Molecules and Cells

CrABCA2 Facilitates Triacylglycerol Accumulation in *Chlamydomonas reinhardtii* under Nitrogen Starvation

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The microalga *Chlamydomonas reinhardtii* accumulates triacylglycerols (TAGs) in lipid droplets under stress conditions, such as nitrogen starvation. TAG biosynthesis occurs mainly at the endoplasmic reticulum (ER) and requires fatty acid (FA) substrates supplied from chloroplasts. How FAs are transferred from chloroplast to ER in microalgae was unknown. We previously reported that an *Arabidopsis thaliana* ATP-binding cassette (ABC) transporter, AtABCA9, facilitates FA transport at the ER during seed development. Here we identified a gene homologous to *AtABCA9* in the *C. reinhardtii* genome, which we named *CrABCA2*. Under nitrogen deprivation conditions, *CrABCA2* expression was upregulated, and the CrABCA2 protein level also increased. *CrABCA2* knockdown lines accumulated less TAGs and *CrABCA2* overexpression lines accumulated more TAGs than their untransformed parental lines. Transmission electron microscopy showed that CrABCA2 was localized in swollen ER. These results suggest that CrABCA2 transports substrates for TAG biosynthesis to the ER during nitrogen starvation. Our study provides a potential tool for increasing lipid production in microalgae.

Keywords: ABC transporter, *Chlamydomonas reinhardtii*, endoplasmic reticulum, triacylglycerol accumulation

INTRODUCTION

Algal biodiesel provides an alternative energy source that does not increase the atmospheric carbon dioxide level as much as fossil fuels (Beer et al., 2009; Hu et al., 2008; Radakovits et al., 2010; Scott et al., 2010; Stephens et al., 2010; Wijffels and Barbosa, 2010). Microalgae produce lipids and accumulate them under stress conditions, such as nitrogen starvation. Many studies have investigated ways to increase lipid productivity in microalgae, by improving culture systems or developing more efficient ways to extract their lipids (Georgianna and Mayfield, 2012; Higgins and Vander-Gheynst, 2014; Hu et al., 2008; Radakovits et al., 2010; Torri et al., 2011). However, the economics of microalgal oil production are far from supporting its actual use in industry. To overcome this economic hurdle, it is necessary to understand lipid biosynthesis in microalgae, identify the genes involved in the process, and use these to create improved algal strains.

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The green microalga *Chlamydomonas reinhardtii* is a model organism for microalgal studies, in regard to topics such as flagella structure and function and photosynthesis (Harris, 2001). Its genome sequence has been reported (Merchant et al., 2007), and a genomics database for the species is continuously updated in Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Rigorous genetic analysis of protein functions is possible because *C. reinhardtii* has a sexual life cycle (Harris, 1989), as well as undergoing asexual division. Furthermore, *C. reinhardtii* accumulates a large amount of neutral lipids (20-45% of dry weight) under nitrogen starvation (-N) conditions (Goodson et al., 2011; Wang et al., 2009). The species has thus been used to study lipid biosynthesis and accumulation, and some regulatory proteins and enzymes involved in the process have been reported (Boyle et al., 2012; Nguyen et al., 2011). For example, overexpression of a Doftype transcription factor is known to increase lipid production (Ibáñez-Salazar et al., 2014; Salas-Montantes et al., 2018). The NRR1 transcription factor regulates many genes under nitrogen starvation conditions (Boyle et al., 2012). CHT7, a DNA-binding protein, acts as a repressor of cellular quiescence (Tsai et al., 2014), and thus might be a useful molecular tool for increasing biomass productivity. Acyltransferases and major lipid droplet protein (MLDP) are involved in lipid metabolism (Boyle et al., 2012; Chen and Smith, 2012; Li et al., 2010; Tsai et al., 2015). Lysophosphatidic acid acyltransferases (LPAATs) are involved in triacylglycerol (TAG) production in the chloroplast and endoplasmic reticulum (ER) (Kim et al., 2018; Yamaoka et al., 2016). However, many aspects of microalgal lipid biosynthesis and storage remain unknown.

ATP-binding cassette (ABC) transporters participate in the transport of small molecules between organelles (Dean et al., 2001; Hwang et al., 2016; Pohl et al., 2005; Roth et al., 2003). In animals, many proteins in the ABCA subfamily transport lipids within cells, and mutations of the corresponding genes cause severe diseases (Piehler et al., 2002; Tarling et al., 2013). In plants, an *Arabidopsis thaliana* ABCA9 (AtABCA9) has an important role in TAG biosynthesis in the seed. AtABCA9 facilitates the transport of lipid precursors, acyl-coenzyme A molecules, and fatty acids (FAs) to the ER, thereby increasing neutral lipid biosynthesis in seeds (Kim et al., 2013). *C. reinhardtii* has 69 ABC transporter coding sequences in its genome (Hwang et al., 2016). We hypothesized that ABCA subfamily transporter proteins have an important role in lipid biosynthesis in *C. reinhardtii*, as in *A. thaliana*. In this study, we examined the function of one ABCA transporter, encoded by Cre14.g613950, which we named CrABCA2. Our results suggest that CrABCA2 is a transporter of lipidic molecules in *C. reinhardtii* and is involved in lipid biosynthesis and accumulation during nitrogen starvation.

MATERIALS AND METHODS

Culture conditions

C. reinhardtii strain C9 (CC-408 wild type, mt-) and the *crabca2-1* mutant were from the Fukuzawa Laboratory at Kyoto University (Yamano et al., 2015). *C. reinhardtii* strain CC-4533 (cw15, mt-) (http://www.chlamycollection.org) and the *crabca2-2* (LMJ.RY0402.160375) and *crabca2-3* (LMJ.RY0402.178253) mutants were obtained from the Chlamydomonas Genetic Center (USA) (https://www.chlamycollection.org/products/clip-strains/) (Li et al., 2016). *C. reinhardtii* strain UVM4 was provided by Dr. R. Bock (MPI-MP, Germany). For isolation of genomic DNA and total RNA, strains were grown to the mid-exponential-growth phase in Tris acetate phosphate (TAP), pH 7.0 medium at 23°C under continuous illumination at 40 μmol photons $m^{-2} s^{-1}$. The cultures were shaken continuously on an orbital shaker at 180 rpm. To induce TAG biosynthesis, cells were collected by centrifugation (500*g*, 5 min, 25°C), washed with TAP medium lacking any nitrogen source (TAP -N), and resuspended in TAP -N.

Phylogenetic analysis

Amino acid sequences were obtained from Phytozome v12.1 (https://phytozome.jgi.doe.gov) and TAIR (https://www.arabidopsis.org), and were aligned using the Mafft algorithm (Katoh and Standley, 2013). The phylogenetic tree was constructed using MEGA 7.0 (Kumar et al., 2016) with 1,000 bootstrap replicates, using the maximum-likelihood method based on the JTT matrix-based model (Jones et al., 1992). The initial tree for the heuristic search was generated automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then the topology with superior log likelihood value was selected.

Vector construction and nuclear transformation

The cDNA sequence of *CrABCA2* (Cre14.g613950) was amplified using the gene-specific primers EcoRI-CA2F and KpnI-CA2R. The polymerase chain reaction (PCR) was carried out using high-fidelity KOD Hot Start DNA Polymerase (Toyobo, Japan). The amplified DNA fragment was cloned as an EcoRI-KpnI fragment into the vector pChlamy4 (Kong et al., 2018), which contains the *ble* gene conferring zeocin resistance (Stevens et al., 1996), to generate the plasmid pChlamy4-cABCA2. Nuclear transformation was performed by electroporation, following a previously described method (Kong et al., 2017). Transgenic strains were selected directly on TAP/agar plates containing zeocin (15 mg/L), and the plates were incubated under continuous fluorescent light (20 μmol $m^{-2} s^{-1}$) at 25°C.

Nucleic acid extraction and expression level analysis

C. reinhardtii genomic DNA was isolated using the phenol-chloroform extraction method (Jang et al., 2015). Total RNA was extracted using homemade Trizol reagent. To obtain cDNA to use as the template for quantitative reverse transcription PCR (qRT-PCR), 4 μg RNA was subjected to reverse transcription with SuperiorScriptIII reverse transcriptase (Enzynomics, Korea). For RT-PCR, the PCR product of the housekeeping gene *RPL17* was employed as a loading control using previously reported primers (Lee et al., 2008). To estimate gene expression levels, qRT-PCR was conducted using TB Green Premix Ex Taq (Takara; http://www.takara-bio.com). The qRT-PCR results for *ABCA2* were normalized based on the level of *RPL17* expression.

Lipid analysis

We followed a previously reported method for TAG analysis (Yamaoka et al., 2019) with a few modifications. Briefly, mid-log-phase cells were transferred to TAP -N medium, grown for 2 days, collected by centrifugation (500*g*, 25°C, 5 min), and subjected to total lipid extraction. The lipid extracts were dissolved in a small amount of chloroform, applied to a thin-layer chromatography (TLC) plate (TLC silica gel 60, #105553; Merck-Millipore, Germany), and separated by using a solvent mixture for neutral lipid separation (80:30:1 [v/v/v] hexane/ether/acetic acid). Lipid spots on the plate were visualized by spraying with 0.01% (w/v) primuline reagent dissolved in 80% acetone. The TAG spot was scraped off the TLC plate and transesterified to FA methyl esters at 95°C for 15 min using 2.5% (v/v) sulfuric acid in methanol. The resultant FA methyl esters were analyzed and quantified using a gas chromatograph (GC-FID, GC-2010; Shimadzu, Japan) equipped with an HP-INNOWax capillary column (30 m × 0.25 mm, 0.25 μm film thickness; Agilent Technologies, USA).

Antibody generation and immunoblotting

CrABCA2 antibody was generated by YounginFrontier (Korea), and purified in our laboratory. A synthetic peptide containing 18 amino acids from the C-terminus of CrABCA2 (GTPAMYPGYNPSPVDSRN) were injected into two rabbits. After the third boost, rabbits were sacrificed to obtain the serum. The CrABCA2 polyclonal antibody was purified following an affinity purification protocol (Brown et al., 2015). To extract total proteins, *C. reinhardtii* cells were harvested using centrifugation (500*g*, 25°C, 5 min) and resuspended in tricine-KOH buffer (50 mM, pH 8.0) containing 150 mM NaCl and 1× protease inhibitor cocktail tablet (Roche, Switzerland). Cells were broken by sonication and unbroken cells were removed by centrifugation (9,300*g*, 4°C, 10 min), as previously reported (Nguyen et al., 2013). Protein amounts were quantified by Bradford assay (Bradford, 1976). Thirty micrograms of total protein was loaded onto an 8% acrylamide/bisacrylamide SDS gel and separated by electrophoresis at 80 V for 2 h. Separated protein bands were blotted on to nitrocellulose membranes. The membranes were blocked in 1× TBST (0.1% [v/v] Tween 20 in 1× TBS) with 7.5% skim milk for 1 h at room temperature, washed three times in 1 × TBST for 5 min each, and then incubated overnight at 4°C with anti-CrABCA2 polyclonal antibody (1:3,000) on a rotary incubator. The membranes were then washed three times with 1× TBST and subjected to a secondary incubation with anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Invitrogen, USA). After three washes with 1× TBST for 5 min each, chemiluminescence was detected by applying the SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, USA) and detecting the signal with a LAS-4000 imaging system (Fuji, Japan).

Membrane fractionation

Membrane fractionation and sucrose gradient fractionation were performed following a method previously described (Aksoy et al., 2013) with a few modifications. Briefly, *C. reinhardtii* cells were cultured until the logarithmic growth phase (optical density at 750 nm = 0.3-0.5) in 500 ml TAP medium using a 2 L Erlenmeyer flask. Cells were pelleted by centrifugation (500*g*, 4°C, 10 min) and resuspended in 30 ml of homogenization solution (250 mM sorbitol, 50 mM Tris-acetate [pH 7.5], 1 mM EGTA-Tris [pH 7.5], 2 mM DTT, 1× protease inhibitor cocktail tablet [PIC; Roche], and 4 mM EDTA or $MgCl_2$). The cells were homogenized by repeated pulse sonication, 10 s each separated by 10-s standby intervals on ice, and then the cell homogenates were subjected to serial centrifugations, which precipitated organelles including nuclei (500*g*, 10 min), chloroplast (3,000*g*, 10 min), and mitochondria (20,000*g*, 30 min) at 4°C. The supernatant from the previous step was centrifuged at 100,000*g* for 4 h at 4°C (SW28 rotor; Beckman Coulter, USA) to obtain microsomes. The microsome pellet was resuspended in fractionation buffer containing 5% (w/v) sucrose, 20 mM Tris-acetate (pH 7.5), 0.5 mM EGTA-Tris, 1× PIC, and 4 mM of EDTA or $MgCl_2$, placed onto a sucrose gradient (10-50% sucrose linear gradient, 20 mM Tris-HCl [pH 7.5], 0.5 mM EGTA-Tris, 1× PIC, and 4 mM EDTA or $MgCl_2$), and centrifuged at 100,000*g* for 16 h at 4°C (SW41Ti rotor; Beckman Coulter). The fractionated proteins were subjected to immunoblotting using primary antibodies for anti-CrABCA2 (1:3,000) or anti-BiP (1:5,000) (AS09481; Agrisera, Sweden), and then HRP-conjugated goat anti-rabbit IgG (1:5,000) as the secondary antibody.

Immunogold labeling of CrABCA2 and CrDGAT2

High-pressure freezing, freeze-substitution, low-temperature embedding, and preparation of serial section ribbons were carried out according to a protocol described (Kang, 2010). Briefly, cells were harvested by gentle centrifugation (500*g*, 23°C, 5 min) and resuspended in TAP medium containing 0.15 M sucrose. The cell slurry samples were cryofixed with an HPM100 high-pressure freezer (Leica Microsystems, Austria) and incubated in freeze-substitution medium (anhydrous acetone with 0.25% glutaraldehyde and 0.1% uranyl acetate) for two days at –80°C. After raising the incubation temperature to –50°C (1°C/h), the freeze-substitution medium was washed with anhydrous acetone and the samples were embedded in HM20 resin (Ted Pella, USA) over two days at –50°C. The resin was cured by UV illumination (24 h) at –50°C. We used an AFS2 machine (Leica Microsystems) for freeze-substitution, resin embedding, and polymerization. Immunogold labeling was performed as described previously (Wang et al., 2017) with our homemade antibody against CrABCA2 and an anti-CrDGAT2A antibody (AS121874, Sweden) purchased from Agrisera (Liu et al., 2017; Wase et al., 2015).

Statistical analysis

All data were expressed as mean ± SEM. Statistical significances for measurements were calculated using Student's *t*-tests and defined as $*P < 0.05$. All statistical calculations were performed using Microsoft Excel 2016 (Microsoft, USA).

RESULTS

Selection of a candidate ABCA transporter involved in FA transport

The *C. reinhardtii* genome database (Phytozome v12) contains 69 ABC transporter coding genes (Hwang et al., 2016). To select candidate *C. reinhardtii* FA transporter proteins, we first identified all *C. reinhardtii* ABCA subfamily proteins, by performing BLASTP using the amino acid sequences of the 12 *A. thaliana* ABCA proteins as queries. This search identified five proteins, Cre03.g154000, Cre14.g613950, Cre14.g618400, Cre16.g674500, and Cre17.g721000, which were annotated as ABC1 homologs (AOH) or ABC2 homologs (ATH). We named these CrABCA1 to CrABCA5 based on the order of their positions on the chromosomes. To determine which of the five was closest to AtABCA9, a FA transporter in *A. thaliana*, we constructed a phylogenetic tree based on the amino acid sequences of the 12 *A. thaliana* and 5 *C. reinhardtii* ABCA proteins using MAFFT tool (http://mafft.cbrc.jp/alignment/server/index.html). The result was drawn using the program phMEGA7 (Katoh and Standley, 2013; Tamura et al., 2013) (Fig. 1A). In this phylogenetic tree, CrABCA2 and CrABCA4 were grouped with AtABCA9 (Fig. 1A, group 2). CrABCA2 showed 36.7% amino acid sequence identity and 51.1% similarity to AtABCA9, whereas CrABCA4 showed 19% sequence identity and 29.6% similarity to AtABCA9.

We then compared the transcriptome data for the five ABCA transporter coding genes in *C. reinhardtii* under nitrogen starvation (Blaby et al., 2013; Boyle et al., 2012; Schmollinger et al., 2014). *CrABCA2* and *CrABCA3* had higher transcription levels under these conditions than the other three genes (Fig. 1B). We chose CrABCA2 for further study since it was close to AtABCA9 in the phylogenetic tree and was highly expressed under nitrogen starvation.

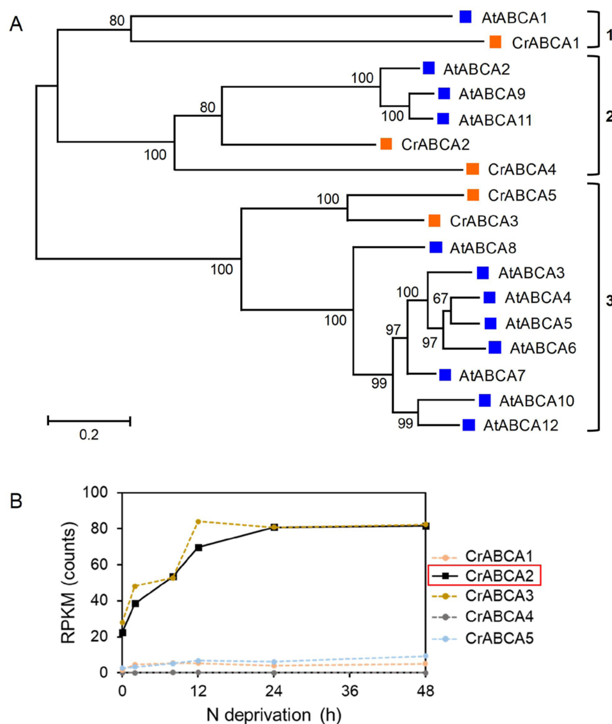

Fig. 1. Selection of candidate ABCA transporters in *Chlamydomonas reinhardtii* important for lipid accumulation under nitrogen starvation. (A) Two CrABCA proteins are close to AtABCA9 in a phylogenetic tree containing all *A. thaliana* and *C. reinhardtii* ABCA proteins. The tree was constructed with the highest log likelihood (-11,722.80) inferred from a maximum-likelihood analysis. Scale bar represents 0.2 amino acid substitutions per site. Numbers at nodes represent the percentage of 1,000 replicate bootstrap trees. Blue squares, *A. thaliana*; Orange squares, *C. reinhardtii*. (B) Transcript levels of two of the five ABCA transporter coding sequences in *C. reinhardtii* were induced under nitrogen starvation. Data are based on Boyle et al. (2012). RPKM, reads per kilo base per million mapped reads.

CrABCA2 transcript and CrABCA2 protein levels increase under nitrogen starvation

If *CrABCA2* were important for lipid accumulation during nitrogen starvation, its expression might increase under exposure to this stress. Indeed, the transcript level of *CrABCA2* increased under nitrogen starvation in both the C9 and CC-4533 parental lines of *C. reinhardtii* (Fig. 2A). The abundance of the CrABCA2 protein also increased: after 24 h of nitrogen starvation, the CrABCA2 level was much higher than that before nitrogen starvation in both the C9 and CC-4533 lines, as revealed by immunoblotting using an antibody against CrABCA2 (Fig. 2B).

Isolation of *crabca2* mutants

To investigate the function of CrABCA2, we searched for *CrABCA2* mutants of *C. reinhardtii*. We isolated the first mu-

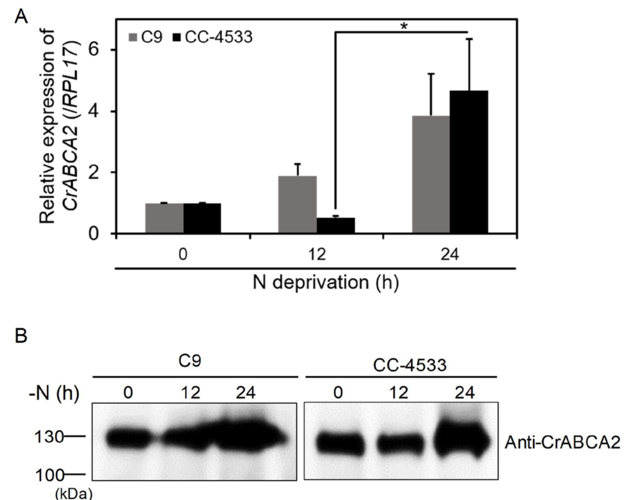

Fig. 2. *CrABCA2* transcript and CrABCA2 protein levels increase under nitrogen starvation. (A) Time-dependent induction of *CrABCA2* transcript levels under nitrogen starvation in *C. reinhardtii* parental lines (C9, CC-4533). The housekeeping gene *RPL17* was used as an internal standard. n = 4. $*P < 0.05$, Student's *t*-test. Error bars represent SE. (B) CrABCA2 protein levels increased under nitrogen starvation (-N) in the two *C. reinhardtii* parental lines.

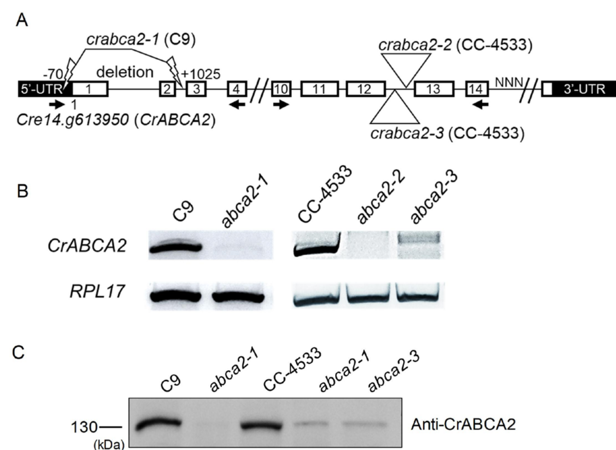

Fig. 3. Isolation and genotyping of *crabca2* mutants. (A) Insertion sites of the *AphVIII* cassette in the three *crabca2* mutants. A deletion (from position -70 nt to +1025 nt from the ATG start codon, including the first exon, the first intron, and the second exon) results from the insertion of the cassette in *crabca2-1*. Triangles, insertional positions of *AphVIII* cassette in *crabca2-2* and *crabca2-3*, respectively; Black arrows, primers used for RT-PCR genotyping. (B) RT-PCR analysis of *CrABCA2* expression in the parental lines (C9, CC-4533) and *crabca2* mutants (*crabca2-1*, *crabca2-2*, and *crabca2-3*). The housekeeping gene *RPL17* was used as an internal standard. (C) Immunoblot analysis of *CrABCA2* protein levels in *crabca2* mutants and their parental lines after 24 h of nitrogen deprivation.

tant, *crabca2-1*, from our random insertional mutant library (Yamano et al., 2015), which contained C9-derived mutants harboring an antibiotic marker gene *AphVIII*, using a PCR-based screening method (Gonzalez-Ballester et al., 2011). The mutant *crabca2-1* has a large deletion between positions –70 nt and +1025 nt from the ATG start codon, which includes the first and second exons of *CrABCA2*, due to the insertion of the *AphVIII* marker gene (Fig. 3A). The *crabca2-1* cells did not produce *CrABCA2* transcripts or CrABCA2 proteins, making this a null mutant of *CrABCA2* (Figs. 3B and 3C). We obtained the second and third mutant alleles of the same locus (Cre14.g613950) from the CLiP mutant library (Li et al., 2016), which is based on the CC-4533 background, and named them *crabca2-2* and *crabca2-3*. These both have the *AphVIII* marker gene integrated into the 12th intron of *CrABCA2* (Fig. 3B). Each had a significantly lower CrABCA2 protein level than CC-4533, making these *CrABCA2* knockdown lines (Fig. 3C).

Cellular oil content correlates with *CrABCA2* expression level

We then analyzed TAG content in *crabca2-2, crabca2-3* and their parental line CC-4533 2 days after the onset of nitrogen starvation, using gas chromatography with flame ionization detector (GC-FID). The *crabca2-2* and *crabca2-3* mutants accumulated 20% and 30% less TAGs, respectively, than their parental line CC-4533 (Fig. 4A).

We subsequently generated *CrABCA2*-overexpressing *C.*

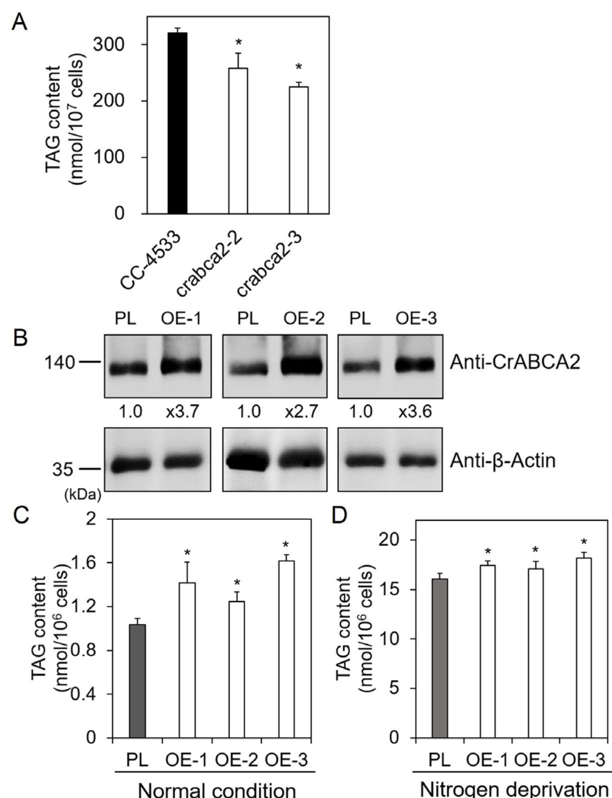

Fig. 4. TAG contents of *crabca2* mutants and *CrABCA2* overexpression lines. (A) TAGs content in one parental line (CC-4533) and its descendant *crabca2* mutants (*crabca2-2* and *crabca2-3*) after 2 days of nitrogen starvation. n = 9. $*P < 0.05$, Student's *t*-test. (B) Immunoblot of CrABCA2 in *CrABCA2*-overexpressing lines (OE-1, OE-2, and OE-3) compared to the UVM4 background (parental line, PL). β-Actin was used as loading control. (C and D) TAG content in cells grown in normal TAP medium (C) and grown first in TAP medium and then under nitrogen deprivation conditions for 1 day (D). n = 6 for each condition. $*P < 0.05$, Student's *t*-test. Error bars represent SE.

reinhardtii lines in a UVM4 strain background (parental line), which can express introduced genes at high levels (Neupert et al., 2009). Expressing the cDNA of CrABCA2 under the control of a constitutive promoter (*pHSP70/RbcS2*) yielded three *CrABCA2* overexpression lines (OE-1, OE-2, and OE-3). The transformants had elevated levels of CrABCA2 protein (3.7-, 2.7-, and 3.6-fold that of the parental line, respectively), as detected by immunoblotting using anti-CrABCA2 antibody (Fig. 4B). The *CrABCA2* overexpression lines accumulated increased amounts of TAG under both nitrogen replete and nitrogen starvation conditions (Figs. 4C and 4D). Under nitrogen-replete conditions, overexpression lines OE-1, OE-2, and OE-3 exhibited 36%, 20%, and 56% higher TAG levels, respectively, than parental line (Fig. 4C). After 1 day of incubation in medium lacking nitrogen, the overexpression lines accumulated 8%, 6%, and 13% more TAG than parental line (Fig. 4D).

CrABCA2 is localized to the ER

We suspected that CrABCA2 was localized to the ER, like AtABCA9 (Kim et al., 2013), since it had high amino acid sequence similarity to AtABCA9 and its overexpression, like that of AtABCA9, increased cellular lipid content (Kim et al., 2013). To test this hypothesis, we prepared microsomal fractions of *C. reinhardtii* using fractionation media with two different compositions: 4 mM $MgCl_2$ or 4 mM EDTA. In the presence of EDTA, ribosomes are detached from the ER membranes, shifting ER-localized proteins to lighter membrane fractions. As expected, CrABCA2 protein bands shifted to lower sucrose concentration fractions in the presence of 4 mM EDTA (Fig. 5B) than in the presence of 4 mM $MgCl_2$ (Fig. 5A). This shift pattern matched that of an ER marker protein, CrBiP, a result that strongly supported the proposed ER localization of CrABCA2.

We further investigated the localization of CrABCA2 through transmission electron microscopy (TEM) and immunogold labeling using an anti-CrABCA2 antibody (Figs. 6A and 6B). The immunogold labeling showed that CrABCA2-specific gold particles were associated with swollen compartments connected to the ER (Fig. 6B). Since these CrABCA2-positive ER regions appeared different from regular tubular/cisternal ER elements, we localized CrDGAT2A, an enzyme essential for TAG biosynthesis in the ER membrane by immunogold labeling to confirm their identity (Boyle et al., 2012; Shockey et al., 2006). CrDGAT2A-specific gold particles were located in structures matching those with CrABCA2

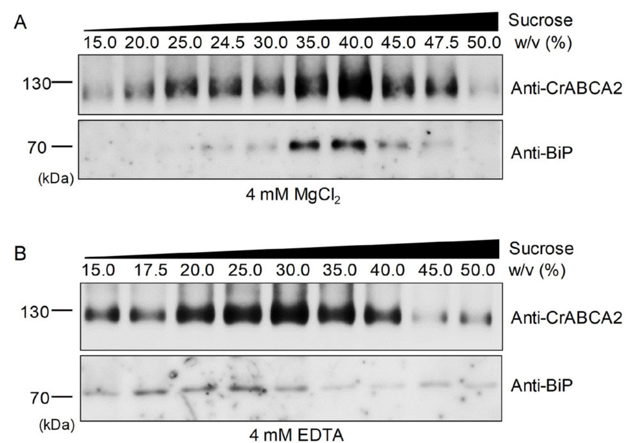

Fig. 5. Localization of CrABCA2 at the endoplasmic reticulum. Immunoblots with antibody against CrABCA2 of a microsome preparation obtained by serial centrifugation and separation through a sucrose density gradient from 15% to 50% sucrose in the presence of either 4 mM $MgCl_2$ (A) or 4 mM EDTA (B). Separated membrane fractions were subjected to immunodetection. BiP was used as marker for ER.

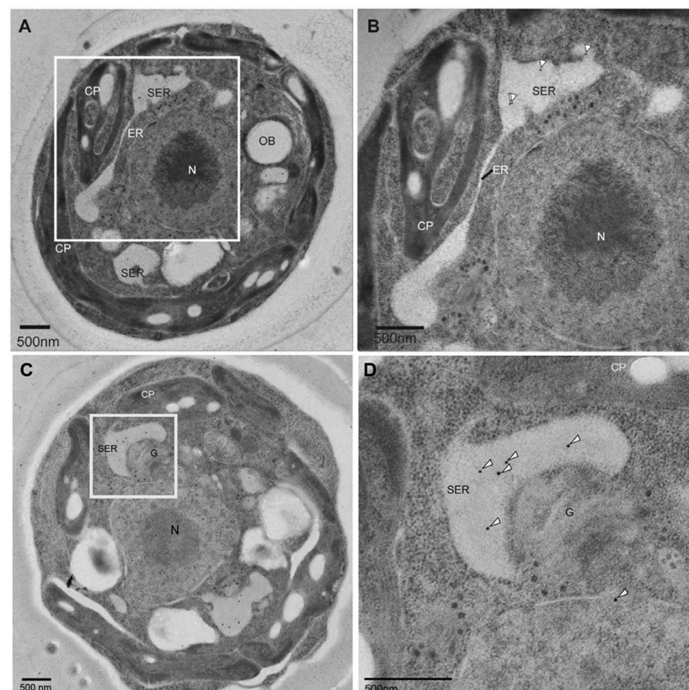

Fig. 6. Immunogold labeling of CrABCA2 indicates localization of CrABCA2 in the swollen ER. *C. reinhardtii* cells of C9 strain incubated in TAP medium without any nitrogen source for 6 h were preserved by high-pressure freezing and freeze-substitution. The sections through *Chlamydomonas* cell immunolabeled with antibodies against CrABCA2 (A) or CrDGAT2A (C) are shown. (B and D) Higher-magnification micrographs of the boxed areas in Figures 6A and 6C, respectively. Gold particles (15 nm) are marked with white triangles. Scale bars = 500 nm. CP, chloroplast; ER, endoplasmic reticulum; SER, swollen ER; N, nucleus; OB, oil body; G, Golgi.

(Figs. 6C and 6D). The TEM images of serial sections of the cells (Supplementary Fig. S1) revealed that CrABCA2 and CrDGAT2A were located in similar structures continuous with the rough ER, further supporting the ER localization of CrABCA2. CrABCA2 and CrDGAT2A have transmembrane domains but their epitopes were seen in the ER membrane as well as its lumen (Fig. 6, Supplementary Fig. S1) for reasons that we cannot specify.

DISCUSSION

Importance of CrABCA2 in lipid accumulation under stress

Here we report that *CrABCA2*, a homologue of *AtABCA9*, is important for lipid accumulation under nitrogen starvation conditions. Several lines of our experimental results support this conclusion. First, among the five ABCA genes in the *C. reinhardtii* genome, *CrABCA2* encodes the protein that has the highest amino acid sequence similarity, and is closest in the phylogenetic tree, to AtABCA9, which is reported to facilitate lipid accumulation in *A. thaliana* seeds (Kim et al., 2013). Second, the transcript and protein levels of CrABCA2 were highly induced under nitrogen starvation (Figs. 1B and 2), which is known to induce lipid accumulation. Third, the amount of TAG that accumulated in cells correlated with *CrABCA2* expression: *CrABCA2* knockout cells had low, whereas *CrABCA2* overexpression lines had high, TAG levels compared to their respective parental lines (Fig. 4). Fourth, CrABCA2 was localized to the ER (Figs. 5 and 6, Supplementary Fig. S1), the site of TAG biosynthesis. Taken together, our results strongly support the status of CrABCA2 as an important factor in TAG biosynthesis and a highly possible transporter of FAs, the precursors of TAGs.

Localization of CrABCA2 at swollen ER

We investigated the subcellular localization of CrABCA2 using two independent methods: membrane fractionation and immunogold labeling of CrABCA2. Both methods indicated that CrABCA2 was localized to the ER (Figs. 5 and 6). Furthermore, the EM micrographs clearly showed that CrABCA2 localized to swollen ER (Fig. 6, Supplementary Fig. S1). The identity of the bloated ER was determined by the localization of CrDGAT2A, a TAG biosynthesis enzyme localized to the ER membrane in similar structures (Fig. 6, Supplementary Fig. S1). The localization of CrABCA2 in swollen ER is not surprising since nitrogen starvation puts severe stress on *C. reinhardtii*, and swollen ER is frequently observed in severely stressed cells (Chavez-Valdez et al., 2016). For example, the cortical ER in yeast cells often swells and separates from the plasma membrane under lipid-induced stress (Pineau et al., 2009).

How CrABCA2 might be regulated under the nitrogen deficiency condition

We observed that *CrABCA2* was highly induced at both the transcript and protein levels under nitrogen (N) starvation (Fig. 2), confirming a previous observation (Fig. 1B; Boyle et al., 2012). How such an induction is achieved is an interesting question. Although we do not have data to answer this question, the simplest explanation for this phenomenon would be as follows: N starvation activates transcription factors that upregulate *CrABCA2* transcription, in turn increasing the CrABCA2 protein level. Previously published literature and information stored in databases could be used to select candidate transcription factors that might upregulate *CrABCA2* transcription. For example, the MYB coiled-coil domain transcription factor PSR1 was identified as a pivotal switch that triggers cytosolic lipid accumulation under conditions that trigger lipid accumulation in *Chlamydomonas* (Ngan et al., 2015). The loss-of-function mutant strain *psr1* exhibited a 50-90% reduction in lipid accumulation compared to the parental line when sulfur (S), phosphorus (P), or N was deficient (Ngan et al., 2015). However, the *PSR1* transcript level peaked at 2 h after the onset of N starvation and then disappeared, in contrast to the slow and steady increase observed in *CrABCA2* transcript level. Another candidate is NRR1, a SQUAMOSA promoter binding domain protein, suggested to be a master regulator of lipid accumulation in *Chlamydomonas* (Boyle et al., 2012). *NRR1* expression level is associated with the expression patterns of three acyltransferases (*DGAT1, DGTT1,* and *PDAT1*) under N starvation, and the *nrr1* knockout accumulated about 50% less TAG than its parental line under the same condition (Boyle et al., 2012). Moreover, the pattern of *NRR1* expression increase resembles that of *CrABCA2*, remaining high until 24 h after the onset of N starvation. Many other transcription factors, belonging to the *bZIP, MYB, GATA,* and *AP2* families, were up-regulated at the time of TAG synthesis, after 6-24 h of N starvation, in a transcriptomic analysis (Gargouri et al., 2015). Among them, we speculate that the best candidates are *GATA3* (Cre10.g435450) and *AP2-13* (Cre01.g009650), since they are co-expressed with *CrABCA2* at 12 h and 24 h after the onset of N starvation (data from the Algae Gene Coexpression Database, ALCOdb; http://alcodb.jp/). Further studies are needed to test whether any of these transcription factors indeed regulate *CrABCA2* expression.

Application potentials of CrABCA2

TAGs are synthesized at the ER, but their FA precursors are synthesized at plastids. There are thus two FA transport steps necessary for TAG biosynthesis: first, efflux from the plastid, and second, uptake into the ER. The observation that overexpression of *CrABCA2* increased cellular TAG content suggests that the FA pool in the ER was not at a saturated level in the parental cell line, and *CrABCA2* overexpression could therefore increase oil production in microalgae by increasing the second step of the transport process. The overexpression of proteins involved in the first step of transport—CrFAX1 and AtFAX1, FA exporting proteins localized to the chloroplast membrane—is also known to increase lipid yield (Li et al., 2015; 2019). AtFAX1 is crucial for the biosynthesis of FA-derived compounds (such as lipids, ketone waxes, and pollen cell wall material) in *A. thaliana*, and *AtFAX1*-overexpressing Arabidopsis lines show an increased TAG content, whereas *fax1* knockouts show a significant decrease in TAG level (Li et al., 2015; 2019). Moreover, overexpression of *CrFAX1*, which is predicted to be a homologue of *AtFAX1*, increases TAGs accumulation in *C. reinhardtii* (Li et al., 2015; 2019).

In summary, our study reveals that CrABCA2 has important

functions in lipid accumulation under nitrogen starvation conditions. Furthermore, it demonstrates that genetic engineering to boost the step mediated by the CrABCA2 transporter can increase cellular oil levels. CrABCA2 overexpression in commercially valuable lines of microalgae might be a useful way to increase the production of FA-derived compounds in these organisms.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

Disclosure

The authors have no potential conflicts of interest to disclose.

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