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Enhancement of lipid productivity by ethyl methane sulfonate-mediated random mutagenesis and proteomic analysis in *Chlamydomonas reinhardtii*

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Abstract−Microalgae-derived biomass has been considered as the most promising candidate for next generation biofuel due to its sustainability and biodegradability. In this study, microalgal strain *Chlamydmonas reinhardtii* was randomly mutagenized by using a chemical mutagen, ethyl methane sulfonate (EMS) to create mutants showing enhanced lipid production. We identified three random mutants that displayed high lipid production in the screening using Nile red staining. Among those, mutant #128 was selected as candidate for further studies. Our flow cytometry and confocal microscopy analysis revealed that mutant #128 contains larger and more abundant lipid bodies than that of wild-type. Moreover, mutant #128 showed 1.4-fold increased fatty acid methyl ester (FAME) content compared to wild-type under nitrogen depleted condition. In addition, mutant #128 grew faster and accumulated more biomass, resulting in high lipid production. 2D gel electrophoresis and MALDI-TOF analysis used for gene targeting revealed that β-subunit of mitochondrial ATP Synthase and two-component response regulator PilR may be involved in enhanced characteristics of mutant #128. These results show the possibilities of EMS mediated random mutagenesis in generation of mutants to produce high amount of lipid as well as further study for molecular mechanism of mutants.

Keywords: Ethyl Methane Sulfonate (EMS), Random Mutagenesis, *Chlamydmonas reinhardtii*, Lipid Productivity, Proteomics

INTRODUCTION

Renewable energy produced by biomass has recently been considered as one of the most important resources to replace the use of petroleum-based fuels due to the lack of environmental risks and pollution [1,2]. Among the various alternative energy sources, microalgae have many advantages as a biodiesel resource, including higher photosynthetic efficiency, faster growth rate, and biomass productivity compared to crops and land plants [3-5]. In addition, most importantly, the ability to accumulate oil in microalgae is up to 25-fold higher than that in other conventional crops or land plants [6]. However, despite the advantage of microalgae for biodiesel production, several technical barriers still need to be overcome before we can use microalgae as an economically viable biodiesel feedstock [7]. To advance the utilization of microalgae in biodiesel production, it is essential to improve many characteristics such as lipid quantity and quality, growth rate, and resistant ability against environmental disturbances [8]. Therefore, phenotypic improvement by genetic manipulation in microalgae is emerging as an important technique to overcome such barriers.

As an effort to facilitate genetic manipulation, progress in microalgal genomics and transcriptomics has been achieved during the last decade [9-12]. Moreover, genetic tools have been developed to transfer DNA into microalgal cells or to display stable expression of transgenes using proper codon usage and strong promoter [13,14]. Among more than 30 transformable strains, the photosynthetic unicellular green alga, *Chlamydomonas reinhardtii*, has been selected as a model organism because of its tractability of genetic studies, rapid growth and full genome sequence [15,16]. Some progress in homologous recombination has been made with the nuclear genome of *C. reinhardtii* [17]. However, it has still been limited to generating targeted gene knockouts through homologous recombination in microalgae, because the efficiency remains low, and nuclear transformation of microalgae generally results in the random integration of transgenes [14,18]. To circumvent the current lack of efficient homologous recombination in microalgae, gene inactivation can be achieved through random mutagenesis.

Random mutagenesis has many advantages in that it needs only little genetic information of an organism, and its process is easy. Especially, EMS mediated random mutagenesis is known as a suitable and powerful method that induces point mutations by modulating A-T to G-C in a DNA [19]. There are several examples of successful random mutagenesis to generate mutants that show increased lipid accumulation. For example, *C. reinhardtii* starchless mutant, which is defective in ADP-glucose pyrophosphorylase, resulted in 10-fold overproduction in TAG [20,21]. Moreover, it has been reported that random mutants can be generated by ethyl methane sulfonate (EMS), chemical mutagen for isolating pigment mutants, autoflocculating mutants and high producer of bioethanol in another eukaryote *Saccharomyces cerevisiae*, *Cyclotella* sp. and cyanobac-

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‡This paper is dedicated to commemorate Prof. Ji-Won Yang (KAIST) for his retirement.

terium *Arthrospira platensis*, respectively [19,22,23]. In addition, there was an attempt to increase biomass and lipid production with chemically induced mutants from *Nannochloropsis* sp., but no significant variation in major fatty acid composition was found among the tested mutants and the wild type parent [24]. There are still bottlenecks in the use of chemical random mutagenesis as a genetic approach, because it is difficult to identify either the genes involved with phenotypes of mutants or the mechanism.

In this study, we generated random mutants by EMS treatment, and screened mutants for high lipid contents and faster growth rate compared wild-type. To identify genes involved in phenotypes of mutant, we further conducted proteomics analysis including two-dimensional gel electrophoresis and MALDI-TOF analysis. Results from our analyses will be discussed.

MATERIALS AND METHODS

1. Strains and Culture Conditions

Chlamydomonas reinhardtii cc-124 was purchased from Chlamydomonas Resource Center in USA. For culture media, Tris-acetate phosphate (TAP) medium and nitrogen-depleted TAP media (TAP-N) without NH_4Cl were used. Cells were grown in mixotrophic condition at 25 ºC, at 150 μmol photons/$m^2 \cdot s$ light continuous illumination with shaking at 150 rpm. Analysis points were at 36 h for exponential phase and 132 h for stationary phase. At 132 h, cultures were centrifuged at 7,000 rpm for 5 min at room temperature and transferred into TAP-N medium for further 72 hours. So, at 204 h, the effects of N-starvation were analyzed. Optical density was measured with UV/Vis spectrophotometer (BECKMAN COULTER DU 730 Life Science) and absorption was read at 750 nm.

2. Random Mutagenesis by EMS

C. reinhardtii cc-124 was grown to early exponential phase. Cell suspensions at 10^7 cells/ml were subjected to random mutagenesis by various concentrations of EMS (Sigma-Aldrich, USA) i.e. 20, 30 and 40 μl/ml for 2 hours with gentle agitation. Then, EMS-treated samples were washed three times with TAP medium, and resuspended in 20 ml of fresh TAP medium. After two days, equal numbers of cells were spread uniformly on TAP agar plates. Colonies appeared in two weeks.

3. Mutant Screening

Each colony generated by EMS mutagenesis was transferred into round-bottom tubes containing 5 ml of TAP medium. After seven-day cultivation, colonies underwent photoluminescence (abbreviated as PL) measurement. The Nile red staining protocols in this study were adopted from a previous study [25]. In brief, 50 μl of each culture was stained with 5 mg/L Nile red and left in the dark at 40 ºC. Nile red is a lipophilic dye that stains neutral lipids into yellow-gold fluorescence. Spectrophotofluoremeter (SHIMADZU RF-5103PC, JAPAN) was used with excitation at 488 nm and emission at 575 nm, specific for Nile red fluorescent dyes.

4. Fluorescence and Confocal Microscopy Analyses

Cells stained with Nile red using the above method were centrifuged at 13,000 rpm for 1 min at 4 ºC. The supernatant was discarded to make the sample more concentrated for fluorescence microscopy. Nile red-stained cells were examined using Leica DM 2500 with ebq 100-04 lamp. For confocal microscopy and flow cytometry, BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen) was used to stain non-polar lipids. 90 μl of each culture was stained with 10 μl of 5 mM BODIPY for 10 min in the dark at room temperature. Samples were centrifuged at 13,000 rpm for 1 min at 4 ºC and supernatant was discarded. Images were acquired using Laser scanning confocal microscope (Eclipse C1si, Nikon, Japan) with excitation at 488 nm and emission at 570-590 nm in order to spot microalgal lipid bodies. Confocal microscope images were taken at stationary phase (132 h) and after N-starvation (204 h).

5. Fatty Acid Analyses

A high speed flow cytometer, MoFlo XDP (Beckman Coulter) was used for the analysis of stained cell. The emission signal was measured by four channels upon excitation of 488 nm Argon laser. FL1 channel was centered at 530/40 nm, FL2 at 580/30 nm, FL3 at 630/30 nm, and FL5 at 740 LP. From the FACS analysis, the mean fluorescence intensity values and images were recorded by using SUMMIT Software Version 5.2. Coulter Isoton II diluent fluid was used in all experiments as flow cytometry sheath fluid.

To measure FAME contents, cell cultures were harvested by centrifugation at 7,000 rpm for 5 min at 20 ºC and microalgal biomass was lyophilized for four days. 10 mg of lyophilized biomass was mixed with chloroform-methanol (2 : 1 v/v) solvent mixture as Folch's method. 1 ml of internal standard solution (100 mg heptadecanoic acid/200 ml chloroform) was added as a reference. The extracted lipid was transesterified into fatty acid methyl esters (FAMEs): 1 ml of methanol was added to extract the lipid. Transesterification was allowed to occur for 20 min at 100 ºC. After sufficient cooling at room temperature, 1 ml NaOH was added and centrifuged at 7,000 rpm for 5 min at 20 ºC to separate the organic phase from the water phase. The FAME contents in the organic phase were extracted by syringe.

Table 1. Fatty acid composition as % of total fatty acids of wild type and mutant #128

	Fatty acid (% total fatty acids)					
	WT			#128		
	Exponential	Stationary	N-depleted	Exponential	Stationary	N-depleted
C14:0	3.00±0.48	0.44	0.35±0.08	1.93±0.17	1.98±0.17	0.27±0.14
C16:0	29.30±5.32	37.16±3.47	46.12±3.93	29.03±0.52	38.06±4.61	41.36±3.95
C16:1	1.70±0.02	0.91±0.23	3.47±0.83	1.56±0.07	0.95±0.22	5.17±1.09
C18:0	22.09±2.80	16.70±0.95	4.77±1.65	14.90±1.18	9.65±1.29	4.20±1.16
C18:1-3 (n)	16.12±0.82	10.30±0.14	26.99±4.73	14.41±0.41	16.05±1.23	34.12±4.14
C18:3n3	23.35±0.82	24.90±0.76	11.34±0.89	27.49±2.58	19.69±4.99	11.27±2.20
Others	4.45±0.19	11.10±0.58	9.03±0.72	10.40±0.29	7.18±1.03	5.13±0.35

2 μl of FAMEs was analyzed by gas chromatography (HP5890, Agilent) with a flame ionized detector (FID) and INNOWAX capillary column (Agilent, 30 m×0.32 mm×0.5 μm). Gas chromatography was conducted at exponential phase (36 h), stationary phase (132 h) and after N-starvation (204 h).

6. Proteomic Analysis

N-starved *C. reinhardtii* cc-124 and mutant #128 strain were lyophilized for two-dimensional gel electrophoresis analysis. Lyophilized samples were homogenized directly by motor-driven homogenizer (PowerGen125, Fisher Scientific) in sample lysis solution composed of 7 M urea, 2 M thio-urea containing 4% (w/v) 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT) and 2% (v/v) pharmalyte and 1 mM benzamidine. Proteins were extracted for one hour at room temperature with vortexing. 100 μg of proteins was purified by elimination of insoluble fraction through centrifugation. These proteins were used for two-dimensional gel electrophoresis and protein concentration was assayed by Bradford method [26].

IPG dry strips (4-10 NL IPG, 24 cm, Genomine, Korea) were equilibrated for 12-16 hours with 7 M urea, 2 M thio-urea containing 2% 3-[(3-cholamidopropy)dimethyammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), 1% pharmalyte and respectively loaded with 100 μg of sample. Isoelectric focusing (IEF) was done at 20 °C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply. The voltage was linearly increased from 150 to 3,5000 V during 3 hours for sample entry followed by constant 3,500 V, with focusing complete after 96 kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20× 24 cm, 10-16%). goo using Hoefer DALT 2D system (Amersham Biosciences) following manufacturer's instruction. 2D gels were run at 20 °C for 1,700 Vh. And then 2D gels were silver stained as described by Oakley et al. (1980), but fixing and sensitization step with glutaraldehyde was omitted.

Using PDQuest (version 7.0, BioRad) software, quantitative analysis of digitized images was performed. Each spot was normalized by total valid spot intensity. Comparing the protein spots of *C. reinhardtii* cc-124 and mutant #128, spots with over two-fold increase in its expression level were selected.

Protein spots were enzymatically digested in-gel using Promega-modified porcine trypsin [27]. Gel pieces were washed with 60% acetonitrile to remove SDS, salt and stain. Dehydrated spots were then vacuum-dried to remove solvent, and then were rehydrated with trypsin solution (8-10 ng/μl) in 50 mM ammonium bicarbonate (pH 8.7) and incubated for 8-10 h at 37 °C.

RESULTS AND DISCUSSION

1. EMS-mediated Mutagenesis and Mutant Screening

To create mutants producing high amount of lipid, wild-type cc-124 strain was randomly mutagenized by EMS treatment. Cells grown by small scale cultivation were then screened by fluorescence intensities of Nile red, a lipophilic fluorescent dye that stains neutral lipids. From our mutagenesis, we isolated three mutants whose fluorescence intensities were increased compared to wild-type. Mutant #128 showed three-fold increased fluorescence intensity, and mutant #132 and mutant #142 showed approximately 2.4-fold increased fluorescence intensity respectively, when comparing mutants with wild-type (Fig. 1). This not only indicates that EMS is useful for random mutagenesis, but also that measurement of fluorescence intensity of cells stained by Nile red is effective for selection of mutants which have high amount of neutral lipids. After mutant screening, we selected mutant #128 as candidate for in-depth study due to its high lipid contents.

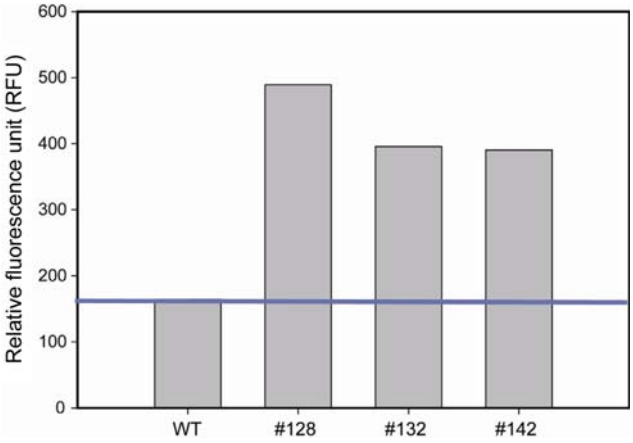

Fig. 1. Comparison of relative fluorescence of Nile red stained wild type and high lipid candidate mutants measured by spectrophotofluoremeter.

2. Analysis of Neutral Lipid Content

To confirm the high lipid content of mutant #128, neutral lipid content at exponential, stationary and N-starved condition was analyzed by fluorescence measurement after staining cells with Nile red. Our results revealed that neutral lipid content of mutant #128 was approximately two-fold increased compared to wild-type under N-depleted condition as well as at exponential phase and stationary phase (Fig. 2(a) and (b)). Interestingly, however, the fluorescence signal by neutral lipid staining was notably increased in both wild-type and mutant #128 grown at N-depleted condition, not only indicating that neutral lipid body is accumulated under N-depleted condition, but also suggesting that mutant #128 produces two-fold increased neutral lipid. This corresponds to previous reports that showed certain nutrient depletion induces accumulation of lipid [28, 29].

For the measurement of fluorescence intensity, another lipophilic fluorescent dye, BODIPY 505/515, was employed. Recently, it has been reported that BODIPY 505/515 is more widely applicable and accurate dye than Nile red, because a narrow emission spectrum of BODIPY 505/515 is more suitable for confocal imaging [30]. BODIPY 505/515 stained lipid bodies with intense green fluorescence, while chlorophyll auto fluorescence appeared red. Therefore, chloroplastic fluorescence and lipid body fluorescence are clearly distinguishable from confocal microscope images [31]. Moreover, BODIPY 505/515 can retain continuous emission under the light condition up to 30 minutes, proving its outstanding photo-stability and resistance to photo-bleaching phenomenon [30]. Our confocal microscopy revealed that mutant #128 contains slightly bigger lipid bodies than that of wild-type at stationary phase. However, lipid

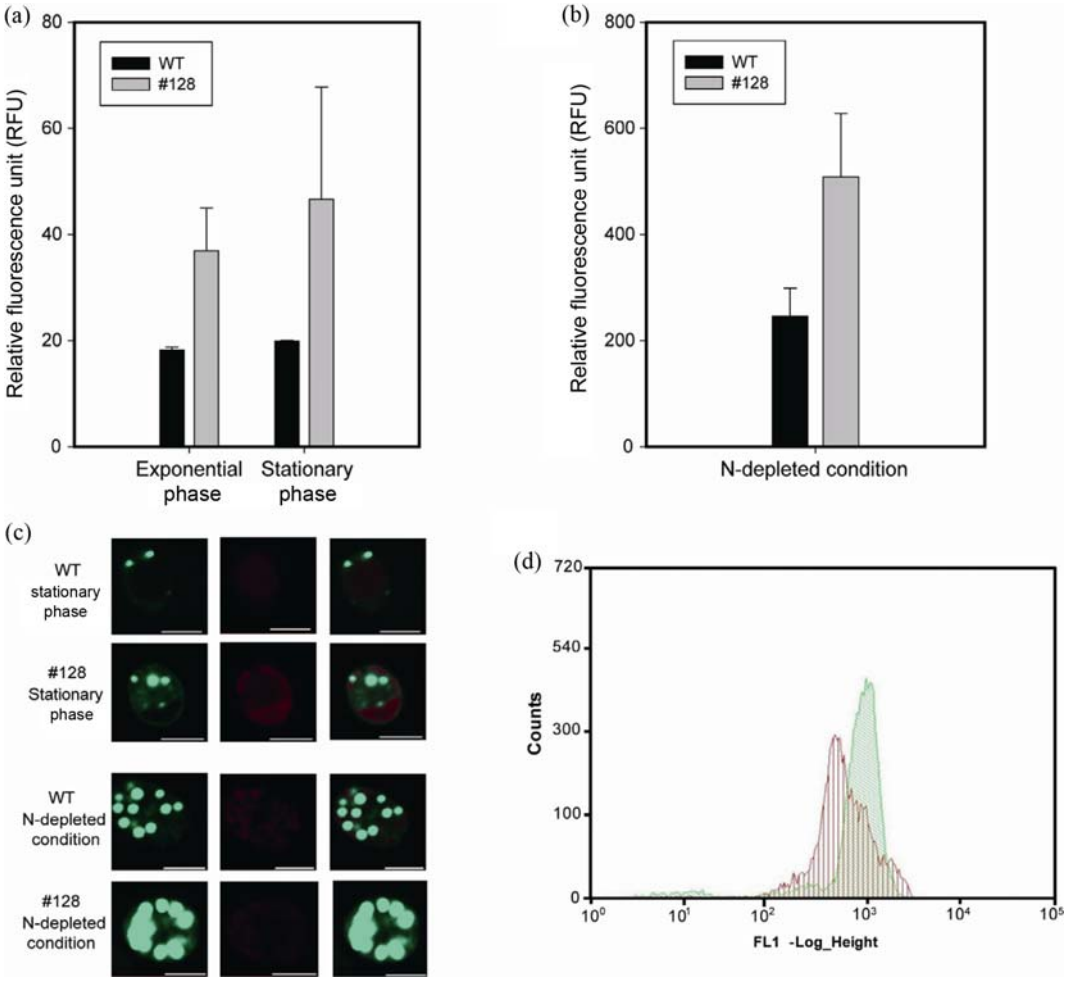

Fig. 2. Comparison of neutral lipid between wild type and mutant #128. Relative fluorescence of Nile red stained at exponential and stationary phases (a) and after nitrogen-starvation (b). Confocal microscopy after Bodipy staining (c) and FACS analysis (d).

bodies seem to have accumulated in mutant #128 after N-starvation, resulting in production of much bigger lipid bodies than that of wild-type, whereas the portion of chloroplast was reduced (Fig. 2(c)). Previously, it was reported that N-starvation triggers two effects: cell differentiation into gametes and accumulation of starch [32]. Recently, another N-starvation response was elucidated, namely the autophagy program, where cells destroy their cytoplasmic, chloroplastic ribosomes and chloroplast membranes [33]. In addition to microscopy, flow cytometric analysis was carried out after BODIPY 505/515 staining. Our analysis demonstrated that the fluorescence peak of mutant #128 is shifted to the right, indicating higher fluorescence signal of the cells (Fig. 2(d)). All these results clearly indicate that mutant #128 generated by EMS random mutagenesis accumulates higher amounts of lipid by accumulating neutral lipid in lipid bodies.

3. Analysis of FAME Content

To assess if accumulation of lipid bodies in mutant #128 directly results in enhancing lipid content, we first analyzed the fatty acid methyl ester (FAME) yield of wild type and mutant #128 at exponential and stationary phase as well as after N-starvation. Our results demonstrated that the FAME yield was approximately 1.4-fold increased in the mutant #128 compared to wild-type under N-depleted condition, whereas FAME yield in the mutant #128 was slightly increased at exponential phase and stationary phase (Fig. 3(a)). This indicates that mutant #128 produces not only more neutral lipid bodies (Fig. 2), but also more FAME. In addition to total FAME content, mutant #128 displayed significant change in FAME composition. As shown in Fig. 3(b) and Table 1, mutant #128 contains higher yield of palmitic acid (C16:0), palmitoleic acid (C16:1), the combination of oleic (C18:1n9c), linoelaidic (C18:2n6t), and γ-linolenic (C18:3n6c) acids, and α-linoleic acid (C18:3n3). One of the most prominent increases occurred in palmitic acid (C16:0), where wild type's content was 0.584 mg/10 mg cell and #128's content was 0.724 mg/mg cell after N-starvation. Moreover, the yield of combined oleic (C18:1n9c), linoelaidic (C18:2n6t), and γ-linolenic (C18:3n6c) acids increased from 0.343 mg/mg cell in wild type to 0.584 mg/10 mg cell in the mutant #128. In addition, when displaying fatty acid composition as % of total fatty acids, mutant #128 synthesizes higher amount of palmitic acid (C16:0), and combination of oleic acid (C18:1n9c), linoelaidic acid (C18:2n6t), and γ-linolenic acid (C18:3n6c). After N-starvation, palmitoleic acid (C16:1) also becomes a higher portion in mutant #128. Moreover, the compositional trends of various fatty acids throughout the growth phases were figured out. Myristic acid (C14:0), stearic acid (C18:0), combination of oleic (C18:1n9c),

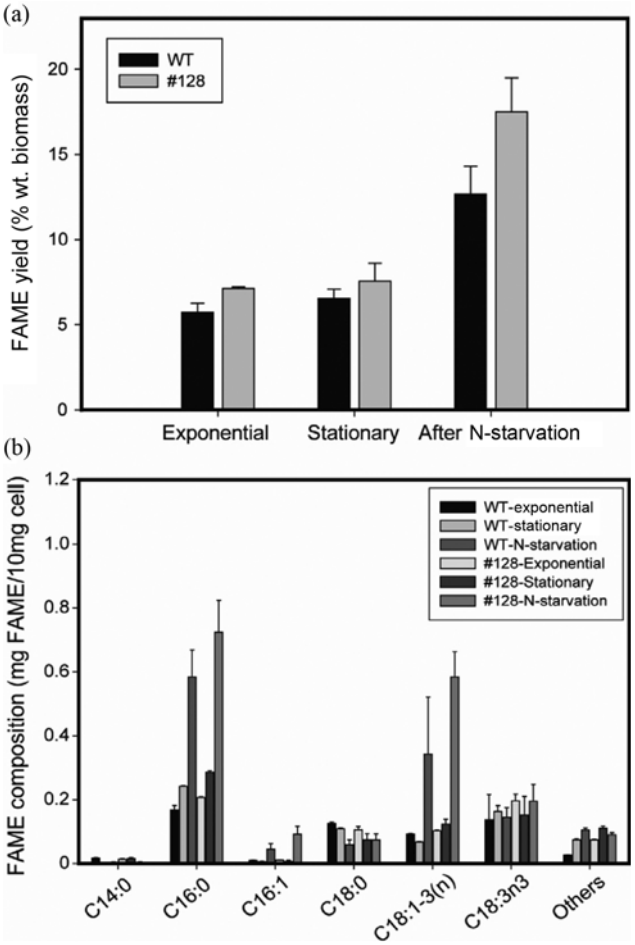

Fig. 3. FAME yield of wild type and mutant #128 at exponential and stationary phases and after nitrogen-starvation (a). Of fatty acid composition (as mg FAME/10 mg cell) of wild type and mutant #128 at exponential and stationary phases and after nitrogen-starvation (b).

linoelaidic (C18:2n6t), and γ-linolenic (C18:3n6c) acids and others show a decreasing trend from exponential to N-depleted phase, in both wild type and mutant #128. Conversely, other fatty acids such as palmitic acid (C16:0), palmitoleic acid (C16:1) and α-linoleic acid (C18:3n3) demonstrated an increasing trend as growth progressed. It has been reported that microalgae accumulate a high amount of neutral lipid and C18 fatty acids as a protection mechanism under stress condition [34]. Therefore, N-starvation would be a helpful condition to apply, when higher quantities of certain fatty acids (C16:0, C16:1, C18:3n3) are desired. Fatty acid in microalgae is produced in optimal growth conditions as building blocks for various types of lipids. Fatty acids can be classified into medium-chain (C10-14), long-chain (C16-18) and very-long-chain (≥C20) fatty acids [35]. The most commonly produced fatty acids in algae are saturated (C16:0) and mono-unsaturated fatty acids (C18:1) in general required for high quality of diesel [36]. Therefore, our results strongly suggest that mutant #128 generated by EMS random mutagenesis not only produces high amount of total FAME, but also contributes to enhance the quality of biodiesel.

4. Analysis of Growth and Lipid Productivity

In addition to the quantity and quality of lipids produced by microalgal cells, growth is another critical factor for efficient production of biodiesel. It has been reported that biodiesel production from algae should optimize two input parameters: the amount of lipid produced per cell and the number of cells per unit of culture per unit of time [29]. In other words, cellular growth rate as well as lipid content is important for biodiesel production from microalgae. To determine the lipid productivity, we analyzed the growth rate dry cell weigh (DCW), and found that the growth rate of the mutant #128 was higher than that of the wild-type, resulting in higher production of biomass (Fig. 4). Most importantly, when considering FAME content and biomass production, lipid productivity of mutant #128 was approximately 25% increased compared to wild-type (Table 2). Note that the growth rate of cells is in general inversely proportional to the amount of lipid content, because cells use considerable energy for growth events such as cell division rather than lipid accumulation [37]. Therefore, our results are unusual, but suggest the possibilities for improvement of algal cell strains in growth as well as lipid content. It is unclear why mutant #128 displayed enhanced phenotypes in both biomass and lipid production. One possible interpretation is that mutant #128 can be multiply mutagenized by EMS treatment, resulting in multiple phenotypic changes.

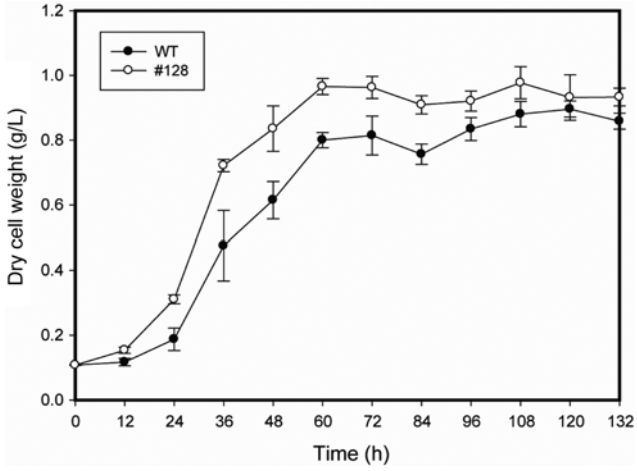

Fig. 4. Growth profile of wild type and mutant #128 measured by dry cell weight.

Table 2. Lipid productivity of wild type and mutant #128

	FAME yield (%)	Biomass concentration (g L^{-1})	Specific growth rate (h^{-1})	Lipid productivity (g L^{-1} d^{-1})
WT	6.53	0.859	0.015	1.019
#128	7.56	0.933	0.016	1.282

5. Proteomic Analysis

In an effort to understand phenotypic changes of mutant #128 at molecular level, the proteomics approach by using two-dimensional gel electrophoresis was applied for N-starved samples to clarify which proteins or metabolic pathways resulted in its phenotypes. Proteins were separated based on their charge by isoelectric focusing (IEF) in the first dimension and based on molecular weight (SDS-PAGE) in the second dimension. Spot analysis was performed by using PDQuest, where 114 spots with more than two-fold difference be-

Table 3. Proteins highly up-regulated in mutant #128

SSP[a]	Protein score[b]	Matched protein	Matched species	SC (%)[c]
1604	151	Beta subunit of mitochondrial ATP Synthase	*Chlamydomonas reinhardtii*	47
1605	207	Beta subunit of mitochondrial ATP Synthase	*Chlamydomonas reinhardtii*	62
5103	78	Hypothetical protein	*Sorghum bicolor*	33
5404	94	Two-component response regulator PilR	*Salinisphaera shabanensis* E1L3A	25

[a]SSP=standard spot

[b]Protein score=−10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 73 are significant for SSP 1604, 1605, and 5103. Protein score greater than 86 is significant for SSP 5404 ($p<0.05$)

[c]Percent of protein sequence covered by matched peptides

tween wild type and mutant #128 were selected. Among the 114 spots, 11 proteins displaying the highest differences in expression level were selected for further MALDI-TOF analysis. Our analysis revealed that four proteins could be identified with protein scores considered significant. Protein 1604, 1605 and protein 5404 were highly up-regulated in the mutant #128 compared to wild-type. Protein 1604 and 1605 were identified as β-subunit of mitochondrial ATP synthase (Table 3). Previously, it was reported that β-subunit of mitochondrial ATP synthase is abundant in *C. reinhardtii* starchless mutant BAF-J5, which stores lipids up to 65% of dry cell weight when grown under nitrogen starvation and photoheterotrophically [38]. Moreover, it also has been reported that ATP synthase of *C. reinhardtii* is involved in light sensitivity, but not in lipid production [39]. Therefore, beta subunit of mitochondrial ATP is a worthwhile protein for further studying related to lipid synthesis mechanism.

On the other hand, protein 5404 was identified as two-component response regulator PilR (Table 3). This protein has not been reported in other proteomic studies yet. However, it is widely known as a transcriptional activator that induces transcription of pilin genes in *Pseudomonas aeruginosa* [40]. Although this protein has been identified from prokaryote, there are many experimental evidences that many eukaryotic proteins are similar to bacterial two-component regulators [41,42]. Moreover, it is widely known that two-component response regulator is involved in singling pathway for many physiological functions as response mechanisms against environmental stresses such as nutrient depletion [43]. Therefore, studying the two-component response regulator is likely valuable to understand lipid metabolism and signaling mechanism of *C. reinhardtii* under stress condition including N-starvation.

CONCLUSION

Here, we report random mutant #128 created by EMS treatment. Mutant #128 showed characteristically enhanced phenotypes compared to wild-type in FAME contents as well as growth. Especially, mutant #128 displayed approximately 25% increased lipid productivity. Moreover, proteomics analysis, which was performed to identify genes related to phenotypes of mutant #128, suggests that β-subunit of mitochondrial ATP synthase and two-component response regulator may involved in high lipid productivity and faster growth of mutant #128. Taken together, we suggest that improved mutants in lipid productivity and growth rate can be generated by EMS mediated random mutagenesis, and either the genes involved or the mechanism can be identified by proteomics analysis for further studies.

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