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ORIGINAL ARTICLE

Characterization of a *Chlamydomonas reinhardtii* Mutant Defective in a Maltose Transporter

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Abstract Microalgae are potential sources of energy and high-value materials. To decipher the process of energy metabolism in green algae, we created a mutant pool of strain CC-503 of the model green microalga *Chlamydomonas reinhardtii*, by random insertion of an antibiotic resistance gene, and screened the pool for lines with altered carbon metabolism. We identified a mutant that harbored the antibiotic resistance gene in *CrMEX1,* a putative *Maltose Exporter-Like protein 1* (*Cre12.g486600.t1.2*). The mutant had reduced levels of *CrMEX1* expression and, similarly to the Arabidopsis *mex1* knockout mutant, which cannot export maltose from the chloroplast, it over-accumulated starch granules in the chloroplast. The mutant's lipid levels were slightly higher than those of the wild type, and its initial growth kinetics were not significantly different from those of the wild type, but the mutant culture did not reach the same high cell density as the wild type in acetate-containing culture medium under continuous light. These results suggest that *CrMEX1* encodes a maltose transporter protein, and that export of photoassimilates from chloroplasts is necessary for normal *Chlamydomonas* growth, even under continuous light with an ample supply of carbon in the form of acetate.

Keywords: Chloroplast, Maltose exporter, Mutant screening, Starch accumulation

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Introduction

Microalgae are important sources of organic carbon on a global scale. The carbon fixed by microalgae through photosynthesis accounts for about 40% of the total carbon produced globally (Falkowski 1994). Therefore, understanding the process of energy metabolism and carbon flow in microalgae is of the utmost importance. Microalgae store fixed carbon as starch, an important energy storage molecule (Ball et al. 1990; Reynolds 2006). Much is known about starch storage and mobilization in terrestrial plants; a portion of the photoassimilates generated by photosynthesis is stored as starch in chloroplasts during the day, and starch is converted to maltose and exported from the chloroplast to the cytosol during night, where it is used as an energy source (Hostettler et al. 2011). Maltose export depends on a transporter at the chloroplast membrane. *Arabidopsis thaliana* maltose exporter (AtMEX1) was the first maltose exporter to be identified in photosynthetic organisms, and *AtMEX1* expression complemented an *E. coli* maltose transporter knockout mutation (Niittyla et al. 2004). Furthermore, the Arabidopsis maltose transporter mutant *atmex1* accumulates starch in chloroplasts, and is inhibited in growth (Niittyla et al. 2004). A maltose transporter has also been identified in *Oryza sativa* (rice), and the gene encoding this transporter, *OsMEX1,* was shown to rescue the *atmex1* mutation (Ryoo et al. 2013). *Chlamydomonas reinhardtii* contains a *Maltose Exporter Like-1* (*MEX1*) gene that seems to be orthologous to *AtMEX1*. However, the function of maltose transporters in microalgae is poorly understood. Recently, a *crmex1* allele was identified in a forward-genetic screen for mutants with altered starch metabolism (Tuncay et al. 2013). However,

high starch levels were the only phenotype reported for *crmex1* to date. In this study, we isolated another *crmex1* allele (designated *crmex1-2*) by random insertion of an antibiotic-resistance gene. We characterized the storage carbon compounds in this mutant, and identified how it differed from the wild type in terms of morphology and growth.

Results and Discussion

Isolation of *crmex1-2*

To understand energy metabolism in green algae, we generated random insertional mutants of *Chlamydomonas* strain CC-503 (*cw92*, cell wall-less). Transgenic lines that exhibited hygromycin resistance were analyzed for their ability to accumulate triacylglycerol (TAG) in medium lacking a nitrogen source, using Nile Red fluorescence staining. Nile Red emits fluorescence when bound to neutral lipids (e.g., TAGs) and, when fluorescence is normalized by optical density at 750 nm (FI/OD_{750}), reflects the level of neutral lipids accumulated in the cell. From 15,000 independent mutant lines, we selected 20 lines that exhibited low Nile Red FI/OD_{750} values. In this study we characterized one of these lines, which harbors an insertion in the fourth exon of *Maltose Exporter Like-1* (*MEX1*, gene locus Cre12.g486600, *Chlamydomonas reinhardtii* v5.5 genome in the Phytozome 10.2 Database, http://www.phytozome.net) and has reduced levels of *CrMEX1* expression (Fig. 1A, B, C).

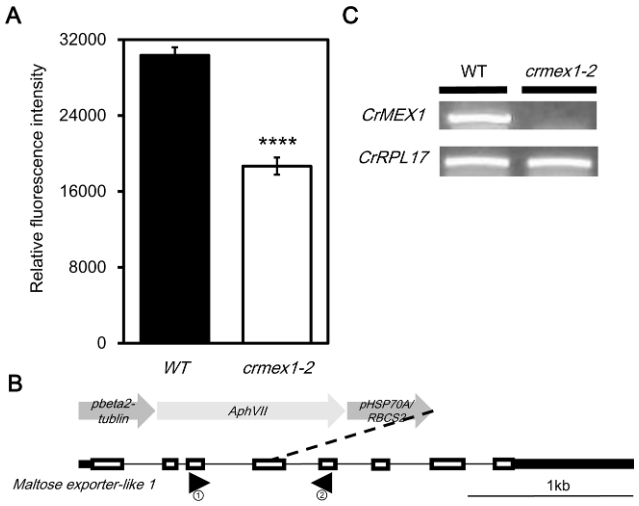

Fig. 1. Isolation of the *crmex1-2* mutant. (A) Nile Red fluorescence/O.D. 750 of wild type and *crmex1-2.* (B) Insertion site of the marker gene *AphVII* in the fourth exon of *CrMEX1*; ① and ② represent primer binding sites. (C) Transcript levels of *CrMEX1* in the wild type and *crmex1-2*, analyzed by reverse transcription polymerase chain reaction (RT-PCR) using primers shown in ① and ② in (B). ****$P < 0.0005$, Student's *t*-test. Error bars represent standard errors.

Over-Accumulation of Starch Granules in the *crmex1-2* Mutant

Transmission electron microscopy revealed that the number of starch granules in plastids was greatly increased in the *crmex1-2* mutant compared with the wild type (CC-503) (Fig. 2 right panels). The starch granules were scattered throughout the chloroplast of *crmex1-2*, even occurring in the lobe zone (Fig. 2, bottom right panel), which is the light harvesting site for photosynthesis (Schottkowski et al. 2012). *C. reinhardtii* was reported to normally store starch around the pyrenoid of the chloroplast (Harris 1989). Pyrenoid area is enriched with RUBISCO (Borkhsenious et al. 1998), and high in carbon concentrating mechanism (Ramazanov et al. 1994; Fukuzawa et al. 2001). The pyrenoid of *crmex1-2* was enlarged compared to those of the wild type (Fig. 2). Biochemical analysis revealed that the *crmex1-2* mutant contained three times more starch than the wild type CC-503 (Fig. 3A), consistent with findings reported for the *crmex1-1* mutant (Tuncay et al. 2013). These results suggest that starch production is increased in the *crmex1-2* mutant, and

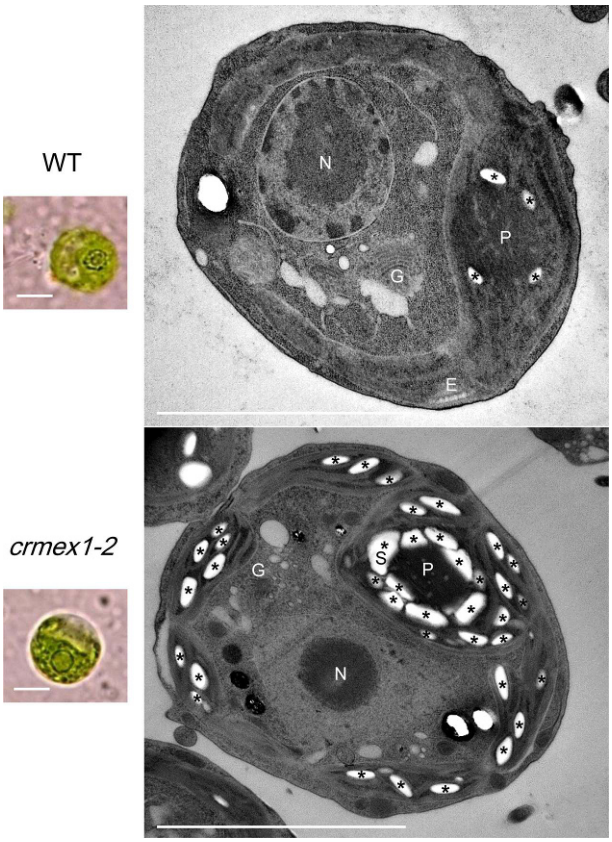

Fig. 2. Transmission electron microscopy images of wild-type and *crmex1-2 Chlamydomonas*. Left panels: Bright field images of cells fixed with Lugol's solution, which stains starch granules. Right panels: Transmission electron microscopy images. E, eyespot; G, Golgi apparatus; N, nucleus; P, pyrenoid; S, starch granule. Starch granules are marked also with asterisks (*). Bar = 5 μm.

that starch is synthesized in broad regions of the chloroplast, and not exclusively in pyrenoid.

The *crmex1-2* Mutant has a High Optical Density and a slight increase in TAGs

The *crmex1-2* mutant exhibited low Nile Red FI/OD_{750} values after 3 d of nitrogen deprivation (Fig. 1A), which, at first sight, seemed to indicate a decrease in lipid accumulation by the mutant. However, biochemical lipid analysis revealed that the levels of total fatty acids and TAGs were slightly higher in *crmex1-2* than in the wild type (Fig. 3B, C). To resolve this apparent discrepancy, we compared the optical densities at 750 nm (OD_{750}) of the same number of cells, and found that the values were much higher for the mutant than for the wild type (Fig. 3D). We suspect that the excess of starch granules contributed to the high OD_{750} of the mutant. Indeed, *crmex1-2* hyper-accumulated starch granules when cultured in nitrogen-deficient medium (data not shown), similarly as under normal conditions (Fig. 2), and *crmex1-1* was shown to contain increased levels of starch regardless of the nitrogen levels in the medium (Tuncay et al. 2013). Thus, the low Nile Red intensity/OD_{750} value was due to the high optical density of *crmex1-2*, and did not indicate a decrease in lipid levels. Therefore, knockout of the maltose transporter did not interfere with TAG accumulation under the nitrogen-deficient conditions we used.

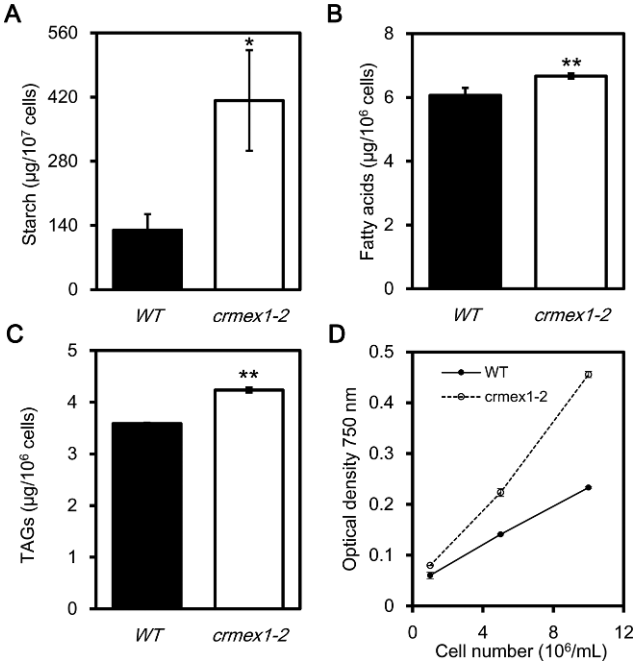

Fig. 3. Starch and lipid contents of wild-type and *crmex1-2 Chlamydomonas*. (A) Total starch amount in wild type and *crmex1-2*. Cells were cultured in TAP-N medium for 5 d, until the saturation phase; N = 2, n = 3. (B) Total fatty acids and (C) TAGs. wild-type and *crmex1-2* cells were harvested after 3 d of culture in TAP-N medium under continuous light. N = 2, n = 3. (D) Optical density at 750 nm of wild-type (CC-503) cells and the same number of *crmex1-2* cells. Cells were diluted and counted using a hemocytometer. *$P < 0.1$, **$P < 0.05$, Student's *t*-test. Error bars represent standard errors from six replicates.

Reduced Growth of *crmex1-2*

To examine the physiological effects of eliminating the maltose transporter, we compared the growth kinetics of the mutant *crmex1-2* with those of CC503, the parental strain of *crmex1-2*. The cell number of the two cultures increased at similar rates until the mid-log phase, but the final cell number of *crmex1-2* was just 70% that of the wild type (Fig. 4A). Furthermore, the mutant *crmex1-2* was smaller in cell size compared to that of the parental strain (Fig. 4B). Interestingly, the mutant *crmex1-2* was high in granularity (Fig. 4C), most likely due to the starch granules accumulated

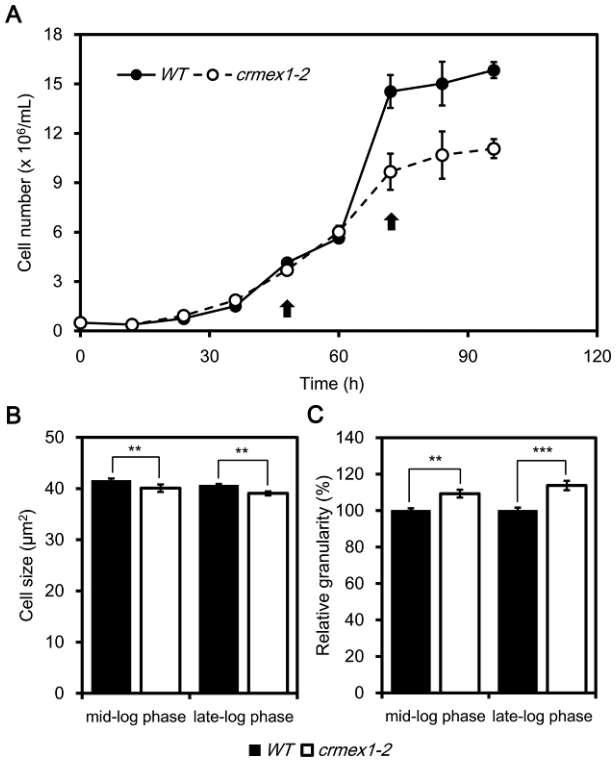

Fig. 4. Effect of *crmex1* knockout on cell number, cell size and granularity. (A) Growth curve of wild type and *crmex1-2*. Cells were fixed with Lugol's solution and counted from inoculation (0 h) until 96 h using a hemocytometer. N = 3, n = 3. (B) Cell size and (C) cell granularity analyzed using flow cytometry. Cells were grown in TAP medium under continuous light, and then harvested at the mid-log phase and the late-log phase marked in (A) by black arrows. Cell size was determined by measuring the amount of the laser beam that passes around the cell, and comparing it with the value obtained using the beads with a known size. Cell granularity was determined by the amount of the laser beam that bounces off of things inside the cell (http://ricfacility.byu.edu/Research/CellSizeGranularity.aspx). **$P < 0.05$, ***$P < 0.005$, Student's *t*-test. Error bars represent standard errors.

in the chloroplast (Fig. 2, bottom right panel).

Thus, *CrMEX1* expression seems to be necessary for optimal growth and/or division of the algal cell. During the night, when there is no energy supply via photosynthesis, the maltose transporter is the major route through which starch is remobilized from chloroplasts and moves to the cytosol, providing energy for the cytosol of both photosynthetic and non-photosynthetic organs. Thus, the Arabidopsis *mex1* mutant is dwarfed when grown under day/night cycles (Niittyla et al. 2004). However, it seems unlikely that *Chlamydomonas* cells in our culture lacked an energy source, since they were grown under continuous light with acetate supplementation. We speculate two possible reasons why the cells needed the maltose transporter for optimal growth/division. Firstly, the over-accumulation of starch granules might exert a negative effect on chloroplast function. For instance, the excess starch might disrupt the synthesis of other metabolites needed for cellular function. Secondly, glucose released from maltose in the cytosol could be a more effective source of carbon than acetate for biomass production, especially in actively dividing *Chlamydomonas* cells. In plants, the conversion of acetate to carbohydrates requires the glyoxylate cycle (Canvin et al. 1961). For rapid biomass production and cell division, particularly in the late-log phase, *Chlamydomonas* cells may

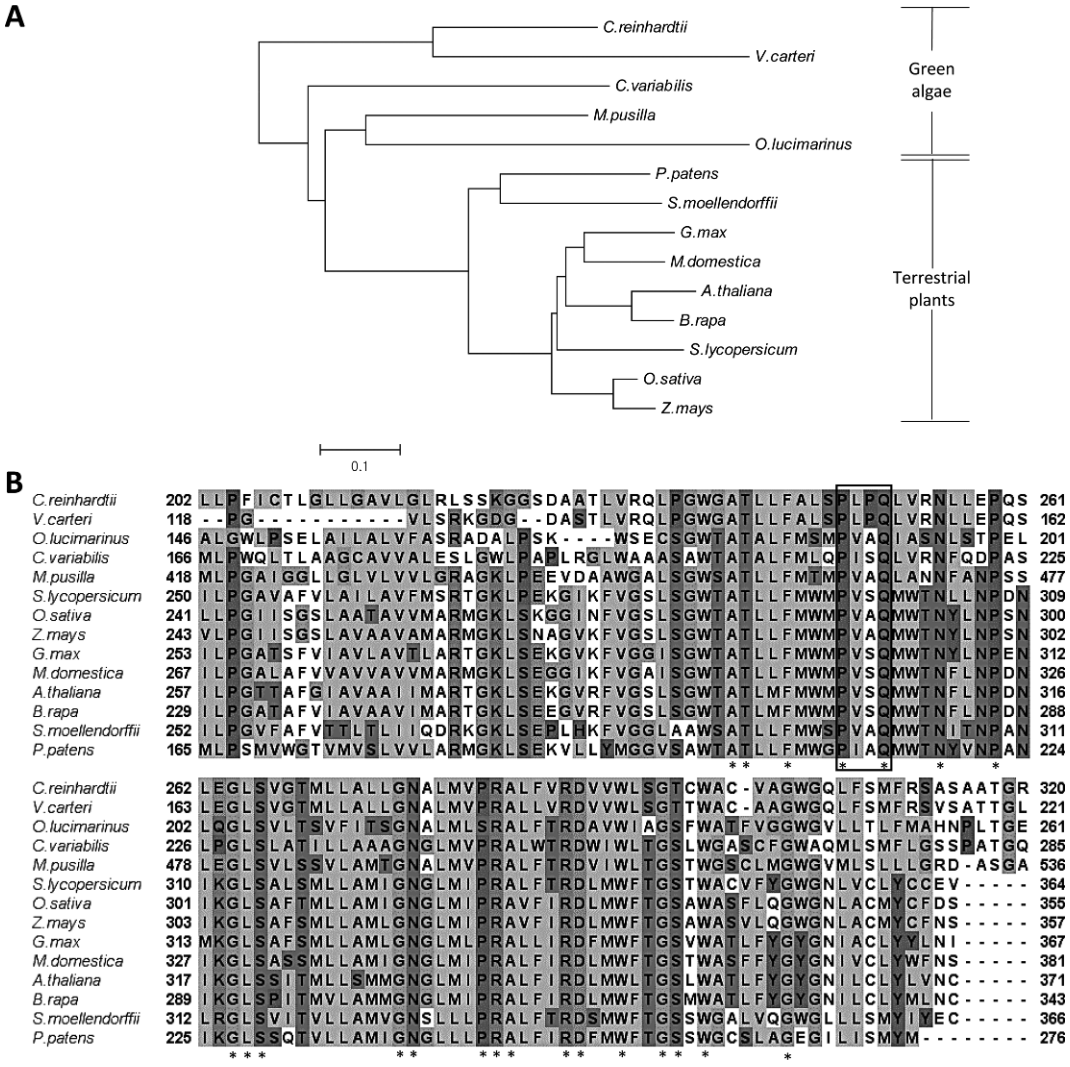

Fig. 5. Amino acid sequence analysis of CrMEX1 and its orthologs. (A) Phylogenetic analysis of 14 maltose exporter-like protein 1 sequences from *Chlamydomonas reinhardtii* (Phytozome accession number, Cre12.g486600), *Volvox carteri* (Vocar20000849m), *Ostreococcus lucimirinus* (eugene.0900010288), *Micromonas pusilla* (MicpuC2.SAR_EuGene.0000030121), *Solanium lycopersicum* (Solyc04g064720.2), *Oryza sativa* (LOC_Os04g51330), *Zea mays* (GRMZM2G156356), *Glycine max* (Glyma.12G013400), *Malus domestica* (MDP0000088105), *Arabidopsis thaliana* (AT5G17520), *Brassica rapa* (Brara.B00717), *Selaginella moellendorffii* (184196), *Physcomitrella patens* (Phpat.024G002500), and *Chlorella variabilis* (Genbank number, XP_005844913.1). Scale bar indicates 0.1 substitutions per amino acid site. (B) Amino acid alignment (bottom) of the C-terminal region, showing many amino acid residues that are conserved among many maltose exporter-like proteins. Gray boxes, similar or identical amino acids; black box, conserved domain (PQ loop); asterisks, highly conserved amino acid residues.

require a sufficient supply of sugar derivatives, such as ribose 5-phosphate, which is needed for the synthesis of nucleotides (via the reductive pentose phosphate pathway) and various monosaccharides involved in cell wall synthesis (via the oxidative pentose phosphate pathway). In *crmex1-2*, the export of photoassimilated carbons to the cytosol, most likely in the form of maltose, may be compromised, resulting in less than optimal division of the cells at the late-log phase.

CrMEX1 is Homologous to MEX1 from Other Organisms

Next, we investigated whether CrMEX1 harbors any important domains by performing a phylogenetic analysis (Fig. 5A) and multiple amino acid sequence alignments (Fig. 5B). We obtained the amino acid sequences of maltose transporters from 14 organisms, including *Chlamydomonas reinhardtii* (Cre12.g486600), *Volvox carteri* (Vocar20000849m), *Ostreococcus lucimirinus* (eugene.0900010288), *Chlorella variabilis* (XP_005844913.1), *Micromonas pusilla* (MicpuC2.SAR_EuGene.0000030121), *Solanium lycopersicum* (Solyc04g064720.2), *Oryza sativa* (LOC_Os04g51330), *Zea mays* (GRMZM2G156356), *Glycine max* (Glyma.12G013400), *Malus domestica* (MDP0000088105), *Arabidopsis thaliana* (AT5G17520), *Brassica rapa* (Brara.B00717), *Selaginella moellendorffii* (184196), and *Physcomitrella patens* (Phpat.024G002500). The presence of MEX1 homologs in these many photosynthetic organisms, including algae, moss, and plants, is consistent with the

Table 1. Amino acid sequence similarity (%) between MEX1-like proteins. Whole protein sequences were blasted using BlastP

		Green algae				
	Query / Subject	*C. reinhardtii*	*V. carteri*	*C. variabilis*	*M. pusilla*	*O. lucimarinus*
Green algae	*C. reinhardtii*	100	88	60	52	54
	V. carteri	88	100	60	66	60
	C. variabilis	62	60	100	56	54
	M. pusilla	56	62	56	100	53
	O. lucimarinus	52	60	50	50	100
Land plants	*P. patens*	55	64	60	53	54
	S. moellendorffii	53	59	57	51	53
	A. thaliana	53	59	56	53	54
	B. rapa	56	63	56	54	54
	G. max	57	51	56	52	53
	M. domestica	52	64	56	52	53
	S. lycopersicum	53	69	53	52	55
	O. sativa	54	55	56	54	53
	Z. mays	54	57	56	54	53

Land plants								
P. patens	*S. moellendorffii*	*A. thaliana*	*B. rapa*	*G. max*	*M. domestica*	*S. lycopersicum*	*O. sativa*	*Z. mays*
55	54	53	56	58	52	54	56	54
55	60	59	63	56	64	69	57	58
60	57	56	56	56	56	53	56	56
55	53	53	54	51	51	53	54	54
54	53	54	54	53	53	55	54	53
100	76	74	74	75	74	72	76	75
76	100	64	62	65	68	69	62	65
74	64	100	84	83	81	73	82	82
74	62	84	100	82	78	79	78	74
75	65	83	82	100	83	76	76	82
72	67	81	78	78	100	84	79	75
72	69	73	79	76	84	100	77	71
76	62	82	78	76	85	77	100	87
75	65	82	74	82	83	71	87	100

C. reinhardtii, Chlamydomonas reinhardtii; V. carteri, Volvox carteri; C. variabilis, Chlorella variabilis; M. pusilla, Micromonas pusilla; O. lucimirinus, Ostreococcus lucimirinus; P. patens, Physcomitrella patens; S. moellendorffii, Selaginella moellendorffii; A. thaliana, Arabidopsis thaliana; B. rapa, Brassica rapa; G. max, Glycine max; M. domestica, Malus domestica; S. lycopersicum, Solanium lycopersicum; O. sativa, Oryza sativa; and Z. mays, Zea mays.

observation that MEX1 is a GreenCut protein that is conserved specifically in plastid-containing organisms (Karpowicz et al. 2011; Heinnickel et al. 2013). Interestingly, maltose transporter proteins are unique in each organism, and do not form a multi-member family.

The amino acid sequence of CrMEX1 was most similar to that of the MEX1-like gene in *Volvox carteri* (Fig. 5A, Table 1). Interestingly, the MEX1 amino acid sequences of the two green algae clustered to a separate group from those of the other green algae, *Chlorella variabilis, Micromonas pusilla,* and *Ostreococcus lucimirinus.* Furthermore, MEX1 amino acid sequences of the terrestrial plants were grouped separately from those of all green algae. Thus, the amino acid sequences of MEX1 in green algae exhibited low similarity with those of the terrestrial plants (Table 1).

Chlamydomonas MEX1 is predicted to have six or seven membrane-spanning helices, according to our analysis using TMHMM, Prediction of transmembrane helices in proteins, whereas Arabidopsis MEX1 is predicted to have nine membrane-spanning helices (Niittyla et al. 2004). Other plant *MEX1-like* genes, including those of *Glycine max* and *Zea mays,* are also predicted to contain eight or nine membrane-spanning helices, according to our analysis using TMHMM.

No functional motif has yet been reported for the maltose transporters of photosynthetic organisms. We attempted to identify domains conserved among the MEX1 proteins. All MEX1 proteins we analyzed, including CrMEX1, had a PQ loop region (boxed in Fig. 5B), but we could not identify any other established functional domains. However, conserved amino acid residues were concentrated in the C-terminal region of many MEX proteins, including CrMEX1 (Fig. 5B, marked with asterisks). The functional importance of these conserved amino acid residues might be worth investigating in future studies. Taken together, our findings reveal that CrMEX1 shares many features with MEX1 proteins from other photosynthetic organisms, and suggest that CrMEX1 is a maltose transporter important for normal growth of *Chlamydomonas*.

Materials and Methods

Culture Conditions

Chlamydomonas reinhardtii CC-503 (*cw92*, mt^+) was used in this work. To isolate genomic DNA and total RNA, cells were grown to the mid-log growth phase in TAP (Tris acetate phosphate, pH 7.0) medium at 25ºC under continuous illumination at 25 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 (3,000 *g*, 16ºC, 5 min), washed with TAP medium without a nitrogen source (TAP–N), and finally resuspended in TAP–N medium of the same volume.

To construct growth curves, the wild type (CC-503) and *crmex1-2* mutant were pre-cultured in TAP medium until the mid-log phase and fresh TAP medium was inoculated with 0.5×10^6 cells of each cell line. Using a hemocytometer placed on the microscope stage, the cell number was determined every 12 h, until cells reached the stationary phase (4 d). To measure cell size and granularity, cells were grown under the same conditions, and harvested at the mid-log phase and the late-log phase. Cell size and granularity were analyzed using BD LSR Fortessa flow cytometry (BD bioscience, San Jose, CA, USA) with 6 μm diameter bead (BD Calibrate bead; BD bioscience, San Jose, CA,USA) as reference. For each sample, 10,000 events were counted.

Mutant Screening

Cells of the wild-type strain CC-503 were transformed using electroporation as previously described Shimogawara et al. (1998), with a linearized plasmid containing an overexpression promoter (*pHSP70A/RBCS2*) fused with an antibiotic resistance gene (*pβ2TUB-APH7*). After 8 h of recovery, cells were spread onto agar-solidified TAP medium containing 10 μg/mL hygromycin B (Sigma-Aldrich). Colonies were picked into 96-well plates (SPL Lifescience, Korea) each containing 200 μL of TAP medium and grown for 3 d. To induce TAG biosynthesis by nitrogen deprivation, 10 μL of each culture was transferred to a new 96-well plate containing TAP-N medium and grown for 3 d under continuous light (25 μmol photons $m^{-2}\,s^{-1}$) at 25ºC. Neutral lipids were visualized by staining the cells with a lipid dye, Nile Red (Sigma-Aldrich, St. Louis, MO, USA); 2 μL of Nile Red stock solution (0.1 mg/mL in 100% acetone) was added to each well. Nile Red fluorescence (488 nm excitation/565 nm emission) and the optical density at 750 nm were measured using a plate reader (Infinite TECAN M200 pro, Männedorf, Switzerland), and the fluorescence value was normalized by the latter, which is usually proportional to cell number.

Nucleic Acid Extraction

Genomic DNA was isolated by phenol chloroform extraction (Sambrook et al. 1989). Inverse PCR was conducted following the method described in Kim et al. (2011) with primers optimized for the plasmid. To identify the insertion site, nested PCR was conducted using primer sets Cr_nF1 (5'-aacccgaacagattgatacccgcc-3') and Cr_nR1 (5'-tttgaatatggctttggtagctcg-3') for the first round, and Cr_nF2 (5'-tatacataaccactcagctagtgg-3') and Cr_nR2 (5'-tagcgcaagaaagagctccaattcgc-3') for the second round. For transcript level analysis, total RNA was isolated using homemade Trizol Reagent. Isolated RNA was subjected to reverse transcription with GoScript Reverse Transcriptase (Promega, Madison, WI, USA) to obtain cDNA, which was later used as the template for RT-PCR. The primer set used to amplify *CrMEX1* transcript was Cr_MEX1_F (5'-caacacattaatgtgcagcc-3') and Cr_MEX1_R (5'-gctggcggtggtgccgcag-3'). The reference gene *CrRPL17* (Lee et al. 2008) was also amplified using previously reported primers, and used to normalize the results for *CrMEX1*. Gel purification of amplified PCR and RT-PCR products was performed using a Gel Purification Kit (Labopass, Cosmo Gentech Co., Seoul, Korea).

Lipid Analysis

To induce TAG biosynthesis, cells were grown in TAP–N medium for 3 d and collected by centrifugation (3,000 *g*, 16ºC, 5 min). Lipid extraction, thin layer chromatography (TLC) of neutral lipids, transesterification, and gas chromatography were performed according to Kim et al. (2013).

Transmission Electron Microscopy

Cells were grown for 2 d to the mid-log phase in 50 mL TAP medium and collected by centrifugation (3,000 *g*, 16ºC, 5 min). The cell pellet

was washed with 0.15 M sucrose containing TAP medium and resuspended in 1 mL of TAP medium containing 0.15 M sucrose. Ten microliters of the resuspended cells was rapidly frozen in an HPM100 high-pressure freezer (Leica Microsystems,www.leica-microsystems.com). The frozen samples were freeze-substituted at 80°C in anhydrous acetone containing 2% OsO_4 for 2 d, and excess OsO_4 was removed by washing with pre-cooled anhydrous acetone at -80°C. The samples were slowly warmed to room temperature and embedded in EMBED 812 resin (Product number 18109, TedPella, Redding, CA, USA; www.tedpella.com). Ultramicrotomy and imaging were performed as described by Kang (2010).

Starch Assay

To extract starch, cells were grown in TAP medium for 5 d and harvested by centrifugation (3000 *g*, 16°C, 5 min). Cellular starch was extracted using a Starch Assay Kit (ab83393, Abcam, Cambridge, MA, USA; www.abcam.com), following the manufacturer's protocol. Extracted starch content was measured using an enzymatic and spectrophotometric assay (Smith et al. 2006).

Analysis of Amino acid Sequence Similarity

Amino acid sequences were obtained from Phytozome (http://www.phytozome.net) and NCBI Genbank (http://www.ncbi.nlm.nih.gov/genbank/). The amino acid sequences were aligned using the MUSCLE algorithm (Edgar 2004) and their similarities were analyzed using the Neighbor-Joining method (Saitou et al. 1987). The phylogenetic tree was constructed and alignments were made using MEGA 6.0 (Tamura et al. 2013) and Jalview (http://www.Jalview.org). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl et al. 1965). To predict transmembrane helices, TMHMM (Sonnhammer et al. 1998) was used and conserved protein motifs were predicted using the NCBI CCD Database (http://www.ncbi.nlm.nih.gov/cdd/).

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Author's Contributions

SHJ performed mutant screening and analyses of lipid accumulation and growth of *crmex1-2*; TK, KK and IN performed starch analysis; SHJ and BHK performed TEM; SHJ, YY, WYS, JUH and YL designed the experimental plans, and analyzed data. SHJ, YY, DK, WYS, IN, JUH and YL wrote the manuscript; all the authors agreed on the contents of the paper and post no conflicting interest.

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