Algal Research 17 (2016) 196–201

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Stable nuclear transformation of the industrial alga *Chlorella pyrenoidosa*

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ARTICLE INFO

Article history:
Received 20 July 2015
Received in revised form 18 April 2016
Accepted 5 May 2016
Available online xxxx

Keywords:
Genetic engineering
Electroporation
Chlorella pyrenoidosa
Nuclear transformation
eGFP

ABSTRACT

Chlorella pyrenoidosa is an extensively investigated species. This species has been used as a commercial microalgal feedstock of proteins, lipids, and chlorophyll for nourishment and aquaculture. As such, reliable and easy genetic modification procedures should be developed to improve this industrial microalga. In this study, a pGreen 0029 vector containing an *eGFP* gene under the control of a *Ubiquitin* promoter and *Npt*II, which is a selective marker gene, was constructed to explore and optimize the electroporation method of *C. pyrenoidosa*. The optimal transformation efficiency was approximately 101 ± 7 transformants per μg plasmid and was obtained under the following conditions: 5×10^6/plate cell density in logarithmic phase; 30 μg/mL plasmid; 660 V pulse voltage; 3.5 ms pulse width; and 30 μg/mL G418. The *Npt*II and *eGFP* genes were identified successfully at the DNA level after transformants were screened and purified. High-intensity green fluorescence was observed in the transformants by using fluorescence and laser confocal microscope. Results showed that foreign genes in the transformants were integrated stably and could be expressed. Hence, the efficient transformation system of *C. pyrenoidosa* can be used to biotechnologically improve this important industrial microalgal species and to facilitate systematic functional genomic studies.

1. Introduction

The unicellular green alga *Chlorella* grows rapidly, easily adapts to culture conditions, and contains abundant bioactive components, such as proteins, lipids, and pigments; *Chlorella* has also become a dominant alga of nutraceuticals, aquaculture, alternative renewable energy source [1], carbon fixation [2], and wastewater treatment [3]. This alga can also be used as a platform to produce properly folded valuable proteins, such as vaccine antigens [4,5] and other functional proteins [6,7].

Chlorella pyrenoidosa, a robust industrial species, has been utilized in large-scale production through heterotrophic and outdoor-photolithotrophic cultivation. This species has also been considered as a new food resource by the Food and Drug Administration of China. In the commercial production of algal biomass used for biofuels and high-value bioproducts, high cell productivity is desirable to reduce the costs of downstream processing. Under a stand-alone heterotrophic culture condition, *C. pyrenoidosa* exhibits a competitive growth rate (avg. 2 g/L/h) and can reach >150 g/L dry-cell weight [8], which is comparable to the competitive growth rate of a commercial yeast fermentation system (approximately 130 g/L) [9]. A series of strategies to cultivate microalgae, especially to efficiently produce *Chlorella* biomass and lipid, were proposed by our group [10,11]. In our previous study, *C. pyrenoidosa* was characterized by an almost absent lag phase during heterotrophic growth and a strong tolerance to high glucose concentrations. Therefore, the heterotrophic *C. pyrenoidosa* seed can be used in photoautotrophy to increase the biomass and lipid productivities of photoautotrophic cultures [11]. *C. pyrenoidosa* powder was successfully produced by Jiaxing Zeyuan Company, China, through an industrial trial. Moreover, *C. pyrenoidosa* was subjected to whole genome and transcriptome sequencing by our group for the first time (NCBI Whole Genome Sequencing Project PRJNA171991). However, an effective and stable genetic transformation method of this species has yet to be studied. Thus, reliable and convenient genetic modification procedures should be developed to improve this industrial microalga.

Several methods, including glass bead method [7], *Agrobacterium tumefaciens*-mediated method [12,13], PEG-mediated protoplast transformation [14] and electroporation [6,15,16], have been developed to transform *Chlorella*. Among these methods, electroporation is a preferred and efficient method; this method has also been applied to transformation various microalgae, such as *Chlamydomonas reinhardtii* [17], *Scenedesmus obliquus* [18], and *Nannochloropsis* sp. [19]. However, few of these species are of commercial interest. The present study is aim to develop an efficient method to genetically transform *C. pyrenoidosa* through electroporation; various parameters, such as pulse voltage, pulse time, and plasmid amount, which affect efficiency, were optimized. Our research provides a foundation to develop *C. pyrenoidosa* into an industrial algal model in metabolic engineering to develop carbon fixation and bioenergy; the proposed model is also a new low-cost and highly efficient bioreactor for recombinant protein production.

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http://dx.doi.org/10.1016/j.algal.2016.05.002
2211-9264/

2. Materials and methods

2.1. Cultivation of microalgae.

C. pyrenoidosa (FACHB 9) cells, obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), were cultured in the modified Endo medium [20,21] at 30 °C under continuous illumination (120 μmol/m^2/s) with a rotary shaker (150 rpm) for 3 days. The cells in the early logarithmic growth phase were used as hosts.

2.2. Antibiotic sensitivity of C. pyrenoidosa

The sensitivity of *C. pyrenoidosa* to G418 was investigated. Approximately 5×10^6 cells were plated uniformly in a solid SE medium [22] with different concentrations of G418 ranging from 15 μg/mL to 25 μg/mL, with a gradient of 2.5 μg/mL. The plates were cultured in an illumination incubator under 60 μmol/m^2/s at 30 °C, and the growth of *C. pyrenoidosa* was observed and recorded daily for 15 days.

2.3. Construction of expression vectors for genetic transformation

The binary vector pGreenII 0029 used in this study is a highly efficient vector for the genetic transformation of green algae [6]. Two plasmids, namely, pSoup and pGreenII 0029-*Ubi-Nos*, were kindly provided by Professor Hu, and the biological functions have been described in detail [6]. The expression vector pGreenII 0029-*Ubi-eGFP-Nos* (abbreviation for 0029UeN) was constructed through the following procedures: *eGFP* genes were digested with the double restriction endonucleases, namely, *Spe*I and *Not*I; the digested genes were subsequently ligated into pGreen 0029-*Ubi-Nos.*

2.4. Optimization of the electroporation method

The transformation parameters, namely, pulse voltage, pulse length, and plasmid concentration, were investigated to establish an optimal transformation method. The pulse voltages were set from 0 V to 1060 V with a gradient of 100 V; the pulse lengths were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 ms. The DNA plasmid concentrations were 6, 12, 18, 24, 30, 40, 50, and 60 μg/mL.

2.5. Generation of transgenic C. pyrenoidosa through electroporation

Electroporation was performed in accordance with previously described methods [6] with necessary modifications. *C. pyrenoidosa* in the early logarithmic growth phase was harvested and treated with pre-cooling osmosis (0.2 M mannitol and 0.2 M sorbitol) in an ice-water bath for 40 min and re-suspended in a pre-cooling electroporation buffer (0.2 M sorbitol, 0.2 M mannitol, 0.08 M KCl, 0.005 M $CaCl_2$, and 0.01 M Hepes; pH 7.2) at a concentration of approximately 5×10^6 cells/mL. The treated *C. pyrenoidosa* was immediately mixed with different concentrations of circular plasmid 0029UeN, and a final concentration of 30 μg/mL circular plasmid pSoup and 150 μg/mL salmon sperm DNA (Invitrogen). The plasmids were extracted and purified by using a HighPure Maxi plasmid kit (TIANGEN Biotech, China); the purified plasmids were then dissolved in sterile ddH_2O. The DNA-cell mixture (100 μL) was transferred to a 0.2 cm electroporation cuvette and was kept in an ice bath for 5–10 min. A Bio-Rad Gene Pulser Xcell™ electroporation system (Bio-Rad, USA) was used to generate a transgenic *C. pyrenoidosa*. After electroporation was performed, *C. pyrenoidosa* cells were transferred to a 12-well plate containing 1.5 mL of BBM medium [23] and were cultured in the dark at 30 °C for 24 h. The cultured cells were harvested and re-suspended in 200 μL of sterile ddH_2O; the cells were plated onto SE agar plates containing 30 μg/mL G418. The selection agar plate cultures were incubated and exposed to continuous fluorescent light with 60 μmol/m^2/s at 30 °C for 15 days. The cells treated with the same transformation protocol but without a plasmid was set as the negative control.

2.6. PCR detection of transformants and southern blot analysis

The transformants obtained in the screening plates were inoculated on the liquid SE medium containing 15 μg/mL G418 to expand the culture. Then, the genomic DNA was isolated using the Plant Genome Extraction Kit (TIANGEN Biotech, China). gDNA was used to detect the integration of *Npt*II with the following primers: 1-F (5′-GACGTTGTCA CTGAAGCGGGAAG-3′) and 1-R (5′-GGCGATACCGTAAAGCACGAGGA-3′); gDNA was also used to detect the integration of *eGFP* with the following primers: 2-F (5′-AAGGACGACGGCAACTACAAGACC-3′) and 2-R (5′-CACGAACTCCAGCAGGACCATG-3′) to amplify 498 and 372 bp fragments. Genomic DNAs were also isolated from wild *C. pyrenoidosa* and were used as a negative control; 0029UeN was set as a positive control. For reverse transcription (RT)-PCR detection, RNA was isolated according to the Trizol RNA extraction procedure (Invitrogen, USA). The primers (F: GCTCAACTCCTCCACGCT, R: GTCCTTGCGGATGTCCAC) were used to amplify the *actin* gene fragment (187 bp) as an internal standard.

Eight milligrams of genomic DNA was digested with *Pst*I, which have no recognition sites in the probed region of the *Npt*II gene. The digested DNA was separated on a 0.7% agarose gel, transferred to a positively charged nylon membrane (Roche, Mannheim, Germany), and hybridized with digoxigenin (DIG)-labeled DNA probes in the presence of 50% (v/v) formamide at 37 °C. DNA probe was prepared by amplifying a 400-bp fragment of the *Npt*II gene with a pair of specific primers (Probe F: CCCCTGATGCTCTTCGTCC; Probe R: CTCTGATGCCGCCGTGTT)

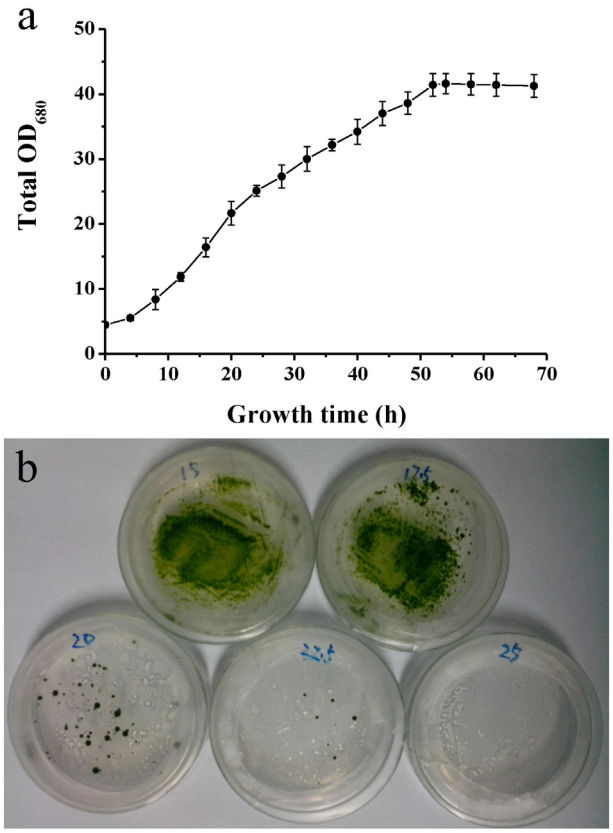

Fig. 1. Growth characteristics of *Chlorella pyrenoidosa.* (a) Growth curve of *C. pyrenoidosa* in liquid Endo medium in 250 mL flask. Cells were cultured at 30 °C under continuous illumination (120 μmol/m^2/s) with a rotary shaker (150 rpm) for about 3 days; (b) Growth state of *C.pyrenoidosa* on culture dish under different concentrations of G418 ranging from 15 μg/mL to 25 μg/mL, with a gradient of 2.5 μg/mL.