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Establishment of an efficient genetic transformation system in *Scenedesmus obliquus*

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ABSTRACT

Scenedesmus obliquus belongs to green microalgae, which is attracting attention as a feedstock for biofuels production and biorefinery as well as in bioremediation of environmental pollutants, making its genetic modifications for more efficient growth and accumulation of aimed metabolites significant. However, the genetic transformation system of *S. obliquus* is still not well established. In the current work, *S. obliquus* was transformed via electroporation using a plasmid containing chloramphenicol resistance gene (*CAT*) as a selectable marker and the green fluorescent protein gene (*gfp*) as a reporter. Using the optimized transformation conditions, the transformation efficiency was 494 ± 48 positive transgenic clones per 10^6 recipient cells, which is more efficient comparing with those reported in other microalgal transformation studies. Green fluorescence was observed after six months of cultivation, and *CAT*-specific products were also detected in the transformants by PCR, Southern blot and RT-PCR analysis. This is the first report on establishing such an efficient and stable transformation system for *S. obliquus*, a prerequisite for both functional genomic studies and strain improvement for other biotechnology applications of this important microalgal species.

1. Introduction

Microalgae have been traditionally used for aquatic feed, and in the recent years, they also have received renewed attention on account of their capacity to offer numerous value-added products (Harun et al., 2010), as well as acting as cell factories for the production of biofuel and recombinant proteins (Amaro et al., 2011; de Morais and Costa, 2007; Huang et al., 2010; Mata et al., 2010; Potvin and Zhang, 2010). *Scenedesmus obliquus* belongs to green microalgae and contains high concentration of protein and has thus been found applications as animal feed additives (Hintz et al., 1966) and for pigment (including astaxanthin) production (Qin et al., 2008; Wiltshire et al., 2000). *S. obliquus* also possesses excellent ability of wastewater treatment, CO_2 sequestration and biodiesel production (Cheng et al., 2010; Ho et al., 2010, 2012; Kumar et al., 2010; Mandal and Mallick, 2009, 2011). The maximum CO_2 consumption rate of *S. obliquus* can reach 1782 mg L^{-1} d^{-1} and their lipid content is up to 55% of the dry cell weight (DCW) (Cheng et al., 2010; Ho et al., 2010, 2012), which indicates the great potential of coupling CO_2 fixation with biodiesel production. Moreover, *S. obliquus* was used to remove heavy metal for bioremediation (Chen et al., 2012; Fayed et al., 1983).

Despite the great economic and environmental importance of *S. obliquus*, genetic studies of *S. obliquus* are very limited, which impedes strain development and improvement of production efficiency using advanced molecular tools. Therefore, there is an urgent need for fundamental and applied research based on genetic manipulation in *S. obliquus*. In microalgae, gene transfer proceeds at a slower rate as compared with bacteria, while genetic transformation systems have been established only in some microalgae species (Hallmann, 2007; Potvin and Zhang, 2010; Kilian et al., 2011; Radakovits et al., 2010, 2012). Absence of the available genetic transformation method is a major bottleneck for genetic engineering of microalgae (Amaro et al., 2011). So far, several methods have been developed for microalgal transformations, including particle bombardment method (Hirata et al., 2011), silicon carbide whiskers method (Dunahay, 1993), *Agrobacterium tumifaciens*-mediated method (Anila et al., 2011; Rajam and Kumar, 2006), glass beads method (Feng et al., 2009; Wang et al., 2010) and

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electroporation method (Shimogawara et al., 1998; Kilian et al., 2011; Niu et al., 2011). Among these methods, electroporation is considered as an efficient method applied in a wide variety of cell types transformations (Brown et al., 1991), including *Chlamydomonas reinhardtii* (Shimogawara et al., 1998; Meslet-Cladie‘re and Vallon, 2011), *Dunaliella salina* (Sun et al., 2005) and *Chlorella vulgaris* (Niu et al., 2011).

In the present study, various genetic transformation methods were attempted and the efficient transformation system for *S. obliquus* genetic transformation by electroporation was successfully demonstrated. Parameters affecting the efficiency of electroporation-based genetic transformation (such as pulse voltage, pulse duration, plasmid amount and concentration of osmosis solution) were optimized, and high efficiency transformation and stable propagation of *S. obliquus* transformants were obtained.

2. Materials and methods

2.1. Cultivation of microalgae

S. obliquus FSP-3 previously isolated from Taiwan (Ho et al., 2010) was used as host strain. The strain was cultivated with a modified version of Detmer's Medium (DM), which consists of ($g\,L^{-1}$): Ca $(NO_3)_2{\cdot}4H_2O$, 1.00, KH_2PO_4, 0.26, $MgSO_4{\cdot}7H_2O$, 0.55, KCl, 0.25, $FeSO_4{\cdot}7H_2O$, 0.02, EDTA·2Na, 0.2, H_3BO_3, 0.0029, $ZnCl_2$, 0.00011, $MnCl_2{\cdot}4H_2O$, 0.00181, $(NH_4)_6Mo_7O_{24}{\cdot}$ $4H_2O$, 0.000018, $CuSO_4{\cdot}5H_2O$, 0.00008. The *S. obliquus* strain was grown at 28 °C and illuminated at a 14/10 h light/dark cycle under a light intensity of roughly 60 $\mu mol\,m^{-2}\,s^{-1}$.

2.2. Antibiotic sensitivity of S. obliquus

The sensitivity of *S. obliquus* to five common antibiotics (ampicillin, streptomycin, gentamicin, kanamycin and chloramphenicol) was investigated. Ethanol, the solvent of chloramphenicol, was also studied as a control. Approximately 1×10^6 cells of *S. obliquus* was incubated in DM medium supplement with antibiotics in the concentration range from 50 to 1000 $\mu g\,mL^{-1}$ and the growth of *S. obliquus* was monitored on a daily basis.

2.3. Construction of expression vector for genetic transformation

The plasmid pCAMBIA 1302 was preserved in School of Life Science and Biotechnology, Dalian University of Technology, and was modified by replacing the hygromycin resistant gene with chloramphenicol resistance gene (chloramphenicol acetyltransferase, *CAT*) before used for transformation. Besides antibiotic selection, the vector also contained the reporter gene encoding green fluorescent protein (GFP) under the control of the CaMV 35S promoter, which used as a marker to visualize the existence of the transformed plasmid in *S. obliquus* cell. To insert the *CAT* gene in pCAMBIA 1302, the coding region of *CAT* was cloned by PCR using plasmid pFL129 as a template with primers designed according to the published sequence (GenBank accession no. NC_005923). Primers used for *CAT* amplification were F01: 5′-ATCTATCTCTCTCGAGATGGAGAAAAAAATCACTG-3′ and R01: 5′-TAT GGAGAAACTCGAGTTACGCCCCGCCCTGCCAC-3′. The conditions of PCR were as follows: 98 °C for 5 min, denaturation (98 °C for 10 s), annealing (55 °C for 5 s) and extension (72 °C for 30 s) for 30 cycles, then a final extension at 72 °C for 10 min, with Prime STAR HS DNA Polymerase (TaKaRa, Dalian, China). PCR product was examined on a 1% agarose gel and further confirmed by sequencing analysis. The plasmid pCAMBIA 1302 was digested by *Xho*I restriction enzyme, then ligated with the PCR fragment of *CAT* gene using the In-Fusion™ Advantage PCR Cloning Kit (TaKaRa, Dalian, China). Plasmids were amplified in *Escherichia coli* (DH5α) and purified using a standard procedure (Sambrook et al., 1989).

2.4. Optimization of the electroporation method

To obtain an optimal transformation method, different transformation parameters including pulse voltage, pulse duration, concentration of osmosis solution and plasmid amount were investigated. Different pulse voltages (1.5, 2, 2.5 and 3 kV) were attempted, and the pulse duration were 3, 4, 5 and 6 ms, respectively. The concentrations of osmosis solution were 0.1, 0.2 and 0.3 $mol\,L^{-1}$, and the concentrations of plasmid DNA were 40, 50, 60 and 70 $\mu g\,mL^{-1}$, respectively.

2.5. Generation of transgenic S. obliquus FSP-3 by electroporation

Cells of *S. obliquus* from the exponential phase were collected and resuspended in cold osmosis solution (0.2 $mol\,L^{-1}$ mannitol, 0.2 $mol\,L^{-1}$ sorbitol and 10% glycerol) at a concentration of approximately 1×10^8 cells mL^{-1}. After 1 min incubation at 42 °C and 5 min on ice, four hundred microliters of competent cells was mixed with 10 $\mu g\,mL^{-1}$ pCAMBIA 1302-CAT and 50 $\mu g\,mL^{-1}$ Salmon sperm DNA (Sigma), then kept on ice for another 5 min. The resuspended cells were transferred into a 2 mm electroporation cuvette (Bio-Rad, USA), and were electroporated using a Bio-Rad GenePulser × cell apparatus (Bio-Rad, USA) (Wang et al., 2007). Electroporated cells were transferred into 10 mL fresh DM medium and were then incubated at 25 °C for 24 h in the dark before plating on DM selection medium containing 100 $\mu g\,mL^{-1}$ chloramphenicol. Meanwhile, cells treated with the same transformation protocol without plasmid were set as the negative control.

2.6. Detection of plasmids introduced into S. obliquus by fluorescence microscopy

Post-electroporated cells were observed with an Olympus IX71 fluorescence microscope (Olympus, Japan) to qualitatively assessed GFP expression. The excitation source for fluorescence was a 100 W Hg vapor arc lamp. Fluorescence in single cells was detected using a fluorescence microscope with an excitation filter at 480 nm and emission at 527 nm. Digital images were captured using an Olympus DP70 digital camera with DP Controller software (Olympus, Japan).

2.7. Determination of GFP gene expression by flow cytometry

Cellular fluorescence from GFP was determined quantitatively with a FACSCanto™ flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 15 mW, 488 nm argon ion laser. All samples were concentrated by centrifugated and resuspended with the fresh medium at a density of approximately 1×10^6 cells mL^{-1}. After filtration, the cells were injected into flow cytometry at a flow rate of 18 $\mu L\,min^{-1}$. GFP was excited by an argon laser and fluorescence was detected using a 488 nm excitation with the detector FL1 (530/30 nm). Typically, 10,000 cells were analyzed per sample. Data acquisition and analysis were performed using Cell Quest software (BD Biosciences, San Jose, CA, USA).

2.8. PCR and Southern blot analysis

Cells in the late-logarithmic phase (approximately 1×10^7 cells mL^{-1}) were collected and genomic DNA was isolated as described (Chen et al., 2001). Two primers (CAT-1: 5′-ATGGAGAAAA-AAATCACTG-3′ and CAT-2: 5′-TAAGCATTCTGCCGACAT-3′) were designed to amplify the complete *CAT* sequence (660 bp). Amplification was performed with PCR programs mentioned above. PCR on