Bioresource Technology 149 (2013) 432–438

Contents lists available at ScienceDirect

Bioresource Technology

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

Phenotypic spectrum of *Parachlorella kessleri* (Chlorophyta) mutants produced by heavy-ion irradiation

Shuhei Ota [a,b,1], Takahiro Matsuda [a,1], Tsuyoshi Takeshita [a], Tomokazu Yamazaki [a,b], Yusuke Kazama [c], Tomoko Abe [c,d], Shigeyuki Kawano [a,b,*]

[a] Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan
[b] Japan Science and Technology Agency, CREST, Kashiwanoha, Kashiwa, Chiba 277-8562, Japan
[c] RIKEN Innovation Center, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
[d] Nishina Center for Accelerator-Based Science, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

HIGHLIGHTS

- A broad spectrum of phenotypes was produced by heavy-ion beam irradiation.
- Lipid production fatty acid profiles and starch contents were altered.
- Heavy-ion beam irradiation shows potential in the breeding of microalgae.

ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form 16 September 2013
Accepted 18 September 2013
Available online 27 September 2013

Keywords:
Algae
Breeding
Parachlorella
Heavy-ion irradiation
Mutation

ABSTRACT

Heavy-ion mutagenesis is a technology used for effective production of genetic mutants. This study demonstrates that algal breeding using a unicellular alga, *Parachlorella kessleri*, by heavy-ion mutagenesis can improve lipid yield in laboratory experiments. The primary screening yielded 23 mutants among which a secondary screening yielded 7 strains, which were subjected to phenotypic assays. *P. kessleri* strains produced by heavy-ion radiation spanned a broad spectrum of phenotypes that differed in lipid content and fatty acid profiles. Starch grain morphology was distinctively altered in one of the mutants. The growth of strain PK4 was comparable to that of the wild type under stress-free culture conditions, and the mutant also produced large quantities of lipids, a combination of traits that may be of commercial interest. Thus, heavy-ion irradiation is an effective mutagenic agent for microalgae and may have potential in the production of strains with gain-of-function phenotypes.

1. Introduction

Heavy-ion beams have higher linear energy transfer (LET) than gamma rays and X-rays; consequently, these beams are used as mutagenic agents in plant breeding programs (Tanaka et al., 2010). Because of its elevated LET, heavy-ion radiation at relatively low doses induces mutations without excrescent abnormalities or other negative phenotypic effects in plants (Tanaka et al., 2010). A major effect of heavy ion beams is pronounced induction of DNA double-strand breaks, which is not the case for gamma- or X-rays (Yokota et al., 2007); high-LET heavy-ion beams also induce large deletions (Hirano et al., 2012).

Heavy-ion beams have advantages in breeding and in forward/reverse genetic protocols. Mutagenesis induced in this manner has been used in genetic studies of several land plants (vascular plants), such as *Arabidopsis thaliana* (Kazama et al., 2008b, 2011; Hirano et al., 2012) and *Nicotiana tabacum* (Kazama et al., 2008a, 2013). Although these procedures have contributed to many advances in the genetics of land plants, few reports of heavy-ion mutagenesis in algae have been published, including those for the red alga *Pyropia yezoensis* (syn. *Porphyra yezoensis*) (Niwa et al., 2009) and the green microalga *Desmodesmus* sp. (Hu et al., 2013).

Micronutrient (N, S, and P) limitation is a major strategy for increased production of lipids or starch yields in algal cells; e.g., cells of *Chlorella*/*Parachlorella* (Brányiková et al., 2011; Mizuno et al., 2013; Li et al., 2013), *Nannochloropsis* (Rodolfi et al., 2009), and *Chlamydomonas* (Moellering and Benning, 2010; Cakmak et al., 2012; Msanne et al., 2012). However, total biomass decreases markedly under N deprivation (Li et al., 2013; Cakmak et al.,

* Corresponding author at: Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan. Fax: +81 4 7136 3679.
E-mail address: kawano@k.u-tokyo.ac.jp (S. Kawano).
[1] These authors contributed equally to this work.

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.09.079

2012; Brányiková et al., 2011). For biofuel production, a desirable strain would be a highly efficient lipid/starch producer without a concomitantly reduced biomass. A possible strategy for reaching this objective would be algal breeding. To this end, several microalgal species have recently been subjected to genetic alteration using heavy ions or UV irradiation (Vigeolas et al., 2012; Hu et al., 2013).

Parachlorella kessleri is a green, coccoid, unicellular alga belonging to the family Chlorellaceae in the class Trebouxiophyceae. It has a haploid life cycle (asexual reproduction) characterized by autospore formation. The species has a rapid growth rate and potential for mass production of lipids and/or starch; thus, *P. kessleri* is a candidate taxon for algal biofuel production (Přibyl et al., 2012; Mizuno et al., 2013; Li et al., 2013). Furthermore, the high rates of growth and its haploid life cycle make this alga suitable for pilot explorations of the effects of mutagenesis induced by heavy-ion-beam irradiation.

Because algal fuels are renewable and potentially carbon–neutral, they are currently viewed as attractive agents for bioenergy production (Georgianna and Mayfield, 2012; Larkum et al., 2012). Microalgal technologies can also be used for the synthesis of high-value added products, such as long-chain polyunsaturated fatty acids (PUFAs) and the strong antioxidant astaxanthin (Guedes et al., 2011). Significant improvements in biomass and biofuel conversion efficiencies, however, are still required to make algal biofuels economically viable (Georgianna and Mayfield, 2012). Improvements through the production of transgenic microalgae are currently hampered by the slow development of transformation techniques for these unicells (Larkum et al., 2012). A viable alternative to current protocols would be isolation and breeding of highly efficient lines by mutagenesis, a process that avoids the problems of transgenic strain usage in outdoor systems (Larkum et al., 2012).

To construct a phenotype spectrum of *P. kessleri* following heavy-ion treatment, heavy-ion irradiated cells were screened for diverse attributes, such as cell size and dry weight, and lipid and starch accumulation were measured. In addition, morphology and ultrastructure were observed by light, fluorescence, and transmission electron microscopy (TEM), and fatty acid profiles were characterized by gas chromatography–mass spectrometry (GC–MS).

2. Methods

2.1. Strain selection and preculture conditions

A strain of *P. kessleri* (NIES-2152) was obtained from the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan (http://mcc.nies.go.jp/). The precultures were grown in 30–40 ml of Tris–acetate–phosphate (TAP) medium (http://mcc.nies.go.jp/medium/ja/tap.pdf) at 23 °C under a 12-h light (L):12-h dark (D) cycle (with a photon flux of ~50 μmol m^{-2} s^{-1}) and no aeration.

2.2. Heavy-ion beam treatment

For the Ar-ion treatment, 200-μl aliquots of a 2-day-old *P. kessleri* culture were transferred into PCR tubes. For the C-ion treatment, 1.0-ml aliquots of a 2-day-old *P. kessleri* culture were transferred into 1.5-ml sampling tubes (Watson, Tokyo, Japan). The samples were kept cold (~4 °C) in the period before heavy-ion-beam irradiation. Cells were irradiated by C ions (LET: 22.6 keV μm^{-1}) or Ar ions (LET: 308 keV μm^{-1}) at doses ranging from 0 to 300 Gy in the RIKEN RI-beam factory (Wako, Saitama, Japan; http://www.rarf.riken.go.jp/Eng/facilities/RIBF.html). The irradiated cells were spread onto TAP-agar plates for isolation of single colonies.

2.3. Screening of mutants

Single colonies were isolated at random from the TAP-agar plates and transferred in 200 μl of TAP medium in 96-well microplates (Iwaki, Tokyo, Japan). For primary screening, approximately 100 isolates were inoculated into 40 ml of TAP medium in glass culture tubes (TEST 30 NP; Iwaki) at initial concentration of 1×10^5 cells ml^{-1} and grown at 23 °C under continual light at a photon flux of 20–30 μmol m^{-2} s^{-1}. As cultures grew, cell numbers, dry weights, and cell diameters were measured (Mizuno et al., 2013). For the primary screening, diverse criteria; e.g., color, cell size, and dry weight, were used. Twenty-three candidate mutants were tested during a second screening for their ability to grow in culture tubes (TEST 30 NP; Iwaki) with 30 ml of TAP medium at 23 °C under 100 μmol photons m^{-2} s^{-1}. Finally, seven mutants were obtained after the screening and were subjected to fuller phenotype assays (Table 1).

2.4. Phenotype assays

2.4.1. Starch assay

To measure starch content, 200-μl aliquots of culture were transferred to each well of 96-well microplates, 5 μl of Lugol's solution (5 g of I_2 and 10 g of KI dissolved in 100 ml of distilled water) were added into each well and the suspensions were mixed. Absorbance was read at 660 nm ($OD_{660\,nm}$) using a microplate reader (Viento nano; DS Pharma Biomedical, Osaka, Japan). The $OD_{660\,nm}$ of an unstained control was used for normalization, and the difference between $OD_{660\,nm}$ of stained and unstained deionized water (DW) functioned as a blank control. Normalization was performed as follows: $(OD_{Lugol\ stained\ sample}–OD_{unstained\ sample})–(OD_{Lugol\ DW}–OD_{DW})$. A serial dilution series of cornstarch Wako, Tokyo, Japan) was produced and used to construct a standard curve.

Before assaying by the iodine method, the correlation between the conventional anthrone assay and the iodine-based quantification was tested. The coefficient of determination (R^2) between the anthrone and iodine data was ~0.9 (Fig. S1), indicating a close linear correlation; hence, the iodine-based assay was used as a convenient and rapid measure of total starch.

2.4.2. Lipid assay

For lipid measurement, 100-μl aliquots of culture were transferred into each well of a black 96-well assay plate (Iwaki); then 100 μl of Nile Red solution (1 mM in dimethyl sulfoxide, DMSO; Polyscience, Inc., Warrington, PA, USA) were pipetted into each well and the suspensions were mixed. After incubation for 10 min at 37 °C, the fluorescence of Nile Red at 570 nm excited by 530 nm were determined from above using an ARVO SX microplate reader (Perkin Elmer Japan, Kanagawa, Japan). A 100-μl aliquot of distilled water was used as a blank control, and values of $OD_{595\,nm}$ were used for normalization.

2.4.3. Gas chromatography–mass spectrometry (GC–MS)

Twenty-milliliter aliquots of 4-day-old cultures in N-limited medium were used for analysis of fatty acid methyl esters (FAME) by GC–MS. Lipid extraction and sample preparation followed (Sheng et al., 2011). The FAMEs were separated and identified on a QP2010 Plus gas chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) fitted with a SP-2340 capillary (Supelco, Bellefonte, PA, USA) and used helium as the carrier gas. The GC oven was initially held at 140 °C for 1 min; subsequently, the temperature was increased at a rate of 4 °C min^{-1} to 220 °C and held there for