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Development of a stable genetic system for *Chlorella vulgaris*—A promising green alga for CO_2 biomitigation

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ABSTRACT

Chlorella vulgaris has been proposed as a promising green alga for CO_2 biomitigation due to its attractive traits of high growth rate, excellent CO_2 fixation ability and broad industrial applications. Nevertheless, the genetic engineering of this alga is still in its infancy. In the present study, a stable and efficient transformation system was established for *C. vulgaris* CBS 15-2075, a robust strain with great potential for CO_2 biomitigation. The antibiotic sensitivity spectrum of this organism was evaluated, and the *nptII* gene was selected as a dominant selectable marker for genetic transformation. The selectable marker, together with an enhanced green fluorescent protein (EGFP) gene, was delivered into *C. vulgaris* protoplasts by using a PEG-mediated method, giving a transformation efficiency of 356 ± 30 cfu per μg vector DNA. Molecular characterization and live-cell fluorescence microscopy demonstrated that the *EGFP* gene was stably integrated into *C. vulgaris* genome and expressed in the cytoplasm of transformed cells. Taken together, we for the first time established a stable and useful genetic toolkit for the industrially important microalga *C. vulgaris*, which will facilitate to a great extent the future rational genetic manipulation for strain improvement to increase CO_2 fixation capacity and may as well provide valuable insights into other *Chlorella* species. Besides, the success in EGFP expression and live-cell fluorescence detection in *C. vulgaris* will be useful in molecular and cell biology for protein subcellular localization.

1. Introduction

Global warming, contributed largely by the increasing level of anthropogenic greenhouse gases (GHG) in the atmosphere, has received great concern worldwide [1]. Carbon dioxide (CO_2) is a major component of GHG primarily released from fossil fuel emissions. The level of CO_2 in the atmosphere is rising alarmingly and has reached to 396 ppm in 2013, approximately 42% higher than the pre-industrial level of 280 ppm [2]. Recently, CO_2 biomitigation has garnered a surge of attention, and the microalgae-mediated biomitigation is of particular interest owing to their unique traits such as great photosynthetic efficiency, rapid growth, and ease of culture for high biomass production [3,4].

There have been many studies reporting the utilization of microalgae for CO_2 biomitigation, including *Chlorella vulgaris* [5,6], *Nannochloropsis oculata* [7], *Dunaliella tertiolecta* [8] and *Scenedesmus obliquus* [9]. Among them, *C. vulgaris* is considered as a promising candidate, as it has good tolerance to high CO_2 concentration (up to 18%), and is capable of maintaining satisfactory growth and CO_2 fixation rate under different CO_2 conditions ranging from 0.04% (*v*/*v*, ambient air) to 18% (*v*/*v*) [6,9–12]. In addition, *C. vulgaris* can accumulate significant amounts of valuable fine metabolites, such as lipids, proteins, carotenoids and vitamins, expanding its applications from biodiesel feedstock to feed and food additives [13–16]. The integrated production of these value-added co-products with CO_2 fixation will benefit, to a great extent, the future development of employing *C. vulgaris* for sustainable CO_2 biomitigation.

The CO_2 fixation ability of *C. vulgaris* is subject to not only external factors such as nitrogen and light supply but also the inherent photosynthetic capacity [17,18]. Finding ways to overcome the photosynthetic capacity is of substantial importance for promoting CO_2 fixation ability. Recently, genetic engineering has proven to be a feasible approach to manipulate photosynthesis towards enhanced CO_2 fixation in plants [19–21], which may provide valuable implications into the engineering of *C. vulgaris* to promote the photosynthetic CO_2 fixation capacity.

The establishment of a stable transformation system is crucial for genetic manipulation of *C. vulgaris*. In a transformation system, a selectable marker is generally regarded as a prerequisite for the selection of positive transformants [22]. Moreover, an efficient and stable transformation method was also required in a transformation system for the integration of exogenous DNA into the nuclear genome. Several

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transformation methods have hitherto been developed for *Chlorella* species, including particle bombardment [23,24], electroporation [25, 26], polyethylene glycol (PEG)-mediated method [27,28] and *Agrobacterium*-mediated method [29]. As far as we know, only one study reported the establishment of a stable transformation system for *C. vulgaris* by particle bombardment [23]. However, particle bombardment bears such limitations as poor repeatability and high cost for specialized equipment, and it is generally considered to be more suitable for algal transient transformation and plastid transformation [30,31]. An alternative transformation method that is cost effective and easy to conduct is in sought for *C. vulgaris*. The PEG-mediated method has proven to be such a method that is easy to perform involving a minimum of handlings, equipment and costs in transformations of various cell types with good reproducibility [32].

In the present study, *C. vulgaris* CBS 15-2075 was selected for transformation development. This strain has been shown to grow robustly under ambient conditions and have the maximum CO_2 fixation rate of 0.2846 g L^{-1} d^{-1}, suggesting a great potential for industrial CO_2 remediation. We first evaluated the sensitivity of *C. vulgaris* CBS 15-2075 to four antibiotics, and picked out an optimal selectable marker, *nptII* gene, for use in the subsequent genetic transformation. Then we employed a reporter, the enhanced green fluorescent protein (EGFP) gene, to evaluate the heterologous expression in *C. vulgaris*. Our study established, for the first time, a PEG-mediated stable nuclear transformation system for the industrially important microalga *C. vulgaris*, which will facilitate the future genetic engineering for strain improvement to increase CO_2 fixation capacity and may as well give valuable implications for other *Chlorella* species. Furthermore, the success in EGFP expression and distinguished fluorescence detection in *C. vulgaris* provides a potent toolkit allowing great applications in molecular and cell biology such as heterologous gene expression and subcellular localization.

2. Materials and methods

2.1. Strains and culture conditions

C. vulgaris CBS 15-2075 used in this study was obtained from Carolina Biological Supply Company, USA. This alga was axenically maintained at 16 °C in the dark in liquid Basal medium [33] consisting of (per liter) 1.25 g KNO_3, 1.25 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g $Na_2EDTA \cdot 2H_2O$, 0.114 g H_3BO_3, 0.111 g $CaCl_2 \cdot 2H_2O$, 0.05 g $FeSO_4 \cdot 7H_2O$, 0.088 g $ZnSO_4 \cdot 7H_2O$, 0.014 g $MnCl_2 \cdot 4H_2O$, 0.016 g $CuSO_4 \cdot 5H_2O$, 7.1 mg MoO_3, and 4.9 mg $Co(NO_3)_2 \cdot 6H_2O$. The pH of the medium was adjusted to 6.1 prior to autoclaving. Cells were passaged once a month by inoculating them to fresh liquid medium at 10% (*v*/*v*).

For culture conditions, in brief, twenty milliliters of liquid Basal medium in 100-mL Erlenmeyer flasks was inoculated at 10% (*v*/*v*) from a stock culture, and cultured for 4 days at 25 °C with orbital shaking at 150 rpm under a continuous photon flux density of 40 μmol m^{-2} s^{-1}. The exponentially growing suspension was then inoculated at 10% (*v*/*v*) into 250-mL flasks containing 50 mL of liquid medium, grown to late exponential phase under the same conditions as described above, and used as seed cultures for further experiments. For antibiotic sensitivity assay, cells were incubated on 1.5% Basal agar plates at 25 °C and illuminated at a 12/12 h light/dark cycle under a photon flux density of 40 μmol m^{-2} s^{-1}. For transformation experiments, cells were incubated under the same conditions in addition to growing on agar plates supplemented with 0.05 M glucose. While for the other experiments, cells were grown in a 250-mL Erlenmeyer flask containing 50 mL of liquid medium under above conditions.

2.2. Antibiotic sensitivity assay

The sensitivity of *C. vulgaris* to four common antibiotics, including spectinomycin, geneticin (G418), kanamycin and chloramphenicol, was investigated. Aliquots of a late-exponential-phase culture containing 1×10^6 cells were respectively plated on Basal agar plates supplemented with different concentrations of G418 (5, 10, 20, 30, 40 and 50 μg/mL) and the other four antibiotics (100, 200, 300, 400 and 500 μg/mL). Medium with no antibiotics was used as a control. Ethanol, the solvent for chloramphenicol stock solution preparation, was also tested as a control. After an incubation period of 2–3 weeks, surviving colonies were scored, and differential sensitivity of *C. vulgaris* in response to four antibiotics was evaluated. The number of cells unable to form colonies under each antibiotic concentration was expressed as an inhibition percentage of the colony number under no antibiotic conditions. Then, LC_{50} values, the median concentrations that are lethal to 50% of the tested organism (*viz.* 50% inhibition), were determined according to the Probit Method [34] and taken as a measure of antibiotic resistance for algal cells.

To test the efficiency of selected antibiotic in liquid media, 30-mL aliquots of an exponentially growing culture in 100-mL flasks were exposed to G418 at various concentrations of 2.5, 5, 10, 20, 30, 40 and 50 μg/mL, respectively. Culture collection with no antibiotics was also used as a control. In a preliminary test (data not shown), it was found that there was a positive linear relationship between the cell numbers and optical density (440 nm) within the range of 0 to 0.8. Thus, cell growth was monitored by the absorbance (*A*) of culture broth at 440 nm after 0, 4, 7, 14 and 21 days. The percent inhibition of cell growth (*I*%) was then calculated by the following equation:

$$I\% = \left(\frac{A_{\text{control}} - A_{\text{treated}}}{A_{\text{control}}}\right) \times 100\%.$$

2.3. Vector construction

The enhanced green fluorescent protein (*EGFP*) gene was amplified by PCR from pET28a-EGFP (preserved in our lab) using EGFPfp (5′-CGCTCTAGAATGGTGAGCAAGGGCGAG-3′, the *Xba*I restriction site is underlined) and EGFPrp (5′-CGCGAGCTCTTACTTGTACAGCTCGTCCATGCC-3′, the *Sac*I restriction site is underlined) primers. The amplified fragment was subcloned into pGM-T vector (Tiangen, China) for sequencing, and a 730-bp *Xba*I/*Sac*I-digested fragment of the *EGFP* gene was then inserted into the *Xba*I/*Sac*I site of binary vector pBI121 (kindly provided by Prof. Junchao Huang, Kunming Institute of Botany, CAS, Kunming, China) to produce the transformation vector pBI-EGFP. Thus, the *EGFP* gene was under the control of Cauliflower mosaic virus (CaMV35S) promoter, which proved to be effective for foreign gene expression in *C. vulgaris* [23], followed by a selectable marker of *nptII* gene conferring resistance to kanamycin and G418 (Fig. 1). Plasmids were propagated in *Escherichia coli* TOP10 (Transgen, China) and purified using a standard procedure [35].

2.4. Protoplast preparation

Cells from a 50-mL exponential-phase culture at a cell density of approximately 1×10^7/mL were harvested by centrifugation at 2000 ×*g* for 5 min. The pellet was gently washed twice with double distilled water, and suspended in 5 mL enzyme mixture composed of 4.0% (*w*/*v*) Cellulase R-10 (Yakult, Japan), 2% (*w*/*v*) Macerozyme R-10 (Yakult, Japan), 0.1% (*w*/*v*) Pectinase (Yakult, Japan), 0.6 M sorbitol and 50 mM $CaCl_2$. Cell suspension was incubated at room temperature for 16 h in the dark with orbital shaking at 70 rpm for protoplast formation.

2.5. PEG-mediated transformation

Transformation of the protoplasts was performed according to the method of Kim et al. [28] with modifications. Protoplasts were centrifuged at 1000 ×*g* for 5 min, and the pellet was gently suspended in 5 mL of the Basal medium containing 0.6 M sorbitol and 0.6 M mannitol.