**Isolation and characterization of a mutant defective in triacylglycerol
accumulation in nitrogen-starved *Chlamydomonas reinhardtii***

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Abbreviations: G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; F6P, fructose 6-phosphate; F16BP, fructose 1,6-bisphosphate; GA3P, glyceraldehyde 3-phosphate; 3-PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; G3P, glycerol 3-phosphate; LysoPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, *sn*-1,2-diacylglycerol; TAG, triacylglycerol.

ABSTRACT

Triacylglycerol (TAG), a major source of biodiesel production, accumulates in nitrogen-starved *Chlamydomonas reinhardtii*. However, the metabolic pathway of starch-to-TAG conversion remains elusive because an enzyme that affects the starch degradation is unknown. Here, we isolated a new class of mutant *bga1*, which expressed an overaccumulation of starch granules and defective photosynthetic growth. The *bga1* was a null mutant of a previously uncharacterized β -galactosidase-like gene (*Cre02.g119700*), which decreased total β -galactosidase activity 40% of wild type. Upon nitrogen starvation, the *bga1* mutant showed decreased TAG accumulation mainly due to the reduced flux of *de novo* TAG biosynthesis evidenced by increased unsaturation of fatty acid composition in TAG and reduced TAG accumulation by additional supplementation of acetate to the culture media. Metabolomic analysis of the *bga1* mutant showed significantly reduced levels of metabolites following the hydrolysis of starch and substrates for TAG accumulation, whereas metabolites in TCA cycle were unaffected. Upon nitrogen starvation, while levels of glucose 6-phosphate, fructose 6-phosphate and acetyl-CoA remained lower, most of the other metabolites in glycolysis were increased but those in the TCA cycle were decreased, supporting TAG accumulation. We suggest that BGAL1 may be involved in the degradation of starch, which affects TAG accumulation in nitrogen-starved *C. reinhardtii*.

1. Introduction

Eukaryotic algae have been focused as bioresource of biodiesel creation because of their high capacity of lipid accumulation and little conflict with land plant cultivation. A primary source of biodiesel is represented by triacylglycerol (TAG), which has three acyl chains bound to the glycerol backbone serving as storage of carbon source and energy.

Chlamydomonas reinhardtii is an established model of eukaryotic microalga. Under nutrient deficiency, *C. reinhardtii* triggers accumulation of TAG at the expense of active cell growth. Among different nutrient starvation tested, including nitrogen (N), phosphorus (P), sulfur (S), iron (Fe) and zinc (Zn) starvation, N starvation best triggers the increase in TAG (Matthew *et al.*, 2009; Kropat *et al.*, 2011; Breuer *et al.*, 2012; Cakmak *et al.*, 2012; Msanne *et al.*, 2012). Based on the current model in *C. reinhardtii* and related species, TAG accumulation occurs at the ER, plastids, and the contact site between plastids and the ER (Fan *et al.*, 2011; Goodson *et al.*, 2011). The final step of TAG biosynthesis is catalyzed by diacylglycerol acyltransferase (DGAT) which transfers an acyl group to *sn*-1,2-diacylglycerol (DAG). *Chlamydomonas* has at least two types of DGAT that take acyl-CoA as acyl donor; type I consists of DGAT1 and type II consists of DGT1, 2, 3, 4 and 5 (Boyle *et al.*, 2012; Msanne *et al.*, 2012; Hung *et al.*, 2013; Sanjaya *et al.*, 2013). Moreover, another type of acyltransferase, phospholipid:diacylglycerol acyltransferase1 (PDAT1), transfers acyl groups from wide range of glycerolipids to TAG (Yoon *et al.*, 2012). Recently, monogalactosyldiacylglycerol (MGDG), a signature lipid class of photosynthetic membranes, was shown as a plastidic source of certain acyl

groups in TAG biosynthesis (Li *et al.*, 2012). A mutation in MGDG lipase, PGD1, that hydrolyzes acyl moiety of MGDG to lyso MGDG, affects TAG accumulation following N starvation. The liberated fatty acid is used for TAG production.

The primary pathway of photo-assimilated carbon source to TAG is complex and not clearly understood yet. However, gene knock out study in *C. reinhardtii* provides numerous information. For example, *starchless6* (*sta6*) mutant, which is defective in small subunit of ADP-Glucopyrophosphorylase, affects starch biosynthesis and hence accumulates large amount of TAG upon N starvation (Mouille *et al.*, 1996). This is considered due to the blockage of carbon flux to storage carbohydrate (Ramazanov and Ramazanov, 2006; Wang *et al.*, 2009; Li *et al.*, 2010b; Li *et al.*, 2010a; Work *et al.*, 2010; Fan *et al.*, 2011; Sanjaya *et al.*, 2011). Indeed, comparative time-course transcriptomics of the *sta6* mutant suggested that glyoxylate pathway and gluconeogenesis are upregulated to promote carbon flux toward hexose-phosphate (Blaby *et al.*, 2013). Thus, metabolomics study using gene knockout models provides ample evidence on the metabolism of primary carbon-containing compounds in *C. reinhardtii*. Because of the limited number of mutants in *C. reinhardtii* affecting TAG biosynthesis, however, we are in need of more gene knock out models with altered TAG profiles to investigate mechanism of TAG accumulation in vivo.

Here, we report a new class of mutant *bga1*, which was isolated while screening transgenic mutants of *C. reinhardtii* which expresses an artificial microRNA designed to target a putative *MONOGALACTOSYLDIACYLGLYCEROL1* (*CrMGD1*; *Cre13.g585301*). The

bga1 did not suppress *CrMGD1* but showed decreased TAG accumulation under N starvation. It showed significantly impaired photosynthetic growth and over-accumulation of starch granules under N-replete condition, showing the phenotype opposite to that of *thesta6*. The *bga1* mutant has an insertional mutation in the open reading frame of *BGAL1*, a previously uncharacterized β -galactosidase-like gene. Cell biological and metabolomic analyses showed that BGAL1 may be involved in the hydrolysis of starch. We suggest that BGAL1 may be involved in the key step of starch degradation, which affects TAG accumulation under nitrogen-starved *C. reinhardtii*.

2. Materials and methods

2.1. Culture condition

C. reinhardtii strain CC-4351 was obtained from Chlamydomonas Resource Center (St. Paul, MN) and used as a wild type. Cells were grown in Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) or high salt (HS) medium (Sueoka, 1960) supplemented with 50 mg L⁻¹ of arginine with air bubbling under 50~60 μ mol continuous white light at 22°C by stirring at 200 rpm. *C. reinhardtii* strain CC-4351 transformed with empty pChlamiRNA2 plasmid (Molnar *et al.*, 2009) was used as empty vector control strain (Hung *et al.*, 2015). Cell number was counted by automatic cell counter (Cellometer[®] AutoT4, Nexcelom Bioscience, Lawrence, MA). For measurement of TAG content under N starvation condition, cells grown in 250 mL TAP medium to the cell density of $\sim 6 \times 10^6$ mL⁻¹ were washed twice with 250 mL TAP (-N) medium by centrifugation. The cell pellets were suspended in TAP (-N) medium by adjusting the cell density to 3×10^6 mL⁻¹ for N-starved growth. For

acetate boosting, cell pellets were suspended in TAP (-N) supplemented with 20 mM potassium acetate as previously described (Goodson *et al.*, 2011). The strains used are listed in Table S1.

2.2. Plasmid constructs and transformation

The 90-mer oligonucleotides for artificial miRNA designed to target a putative *CrMGD1* specifically (YN1253 and YN1254) were annealed and ligated into the *SpeI* site of the pChlamiRNA2 to produce pKK501. The plasmids pChlamiRNA2 and pKK501 were linearized by digesting with *HindIII* and transformed into strain CC-4351 using glass beads as described (Kindle, 1990; Molnar *et al.*, 2009). Control (CHC041) and the *bga1*(CHC042) mutant were screened on solid TAP media with 2% agar. The primers and plasmids used are listed in Table S2 and Table S3, respectively.

2.3. Isolation of the *bga1* mutant

Genomic DNA of CC-4351 and transgenic strains was extracted from exponentially growing cells by lysis buffer (1% SDS, 200 mM NaCl, 40 mM Tris-HCl pH 8.0, 20 mM EDTA) and phenol (Newman *et al.*, 1990). The flanking sequences of transgene insertion position were determined by thermal asymmetric interlaced (TAIL)-PCR as described in (Dent *et al.*, 2005) with 3 specific and 4 arbitrary degenerate primers for primary, secondary, and tertiary reactions. Sequences of the primers are listed in Table S4.

2.4. Genotype analysis and prediction of chloroplast transit peptides Genomic DNA was isolated from cells of the wild type and the *bga1* mutant. The primers CH428 and CH456 were designed to amplify a 616-bp

fragment from the wild type genetic background, and primers CH455 (specific to pChlamiRNA2) and CH456 (specific to *BGAL1*) were used to amplify a 602-bp fragment from the *bga1* mutant. The putative chloroplast transit peptides were predicted by ChloroP 1.1 Server (<http://www.cbs.dtu.dk/services/ChloroP/>) (Emanuelsson *et al.*, 2000).

2.5. RNA extraction and qRT-PCR analysis

Total RNA was extracted by use of TRI reagent (Ambion) including DNase treatment and reverse-transcribed with SuperScript III (Invitrogen, Carlsbad, CA) for cDNA synthesis. Quantitative RT-PCR involved the ABI 7500 Real Time PCR System (Applied Biosystems) with the oligonucleotide primers for *ACT1* (*Cre13.g603700*; CH577 and CH578), *MGD1* (*Cre13.g585301*; CH890 and CH891) and *BGAL1*(*Cre02.g119700*; CH964 and CH965). Gene expression was normalized to that of *ACT1* (*Cre13.g603700*). Data were averaged by 3 technical replicates in the same run and 3 biological replicates in separate runs.

2.6. Quantification of chlorophyll contents

Chlorophyll contents were analyzed as described (Arnon *et al.*, 1954). Briefly, *C.reinhardtii* cells were grown in TAP or TAP (-N) media and harvested by centrifugation. Chlorophyll was extracted by 80% acetone with vigorous vortex and centrifugation, and the supernatant was separated to measure absorption at 645nm and 663 nm.

2.7. Measurement of oxygen evolution

Oxygen evolution was measured with a Clark type oxygen electrode (YSI model 5331 oxygen probe) fitted with a water-jacketed cell as described previously (Hung et al., 2007).

2.8 Quantification of starch contents

Starch contents were analyzed as previously reported (Doebbe et al., 2010) using an assay kit (Cat. No. 10-207-748-035, Roche). Cells were cultured in N-replete condition to a density of $\sim 3 \times 10^6$ cells mL⁻¹ before transferring to N-starved condition to a final cell density of 2×10^6 cells mL⁻¹. Samples were taken at the time of transferring to N-starved condition (0 day), 1 day, and 5 days after the transfer.

2.9 Lipid extraction and analysis

C. reinhardtii cells were grown in TAP medium at 22°C and harvested by centrifugation. Cell pellets were treated with pre-heated isopropanol containing 0.01% butylated hydroxytoluene at 75 °C for 15 min to inactivate phospholipase activity. Total lipids were extracted as described (Folch *et al.*, 1957). Neutral lipids were separated from total lipid extracts by one-dimensional thin-layer chromatography (TLC) (Silica Gel 60 F254 plate, Merck) with the solvent system of hexane:diethylether:glacial acetic acid (40:10:1, v/v/v) and visualized by primuline staining. Lipid analysis was conducted as described (Nakamura *et al.*, 2003). Fatty acid methyl esters derived from TAG were analyzed by gas chromatography (GC-2010; Shimadzu, Kyoto, Japan) equipped with a ULBON HR-SS-10 column (Shinwa Chemical Industries, Kyoto, Japan).

2.10. Transmission electron microscopic (TEM) observation

C. reinhardtii wild type control and *bga1* cells were grown to a density of $\sim 3 \times 10^6 \text{ mL}^{-1}$ in TAP medium at 22°C. N deficiency was induced by collecting the cells by centrifugation (5 min at 3,000xg), washing twice with the TAP medium without N (TAP-N) and subsequently adjusting cell density to $2 \times 10^6 \text{ mL}^{-1}$ in TAP-N media. Samples were taken at 1 day after the transfer to TAP-N media. Samples at day 0 were taken before transferring to N-starved condition. For TEM observation, samples were frozen using high pressure freezer EMPACT2 (Leica, Vienna, Austria) at pressure of 2,000-2,050 bar. Freeze substitution was performed in anhydrous acetone (containing 1% OsO_4 and 0.1% UA) using Leica EM AFS2 (automatic freeze substitution). The samples were kept at -80 °C for 3 days, then at -60°C, -20°C and 0°C for 1 day each, then moved to the room temperature. After twice rinsing for 12 hours with acetone, the LR White resin was used for infiltration and embedding. Ultrathin sections (70-90 nm) were obtained using a Reichert Ultracut S or Leica EM UC7 (Leica, Vienna, Austria) and collected using 100 mesh copper grids. The sections were stained with 5% uranyl acetate in 50% methanol for 10 min and 0.4% lead citrate for 4-6 min. Sections were observed using a TEM (CM 100, Philips) at 80 KV and the images were taken with Gatan Orius CCD camera.

2.11. β -galactosidase activity assay

C. reinhardtii wild type control and the *bga1* cells were grown to a density of $1 \sim 4 \times 10^6 \text{ mL}^{-1}$ in TAP medium at 22°C. Fifteen milliliter of culture was harvested by centrifugation (3,000xg, 5 min at 4°C). Cells were disrupted by

vortexing twice for 30 sec with 0.5mL Zirconia/Silica beads (BioSpec, Cat.11079105z) and 0.8mL of lysis buffer (100 mM sorbitol, 50 mM potassium acetate, 20 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride). The protein concentration of the supernatant of cell lysate was measured by BCA Protein Assay Kit (Pierce, Thermo Scientific) and the β -galactosidase activity was assayed using β -Galactosidase Assay Kit (Agilent Technologies) with O-Nitrophenyl- β -D-Galactopyranoside (ONPG) as the substrate. The β -galactosidase activity was expressed in units/mg of lysate (Nielsen *et al.*, 1983). Data were averaged by 3 biological replicates with SD.

2.12. Sample preparation for metabolomics analysis

C. reinhardtii wild type control and the *bga1* cells were grown to a density of $\sim 3 \times 10^6$ mL⁻¹ in TAP medium at 22 °C with the light intensity of 50-60 μ mol photons m⁻² s⁻¹. N deficiency was induced by collecting the cells by centrifugation (5 min at 3,000xg), washing twice with the TAP medium without N (TAP-N) and subsequently adjusting cell density to 2×10^6 mL⁻¹ in TAP-N media. Cells were grown for 5 days then harvested by centrifugation (3,000xg for 5 min, 4 °C). The supernatant was removed and the wet cell weight was measured. Samples were frozen by liquid nitrogen and kept under -80 °C till use. Based on the wet weight measured, an internal standard D-fructose-[¹³C₆] 1,6-bisphosphate sodium salt hydrate (Cat. No. F792571, Toronto Research Chemicals, Ontario, Canada) was added to the final concentration of 0.96 mM. Ice-cooled extraction solvent (3.75 ml of dH₂O, 7.5 ml of methanol, 3.75 ml of chloroform and 3 mL of 0.5 mm Zirconia/Silica beads) was added to ~ 3 g of

sample and cells were disrupted by vigorous vortex for 30 sec for 3 times. Supernatant was separated by quick spin-down at 3,000xg, incubated at -30 °C for 30 min, then centrifugated at 3,000xg for 10 min at 4 °C. Obtained supernatant was mixed by vortexing with 3.75 ml of chloroform to remove pigments. Colorless supernatant was lyophilized and kept under -80 °C for mass spectrometry assay. The lyophilized samples were dissolved in 10mM ammonium formate, pH 3.0, and centrifuged at 15,000xg at 4 °C for 20 min. The supernatant was transferred to a sample vial for metabolites analysis.

2.13. Metabolite analysis

Amount of fructose 1,6-biphosphate (F16BP), dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3-PGA), phosphoenolpyruvate (PEP), glyceraldehyde 3-phosphate (GA3P), acetyl-CoA, citrate, aconitic acid, isocitrate, succinate, malate and ATP were analyzed by Ion-Pair Chromatography (LCQ Deca XP Plus, ThermoFinnigan, San Jose, CA). LCQ mass spectrometry was performed with Agilent 1200 series binary HPLC system (Agilent Technologies, Palo Alto, CA) coupled to a LCQ Deca XP+ ion trap (Thermo Scientific) equipped with an electrospray ionization interface. The mass spectrometry was set to one full MS scan (m/z 50 to 850). Chromatographic separation was performed by passing aliquots through Agilent ZORBAX Eclipse XDB-C8 2.1×12.5 mm pre-column (Agilent Technologies), before separation with a column (ZORBAX Eclipse XDB-C18, 2.1×150 mm, 5 μ m; Agilent) at room temperature using a multistep gradient with online-degassed eluent A (10 mM tributylamine aqueous solution, adjusted

to pH 5 with 15 mM acetic acid and 2% methanol): 0 to 5 min, 99.9% to 95% A; 5 to 25 min, 95% to 73% A; 25 to 30 min, 73% to 68% A; 30 to 35 min, 68% to 1% A; 35 to 36 min, 1% to 1% A; 36 to 37 min, 1% to 99.9% A. The flow rate was $0.5 \text{ mL}^{-1} \text{ min}^{-1}$. The volume of injected sample was 10 μL . After separation, compounds were ionized by electrospray ionization and detected by the LCQ that was operated in negative ion mode with the spray voltage of 4.5 kV, sheath gas at 30 (arb) and capillary temperature at 300°C. Prior to injection, a mixture of 25 ppm (final concentration) of reference compounds and 166.6 ppm of D-fructose- $^{13}\text{C}_6$ 1,6-Bisphosphate was added to the sample to correct matrix effects on these analytes in the analysis. The mass spectrometry data were analyzed using software Xcalibur 2.2 (Thermo Scientific).

Amount of glucose 6-phosphate (G6P), glucose 1-phosphate (G1P) and fructose 6-phosphate (F6P) were analyzed with ACQUITY UPLC system (Waters, Manchester, UK) equipped with ACQUITY UPLC BEH amide column (130Å, 1.7 μm , 2.1 mm X 100 mm). The column temperature was set at 25 °C. The UPLC mobile phase consisted of 40% acetonitrile/10mM ammonium acetate, pH9.0 (solution A) and 95% of acetonitrile/10mM ammonium acetate, pH9.0 (solution B). The linear gradient increased: 0 to 1 min, 35% to 35% A; 1 to 5 min, 35% to 55% A; 5 to 5.01 min, 55% to 35% A; 5.01 to 9 min, 35% to 35% A. The injection volume was 1.0 μL . The mass spectrometric data were collected by a SYNAPT™ High Definition Mass Spectrometry™ G1 (Synapt HDMS™ G1) System (Waters, Manchester, UK) with an electrospray ionization (ESI) interface, ion-mobility and time-of-flight system. An electrospray source was used; sample cone voltage of 40 V capillary was set at 2.5 kV, the source

and desolvation temperatures were 80°C and 250 °C, respectively, and the desolvation and cone gas flow rates were 800 L h⁻¹ and 50 L h⁻¹, respectively. Spectra were collected in the negative ionization (ES⁻). A lock mass calibration of sulfadimethoxin at a concentration of 0.05 mg/L in water/MeOH (50:50, v/v) was introduced by HPLC pump (LC-10ATVP, Shimadzu, Japan) and split the 20 µl/min to lock spray interface. The MS range acquired was 50–1000 m/z with 0.2 sec/scan in centroid mode. Prior to injection, a mixture of 100 ppm (final concentration) of reference compounds and 100 ppm of D-fructose-¹³C₆ 1,6-Bisphosphate were added to the sample to correct matrix effects on these analytes in the analysis. The mass data acquired were imported to Markerlynx (Waters, Manchester, UK) within Masslynx and Targetlynx software (version 4.1) for peak detection and alignment. The retention time and m/z data for each peak were manually determined.

3. Results

3.1. Isolation of a mutant that exhibits defective photosynthetic growth in *C.reinhardtii*

While screening transgenic mutants of *C.reinhardtii* which expresses an artificial microRNA designed to target a putative *MONOGALACTOSYLDIACYLGLYCEROL1* (*CrMGD1*; *Cre13.g585301*), we found a transformant showing pale green color and defective growth (Figure 1A). The pale green phenotype persisted after several generations. However, this mutant (*bga1*, as designated in Figure 2) did not suppress levels of *MGD1* transcript compared with the wild type strain (CC-4351) (Figure 1B). This suggests that the phenotype is caused not by suppression of the

putative *MGD1* but an insertion of a transgene into the genome of *C. reinhardtii*, where a functional gene that accounts for this phenotype is encoded. Chlorophyll contents of the *bga1* were about a half as compared to that of wild type under the photoheterotrophic condition (Figure 1C). Under photoheterotrophic condition (TAP media), the growth of the *bga1* mutant was considerably slower as compared to wild type, which resulted in cell number about 4 times lower than that of wild type in 3 days (Figure 1D). The phenotype was more pronounced under photoautotrophic condition (HS media), where cell growth of the *bga1* mutant was almost stopped (Figure 1E). We measured oxygen evolution of the *bga1* mutant as a parameter of photosynthetic activity, which showed significant reduction compared to that of the wild type (Figure 1F). Thus, the *bga1* mutant carried a mutation affecting photosynthetic growth that was not complemented by exogenously supplied carbon source in the culture media.

3.2. A β -galactosidase-like gene was disrupted in the *bga1* mutant

To identify the position of transgene insertion in the genome, we performed TAIL PCR and found the insertion within the open reading frame of a previously uncharacterized gene *Cre02.g119700*, which contains a putative β -galactosidase-like motif (Figure 2A). We designated this gene *β -galactosidase-like1* (*BGAL1*). PCR-based genotyping confirmed that the *bga1* was homozygous (Figure S1A). Moreover, *BGAL1* transcripts were undetectable in the *bga1* mutant by qPCR (Figure 2B), indicating that *bga1* is a null mutant of *BGAL1*. Global BLAST search revealed that *BGAL1* is highly unique in microalgae, as proteins sharing overall high homology can be found in Volvox,

Chlorella and Trichodesmium (see Figure S2 for phylogenetic relationship among them). Motif search by Pfam database revealed that a region ranging from amino acid residues 115 to 312 shares homology with glycosyl hydrolase 42 (GH42) superfamily represented by *bgaB*, a β -galactosidase of *Bacillus subtilis* (Figure S1B). The ChloroP 1.1 program predicted that N-terminal amino acid residues 1-75 of BGAL1 contains a possible chloroplast transit peptide (Figure S1B). To investigate whether BGAL1 is responsible for the major β -galactosidase activity, we performed enzyme activity assay using total crude protein extract from the *bga1* mutant. As compared to the wild type, β -galactosidase activity was reduced by 40% in the *bga1* mutant (Figure 2C). Taken together, these results indicate that the *bga1* is a knockout mutant of *BGAL1* (*Cre02.g119700*), which is responsible for the primary β -galactosidase activity in *C. reinhardtii*.

3.3. Observation of cell ultrastructure in the *bga1* mutant

Reduced β -galactosidase activity in the *bga1* mutant (Figure 2C) suggests an accumulation of carbohydrate, which may have relevance to the growth defect (Figure 1). To investigate the defect in the *bga1* at cellular level, we observed the ultrastructure of the *bga1* mutant cells by transmission electron microscope. A remarkable difference between the *bga1* and wild type was the accumulation of starch granules within the chloroplasts: As compared with wild type (Figure 2D), massive accumulation of starch was observed in the *bga1* mutant (Figure 2E). A closer look at the organelle structure showed that thylakoid membranes as well as other sub-organelle compartment of chloroplasts be indistinguishable between wild type (Figure 2F) and

the *bga1* (Figure 2G). This observation suggests that defective photosynthetic growth observed in the *bga1* is likely caused by over-accumulation of starch.

3.4. N starvation compromised growth defect of the *bga1* mutant

Over-accumulation of starch granules suggests that carbon flux is affected in the *bga1*. Because TAG serves as an alternative fate of photo-assimilated carbon source (Msanne *et al.*, 2012; Blaby *et al.*, 2013; Wang *et al.*, 2015), we next observed the phenotype of the *bga1* under N starvation.

We performed time-course observation of cell growth in response to N starvation. Upon entry into N deprivation, wild type cells nearly stopped their growth. Both the *bga1* and wild type showed degradation of chlorophyll as evidenced by the changes in the green pigment color of culture (Figure 3A). We found that cell number was not significantly different between the *bga1* and wild type until 7 days after switching to N starvation (Figure 3B), indicating that the difference in growth phenotype between *bga1* mutant and wild type was less obvious under N starvation than under N-replete condition. Interestingly, the degradation of chlorophyll contents occurred more rapidly in wild type than the *bga1*, so the difference in chlorophyll contents between wild type and the *bga1* became indistinguishable by 3 days after N starvation (Figure 3C). Thus, the difference in growth phenotype of *bga1* mutant and wild type was less obvious under N starvation.

3.5. Observation of cell ultrastructure in N-starved *bga1* mutant

To investigate accumulation of starch granules and lipid droplets in N-starved *bga1* mutant, we performed time-course observation of cell ultrastructure following N starvation by transmission electron

microscopy. Before switching to N-starved condition, compared to the wild type cell (Figure 4A), much more starch granules were observed in the *bga1* mutant (Figure 4B). At 1 day after switching to N-starved condition, the starch granules and lipid droplets were both accumulated in wild type (Figure 4C). However, in the *bga1* mutant, the starch granules were more significantly accumulated than lipid droplets (Figure 4D). Indeed, starch contents were significantly increased in the *bga1* mutant compared to wild type (Figure 4E). These observations suggest that the *bga1* mutant has a metabolic defect in conversion of starch into the storage lipid, thus mainly accumulates starch granules rather than lipid droplets under N starvation.

3.6. Triacylglycerol accumulation was defective in N-starved *bga1*

To investigate whether TAG level is conversely decreased in the *bga1* because of the over-accumulation of starch, we performed time-course TAG analysis following N starvation. Wild type cells accumulated TAG massively by 7 days after N starvation; however, the *bga1* showed much lower level of TAG accumulation as indicated by the intensity of spots corresponding to TAG on the thin-layer chromatography (TLC) plate (Figure 5A). We quantified TAG levels at different time points after N starvation (Figure 5B). By 1 day after N starvation, TAG levels in the *bga1* were significantly lower by 63% of wild type. By 7 days, TAG levels in the *bga1* were about one-third of that in wild type. We next analyzed the fatty acid composition of TAG accumulated at 7 days after N starvation. As shown in Figure 5C, fatty acid composition of TAG in the *bga1* was significantly different from that in wild type. The major changes involved decrease in C18:1 Δ 9 but increase in polyunsaturated fatty acids such

as C16:4 and C18:3 ω 6. Moreover, saturated fatty acid species (C16:0 and C18:0) and monounsaturated fatty acid (C18:1 Δ 11) showed clear increase, and C16:1 and C16:2 exhibited considerable decrease in the *bga1*. The increase in unsaturation of fatty acid in the *bga1* is likely due to the reduced flux of de novo TAG biosynthesis, because primary neosynthesis of TAG incorporates saturated and C18:1 Δ 9 fatty acid species (Fan *et al.*, 2011). To examine the metabolic capacity of de novo TAG biosynthesis in the *bga1*, we analyzed time-course TAG profiles following acetate supplementation. Because acetate is an initial precursor of fatty acid synthesis, this “acetate boost” triggers rapid induction of TAG biosynthesis, which results in cells filled with lipid droplets composed of TAG (Goodson *et al.*, 2011). Following acetate boost, TAG levels were not increased significantly in the *bga1* as compared to those in the wild type (Figures 5B and 5D), suggesting that the *bga1* mutant has slightly reduced capacity of de novo TAG biosynthesis.

3.7. Metabolomic analysis of N-replete and N-starved *bga1*

To investigate the global metabolic defect in the primary carbon metabolism in the *bga1* mutant, we performed metabolomic analysis of some of major metabolites in the glycolysis pathway and tricarboxylic acid cycle (TCA cycle), and compared the profile between wild type and the *bga1* mutant under N-replete (Figure 6) or N-starved (Figure 7) conditions. Under N-replete growth condition, the *bga1* mutant showed significantly reduced levels of metabolites following the hydrolysis of starch, such as fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (F16BP) and glyceraldehyde 3-phosphate (GA3P). In addition, dihydroxyacetone phosphate (DHAP), which is a substrate for

glycerolipid biosynthesis, was not detectable. Moreover, acetyl-CoA level was significantly decreased, suggesting a reduced synthesis of fatty acid. On the other hand, metabolites related to the TCA cycle were not affected except reduced levels of citrate and isocitrate in the *bga1* mutant compared with the wild type. These profiles support accumulation of starch and suggest reduced capacity of TAG biosynthesis.

Upon N starvation, these profiles altered dramatically. In the *bga1* mutant, some of the metabolites involved in the reaction from glucose 6-phosphate (G6P) to TAG biosynthesis were increased, whereas those in the TCA cycle were decreased (Figure 7). In the glycolysis pathway, levels of F16BP, GA3P and DHAP were increased to be as high as those of WT. Remarkably, GA3P and DHAP were not detectable under N-replete condition (Figure 6) but their levels were similar between wild type and the *bga1* mutant upon N starvation (Figure 7). On the other hand, levels of G6P and F6P were lower in the *bga1* mutant, which are consistent with the changes in N-replete growth condition. In addition, levels of phosphoenolpyruvate (PEP) were decreased but pyruvate were increased in the *bga1* mutant upon N starvation. In the TCA cycle, levels of all metabolites analyzed showed significant decrease in the *bga1* mutant upon N starvation. This suggests decreased energy production, which may be relevant to decreased ATP level in the *bga1* mutant upon N starvation. Of note, acetyl-CoA level was significantly lower even under N starvation, suggesting consistent defect in the supply of *de novo* fatty acid biosynthesis both in N-replete and N-starved conditions. Thus, the *bga1* mutant showed characteristic metabolite profiles; while consistent decrease

was observed for the levels of G6P, F6P and acetyl-CoA regardless of the growth condition, most of the other metabolites in glycolysis were increased but those in the TCA cycle were decreased upon N starvation.

4. Discussion

The *bga1* mutant was characterized for its enhanced accumulation of starch granules under N-replete growth condition (Figure 2). Starch is the major pool of photo-assimilated carbon source whose synthesis and degradation occur simultaneously under light condition in *C. reinhardtii* (Klein, 1987). The *bga1* mutant has an insertional mutation in the open reading frame of a β -galactosidase-like gene (Figure 2A). Upon N starvation, at which conversion of starch into TAG is stimulated in green algae such as *Pseudochlorococcum* sp. and *C. reinhardtii* (Li *et al.*, 2011; Gardner *et al.*, 2013), the *bga1* mutant displayed further accumulation of starch granules at the expense of TAG accumulation. While results shown here support the conclusion that BGAL1 is involved in the hydrolysis of starch and TAG accumulation during N starvation, we still await genetic complementation of the *bga1* mutant to confirm these effects genetically. Since *C. reinhardtii* is haploid and notorious for having multiple insertions and other genetic rearrangement, there is still the possibility that the effect observed in this study might be due to second site mutations.

The growth phenotype of the *bga1* was more pronounced under photoautotrophic condition (Figure 1E) than photoheterotrophic condition (Figure 1D) under N-replete condition. In the former condition, supply of carbon

source relies on photosynthesis. Since starch as a principal carbon sink takes part in the photoprotection against oxidative damage (Krishnan *et al.*, 2015), reduced photosynthetic growth in the *bga1* may be related to starch accumulation. Indeed, the phenotypic difference between the *bga1* and wild type was much attenuated under N-starved condition (Figure 3), at which overall photosynthetic capacity is greatly reduced (Juergens *et al.*, 2015). The deduced amino acid sequence of BGAL1 possesses a predicted N-terminal chloroplast transit peptide (Figure S1B). Compared with wild type, microscopic observation showed that the *bga1* accumulated more starch granules in the chloroplasts under photoheterotrophic condition (Figure 2D and 2E). It is possible that increased starch accumulation occurs in the chloroplasts in the *bga1* mutant, affecting chloroplast function in the maintenance of photosynthesis.

The *BGAL1* encodes β -galactosidase-like enzyme, which contains glycosyl hydrolase superfamily 42 motif. In the *bga1* mutant, β -galactosidase activity in the total cell lysate was 40% of that of wild type (Figure 2C), which suggests that BGAL1 is responsible for the major activity to hydrolyze the non-reducing terminal β -galactoside bonds. Because starch is mainly composed of α -linked glucose, an involvement of β -galactoside in starch degradation was somewhat unexpected from the enzymatic point of view. In *Arabidopsis thaliana*, a homologue of β -galactosidase MUM2/BGAL6 (*At5g63800*) is required for swelling and extrusion of the mucilage on seed hydration by removal of galactose/galactan branches (Dean *et al.*, 2007; Macquet *et al.*, 2007). The AtBGAL10 (*At5g63810*) was suggested to act on nonreducing ends

of xyloglucan for appropriate silique elongation (Sampedro *et al.*, 2012). Recently, a novel agarolytic β -galactosidase was reported in *Vibrio* sp. EJY3 that can act at the nonreducing end of agarooligosaccharides to release galactose (Lee *et al.*, 2014). These evidences suggest that various β -galactosidase homologues show different substrate specificity. It is possible that β -galactosidase is required at some step in hydrolyzing starch in *C. reinhardtii*. In future effort, it may be helpful to conduct in vitro assay using recombinant BGAL1 protein and starch purified from the *bga1* mutant. Analyzing the resulting polysaccharide chain patterns by glycomics technology will elucidate the role of BGAL1, and further the whole metabolic steps, in starch hydrolysis.

The *bga1* mutant showed reduced TAG accumulation under N starvation (Figure 5). The fatty acid composition of TAG in the *bga1* showed significant changes from that in wild type (Figure 5C) but is similar to the profile of *pgd1* mutant (Li *et al.*, 2012). Because PGD1 is the chloroplast-localized lipase involved in the de novo synthesis of TAG, the similar changes in the fatty acid composition between *bga1* and *pgd1* suggests that the *bga1* is defective in de novo TAG biosynthesis as well. TAG is assembled by acyl groups and a glycerol backbone. While acyl groups are provided by the fatty acid synthesis originating from acetyl-CoA, the glycerol backbone is derived from DHAP. In the metabolomic profiling, levels of both acetyl-CoA and DHAP were decreased in the *bga1* mutant under N-replete growth condition (Figure 6). Upon N starvation, DHAP level was recovered in the *bga1* mutant whereas acetyl-CoA level remained lower (Figure 7). However, acetate boost experiment, which stimulates FA synthesis, did not rescue the TAG levels (Figure 5D). This

suggests that reduced TAG levels in N-starved *bga1* mutant is not mainly due to the reduced metabolic flux of fatty acid biosynthesis but presumably for the reduced supply of glycerol backbone.

Our metabolomics profiling of the *bga1* mutant provided further insight into the metabolic alteration underlying the *bga1* mutant. The *sta6* mutant is a widely used mutant of *C. reinhardtii* which increases TAG level because an enzyme for starch biosynthesis is mutated (Zabawinski *et al.*, 2001; Work *et al.*, 2010). A recent metabolomic study of the *sta6* showed increased levels of metabolites related to glycolysis and TCA cycle (Blaby *et al.*, 2013; Krishnan *et al.*, 2015). Our metabolite analysis showed that the *bga1* mutant reduced levels of metabolites in glycolysis and TCA cycle, especially under N starvation (Figure 7). This contrasting difference in metabolite profiles between *bga1* and *sta6* may support the proposal that BGAL1 is involved in the hydrolysis of starch.

5. Conclusion

We isolated and characterized a new class of β -galactosidase BGAL1 in *C. reinhardtii* through isolation of its mutant, which may be involved in the hydrolysis of starch and affect TAG accumulation upon N starvation. This study provides a new mutant model contrasting to *sta6* in investigating N starvation-induced TAG accumulation, as well as metabolic insight into TAG biosynthesis in *C. reinhardtii*.

Author contributions

C.-H.H. performed overall experiments. K. K. designed research and performed molecular construction. Y. N. conceived and designed research, and wrote the

manuscript. All authors commented on the manuscript and approved the contents.

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Figure Legend

Figure 1. Phenotype of the *bga1* mutant under photoheterotrophic and photoautotrophic growth conditions. (A) Plate culture of wild type and the *bga1* mutant in photoheterotrophic growth condition in TAP media. (B) Relative transcript level of *MGD1* in wild type and the *bga1* mutant. Gene expression was normalized to *ACT1*. Data were averaged by 3 technical replicates in the same run and 3 biological replicates in separate runs. (C) Time-course measurement of chlorophyll contents in wild type and the *bga1* mutant under photoheterotrophic growth condition in TAP media. (D-E) Growth of wild type and the *bga1* mutant in photoheterotrophic condition in TAP media (D) or photoautotrophic condition in HS media (E). (F) Measurement of net electron transfer of the *bga1* mutant in TAP media. Data are averaged by 3 biological replicates with SD. Asterisks indicate significance by Student's *t*-test (*P* value: ** < 0.01). WT, wild type; *MGD1*, *monogalactosyldiacylglycerolsynthase 1*.

Figure 2. Isolation and transmission electron microscopy observation of cells of wild type and the *bga1* mutant. (A) Position of transgene insertion in the *bga1* mutant. Positions of primers used for PCR-based genotyping are indicated by arrows. (B) Relative transcript level of *BGAL1* in wild type and the *bga1* mutant. Gene expression levels were normalized to that of *ACT1*. Data were averaged by 3 technical replicates in the same run and 3 biological replicates in separate runs. (C) Total β -galactosidase activity in the cell lysate of wild type and the *bga1* mutant. Data are mean $\pm$ SD from 3 biological replicates. An **asterisk indicates significance by Student's *t* test (*P* < 0.01). (D, E) Whole cell images of wild type (D) and the *bga1* mutant (E). Bars = 1 μ m. (F, G) Magnified view of (F) and (G) indicating stacking of the thylakoid membranes. Bars = 0.4 μ m. Cells were photoheterotrophically cultured in TAP media to a density of $3\sim5 \times 10^6$ cells mL⁻¹ for observation. WT, wild type; *BGAL1*, β -galactosidase 1.

Figure 3. Phenotype of the *bga1* mutant under N starvation. (A) Time-course observation of pigment color in the culture of the *bga1* mutant upon N starvation. (B) Growth of the *bga1* mutant upon N starvation measured by cell number. (C) Decrease in chlorophyll contents of wild type and the *bga1* mutant cells under N starvation. Data are averaged by 3 biological replicates with SD. WT, wild type.

Figure 4. Transmission electron microscopy observation of N-starved cells of wild type (A and C) and the *bga1* mutant (B and D) and quantification of starch contents (E). Cells were cultured in N-replete condition to a density of $\sim 3 \times 10^6$ cells mL⁻¹ before transferring to N-starved cultures to a final cell density of 2×10^6 cells mL⁻¹. Samples were taken at the time of transferring to N-starved condition (A, B), 1 day (C, D) and 5 days after the transfer for microscopy observation. Bars = 1 μ m.

Figure 5. TAG analysis in N-starved *bga1* mutant. (A) A thin layer chromatography image of time-course TAG accumulation in the *bga1* mutant. Amount of lipid loaded on each lane was normalized by the cell number. (B) Time-course profile of TAG levels in the wild type and *bga1* mutant following N starvation. (C) Fatty acid composition of TAG in the wild type and *bga1* mutant at 7 days following N starvation. (D) Time-course profile of TAG levels following N starvation with acetate boost. Data are averaged by 3 biological replicates with SD. Asterisks indicate significance by Student's *t*-test (*P* value; * < 0.05, ** < 0.01). WT, wild type; TAG, triacylglycerol; FA, fatty acid.

Figure 6. Metabolomic profiling of compounds in glycolysis pathway and TCA cycle in wild type and the *bga1* under N-replete condition. Cells were cultured in N-replete condition to a density of $\sim 3 \times 10^6$ cells mL⁻¹. Metabolite levels were shown by fold change in the *bga1* relative to that of wild type in N-replete condition. Data are mean $\pm$ SD from 3 biological replicates. Asterisks indicate significance by Student's *t*-test (*P* value: * < 0.01; ** < 0.0001). G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; F6P, fructose 6-phosphate; F16BP, fructose 1,6-bisphosphate; GA3P, glyceraldehyde 3-phosphate; 3-PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; G3P, glycerol 3-phosphate; LysoPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, *sn*-1,2-diacylglycerol; TAG, triacylglycerol.

Figure 7. Metabolomic profiling of compounds in glycolysis pathway and TCA cycle in wild type and the *bga1* under N-starved condition. Cells were cultured in N-replete condition to a density of $\sim 3 \times 10^6$ cells mL⁻¹ before transferring to N-starved condition to a final cell density of 2×10^6 cells mL⁻¹. Samples were taken at 5 days after the transfer for metabolite analysis. Metabolite profiles were shown by fold change in the *bga1* relative to that of wild type in N-starved condition. Data are mean $\pm$ SD from 3 biological replicates. Asterisks indicate significance by Student's *t*-test (*P* value: * < 0.01; ** < 0.0001). See Figure 6 for abbreviations.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7