Characterization of Chlamydomonas Very High Light-tolerant Mutants for Enhanced Lipid Production

Chonlada Yaisamlee¹, ² and Anchalee Sirikhachornkit¹, ²*

¹ Microalgal Molecular Genetics and Functional Genomics Special Research Unit, Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, THAILAND
² Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University, Bangkok 10900, THAILAND

Abstract: Biodiesel production from microalgae is still not commercially realized due to the high cost of production. High light-tolerance has been suggested as a desirable phenotype for efficient cultivation in large scale production systems under fluctuating outdoor conditions. Nevertheless, it has not been shown if algae with such a phenotype would have better efficiency for lipid production. To determine lipid productivity in high light-tolerant mutants, and to understand the pathways involved in high light-tolerant phenotype, two very high light-tolerant mutants of the green alga Chlamydomonas reinhardtii - CAL028_01_28 and CAL034_01_48 - were selected from eighteen high light-tolerant mutants from the CAL collection. Under high light intensity conditions, and the presence of reactive oxygen species, which are conditions constantly experienced by algae growing in open-pond environments, these strains exhibited higher photosynthetic efficiency and improved survival. The physiological characterization of these mutants revealed that the detoxification of ROS by carotenoids and antioxidant enzymes is crucial for their growth under high light conditions. Neither mutant was affected in terms of its ability to accumulate lipid under nitrogen-depleted condition. More importantly, lipid productivity under high light conditions increased two-fold in these mutants compared to that of the wild-type. Taken together, very high light-tolerant mutants confer a high potential for biofuel production under outdoor conditions, and their improved ability to survive under oxidative stress is an important key for efficient growth under outdoor conditions.

Key words: biodiesel, Chlamydomonas, lipid production, microalgae, high light, oxidative stress

1 Introduction

Biodiesel from microalgae is still not available commercially due to the high cost of production. Most commercial cultivation of microalgae utilize open pond systems as they are easy and inexpensive to construct¹, but one of the disadvantages is uncontrollable light intensity that could lead to oxidative stress and eventually biomass reduction. In outdoor conditions, light intensity gradually increases from sunrise to midday with around 2,000 μmol photons m⁻² s⁻¹ and then gradually decreases towards sunset. Furthermore, light intensity is highest around the equator and decreases with increasing distance from the equator to the poles. Light is absolutely essential for algal growth, and different strains require different amounts of light for maximal growth, whereas excessive light is harmful. When excess light is absorbed, damage to the photosynthetic apparatus may be induced, which results in the reduction of photosynthetic capacity and changes in pigment concentrations², ³. Exposure of photosynthetic organisms to strong light, which results in the inhibition of photosystem II (PSII) activity, is referred to as photoinhibition. Photoinhibition is induced by production of reactive oxygen species (ROS), which directly inactivate the PSII reaction center. In the event of continuous exposure to strong light, the excessive production of ROS results in progressive oxidative damage and ultimately cell death⁴, ⁵.

Therefore, one of many desirable phenotypes for lipid production in large scale production systems under fluctuating outdoor conditions is high light-tolerance⁶-⁸. In the well-studied model green alga Chlamydomonas reinhardtii, high light-tolerant mutants have been generated in several studies⁹-¹¹. Nevertheless, none of the high light-

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; HL, high light; H₂O₂, hydrogen peroxide; IC₅₀, the half maximal inhibitory concentration; LL, low light; MDA, malonaldehydes; ROS, reactive oxygen species; RB, rose bengal; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; WT, wild type

*Correspondence to: Anchalee Sirikhachornkit, Department of Genetics, Faculty of Science, Kasetsart University, Bangkok, 10900, THAILAND
E-mail: anchalee.si@ku.ac.th
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tolerant mutants have been studied in terms of biodiesel production. In this work, we utilized the high light-tolerant mutants of the CAL collection available from the Chlamydomonas stock center\(^5\). Two of the mutants exhibited very high light-tolerant phenotypes. We demonstrated that these mutants possessed higher lipid productivity under high light conditions, and that the upregulation of antioxidant systems leading to increased oxidative stress resistance, is an important component of high light-tolerance.

2 Experimental

2.1 Strains and culture conditions

*Chlamydomonas reinhardtii* wild-type strain 4A+ was obtained from Dr. Krishna Niyogi (University of California, Berkeley) and high-light tolerant mutant strains (CAL021_01_11, CAL021_01_35, CAL021_01_46, CAL028_01_28, CAL028_01_38, CAL028_02_01, CAL028_02_20, CAL028_02_27, CAL028_02_28, CAL028_02_33, CAL028_02_36, CAL028_02_37, CAL028_02_43, CAL028_03_15, CAL028_03_18, CAL028_04_05, CAL030_02_17, CAL034_01_37 and CAL034_01_48) were from the Chlamydomonas Resource Center\(^9\). For control condition, cells were grown in Tris-acetate-phosphate (TAP) medium on a shaker at 120 rpm, 25°C with constant illumination at 50 μmol photons m\(^{-2}\)s\(^{-1}\). For stress treatments, cells were exposed to different light intensities for 2 weeks.

2.2 Growth conditions

In order to compare the growth of the cells under different conditions, cultures were diluted to a density of 0.5 × 10\(^6\) cells mL\(^{-1}\). These cultures were incubated under different stresses. Sampling was performed every day by taking 1 mL of each culture and measuring the optical density at 750 nm. For photosynthesis efficiency, one milliliter of each culture was collected and dark-adapted for 30 min. Photosynthesis efficiency was monitored by measurement of F\(_{v}/\text{F}_\text{m}\) value using AquaPen AP100 (Photon systems instrument, Czech Republic). To determine the effects of H\(_2\)O\(_2\) and RB on the growth of cells, IC\(_{50}\) values were calculated by using The IC\(_{50}\) Tool Kit.

Cell growth also was monitored by gravimetric determination of algal biomass dry weight. One hundred milliliters of culture were harvested and centrifuged at 7500 rpm for 5 min (Beckman Allegra 25R Refrigerated Centrifuge, USA). Then, the supernatant was discarded and the pellet was dried at 105°C for 24 hours, cooled down at room temperature and weighted. Biomass productivity (P\(_\text{biomass}\)) was determined based on previously reported equation\(^{12}\).

2.3 Lipid content

Total lipid was quantified by Vanillin assay\(^{13}\) with some modifications of sample preparation. Two-hundred microliters of sulfuric acid was added to 1.5 × 10\(^6\) cells in each microtube. Productivity of lipids under nitrogen deprivation were calculated following previously reported equation\(^{12}\).

Neutral lipids were quantified using Nile Red. Cells were diluted to a density of 2 × 10\(^6\) cells mL\(^{-1}\) in a microtube. Then, 50 μL of dimethyl sulfoxide and 2.5 μL of Nile red were added to the tube containing 950 μL of cell culture. Samples were vortexed and kept in dark for 10 min. Two-hundred microliters of samples were transferred to a 96-well plate and visualized under Microplate Reader (TECAN SparkControl v1.1.13.0, Switzerland) using the excitation/emission wavelength at 528/576 nm\(^{14}\).

2.4 Pigment content

One milliliter of each sample was collected by centrifugation at 5000 rpm for 10 min. Then, the supernatant was discarded and 1 mL of 80 % acetone was added. Cells were lysed by vortexing until pellets were white. The tubes were then centrifuged to remove cell debris. Absorbance was measured at 470, 646 and 663 nm to calculate the amount of chlorophyll and carotenoid\(^{15}\). For all measurements, an absorbance of 720 nm was used to correct for contaminated color compound\(^{16}\).

2.5 Lipid peroxidation

Ten milliliters of sample were collected in duplicates by centrifugation at 5000 rpm for 10 min. The supernatant was discarded. One milliliter of –TBA solution containing 20.0% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene was added to one sample tube. One milliliter of + TBA solution containing the above plus 0.65% thiobarbituric acid (TBA) was added to the other sample tube. Samples were mixed and heated at 95°C for 25 min. Samples were cooled at room temperature and centrifuged at 12000 rpm for 10 min. Finally, supernatant was used for measurement of optical density at 440, 532 and 600 nm to calculate malondialdehyde (MDA) level. The amount of
MDA was calculated as described\(^\text{17}\).

### 2.6 Enzyme activity assays

Cells were centrifuged at 8000 rpm for 10 min and then broken down by sonication in 3 mL of cold extraction buffer, which includes 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM MgCl\(_2\) and 1 % w/w polyvinylpyrrolidone. In the case of ascorbate peroxidase, 1 mM ascorbate acid was added to extraction buffer. The sample was centrifuged at 8000 rpm at 4°C for 10 min. Supernatant was used for the next step depending on type of enzymes.

Superoxide dismutase (SOD) activity was measured by ability to convert the photoreduction of nitroblue tetrazolium (NBT) to NBT-diformazan, which absorbs light at 560 nm\(^\text{18}\). The reaction was carried out in 50 mM potassium phosphate buffer (pH 7.6), 0.1 mM EDTA, 13 mM methionine, 75 μM NBT and 190 μL enzyme extract. Riboflavin was added last and the tubes were shaken and placed under constant illumination at 50 μmol photons m\(^{-2}\) s\(^{-1}\) for 10 min. Finally, solution was used for measurement of optical density at 560 nm. SOD activity was determined by % inhibition of NBT reduction.

Catalase (CAT) activity was assayed by measuring the rate of disappearance of H\(_2\)O\(_2\) using the absorbance at 240 nm for 180 s with some modifications\(^\text{19}\). The reaction was carried out in a tube containing 50 mM potassium phosphate buffer (pH 7.0), 0.036 % w/w H\(_2\)O\(_2\) and 35 μL enzyme extract. Activity was calculated using the extinction coefficient of 0.036 mM\(^{-1}\) cm\(^{-1}\).

Ascorbate peroxidase (APX) activity was determined through the decrease in absorbance of ascorbate at 290 nm for 180 s with some modifications\(^\text{20}\). The reaction was carried out in 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H\(_2\)O\(_2\) and 20 μL enzyme extract. Activity was calculated using 2.8 mM\(^{-1}\) cm\(^{-1}\) as the extinction coefficient.

**Fig. 1** Growth of high-light tolerant mutants compared with wild-type strain. (A) All strains (1 = CAL021_01_35, 2 = CAL021_01_46, 3 = CAL028_01_28, 4 = CAL028_01_38, 5 = CAL021_01_11, 6 = CAL028_02_36, 7 = CAL28_02_37, 8 = CAL028_02_20, 9 = CAL028_02_33, 10 = CAL028_02_28, 11 = CAL028_02_27, 12 = CAL028_03_15, 13 = CAL028_03_18, 14 = CAL028_04_05, 15 = CAL030_02_17, 16 = CAL039_01_37, 17 = CAL034_01_48, 18 = CAL034_01_48 and 19, 20 = WT) were spotted onto TAP medium plates and exposed to light intensity of 50 and 1,000 μmol photons m\(^{-2}\) s\(^{-1}\) for 2 weeks. (B) Growth of CAL028_01_28, CAL034_01_48 and WT under low light (50 μmol photons m\(^{-2}\) s\(^{-1}\)) and high-light condition (700 μmol photons m\(^{-2}\) s\(^{-1}\)).
3 Results

3.1 Effect of light intensity on growth

Tolerance to high light stress is a desired phenotype for microalgae that are intended to be used for outdoor cultivation. To select the best mutants for survival under high light intensity conditions, eighteen high light-tolerant mutant strains from the CAL collection were obtained. Cells were spotted and placed directly under low light and high light intensities. All strains were able to grow normally under a light intensity of 50 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), but could not survive under high light intensity condition of 1,000 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) with the exception of the CAL028_01_28 and CAL034_01_48 strains (Fig. 1A). To confirm the viability of the high light-tolerant phenotype, these two strains were retested under high light intensity condition of 700 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \). WT cells did not survive this condition, but these two strains were still able to grow (Fig. 1B). This result showed that CAL028_01_28 and CAL034_01_48 exhibited a very high light-tolerant phenotype.

To further investigate the phenotype of these mutants, we monitored their growth rate under high light intensity condition over a period of 5 days. Interestingly, the mutants exhibited a lower growth rate compared to the WT under control light condition of 50 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) (Fig. 2A; left). However, both mutants exhibited a robust growth phenotype under high light condition, showing higher growth rates starting at day 2 compared to the WT (Fig. 2A; right). The photosynthetic efficiency expressed as the \( F_{v}/F_{m} \) values of all strains showed a similar trend under low light intensity condition (Fig. 2B; left). Under high light intensity conditions, the \( F_{v}/F_{m} \) values of all strains decreased from 0.7 to 0.3 after exposure to high light intensity for a day. Nevertheless, the \( F_{v}/F_{m} \) values of the mutants showed a better recovery compared to that of the WT after day 2 (Fig. 2B; right).

3.2 Effect of oxidative stress on growth

Other than high light tolerance, oxidative stress tolerance is another important phenotype that is beneficial for outdoor cultivation. To evaluate growth under oxidative stress conditions, the singlet oxygen dye, rose bengal (RB), and \( \text{H}_{2}\text{O}_{2} \) were added to a growth medium. The results revealed that there was an obvious difference in the growth of the WT strain under control condition compared to under oxidative stress conditions. The presence of chemicals readily inhibited the growth of WT, starting from early time points (Fig. 3A). In contrast, there was only a slight difference in the growth of mutants between control and oxidative stress conditions (Figs. 3B and 3C). Interestingly, CAL028_01_28 grew noticeably well in a medium containing RB starting from day 3. On the other hand, there was no difference in terms of growth under \( \text{H}_{2}\text{O}_{2} \) until day 3, but the growth slightly decreased at day 4 and day 5 when compared with the control condition (Fig. 3B). In the case of CAL034_01_48, the only difference was a slightly lower cell density in the medium with \( \text{H}_{2}\text{O}_{2} \) on day 1 and day 3 (Fig. 3C). Based on the IC50 values, it was clear that CAL028_01_28 exhibits a greater tolerance to both hydrogen peroxide and singlet oxygen compared to the WT and

![Fig. 2](image-url) Growth and chlorophyll fluorescent parameter under low and high light intensities. Cell density (A) and \( F_{v}/F_{m} \) (B) of wild-type strain (WT), CAL028_01_28, and CAL034_01_48 during 5-d cultivation period under low light condition at 50 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) (left) and high-light condition at 700 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) (right). All data are means ± SD of three biological replicates. Significant differences between WT and CAL028_01_28 are indicated by asterisks (*) while significant differences between WT and CAL034_01_48 are indicated by plus signs (+) \( p < 0.05 \).
CAL034_01_48 strains (Tables 1 and 2).

To evaluate the degree of damage resulting from high light and oxidative stress, a lipid peroxidation assay (TBARS) was performed. The maximum accumulation of MDA levels was observed in WT in the presence of chemicals combined with high light (Fig. 4). In the case of CAL028_01_28, the MDA level slightly increased when placed in the medium with H_2O_2 and in the presence of either chemical combined with high light. For CAL034_01_48, increased MDA levels were observed under all conditions except under H_2O_2 when it was found that the MDA level was much lower when compared with the

Table 1  Toxicity test of hydrogen peroxide on WT and mutant strains for 5 days.

| Strains       | Value (mM)       |
|---------------|------------------|
| WT            | 1.99 ± 0.02839   |
| CAL028_01_28  | 1.45 ± 0.08348*  |
| CAL034_01_48  | 1.97 ± 0.2698    |

All data are means ± SD of three biological replicates. Significant differences between WT and mutants are indicated by asterisks (*) (p < 0.05).

Table 2  Toxicity test of rose bengal on WT and mutant strains for 5 days.

| Strains       | Value (µM)       |
|---------------|------------------|
| WT            | 2.22 ± 0.129     |
| CAL028_01_28  | 1.97 ± 0.2698*   |
| CAL034_01_48  | 2.17 ± 0.05204   |

All data are means ± SD of three biological replicates. Significant differences between WT and mutants are indicated by asterisks (*) (p < 0.05).
other two strains. When compared to WT, both CAL028_01_28 and CAL034_01_48 exhibited lower MDA levels under RB combined with high light. In addition, the level of MDA of CAL028_01_28 under H2O2, combined with high light, was also significantly lower than that of the WT.

To investigate if increased high light and oxidative stress tolerance of the mutants are the results of ROS detoxification, the activities of antioxidant enzymes were assessed. We observed that WT exhibited increased superoxide dismutase (SOD) activity under high light and the presence of RB, and increased ascorbate peroxidase (APX) activity under high light (Figs. 5A-5C). CAL028_01_28 exhibited increased activity of catalase (CAT) under high light and RB conditions, and increased APX activity under all conditions. CAL034_01_48 displayed higher activities in terms of both CAT and APX in the presence of H2O2. When compared between WT and the mutants, CAL028_01_28 exhibited higher CAT and SOD activities under control condition, whereas CAL034_01_48 exhibited higher APX activities in the presence of H2O2.

3.3 Effect of oxidative stress on pigments
The level of photosynthetic pigments is another parameter for assessing stress levels in plants. Both mutants showed a slightly lower chlorophyll a/b ratio compared to that of the WT under low light condition (Fig. 6A). Inter-
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Interestingly, only CAL034_01_48 exhibited a higher chlorophyll a/b ratio under high light condition. When considering each strain in different conditions, WT exhibited an increase in the chlorophyll a/b ratio under RB. Interestingly, this ratio was elevated in CAL034_01_48 under all stress conditions, whereas the value of CAL028_01_28 increased under high light condition and the presence of RB.

For the accessory pigment and antioxidant carotenoids, the total carotenoids of CAL028_01_28 was about two-times higher than that of the WT and CAL034_01_48 under low light condition (Fig. 6B). This level was not affected when this strain was placed under high light condition, but was significantly decreased when exposed to RB and H2O2. For CAL034_01_48, the carotenoid level elevated under high light condition but decreased under RB and H2O2 conditions.

3.4 Lipid production under nitrogen depletion and high light conditions

Algae-based biofuels have been highlighted as one of the current alternative sources of renewable energy. Nitrogen starvation is one of the most widely-used conditions for inducing lipid production. We found that the total lipid accumulation under nitrogen starvation condition was elevated to a similar level in all strains (Fig. 7A).

To explore the efficiency of lipid production under outdoor cultivation conditions, cells were induced under high light intensity condition for 2 days. The biomass productivity of CAL028_01_28 was significantly higher than that of the WT (Fig. 8A). The total lipids accumulated was at about the same level in all strains (Fig. 8A). Lipid productivity was then calculated as the product of the lipid content and biomass productivity. Both mutants exhibited approximately a two-fold increase in lipid productivity compared to the WT (Fig. 8B). To further investigate spe-
cifically whether neutral lipids were increased in the mutants under high light, lipid droplet formation was quantified using Nile Red fluorescence. The result clearly showed a higher accumulation of neutral lipids in both mutants compared to the WT (Fig. 8C).

4 Discussion

Algae cultivated under outdoor conditions inevitably experience high light stress, which reduces growth and biomass yield. Many studies have attempted to find micro-algal strains, either by natural isolation or by genetic engineering, that have high survival rates in outdoor ponds, in order to reduce the cost of biofuel production from micro-algae. Under high light conditions, the photosynthesis apparatus in algal cells is oversaturated with light energy, which significantly reduces the light use efficiency and could lead to photoinhibition. Potential solutions have been proposed to increase the light use efficiency such as reducing pigmentation or antenna size21–25. The use of high light-tolerant strains for outdoor cultivation is also a direct way to increase biomass yield. In the case of the model green alga Chlamydomonas reinhardtii, high light-tolerant strains have been isolated by many researchers9–11, but lipid productivity and the pathways involved in high light-tolerance conditions have not been determined.

In this study, C. reinhardtii high light-tolerant strains from the CAL collection were selected as representative of high light-tolerant strains9. CAL028_01_28 and CAL034_01_48 were chosen for detailed investigation. Growth inhibition due to high light levels was observed in the WT (Fig. 2A). Faster growth kinetics accompanied by higher biomass productivity were observed in the mutants, suggesting a more efficient light utilization, which was also evident from the efficiency of photosynthesis (Fv/Fm) (Fig. 2B). These results also revealed that the mutants were better at dealing with excessive light energy and reducing photoinhibition. Under high light condition, the cellular chlorophyll a/b ratio increased in CAL034_01_48, indicating that this mutant employed antenna size reduction as one of the main pathways for its high light-tolerance. Nevertheless, this reduction was not observed in CAL028_01_28, indicating that this mutant relied on alternative photoprotective mechanisms for survival.

Other than the ability to grow under high light intensity condition, the two mutant strains exhibited improved growth in the presence of ROS-generating chemicals such as RB or H2O2 (Figs. 3B and 3C). A lower degree of damage from oxidative stress was observed, especially when placed under high light combined with the presence of chemicals (Fig. 4). ROS scavenging in the cells is performed by antioxidants and antioxidant enzymes. In fact, this was found to be the main reason for the robust growth of CAL028_01_28 under high light condition. The baseline levels of the CAT and SOD activities of CAL028_01_28 were over twice as high as those of the WT and CAL034_01_48, whereas the APX activity was induced several-fold when this mutant was placed under high light condition or in the presence of ROS-generating chemicals (Fig. 5). In addition, carotenoids, multi-role molecules that can act as electron donors, and physical quenchers of ROS with an important role in the non-photochemical quenching (NPQ) mechanism, were also found to accumulate at about twice the amount in CAL028_01_28 compared with in the WT and CAL034_01_48 (Fig. 6B). Interestingly, the level of carotenoids in CAL034_01_48 was substantially induced to the same level as CAL028_01_28 under high light condition. This result showed that carotenoids is crucial for the high light-tolerant phenotype of both mutants.

Many factors, including excessive light, high temperature, and especially nutritional stresses, lead to increased lipid accumulation26–32. Enhanced lipid production can be induced by nitrogen starvation as the excess carbon from photosynthesis is shifted into storage molecules such as triglycerides37, 33, 34. Increased neutral lipid accumulation was observed in both mutants (Fig. 8C). It is possible that improved lipid accumulation is a result of preventing lipid loss from peroxidation35–37, which corresponds to smaller levels of lipid peroxidation in the two mutants (Fig. 4).

In order to improve the efficiency of large-scale biofuel production under outdoor conditions, it is important to optimize productivity by considering factors influencing growth and lipid accumulation. Light intensity has been suggested as a way to enhance algal lipid accumulation using strong illumination, as lipid biosynthesis is a way to relieve excess energy from light driven processes38, 39. In inducing lipid production with the use of high light levels, all strains showed improved lipid productivity compared with low light growth (Fig. 8B). Nevertheless, the two mutants exhibited a two-fold higher level of lipid productivity compared to the WT under high light condition. This can be explained by the fact that the excess energy from high light levels is better managed to minimize cellular damage from ROS in these mutants, so that the energy is efficiently used for cell growth and lipid synthesis, whereas high light intensity inhibits WT cell growth. These finding clearly emphasize the importance of high light-tolerance as a critical element for choosing algal strains for efficient biofuel production in outdoor conditions.

5 Conclusion

In this work, we demonstrated that very high light-tolerant stains indeed exhibit improved lipid productivity under high light condition, which is an important factor that
limits algal growth under outdoor cultivation. Two very high light-tolerant \textit{C. reinhardtii} strains from the CAL collection were studied. Other than better growth under high light condition, they also displayed improved growth under oxidative stress conditions. This was supported by the decrease in cell damage due to oxidative stress in the mutant cells, resulting from better utilization of light absorbed and the enhanced antioxidant levels and antioxidant enzyme activities. Our results offer new opportunities for future investigations of algal biofuel under fluctuating light conditions.

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