Mastercycler ep Gradient PCR (Eppendorf, Germany). The program was optimized as follows for both sets of primers: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified PCR products were visualized on a 0.8% agarose gel stained with EtBr. The pGreen0179:*egfp* plasmid and untransformed algae genomic DNA were used as positive and negative controls, respectively.

Southern hybridization

The integration of transgene in *C. vulgaris*, PCR positive for *egfp* and *hptII* genes, was detected by southern hybridization analysis. Ten micrograms of genomic DNA was isolated, from randomly selected three individual transformants and untransformed *C. vulgaris*. The DNA was digested with *HindIII* and resolved on a 0.8% agarose gel. The resolved DNA was blotted onto a Hybond-N⁺-membrane (Amersham Biosciences, USA) and fixed by cross-linking with UV (Stratagene, UK). The blot was hybridized with a 605 bp *hptII* probe, synthesized using *hptII* gene-specific primers and PCR DIG probe synthesis kit (Roche Diagnostics Corporation, USA). The hybridizations were detected using a DIG Nucleic Acid Detection Kit (Roche Diagnostics Corporation, USA) according to manufacturer's protocol.

Results

The pGreen0179 vector was reconstituted with an *egfp* expression cassette (Fig. 1). Restriction digestion with *BglIII* released an approximately 4.5 Kb T-DNA fragment, constituting *hptII* cassette and *egfp* cassette (Fig. 2a), confirming the reconstitution.

Fig. 1 pGreen0179 vector reconstituted with *egfp* gene expression cassette

gh:TAT complex

The electrophoresis of various complex combinations of TAT peptide and *gh* revealed that, with an increase in peptide concentration, the amount of displaced linear *gh* on the electrophoresis gel increased, but its fluorescence decreased. At the ratio above 1:4 (*gh*:TAT), the DNA did not display any fluorescence on gel, suggesting complete inhibition of EtBr staining by the bound TAT peptide. Therefore, 1 µg of *gh* complexed with TAT peptide in the N/P ratio of 1:4 has been used in further studies (Fig. 2b).

Transfection of *C. vulgaris*

The cells transfected with *gh* alone or in combination with TAT peptide displayed neither fluorescence when evaluated under confocal microscope, nor survived or regenerated as colonies on selection media. The cells treated with detergent TX-100 preceding transfection with *gh*:TAT complex showed fluorescence when observed under confocal microscope, thereby confirming the translocation and expression of the *egfp* gene in the *Chlorella* cells (Fig. 2c).

The transformed culture with *egfp*-expressing cells gave rise to hygromycin-resistant colonies on regeneration medium. The number of colonies on selection plates was noticed to increase with increase in percentage of TX-100. The highest colony-forming units (cfu) was observed in cultures treated with 0.1% TX-100 (345.3 ± 15.8 cfu); however, with further increase in TX-100 concentration, the colonies gradually decreased (Table 1). The number

Fig. 2 TAT peptide-mediated genetic transformation of *Chlorella vulgaris*. **a** Schematic representation of the T-DNA (*gh*) used for transfection of *C. vulgaris*. **b** Complexing *gh* with TAT peptide in different molar ratios; M – 1 kb ladder (Fermentas), 1—linear dsDNA *gh*, 2—*gh*:TAT (1:0.5), 3—*gh*:TAT (1:1), 4—*gh*:TAT (1:2), 5—*gh*:TAT (1:3), 6—*gh*:TAT (1:4), 7—*gh*:TAT (1:5). **c** Fluorescence signals as

visualized under confocal microscope; 1—cells transfected with *gh*, 2—cells transfected with *gh*:TAT, 3—cells treated with Triton X-100 and transfected with *gh*:TAT. **d** The hygromycin-resistant colonies cultured for 14 days on BG11 media with hygromycin (50 mg l^{-1}), along with untransformed cultures

Table 1 Influence of Triton X-100 concentration on transformation efficiency of *C. vulgaris*

Sl. no.	TX-100 concentration (%)	Mean no. of colonies on selection medium	Transformation efficiency (%)
1	0	0	0
2	0.05	151 ± 9.3	0.0755
3	0.10	345.3 ± 15.8	0.172667
4	0.15	301.7 ± 18	0.150833
5	0.20	125 ± 10.6	0.062833
6	0.25	59 ± 7.9	0.0295
7	0.30	20 ± 3.55	0.01

The freshly harvested microalgae culture ($\sim 2 \times 10^5$ cells) was treated with different concentrations of Triton X-100 for 5 min, followed by two consecutive washes with fresh BG11 medium. The washed microalgal cells were suspended with *gh*:TAT for 12 h before plating the culture on selection plates

Transformation efficiency (%) = number of colonies formed on selection medium plates/number of transfected cells $\times 100$. Mean values of three separate trials ($\pm$) with standard errors

of colonies further increased with increase in transfection duration and the highest was seen after transfection for 48 h (1126 ± 28.9 cfu), which gradually decreased with further increase in transfection duration (Table 2).

The regenerated colonies were picked and successively cultured on fresh selection plates after every 14 days along with negative control (untransformed *C. vulgaris*). The survival of the transformed cultures further confirmed the

Table 2 Influence of transfection duration on transformation efficiency of *C. vulgaris*

Sl. no.	Transfection duration (h)	Mean no. of colonies on selection medium	Transformation efficiency (%)
1	12	345 ± 15.8	0.172667
2	24	590 ± 18.8	0.295
3	36	884 ± 29.7	0.442
4	48	1126 ± 28.9	0.563167
5	60	979 ± 41	0.4895
6	72	943 ± 52	0.471667

The freshly harvested microalgae culture ($\sim 2 \times 10^5$ cells) was treated with 0.1% Triton X-100 for 5 min, followed by two consecutive washes with fresh BG11 medium. The washed microalgal cells were suspended with *gh*:TAT for various durations before plating the culture on selection plates

Transformation efficiency = number of colonies formed on selection medium plates/number of transfected cells $\times 100$. Mean values of three separate trials ($\pm$) with standard errors

stability of the integrated *gh* in *C. vulgaris*; however, the control cultures turned pale within 72 h indicating mortality (Fig. 2d).

PCR analysis of the cultures

PCR analysis with *hptII* gene-specific primers successfully amplified a 605 bp gene fragment in all the 14 *C. vulgaris* lines (Fig. 3a), and *egfp* primers amplified a 709 bp gene fragment of *egfp* gene in 11 out of 14 *C. vulgaris* lines

Fig. 3 Molecular analysis of *C. vulgaris* transformants. **a** PCR analysis of genomic DNA isolated from hygromycin-resistant cultures and untransformed *C. vulgaris* cultures with *hptII* gene-specific primers; *M* 1 kb plus ladder (Fermentas), *1–14* Hygromycin-resistant *C. vulgaris* cultures, *N* untransformed *C. vulgaris*, *P* pGreen 0179-*egfp*

plasmid. **b** PCR analysis with *egfp* gene-specific primers. *M* 1 kb plus ladder (Fermentas), *1–14* Hygromycin-resistant *C. vulgaris* cultures, *N* untransformed *C. vulgaris*, *P* pGreen 0179-*egfp*. **c** Southern hybridization analysis of *C. vulgaris*. *M* λ DNA/*Hind*III ladder, *N* untransformed *C. vulgaris*, *1–3* Transformed *C. vulgaris*

(Fig. 3b). The PCR analysis for untransformed wild-type *C. vulgaris* cultures did not result in any amplicons, indicating the complete T-DNA integration in 11 of 14 hygromycin-resistant *C. vulgaris* cell lines.

Southern hybridizations assay

The Southern hybridization analysis further confirmed the integration of transgene in the transformed PCR positive *C. vulgaris*. One or more copies of the transgene were detected in the analyzed cultures (Fig. 3c).

Discussion

HIV-TAT peptide is gaining popularity as a non-toxic transporter. It has been successfully used for delivering nucleic acids (DNA and dsRNA) into mung bean, immature wheat embryos, soybean root tips, tobacco suspension cells and protoplasts (Chugh and Eudes 2007; Gump and Dowdy 2007; Zou et al. 2017), and transformation of Triticale (Ziemienowicz et al. 2012). TAT acts as an excellent carrier as TAT with positive charge binds to carrying negative charge via electrostatic interactions by non-covalent bonding (Chen et al. 2007). Their entry into cells has been postulated to occur via direct membrane translocation, macropinocytosis or an amalgamation of multiple pathways (Liu et al. 2013).

In *Chlorella*, earlier attempts to translocate CPP (R9), bound to nucleic acid or protein, have been futile in contrary to the successful translocation observed in other microalgal species. This is probably due to the cell wall structure that varies among the microalgae (Liu et al. 2008; Wei et al. 2015). This suggests that it is necessary to impregnate the outer cell wall of *C. vulgaris* that inhibits the entry of exogenous DNA into the cell to achieve genetic transformation. In our studies too, *gh*:TAT alone could not transform *C. vulgaris*. Therefore, to overcome the cellular barriers, we considered using surface detergent TX-100, a nonionic surfactant reported to permeabilize algal cells (Corre et al. 1996; Tear et al. 2013).

TX-100 is a popular non-ionic detergent used to lyse cells and extract cellular organelles and proteins. It is also reportedly used for transfection of cells. However, exposure of cells to high concentrations of TX-100 or for long durations results in cell death (Koley and Bard 2010). Hence, in the present study, we used TX-100 in diluted concentrations so as to ensure the recovery of *C. vulgaris* cells and facilitate the uptake of CPPs.

Efficient binding of CPP to DNA is a prerequisite for efficient uptake and delivery of nucleic acids to the cell. The binding ratio of DNA and CPP is calculated by the N/P ratio. Earlier studies with gel retardation and DNase activity demonstrated that DNA mobility was retarded beyond TAT dimer:DNA, N/P ratio of 4:1, which also protected DNA from DNase enzyme activity (Chugh et al. 2009). Similarly,

Kim et al. (2003) reported that R15 peptide DNA complex of N/P ratio beyond 3:1 protected the DNA against DNaseI, suggesting that CPPs, apart from binding to DNA with high affinity, also shielded them from nuclease enzymes. Similarly, to determine the optimal concentration of HIV-TAT peptide required for complex formation with *gh*, different molar ratios of peptide were used. Gel electrophoresis image revealed that, compared to the DNA (*gh*) alone, various degrees of retardation were observed as the concentration of peptide increased. It was also observed that the DNA mobility was completely inhibited above the molar ratios of 1:4, indicating that DNA was completely bound to TAT peptide at this particular concentration ratio. Hence, the ratio of 1:4 (*gh*:TAT) was used in our studies. Following translocation across the cell membranes, the uptake of the *gh* into the nucleus is attributed to the affinity of the HIV-TAT peptide to the nucleus (Chugh and Eudes 2007; Wei et al. 2015). Once inside the nucleus, the linear DNA can integrate into the genome by the homologous recombination process or non-homologous end joining into a region of active replication or transcription or simple broken DNA (Ziemienowicz et al. 2008). However, mechanism underlying the integration of exogenous gene into the nuclear genome of *C. vulgaris* is yet unclear.

The treatment of *C. vulgaris* cells with TX-100, followed by transfection with *gh*:TAT (1:4) mixture, resulted in the transformation of *C. vulgaris* cells with *gh*, as evaluated by fluorescent microscopy. The primary analysis by a fluorescent microscope revealed that the red auto-fluorescence of the chloroplasts in the algae was masked in few of the cells treated with *gh*:TAT, which displayed an intense green fluorescence. This confirmed the integration and expression of linear T-DNA in *C. vulgaris*. Similarly, Yang et al. (2015) observed that transformation and expression of transgene *egfp* masked the auto-fluorescence in *C. vulgaris*. The transformed *Chlorella* cells were evaluated for transformation efficiency by screening on BG11 medium supplemented with Hygromycin (50 mg l^{-1}), a concentration previously reported for efficient screening of *C. vulgaris* transformants (Chow and Tung 1999). From the regenerating, hygromycin-resistant colonies, we randomly selected 14 colonies and restreaked and sub-cultured them thrice to evaluate the stability of the integrated transgene. PCR analysis was carried out for these 14 cultures using *hptII* and *egfp* gene-specific primers to further confirm the presence of transgenes in the hygromycin-resistant colonies; all the 14 colonies were positive for the presence of *hptII* gene. However, three colonies were negative for *egfp*, suggesting an incomplete integration of linear DNA into the cells probably due to the truncation of the *gh* during transfection. Similarly, the PCR analysis was carried out by Niu et al. (2011). Southern hybridization with probe for *hptII* gene probe, further confirmed the stable integration of the transgenes in the transformed cultures. In

the present study the highest transformation efficiency was observed to be $1.1 \times 10^3 \pm 0.029 \text{ cfu } \mu\text{g}^{-1}$ when 2×10^5 cells were treated with 0.1% Triton X-100 for 5 min followed by transfection for 48 h with linear T-DNA complexed to TAT peptide. Comparatively Kumar et al. (2018) reported transformation efficiency of $1.67 \times 10^4 \pm 0.08 \text{ cfu } \mu\text{g}^{-1}$ plasmid for pSK397 and $1.77 \times 10^4 \pm 0.16 \text{ cfu } \mu\text{g}^{-1}$ plasmid for pCAMBIA1302 by electroporation when 2×10^6 cells were transformed, which is the highest reported till date.

Agrobacterium mediated transformation of microalgae involving maintenance of *agrobacterium* strains, followed by co-cultivation with *chlorella*, and finally elimination of *agrobacterium* before selection of transformants is quite tedious (Cha et al. 2012). PEG- CaCl_2 method requires preparation of protoplasts prior to transformation (Kim et al. 2002). Microprojectile bombardment and electroporation techniques for transformation are quite simpler, and result in higher transformation rates, but they do require complex expensive apparatus such as helium-driven guns and pulse generators (El-Sheekh 1999; Niu et al. 2011). Whereas the TAT peptide-mediated transformation of *C. vulgaris*, we describe here is a simpler, less tedious alternative, which also does not require any special apparatus.

We believe that this methodology will open up avenues to easily manipulate microorganisms, in academics as well as for industrial applications.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

- Cha TS, Yee W, Azia A (2012) Assessment of factors affecting *Agrobacterium*-mediated genetic transformation of the unicellular green alga, *Chlorella vulgaris*. World J Microbiol Biotechnol 28:1771–1779
- Chen CP, Chou JC, Liu BR, Chang M, Lee HJ (2007) Transfection and expression of plasmid DNA in plant cells by arginine-rich intracellular delivery peptide without protoplast preparation. FEBS Lett 581:1891–1897
- Chow KC, Tung WL (1999) Electroporation of *Chlorella vulgaris*. Plant Cell Rep 18:778–780

- Chuah JA, Yoshizumi T, Kodama Y, Numata K (2015) Gene introduction into the mitochondria of *Arabidopsis thaliana* via peptide-based carriers. *Sci Rep* 5:7751
- Chugh A, Eudes F (2007) Translocation and nuclear accumulation of monomer and dimer of HIV-1Tat basic domain in triticales mesophyll protoplasts. *Biochim Biophys Acta* 1768:419–426
- Chugh A, Amundsen E, Eudes F (2009) Translocation of cell-penetrating peptides and delivery of their cargoes in triticales microspores. *Plant Cell Rep* 28:801–810
- Converti A, Casazza AA, Ortiz EY (2009) Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chem Eng Process* 48(6):1146–1151
- Corre G, Templier J, Largeau C, Rousseau B, Berkalo C (1996) Influence of cell wall composition on the resistance of two *Chlorella* species (Chlorophyta) to detergents. *J Phycol* 32:584–590
- Dawson HN, Burlingame R, Cannons AC (1997) Stable transformation of *Chlorella*: rescue of nitrate reductase-deficient mutants with the nitrate reductase gene. *Curr Microbiol* 35:356–362
- El-Sheekh MM (1999) Stable transformation of the intact cells of *Chlorella kessleri* with high velocity microprojectiles. *Biol Plant* 42:209–216
- Gomma AE, Lee SK, Sun SM, Yang SH, Chung G (2015) Development of stable marker-free nuclear transformation strategy in the green microalga *Chlorella vulgaris*. *Afr J Biotechnol* 14(37):2715–2723
- Gump JM, Dowdy SF (2007) TAT transduction: the molecular mechanism and therapeutic prospects. *Trends Mol Med* 13(10):443–448
- Hyman JM, Geihe EI, Trantow BM, Parvin B, Wender PA (2012) A molecular method for the delivery of small molecules and proteins across the cell wall of algae using molecular transporters. *Proc Natl Acad Sci USA* 109:13225–13230
- Kang S, Suresha A, Kim YC (2017) A highly efficient cell penetrating peptide pVEC-mediated protein delivery system into microalgae. *Algal Res* 24:360–367
- Kim D, Kim YT, Cho JJ, Bae J, Hur S, Hwang I, Choi T (2002) Stable integration and functional expression of flounder growth hormone gene in transformed microalga, *Chlorella ellipsoidea*. *Mar Biotechnol* 4:63–73
- Kim HH, Lee WS, Yang JM, Shin S (2003) Basic peptide system for efficient delivery of foreign genes. *Biochim Biophys Acta* 1640:129–136
- Koley D, Bard AJ (2010) Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). *Proc Natl Acad Sci USA* 107:16783–16787
- Kumar M, Jeon J, Choi J, Kim SR (2018) Rapid and efficient genetic transformation of the green microalga *Chlorella vulgaris*. *J Appl Phycol* 30(3):1735–1745
- Liu BR, Chou J-C, Lee H-J (2008) Cell membrane diversity in noncovalent protein transduction. *J Membr Biol* 222:1–15
- Liu BR, Huang YW, Lee HJ (2013) Mechanistic studies of intracellular delivery of proteins by cell-penetrating peptides in cyanobacteria. *BMC Microbiol* 13(1):57
- Niu YF, Zhang MH, Xie WH, Li JN, Gao YF, Yang WD, Liu JS, Li HY (2011) A new inducible expression system in a transformed green alga, *Chlorella vulgaris*. *Genetic Mol Res* 10(4):3427–3434
- Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
- Sambrook J, Fritschi EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
- Suresh A, Kim YC (2013) Translocation of cell penetrating peptides on *Chlamydomonas reinhardtii*. *Biotechnol Bioeng* 110:2795–2801
- Tear CJ, Lim C, Wu J, Zhao H (2013) Accumulated lipids rather than the rigid cell walls impede the extraction of genetic materials for effective colony PCRs in *Chlorella vulgaris*. *Microb Cell Factories* 12:106
- Watts A, Singh SK, Bhadouria J, Naresh V, Bishoyi AK, Geetha KA, Chamola R et al (2017) *Brassica juncea* lines with substituted chimeric GFPcENH3 give haploid and aneuploid progenies on crossing with other lines. *Front Plant Sci* 7:2019
- Wei Y, Niu J, Huan L, Huang A, He L, Wang G (2015) Cell penetrating peptide can transport dsRNA into microalgae with thin cell walls. *Algal Res* 8:135–139
- Yang B, Liu J, Liu B, Sun P, Ma X, Jiang Y et al (2015) Development of a stable genetic system for *Chlorella vulgaris*—a promising green alga for CO₂ biomitigation. *Algal Res* 12:134–141
- Ziemienowicz A, Tzfira T, Hohn B (2008) Mechanisms of T-DNA integration. *Agrobacterium: from biology to biotechnology*. Springer, New York, pp 396–441
- Ziemienowicz A, Shim YS, Matsuoka A, Eudes F, Kovalchuk I (2012) A novel method of transgene delivery into triticales plants using the *Agrobacterium* transferred DNA-derived nano complex. *Plant Physiol* 158:1503–1513
- Zou L, Peng Q, Wang P, Zhou B (2017) Progress in research and application of HIV-1 TAT derived cell-penetrating peptide. *J Membr Biol* 250(2):115–122