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Method for assembling and expressing multiple genes in the nucleus of microalgae

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Abstract The green alga, *Chlamydomonas reinhardtii,* is a model organism used in the study of photosynthesis and biotechnological research. Despite its importance, a complete set of genetic tools has yet to be developed. Here, we report the development of a new method for constructing a multi-gene pathway in *Saccharomyces cerevisiae* and integrating the assembled pathway into the nuclear genome of *C. reinhardtii*. To demonstrate the use of this method, we assembled and functionally expressed up to three reporter proteins (Ble, AphVIII, and GFP) simultaneously in the nucleus of *C. reinhardtii*. This new molecular tool should aid efforts to engineer microalgae for biofuel and biopharmaceutical production.

Keywords *Chlamydomonas reinhardtii* ·
Genetic tools for microalgae · Microalgae ·
Nuclear genome engineering · Synthetic biology

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Introduction

The eukaryotic green alga, *Chlamydomonas reinhardtii,* is a model organism used in the study of photosynthesis, flagellar function, and lipid metabolism (Harris 1989). Compared to other unicellular microalgae, *C. reinhardtii* is a particularly attractive species because of its relatively fast growth rate, simple nutrient requirements, well characterized genetics, and genetic tractability. All three genomes (nuclear, chloroplast, and mitochondrial) of *C. reinhardtii* have been sequenced and transformation methods that target each of these genomes have been developed (Meslet-Cladiere and Vallon 2011). Despite these advances, new genetic tools are still needed to harness the full potential of *C. reinhardtii*, especially in light of the surging interest in using microalgae as a source of biofuels, therapeutic proteins, fish and animal feed, and nutraceuticals (Pulz and Gross 2004).

To date, most studies have focused only on introducing single transgenes into the nuclear genome of *C. reinhardtii* to enhance metabolite production (Cordero et al. 2011; Leon et al. 2007; Radakovits et al. 2010). The introduction of more sophisticated biochemical pathways into the nuclear genome of *C. reinhardtii* has likely been hampered by the inherent limitations of traditional molecular cloning. The most complex pathway to be integrated and expressed in the nuclear genome of *C. reinhardtii* is the poly-3-hydroxybutyrate (PHB) biosynthetic pathway (Chaogang et al.

2010). To achieve PHB production, these authors had to co-transform *C. reinhardtii* with two different expression vectors containing the genes *phbB* and *phbC*. This example highlights the current difficulties of introducing multi-gene pathways into the nuclear genome of *C. reinhardtii* and indicates the need for a new, more robust method.

We have reported a new method for assembling and integrating biochemical pathways into the chloroplast genome of *C. reinhardtii* (Noor-Mohammadi et al. 2012). In this present study, we present a system based, in part, on the DNA Assembler method (Shao and Zhao 2009) for constructing and integrating multi-gene pathways into the nuclear genome of *C. reinhardtii*. As a proof of concept, we used this approach to assemble, integrate, and functionally express multiple reporter genes in the nucleus of *C. reinhardtii*.

Materials and methods

Strains and culture conditions

Chlamydomonas reinhardtii strain 137c was obtained from Life Technologies (Carlsbad, CA). The plasmid pRS426 was a gift from Dr Huimin Zhao at the University of Illinois at Urbana-Champaign. Plasmids pSP124S, pKS-aphVIII-lox, and pKScGFP were obtained from the Chlamydomonas Center (Duke University, Durham, NC). All algal cultures were grown in Tris/acetate/phosphate (TAP) medium at 23 °C under constant fluorescent white light [80 µmol m^{-2} s^{-1} as measured by a Field Scout Quantum Meter (Plainfield, IL)] on a rotary shaker at 100 rpm. Microalgae transformants were selected on 20 µg zeocin/ml or 20 µg zeocin/ml + 10 µg paromomycin/ml, when appropriate. *Saccharomyces cerevisiae* YSG50 was grown at 30 °C in YPAD medium containing 1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose and 0.01 % adenine hemisulphate. *Escherichia coli* strain DH5α was grown at 37 °C in LB medium.

Plasmid construction

Plasmids pRS426-ble, pRS426-ble-aphVIII, and pRS426-ble-aphVIII-gfp were constructed using the DNA Assembler method (Supplementary Fig. 1) (Shao and Zhao 2009). The 5′-untranslated region (UTR; *rbcS2*,

208 bp; *β$_2$-tubulin*, 331 bp; *psaD*, 823 bp) and 3′-UTR (*rbcS2*, 227 bp; *COP*, 162 bp; *psaD*, 392 bp) used to construct the gene expression cassettes were PCR-amplified from the plasmids pSP124S, pKS-aphVIII-lox, and the nuclear genome of *C. reinhardtii*. The structural genes *ble* (527 bp), *aphVIII* (804 bp), and *gfp* (717 bp) were amplified from pSP124S, pKS-aphVIII-lox, and pKScGFP, respectively. Construction primers for the plasmids are listed in Supplementary Table 1. Constructed plasmids were verified to be correct restriction digestion analysis.

Nuclear transformations

For nuclear transformations, 230 µl frozen *C. reinhardtii* cells (GeneArt Chlamydomonas Engineering Kit, Life Technologies, Carlsbad, CA, USA) were thawed in a 37 °C water bath then mixed with 4 ml of room temperature Gibco TAP medium in a 6-well plate and grown under light for 3–6 days. Once the culture reached an OD$_{750}$ >0.6, cells were diluted in fresh medium to give an OD$_{750}$ of 0.06. After 20–24 h (OD$_{750}$ = 0.3–0.5), 15 ml of algae culture was harvested by centrifugation at $2500 \times g$ for 10 min and then resuspended in 250 µl TAP/40 mM sucrose. 2 µg of *Hin*dIII-linearized plasmid was added to the cells and the mixture was transferred to a 4 mm electroporation cuvette and incubated at room temperature for 5 min. The following electroporation parameters were used for the transformation: 600 V, capacitance 50 µF, and resistance set to infinity. Transformed cells were mixed with 5 ml TAP/40 mM sucrose and incubated under continuous light for recovery. After 24 h, cells were harvested and resuspended in fresh TAP/40 mM sucrose and then plated on TAP/agar plates containing 20 µg zeocin/ml or 20 µg zeocin/ml + 10 µg paromomycin/ml. The plates were placed under constant light for 5–7 days until transformed algae colonies appeared. PCR analysis was performed to verify gene integration for each transgenic strain.

Functional analysis of the transgenic strains

Transgenic strains were grown in 50 ml liquid TAP medium under light with antibiotics to analyze growth. Transformants were grown in TAP media containing either 20 µg zeocin/ml (*ble* strains) or

20 µg zeocin/ml + 10 µg paromomycin/ml (*ble-aphVIII* and *ble-aphVIII-gfp* strains). Cell growth was monitored by measuring the OD_{750} using a Thermo Scientific NanoDrop 2000c and correlated to dry cell weight. GFP fluorescence of the *ble-aphVIII-gfp* transformants was measured using a Safire Tecan microplate reader. To analyze the GFP transformants, 3 ml cells grown to saturation were centrifuged at $17,000 \times g$ for 2 min, re-suspended in 300 µl fresh TAP medium, then 150 µl suspension was used to measure relative fluorescence using the plate reader at 488 nm excitation and 530 nm emission.

Western blot analysis

Algal cell cultures were grown to late growth phase in 40 ml of TAP medium. Cells were harvested by centrifugation and resuspended in 0.5 ml 20 mM Tris/HCl pH 8.0. The resuspended cells were lysed by sonication using a Misonix sonicator (Farmingdale, NY, USA) with the amplitude set at 30 % and with a pulse sequence of 10 s on and 10 s off, for 5 min. The insoluble cellular material was removed by centrifugation at $14,000 \times g$ for 60 min. Samples were first boiled for 7 min and separated on a 12 % SDS-PAGE and then transferred onto a methanol-treated PVDF membrane as described by Pourmir et al. (2013). The PVDF membrane was blocked with 5 % (v/v) milk in TBST buffer (20 mM Tris, 150 mM NaCl, 0.02 % Tween 20, pH 7.5) for 1 h at room temperature. The blocked membrane was incubated at 4 °C overnight in TBST solution containing 1:1000 dilution of a rabbit anti-FLAG primary antibody (Cell Signaling Technology, Danvers, MA, USA), washed three times with TBST for 5 min, incubated with 1:2000 dilution of a horse-radish peroxidase conjugated anti-rabbit IgG secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 h, then the membrane was washed with TBST for 30 min. A working solution of Pierce ECL Substrate (Thermo Scientific, Rockford, IL, USA) was prepared according to the manufacturer's instructions and added to the membrane for 1 min. The membrane was removed from the substrate and analyzed by an Alpha Innotech FluorChem HD2 imager and the software AlphaEaseFC.

Results and discussion

Overview of the method

An overview of the method is shown in Fig. 1. Expression cassettes consisting of a 5′-UTR, structural gene, and 3′-UTR were first assembled via overlap extension PCR (OE-PCR) (Horton et al. 2013). The expression cassettes were transformed into the yeast strain *S. cerevisiae* YSG50, where in vivo homologous recombination is used to assemble the biochemical pathway into the vector pRS426. The assembled plasmid was isolated from yeast then transformed into the nuclear genome of *C. reinhardtii*.

Construction of the expression cassettes

Expression of both homologous and heterologous genes in the nucleus of *C. reinhardtii* is heavily influenced by promoter and regulatory elements. The most commonly used promoters for nuclear expression are those from the *rbcS2, psaD, HSP70A,* and β_2-*tubulin* genes (Griesbeck et al. 2006; Specht et al. 2010). High levels of transcription and expression have also been achieved using chimeric constitutive promoters and by placing endogenous intronic sequences in transgenes (Lumbreras et al. 1998). Inducible promoters, such as *NIT1, CA1,* and *CYC6,* that allow for more controllable expression have been developed (Ferrante et al. 2008). For this work, we selected the *rbcS2, psaD,* and β_2-*tublin* promoters. A unique 5′-UTR and 3′-UTR was chosen to flank each gene to minimize the potential for unintentional homologous recombination during assembly in yeast. Expression cassettes consisting of a 5′-UTR, gene (*ble, aphVIII,* or *gfp*), and 3′-UTR were PCR-amplified, then assembled using OE-PCR. Assembled expression cassettes along with linearized pRS426 were co-transformed into yeast. The resulting fully assembled vectors contained the one-gene, two-gene, and three-gene pathways (Fig. 2). Correct assembly of these three vectors was confirmed by restriction digestion analysis (Supplementary Fig. 2).

Integration into the nuclear genome of *C. reinhardtii*

The assembled plasmids pRS426-ble, pRS426-ble-aphVIII, and pRS426-ble-aphVIII-gfp were each

Fig. 1 Overview of the method. Assembly of the biochemical pathway occurs via homologous recombination in *S. cerevisiae*. The assembled plasmid is isolated from yeast, then transformed into the microalga *C. reinhardtii* where it integrates into the nuclear genome
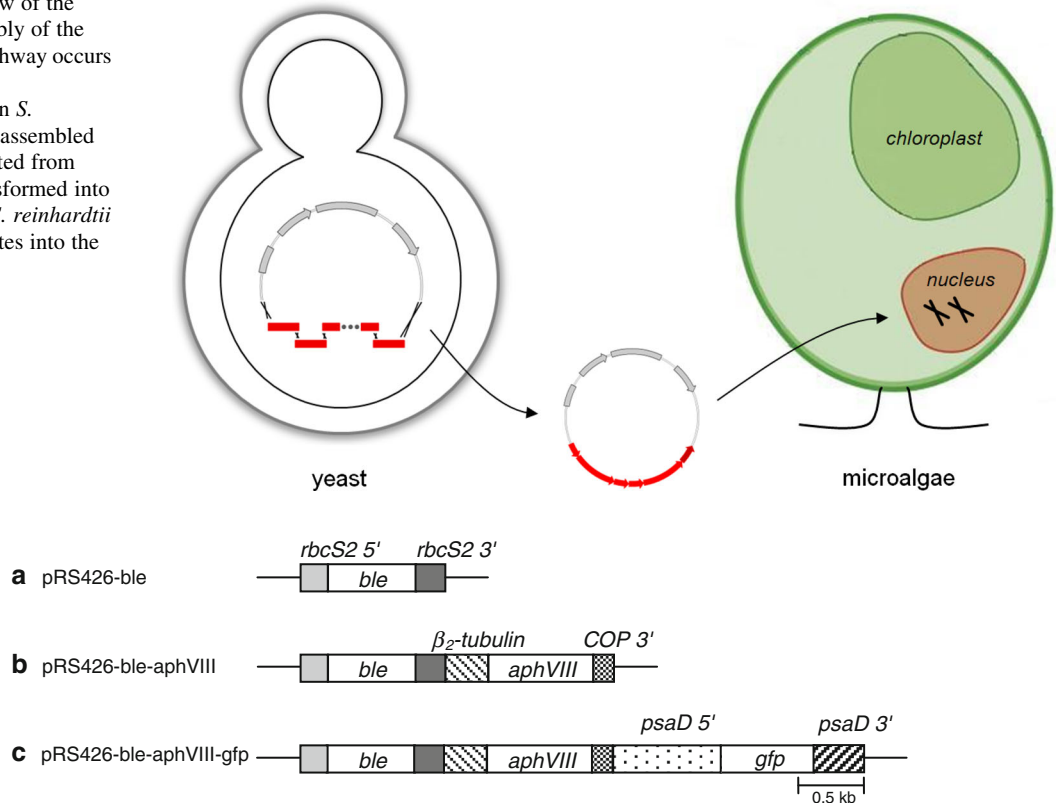

Fig. 2 Expression cassettes used for heterologous gene expression from the nuclear genome of *C. reinhardtii*. Expression cassettes were each assembled into the vector pRS426. Genes are flanked by 5′- and 3′-untranslated regions. Constructs used in this study include: **a** pRS426-ble, **b** pRS426-ble-aphVIII, and **c** pRS426-ble-aphVIII-gfp

linearized by restriction enzyme digestion and transformed into *C. reinhardtii* by electroporation. Transformants were selected on TAP plates supplemented with the antibiotic zeocin and screened by PCR to verify integration of each of the genes (Supplementary Fig. 3). Transformation efficiencies for the three constructs are shown in Table 1. The % gene-positive clones identified seemed to decrease with increasing pathway size.

Functional analysis of the engineered strains

The functional expression of the integrated *ble*, *ble-aphVIII*, and *ble-aphVIII-gfp* pathways were analyzed by growing the transformant strains in batch cultures of TAP medium supplemented with either zeocin or zeocin plus paromomycin. As shown in Fig. 3a, the *ble* transformant was able to grow in the presence of the antibiotic zeocin, whereas wild-type

Table 1 Transformation data for the constructs used in this study

Construct	Number of transformants per µg DNA[a]	% gene-positive[b]
pRS426-ble	59	100
pRS426-ble-aphVIII	43	72
pRS426-ble-aphVIII-gfp	41	34

[a] Transformations were performed twice on separate days; 2 µg of linear DNA was used per electroporation

[b] Numbers represent the calculated percentage of clones that were gene-positive for two independent transformation experiments per construct

C. reinhardtii did not grow. Similarly, the *ble-aphVIII* and *ble-aphVIII-gfp* transformant strains grew in the presence of zeocin plus paromomycin, while no growth was observed from wild-type *C. reinhardtii*

(Fig. 3b). These results demonstrate the functional expression of the *ble* and *aphVIII* genes in the engineered strains of *C. reinhardtii*.

To analyze GFP expression, six clones of *ble-aphVIII-gfp* were randomly chosen and the fluorescence of each of these clones was measured using a microplate reader. Six clones were chosen for analysis because heterologous genes have been shown to be inserted randomly into the nuclear genome of *C. reinhardtii*, resulting in a wide range of heterologous gene expression levels (Slaninová et al. 2008). *Chlamydomonas* naturally produces a variety of highly fluorescent pigments, such as chlorophylls and flavonoids, which results in a high level of auto-fluorescence (Rasala et al. 2013), thus the clones were analyzed relative to wild-type *C. reinhardtii*. As shown in Fig. 4a, fluorescence above auto-fluorescence was detected in four of the six clones tested (C1, C2, C4, and C5). The variability of fluorescence across the four clones ranged from 1.1 to 1.4 times more fluorescence than wild-type *C. reinhardtii*.

In addition to growth and fluorescence analysis, the *ble-aphVIII-gfp* clones were also subjected to western blot analysis to detect GFP protein accumulation in the nucleus of *C. reinhardtii*. As shown in Fig. 4b, three of the clones (C1, C2, and C5) had clearly discernible bands indicating GFP accumulation. Interestingly, despite displaying a 10 % increase in relative fluorescence compared to wild-type (Fig. 4a), the clone C4 did not have a detectable GFP band on the western
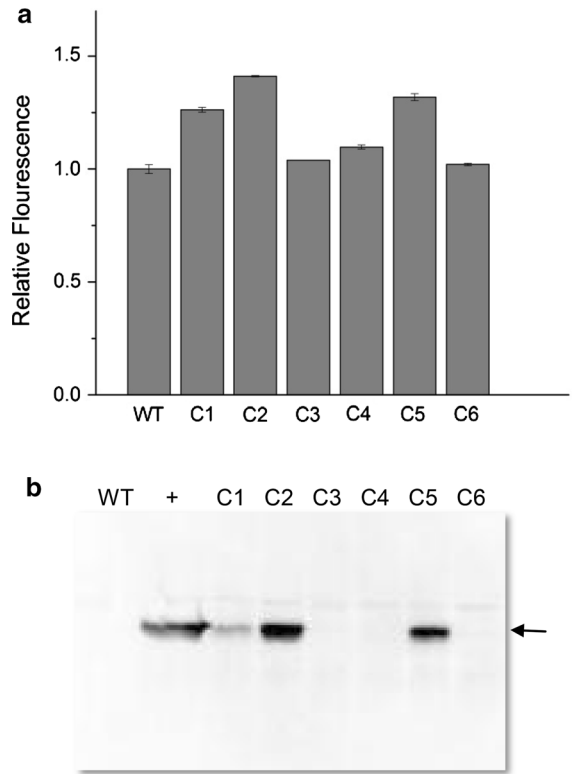

Fig. 4 **a** Quantification of the relative GFP fluorescence of six selected *ble-aphVIII-gfp* clones (C1-C6). The *ble-aphVIII-gfp* clones expressing GFP are shown relative to wild-type (WT) *C. reinhardtii*. **b** Western blot analysis of the six ble-aphVIII-gfp clones. WT *C. reinhardtii* and a 16S/GFP *C. reinhardtii* chloroplast transformant (unpublished work) were included as negative and positive controls, respectively
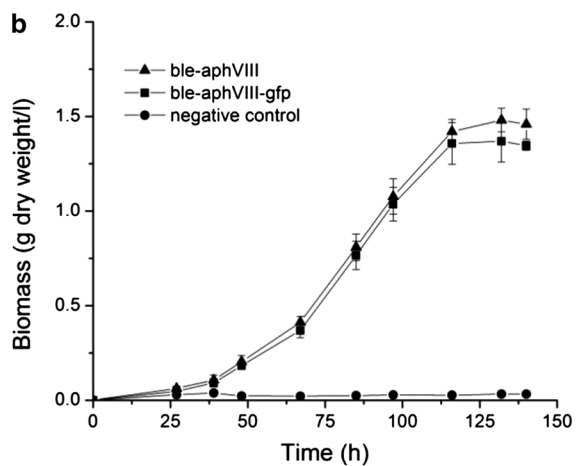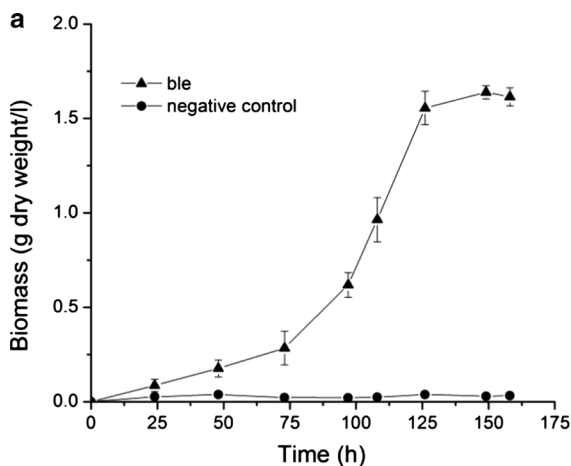

Fig. 3 Growth of the engineered strains in TAP medium supplemented with the antibiotics **a** zeocin and **b** zeocin + paromomycin. Wild-type *C. reinhardtii* served as a negative control

blot; however, the other three clones seem to show a strong correlation between relative fluorescence and GFP band intensity.

Conclusions

This study presents the design and application of a new system for expressing biochemical pathways in the nucleus of *C. reinhardtii*. Using this method, it should be possible to introduce larger and more complex biosynthetic pathways into the nuclear genome of *C. reinhardtii* and to optimize the expression of each gene in the pathway to maximize the production of a target compound. This new molecular tool should be useful for engineering microalgae for biofuel and biotechnological applications.

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