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Photoautotrophic production of macular pigment in a *Chlamydomonas reinhardtii* strain generated by using DNA-free CRISPR-Cas9 RNP-mediated mutagenesis†

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ABSTRACT: Lutein and zeaxanthin are dietary carotenoids reported to be protective against age-related macular degeneration. Recently, the green alga *Chlamydomonas reinhardtii* has received attention as a photosynthetic cell factory, but the potential of this alga for carotenoid production has not yet been evaluated. In this study, we selected the *C. reinhardtii* CC-4349 strain as the best candidate among seven laboratory strains tested for carotenoid production. A knock-out mutant of the zeaxanthin epoxidase gene induced by preassembled DNA-free CRISPR-Cas9 ribonucleoproteins in the CC-4349 strain had a significantly higher zeaxanthin content (56-fold) and productivity (47-fold) than the wild type without the reduction in lutein level. Furthermore, we produced eggs fortified with lutein (2-fold) and zeaxanthin (2.2-fold) by feeding hens a diet containing the mutant. Our results clearly demonstrate the possibility of cost-effective commercial use of microalgal mutants induced by DNA-free CRISPR-Cas9 ribonucleoproteins in algal biotechnology for the production of high-value products.

KEYWORDS: *Chlamydomonas reinhardtii*; CRISPR-Cas9; lutein; zeaxanthin; algal biotechnology

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Introduction

Carotenoids are isoprenoids generally regarded as natural pigments; their distinctive characteristic colors range from yellow to red and they are present in many flowers, fruits, and vegetables (Cazzonelli 2011; Humphries and Khachik 2003). Carotenoids are comprised of 40 carbon atoms with 8 isoprene units and are synthesized by all photosynthetic organisms including cyanobacteria, algae, and higher plants (Takaichi 2011). Carotenoids are classified into two groups: carotenes, which are purely unsaturated hydrocarbons, and xanthophylls (including neoxanthin, violaxanthin, antheraxanthin, lutein, and zeaxanthin), which are oxygenated carotenoids. In photosynthesis, carotenoids serve as accessory antenna pigments by capturing light energy at wavelengths that chlorophyll cannot absorb (Nisar et al. 2015). Carotenoids protect organisms by absorbing excess light energy and dissipating it as heat (Niyogi et al. 1997a). Among the carotenoids, lutein and its stereoisomer zeaxanthin have received attention, because they play a critical role in maintaining eye health (Abdel-Aal et al. 2013). Lutein and zeaxanthin are referred to as macular pigments, because they are specifically accumulated in the macula of the human retina (Landrum and Bone 2001). Zeaxanthin is dominant in the macula (near the center of the retina), while lutein is widely distributed throughout the retina. The macular pigments in the retina prevent damage from oxidative stress by attenuating high-energy blue light (Ma and Lin 2010). Adequate intake of products containing a high amount of macular pigments, such as spinach or collards, reduces the risk of age-related macular degeneration (AMD), the leading cause of irreversible blindness in adults (Abdel-Aal et al. 2013; Moeller et al. 2000). Although lutein is abundant in green leafy vegetables, the content of zeaxanthin is relatively low, making it difficult to use leafy vegetables commercially as zeaxanthin sources (Perry et al. 2009).

Recently, microalgae have received attention as photosynthetic cell factories that convert carbon dioxide to high-value products such as biofuels, antioxidants, fatty acids, and

carotenoids (Koller et al. 2014; Sasso et al. 2012). Over the past several years, large-scale commercial production of carotenoids in strains of green algae has been successfully developed, including production of β-carotene in *Dunaliella*, astaxanthin in *Haematococcus*, and lutein in *Chlorella* (Spolaore et al. 2006). Compared to terrestrial plant-based sources, microalgae have a clear advantage for carotenoid production (Gong and Bassi 2016). Microalgae have a greater specific carotenoid content than any alternative source. For example, lutein production by some *Scenedesmus* strains (4mg g^{-1}) is up to 13 times that of the predominant producer, marigold flowers (usually 0.3 mg g^{-1}) (Ho et al. 2014). The content of β-carotene in *Dunaliella* was reported a 10% of its dry biomass (García-González et al. 2005). In addition to high carotenoid content, the advantages of microalgae cultivation include rapid growth, high productivity, and a short harvesting cycle (Lin et al. 2015). Microalgae also can be produced on non-arable lands such as wet-lands or deserts using non-potable water (waste and sea water) without seasonal variation in growth. Despite these advantages, wild type microalgae are not suitable for macular pigment production. Microalgae are generally rich in lutein, whereas they rarely contain zeaxanthin found under non-stress conditions as zeaxanthin content is controlled by the intensity of light or heat (Jin et al. 2003b). Therefore, genetic engineering of carotenoid biosynthesis to optimize lutein and zeaxanthin content is important for industrial production of macular pigments (Bajhaiya et al. 2016; Varela et al. 2015). Over the past two decades, there have been attempts to generate carotenogenic mutants using mutagens and to develop these mutants as industrial strains. By using EMS mutagenesis, a zeaxanthin-accumulating mutant in Dunaliella salina was isolated which has impaired zeaxanthin epoxidation (Jin et al. 2003a). However, there are only a few cases where carotenoid mutants have been successfully obtained by random mutagenesis (Gong and Bassi 2016), and the development of an effective transformation system for genetic manipulation in industrial algal strains remains in its early stage.

As a model organism with a vast variety of techniques and tools for metabolic engineering, *Chlamydomonas reinhardtii* is promising for industrial production of macular pigments. For decades, *C. reinhardtii* has been used as a versatile system to study cellular mechanisms for research in cell biology and for strain improvement, since it offers many advantages such as fast growth rates, easy transformation, and simplicity of culture. Recent studies that used *C. reinhardtii* have advanced in the production of high-value products including biofuels, therapeutic proteins, and nutraceuticals on a laboratory scale (Rosales-Mendoza et al. 2012; Scaife et al. 2015; Scranton et al. 2015). In addition, a variety of carotenoid mutants have been found over the long history of research on *C. reinhardtii*, leading to the discovery of genes related to biosynthesis of lutein and zeaxanthin (Baroli et al. 2003; Niyogi et al. 1997b; Polle et al. 2001). Therefore, *C. reinhardtii* has an excellent potential to be used as a macular pigment cell factory for commercial exploitation.

To achieve this goal, several major challenges must be overcome. Firstly, a strain with higher pigment productivity under photoautotrophic growth conditions than that of the currently available strains is needed. To identify such a strain, it is necessary to screen natural *Chlamydomonas* strains for traits suitable for commercial scale production. Secondly, new technology is necessary to develop high-pigment producing, non-GM algae, because existing controversies restrict the industrial utility of genetically modified algal strains. Third, new approaches that can reduce the cost of biomass harvesting and pigment extraction are needed. These issues remain major obstacles that will contribute significantly to the overall production costs (Kim et al. 2013; Park et al. 2015). To solve these problems, we analyzed a widely used wild type strains of *C. reinhardtii* to find an optimized strain for carotenoid production. To achieve simultaneous accumulation of lutein and zeaxanthin, we have knocked out the gene for zeaxanthin epoxidase (*ZEP*) using a preassembled Cas9 protein–gRNA ribonucleoprotein (RNP) delivery method (Baek et al. 2016) and we evaluated the industrial feasibility of macular

pigment–accumulating mutants by measuring pigment contents and productivity under photoautotrophic growth conditions. Finally, to reduce the cost of pigment extraction, we attempted to produce chicken eggs by feeding laying hens with strains with enhanced pigment production. We discuss this approach with respect to its commercial use and cost-effective application in algal biotechnology for the production of high-value products as well as macular pigments.

Materials and Methods

Algal strains and culture conditions

C. reinhardtii strains CC-4349 (cw15 mt-) was obtained from Dr. Jae-Hyeok Lee (University of British Columbia). All strains are also available as CC-124, CC-125, CC-620 (R3+), CC-621 (NO-), CC-4051 (4A+), and CC-4533 (cw15 mt-) from the *Chlamydomonas* Resource Center (CRC). Cells were maintained photoheterotrophically in Tris-acetate phosphate (TAP) medium or photoautotrophically in high-salt (HS) medium under continuous white fluorescence light (70 μmol photons m^{-2} s^{-1}) at 25°C with shaking on an orbital shaker at 90 rpm.

Growth analysis

Growth experiments were performed in a bubble column photobioreactor composed of 4-cm-diameter and 50-cm-tall glass cylinders. *C. reinhardtii* cells were grown photoautotrophically in 400 mL HS medium under continuous light (200 μmol photons m^{-2} s^{-1}) at 25°C. Cultures were bubbled with 5% CO_2 at 80 mL $minute^{-1}$. The initial cell concentration for each culture was 1×10^6 cells ml^{-1}. Microalgal growth was measured by the increase in cell number and dry biomass weight. Cells were counted every 12 hours using a hemocytometer under a light microscope. The specific growth rate (μ) in the exponential growth phase was calculated using

the following equation: $\mu = (\ln x_2 - \ln x_1) / (t_2 - t_1)$, where x_2 and x_1 are cell numbers at times t_2 and t_1, respectively. To measure the dry biomass weight, cells were collected by centrifugation at 3000 *g* for 5 minutes in pre-weighed 25 ml glass vials. Supernatants were discarded, and the cell pellets in the glass vials were dried for 48 hours at 65°C in a drying oven prior to weighing.

Total carotenoid and chlorophyll determination

Pigments were extracted in 80% acetone, and cell debris was removed by centrifugation at 16,000 *g* for 5 minutes. The absorbance of the supernatant was measured with an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, USA). Total carotenoid and chlorophyll concentrations of the samples were determined according to an established method (Lichtenthaler 1987).

Cell disruption test

To evaluate cell fragility, the effects of chemical and physical shock on cell wall integrity were investigated according to a modified protocol (Miyake et al. 2014). Cells of all strains were cultivated to the exponential phase in TAP medium under 70 μmol photons m^{-2} s^{-1} light at 25°C with shaking at 90 rpm and diluted to OD_{750} of 1.0. Four experimental methods were used to test cell disruption. The effect of detergent was tested for 90 seconds in TAP media containing 0.1% Triton X-100 and 0.1% NP40. Glass bead vortexing was performed in 10 mL glass tubes with 0.3 g of acid washed glass beads (425–600 μm, Sigma, USA) for 5 minutes at maximum speed. Sonication was performed for 30 seconds with 1 second pulses and 1 second intervals, (i.e., 15 times in total) at 60% power. All treated cells were collected by centrifugation at 3000 *g* for 2 minutes, and the harvested pellet was gently re-suspended in TAP medium. The optical density of the supernatant was measured at 750 nm and compared with that of the control (centrifuged and re-suspended cells that had not undergone any stresses).

Pigment analysis (HPLC)

Cells were grown photoautotrophically in 400 mL HS media with 5% CO_2 under 200 μmol photons m^{-2} s^{-1} continuous light and were harvested in the early stationary phase. The pigments were extracted from the cells in 90% (v/v) acetone, and the supernatant was filtered (0.2 μm nylon filter) and subjected to HPLC analysis. HPLC analysis was conducted with a Shimadzu Prominence HPLC model LC-20AD equipped with a Waters Spherisorb 5.0 μm ODS1 4.6 × 250 mm cartridge column. Pigments were separated in a mixture containing 14% 0.1 M Tris-HCl pH 8.0, 84% acetonitrile, and 2% methanol from 0 to 15 minutes and 68% methanol and 32% acetonitrile from 15 to 19 minutes. A post-run was performed for 6 minutes with the initial solvent mixture. The flow rate was 1.2 mL/minute. Pigments were detected at 445 nm and 670 nm. The concentrations of individual pigments were determined from the HPLC profiles calibrated with chlorophyll and carotenoid standards (DHI, Denmark).

Genotype characterization

To confirm the insertions and deletions, the target regions of *ZEP*–targeted RNPs were PCR-amplified with specific primers (5′-CTACGTCGCCTTACTGTGTG-3′ and 5′-GATTGCCTACTCACCACTCG-3′). The PCR products were separated by electrophoresis on a 2% agarose gel. To analyze potential off-target mutations in *ΔZEP* mutants, we used Cas-OFFinder (Bae et al. 2014) to list potential off-target sites that differed from on-target sites by up to 4 nucleotides and identified 11 potential off-target sites. We performed the targeted deep sequencing for each mutant and analyzed the NGS data of them by using Cas-Analyzer (Park et al. 2017). Wild type cells were used as a negative control.

Egg production and analysis

Six 15-week old laying hens (hy-line Brown) were randomly allocated to three groups and fed a standard diet for laying hens (Purina feed, Korea) for the first week (Table SI). Afterwards, the control group was fed an only the standard diet, while the WT and *ΔZEP1* groups were fed a diet supplemented with 3 g kg^{-1} (0.3%) of dried *C. reinhardtii* CC-4349 (wild type or *ΔZEP1* mutant strains, respectively). Each group was kept in commercial-type wire cages in a room with a 16/8 hours light/dark cycle. Feed and water were supplied ad libitum. About 30 days later, all groups began to produce eggs. Six eggs from each group were randomly collected to analyze pigment concentration. Pigments were extracted from egg yolks in 90% (v/v) acetone, and the supernatants were filtered (0.2 μm nylon filter) and subjected to HPLC analysis.

Results and Discussion

Strain screening for industrial pigment production in *Chlamydomonas reinhardtii* under photoautotrophic conditions

C. reinhardtii has long been used as a model organism, and a wide variety of laboratory strains are currently in use. Despite sharing a recent common ancestor, it is clear that the laboratory strains of *C. reinhardtii* have considerably different biological features including starch and oil accumulation (Siaut et al. 2011). To screen for the best strain for pigment production, we analyzed photoautotrophic growth and carotenoid production in seven common laboratory strains: CC-124, CC-125, CC-620, CC-621, CC-4051, CC-4349, and CC-4533. To achieve high productivity, 5% CO_2 was added. Cell growth curves of these strains in a bubble column are shown in Figure 1. After almost 36 hours of culture, most strains the reached stationary phase. CC-4349 grew remarkably better than other strains at all growth stages (Fig. 1). Not only did it grow at a faster rate, but CC-4349 also achieved a higher cell density. During the

exponential phase, its specific growth rate was 0.12 h^{-1}; in the stationary phase, its cell concentration was 3.57×10^7 cells mL^{-1} (Table I). Among all strains tested, CC-4349 also showed the highest biomass concentration (1.26 g L^{-1}) and productivity (0.84 g L^{-1} d^{-1}) in the stationary phase. Under similar culture conditions, strains of the industrial alga *Chlorella* showed specific growth rates between 0.02 and 0.11 h^{-1} (Cordero et al. 2011) and culture biomass productivity values ranging from 0.19 to 0.84 g L^{-1} d^{-1} (Cordero et al. 2011; Del Campo et al. 2000). Our data showed that *C. reinhardtii* strains, including CC-4349, have biomass productivity levels that are competitive enough for used industrial use.

Pigment contents and productivity were also measured in the early exponential phase under the same conditions used for the growth and biomass tests. Interestingly, the carotenoid level of the CC-4349 strain was the highest among the strains tested (9.43 mg L^{-1}), although its carotenoid on a per cell basis was considerably lower than that of any of the other strains (Fig. 2A, 2B). Similar results were obtained for chlorophyll levels in the same strains (Fig. S1). To investigate the differences in growth rates and biomass, we analyzed the median cell size in the late stationary phase (Fig. S2). CC-4349 showed the smallest median cell size value among the strains examined; this characteristic of CC-4349 is thought to be related to its high growth rate (Lloyd 2013), which resulted in the highest biomass content (Fig. S3). As a result, when the carotenoid contents per dry weight were compared, CC-4349 was found to produce most abundant carotenoids (Fig. 2C).

During pigment extraction process, cell disruption is an essential pre-step for high yield, although it introduces additional processing costs and energy expenditure (Gong and Bassi 2016; McMillan et al. 2013). To identify the strain most vulnerable to cell disruption, we investigated the effects of various chemical and physical stresses on cell wall integrity. Among the tested strains, CC-4349 was most susceptible strain to all disruption tests (Fig. 3), which included detergent treatment (NP40, Triton X-100), vortexing with glass bead beating, and

sonication; its high susceptibility is likely caused by a known defect in cell wall production (Davies and Plaskitt 1971). Whether the fast growth and small size of CC-4349 cells are also caused by this defect is currently unclear. Ultimately, we choose CC-4349 for further work to increase macular pigment production via genetic manipulation, since this strain showed the highest specific growth rate and carotenoid productivity. This is the first report to evaluate pigment productivity under photoautotrophic conditions in a *C. reinhardtii* strains and to show the potential of industrial of this species for pigment production.

ZEP gene knockout via DNA-free CRISPR-Cas9 RNP delivery in the *C. reinhardtii* CC-4349 strain

In our previous study, we established an one-step method of gene knock out using preassembled DNA-free CRISPR-Cas9 RNP in the *Chlamydomonas* CC-4349 strain and thereby, we constructed three *ZEP* knock-out cell lines (*ΔZEP1, ΔZEP2, ΔZEP3*) by targeting the end region of the first exon with each single guide RNA (sgRNA) (Baek et al. 2016). It had been reported that these mutants constitutively accumulated zeaxanthin, and sequencing showed various patterns of insertions and deletions (indels) at the expected positions of genomic DNA. These results suggested the possibility of macular pigment production for industrial use, and so we selected these mutants for further analysis. The coloration of *ΔZEP* mutants was more yellow green than that of wild type under the 300 μmol photons m^{-2} s^{-1} light photoautotrophic conditions (Fig. S4A). To confirm insertions or deletions found in the *ΔZEP* mutants, we compared the lengths of PCR fragments amplified that comprised the target loci and observed different sizes of PCR product (Fig. S4B). We also examined the presence of the ZEP protein by western blot analysis and confirmed that all of the Δ*ZEP* mutants lacked ZEP (Fig. S4C). To investigate whether the *ZEP*–targeted RNPs induced off-target mutations in the *ΔZEP* mutants, mutation frequencies at on-target and potential off-target sites of the *ZEP* gene-specific sgRNA were measured by targeted deep sequencing. Indel frequencies did not exceed

the sequencing error rates at the sites (Table SII), suggesting that the DNA-free Cas9 RNP delivery method reduced off-target effects because the CRISPR-Cas9 protein is only transiently active and is then degraded by endogenous proteases in cells (Kim et al. 2014). Furthermore, the resulting transformants could be exempt from genetically modified organism (GMO) regulations because of the absence of foreign DNA fragments (Kim and Kim 2016), potentially enabling their wide commercial use. A comparison of pigment compositions of wild type and *ΔZEP* mutants grown under different irradiance conditions is shown in Figure 4 and Table SⅢ.

Lutein content of the Δ*ZEP* mutants was similar to that of the wild type; however, the amount of zeaxanthin was significantly higher in Δ*ZEP* mutants grown under high light (mean, 365.95 mmol mol^{-1}) and even low light (264.48 mmol mol^{-1}) than in the high light–grown wild type (115.68 mmol mol^{-1}). Thus, these characteristics of the Δ*ZEP* mutants would reduce the cost of macular pigment production.

Macular pigment production in *ΔZEP* mutant lines under photoautotrophic conditions

We compared the cell growth and macular pigment contents of the *ΔZEP* mutants and wild type under 200 μmol photons m^{-2} s^{-1} light in bubble columns with 5% CO_2 (Fig. 5A). All strains entered into the stationary phase after 36 hours, and the cell growth curves of *ΔZEP* mutants were similar to that of the wild type. Although the level of lutein in the mutants was also similar to the wild type level (Fig. 5B), the level of zeaxanthin produced by the Δ*ZEP* mutants(mean, 2.26 mg L^{-1}) was significantly higher than that of the wild type (0.05 mg L^{-1}) (Fig. 5C). These results show that the *ΔZEP* mutants are able to produce large amounts of both lutein and zeaxanthin even under moderate light intensities. Comparison of the biomass and macular pigment productivity of the wild type and *ΔZEP* mutants during the early stationary phase is shown in Table II. Biomass of the Δ*ZEP* was lower than that of the wild type. However, Δ*ZEP* mutants had a higher macular pigment content per dry weight in comparison with the

wild type; zeaxanthin content was at least 50 times that of the wild type. In addition, since CC-4349 is a fast-growing strain, the Δ*ZEP* mutants showed high levels of daily macular pigment productivity. Compared to other microalgae in similar autotrophic culture conditions, the lutein productivity of lutein in *ΔZEP1* (2.31 mg L^{-1} d^{-1}) was similar to those of *Chlorella zofingiensis* (1.80 mg L^{-1} d^{-1}) and *Chlorella sorokiniana* (2.53 mg L^{-1} d^{-1}) (Cordero et al. 2011; Del Campo et al. 2000). Although few studies have reported zeaxanthin productivity in microalgae, zeaxanthin content of Δ*ZEP1* (2.21 mg g^{-1}) was markedly higher than those of other microalgal strains (between 0.09 and 1.11 mg g^{-1}) (Cordero et al. 2011; Paliwal et al. 2016). Taken together, these results suggest that CRISPR-Cas9 RNP-mediated *ZEP* mutagenesis can be used to generate strains for producing macular pigments.

Production of macular pigment-enriched eggs using CRISPR-Cas9 RNP-induced *ZEP* mutants as dietary supplements

Downstream processes of pigment extraction, purification, and storage are the most costly steps in microalgal carotenoid production on an industrial scale (Gong and Bassi 2016). To increase the efficiency of downstream processing, a cost-effective method must be developed. Currently, it is more efficient to obtain products with increased macular pigment content by using algae as an animal feed rather than by directly extracting the pigments from the microalgae. In particular, chicken eggs are an ideal dietary source of macular pigments. Although the amount of macular pigments in the yolk is relatively small compared to other sources such as marigold or spinach, the fatty acid composition of yolk improves the bioavailability of macular pigments, making eggs the most effective product in terms of macular pigment bioavailability (Chung et al. 2004). Indeed, daily consumption of eggs significantly increases serum lutein and zeaxanthin concentrations and helps to prevent adult cataracts and age-related macular degeneration (Goodrow et al. 2006; Vishwanathan et al. 2009). Therefore, as an alternative route to bypass the downstream processes mentioned above, we attempted to produce macular

pigment–enriched eggs by feeding laying hens a diet containing powder prepared from *C. reinhardtii* WT or Δ*ZEP* mutants. The yolk color of the control group was yellow, while that of the WT group was light yellow and that of the Δ*ZEP* group was orange (Fig. 6A). Large increases in the macular pigment concentrations of yolks in the WT and Δ*ZEP* groups were confirmed by HPLC analysis (Fig. 6B). Although the lutein concentration of egg yolk in the WT group (22.94 μg mL^{-1}) was much higher than that of the control group (12.49 μg mL^{-1}), the concentrations of zeaxanthin were not significantly different between the WT group (11.06 μg mL^{-1}) and the control group (11.65 μg mL^{-1}). However, the lutein and zeaxanthin concentrations of yolks in the Δ*ZEP* group (25.48 μg mL^{-1} and 25.63 μg mL^{-1}, respectively) were more than twice those of the control group (Fig. 6C). Considering the volume of egg yolks in the ΔZEP mutant fed group, the amounts of total lutein and zeaxanthin in the whole yolk were about 254.8 μg and 256.3 μg per egg, respectively. This result suggests that dietary supplements with Δ*ZEP* mutants could efficiently increase the concentrations of lutein and zeaxanthin in egg yolk. Due to the high macular pigment content of the Δ*ZEP* mutants, the addition of only a small amount (0.3%) of dried Δ*ZEP* mutant into the feed resulted in the same increase in macular pigment concentration in yolk as the addition of 2% dried *Chlorella* (Jeon et al. 2012). An increased lutein content has been reported in eggs produced by laying hens fed diets with crude extracts or dried biomass of marigold, spinach, and microalgae (Jang et al. 2014; Jeon et al. 2012; Karadas et al. 2006). To our knowledge, there have been no reports demonstrating increases in both lutein and zeaxanthin in eggs using microalgal mutants with increased macular pigment content. The eggs developed in this study will be beneficial in terms of protection against diseases caused by insufficient intake of lutein and zeaxanthin. Moreover, this approach demonstrates the commercial value and potential utility of microalgal mutants generated by using DNA-free CRISPR-Cas9 RNP.

Conclusions

We determined that the CC-4349 strain of *Chlamydomonas reinhardtii* was the best candidate for carotenoid production among seven laboratory strains tested. The production of macular pigment from *ZEP* knock out mutant of CC-4349 strain, which was generated by preassembled DNA-free CRISPR-Cas9 ribonucleoproteins, demonstrated the possibility of commercial application of this strain under photoautotrophic conditions. Furthermore, this mutant was successfully used as a feed supplement to produce macular pigment–enriched eggs. Since algal varieties obtained by using this transformation method might be exempt from current GMO regulations, our approach could be widely applicable in algal biotechnology for production of high-value bio-products.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Tables

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Figure 2. Total carotenoid contents in common laboratory strains of *C. reinhardtii*. Cells cultured in bubble columns were analyzed at the early exponential stage. (**A**) Total carotenoid content per cell. (**B**) Total carotenoid content per L of culture. (**C**) Comparison between total carotenoid contents and biomass. Values represent the mean and standard error of three independent experiments.

Figure 3. Cell disruption test in laboratory strains of *C. reinhardtii*. Cells were subjected to (**A**) 0.1% NP40, (**B**) 0.1% Triton X-100 treatment, (**C**) glass bead vortexing, or (**D**) sonication, harvested by centrifugation, resuspended, and the optical density of supernatants was measured. Values represent the mean and standard error of three replicates (three independent experiments).

Figure 4. Lutein and zeaxanthin level in WT and CRISPR-Cas9 RNP-mediated *ZEP* gene knockout mutants of the *C. reinhardtii* CC-4349 strain. *ΔZEP* mutant lines were grown under LL, low light (50 μ mol photons m^{-2} s^{-1}); ML, medium light (300 μ mol photons m^{-2} s^{-1}); HL, high light (600 μ mol photons m^{-2} s^{-1}) in TAP medium. Pigments were analyzed by HPLC. Data were normalized to chlorophyll a. The values represent the mean and standard error of three replicates (three independent experiments).

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(**C**) Time course of the zeaxanthin concentrations. Cells were cultured in HS medium at 25°C with air containing 5% CO_2 under continuous white light (200 μmol photons m^{-2} s^{-1}). At each time point, values represent the mean and standard error of four replicates (three independent experiments). Where the error bars are not shown, they were smaller than the symbol size.

Figure 6. The effects of *C. reinhardtii* wild type (WT) and *ΔZEP* mutant powder added to the diet fed to laying hens on the macular pigment contents of egg yolk. (**A**) Comparison of egg yolk color of the control (basal diet), WT, and *ΔZEP1* groups. (**B**) HPLC profiles of macular pigments extracted from egg yolk with acetone. Lut, lutein; Zea, zeaxanthin. (**C**) Macular pigment content in the yolk. Values represent the mean and standard error of three replicates (six independent experiments).

Table I. Specific growth rate, doubling time, maximum biomass, and biomass productivity of common laboratory strains of *C. reinhardtii* under photoautotrophic conditions. The values represent the mean and standard error of four replicates (three independent experiments).

Strain Name	Alternate Names	Mating Locus	Specific growth rate (μ, h^{-1})	Doubling time (h)	Maximum biomass ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ d^{-1}$)
CC-124	137c mt–	mt-	0.08 ± 0.00	8.81 ± 0.44	1.25 ± 0.10	0.84 ± 0.07
CC-125	137c mt+	mt+	0.09 ± 0.01	7.66 ± 0.52	1.19 ± 0.10	0.80 ± 0.07
CC-620	R3+	mt+	0.07 ± 0.00	9.77 ± 0.39	1.03 ± 0.04	0.69 ± 0.03
CC-621	NO-	mt-	0.08 ± 0.00	8.97 ± 0.52	1.14 ± 0.19	0.76 ± 0.12
CC-4051	4A+	mt+	0.09 ± 0.00	8.19 ± 0.36	0.95 ± 0.06	0.63 ± 0.04
CC-4349	Goodenough 330A	mt-	0.12 ± 0.00	5.93 ± 0.19	1.26 ± 0.03	0.84 ± 0.02
CC-4533	Jonikas CMJ030	mt-	0.07 ± 0.01	12.06 ± 1.73	1.11 ± 0.02	0.74 ± 0.01

Table II. Biomass productivity (g L^{-1} d^{-1}), and macular pigment content (mg g^{-1}), and productivity (mg L^{-1} d^{-1}) of the wild type and Δ*ZEP* mutant lines. Values represent the mean and standard error of three replicates (three independent experiments).

	Biomass	**Lutein**		**Zeaxanthin**	
	g L^{-1} d^{-1}	**mg g^{-1}**	**mg L^{-1} d^{-1}**	**mg g^{-1}**	**mg L^{-1} d^{-1}**
WT	0.90 ± 0.01	2.71 ± 0.04	2.44 ± 0.04	0.04 ± 0.00	0.04 ± 0.00
ΔZEP1	0.75 ± 0.02	3.08 ± 0.07	2.31 ± 0.04	2.21 ± 0.03	1.66 ± 0.06
ΔZEP2	0.71 ± 0.01	3.03 ± 0.12	2.16 ± 0.07	2.00 ± 0.06	1.42 ± 0.04
ΔZEP3	0.61 ± 0.02	3.08 ± 0.10	1.86 ± 0.04	2.37 ± 0.04	1.44 ± 0.06

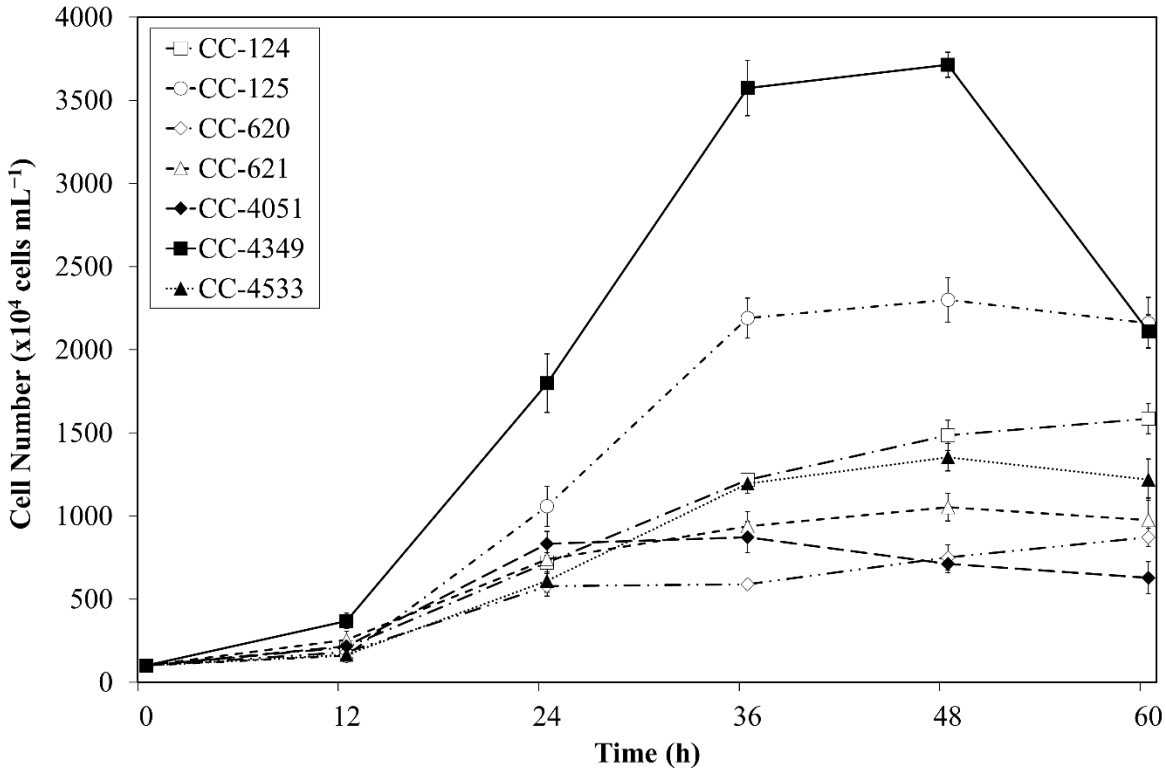


Figure 1

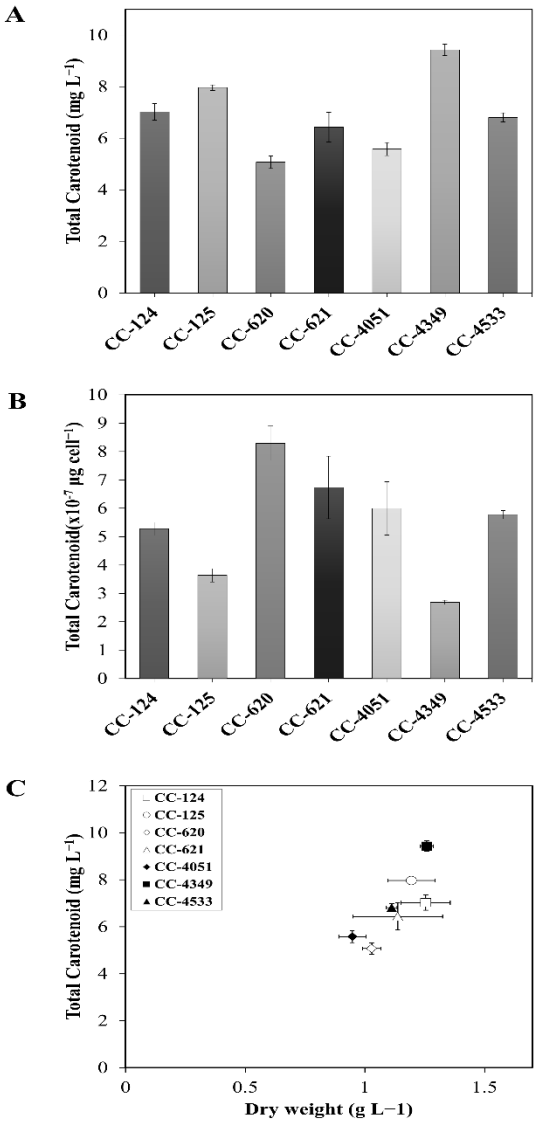


Figure 2

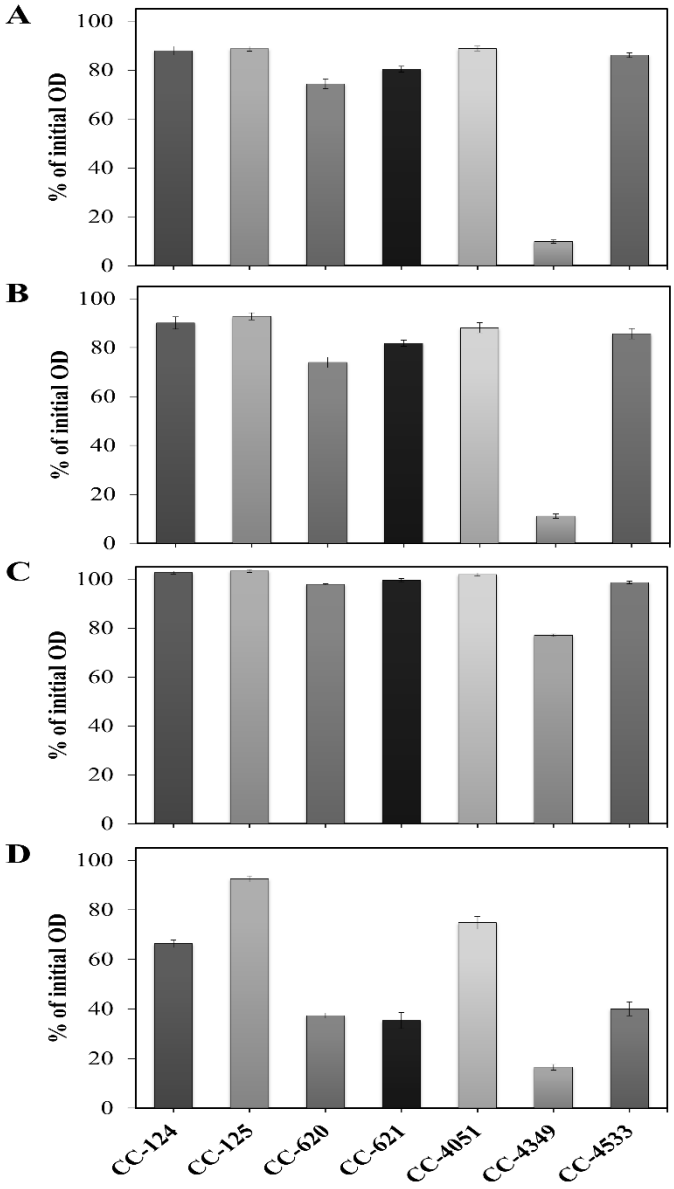
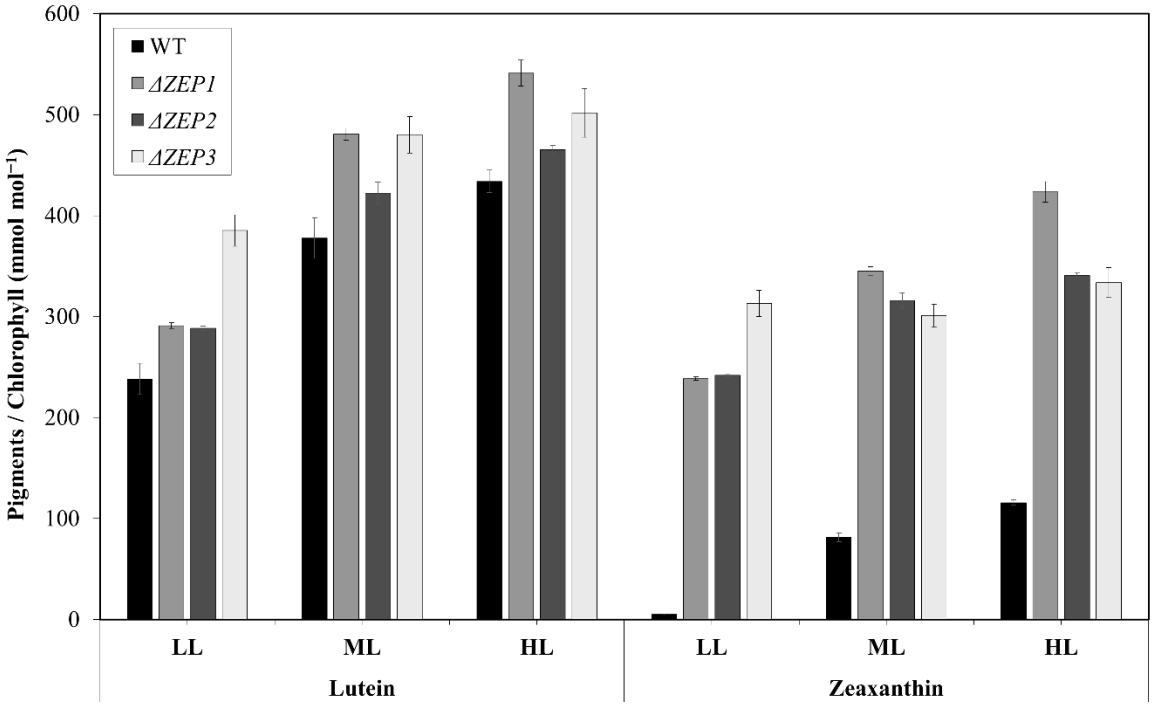


Figure 3



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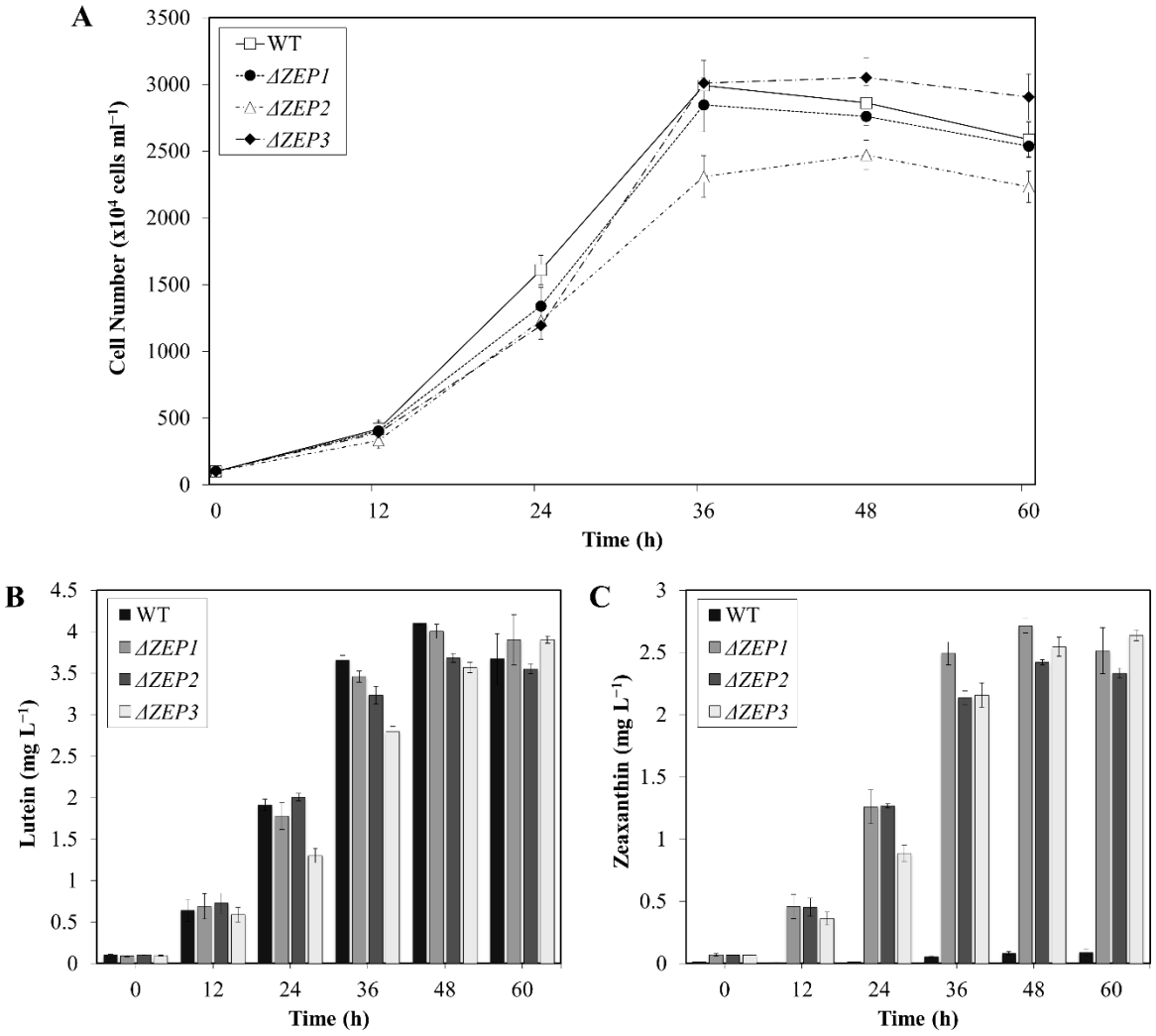

Figure 5

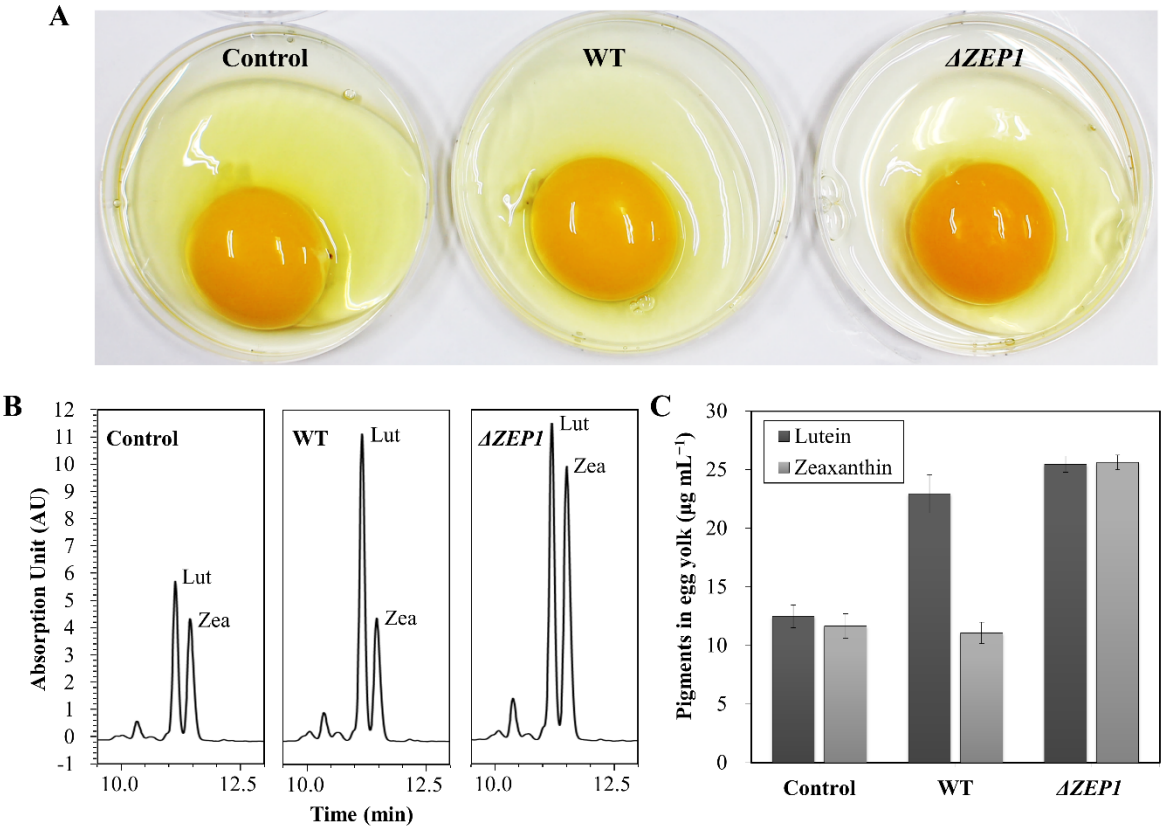

Figure 6