

Increasing lipid productivity in *Chlamydomonas* by engineering lipid catabolism using the CRISPR-Cas9 system

Thu Ha Thi Nguyen

Hanyang University

Seunghye Park

Hanyang University College of Natural Science <https://orcid.org/0000-0002-3276-6110>

Jooyeon Jeong

Hanyang University

Ye Sol Shin

Korea University

Sang Jun Sim

Korea University

EonSeon Jin (✉ esjin@hanyang.ac.kr)

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Abstract

Background

Currently, most of the attention in renewable energy industry is focused on the development of alternative, sustainable energy sources. Microalgae are a promising feedstock for biofuel production in response to the energy crisis. The use of metabolic engineering to improve yields of biofuel-related lipid components in microalgae, without affecting cell growth, is now a promising approach to develop more sustainable energy sources and to make this approach more economically feasible.

Results

The CRISPR-Cas9 system was successfully applied to generate a target-specific knockout of the *ELT* gene in *Chlamydomonas reinhardtii*. The target gene encodes an enzyme involved in lipid catabolism, in which the knockout phenotype impacts fatty acid degradation. As a result, the knockout mutants show up to 28.52% increased total lipid accumulation in comparison with the wild-type strain. This is also accompanied by a shift in the fatty acid composition with an increase of up to 27.2% in the C18:1 proportion. These changes do not significantly impact cell growth.

Conclusion

This study provides useful insights for the improvement of the oleaginous microalgae strain for biodiesel production. The acquired *elt* mutants showed improved lipid accumulation and productivity without compromising the growth rate.

Background

Global CO₂ emissions have risen while fossil fuel abundance has drastically fallen. There is now an increasingly urgent need to develop alternative fuels. Natural biofuels are promising sources of such alternative fuels. However, if biofuel production is to be a reality, the entire production process must be improved to reduce costs and increase efficiency [1]. Microalgae and cyanobacteria are promising biological sources of various fuel-relevant molecules including lipids, ethanol, and hydrocarbons [2]. Eukaryotic microalgae have received a great deal of attention due to their ability to produce triacylglycerol (TAG), especially when exposed to nutrition-deprived conditions. These neutral lipid compounds can be converted into fatty acid methyl esters (FAMES), the primary component of biodiesel, through transesterification processes, or they can be refined into other fuel constituents [3]. Total lipids and other biomass compositions can be converted into crude oil alternatives through thermochemical processes, such as hydrothermal liquefaction [4]. However, the economic feasibility of using microalgae as a biofuel source is dependent upon making improvements in the entire production process [5]. One of the most influential improvements would be to increase lipid yields [6]. Increasing the lipid yield, without hindering growth, in microalgae is the key to achieving economic feasibility. In comparison to traditional approaches, which are mainly based on identifying high-lipid-producing strains [7], recently developed

techniques for metabolic engineering offer alternative methods of improving lipid production in microalgal strains.

In recent years, various strategies for increasing lipid production have been proposed, in particular, random mutagenesis, genome editing, and metabolic pathway engineered to increase lipid biosynthesis, as well as blocking the competing pathways of carbohydrate metabolism and lipid degradation processes. However, as a result of disrupting cellular metabolic pathways, the engineered strains that yield high lipid content often suffer from a reduced growth rate [8, 9]. When applied to large-scale economic cultivation, high growth rates and high biomass production are essential [10]. At the same time, engineering advancements that can improve lipid yield, without impacting on growth rate, can significantly decrease production cost and fortify the economic viability of microalgae-derived biofuels [6]. Among the considered strategies, the inhibition of competing pathways has been recognized as a potential alternative to enhance cellular lipid yield. Some have suggested that inhibition of lipid catabolism, more specifically the enzymes that catalyze the release of free fatty acids from lipids, may increase lipid accumulation. In microalgal cells, lipid catabolism facilitates membrane re-organization by providing acyl groups, which are required for remodeling membranes and reorganizing the photosynthetic system [11, 12]. However, recent study has shown that unlike the disruption of carbohydrate pools, which play a role in primary carbon storage product in a wide range of microalgae [13], knockdown of genes involved in lipid catabolism may have less of an impact on the growth rate in cell culture [14].

Our study was performed on *Chlamydomonas reinhardtii*, a model microalgal species with a fully sequenced genome [15]. Microalgae have long been recognized as an ideal source of biofuels and recent technological developments have allowed for genomic manipulation of these microorganisms [16, 17]. Using the optimized genetic editing tool, CRISPR-Cas9, here we report the knockout of Cre01.g000300, a gene encoding an enzyme which belongs to the multifunctional ELT family, and we characterize the resulting mutants. The knockout strains show an increase in cellular lipid accumulation during both nutrient-replete and nutrient-deplete cultivating conditions. Our results demonstrate that targeted knockout of this catabolic enzyme in eukaryotic microalgae may be an effective strategy to enhance the outcome of the desired products, while minimize the negative impacts on culture growth rate.

Results

Characterization of the Cre01.g000300

A Basic Local Alignment Search Tool (BLAST) search result indicated that Cre01.g000300 encodes an enzyme that is closely related to a member of the esterase/lipase/thioesterase 1 (ELT1) family of acyltransferases. ELT is composed of two conserved domains, one of which has hydrolase activity, while the other has acyltransferase activity [18]. Each domain has a relatively high sequence similarity compared to organisms (Fig. 1). A GXSXG motif in hydrolase domain and H(X)4D near acyltransferase domain were also found (Fig. 1), and these are residues for catalytic activity domains in earlier studies [14, 19]. Lipid catabolism in microalgae. *New Phytologist*. 218: 1340–1348). The alpha/beta hydrolase

domain-containing proteins are structurally related to various catalytic activities. Acyltransferases catalyze acylation in the lipid synthesis pathway, allowing for transformation from a long-chain acyl-CoA to Coenzyme A [20]. Acyl-CoA molecules are used as substrates for the synthesis of membrane glycolipids, triacylglycerols, and other acylated molecules. Acyl-CoA can also be directed to beta-oxidative catabolism [21]. Moreover, acyl-CoA can be utilized by a number of lipid metabolic enzymes including endogenous lipase/acyltransferase. Thus, the availability of acyl-CoA plays a fundamental role in determining the quantity and composition of membrane lipids and storage lipids. The manipulation of the gene encoding acyl-CoA can, therefore, be genetically manipulated to improve cellular lipid production.

Targeted knockout of ELT using CRISPR-Cas9

elt knockout mutants were generated in *C. reinhardtii* using CRISPR-Cas9 technology. The sgRNA used to generate the mutation in the target gene was designed by using CasDesigner (www.rgenome.net). Gene editing was accompanied with the introduction of hygromycin resistance in the subsequent mutants allowing for the selection of successfully mutated colonies on hygromycin selection agar plates. Mutations were assessed by sequencing with the primers listed in Table 1. Three mutant strains, elt-1, elt-2, and elt-3, were selected by colony PCR and confirmed by Sanger sequencing. Results show short random DNA fragment insertions with the cassette in these strains (Fig. 1c). Southern blot analysis of genomic DNA digested with PstI and KpnI was carried out to confirm the number of inserted hygromycin resistance cassettes in the generated mutants. The result showed a single band in all mutants (Fig. 2b), which suggested that each mutant had a single copy of this cassette in their genome.

Table 1
Primers used in PCR and Southern blot analysis

Gene	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
Colony PCR	ACAAGCCTTCCATCGCACCC	AGGGAGGTGGGTCGTAGTGT	330
Hygromycin resistance probe	CTACCTGGTGATGAGCCGGA	GTCGGTGAAGTCGACGATCC	402
RACK1	AGGTCTGGAACCTGACCAACT	AAGCACAGGCAGTGGATGA	199
ELT	CGGTCTCGGTTCAACACTCT	CTTTGTACTCGGCGTCCTTG	570
ELT_qPCR_1	GGGCGAGAAGTGGACGTGGC	GATCTCGCGCTCGGACAGGC	178
ELT_qPCR_2	TAGCCTGGGCGGCTACCTGG	CTCTGGCGCCTTGGGCACTC	162

Comparative growth properties of elt knockout mutants

Enhancing the desired metabolic products, leading to uncompromised biomass accumulation is an essential factor when applying microalgae in large-scale cultivation for biofuels [22]. To this end, we

evaluated the growth and photosynthetic efficiency of the generated mutant strains. The mutant and wild-type (WT) strains were cultured in optimal condition for growth and cell densities were measured every 24 h during the 3 days of growth. The growth of all three elt mutants were faster and reached the maximum density earlier than the WT strain (Fig. 3a). Additionally, elt-2 also had a higher cell density than the WT and the two other mutant strains (Table 2). The calculated specific growth rate of 0.672, 0.540, 0.686, and 0.572 were recorded for WT, elt-1, elt-2, and elt-3, respectively. The biomass accumulation of elt-2 (Table 2) was as high as WT, suggesting that the growth of elt-2 was not disturbed. The value of F_v/F_m is a sensitive indicator of maximum quantum yield of photosystem II (PSII). A lower F_v/F_m value may indicate stress and/or photoinhibition [23]. There was no remarkable change in F_v/F_m values between the mutant and WT strains (Fig. 3b) which indicates that the knockout event did not impact the photosynthetic performance of the generated mutants.

Table 2
Comparative growth properties of wild-type (WT) and mutant strains.

	Maximum specific growth rate (μ_{max})	Biomass concentration (mg/mL)	Cell number ($\times 10^4$ cells/mL)
WT	0.672	9.85 \pm 0.12	2.42 \pm 0.03
elt-1	0.540	8.80 \pm 0.57	1.98 \pm 0.09
elt-2	0.686	9.98 \pm 0.02	2.54 \pm 0.14
elt-3	0.572	9.48 \pm 0.15	2.25 \pm 0.12

Table 3
Lipid content and productivity of wild-type (WT) and mutant strains in growth phase and nitrogen-deprivation.

	Lipid content (mg/g DCW)		Lipid productivity (mg/L/day)	
	Normal	N-starvation	Normal	N-starvation
WT	179.92 \pm 8.82	218.42 \pm 12.78	40.72 \pm 2.42	46.75 \pm 2.33
elt-1	226.97 \pm 10.02	264.14 \pm 17.92	58.33 \pm 0.95	67.66 \pm 1.12
elt-2	212.59 \pm 7.68	287.07 \pm 22.56	61.03 \pm 1.27	78.12 \pm 1.87
elt-3	223.02 \pm 6.05	263.94 \pm 4.87	52.62 \pm 1.54	58.73 \pm 0.73

elt mutants show an increase in total lipid accumulation

Nile Red staining was used to estimate the relative lipid content while BODIPY staining was used to visualize neutral lipid droplets in the mutants. Regarding the Nile Red data, after being transferred to

nitrogen-deprived phase, the fluorescence signal from the mutants was 19.4% higher than the WT strain (Fig. 5a). BODIPY visualization also demonstrated an increase in the number and size of lipid droplets in mutant strains (Fig. 5b). Work from other groups has shown that nutrient deprivation causes a disturbance in cell growth resulting in decreased biomass accumulation. Under nitrogen limited conditions, WT *C. reinhardtii* drives its partial carbon flux from carbohydrate compounds into lipid biosynthesis [24]. Total lipid content was extracted for measurement during the three days of growth phase and three days of nitrogen starved lipid inducing phase. In both phases, all mutants showed a significantly higher value of lipid content than the WT strain, with mutant elt-2 accumulating the highest cellular lipid content (~ 28.52%), followed by elt-1 (~ 26.33%) and elt-3 (~ 25.87%; Fig. 5c). We also analyzed the activity level of enzyme encoded by Cre01.g000300 in both exponential growth phases and in the nitrogen starvation phase. Like other physiological parameters, however, there were slight differences between the strains. Despite the similar genotypes, elt-2 demonstrated the most equivalent growth profile to the WT strain as well as the highest increase in lipid content among three mutants.

Fatty acid composition analysis

The fatty acid (FA) profiles of the WT and mutant strains after three days of nitrogen starvation were analyzed using gas chromatography. The contents of different fatty acid classes are listed in the Table 4. The fatty acid profile of the WT strain was dominated by palmitic acid (C16), which is similar to the composition of oil extracted from higher plants [25]. The mutant strains exhibited similar FA composition and had a noticeable shift between the proportion of oleic acid (C18:1) and alpha-linoleic acid (C18:3). The mutants showed approximately 27.2% of C18:1 increase and 14.8% of C18:3 decrease compared to the WT. No significant changes were observed in the proportion of palmitic acid and saturated stearic acid (Fig. 6).

Table 4

The content of fatty acid classes (mg/g) in wild-type (WT) and the representative mutant strain.

	Nitrogen repletion (growth phase)		Nitrogen depleted condition	
	WT	elt mutants	WT	elt mutants
Myristic acid (tetradecanoic acid) C14:0	0.49 ± 0.02	0.50 ± 0.02	0.45 ± 0.07	0.49 ± 0.02
Palmitic acid (hexadecanoic acid) C16:0	37.64 ± 0.40	37.04 ± 3.21	57.33 ± 0.38	73.02 ± 3.66
Stearic acid (octadecanoic acid) C18:0	4.15 ± 0.08	3.98 ± 0.34	5.49 ± 0.06	7.10 ± 0.54
Oleic acid C18:1N9	5.38 ± 0.08	4.95 ± 0.46	22.42 ± 0.16	36.77 ± 2.01
Linolelaidic acid C18:2N6	20.12 ± 1.82	19.29 ± 2.33	28.87 ± 0.64	37.70 ± 3.12
Alpha-linolenic acid (ALA) C18:3N3	25.48 ± 0.56	25.86 ± 1.28	21.58 ± 0.27	24.58 ± 2.76
Arachidic acid (eicosanoic acid)	-	-	0.27 ± 0.01	0.34 ± 0.05
Eicosenoic acid	-	-	0.42 ± 0.01	0.80 ± 0.03

Discussion

We have successfully applied CRISPR-Cas9 technology to generate ELT knockout mutants in *C. reinhardtii* [26]. This method is advantageous over traditional techniques because generation of knockdown or knockout mutants by random DNA mutagenesis is labor-intensive and time-consuming. We were also able to succeed in manipulating the genomes of different microalgae species, including *N. oceanica* [27], *N. gaditana* [28], *P. tricornutum* [29], and *T. pseudonana* [30] using CRISPR-Cas9. The targeted knockout of ELT was generated by recruiting the RNP complex co-operating with gene for selection by a knock-in with a hygromycin cassette at the site of interest. The insertion of the hygromycin resistance cassette was confirmed by Southern blot analysis (Fig. 2b). This insertion facilitates the selection of successfully mutated colonies on hygromycin selection plates. Such advantages allow this method to significantly reduce the time spent on screening and selecting for putative mutants [31].

There was little difference in the growth pattern among elt strains, and there were few differences in the maximum cell numbers between WT and elt mutants (Fig. 3). Notably, the density of elt mutant cells increased and reached the maximum more rapidly than the wild-type strain under nitrogen deprived conditions. This proves that the growth of elt was not compromised and, therefore, does not affect productivity under nitrogen deprived condition.

In a review, Park et al. (2019) proposed that improving lipid content, while maintaining efficient growth, holds the key to achieving economic feasibility of microalgae-derived biofuel production because biomass, from which lipids are extracted, is a critical factor for lipid productivity [32]. The inhibition of lipid catabolism, particularly of the enzymes catalyzing the release of free fatty acids from lipids, may increase lipid accumulation [14]. Previous studies have found that, unlike the disruption of carbohydrate pools, which play role as primary carbon storage product in wide range of microalgae (Chauton et al., 2013), the knockdown effect in lipid catabolism has a smaller impact on growth rate (Trentacoste et al., 2013). Another report tried to use a similar strategy to support this hypothesis. In a previous study on *Chlamydomonas*, a phospholipase knockout was generated by CRISPR-Cas9. The resultant mutant showed an increase of up to 62.25% in the total lipid content [33]. The lipase mutant strains showed uncompromised growth, as well as high biomass production, thereby suggesting that it may be possible to obtain a microalgal strain with simultaneous rich oil content and high lipid productivity. Such mutants would be applicable for efficient industrialized production of algal biofuels.

The lipase knockout effects

ELT1 (Cre01.g000300) encodes an alpha/beta hydrolase, which shares a highly conserved domain with Thaps3_264297 (E-value of 1.3×10^{-46} ; [34]. Identification of the 58/alpha/beta hydrolase domain 5 (CGI-58/ABHD5), a homologue of Thaps3_264297, by comparative genomics showed that it has lipid hydrolase activity along with a signature motif, which is found in the ELT family [34]. In human, a CGI-58 mutation results in Chanarin-Dorfman syndrome, a rare autosomal recessive disease of lipid metabolism with the accumulation of an abnormally large number of cytosolic lipid droplets in various tissues, such as the skin, liver, and leukocytes [35]. The knockout of a similar homolog was obtained in *A. thaliana* and caused a Chanarin-Dorfman-like phenotype with a high lipid phenotype [36]. The *elt* knockout mutants showed a higher level of total lipid accumulation than the WT. In both phases of cultivation (Fig. 5c). To confirm the knockout effects on the target gene expression, we performed qRT-PCR. It seems that the mutated ELT gene is still transcribed in the knockout mutants, and regulated in the same manner as it is in the WT. However, proper processing of the mutated transcripts might be disturbed due to the inserted cassette in front of the protospacer adjacent motif (PAM) site in the first exon (Fig. 2C, Additional file 1: Figure S1). Nonetheless, we found that lipase activity in the mutant cells is significantly reduced (Fig. 4). Therefore, these result shows that ELT gene knockout in *C. reinhardtii* affected lipid catabolism, in which lipid triglycerides are hydrolyzed into glycerol and free fatty acids. Another study that tried the same strategy using RNA interference (RNAi), also shows the same effect. The knockdown of ELT led to a 3.3-fold increase in lipid content in normal growth condition, and a more than 4-fold increase in nutrition-deplete condition, in *Thalassiosira pseudonana* [14]. In terms of technical approach, compared to RNAi mediated knockdown, which depends on constant transcription of an RNAi coding transgene segment, targeted gene editing is advantageous because it permanently knocks out the expression of a functional gene product.

The fatty acid profile of mutants showed a noticeable shift in the FA proportion, from oleic acid (C18:1) to alpha-linoleic acid (C18:3) with no significant changes in saturated FA components and no changes in

the length of carbon chains. A study on acyltransferases showed that this enzyme group determined the fatty acid composition of glycerolipids [37], this study also indicated that acyltransferases took part in the regulation of TAG biosynthesis. In the microalgae, *Nannochloropsis oceanica*, the changes in acyltransferase activity resulted in changes in C16:0 and C18:1 in the TAG sn-1/sn-3 positions, which could be explained by the difference in the substrate preference of this enzyme [38]. The elt mutants showed an increase in the proportion of unsaturated FA C18:1. Some studies have suggested that the C18:1 component is beneficial for the balance between oxidative stability and low-temperature properties. These characteristics help to promote biodiesel quality [39]. Modifying the fatty acid composition is a potential approach to improve biodiesel properties by enrichment of components with more favorable properties.

Conclusion

In this study, we present a knockout mutant successfully generated using the CRISPR-Cas9 system. By targeting the multifunctional enzyme ELT, the acquired mutants demonstrated an improvement in lipid accumulation as well as lipid productivity without compromising the growth rate. The expression of the target gene was also tested to confirm the relation between the genotype and phenotype of the mutants. Our results show that disrupting lipid catabolism may be an efficient strategy to obtain microalgae strains with the desired characteristics for biofuel production.

Materials And Methods

Culture condition

Chlamydomonas reinhardtii strain CC-4349 [40] was cultured in Tris-acetate phosphate (TAP) medium on a rotary shaker (120 rpm) at 25°C under continuous illumination with a light intensity of 80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. In order to investigate the lipid content under conditions of nitrogen deprivation, the cell cultures were harvested by centrifugation at 3200 rpm for 10 minutes. The pelleted cells were resuspended in nitrogen-deficient medium. All experiments were performed in biological triplicates and all data is indicated as the mean \pm standard error.

Delivery of the RNP complex and selection marker into the cell

The RNP complex was delivered into microalgae cells following as described by [26] with a minor modification. The cells were harvested at the exponential phase by centrifugation at 3200 rpm for 5 minutes. The Cas9 protein was dissolved in storage buffer (20 mM HEPES, 150 mM KCl, 1 mM DTT, and 10% (v/v) glycerol). Small guide RNAs (sgRNA), which were designed to recognize target sequence, were resuspended in distilled water and mixed with the Cas9 protein to form the RNP complex. Before electroporation, the RNP complex and the harvested cells were incubated together at room temperature for 10 minutes. To facilitate the delivery of the RNP complex into the microalgal cells a Bio-Rad Gene Pulse XCell Electroporator was used to generate an electric pulse with the following parameters: 600 V, 50

μF , 200 Ω . After the electro-transformation, the cells were suspended in TAP medium supplemented with 40 mM sucrose for recovery. The treated cells were incubated under dim light for 12 h, and then spread on 1% (w/v) agar TAP plates supplemented 50 $\mu\text{g}/\text{mL}$ hygromycin for colony selection.

Determining mutant strains

The colonies that grew on hygromycin TAP agar plates were subjected to PCR. The colonies were dissolved in 40 μL lysis buffer, and incubated at 58°C for 90 minutes, then raised to 95°C for 60 minutes. The lysate supernatant was then used as colony PCR template. The colony PCR was performed using Taq polymerase with the following thermocycles: 98°C for 10 seconds, 60°C for 10 seconds, and 72°C for 1 minutes for 32 cycles. The mutants were identified based on gel electrophoresis of the PCR products. For Southern blot and sequencing, the genomic DNA of the identified mutants were then extracted using chloroform:isoamyl alcohol (24:1) and precipitated with ethanol [40]. The target sequences were amplified by PCR using the specific primers (Table 1). The corresponding band was then eluted and purified for Sanger sequencing (Macrogen, South Korea).

Southern blot analysis

The southern blot protocol followed as previously described [40]. The extracted genomic DNA of the identified mutants was digested with PstI and KpnI (Takara, Japan) and electrophoresed on a 0.8% (w/v) agarose gel running at 75 V for 2 hours. The DNA bands were then transferred to a positively-charged nylon membrane (Hybond-N, GE Healthcare, UK). Probes were synthesized by PCR amplification of part of the hygromycin resistance cassette (Supple Fig. 1) and labeled with alkaline phosphatase (AlkPhos Direct Labeling Module, GE Healthcare, UK). The membrane was hybridized with the labeled probes at 66°C overnight. Successful probe-to-membrane hybridization was detected by exposure to X-ray film after treatment with CDP-Star according to the manufacturer's instruction (CDP-Star Detection Reagent, GE Healthcare, UK)

Determination of growth rate and dry biomass

Cell density was measured by absorbance of liquid culture at 750 nm using a Ultrospec 2100 Pro spectrophotometer (GE Healthcare, UK) and cell numbers were counted on a haemocytometer (Paul Marienfeld GmbH & Co, Germany). Microalgal growth rate was measured during the growth phase with the increase of cell number as a log function of time. The growth rate (μ) was calculated by the following equation:

$$\mu = \frac{\ln P_1 - \ln P_0}{t_1 - t_0}$$

where P_1 and P_0 were the counted cell densities at time t_1 and t_0 , when the cultivation ended and started, respectively.

To measure dry biomass, 5 mL of cell culture were filtered through pre-weighed cellulose ester membrane (Merck Millipore, USA), dried over night at 65°C, and weighed.

Lipase activity assay

A lipase activity test was performed using the Lipase Activity Assay Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Cells were harvested at the exponential phase and 3 days after nitrogen starvation, 2×10^6 cells were harvested for each sample and incubated with 4 volumes of ice-cold lipase assay buffer and sonicated to obtain the cell lysate. The standard curve was generated by dilution of a 1 mM glycerol standard to different concentrations (0, 2, 4, 6, 8, and 10 nmole/well). The sample was added with reaction mix in each well and incubated at 37°C for 2–3 min before measuring absorbance at 570 nm (i.e. $(A_{570})_{\text{initial}}$). The sample plate was then incubated for 60–90 min in the dark and absorbance was again measured at 570 nm (i.e. $(A_{570})_{\text{final}}$). The value ΔA_{570} was measured as the change between $(A_{570})_{\text{initial}}$ and $(A_{570})_{\text{final}}$. The amount of glycerol generated by the lipase was determined by comparing the ΔA_{570} of each sample to the standard curve, and lipase activity of each sample was calculated by following equation:

$$\text{lipase activity} = \frac{B \times \text{sample dilution factor}}{\text{Reaction time} \times V}$$

where B is amount (nmole) of glycerol generated within a reaction time, V is sample volume (mL) added to each well. Lipase activity is reported as nmole/min/mL = milliunit/mL.

RNA extraction and qRT-PCR

Total RNA extraction was carried out by using the RNeasy Plant mini kit (QIAGEN) and the concentration of extracted RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The semi-quantified RNA was used as template to synthesize cDNA using reverse-transcription master mix (Takara, Japan). The RACK1 gene of the WT strain was used for normalization and quantitative reverse transcriptase PCR (qRT-PCR) was performed with the specific primers listed in Table 1.

Total lipid extraction

Cells were harvested at two different stages: 1) cells at exponential phase in TAP medium and, 2) cells at stationary phase in nitrogen deprived TAP medium. Total lipid was extracted as previously described [41] with some modification. The harvested cells were resuspended in distilled water and then diluted with 15 mL of 2:1 (v/v) methanol/chloroform solution. The cells were incubated at room temperature while shaking at 120 rpm for 12 h. Next, 5 mL of distilled water and 5 mL of chloroform were added to the mixture, which was then vigorously vortexed. Two different phases were separated following centrifugation at 3200 rpm for 10 minutes. The chloroform phase, containing lipid fractions, was

transferred to a pre-weighed glass vial. The weight of lipid was determined by the measuring the difference between the weight of the glass vial after the chloroform had completely evaporated and the empty glass vial.

Nile Red staining

After three days of nitrogen starvation, the cells were harvested for Nile Red staining. The Nile Red solution (9-diethylamino-5H-benzo [a] phenoxa-phenoxazine-5-one; Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 µg/mL. Next, 100 µL of Nile Red solution was mixed with 400 µL of the cell sample using 4×10^6 cells/mL and the mixture was vortexed thoroughly for 10 seconds and incubated at 37°C for 30 minutes. The samples were then transferred to a 96-well black plate (SPL Life Sciences, Korea) and fluorescence emission signal was then detected with an excitation wavelength of 530 nm and an emission wavelength of 595 nm. Each treatment was performed in triplicate.

BODIPY staining

BODIPY 505/515 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene) staining was performed to visualize the accumulation of lipid droplets. The process was performed as previously described [42] and cells were observed under a fluorescence microscope (Nikon Y-TV55, Japan).

Fatty acid profile analysis

The FAME profile was analyzed using gas chromatography as previously described [43] with several optimization steps. Lyophilized cells were treated with 2 mL of methylation mixture (MeOH: Benzene: DMP (2,2-Dimethoxy-propane): H₂SO₄, 39: 20: 5: 2) and 1 mL of heptane. The mixture was maintained at 95 °C for 2 hours for extraction. After cooling at room temperature, the separated organic phase was collected. Pentadecanoic acid (Sigma-Aldrich, MO, USA) was supplied as an internal standard. FAMES were analyzed using a gas chromatograph (Agilent technology, USA) equipped with a flame ionization detector and DB-23 column (60 m × 0.25 mm × 0.25 µm; Agilent technology, USA) with helium used as carrier gas. For FAME analysis, a sample volume of 1 µL was injected with a split ratio of 1:10. The temperature was set at 250 °C and 280 °C for the injector and detector, respectively. Initially, the oven temperature was held 50 °C for 1 min then gradual increased to 250 °C and held for 5 min. This FAME analysis was supported by the National Instrumentation Center for Environmental Management (NICEM).

Abbreviations

ELT:esterase/lipase/thioesterase; CRISPR:clustered regularly interspaced short palindromic repeats; FAME:fatty acid methyl esters; TAG:triacylglycerol

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contribution

E.S Jin and S.J Sim conceived and designed the analysis. T.H.T Nguyen, J.Y Jeong, and Y.S Shin performed the experiments. T.H.T Nguyen and S.H Park analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

No conflicts, informed consent, or human or animal rights are applicable to this study.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare no conflict of interest in this study.

Author details

¹Department of Life Science, Research Institute for Natural Sciences, Hanyang University, 222, Wangsimni-ro, Seongdong-gu, Seoul, Republic of Korea

²Department of Chemical and Biological Engineering, Korea University, 145, Anam-ro, Seoungbuk-gu, Seoul 02841, Republic of Korea

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Figures

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AT4G24160.1      52 PNSLRWIPTSTDYIIAAEKRLLSILKTPYVQEQVSIQSGPPGSKIRWFRSTSNESRYINT
AT4G24160.2      52 PNSLRWIPTSTDYIIAAEKRLLSILKTPYVQEQVSIQSGPPGSKIRWFRSTSNESRYINT
Cre01.g000300.1 121 TRYTRWRQTSRQEGKDAEYKVMRLFPAGPLQASDVPVPGP-----REDFLHT
XP_005644956.1   1 -----MHT
PRW58725.1      1 ---MRWRPTSHDEGVYERKLLDMLRN---TYEARDVRVPGP-----KEEFVHT
consensus        . . . . . *

AT4G24160.1      112 VTFDAKE-GAPTLVMVHGYGASQGGFFRNFDALASRFRVIAIDQLGWGGSSRPDFTCRST
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Cre01.g000300.1 170 LSGGQQG--VDPPVVMMPGYGAGVGFYRNFPSLAQRLRLFAVDWLGTGLSGRPAFRAKGR
XP_005644956.1   4 ISAGAP--DAPPLVLPVPGYGAAFYRNLAGLASHFRTHAVDLLGTGMSGRPSFPARSR
PRW58725.1      44 VTSGLHNTDSPAFAVAPGYSTGSSFLFKLFDGLSSAFRLYRIDLLGTGLSGRPPFRVRNR
consensus        . . . * * . * * . . * . . . * . . * * * * * * * * * * . .

AT4G24160.1      171 EETEAWFIDSFEEWRKAQNLS--NFILLGHSFSGYVAAKYALKHPEHVQHLILVGSAGFS
AT4G24160.2      171 EETEAWFIDSFEEWRKAQNLS--NFILLGHSFSGYVAAKYALKHPEHVQHLILVGSAGFS
Cre01.g000300.1 229 EDAEDFFINSLVTRRAAGLEGSKMLVGHSLGGYLAATYALRHPEHVQHLVLCVAGVP
XP_005644956.1   62 EEAEGLFDALSRLWREAMGVD--KMLVGHSLGGYLAASYALQHPHVQHLVLCVAGVP
PRW58725.1      104 EEAERFFVDSLDLWRQEQGLD--KMLVGHSMGGYLSACYALRHPERVQHLILMCPAGVP
consensus        * . * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

AT4G24160.1      229 AEADAKS-EWLTKFRATWKGAVLN---HLWESNFTPQKLVRLGPGWGPGLVNRYTTARFG
AT4G24160.2      229 AEADAKS-EWLTKFRATWKGAVLN---HLWESNFTPQKLVRLGPGWGPGLVNRYTTARFG
Cre01.g000300.1 289 KAPEDWER-QWLGEKWTWRGQLFKVFMRGWEAGITPGSIIIRGLGPGWQSLVYKYVANRFS
XP_005644956.1 120 SR-----WSVRGQLYHLSRTLWESGATPGAVIRSLGPGWGPALIQKYARNRFR
PRW58725.1      162 GKPADWQIPAILRDPWTWRGMLFRFATRVEWGVTPGSIIVRCWGPWGPGLTKKYCRSRFT
consensus        . . . . . * . . . * * . . * * * * * * * * * * * * * *

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AT4G24160.2      285 AHSEGTGLTEEEAKLLTDYVYHTLAAKASGELCLKYIFSFQAFARKPLIQ-----
Cre01.g000300.1 348 H--HGEGLSEREIEVFKQYFYHIAAAPGSGEFALRHLLAPGAWAHSPLERLHELKVPVT
XP_005644956.1 167 ---EGMGLSTEEVAAFEYFYHIMAARGSGEHALRHLLAPFARAKHPLERLHDLKVPVS
PRW58725.1      222 ---RQQQLTDAEVEVFIEIYHYHTLGKTGSGEFALRHILEPFAWRAPLERMAELDVPIT
consensus        . . * . * . . * * * . . * * * . . * . . * * . . . . .

AT4G24160.1      345 FIYGMNDWMNYQGAVEARKSMKVP-----CEIIRVPQGGHFVFIIDNP-IGFHSAVLY
AT4G24160.2      335 -----
Cre01.g000300.1 406 FIYGKHDWMRPEHAVRLCEQLRTERAPRAAHDLSVEIIDDAGHFVFLDQF-ELFDKALMS
XP_005644956.1 224 FIYGDSDWMEPAAGQRVAR-----AVDLVERAGHYAFLDQFQEFLLQKLLSQ
PRW58725.1      279 FIYGEHDWMDAKAGRRVVAALSKQRAAAHPGDLKVMITPDAGHYPAIDQF-RGVLLQLAD
consensus        . . . . . * . . . . .

AT4G24160.1      396 ACRKFISQDSSHD-----QQLLDGL
AT4G24160.2      335 ---RYVHQKHC-----
Cre01.g000300.1 465 VVAPYLKTASQRSSANAYAAAGTSVHVVAPEAGAAAAAAGGAPLSGAAATSWGFAASGA
XP_005644956.1 270 TLTAFPDWDAKRQ-----KPDQ-----ASG-
PRW58725.1      338 ACGSYLPAAAREA-----LLAEAG-----RQLPHAA
consensus        . . . . .

AT4G24160.1      416 RLV-----
AT4G24160.2      -----
Cre01.g000300.1 525 GQGQAQQQQEEAVDAEGVQLEVGEEDESGAARPAVSGGGRVVR
XP_005644956.1 -----
PRW58725.1      364 VQSDSKEELEREMGRNPLEAAAHEASEL-----
consensus        . . . . .

```

Figure 1

Alignment of protein sequence encoded by Cre01.000300 and the homologs described in other species. The conserved domains and catalytically active motifs from previous reports (refer to the text) are highlighted and marked with red boxes. The sequence alignment was performed using ClustalX2

software, with the sequences of ELT protein from different organisms, AT4G24160.1 and AT4G24160.2 from Arabidopsis, XP_005644956.1 from *Coccomyxa subellipsoidea*, and PRW58725.1 from *Chlorella sorokiniana*.

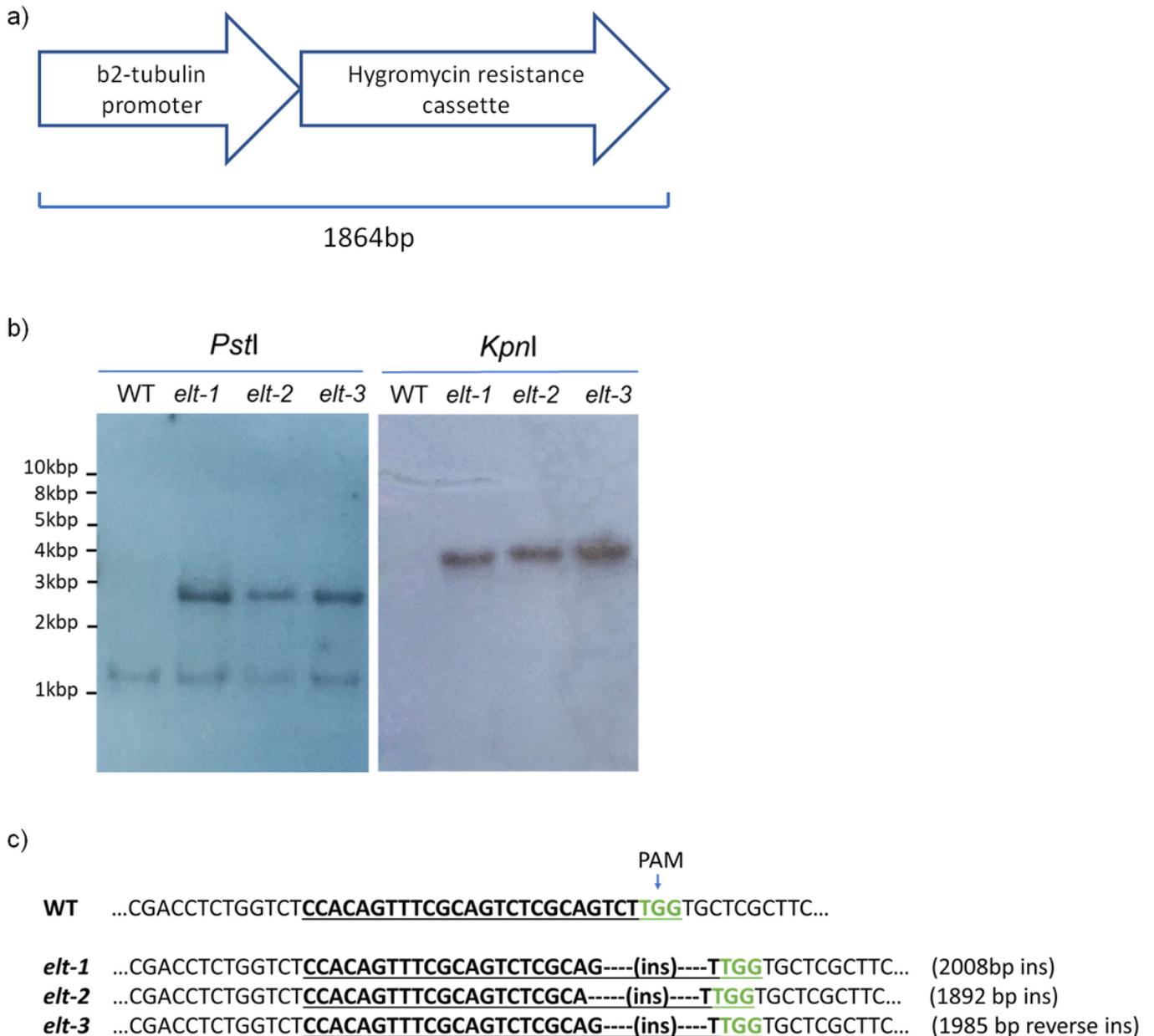


Figure 2

The map of hygromycin resistance cassette and the characterization of *elt* knockout mutant strains. (a) Map of hygromycin resistance cassette used as marker gene to assess the ELT gene in *C. reinhardtii*. (b) Southern blot analysis of genomic DNA of WT and mutant strains. Genomic DNA were digested by *Pst*I and *Kpn*I. Blotting and hybridization were performed as described in the materials and methods. (c) The genotype of *elt* mutants determined using Sanger sequencing.

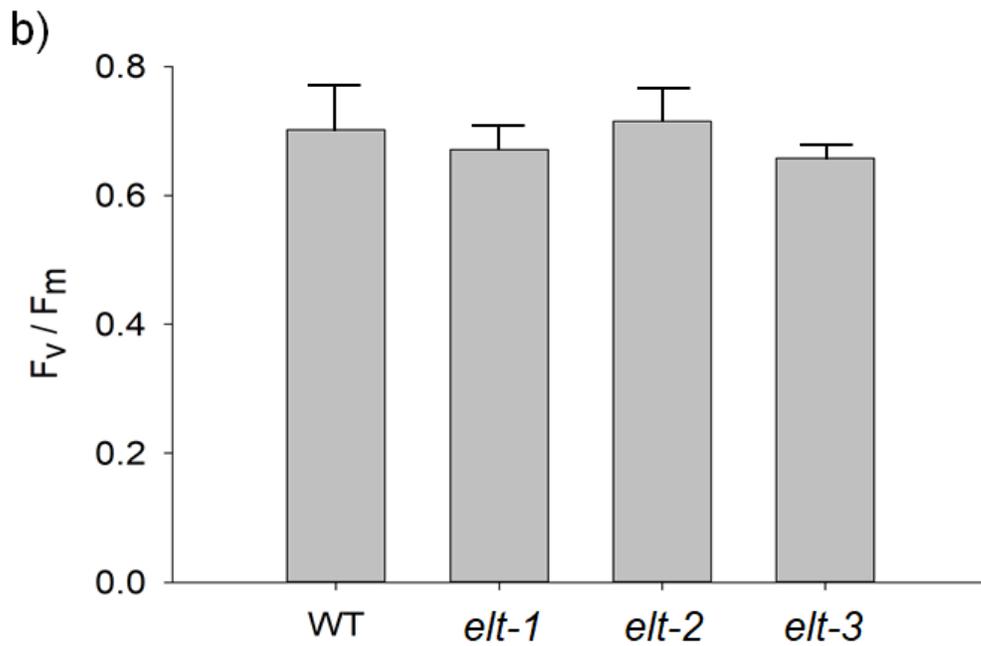
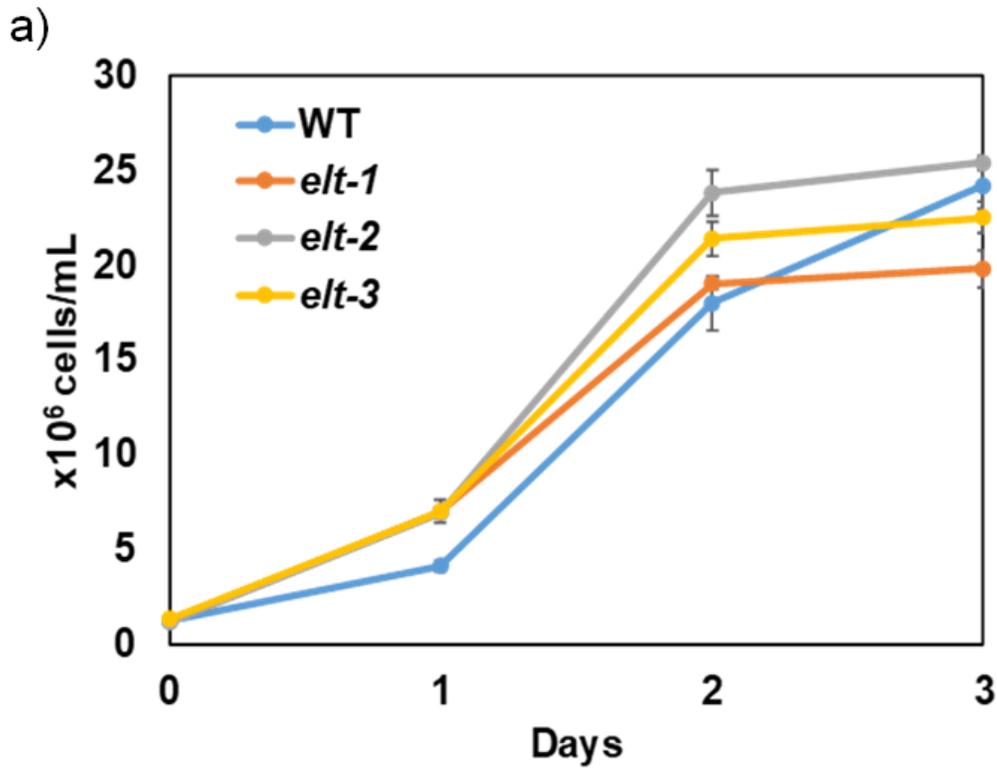


Figure 3

The growth and photosynthetic performance of the mutants and WT strain. (a) The growth curves of mutants and WT strains. (b) Maximum quantum yield of photosystem II, calculated as F_v/F_m . The values indicate the mean \pm standard error of the three biological replicates of each strain.

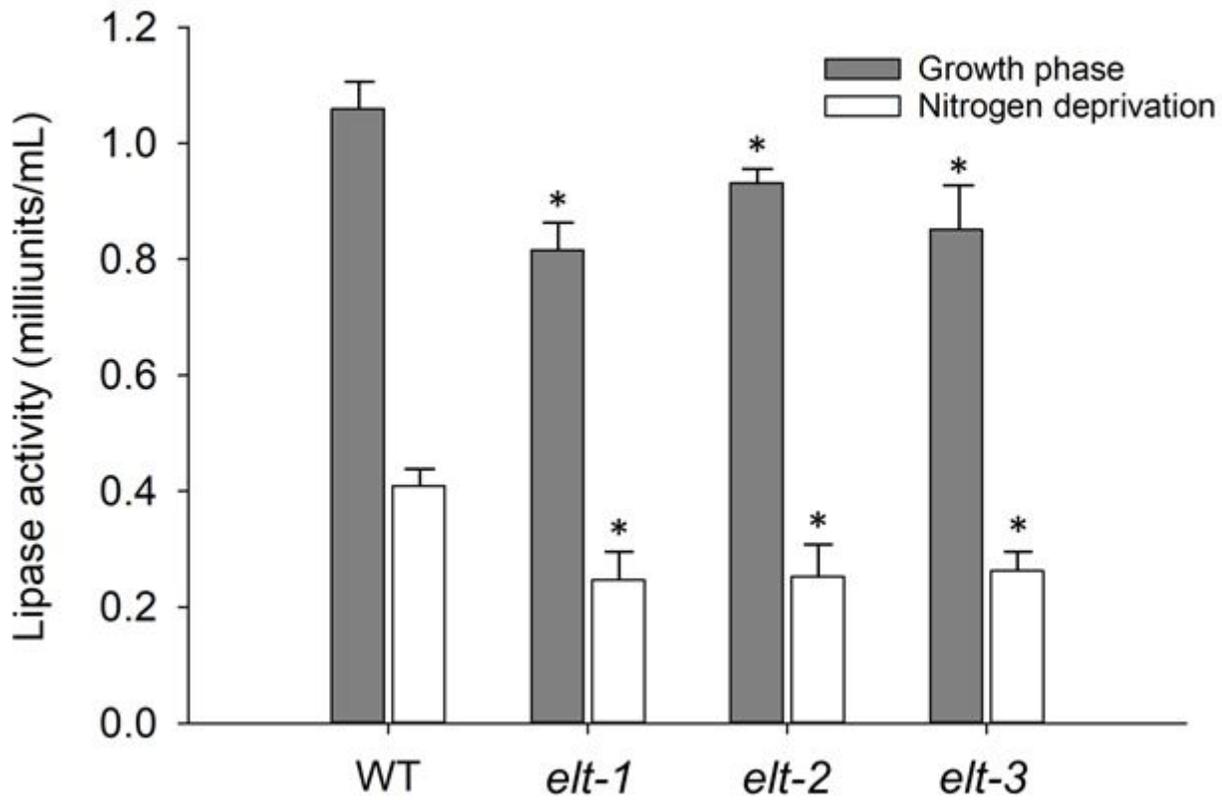


Figure 4

Enzymatic activity assay on mutants and WT strain. (a) The colorimetric observation, in which the color intensity indicates the concentration of product generated by lipase reaction. (b) Lipase activity of mutants and WT strains in exponential phase and nitrogen deprivation.

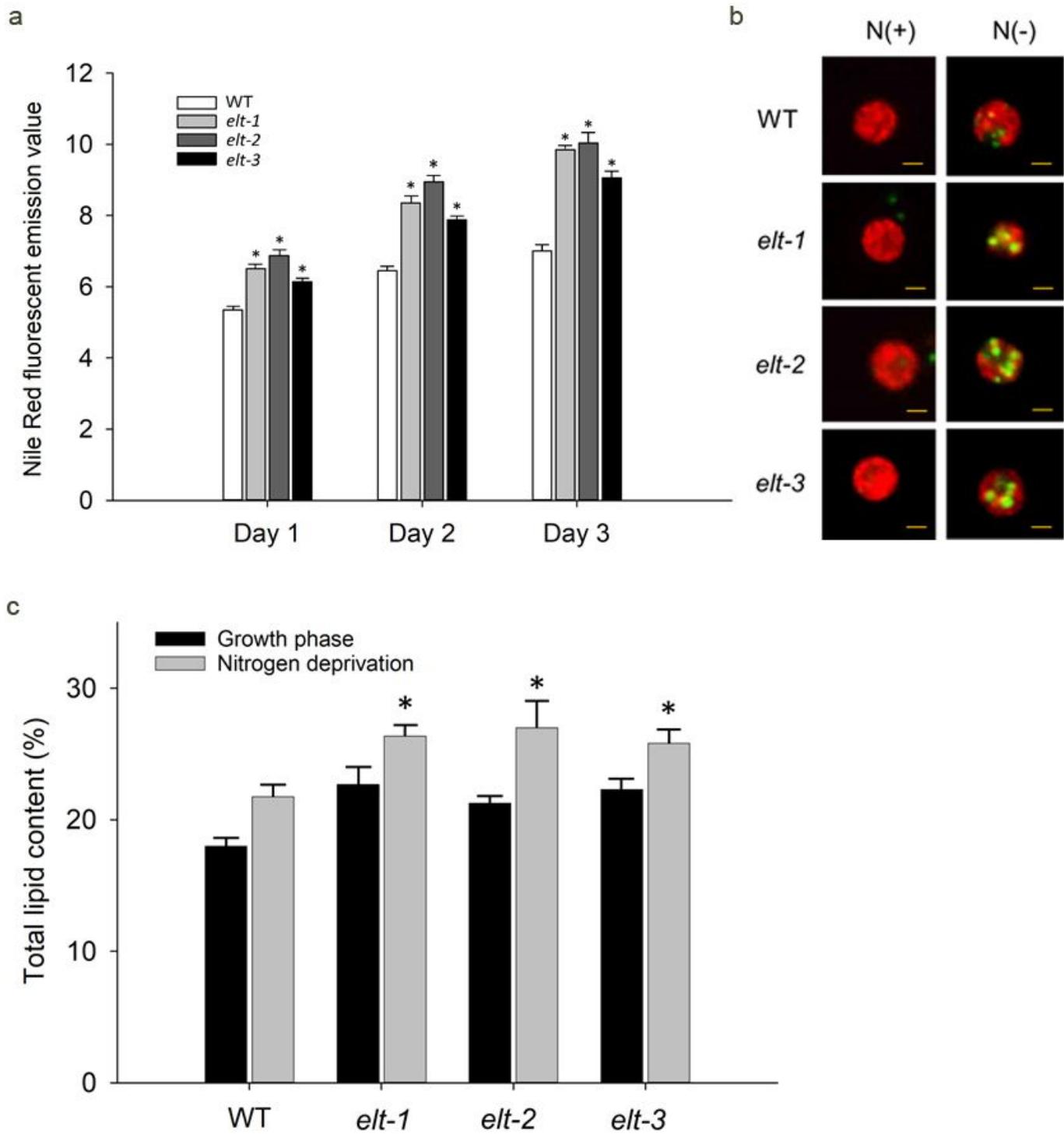


Figure 5

Lipid accumulation and relative gene expression in the mutants and WT strain. (a) Relative value of Nile Red staining fluorescent emission. (b) BODIPY staining images of mutants and WT strains in growth phase and 3 days after nitrogen deprivation, size bar 5 μ m. (c) Percentage of total lipid content of the mutants and WT strains in the nitrogen replete and depleted conditions. The data indicates the mean \pm

standard error of the three biological replicates of each strain. Statistical analyses were performed using Student t-test, *P < 0.05.

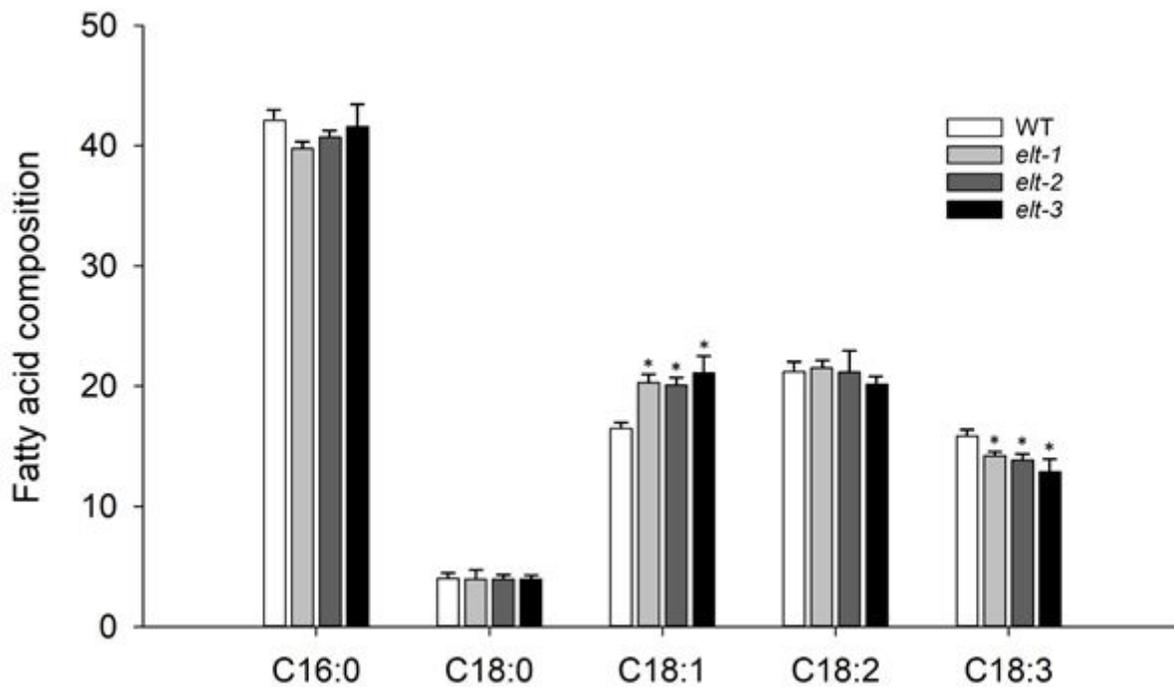


Figure 6

Analysis of fatty acid profile in mutants and WT strains after three days of nitrogen starvation. The data are indicated as the mean \pm standard error of the three biological replicates of each strain. Statistical analyses were performed using Student t-test, *P < 0.05.

Supplementary Files

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