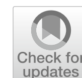
World Journal of Microbiology and Biotechnology (2019) 35:119
https://doi.org/10.1007/s11274-019-2695-9

ORIGINAL PAPER



High efficiency transformation by electroporation of the freshwater alga *Nannochloropsis limnetica*

Yiwen Chen[1,2] · Hanhua Hu[1]

Received: 10 April 2019 / Accepted: 12 July 2019 / Published online: 22 July 2019
© Springer Nature B.V. 2019

Abstract
The microalgal genus of *Nannochloropsis* is considered one of the most promising organisms for the production of biofuels due to their high lipid content. Transformation systems for marine *Nannochloropsis* species have been established in the recent decade, however, genetic manipulation of *Nannochloropsis limnetica*, the only known freshwater species in this genus, is not yet available. Based on established marine *Nannochloropsis* species electrotransformation protocol, nuclear genetic transformation was established in *N. limnetica*, meanwhile the appropriate antibiotic selection concentration and electric field strength of electroporation were determined. For the selection of transformants in *N. limnetica* on plates, 0.07 μg mL^{-1} of zeocin or 5 μg mL^{-1} of hygromycin B was proved sufficient, and the transformation efficiency was $< 2 \times 10^{-8}$ with a single pulse ranging from 2200 to 2600 V using 2-mm electroporation cuvettes. Pretreatment of *N. limnetica* with 10 mM lithium acetate and 3 mM dithiothreitol before electroporation increased transformation efficiency hundreds of times, and the highest transformation efficiency of $10–11 \times 10^{-6}$ was obtained with an electric field strength of 12,000 V/cm. Our results help to expand the biotechnological applications of this freshwater species and provide means for successful electrotransformation of other microalgae as well.

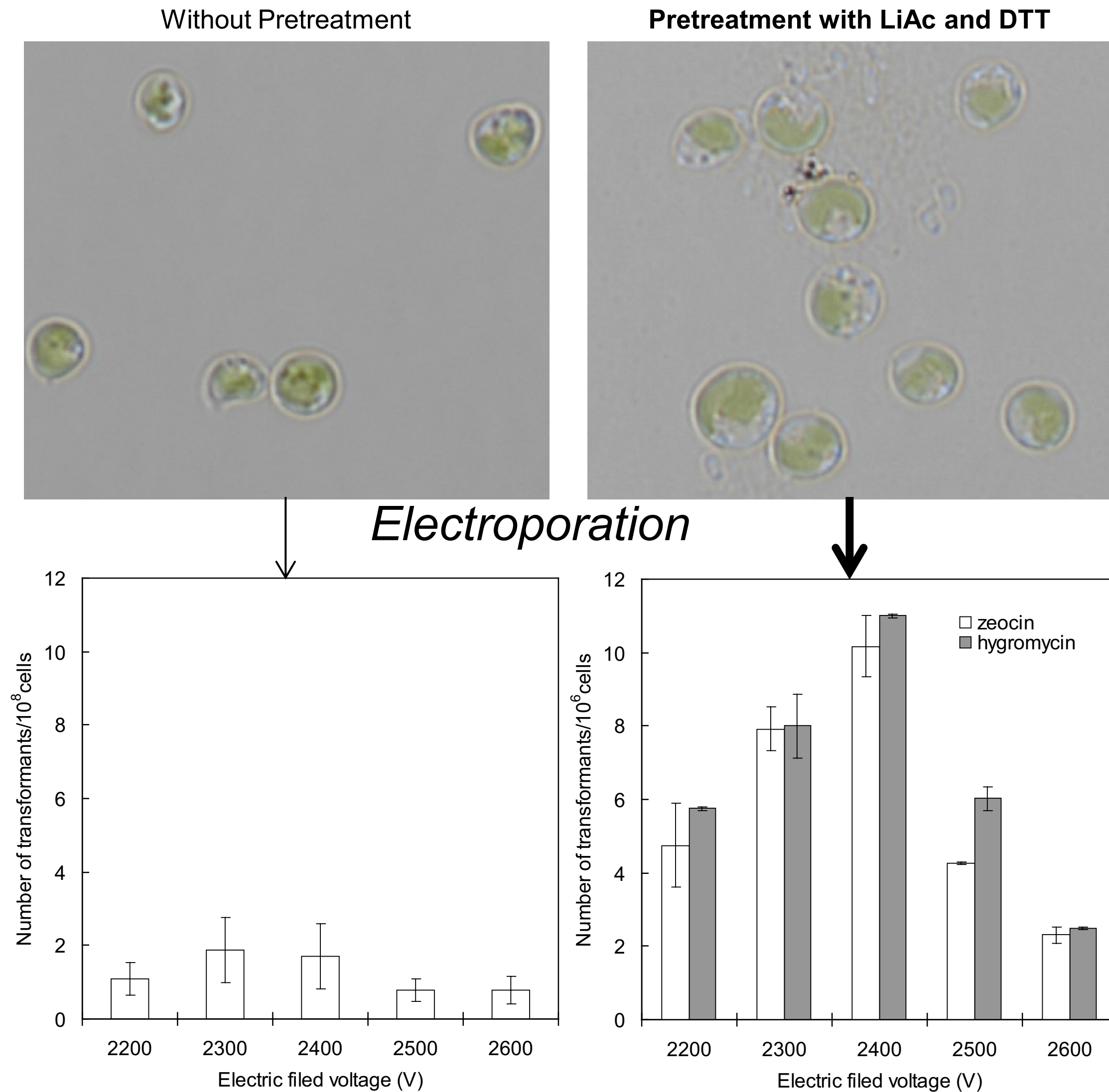
Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11274-019-2695-9) contains supplementary material, which is available to authorized users.

✉ Hanhua Hu
hanhuahu@ihb.ac.cn

[1] Key Laboratory of Algal Biology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

Graphic abstract
High-efficiency transformation of freshwater *Nannochloropsis*pretreatment of *N. limnetica* with 10 mM lithium acetate and 3 mM dithiothreitol before electroporation increased transformation efficiency hundreds of times.



Keywords Biofuels · Electroporation · Freshwater microalgae · *Nannochloropsis limnetica* · Nuclear transformation

Introduction

The microalgal genus of *Nannochloropsis* belongs to Eustigmatophyceae. It is highly appreciated in aquaculture due to the high polyunsaturated fatty acid (mainly eicosapentaenoic acid) contents (Sukenik 1998; Chini Zittelli et al. 1999; Gbadamosi and Lupatsch 2018). Meanwhile, species of this genus are considered the most promising candidates for biofuel production since they can produce high biomass and triacylglycerol productivities (Rodolfi 2009; Soria-Verdugo et al. 2018). There are seven known species of *Nannochloropsis*, including *N. gaditana*, *N. salina*, *N. oculata*, *N. oceanica*, *N. australis*, *N. granulata and N. limnetica* (Andersen et al. 1998; Fawley et al. 2015). At present, the whole genome sequences of almost all of the marine *Nannochloropsis* species have been published, and their genetic transformation systems have been established (Kilian et al. 2011; Radakovits et al. 2012; Vieler et al. 2012; Li et al. 2014; Wang et al. 2014).

Nannochloropsis limnetica is the only freshwater species of genus *Nannochloropsis*, and was first isolated from a small inland pond in Germany (Krienitz et al. 2000). This

freshwater species was found to be present in many freshwater habitats and was of excellent food quality in freshwater ecosystems (Krienitz and Wirth 2006). Similar to marine species of the genus, *N. limnetica* is also promising microalga for biodiesel feedstock production and diet for freshwater aquaculture (Ma et al. 2014; Freire et al. 2016; Soria-Verdugo et al. 2018). In addition, freshwater species have some advantages as a diet for marine aquaculture thanks to the absence of marine pathogens (Freire et al. 2016). Moreover, the freshwater *N. limnetica* could be of interest for specific applications such as wastewater treatment (Freire et al. 2016). Therefore, establishing genetic tools in *N. limnetica* is of importance for developing it into a molecular model species in freshwater microalgal biotechnology and ecology.

Although the genome of *N. limnetica* has not yet been sequenced, the extensive analysis of sensitivity of the alga to a range of antibiotics has been reported and a protocol using lysozyme to obtain viable *N. limnetica* protoplasts is available by Noda et al. (2017). Unfortunately, no transformants have been obtained yet in *N. limnetica* by electroporation and biolistic bombardment to normal or permeabilized cells (Noda et al. 2017). In this study, we established a simple method for high efficiency nuclear transformation of *N. limnetica* by electroporation directly to wild-type cells pretreated with lithium acetate (LiAc) and dithiothreitol (DTT).

Materials and methods

Strain and growth conditions

Nannochloropsis limnetica KR 1998/3, a kind gift from Lothar Krienitz, was isolated form Dorfteich Schwarz, Germany (Krienitz et al. 2000). The strain was cultured in BG11 medium at 22 °C under a 12 h light/12 h dark photoperiod with the photon flux density of about 50 µmol photons m^{-2} s^{-1}. The same medium was used for preparing 1.2% agar plates.

Plasmid construction

Antibiotic sensitivity to zeocin and hygromycin B were tested for *N. limnetica* and accordingly plasmids containing bleomycin-resistant gene (*sh ble*) or hygromycin B-resistant gene (*hpt*II) were constructed in the study. Plasmids pPha-T1-NlTUBp and pPha-NoVCPp-eGFP contained the *sh ble* gene driven respectively by endogenous *N. limnetica β-tubulin* (*NlTUB*) promoter and exogenous *Nannochloropsis oceanica violaxanthin/chlorophyll a-binding protein* (*NoVCP*) promoter. *NlTUB* promoter (*NlTUB*p) region was amplified from *N. limnetica* genomic DNA using the forward and reverse primers *NlTubAor_fw* and *NlTubBal_rv* (Li et al. 2014). The PCR product was digested with *Aor*13H I and *Bal* I and ligated in front of the *sh ble* in the pPha-T1 plasmid replacing the *Phaeodactylum tricornutum fucoxanthin chlorophyll a/c-binding protein* (*FCP*) *B* gene promoter (*FcpB*p) to generate the pPha-T1-NlTUBp plasmid (Fig. 1a). The 1135 bp promoter sequence of the *NoVCP2* gene (*NoVCP2*p) and the 643 bp terminator sequence of the *NoVCP1* gene (*NoVCP1*t) were amplified from *N. oceanica* MBIC10090 genomic DNA by PCR using primer sets *Vcp2Bal-fw* and *Vcp2Kpn-rv* for the promoter, and *Vcp1Kpn-fw* and *Vcp1Pst-rv* for the terminator (Kilian et al. 2011). The resulting PCR products were digested and cloned into the *Pst*I-*Bal*I sites of pPha-T1 to create pPha-NoVCPp. eGFP gene (*egfp*) was amplified by PCR from

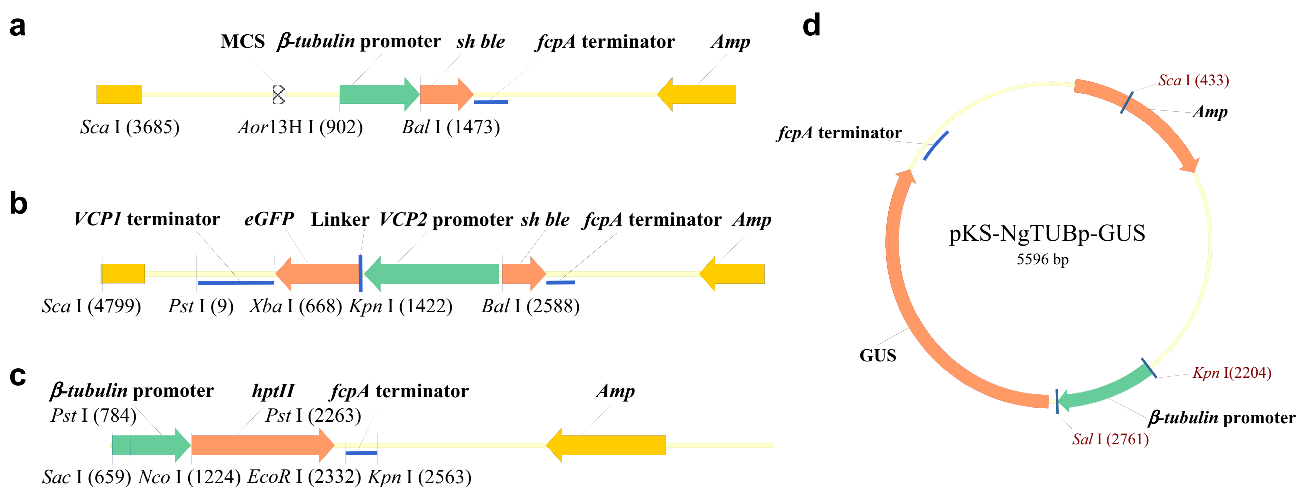


Fig. 1 Schematic representations of the expression constructs used for electrotransformation in this study. Restriction sites used for cloning and linearization are indicated. **a** *Sca*I linearized pPha-T1-NlTUBp (4471 bp); **b** *Sca*I linearized pPha-NoVCPp-eGFP (5586 bp); **c** *Sac*I linearized pKS-NlTUBp-Hyg (4760 bp); **d** circular plasmid pKS-NgTUPp-GUS (5596 bp)

pEGFP-N1 (GenBank accession number U55762) using the sense primer *egfpKpn-fw* and the antisense primer *egfpXba-rv*. The PCR product was digested with *Kpn*I and *Xba*I and cloned into the *Kpn*I–*Xba*I sites of pPha-NoVCPp to generate the pPha-NoVCPp-eGFP expression vector (Fig. 1b). In this vector, *egfp* is expressed under control of the *NoVCP2* bidirectional promoter and *NoVCP1* terminator. At the opposite end of the bidirectional *NoVCP2* promoter, the selection marker gene *sh ble* conferring resistance to zeocin was fused in reverse complementary orientation to *egfp* and flanked at the 3′ end with the *P. tricornutum FCPA* gene terminator (*FcpA*t) fragment.

Plasmid pKS-NlTUBp-Hyg contained the *hptII* gene expressed under control of the endogenous *N. limnetica TUB* promoter and exogenous *P. tricornutum FCPA* terminator. The 221 bp terminator sequence was amplified from *P. tricornutum* genomic DNA by PCR using primers *FcpAEco-fw* and *FcpAKpn-rv* (Siaut et al. 2007). The resulting *FcpA*t PCR product was cloned into the *Eco*RI–*Kpn*I sites of pBluescript KS+ (Stratagene) to generate pKS-FcpAt. *NlTUB* promoter and *hptII* gene were amplified from *N. limnetica* genomic DNA and vector pZC1 (GenBank accession number FM200848), respectively, with the primer sets *NlTubSac_fw* and *NlTubNco*_rv, and *HptIINco_fw* and *HptIIPst_rv*. These two fragments were digested with *Sac*I and *Nco*I or *Nco*I and *Pst*I and then ligated into *Sac*I–*Pst*I digested pKS-FcpAt vector to generate pKS-NlTUBp-Hyg (Fig. 1c).

For the construction of pKS-NgTUPp-GUS (Fig. 1d), a non-selective vector, *Nannochloropsis granulata* (MBIC10054) *TUB* (*NgTUB*) promoter was obtained by PCR using the primers *NgTubKpn-fw* and *NgTubSal-rv*, and then replaced the *fcpB* promoter of plasmid pFcpBp-GUS (Falciatore et al. 1999) using standard molecular cloning procedures. Primers used for vector construction are shown in Table 1.

Electroporation transformation protocol

Nannochloropsis limnetica was grown in liquid medium for about 10 days to reach mid-log phase ($OD_{730}=0.6$–0.7), harvested by centrifugation at 4000×*g* for 10 min at 4 °C, washed four times with 375 mM sorbitol, and resuspended to a final concentration of about 3.3×10^9 cells mL^{-1} in 150 µL ice-cold 375 mM sorbitol. The suspension was transferred to 2-mm electroporation cuvette and mixed with 4 µg of linearized plasmid DNA and 40 µg (10 µg μL^{-1}) of salmon sperm DNA (as carrier), and incubated on ice for 30 min. The standard electroporation was performed by a single pulse at 2.4 kV, 600 Ohms, and 50 µF in a Bio-Rad Gene Pulser Xcell Electroporation System except as noted. After electroporation, the cell suspension was transferred to 15-ml conical Falcon tubes containing 10 ml BG11 medium and incubated in low light (~30 µmol photons m^{-2} s^{-1}) for 12–24 h without shaking. Then cells were harvested, resuspended and plated onto solid medium containing antibiotics

Table 1 PCR primers used in this study

Primer	Sequence (5′→3′)
NlTubAor_fw	ATGCtccggaATCATATCGTGCCACAGCAGATTGG
NlTubBal_rv	AGCTtggccaTGGTTGAGACTAGTTGGAGGGAGG
Vcp2Bal-fw	ACGTtggccaTACTTAAGAAGTGGTGGTGGTG
Vcp2Kpn-rv	CGGggtaccACTTGAGAGAGTGGTGGAGTTG
Vcp1Kpn-fw	CGGggtaccggggatcctctagagattGCTTCTGTGGAAGAGCCAGTGGTAG
Vcp1Pst-rv	AActgcaggtcgacgattCTGATCTTGTCCATCTCGTGTGCC
egfpKpn-fw	GGggtaccGGACCTAGGGGAGGAGGAGGAGGAATGGTGAGCAAGGGCGAG
egfpXba-rv	GCtctagaTTACTTGTACAGCTCGTCCATGC
FcpAEco-fw	GTAgaattcTGAGCTACCTCGACTTTGGCT
FcpAKpn-rv	CGAggtaccTGAAGACGAGCTAGTGTT
NlTubSac_fw	GCgagctcATCATATCGTGCCACAGC
NlTubNco_rv	CATGccatggTGGTTGAGACTAGTTGGAG
HptIINco_fw	CATGccatggATGGGTAAAAAGCCTGAACTC
HptIIPst_rv	AActgcagTTATTCCTTTGCCCTCGG
NgTubKpn-fw	ATGCggtaccATCATATCGTGCCACAGCAGATTGG
NgTubSal-rv	AGCTgtcgacTGGTTGAGACTAGTTGGAGGGAGG
ble298-rv	GCTCGCCGATCTCGGTCATG
NlH4-fw	ACATCCAGGGCATTACCAAG
NlH4-rv	GCGTGCTCTGTGTAGGTGAC

Underlined sequences indicate restriction sites used for cloning, and the sequence in box indicates the linker fragment introduced before GFP coding sequence

as described previously (Li et al. 2014). Colonies appeared after 3–4 weeks and were picked after 4–5 weeks. The transformation efficiency was calculated by dividing obtained transformant cell number by the number of cells plated in this transformation reaction.

For the linearization of circular plasmids, pPha-T1-NlTUBp and pPha-NoVCPp-eGFP were digested with *Sca*I, and pKS-NlTUBp-Hyg with *Sac*I (Fig. 1). After digestion, the linearized plasmids were purified on OMEGA Gel & PCR clean up kit D2000 (OMEGA, USA). For the cotransformation, circular plasmid pKS-NgTUPp-GUS (containing the complete *uidA* gene) and the linearized pPha-T1-NlTUBp were used, and both were 2 µg.

Sensitivity to antibiotics after electroporation

Antibiotic tests on agar plates were carried out for the wild-type *N. limnetica* after electroporation with various voltages. Cells were grown, harvested, washed and suspended as mentioned above. For each electroporation, the 150 µL suspension containing 5×10^8 cells was transferred into the 2-mm cuvettes and was electroporated at different electric field strengths ranging from 2000 to 2900 V with 50 µF and 600 Ohms. After the pulse, the cells were suspended in 10 mL BG11 medium and allowed to recover for 12–24 h at 22 °C in low light without shaking. The cell suspensions then were diluted and plated onto 70 mm plates (each with 800 cells in 200 µL) of solid medium with different amounts of antibiotics (zeocin: 0.01, 0.03, 0.07, 0.1, 0.3, 0.5, 1 or 2 µg mL^{-1}; or hygromycin B: 3, 4, 5, 7, 9, 10, 15 or 20 µg mL^{-1}) or without antibiotics. Each test was repeated in triplicate. All plates were kept for 3 weeks under the conditions mentioned above and then colonies were counted by visual inspection. The survival rate was calculated as the mean number of colonies in the plates after 3-week culture divided by 800 cells.

Pretreatment of cells with LiAc and DTT

Before being washed with sorbitol, the harvested cell pellets (5×10^8) were resuspended in 10 mL BG11 medium. Twenty microliters of 5 M LiAc and 20 µL of 1.5 M DTT were added to the suspension, and mixed with rotation for 30 min at 10 °C according to Jaeger et al. (2017). Following pretreatment, the cells were pelleted, resuspended in 1.5 ml Eppendorf tube, and washed as described above.

PCR and Southern blot analysis

Colonies picked from the antibiotic plates were grown to late-logarithmic phase in liquid BG11 with 0.05 µg mL^{-1} zeocin or 5 µg mL^{-1} hygromycin B then cells were collected, and genomic DNA was isolated as described previously (Radakovits et al. 2012). For the genomic PCR, primer pairs *NlTubSac_fw* and *ble298-rv* were used for amplifying the TUB promoter (562 bp) and a part of *sh ble* gene (298 bp), while primers *HptIINco_fw* and *HptIIPst_rv* were used to amplify *HptII* gene (1029 bp). Primers *NlH4-fw* and *NlH4-rv* were used for amplifying a part of (154 bp, nucleotides 77–230 in the coding region) the *N. limnetica histone H4* gene.

The Southern blot was performed as previously described (Li et al. 2014) with some modification. Briefly, 10 µg genomic DNA from two different zeocin-resistant transformants and wild-type cells of *N. limnetica* was completely digested with the *Hin*dIII, *Bam*HI, or *Eco*RI, and the DNA fragments were separated by electrophoresis on 0.7% agarose gels. The DNA was then transferred onto an Immobilon Ny + membrane (Millipore) and used for hybridization with the Digoxigenin labeling of the *sh ble* DNA probe. The labeling and hybridization were performed using the DIG DNA labeling and detection Kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions.

GFP and GUS assay

For GFP expression analysis, the samples were observed by confocal microscopy (Leica TCS SP8 laser scanning confocal microscope). Excitation wavelength was 488 nm, and emission was detected with two photomultiplier tubes at 500–520 nm (green GFP fluorescence) and 625–720 nm (red chlorophyll autofluorescence) respectively. GUS staining was performed according to Jefferson et al. (1987) (Wu and Letchworth 2004) with minor modifications. The GUS transformants were grown to logarithmic phase and then 1.5 ml culture was collected by centrifugation. The cell pellet was washed with distilled water once and suspended in 100 µL of GUS staining solution (50 mM Na-phosphate buffer, pH 7.0; 10 mM Na_2EDTA; 0.1% Triton X-100; 0.5 mM $K_3Fe[CN]_6$; 0.5 mM $K_4Fe[CN]_6$; 2 mM X-Gluc), and incubated for 12 h at 37 °C. After staining, the samples were centrifuged at 6000×*g* for 5 min and the supernatant discarded, and washed once with 70% ethanol and twice with 100% ethanol until the negative controls (wild-type cells) became white. Stained cells were observed by microscopy and photographed.

Results

Transformation based on marine species electroporation protocol

Based on the electroporation protocol for marine *Nannochloropsis*, we tried to transform the freshwater *N. limnetica* with linearized pPha-T1-NlTUBp at 2200 V using 2-mm

electroporation cuvettes. In each transformation reaction, a total of 5×10^8 cells was used and placed on three plates containing 2 μg mL^{-1} zeocin. After 4–5 weeks, 1–2 resistant colonies to the most were obtained on each plate with a transformation efficiency of about 1.1×10^{-8} (Fig. 2a), less than 1% of that for marine species. In order to improve the efficiency, we tried to increase the electric field strengths (2300, 2400, 2500, 2600 and 2900 V) and lower the antibiotic concentration (0.1, 0.2, 0.3 and 0.5 μg mL^{-1} zeocin). However, enhancing electric field strengths did not improve the efficiency significantly, and 1–3 resistant colonies to the most appeared on each plate at electric field strengths ranging from 2300 to 2600 V (Fig. 2a). Increasing the electric field strengths to 2900 V caused cells to turn white and die within 10 days. Resistant colonies did not increase when the zeocin concentration was lowered to 0.5 μg mL^{-1} (Supplemental Fig. S1). Even pulse at 2400 V and selection on 0.1–0.3 μg mL^{-1} zeocin-containing plates gave only one resistant colony. In addition, pulse with electric field strength under 2200 V (500–2100 V) could not obtain resistant colonies regardless of the antibiotic concentration (0.1–2 μg mL^{-1} zeocin).

Survival rate of electroporated cells at different antibiotics

Electroporation increases the permeability of the cell membrane and therefore the electroporated cells may be more sensitive to the antibiotics. To optimize the antibiotic concentration for selection of *N. limnetica*, wild-type *N. limnetica* was electroporated at different electric field strengths (2300–2900 V in 2-mm cuvettes) and then the survival rate was determined against different concentrations of zeocin and hygromycin B (Table 2). The survival rate of electroporated cells was < 50% on the plates without any antibiotics, and it decreased from 42 to 31%

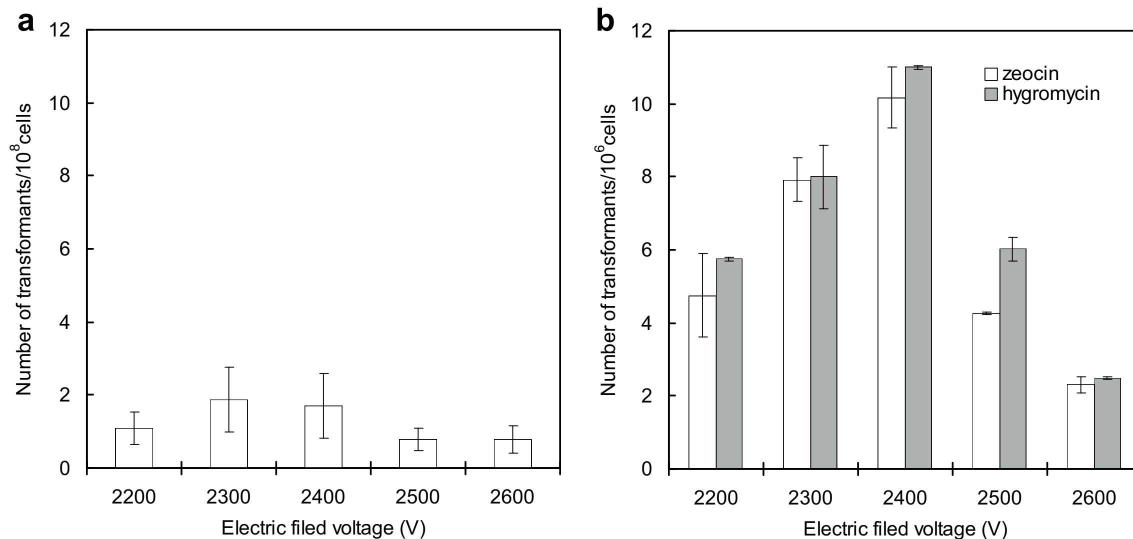


Fig. 2 Transformation efficiency as a function of electric field voltage. **a** *Sca*I-digested pPha-T1-NlTUBp was electroporated into *N. limnetica* without pretreatment and transformants were selected on 2 μg mL^{-1} zeocin plates. **b** *Sca*I-digested pPha-T1-NlTUBp and *Sac*I-digested pKS-NlTUBp-Hyg were electroporated into *N. limnetica* pretreated with LiAc and DTT, and transformants were selected on 0.1 μg mL^{-1} zeocin and 10 μg mL^{-1} hygromycin B plates respectively

Table 2 Survival rate (%) of wild-type cells under different antibiotic concentrations after electroporation with different voltages (data from three biological replicates)

Antibiotic	Concentration (μg mL^{-1})	2300 V	2400 V	2500 V	2600 V	2900 V
	0	41.8 ± 2.0	40.5 ± 2.0	34.6 ± 3.7	31.2 ± 1.3	0
Zeocin	0.01	13.9 ± 4.2	12.5 ± 4.8	10.6 ± 1.0	7.6 ± 1.1	0
	0.03	0.13 ± 0.14	0.13 ± 0.22	0.25 ± 0.22	0.03 ± 0.07	0
	0.07–2	0	0	0	0	0
Hygromycin B	3	3.4 ± 0.8	2.8 ± 2.8	4.1 ± 1.6	3.1 ± 0.3	0
	4	4.4 ± 0.8	3.1 ± 0.4	5.8 ± 1.4	2.8 ± 0.5	0
	5–20	0	0	0	0	0

with the increase of electric field strengths from 2300 to 2600 V. *N. limnetica* could not survive when electroporation was performed with electric field strength up to 2900 V.

Nannochloropsis limnetica is very sensitive to both zeocin and hygromycin B. Compared with the control (without antibiotics), the survival rate of electroporated cells (2300–2600 V) on 0.01 μg mL^{-1} zeocin plates decreased by 67–76%, and decreased by over 99% on 0.03 μg mL^{-1} zeocin plates. For the electroporated cells on hygromycin B plates, the survival rate decreased by 83–93% with 3–4 μg mL^{-1} hygromycin B. Electroporated wild-type cells died within 10 days on the plates containing up to 0.07 μg mL^{-1} zeocin or 5 μg mL^{-1} hygromycin B regardless of the electric field strengths. These data showed that 0.07 μg mL^{-1} of zeocin or 5 μg mL^{-1} of hygromycin B would be enough for the selection of transformants in *N. limnetica* and thus we used 0.1 μg mL^{-1} of zeocin and 10 μg mL^{-1} of hygromycin B respectively in the following experiments.

Increased transformation efficiency of *N. limnetica* pretreated with LiAc and DTT

Much lower transformation efficiency obtained in the freshwater *Nannochloropsis* might be attributed to the lower permeability of cell membrane/cell wall compared with marine species. In fact, algal species display a dramatic variability in the composition and structure of their cell walls. This variability can even be evidenced within a genus, a species and even within a strain or depending on the life stage of the cell (Baudelet et al. 2017). Eliminating or weakening the cell wall will increase permeability of cell and thus might improve transformation efficiency. As a simpler approach, weakening the cell wall could be achieved by chemical pretreatment. Usually, chemical pretreatment can weaken or even disrupt the chemical bindings between the components of the cell wall, although Suchodolskis et al. (2011) found that pretreatment by lithium acetate and dithiothreitol increased stiffness and roughness of the cell wall in *Saccharomyces cerevisiae*.

In this study, the transformation efficiency of *N. limnetica* was enhanced dramatically by electroporation after inclusion of a LiAc and DTT pretreatment step. As seen in Fig. 2b, *N. limnetica* transformation efficiency was enhanced by approximately 300–600-fold when the cells were treated with LiAc and DTT prior to electroporation. The highest transformation efficiency of $10–11 \times 10^{-6}$ (Fig. 2b) was obtained with an electric field strength of 12,000 V/cm, which was comparable with that reported in marine *Nannochloropsis* (Li et al. 2014).

Confirmation of stable transformation

Resistant colonies were inoculated into BG11 medium and the cultures were sub-cultured regularly at 2 week intervals for over 1 year. The transformants were also maintained on medium containing zeocin (0.05 μg mL^{-1}) or hygromycin B (5 μg mL^{-1}) and their growth was observed to verify the stability of transformants. Wild-type could not survive in the media with antibiotics, while transformants exhibited robust growth but different sensitivity to varying concentrations of zeocin (Supplemental Fig. S2). In addition, the integration of the transgene into the genomic DNA was investigated by PCR using primer pairs *NlTubSac_fw* and *ble298-rv* (TUB promoter and a part of *sh ble* gene, 860 bp) or *HptIINco_fw* and *HptIIPst_rv* (full length of gene *HptII*, 1029 bp). Figure 3a shows the PCR results of wild-type (wt) and randomly picked zeocin or hygromycin B resistant colonies. All of the resistant cell lines and plasmid (positive control) contained a DNA fragment of the correct size while no amplified product was detected in wild-type samples. These results suggested that the exogenous antibiotic genes have been integrated into the genome of *N. limnetica*.

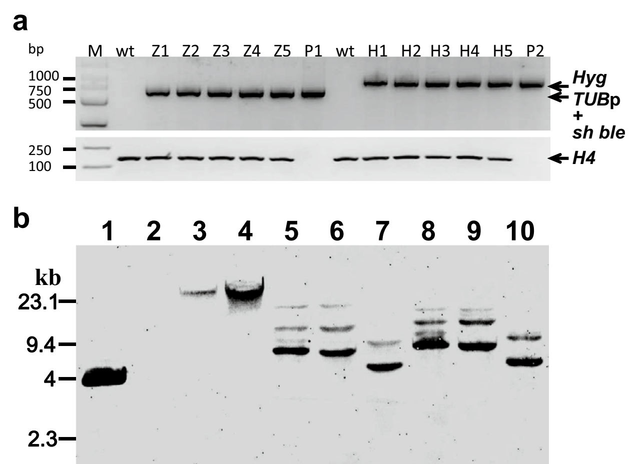


Fig. 3 Confirmation of *N. limnetica* transformation. **a** PCR amplification of *β-tubulin* promoter region and partial *sh ble* gene sequence (860 bp) from genomic DNA of zeocin-resistant transformants (Z1–Z5), and amplification of *hptII* gene sequence (1029 bp) from genomic DNA of hygromycin B-resistant transformant (H1–H5). M: DL2000 DNA marker (Takara); wt: wild-type *N. limnetica*; P1: plasmid pPha-T1-NlTUBp; P2: plasmid pKS-NlTUBp-Hyg. *H4* serves as an internal control for PCR amplification (154 bp). **b** Southern blot analysis of zeocin-resistant and wild-type (wt) *N. limnetica* strains. Southern blot probed with DIG-labeled *sh ble* DNA. Lane 1, plasmid pPha-T1-NlTUBp (10 ng); lane 2, wt genomic DNA (10 μg); lanes 3 and 4, genomic DNA (10 μg) from transformed strains Z1 and Z3 respectively; lanes 5–7, genomic DNA (10 μg) from transformed strains Z1 digested with *Hin*dIII, *Bam*HI and *Eco*RI respectively; lanes 8–10, genomic DNA (10 μg) from transformed strains Z3 digested with *Hin*d III, *Bam*H I and *Eco*R I respectively

To further confirm the integration and define the copy number of antibiotic genes, Southern blot analysis was performed for two zeocin resistant transformants using the *sh ble* gene as a probe (Fig. 3b). The digestion of DNA with restriction enzymes *Hin*dIII, *Bam*HI or *Eco*RI yielded 2–4 hybridizing bands of different sizes in transformants. DNA samples without digestion gave one band in the two transformants and while no bands were observed in DNA extracted from wild-type (Fig. 3b). The Southern blot analysis clearly indicated that the transgene is incorporated into the genome of *N. limnetica*. Since multiple bands and different band sizes were observed, it was indicated that the integration of introduced genes was probably random.

Expression of the exogenous reporter genes

In order to express reporter genes, *eGFP* and *GUS* were introduced respectively by electroporation and their expression was driven by exogenous *N. oceanica VCP* promoter and *N. granulata* TUB promoter. Expression of the *eGFP* gene in the *N. limnetica* transformants was visualized by fluorescence microscopy. Cells were almost filled with green fluorescence and the fluorescence intensity was less in plastid, showing that GFP protein might be expressed in the cytosol (Fig. 4a). To introduce non-selectable pKS-NgTUPp-GUS plasmid into *N. limnetica* cells, pKS-NgTUPp-GUS and linearized pPha-T1-NlTUBp (confers resistance to zeocin) were co-transformed by electroporation as described before (Li et al. 2014; Zhang and Hu 2014). Co-transformation efficiency achieved was over 50%, which is comparable with that of our previous studies on *P. tricornutum* (Zhang and Hu 2014) and *N. salina* (Li et al. 2014). GUS activity can be easily detected with the naked eye, and microscopy further confirmed the in vivo GUS expression in *N. limnetica* (Fig. 4b).

Discussion

Stable transformation systems for *N. oceanica* and *N. gaditana* have been established several years ago (Kilian et al. 2011; Radakovits et al. 2012; Vieler et al. 2012). We reported the genetic transformation approaches in other three marine *Nannochloropsis* species *N. granulata*, *N. salina*, and *N. oculata* (Li et al. 2014). Successful and efficient transformation of these marine *Nannochloropsis* with the robust cell wall requires unusually high electric field strengths (around 11–12 kV/cm). In addition, 2 or 3 μg ml^{-1} zeocin was usually used for screening transformants in marine *Nannochloropsis* (Kilian et al. 2011; Radakovits et al. 2012; Li et al. 2014). Noda et al. (2017) tested the effects of 11 antibiotics on growth of *N.*

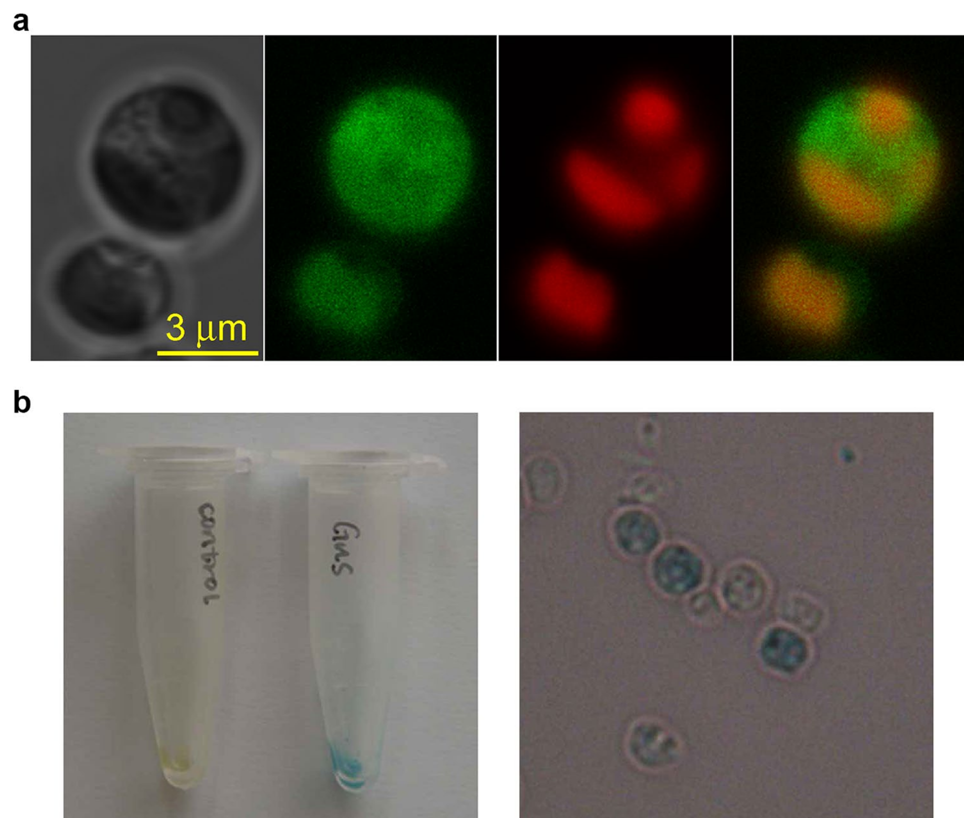


Fig. 4 Expression of *eGFP* and *GUS* gene in *N. Limnetica*. **a** Fluorescent microscope images of the transformant expressing *eGFP* under the control of the exogenous *N. oceanica VCP* promoter. Panels show microscopical images of transmitted light, GFP fluorescence, chlorophyll autofluorescence, merged image of chlorophyll and GFP fluorescence from left to right. **b** GUS histochemical assay of the wild type (control) and transformed (GUS) cells with naked eye (left) and microscope (right). *GUS* was expressed under the control of the exogenous *N. granulata TUB* promoter

limnetica and two marine *Nannochloropsis*, and showed that the freshwater *Nannochloropsis* exhibited a much higher sensitivity to antibiotics. They tested four concentrations of zeocin (1, 5, 10, or 40 μg mL^{-1}), and found that 1 μg mL^{-1} resulted in 50% and 41% reduction respectively in growth of *N. limnetica* and *N. oceanica* in liquid cultures, while 5 μg mL^{-1} caused the cells to turn white and resulted in cell death on agar plates after 14 days. Furthermore, they observed that 5 μg mL^{-1} hygromycin resulted in the death of all *N. limnetica* cells in 14 days on the plates (Noda et al. 2017), while 50 μg mL^{-1} hygromycin B was used for the selection of transformants in *N. oceanica* (Vieler et al. 2012). We determined the optimal selection concentration of zeocin and hygromycin B for *N. limnetica*, which is much lower than the marine *Nannochloropsis*. However, high electric field strengths (11.5–13 kV/cm) with lower antibiotic concentrations did not give rise to efficient transformation in the freshwater species *N. limnetica*.

It is known that in eukaryotic algae, the rigid cell wall could be a barrier to transformation. Although Noda et al. (2017) observed that the cell wall of *N. limnetica* was thinner than in *N. oceanica*, and Fietz et al. (2005) found *N. limnetica* cells were less permeable in exponential growth phase. Pretreatment with lithium acetate and DTT can weaken cell wall thus greatly enhance transformation efficiency in yeast *Pichia pastoris* (Wu and Letchworth 2004), bacterium *Lactococcus lactis* (Papagianni et al. 2007) and green alga *Monoraphidium neglectum* (Jaeger et al. 2017). In this study, based on an established marine *Nannochloropsis* species electrotransformation protocol, a tremendous improvement of transformation efficiency was achieved in *N. limnetica* cells treated with LiAc and DDT prior to electroporation. The described strategy for improvement of transformation efficiency can also provide some tips for the development of an efficient electrotransformation system in other freshwater microalgae as well.

Acknowledgements This work was supported by the National Key R&D Program of China (Grant No. 2018YFD0901500) and National Natural Science Foundation of China (Grant No. 91751117).

Author contributions HH, taking responsibility for the integrity of the work as a whole, designed the research, analyzed the data and wrote the manuscript. YC performed the experiments and analyze the data. All authors agree on the authorship and submission of the manuscript for peer review.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Informed consent No informed consent, human or animal rights applicable.

References

Andersen RA, Brett RW, Potter D, Sexton JP (1998) Phylogeny of the Eustigmatophyceae based upon 18S rDNA, with emphasis on *Nannochloropsis*. Protist 149:61–74

Baudelet P-H, Ricochon G, Linder M, Muniglia L (2017) A new insight into cell walls of Chlorophyta. Algal Res 25:333–371

Chini Zittelli G, Lavista F, Bastianini A, Rodolfi L, Vincenzini M, Tredici MR (1999) Production of eicosapentaenoic acid by *Nannochloropsis* sp. cultures in outdoor tubular photobioreactors. J Biotechnol 70:299–312

Falciatore A, Casotti R, Leblanc C, Abrescia C, Bowler C (1999) Transformation of nonselectable reporter genes in marine diatoms. Mar Biotechnol 1:239–251

Fawley MW, Jameson I, Fawley KP (2015) The phylogeny of the genus *Nannochloropsis* (Monodopsidaceae, Eustigmatophyceae), with descriptions of *N. australis* sp. nov. and *Microchloropsis* gen. nov. Phycologia 54:545–552

Fietz S, Bleiß W, Hepperle D, Koppitz H, Krienitz L, Nicklisch A (2005) First record of *Nannochloropsis limnetica* (Eustigmatophyceae) in the autotrophic picoplankton from Lake Baikal. J Phycol 41(4):780–790

Freire I, Cortina-Burgueño A, Grille P, Arizcun MA, Abellán E, Segura M, Sousa FW, Otero A (2016) *Nannochloropsis limnetica*: A freshwater microalga for marine aquaculture. Aquaculture 459:124–130

Gbadamosi OK, Lupatsch I (2018) Effects of dietary *Nannochloropsis salina* on the nutritional performance and fatty acid profile of Nile tilapia, *Oreochromis niloticus*. Algal Res 33:48–54

Jaeger D, Hübner W, Huser T, Mussgnug JH, Kruse O (2017) Nuclear transformation and functional gene expression in the oleaginous microalga *Monoraphidium neglectum*. J Biotechnol 249:10–15

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6(13):3901–3907

Kilian O, Benemann CSE, Niyogi KK, Vick B (2011) High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. Proc Natl Acad Sci USA 108(52):21265–21269

Krienitz L, Wirth M (2006) The high content of polyunsaturated fatty acids in *Nannochloropsis limnetica* (Eustigmatophyceae) and its implication for food web interactions, freshwater aquaculture and biotechnology. Limnologica 36(3):204–210

Krienitz L, Hepperle D, Stich H-B, Weiler W (2000) *Nannochloropsis limnetica* (Eustigmatophyceae), a new species of picoplankton from freshwater. Phycologia 39(3):219–227

Li F, Gao D, Hu H (2014) High-efficiency nuclear transformation of the oleaginous marine *Nannochloropsis* species using PCR product. Biosci Biotechnol Biochem 78(5):812–817

Ma Y, Wang Z, Yu C, Yin Y, Zhou G (2014) Evaluation of the potential of 9 *Nannochloropsis* strains for biodiesel production. Bioresource Technol 167:503–509

Noda J, Mühlroth A, Bučinská L, Dean J, Bones AM, Sobotka R (2017) Tools for biotechnological studies of the freshwater alga *Nannochloropsis limnetica*: antibiotic resistance and protoplast production. J Appl Phycol 29(2):853–863

Papagianni M, Avramidis N, Filioussis G (2007) High efficiency electrotransformation of Lactococcus lactis spp lactis cells pretreated with lithium acetate and dithiothreitol. BMC Biotechnol 7:15

Radakovits R, Jinkerson RE, Fuerstenberg SI, Tae H, Settlage RE, Boore JL, Posewitz MC (2012) Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. Nat Commun 3:686

Rodolfi L (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol Bioeng 102(1):100–112

Siaut M, Heijde M, Mangogna M, Montsant A, Coesel S, Allen A, Manfredonia A, Falciatore A, Bowler C (2007) Molecular toolbox for studying diatom biology in *Phaeodactylum tricornutum*. Gene 406(1–2):23–35

Soria-Verdugo A, Goos E, García-Hernando N, Riedel U (2018) Analyzing the pyrolysis kinetics of several microalgae species by various differential and integral isoconversional kinetic methods and the Distributed Activation Energy Model. Algal Res 32:11–29

Suchodolskis A, Feiza V, Stirke A, Timonina A, Ramanaviciene A, Ramanavicius A (2011) Elastic properties of chemically modified baker's yeast cells studied by AFM. Surf Interface Anal 43(13):1636–1640

Sukenik A (1998) Production of eicosapentaenoic acid by the marine eustigmatophyte Nannochloropsis sp. In: Cohen Z (ed) Chemicals from microalgae. Taylor and Francis, London, pp 41–56

Vieler A, Wu G, Tsai CH, Bullard B, Cornish AJ, Harvey C, Reca IB, Thornburg C, Achawanantakun R, Buehl CJ, Campbell MS, Cavalier D, Childs KL, Clark TJ, Deshpande R, Erickson E, Ferguson AA, Handee W, Kong Q, Li X, Liu B, Lundback S, Peng C, Roston RL, Sanjaya Simpson JP, TerBush A, Warakanont J, Zäuner S, Farre EM, Hegg EL, Jiang N, Kuo MH, Lu Y, Niyogi KK, Ohlrogge J, Osteryoung KW, Shachar-Hill Y, Sears BB, Sun Y, Takahashi H, Yandell M, Shiu SH, Benning C (2012) Genome, functional gene annotation, and nuclear transformation of the Heterokont oleaginous alga *Nannochloropsis oceanica* CCMP1779. PLoS Genet 8(11):e1003064

Wang D, Ning K, Li J, Hu J, Han D, Wang H, Zeng X, Jing X, Zhou Q, Su X, Chang X, Wang A, Wang W, Jia J, Wei L, Xin Y, Qiao Y, Huang R, Chen J, Han B, Yoon K, Hill RT, Zohar Y, Chen F, Hu Q, Xu J (2014) *Nannochloropsis* genomes reveal evolution of microalgal oleaginous traits. PLoS Genet 10(1):e1004094

Wu S, Letchworth GJ (2004) High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. Biotechniques 36(1):152–154

Zhang C, Hu H (2014) High-efficiency nuclear transformation of the diatom *Phaeodactylum tricornutum* by electroporation. Mar Genomics 16:63–66

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.