Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

The G-protein alpha-subunit gene *CGA1* is involved in regulation of resistance to heat and osmotic stress in *Chlamydomonas reinhardtii*

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Received July 4, 2016; **Accepted** February 20, 2017; **Published** February 28, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.2.5

Abstract: In eukaryotic cells, many important functions of specific G-proteins have been identified, but microalgal G-proteins are poorly studied. In this work, we characterized a gene (*CGA1*) encoding the G-protein α-subunit in *Chlamydomonas reinhardtii*. Independent knockdown mutants of *CGA1* were generated via RNA interference (RNAi). *CGA1* expression levels were consistently and significantly reduced in both independent *CGA1* mutant cell lines (*cga1*). Both *cga1* mutants had a higher survival rate at 35°C in comparison with the wild type. This stronger resistance of the *cga1* mutants became more evident during simultaneous exposure to heat and osmotic stress. The stronger resistance of the *CGA1* knockdown mutants to the two stressors was accompanied with significant morphological alterations—both cell size and cell wall thickness were different from those of the wild type. This finding supports the roles of *CGA1* in *C. reinhardtii* morphology in response to stressors. To further understand biochemical mechanisms of the *CGA1*-mediated resistance, we thoroughly analyzed the level of reactive oxygen species (ROS) and the expression of several heat shock proteins or MAP kinase genes as possible downstream effectors of *CGA1*. Our data clearly indicated that *CGA1* is implicated in the regulation of resistance to heat or osmotic stress in *C. reinhardtii* via *HSP70A* and *MAPK6*. Because the G-protein α-subunit is highly conserved across microalgal species, our results should facilitate future biotechnological applications of microalgae under extreme environmental conditions.

Key words: *CGA1*; ROS; RNAi; Heat stress; Osmotic stress.

Introduction

Every eukaryotic organism including microalgae should perceive various environmental cues and react correctly in order to survive or adapt under different circumstances. To this end, an extensive web of signaling pathways within cells is mainly responsible for creating responses to external environmental stimuli. G-protein-mediated signaling is a major signaling pathway in eukaryotic organisms and plays a pivotal role in processing of the information from the external environment. Therefore, substantial efforts have been devoted to elucidation of G-protein signaling across different species.

The genes encoding heterotrimeric GTPases (G-proteins) have been characterized in various eukaryotic cells, and varied functional roles of G-proteins have been revealed (1, 2). Well-conserved heterotrimeric G-proteins in eukaryotic cells are composed of three subunits: α, β, and γ. The receptors called G-protein-coupled receptors (GPCRs), which span the lipid bilayer of the cell membrane seven times, drive activation of heterotrimeric G-proteins. When signaling molecules bind to GPCR, GPCR undergoes a conformational change, which in turn allows it to interact with specific heterotrimeric G-proteins for subsequent activation of these proteins. In the inactivated state, the G_α subunit is bound to GDP. By contrast, the G_α subunit replaces GDP with GTP when stimulated by GPCR. This "on" state of the G_α subunit enables dissociation of the heterotrimeric G-protein into the G_α subunit and the βγ dimer complex. Both the G_α subunit and the βγ dimer can regulate a wide variety of downstream proteins that transmit important signals within eukaryotic cells. For example, both the G_α subunit and the βγ complex affect multiple signaling pathways such as those involving adenylyl cyclase, phosphodiesterase, and ion channels (3, 4, 5).

In plants, the first α-subunit of a G-protein was isolated and cloned from *Arabidopsis thaliana* (AtGPA1) in 1990 (6), and then researchers started cloning G proteins from many other species (7, 8). The physiological functions of the G_α subunit were determined using transgenic strains and loss-of-function mutations. G-proteins in plants perform multiple functions in oxidative stress response, defense against fungal pathogens, stomatal opening/closure, seed germination, sugar perception, and several cryptochrome/phytochrome-mediated responses (5). In rice, a G-protein α-subunit-deficient line was found during screening for defective gibberellin (GA) responses (9). In addition, several results suggest that either cytokinin (10) or the abscisic acid (ABA) signaling is activated via a G-protein pathway (11).

Microalgae are a diverse group of photosynthe-

tic microorganisms, and the majority can be classified as eukaryotic organisms. Moreover, land plants have derived from microalgae as an evolutionary ancestor. Therefore, most of the key signaling pathways such as G-protein signaling may be conserved between microalgae and plant species. Despite the crucial importance of microalgal G-proteins, functional characterization has not yet been performed.

In this study, we characterized a gene (*CGA1*) encoding a G-protein α-subunit in *Chlamydomonas reinhardtii*. The green alga *C. reinhardtii* is a model organism that has been extensively studied in the pursuit of biological knowledge about chloroplast biology, photosynthesis, flagellar motility, and lipid metabolism for biodiesel production (12). Moreover, *C. reinhardtii* has been widely exploited in technical applications because of its amenability to genetic manipulations (13). Accordingly, we selected *C. reinhardtii* as a target microalgal organism for characterization of the G-protein α-subunit. Here, we report isolation and functional characterization of a cDNA clone encoding a G_α subunit (*CGA1*) from *C. reinhardtii*. The deduced amino acid sequence shares high homology with other known eukaryotic G-protein α-subunits, such as those from *A. thaliana* or *Chara braunii*. Independent knockdown mutants of *CGA1* were generated via RNA interference (RNAi). *CGA1* expression levels were consistently and significantly reduced in both independent *CGA1* mutant cell lines (*cga1*). Both of the *cga1* mutants exhibited a higher survival rate at 35°C in comparison with the wild-type. This stronger resistance of the *cga1* mutants became more evident during simultaneous exposure to heat and osmotic stress. The stronger resistance of the *CGA1* knockdown mutants to the two stressors was accompanied with significant morphological alterations—both cell size and cell wall thickness were different from those of the wild type. To understand the biochemical mechanisms of the *CGA1*-mediated resistance, we thoroughly analyzed the level of reactive oxygen species (ROS) and the expression of several heat shock proteins as possible downstream effectors of *CGA1*. Our data clearly indicate that *CGA1* is associated with the regulation of resistance to heat or osmotic stress in *C. reinhardtii* via *HSP70A* and *MAPK6*. Because the G-protein α-subunit must be highly conserved across different microalgal species, our results should facilitate future biotechnological applications of microalgae under stressful environmental conditions.

Materials and Methods

C. reinhardtii strains and culture conditions

We used *C. reinhardtii* strain CC-125 as a wild-type control. The independent G_α mutants (*cga1-1, cga1-2*), whose expression of *CGA1* (encoding the G-protein α-subunit) is significantly down-regulated by RNAi, were generated and validated. The strains were routinely maintained in Tris-acetate-phosphate (TAP) agar plates, and inoculums were prepared with the certain number of cells (2×10^6 cells/ml) from 4-day-old exponentially growing seed culture. Basically, the strains were cultivated in 500-mL Erlenmeyer flasks containing 150 mL of the sterile TAP medium at 25˚C. To impose heat stress, cultivation temperature was raised

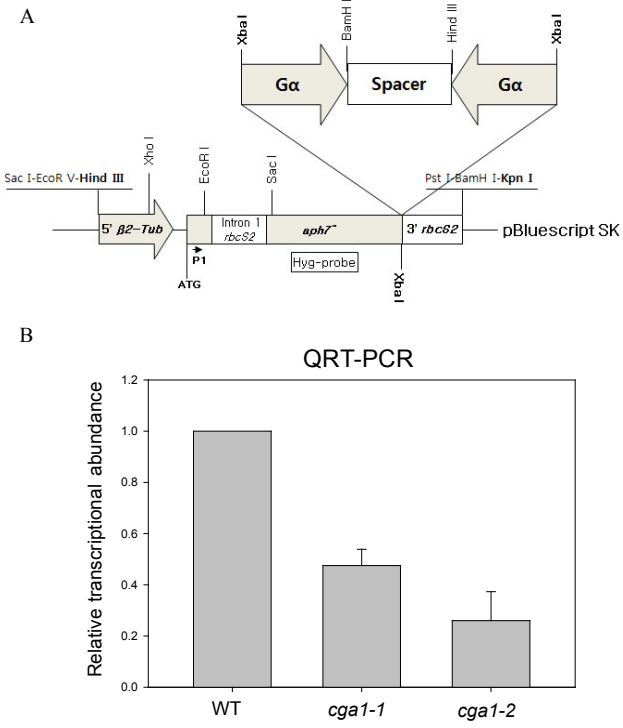

Figure 1. The strategy for generation of a gene knockdown construct and validation of independent *CGA1* knockdown mutants by means of quantitative real-time PCR (qRT-PCR). A. The *CGA1* knockdown construct was synthesized according to the small hairpin RNA interference (shRNAi) principle (24). In order to make the effect of RNA interference, the coding region of *CGA1* was repeated inversely including the spacer region. The resultant construct generates the structure of hairpin of *CGA1*, thereby causing the down-regulation of *CGA1* transcripts. B. *CGA1* downregulation in the *CGA1* knockdown mutants was confirmed by qRT-PCR with *CGA1*-gene specific primers (Table 1). Two independent mutants were obtained and designated as *cga1-1* and *cga1-2*.

to 35°C. Osmotic stress was given by supplementing 0.33 M sorbitol additionally to the sterile TAP medium. The culture flask was shaken at 200 rpm to ensure sufficient aeration. Continuous illumination was provided at the average intensity of 150 μE m^{-2} s^{-1}. All assays were repeated at least three times.

Vector construction and transformation

The gene knockdown construct for *CGA1* (XM_001691429.1) was created according to the principle of small hairpin RNA interference (shRNAi), with a palindromic sequence (14). The coding region of *CGA1* cDNA was composed of the palindromic repeat region 5′-TCTAGAATCCTCTACAAGCTGAAGC-TGGGCGAAATCGTGACGACTATTCCCAC-CATCGGCTTCAACGTGGAGACTGTGGAGTA-CAAGAACATTAGCTTCACCGTGTGGGATGTCG-GTGGCCAGGACAAGATCCGCCCTCTGTGGCG-GCACTACTTCCAGAACACTCAGGGCCTCATT-3′ and the spacer region 5′-AATTCAGATGTAGGAGG-CACAGGAAGAGCAAAAGGCTTATGAAGATT-TACGGAGAAGCAAATTTGCATGGCTGGTACA-TGGGGCGGGAGGGAGCGGGCGCAGGGCCG-GCGGGCATGTGCGTGATCATGAGGCAAGG-CACCAGCAGAAGCAGATGCTATGTAACAACG-CAAGGGCAAGAGTGTCAGAAGTCACGAGGAA-TAGAAGGATGCTTGAGGATGCATCACGAATT-3′

Table 1. The list and description of the primers.

Target	Primer name	Nucleotide sequence (5′→3′)
G_α	Ga1F3	AGCTGAAGCTGGGCGAAATCGTGACG
	Ga1R3	ACTGCAAGGAAGGCTGATGTGGACCC
	p300F01	ACTTCGCCCGCGAACTGCTCGCCTTCA
	Ga1R1	GTGTTCTGGAAGTAGTGCCGCCACAG
HSP70A	HSP70AF1	GCCCGAGATCGTTGTCTCCTACAAG
	HSP70AR1	CGGGGTTGATCGACTTGTTCAGCTC
MAPK6	MAPK6F1	CATCCAGGTCAAGGACGTGCTCAAG
	MAPK6R1	GCTTCAGCGCCTGGATGACGTCAAT
Actin	ActF1	CTGGCACCACACCTTCTTCAACGAGC
	ActR1	CAGCTTCTCCTTGATGTCGCGCACGA

(the restriction sites are underlined). This construct was digested with XbaI and inserted into the XbaI site of the expression vector pCr300 (15). The construct also contains the β2-tubulin promoter (β2-Tub), hygromycin resistance gene (*aph700*), and the rbc terminator (Fig. 1A). The completed *CGA1* construct was introduced into the wild-type strain CC-125 according to the existing protocol (16, 17, 18).

The transformants were allowed to grow in the dark without shaking for 2 days and then were transferred onto a selective agar medium supplemented with hygromycin (15 μg mL^{-1}). The plates, including control plates, were subsequently incubated under dim light for 2 weeks (16). Once we obtained hygromycin-resistant transformants, we screened them for mutants showing *CGA1* down-regulation.

RNA preparation and cDNA synthesis

Cells of wild-type and *cga1* independent mutants were grown for 4 days under normal or heat or osmotic stress condition. Then, wild-type and *cga1* independent mutants were harvested and subsequently prepared for RNA extraction. Total RNA was extracted from 100 mg of algal culture with the TRIzol Reagent (Invitrogen) in accordance with the manufacturer's protocols. The cDNA was synthesized by means of the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Briefly, 5 μg of total RNA, 10 μL of 2× RT buffer and 20× enzyme mixture were mixed in 1.5 mL reaction tube and incubated for 60 min at 37°C, and then the reaction was stopped by heating for 5 min at 95°C. Resultant cDNA was utilized for *CGA1* cloning and quantitative real-time PCR (qRT-PCR). All primers that we used in this study are listed in Table 1.

The qRT-PCR analysis

cDNA was amplified with the SYBR Select Master Mix for CFX (Applied Biosystems) using Rotor-Gene 6000 Real-Time System (Qiagen). The amplification was carried out by means of 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. For detection of *CGA1* expression, we used the primers 5′-AGCTGAAGCTGGGCG AAATCGTGACG-3′ and 5′-ACTGCAAGGAAGGCTGATGTGGACCC-3′; 5′-CTGGCACC ACACCTTCTTCAACGAGC-3C and 5a-CAGCTTCTCCTTGATGTCGCGCACGA-3C were used for the actin gene (Table 1) as the endogenous control. The gene expression was calibrated by $2^{-\Delta\Delta Ct}$ method. The range of expression was calibrated using $2^{-\Delta\Delta Ct-s}$–$2^{-\Delta\Delta Ct+s}$, where s is the standard deviation of ΔCt value (Ct = Threshold Cycle).

The survival assay

We compared the effects of high temperature (35°C) and osmotic (TAP with 0.33 M sorbitol) stresses on survival between the wild type and the *CGA1* mutants (*cga1-1* and *cga1-2*). For this purpose, the wild type and the *CGA1* mutants (*cga1-1* and *cga1-2*) were first cultured at 25°C in liquid TAP medium until stationary phase. After that, exactly the same concentration of algal cells were either spread on an agar plate or resuspended in the liquid media (TAP, and TAP + 0.33 M sorbitol) and then incubated either at 25°C or at 35°C.

In order to measure survival on agar plates, the wild-type and *cga1* were prepared at the exponential stage of growth. Exactly same numbers of cells (10 X 10^4 cells) were prepared and plated on either normal TAP or osmotic stress media under 25°C or 35°C. After incubating for 7 days, the percentage of survival was calculated by dividing the number of colonies formed on each stress conditions by those on plates incubated on the normal TAP media at 25°C. All assays were repeated three times.

ROS staining

In situ detection of the superoxide radical was performed in algal cultures by means of nitroblue tetrazolium (NBT; cat. # N6876, Sigma–Aldrich) staining, according to a protocol described previously (19). NBT assay is a well-established standard method to measure the extent of reactive oxygen species produced by cells (20, 21). More than three biological repeats were performed. Cultures grown in the TAP, TAP with 0.33 M sorbitol for 5 days were centrifuged and resuspended in 0.2% NBT. All samples were adjusted to certain number of cells (20 × 10^4 cells/ml). After incubation for 12 h, the cells were centrifuged again and resuspended in 10 mL of 50% glacial acetic acid. For quantification, a sonicator was used to lyse the stained cells and the concentration of ROS was assessed by measuring absorbance at 560 nm using a microplate spectrophotometer (PowerWave XS, BioTek).

Transmission electron microscopy (TEM)

A 100-μL culture of each strain wild type, *cga1-1*, and *cga1-2* was fixed according to a modified Karnovsky fixative protocol. The cells were washed once with 0.05 M sodium cacodylate buffer (pH 7.2) and postfixed

with 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2). Subsequently, the samples were washed twice and then stained with 0.5% uranyl acetate at 4°C for 30 min. Dehydration in graded ethanol solutions (10 min in each solution) was performed after staining. A transition step was then performed: two incubations with 100% propylene oxide. Solutions of propylene oxide and Embed 812 were used for infiltration. The samples were cut on an ultramicrotome after polymerization at 60°C for 48 h. Finally, the slices were stained before examination by means of TEM (H-7650, Japan). Cell size as well as cell wall thickness were measured based on the computer program (GATAN ES1000W, USA) installed with TEM. Average values were obtained from more than 10 replications of measurements. The data obtained were subsequently statistically analyzed using SAS (ver. 9.3; SAS Institute Inc., Cary, NC, USA).

G_α inhibitor treatment

In order to further verify the impact of down-regulation of *CGA1* gene in *C. reinhardtii*, we attempted to utilize the inhibitor specifically designated to suppress the activity of G-protein α-subunit. G_α inhibitor was purchased from Novus Biologicals (NBP1-72466, Colorado, U.S.A.). The wild-type strain of *C. reinhardtii* CC-125 was subjected to the treatment of G_α inhibitor. To examine the putative effect of G_α inhibitor to the wild-type *C. reinhardtii*, *C. reinhardtii* CC-125 was routinely maintained in tris-acetate-phosphate (TAP) agar plates. Inoculums were prepared from 4-day-old exponentially growing seed culture. The strains were cultivated in 15-mL test tube containing 3 mL of the sterile TAP medium or osmotic stress medium (TAP with 0.33 M sorbitol) at 25°C or 35°C, respectively. G_α inhibitor was dissolved in Tris-HCl solution and treated with the certain concentration (3.3 μg mL^{-1}). The culture tube was shaken at 200 rpm to ensure sufficient aeration. Continuous illumination was provided at the average intensity of 150 μE m^{-2} s^{-1}. All assays were repeated at least three times.

Results

The *CGA1* gene has low homology with other known eukaryotic cells

We searched for a G-protein α-subunit gene in *C. reinhardtii* in genomic databases (http://genome.jgi-psf.org/chlamy/chlamy.info.html and http://www.ncbi.nlm.nih.gov/pubmed/). This gene encodes a small ARF-related GTPase, in particular, in the sequence starting at nucleotide position 543. However, conserved G_α domain was identified in small ARF-related GTPase. Analysis of the sequence revealed that it is homologous to various G-protein α-subunit genes among various algae and eukaryotic cells. We aligned the corresponding amino acid sequences of *CGA1* from *C. reinhardtii* with the sequences of the *A. thaliana* G-protein α-subunit, *GPA1* (GenBank accession # NP_180198.1), *Chara braunii* G-protein α-subunit, *GPA1* (GenBank accession # AHB52757.1) and *Oryza sativa* G-protein α-subunit isoform X1 (GenBank accession# XP_015639183.1). Analysis of the *CGA1* sequence showed that the CGA1 protein shares 21.55% homology with *GPA1* of *A. thaliana*, 29% with *O. sativa* and 21.55% with *GPA1* of *C. braunii* (according to http://www.ebi.ac.uk/Tools/msa/clustalw2/ and http://www.ch.embnet.org/software/ClustalW.html; Fig. 2). Further *in silico* analysis revealed that five characteristic G box domains of G_α domain are present in *CGA1(GenBank accession # XP_001691481)* of *C. reinhardtii* (Fig. 2). Therefore, we concluded that *CGA1* in *C. reinhardtii* indeed encodes a homologous protein with G_α subunit.

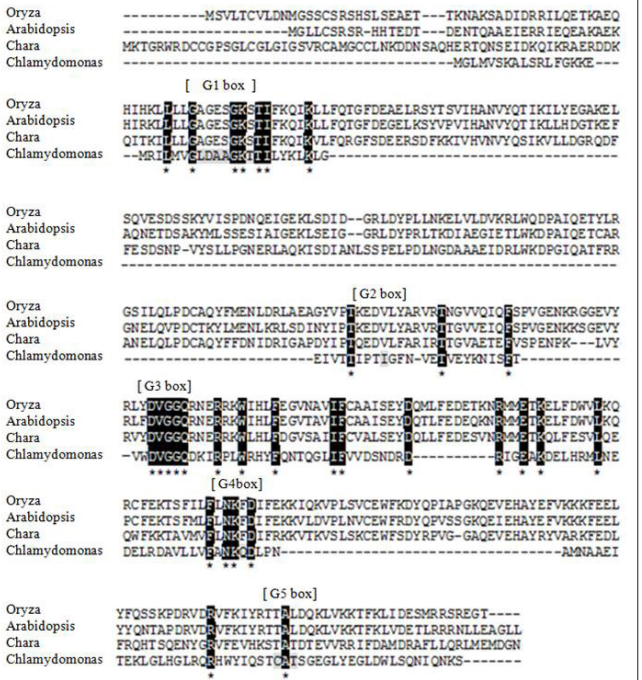

Figure 2. Alignment of the amino acid sequence of the putative G_α subunit protein encoded by *CGA1* in *Chlamydomonas reinhardtii* with known G_α subunit proteins from *Arabidopsis thaliana, Oryza sativa* and *Chara braunii*. *CGA1* in *C. reinhardtii* harbors five characteristic G box domains of typical G_α protein.

CGA1 knockdown mutants were generated via RNAi

To test the functional roles of *CGA1* in *C. reinhardtii*, we generated *CGA1* knockdown mutants using the reverse-genetics approach of RNAi (22, 23). Among 97 hygromycin-resistant transformants, two independent *CGA1* knockdown mutants were identified by PCR screening with primers p300F01 and Ga1R1 (Table 1). The primers p300F01 and Ga1R1 are located in the *CGA1* ORF and in the RNAi vector, respectively, and the resultant *CGA1* knockdown mutants should incorporate the gene knockdown construct containing a hygromycin phosphotransferase (*HPH*) gene as a selection marker. Accordingly, we identified putative knockdown mutants of *CGA1* by size of the amplicons resulting from the *CGA1* gene knockdown construct. The wild-type strain did not yield the mutation-specific PCR band, whereas the *CGA1* knockdown mutants yielded the expected 308-bp (data not shown). Successful transformation was further confirmed with another set of primers, Ga1F4 and Ga1R3 (Table 1), located within the *CGA1* ORF. Wild-type cells showed a normal expression level of the *CGA1* ORF, whereas the *CGA1* gene knockdown mutants showed reduced expression levels of the *CGA1* ORF (Fig. 1A). We designated the two independent mutants as *cga1-1* and *cga1-2*. These *CGA1* mutants, *cga1-1* and *cga1-2* expressed 2.4-fold and 4.2-fold lower levels of *CGA1* mRNA, respectively, than did the wild-type strain according to the qRT-PCR

Table 2. Cell survival on solid media. The wild-type and *cga1* were prepared at the exponential stage of growth. Certain cell numbers (10 x 10^4 cells) were prepared and plated on either normal TAP or osmotic stress media under 25°C or 35°C. After incubating for 7 days, the percentage of survival was calculated by dividing the number of colonies formed on each of stress conditions by those on plates incubated on the normal TAP media at 25°C.

	TAP medium					Osmotic stress			
	25°C		35°C			25°C		35°C	
Wild type	100	0	9.19	0	Wild type	36.49	±4.97	46.76	±3.44
cga1-1	100	0	41.80	0	*cga1-1*	68.03	± 8.11	113.93	±41.73
cga1-2	100	0	53.79	±16.07	*cga1-2*	143.94	±21.43	145.96	±20.18

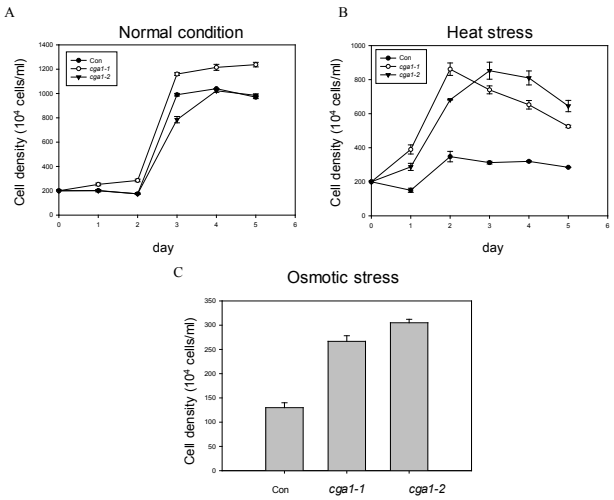

Figure 3. Comparison of growth between the wild type and the *CGA1* knockdown mutants under normal (A), heat (B), or osmotic stress conditions (C). Heat stress was imposed by incubating the wild type and the *CGA1* knockdown mutants at 35°C and osmotic stress was given by 0.33 M sorbitol.

(Fig. 1B).

The *CGA1* knockdown mutants had a high survival rate under conditions of heat or osmotic stress

Once *CGA1* knockdown mutants were generated, we began to compare the various phenotypic characteristics with those of the wild-type progenitor. One phenotypic feature that we identified in the *CGA1* knockdown mutants was the resistance to stressful conditions on agar plates. Down-regulation of *CGA1* increased the survival rate on agar plates under the stressful conditions in comparison with the wild type (Table 2). This resistance to stressors was consistent between the two independent *cga1* knockdown strains (Table 2). Particularly, the *cga1* mutants showed a >16.6-fold higher survival rate than did the wild type at 35°C. Under osmotic stress with 0.33 M sorbitol, survival rates of the two independent *cga1* knockdown strains were increased 2- to 4-fold compared to that of the wild type (Table 2). Osmotic stresses were also imposed with a variety of different sources, including nitrogen surplus, sucrose, NaCl and sorbitol. Regardless of different osmotic sources, there was no significant difference in the trend of higher survival ratio of *CGA1* mutants than those of the wild-type (data not shown). Therefore, the resistance to stressors such as heat or osmotic shock was consistently observed in both independent *CGA1* knockdown strains. To confirm this phenomenon, we also applied the same extent of either heat or osmotic stress during liquid cultivation and obtained similar results: the survival rates of the *cga1* knockdown strains were 2.0-fold and 2.3-fold higher, respectively, than the survival rate of the wild type (Fig. 3). We concluded that the down-regulation of *CGA1* in *C. reinhardtii* rendered the microalgal cells resistant to stressors. Our results clearly showed that the *CGA1* gene in *C. reinhardtii* is a negative regulator of stress responses.

The *CGA1* knockdown mutants had low ROS levels under the stressful conditions

Because ROS generation has been well documented in cellular systems during environmental stress, we speculated that the extent of ROS production during stress must be different between the wild type strain and the *CGA1* knockdown mutants. To test our hypothesis, we measured the levels of ROS in the wild-type strain and *CGA1* knockdown mutants using the NBT staining protocol, which is specifically designed to detect ROS. Consistent with our hypothesis, we clearly detected alterations in ROS concentration between the wild type and the *CGA1* knockdown mutants. Under normal conditions, there was no significant difference in ROS levels between the wild-type strain and the *CGA1* knockdown mutants. In contrast, under stressful conditions, the *cga1* mutants displayed significantly lower ROS levels than the wild type (Fig. 4). The ROS levels were remarkably reduced by the *CGA1* gene downregulation at a high temperature and during osmotic stress. In particular, the ROS level in the *CGA1* knockdown mutants was reduced approximately 3.2-fold and 2.7-fold under the high-temperature and osmotic-stress conditions, respectively (Fig. 4). Our finding about the alteration of

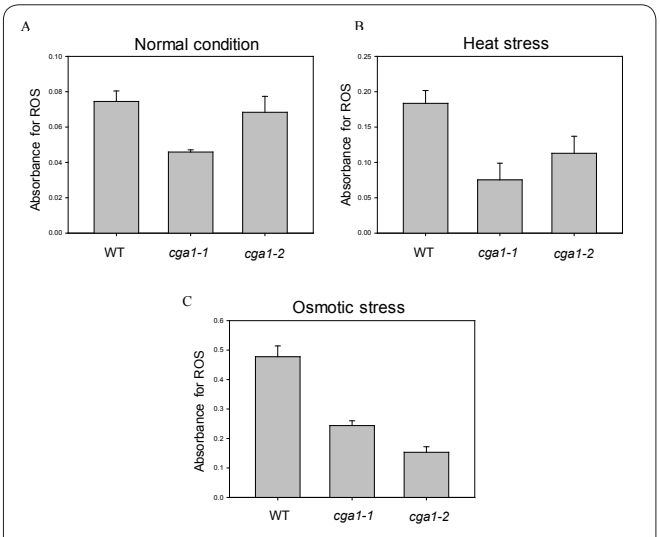

Figure 4. Cellular levels of reactive oxygen species (ROS) were measured and compared between the wild type and the *CGA1* knockdown mutants under normal (A), heat (B), or osmotic-stress conditions (C). The absorbance of ROS was monitored with a certain number of cells (2 X 10^6 cells/ml) for the normalization.

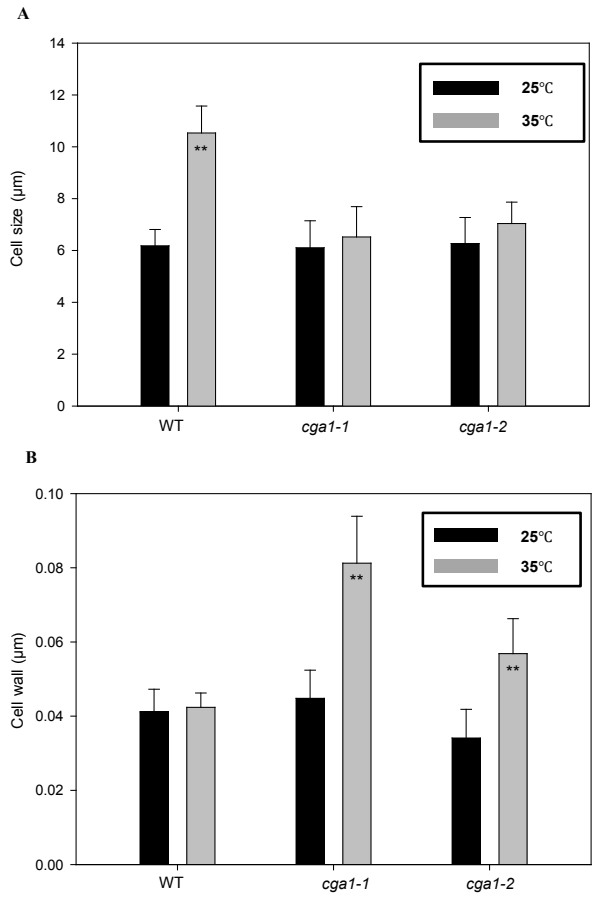

Figure 5. Comparison of cell morphology between the wild type and the *CGA1* knockdown mutants by microscopy. The cell size (A) and cell wall thickness (B) were analyzed under heat stress conditions. Significant statistical difference ($p<0.05$) was highlighted with the mark (**). (C) Transmission electron microscopy was used to compare the wild-type strain and the *CGA1* knockdown mutants and indicates death among the wild-type cells during heat stress; the *CGA1* knockdown mutant (*cga1*) survived.

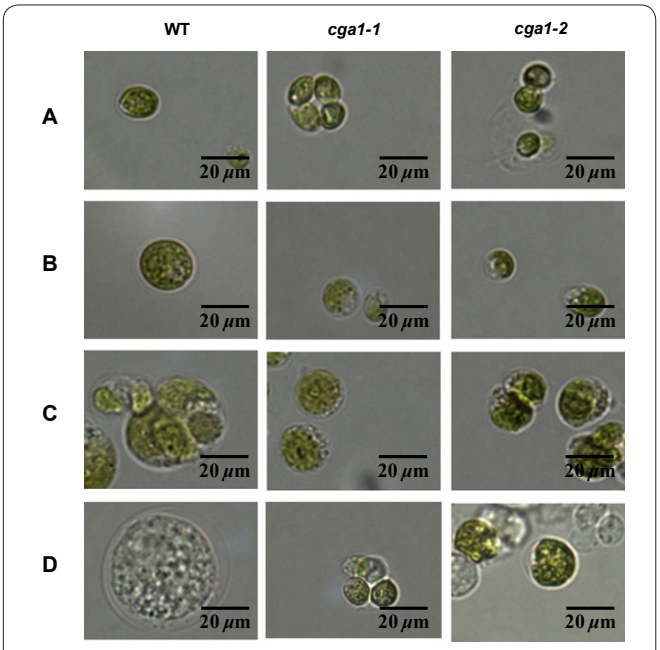

Figure 6. Microscopy images comparing the wild type and the *CGA1* knockdown mutants (*cga1-1* and *cga1-2*) under various stressful conditions. (A) The normal condition, (B) heat, (C) osmotic stress, or (D) simultaneous application of both heat and osmotic stress.

ROS levels as a result of the *CGA1* knockdown raises the intriguing possibility that *CGA1* might implicated in stress signaling in microalgae, via the alteration of cellular levels of ROS.

The *CGA1* knockdown resulted in a significant decrease in cell size but an increase in cell wall thickness under stress

To test whether down-regulation of the *CGA1* gene affects *C. reinhardtii* morphology, we analyzed cellular features under various stressful conditions. Interestingly, the two independent *CGA1* knockdown mutants (*cga1-1* and *cga1*-2) showed significant differences in both cell size and cell wall thickness compared to the wild type under stressful conditions (Fig. 5). Under normal conditions, there was no significant difference in cell size and cell wall thickness between the wild type and the *CGA1* knockdown mutants. The difference in cell size and cell wall thickness became evident after application of either heat or osmotic stress (Figs. 5 and 6). These differences became more apparent when heat and osmotic stress were applied simultaneously (Fig. 6). During both heat and osmotic stress, the wild-type cells showed increased cell size and a thin cell wall, whereas *CGA1* knock-down mutants successfully maintained their regular cell size with a thick cell wall. Furthermore, the wild-type cells eventually turned all white due to the serious damage leading to cell death, whereas the *CGA1* knock-down mutants did not lose the greenish hue completely. Examination by TEM also supported our data regarding the differences in cell size and cell wall thickness between the wild type and the *CGA1* knock-down mutants (Fig. 5C). Our data suggested that the *CGA1* gene in *C. reinhardtii* affected cellular morphology in response to stress, and altered cell morphology in *CGA1* knock-down mutants may help alleviate environmental stress rendering tolerances against stresses such as heat or osmotic stress or both.

Gene expression of *CGA1* showed significantly difference between wild-type and *cga-1* under stress conditions

In order to test the expression patterns of *CGA1*itself, the wild-type strain and two independent *CGA1* mutant strains (*cga1-1* and *cga1-2)* were grown for 4-day under either heat or osmotic stress condition. Total RNA were extracted and subject to *CGA1* expression analysis via qRT-PCR using specific primers (Ga1F3 and Ga1R3). In wild-type, the expression of *CGA1* increased dramatically under stress conditions (19-fold in osmotic stress; 3.1-fold under heat stress), whereas both in *cga1-1* and *cga1-2*, of the extent of *CGA1* transcripts did not altered (Fig. 7A). These results indicate that expression of *CGA1* gene was tightly regulated in response to stress conditions, indirectly suggesting the role of *CGA1* as a modulator of stress response in microalgae.

Genes encoding heat shock proteins (HSPs) and mitogen-activated protein kinase (MAPK) are downstream of *CGA1* in the response to the stressful conditions

To understand the mechanisms of the *CGA1*-mediated stress resistance, we used RT-PCR to analyze the impact of *CGA1* down-regulation on the expression

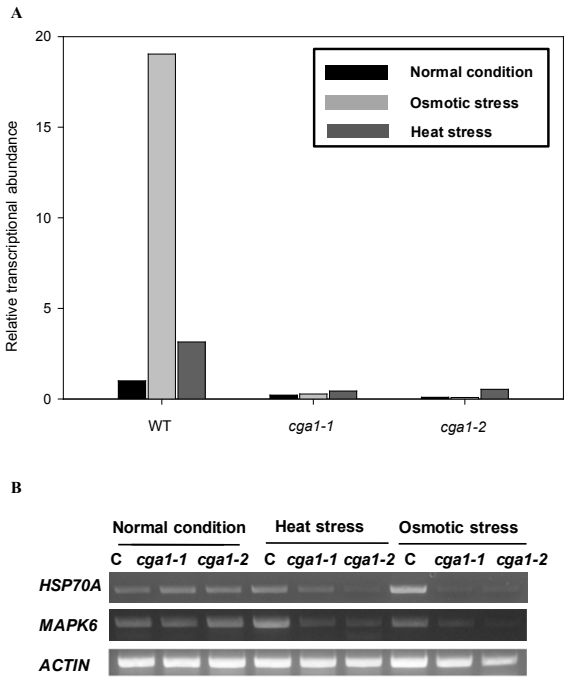

Figure 7. Gene expression of *CGA1* under stress condition and identification of downstream genes of *CGA1*. (A) Gene expression of *CGA1* under either heat and osmotic stress conditions via qRT-PCR using specific primers Ga1F3 and Ga1R3 (Table 1). (B) Differential gene expression of *HSP70A* and *MAPK6* in wild-type and *CGA1* knockdown mutants detected by RT-PCR. Expression levels of *HSP70A* and *MAPK6* fluctuated under either heat or osmotic-stress conditions and were significantly different between the wild type and the *CGA1* knockdown mutants. All samples were prepared with same amount of biomass (100 mg) after 4 day incubation. DNA bands corresponding to housekeeping gene of actin is shown as controls.

of putative downstream genes, such as genes encoding heat shock proteins (hsp) as well as several map kinases. To this end, expression of a wide variety of *C. reinhardtii* genes encoding highly conserved HSPs such as *HSP90B*, *HSP90C*, *HSP70A*, *HSP70B*, *HSP70C*, *HSP70E*, *HSP22A*, *HSP22B*, *HSP22C*, and *HSP22F* was compared between the wild-type strain and the *CGA1* knockdown mutants. Total RNA samples were prepared from microalgal biomass of the wild-type strain and the two independent *CGA1* mutant strains (*cga1-1* and *cga1-2)* grown either under normal conditions or during heat or osmotic stress at stationary stage. RT-PCR analysis revealed that among these putative downstream genes, expression of *HSP70A* was significantly affected by the knockdown of *CGA1*. The expression of *HSP70A* was correspondingly increased upon stress, whereas the expressions of *HSP70A* in *CGA1* mutants were oppositely down-regulated (Fig. 7B). Therefore, it is likely that *CGA1* affected the expression of *HSP70A* as a downstream gene in response to the stressors.

After that, we examined the expression of several genes encoding MAPKs in *C. reinhardtii* as putative downstream genes of *CGA1* because MAPKs are well known as key signaling regulators affecting stress responses in many eukaryotic cells. Among the MAPK genes tested, we identified a gene encoding the highly conserved MAPK6 protein as a likely downstream effector of *CGA1*. As with the *HSP70A* gene, the expression of the *MAPK6* gene correlated with the exposure to stress in the wild-type cells. In contrast, the expression levels of *MAPK6* in the *CGA1* mutants were down-regulated, and this down-regulation was observed consistently in both independent mutants: strains *cga1-1* and *cga1-2* (Fig. 7B). On the basis of these data, we conclude that expression of *MAPK6* was significantly affected by the knockdown of *CGA1*; this phenomenon manifested itself under the stressful conditions. Taken together, our data provide molecular evidence that both *HSP70A* and *MAPK6* are downstream genes of *CGA1*, and they are under positive regulation by *CGA1*; in particular, they respond to stress in *C. reinhardtii.*

Direct G_α inhibitor treatment to the wild-type *C. reinhardtii*

In order to further verify the functional roles of *CGA1* in *C. reinhardtii*, we employed chemical genetics with a small molecule, which binds to specific protein implicating in important signaling pathway. Therefore, the treatment with small molecule of interest leads to alter functions of particular signal transduction in living organisms. To this aim, G_α specific inhibitor (NBP1-72466, Novus) was treated directly to the culture of the wild-type *C. reinhardtii*. The treatments of G_α specific inhibitor were performed under both normal condition, heat and osmotic stress. Consistent with the stress-re-

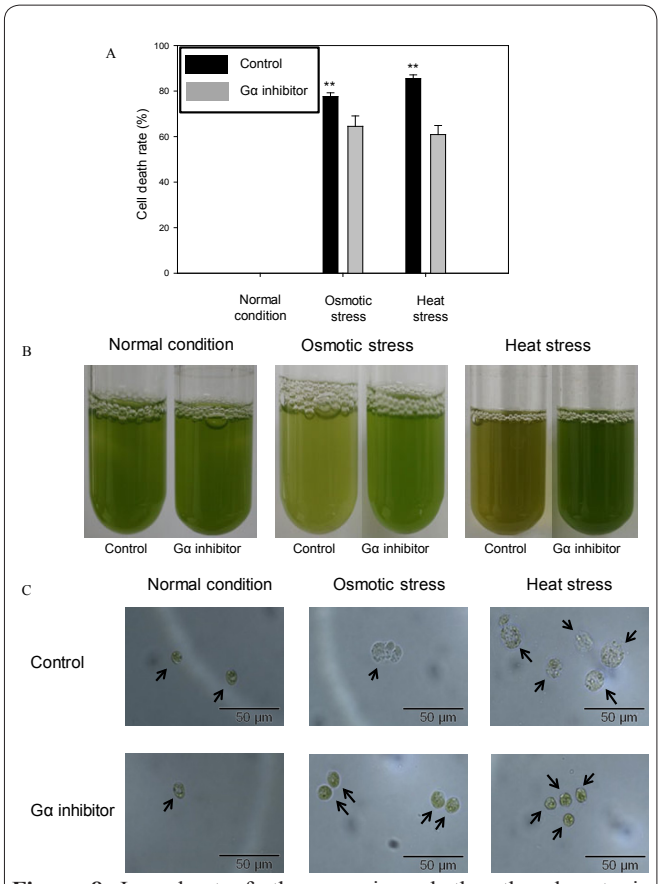

Figure 8. In order to further examine whether the phenotypic characteristics of the *CGA1* knockdown mutants is due to altered *CGA1* expression, we applied specific G_α inhibitor, which must suppress the activity of G_α proteins, to the wild-type. *In situ* treatment of G_α inhibitor to the wild-type resulted in similar resistance against stresses as with the *CGA1* knockdown mutants. Comparison of growth (A) and morphology (B and C) between control and G_α inhibitor treated wild-type *Chlamydomonas* cells under normal, heat, or osmotic stress conditions. Significant statistical difference ($p<0.05$) was highlighted with the mark (**).

sistant phenotypes of *CGA1* knock-down mutants, *C. reinhardtii* with the G_α inhibitor became more resistant to both osmotic and heat stress conditions (Fig. 8). Whereas there was no significant difference between control and G_α inhibitor-treated cells under the normal condition, G_α inhibitor-treated cells displayed significant increases in survival under osmotic and heat stress conditions (Fig. 8). G_α inhibitor treated cells showed 13% lower cell death rate than control under osmotic stress, and 25% lower cell death rate under heat stress (Fig. 8A). Furthermore, the apparent effect of G_α inhibitor treatment became evident when the color tunes of microalgal cultures were compared between control and G_α inhibitor-treated culture under the stresses (Fig. 8B). In the absences of G_α inhibitor, microalgal cultures became weak in chlorophyll and even turned to brownish color. On the other hand, G_α inhibitor treatment helped maintain the greenish color tune typical of microalgae even under the stress conditions (Fig. 8B). The effect of G_α inhibitor treatment became more evident under the microscope, as microalgal cells still did not lose their viability under the stresses compared to those without the treatment (Fig. 8C). With the imposed stresses of either heat or osmotic stress, the control cells displayed increased cell size with cell damages of whitish hues, whereas G_α inhibitor-treated cells maintained regular cell sizes with the greenish hues (Fig. 8C). In conclusion, to further validate whether the phenotypic characteristics of the *CGA1* knockdown mutants is really originated from gene knock-down of *CGA1*, a specific G_α inhibitor, which must suppress the activity of G_α proteins, was applied to the wild-type either under normal, osmotic, or heat stress conditions. *In situ* treatment of G_α inhibitor to the culture of wild-type resulted in similar resistance against stresses as with the *CGA1* knockdown mutants. Therefore, our results again supported the notion that *CGA1* gene belonging to a family of G alpha subunit implicates in stress management response in microalga *C. reinhardtii*.

Discussion

Microalgae are thought to be a promising biomass species due to their rapid growth, high lipid content, and an extensive array of secondary metabolites of high practical value. A wide variety of compounds with beneficial biological activities e.g., antioxidants and potential medications could also be obtained from microalgae. In addition, microalgae may be utilized as environmental agents because they are capable of rapid uptake of nitrogen and phosphate in waste waters. Furthermore, microalgae can fix carbon dioxide during photosynthesis. Therefore, microalgal biomass holds great promise for future biotechnological applications. Due to the importance of microalgae, the scientific community needs to gain a better understanding of the molecular mechanisms governing signal transduction in these cells.

In land plants, the G_α subunit provides an important means of regulation of various cellular functions in response to external signals. Functional roles of G_α subunit in higher plants have been well-reported. In Arabidopsis, the mutation of G_α subunit induced short hypercotyl at etiolated seedling, and fewer lateral root formation under light-grown at seedling. In rice, the mutation of G_α subunit induced dwarf, erected leaf, short panicle and short seed at mature plant (5). Because microalgae are evolutionary ancestors of land plants, it is reasonable to suppose that most of key metabolic pathways or important signaling pathways such as those involving a G-protein must be well conserved between microalgae and plant species. Despite crucial importance of microalgal G-proteins (just as in plants), only limited information is available about the functions of microalgal G-proteins.

In this study, we characterized a gene (*CGA1*) of a G_α subunit in *C. reinhardtii* by means of a gene knockdown strategy involving RNAi. *C. reinhardtii CGA1* shares homology with genes encoding a G_α subunit in other species, such as *A. thaliana* and *C. braunii* (Fig. 2). Although there is some variation in amino acid sequences, probably due to the large evolutionary divergence among these species, we observed high homology between *C. reinhardtii CGA1* and the genes corresponding to the G_α subunit in *A. thaliana* and *C. braunii*. To support the notion that *C. reinhardtii CGA1* encodes G_α subunit, we further performed *in silico* analysis revealing that *C. reinhardtii CGA1* harbors five characteristic G box domains of G_α domain (Fig. 2).

After that, we characterized the *CGA1* gene in *C. reinhardtii* with a reverse-genetics approach based on the phenotypic characteristics of knockdown of *CGA1*. Particularly, we utilized RNAi to determine the functional roles of *CGA1* in *C. reinhardtii*. RNAi is a good tool for functional studies of any gene of interest because it is a well-established powerful method for silencing the expression of a specific gene of interest (24, 25). Using RNAi, we successfully obtained two independent knockdown mutants of *CGA1* showing significant downregulation of *CGA1*, and these strains were designated as *cga1-1* and *cga1-2* (Fig. 1B).

The knockdown of the *CGA1* gene resulted in a higher survival rate in comparison with the wild-type under various stressful conditions, such as an increased temperature and osmotic shock. This tolerance to stressors was consistently observed in both independent *CGA1* knockdown strains. We also applied stress during cultivation either on agar plates or in liquid media. Regardless of the type of culture (solid or liquid medium), the stress tolerance was evident in both independent *CGA1* knockdown strains (Table 2 and Fig. 3). In particular, increased survival of the *CGA1* knockdown strains was clearly evident: it was ~4-fold higher under heat stress conditions in comparison with the wild-type strain (Fig. 3). Therefore, we could conclude that the downregulation of *CGA1* in *C. reinhardtii* renders microalgal cells more resistant to stressors. Our results clearly showed that the *CGA1* gene in *C. reinhardtii* serves as a negative regulator of stress responses involving in stress maintenance to harsh stressful conditions.

To elucidate the mechanisms of the *CGA1*-mediated resistance to stress, we compared the level of ROS between the wild type and the *CGA1* knockdown strains because ROS have been implicated in cellular activities in response to a variety of stressors. As expected, the concentration of ROS was significantly lower in the mutants than in the wild type (Fig. 4). Therefore, it is conceivable that down-regulation of *CGA1* decreased the ROS level, thereby increasing overall tolerance to

stressors. Further research is needed to pinpoint the exact mechanism of the *CGA1*-mediated stress resistance.

The *CGA1* knockdown prevented significant morphological changes under heat and/or osmotic stress conditions (Fig. 5). Under the normal conditions, there was no significant difference in cell size and cell wall thickness between the wild type and the *CGA1* knockdown mutants. In contrast, under stress such as heat, the wild-type cells enlarged, whereas the *CGA1* knockdown strains showed little or no change and both mutants maintained the normal cell size (Fig. 5 and Fig. 6). These differences became more apparent when heat and osmotic stress were applied simultaneously (Fig. 6). In general, in response to the detrimental stressors, microalgal cells enlarge, though the extent of the cell enlargement varies among microalgal species and strains. Likewise, we observed a significant change in cell size under stress in the wild-type strain. By contrast, the *CGA1* knockdown mutants did not show any significant alteration in cell size under either normal or stressful conditions (Fig. 5 and Fig. 6). It is likely that consistent cell size under both normal and stressful conditions in the *CGA1* knockdown mutants reflects the resistance or tolerance to various stressful conditions. Similarly, the *CGA1* knockdown mutants showed a thick cell wall, along with a consistent cell size even under stressful conditions (Fig. 5). Our data clearly show that the *CGA1* gene in *C. reinhardtii* is implicated in changes of cellular morphology in response to the stressors. We can speculate that the prevention of morphological alterations in *CGA1* knockdown mutants must be related to the resistance or tolerance to various stressful conditions. Further research is needed to clearly determine the possible *CGA1*-mediated microalgal morphological changes that are directly linked to the increased resistance to stress. These morphological changes in *CGA1* knockdown mutants probably help to release built-up stress, which is not the case for the wild type.

To further understand the mechanisms of the *CGA1*-mediated resistance, we attempted to identify possible downstream genes of *CGA1* during the response to stress. In search of *CGA1*-mediated resistance mechanisms, we thoroughly analyzed the expression levels of multiple candidate genes as possible downstream effectors of *CGA1*. First, we examined the expression of a wide variety of genes encoding heat-shock proteins in *C. reinhardtii*: *HSP90B*, *HSP90C*, *HSP70A*, *HSP70B*, *HSP70C*, *HSP70E*, *HSP22A*, *HSP22B*, *HSP22C*, and *HSP22F*. In particular, the expression of *HSP70A* turned out to be most sensitive to the stressors applied in this study. The expression of *HSP70A* was significantly altered by the *CGA1* knockdown, in contrast to the wild type. The expression of *HSP70A* in the wild-type strain increased with increasing stress exposure, whereas the expression levels of *HSP70A* in the *CGA1* mutants were downregulated even under stress (Fig. 7). Therefore, *HSP70A* seems to be a downstream gene of the *CGA1*-mediated stress response in *C. reinhardtii*. In addition, we examined the expression of genes encoding mitogen-activated protein kinase (MAPKs) as possible downstream effectors of *CGA1*. The rationale for studying several MAPK genes as possible downstream genes of *CGA1* is that G-protein signaling regulates various physiological and developmental processes in conjunction with the MAPK signal transduction pathway downstream of G-proteins (26, 27, 28, 29). In line with this rationale, we identified one MAPK gene (*MAPK6*) as an actual gene downstream of *CGA1*. The expression of *MAPK6* is significantly influenced by the downregulation of *CGA1*, and this phenomenon became evident under the stressful conditions (Fig. 7). Consequently, here we provide molecular evidence that both *HSP70A* and *MAPK6* are linked to the *CGA1*-mediated stress response mechanism in *C. reinhardtii*. It seems that the expression of *HSP70A* and *MAPK6* is dependent on osmotic and heat stress, respectively (Fig. 7). Another study will be necessary to elucidate the exact metabolic pathways underlying the sensitivity to osmotic and heat stress; this sensitivity is likely to be regulated by the *CGA1*-controlled *HSP70A* and *MAPK6*, respectively.

In addition, with the aim of further validating whether the phenotypic characteristics of the *CGA1* knockdown mutants is really originated from gene knock-down of *CGA1*, we employed the chemical genetic approach with a specific G_α inhibitor, which must suppress the activity of G_α proteins. G_α inhibitor was directly applied to the wild-type either under normal, osmotic, or heat stress conditions. In agreement with the phenotypic characteristics of the independent *CGA1* knockdown mutants, interestingly, the treatment of G_α inhibitor led to increased resistance against stresses, which were also observed in *CGA1* knockdown mutants. These data again supported the notion that *CGA1* gene belonging to a family of G alpha subunit implicates in stress management response in microalga *C. reinhardtii*.

In the process of microalgal fermentation using photobioreactor, it is indispensable to apply sufficient light sources to guarantee the efficient growth of microalgae. Therefore, the operation of photobioreactor, which mostly depends on a closed system, inevitably gives rise to the increase of temperature mostly due to the light illumination. Since the temperature is one of the important factors affecting the growth of microalgae, unwanted increase of temperature in the photobioreactor could led to the detrimental results, totally checking the growth of microalgae of interest. To solve these problems, the installation of equipment, such as water jacket, for lowering temperature is continuously necessary, thereby causing additional cost of the photobioreactor operation. In this regards, we contend that our discovery on microalgal G alpha subunit implicated in stress response will have the possible impact on future applications of photobioreactor at high temperature. By establishing the way to modulate G alpha protein *in vitro* or *in vivo*, it will be possible to find a way to operate the photobioreactor at higher temperature without being burdened with a cooling system. In addition, to compete with unwanted contamination with other eukaryotes, bacteria, and viruses during the process of microalgal cultivation, it will be better to shift of growth condition to unfavorable conditions such as high osmotic conditions to compete with contaminants, only if microalgal species could tolerate against the harsh conditions. Therefore, again, the modulation of microalgal G alpha subunit might provide the foundation for further application of microalgal cultivation under the stresses.

In summary, we characterized a gene (*CGA1*) enco-

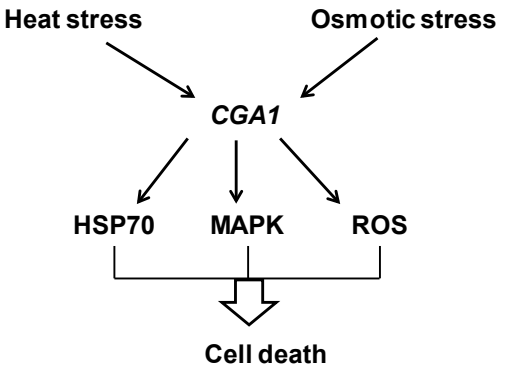

Figure 9. Schematic illustration of the *CGA1*-mediated resistance to stress in *Chlamydomonas reinhardtii*. HSP70: heat shock protein 70, MAPK: mitogen-activated protein kinase, ROS: reactive oxygen species.

ding a G-protein α-subunit in *C. reinhardtii* via RNAi. Independent *CGA1* mutants were obtained, and both *cga1* mutants show higher resistance to heat or osmotic stress, in comparison with the wild type. Under stressful conditions, the *CGA1* knockdown mutants successfully maintain their regular cell size with a thick cell wall, whereas the wild type shows cell enlargement. These morphological differences that are caused by the knockdown of *CGA1* are accompanied with alterations of cellular levels of ROS. Furthermore, we provide molecular evidence that both *HSP70A* and *MAPK6* are associated with the *CGA1*-mediated stress response mechanism in *C. reinhardtii*. All of these data suggest that *CGA1* plays a central role in the regulation of stress response in *C. reinhardtii* by governing cell morphology, cellular ROS levels, and several downstream genes including *HSP70A* and *MAPK6*. Overall illustration of the *CGA1*-mediated stress response is shown in Fig. 8. Further research will be necessary to expand the knowledge about stress resistance mechanisms associated with *CGA1* in *C. reinhardtii*. Because most of signaling pathways in microalgae may be strongly evolutionarily conserved among microalgal species, our results should advance microalgal biotechnological applications for stressful environmental conditions.

Acknowledgments

This research was supported by the Korea Basic Science Institute under the R&D program(Project No. C36703) supervised by the Ministry of Science, ICT and Future Planning. This research was also supported by Korea University Future Research Grant. This work was also supported by the National Research Foundation of Korea(NRF) grant funded by the Korea government(MSIP) (2016R1D1A1B03932773).

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