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

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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; Kazama et al., 2011; Hirano et al., 2012) and *Nicotiana tabacum* (Kazama et al., 2008a; Kazama et al., 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., 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 (Pibyl 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 *Parachlorella 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 $\sim 50 \text{ mol m}^{-2} \text{ s}^{-1}$) and no aeration.

2.2. Heavy-ion beam treatment

For the Ar-ion treatment, 200- l 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 ($\sim 4^{\circ}\text{C}$) in the period before heavy-ion-beam irradiation. Cells were irradiated by C ions (LET: 22.6 keV m^{-1}) or Ar ions (LET: 308 keV m^{-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 $1 \times 10^5 \text{ cells ml}^{-1}$ and grown at 23°C under continual light at a photon flux of $20\text{--}30 \text{ mol m}^{-2} \text{ s}^{-1}$. 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 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. 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 ($\text{OD}_{660 \text{ nm}}$) using a microplate reader (Viento nano; DS Pharma Biomedical, Osaka, Japan). The $\text{OD}_{660 \text{ nm}}$ of an unstained control was used for normalization, and the difference between $\text{OD}_{660 \text{ nm}}$ of stained and unstained deionized water (DW) functioned as a blank control. Normalization was performed as follows:

$(\text{OD}_{\text{Lugol stained sample}} - \text{OD}_{\text{unstained sample}}) - (\text{OD}_{\text{Lugol stained DW}} - \text{OD}_{\text{DW}})$. 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- l aliquots of culture were transferred into each well of a black 96-well assay plate (Iwaki); then 100 l 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- l 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 25 min. The methyl esters were quantified using nonadecanoic acid (19:0) as an internal standard.

2.4.4. Determination of chlorophyll concentration

Total chlorophylls were extracted with DMSO as a solvent, and chlorophyll concentration was determined spectrophotometrically following (Wellburn, 1994).

3. Results and discussion

3.1. Correlation between growth rate and cell weight in heavy-ion-beam-irradiated strains

The primary screening yielded 23 mutants from ~100 isolates derived from single colonies; the secondary screening yielded 7 strains (Fig. 1, Table 1), which were subjected to phenotypic assays, including measurements of lipid and starch accumulation and construction of fatty acid profiles. For the secondary screening,

growth rate, cell dry weight (DW), chlorophyll concentration, and cell diameter were used as selection criteria (Fig. 1, Table 1). The screened mutants were from cells subjected to C- or Ar-ion irradiation within the dose ranges of 10–300 Gy (C ion) or 5–150 Gy (Ar ion).

Survival rates were determined by a colony-forming unit (CFU) assay (Fig. S2). Cells were irradiated with C ions (LET: 22.6 keV m⁻¹) or Ar ions (LET: 308 keV m⁻¹) in a dose range from 0 to 300 Gy. Ar ions were more lethal than C ions (Fig. S2). After an Ar ion irradiation dose of 100 Gy, the CFUs dropped to nearly 0. At a similar C ion dose rate, 30% of cells survived. This outcome indicates that lethality is related to LET, which varies by ion species.

Survival curves are useful in determining the most effective dose for each ion species for mutation induction. A dose resulting in ~90% survivorship is suitable for mutation induction in land plants (Kazama et al., 2008b), whereas a dose with ~10% survivorship is suitable for microorganisms (Matuo et al., 2006). Unfortunately, the relationships between survival rate and mutation frequency in unicellular green algae are currently unknown.

The correlation between relative cell DW and relative growth rate was determined using prescreened *P. kessleri* isolates. Growth rate among the potential candidate

mutants was roughly negatively correlated with DW (Fig. 2), which was also the case for second-round screened mutants (Fig. 1a, b).

Before primary screening, a scatterplot analysis of heavy-ion-irradiated strains was conducted. Cell DW and growth rate were inversely correlated, indicating that heavy-ion treatment affects the basic parameters of cellular function (i.e., growth rate and cell mass in this case). This procedure may also be used for screening of mutants whose basic cellular functions might have been affected in the mutagenic process, but a rapid and accurate method is required for measurements of mutation frequencies; these measurements will contribute to a better understanding of the correlation between the survival rate and mutation rate.

3.2. Microscopic observations of subcellular starch and lipid bodies

The ultrastructure of mutants and the WT was observed using TEM. The starch granules of the WT cells had smooth surfaces and were filled with an electron-dense material (Fig. S3a). In the mutant strain 1A3, the starch granules were irregularly shaped, had rough surfaces, and were filled with electron-transparent material (Fig. S3b). Three other mutants (11A5, 1W15, and PK4) also had electron-transparent starch

granules, but the surfaces were smooth, similar to those of the WT (Fig. S3c, f, h).

Strains 11A5 and PK4 accumulated lipids in oil bodies (Fig. S3f, h).

The present ultrastructural observations showed that the starch phenotype of strain 1A3 was very different from the WT. WT starch granules had smooth surfaces and were filled with an electron-dense material. Starch granules of strain 1A3 were irregularly shaped, had rough surfaces, and contained electron-transparent material. This mutant starch granule form was reminiscent of those occurring in *Chlamydomonas* (Chlorophyta) and *Cryptocodinium cohnii* (Dinoflagellata) soluble starch synthase mutants (Buléon et al., 1997; Dauvillée et al., 2009).

Strain 11A5 in TAP and S-limited TAP media had higher lipid contents than the WT (Fig. S3c). To observe subcellular localization of lipids in living cells, Nile Red-stained cells were observed by fluorescence microscopy (Fig. S4). In the WT, granular bodies were stained with Nile Red and chlorophyll autofluorescence was clearly visible (Fig. S4). In cells of strain 11A5, globule-like structures with high-intensity Nile Red signals were observed; chlorophyll autofluorescence decreased in this mutant (Fig. S4).

3.4. Time-course analyses of lipid and starch accumulation in seven selected mutants

To determine the phenotypes of oil/starch accumulation, high-throughput phenotype assays using 96-well microplates were performed. Production of oil and starch in heavy-ion-irradiated mutants and the WT strain were assayed over time in three different culture media. In this study, full TAP medium, N-limited TAP medium (only 20% of normal N content), and S-limited TAP medium (no S content) were used. Because TAP without N medium noticeably inhibited the growth of cultures, a formula with 20% of the normal N content was used. In the S-limited medium, Cl^- ions were substituted for SO_4^{2-} ions.

In TAP medium, the starch level reached a peak at day 4 of cultivation and subsequently declined in all mutant and WT strains (Fig. 3a). Oil content increased after the starch peak and subsequently plateaued between days 6 and 9 of cultivation (Fig. 3a). Lipid accumulation of strains 11A5 and PK4 continued to rise after day 4. Notably, no plateau phase occurred in cultures of strain PK4 throughout the whole cultivation period (Fig. 3a). Strains 11A5 and PK4 had nearly double the lipid level of the WT after 12 days of cultivation.

The time courses of starch concentration in N-limited medium also peaked at day 4 in all strains, including the WT (Fig. 3b). In comparison with cultures in TAP medium, the starch levels in N-limited medium declined sharply from days 4 to 6 and continued

dropping thereafter (Fig. 3b). Lipid levels rose constantly from the early phases to the termination of the batch cultures. Notably, no lipid content plateau phase was observed in strain 1A3 during the entire cultivation period. Lipid levels of strains 1A3 and PK4 after 12 days of cultivation were nearly 1.5-fold higher than that of the WT.

Under S starvation, the total lipid contents were much higher than in S-replete TAP medium. For example, the WT after 12 days in S-limited TAP had a lipid content 3.2-fold higher than that in cultures raised in complete TAP medium (Fig. 3a, c). Starch level declined from days 2 to 4, peaking at day 6 and declining once more toward the end of the cultivation period (Fig. 3c). Lipid levels rose constantly from the beginning to the end of batch cultures. Strain 1A3 had a plateau phase in lipid level, whereas other strains (including the WT) did not. By day 12, strains 11A5, 1B8, 6A1, and 1W15 had lipid levels ~1.3–1.5-fold higher than that of the WT.

The mutant strain 11A5 exhibited hyperaccumulation of lipids in complete and S-limited medium. Fluorescence microscopy showed that this mutant had enhanced Nile Red signals compared with the WT; fat globules were obvious in the cells. Chlorophyll autofluorescence was barely measureable. This strain accumulated lipids in full medium to the same degree as WT cells under nutrient limitation. The growth of strain 11A5 was defective at later culture stages in comparison with the WT. All of the evidence

suggested that this mutant is likely a phenocopy of the WT under nutrient-limited conditions.

Reports have suggested metabolic interconnections between starch and lipid contents in *Chlorella* (Ramazanov and Ramazanov, 2006) and *Chlamydomonas* (Li et al., 2010). Detailed time-course analyses showed that lipid accumulation lags behind starch accumulation in *Chlamydomonas* cells subjected to N starvation (Fan et al., 2012) and in *Parachlorella* and *Chlorella* spp. subjected to S-limitation (Mizuno et al., 2013). The present study similarly showed that oil accumulation lags behind the buildup of starch content in the WT and all mutants of *P. kessleri* examined, an outcome congruent with the results of (Mizuno et al., 2013). Lipid accumulation occurred subsequent to the decrease in starch levels under all conditions examined. Notably, the accumulation of both starch and lipid was enhanced to some extent under N deprivation.

Two peaks in starch accumulation were observed on days 2 and 6 in *P. kessleri* cultures subjected to S starvation; this was not the case in medium with the full nutrient content or in medium with a reduced N concentration. In *Chlamydomonas* cells, photosystem II (PSII) activity is reduced in S-deprived cultures, but PSI is not affected (Wykoff et al., 1998). PSII activity in N-starved *Chlamydomonas* cultures also decreased, but not as rapidly as in S-starved cultures (Philipps et al., 2012).

Transcriptome analysis of S-deprived *Chlamydomonas* cells demonstrated that the transcript level of *LHCBM1-8* declines after 6 h of S deprivation; a more dramatic decline occurs after 24 h (González-Ballester et al., 2010). LHCBM1-8 is a light-harvesting protein of PSII, suggesting that S deprivation affects its functional structure. Starch metabolism may shift after these changes in the photosynthetic machinery. Therefore, one can reasonably postulate that S starvation interferes with cellular PSII activity in mutants and WT of *P. kessleri*.

3.5. Production of gain-of-function mutants by heavy-ion mutagenesis

The present phenotype assay revealed a broad spectrum of algal traits. Importantly, new phenotypes in lipid production and fatty acid profiles were found. For example, strain 11A5 was remarkable for elevated lipid production in complete medium and in S-limited medium. Lipid traits of this strain were not different from the WT in N-limited medium. In contrast, strain PK4 formed an elevated lipid content in both complete medium and N-limited medium. The growth of strain PK4 was not different from the WT over the culture period (Fig. 1a, data not shown), implying that heavy-ion irradiation

is an effective mutagenic agent for microalgae with the potential to produce mutants with gain-of-function phenotypes.

One or two peaks of starch production were observed on days 2, 4, and 6 of the cultivation period in all mutants and the WT, and in all cases, the starch content decreased toward the end of the experiment. No significant differences in starch production were detected among mutant strains. Thus, lipid metabolism phenotypes were much more varied than starch metabolism phenotypes after heavy-ion irradiation.

Fatty acid compositions of the mutants and WT strains after 4 days of cultivation were analyzed by GC-MS. The relative amounts of α -linoleic acid (C18:3) in strains C300-15 and PK4 were less than half that of the WT (Fig. 4). Furthermore, relative concentrations of fatty acids with long acyl chains (carbon numbers > 18) in the mutants were 1.6-fold higher than in the WT. The proportion of saturated fatty acids to total fatty acids in the WT was 21% (considering only fatty acids in the spectrum from C16 to C18); in strains 1A3, 1B8, C300-15, and PK4, the proportions of saturated fatty acids were 26, 28, 31, and 29%, respectively.

The GC-MS analysis showed that heavy-ion irradiation altered the fatty acid profile of *P. kessleri*. Some strains (C300-15 and PK4) had concentrations of fatty acids with long acyl chains (>C18) that were 1.6-fold higher than in the WT. Nutrients,

temperature, and/or light intensity are major factors affecting triacylglycerol accumulation and fatty acid profiles (Guschina and Harwood, 2006; Hu et al., 2008). It was demonstrated that heavy-ion irradiation can modify the fatty acid profiles or metabolic pathways of microalgal cells. Currently, the production of PUFAs by microalgae is receiving intense commercial attention (Guschina and Harwood, 2006). Unfortunately, it could not be determined whether the long-chain fatty acids detected by GC-MS were saturated or unsaturated. Further detailed studies are certainly warranted.

Conclusions

The present study demonstrated that heavy-ion-irradiated algal cells express a broad spectrum of biochemical phenotypes of potential commercial relevance, particularly those relating to lipid production and fatty acid composition. This mutagenic procedure has potential for the production of genetic mutants with gain-of-function phenotypes. Among the phenotypes produced by heavy-ion mutagenesis, there were several that hyperaccumulated lipids without nutrient limitation; these mutants may advance progress toward viable biofuel production from algal cells.

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Figure legends

Fig. 1. Screening of mutants by diverse criteria. The 23 strains were characterized after primary screening; from these 23, a final list of 7 strains was drawn up on the basis of multiple independent screenings. Mutant and wild-type (WT) strains were cultivated in 30 ml of TAP medium at 23°C in culture tubes under a 12/12-h light/dark cycle (photon flux: $100 \text{ mol m}^{-2} \text{ s}^{-1}$). The bars in blue indicate the mutants selected. Bars in cyan indicate strains selected by other criteria. The WT strain was used as a control for calculating relative values (red bars, WT = 1). (a) Relative growth rate from day 0 to day 4. (b) Relative cell DW in an 8-day-old culture. (c) Relative chlorophyll concentration in an 8-day-old culture. (d) Relative cell diameter in an 8-day-old culture.

Fig. 2. Scatterplot showing the correlation between relative dry weight (DW) per cell at the stationary phase and the relative growth rate. Each symbol refers to an isolate from prescreened colonies after heavy ion (Ar and C ions) irradiation or a wild-type (WT) strain (shown in red). Blue symbols indicate strains examined in this study. The WT strains were used as controls in calculating relative values (WT = 1). Growth rates were

calculated from the numbers of cells on day 0 and day 3 of culture (for strains 1B8, 1W15, C300-15, and PK4, cell numbers on days 0 and 4 were used to calculate the growth rate). See Table 1 for strain codes.

Fig. 3. Phenotype spectrum of lipid and starch accumulation in heavy-ion-irradiated strains and the wild type. The cultures were raised in 200 μ l of TAP medium at 23°C in 96-well microplates under a 12/12-h light/dark cycle (photon flux $\sim 100 \text{ mol m}^{-2} \text{ s}^{-1}$). (a) Time courses of lipid ($n = 6$) and starch ($n = 5$) concentration per $\text{OD}_{565 \text{ nm}}$ in TAP medium; values are means $\pm$ SD. (b) Time courses of lipid ($n = 6$) and starch ($n = 5$) levels per $\text{OD}_{565 \text{ nm}}$ under N limitation (TAP medium with N concentration diluted by 80%); values are means $\pm$ SD. (c) Time course of lipid ($n = 6$) and starch ($n = 5$) levels per $\text{OD}_{565 \text{ nm}}$ under S limitation (TAP medium without S); values are means $\pm$ SD. See Table 1 for strain codes.

Fig. 4. Fatty acid profiles of seven mutants and one wild-type strain. The strains were cultivated in 20 ml of TAP medium at 23°C in a culture flask under a 12/12-h light/dark cycle (photon flux $\sim 100 \text{ mol m}^{-2} \text{ s}^{-1}$). Lipids in 4-day-old cultures were extracted and fatty acid compositions were analyzed by GC-MS. “Others” refers to fatty acids with

long acyl chains (>C18). See Table 1 for strain codes.

Table 1

Parachlorella kessleri: List of mutants obtained by heavy ion irradiation and subjected to phenotypic screening.

Strains	Ion species	Dose (Gy)	First characterizations (compared to the wild type) ^a
1A3	Argon	150	Cell mass: 3.8-fold higher
11A5	Argon	50	Cell mass: 4.7-fold higher
1B8	Carbon	100	Chlorophyll concentration: 1.2-fold higher
6A1	Argon	5	Growth rate: 1.2-fold higher
1W15	Carbon	300	Chlorophyll concentration: 1.5-fold higher
C300-15	Carbon	300	Chlorophyll concentration: same as the wild type
PK4	Carbon	10	Growth rate: 1.1-fold higher

^a See Fig. 1 for details.

Figures

Figure 1

Figure 2

Figure 2(BW)

Figure 3

Figure 3(BW)

Figure 4