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Improvement of biomass and lipid yield under stress conditions by using diploid strains of *Chlamydomonas reinhardtii*

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ABSTRACT

Algal biofuel feedstocks are excellent candidates for sustainable and eco-friendly fuels for the next generation, which can be improved by genetic modifications for their maximal production of biomaterials. However, currently available genetic modifications involve the introduction of foreign DNA into the algal genome, and this may face legal and public conflicts due to the risk of environmental, economic, and/or health problems. In this regard, we employed an old concept of crop improvement that has been accepted in the long history of agriculture, i.e. polyploidization. Polyploidization of crop plants has been selected fortuitously or intentionally not only for increased quality and/or quantity of products, but also for enhanced stress tolerance. We induced diploidy in the model algae *Chlamydomonas* by treating haploid cells with the microtubule inhibitor colcemid, and the resulting diploids were selected for increased colony size and neutral lipid contents. Two of the isolated diploid strains containing doubled DNA contents, named CMD ex1 and CMD ex4, were increased in their cell size and cellular weight. These diploids were excellent in coping with abiotic stresses, including nutritional, oxidative, and cold stresses. Under these conditions, the diploids accumulated two times more biomass and FAME yield compared to the control. To understand underlying mechanisms, we performed RNA-Seq analyses for the diploid under the cold stress. Transcriptomic analyses revealed that the diploids showed enhanced expression of genes involved in photosynthesis, energy metabolism, and translation as well as reduced starch metabolism. Overall, diploids of *Chlamydomonas* showed improvements including increased yields of biomass and FAME and enhanced stress tolerance compared to wild-type organisms. The results demonstrate that polyploidization can be utilized in industrial microalgae for the production of biofuels and other biomaterials not only on a laboratory scale but also in outdoor cultivation, where stress conditions are inevitable.

1. Introduction

Microalgae have been studied extensively due to their potential as third generation biofuel feedstocks [1–3]. Their use could help alleviate or solve energy and environmental problems caused by the excessive use of conventional fuels. Successful commercialization of microalgal biofuel requires improvements in the entire value chain of biofuel production, including algal improvement, cultivation, harvest, extraction, and conversion [4,5]. Among these, the importance of algal improvement is being recognized as critical to overcome the high cost of algal biofuel production [6]. Currently, genetic improvement is the preferable approach, and many methods involve the introduction of heterologous markers and genes into the algal genome [7]. However, this may lead to political and public conflicts due to environmental and health concerns regarding the introduced genes and markers [8].

Genetic strategies for microalgal improvement aim to achieve these

Abbreviations: ACO, aconitase; AGPase, ADP-glucose pyrophosphorylase; CAHs, carbonic anhydrases; CMD, colcemid; DEGs, differently expressed genes; DCW, dry cell weight; FAME, fatty acid methyl ester; FBA, fructose-1,6-biphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GMO, genetically modified organism; LHC, light harvesting complex; PS, photosystem; Ru5P, ribulose-5-phosphate; RuBisCO, ribulose-1,5-biosphosphate carboxylase oxygenase; SBP, sedoheptulose-1,7-bisphosphatse; TK, transketolase; PYKs, pyruvate kinases; TA, transaldolase; TAP, tris acetate phosphate; TPI, triose-phosphate isomerase

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goals: high lipid yield, increased biomass, and stress tolerance. Similar goals have been achieved in traditional crop improvement through the selective breeding of preferred traits, and this process often results in the multiplication of the genome, called polyploidization [9]. Polyploidization of crop plants can also be artificially induced to increase the size, number, and other qualities of their products [10]. Induction of polyploidy for crop improvement dates back to the 1930s, and doubling of the chromosome number is associated with increased biomass and tolerance to various stresses [11]. These improved traits are essential for the maximal production of crops on a large agricultural scale.

Polyploidy in microalgae has not been studied as extensively as it has in plants due to the lack of agricultural or industrial interest in the past. Green algae, including chlorophytes and charophytes, are considered to have a haplontic life cycle during which their genomes are haploids during vegetative growth [12], and this may be true in other microalgae including *Nannochloropsis* [3] and *Gillardia* [13]. Studies of polyploidy in microalgae have mostly focused on the model microalgae *Chlamydomonas* in regard to growth, developmental, and genetic aspects [14–20]. In some species of *Chlamydomonas*, diploids and polyploids can be induced by various methods, including mutagenesis, incomplete zygote development, and the application of stresses [17,18,21,22] in addition to treatment with colchicine [20]. Among these, stress-induced diploids tend to return to haploids, but stable inheritance of diploidy has been observed in other cases [17,20].

In this study, we induced diploidy in haploid cells of *Chlamydomonas reinhardtii* by treating cells with colcemid, a derivative of colchicine that disrupts chromosome segregation and results in polyploidy. Candidates of diploid cells were further selected for increased cell size and confirmed doubled contents of genomic DNA. Further analyses of the diploid strains, named CMD ex1 and CMD ex4, revealed that they were larger and heavier than wild-type organisms. They also showed enhanced tolerance to stresses, including N starvation, cold, and oxidative stresses, which cells can face in outdoor cultivation. To our knowledge, these are the first examples of stable strains of diploids that have been selected for increased lipid and biomass yields and stress tolerance. These traits are important criteria for the commercial production of biomaterials from microalgae, and this concept of polyploidization can be readily applied to industrial strains of microalgae.

2. Materials and methods

2.1. Strains and culture conditions

C. reinhardtii (CC-124) was obtained from the Chlamydomonas Resource Center (http://www.chlamycollection.org/) and was grown in TAP medium under continuous light from five 55 W cool white fluorescent lamps (measured to be 100 μmol photons $m^{-2} s^{-1}$) at 26 °C. Cells were exposed to 1 mM of colcemid (Sigma) at exponential or stationary phase for 4 days and were spread on TAP agar plates. A week later, six large colonies were isolated as diploid candidates from each phase and were named CMD ex1 through ex6 and CMD st1 to st6 (CMD for colcemid; ex for exponential; st for stationary). All candidates were stained with Nile red so that they could be screened for high neutral lipid content, following previously reported procedures [23].

2.2. Quantitation of genomic DNA and measurement of cell size

Candidate strains were cultivated to the stationary phase (where they stop synthesizing DNA) and fixed with 2.5% glutaraldehyde in PBS buffer. Their genomic DNA was stained with 5 μM SYTOX green (Life technologies, USA) for 10 min, and the resulting fluorescence was measured using SpectraMax M2^e microplate readers (Molecular Devices) with excitation at 488 nm and emission at 515 nm. Cell size was measured using a HELOS particle-size analyzer [HELOS (H1483) & QUIXEL, System-Partikel-Technik], and cell size distribution was analyzed. Microscopic image of diploid cells was obtained with a DM2500 microscope equipped with a DFC425C camera (Leica Microsystems, Germany).

2.3. DNA extraction and quantitation

Genomic DNA was extracted from 10^7 cells of each strain by following a modified version of the standard phenol/chloroform extraction method [24–26]. First, 1 μl of the isolated genomic DNA was loaded on 0.8% (w/v) agarose gel and was stained with Loading Star (DyneBio Inc., Korea). The relative intensity of genomic DNA and rRNA was analyzed by the Chemidoc system and ImageLab software (Biorad).

2.4. Cell growth analysis under nutrient, oxidative, and cold stresses

Cells were initially inoculated into the TAP medium for two days, and pre-cultures were started with the same cell density and were grown for three days. Before the experiments, cells were washed with fresh TAP or TAP-N (nitrogen-depleted) media twice by centrifugation at 4000 rpm for 3 min, and the cell density was adjusted to 5×10^6 cells/ml. These cells were cultured for 7 days during which the cell density, dry cell weight (DCW), and neutral lipid contents were measured. For the oxidative stress test, 2.5×10^6 cells/ml of cells was treated with 2 mM hydrogen peroxide, and their cell growth was observed for 2 days. Cell density was estimated by counting cells with a light microscope (Eclipse E200, Nikon) using a disposable hemocytomer C-chip. DCW was measured by filtering an aliquot of culture on pre-weighed GF/C filter paper (Whatman) and dried at 100 °C overnight. Cellular mass was calculated by dividing the DCW by the cell density. For cold stress, cells were cultivated at 10 °C under the same conditions.

2.5. Quantitation of neutral lipids and fatty acid methyl ester (FAME) content

The relative contents of neutral lipids were estimated by staining cells with Nile red. First, 200 μl of cell cultures were mixed with 50 μl of Nile red solution in DMSO (final concentration was 5 μg/ml) for 30 min at 37 °C in the dark. Relative fluorescence was measured using SpectraMax M2^e microplate readers (Molecular Devices) with excitation at 530 nm and emission at 575 nm. For FAME contents, cells were harvested by centrifugation (7000 rpm, 3 min) and washed twice with distilled water. Pelleted cells were lyophilized at − 80 °C for 3 days, and lipids were extracted from 10 mg of lyophilized biomass with chloroform-methanol (2:1, v/v) following the modified Folch method [27]. The extracted lipids were converted into FAMEs via transesterification and then treated with methanol and sulfuric acid at 105 °C for 20 min. The FAME contents in the organic phase were analyzed by gas chromatography (HP6890, Agilent) with a flame-ionized detector and an INNOWAX capillary column (30 m × 0.32 mm × 0.5 μm, Agilent, USA). The identification and quantitation of fatty acids were determined by comparison of the retention time and the peak area with FAME standards.

2.6. RNA preparation and sequencing

For RNA sequencing, CC-124 (WT) and CMD ex 4 (Diploid) were cultivated at 26 °C and 10 °C for 4 days. In order to keep the same initial condition, we cultured cells at 26 °C, inoculated cells at different temperatures of 26 and 10 °C, and sampled at days 1 and 4. Immediately after inoculation, the day 0 sample did not provide enough cells for RNA prep, so we decided to use the inoculating cells (grown at 26 °C) for the day 0 control. RNA samples were prepared with a plant RNA purification kit (Qiagen, Germany) at 0, 1, and 4 days after inoculation. The extracted RNA was stored at − 80 °C and used for library