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Characteristic of *Glutamate Cysteine Ligase* Gene and its response to the salinity and temperature stress in *Chlamydomonas* sp. ICE-L from Antarctica

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Abstract: *Chlamydomonas* sp. ICE-L, an Antarctic ice alga, has high tolerance ability to freezing and salinity. Glutathione (GSH) is an important small antioxidative molecule in the growth and stress responses of plants including algae. We cloned a full-length cDNA encoding glutamate cysteine ligase (ICE-LGCL), the key enzyme of GSH synthesis, from *Chlamydomonas* sp. ICE-L by RT-PCR and rapid amplification of cDNA ends technique (RACE). The cDNA has 2199 bp nucleotides with an open reading frame (ORF) of 1452 bp encoding a polypeptide of 453 amino acids. BLASTP algorithm results showed that ICE-LGCL shared 51%–70% amino acid sequence identity with the reported GCLs and shared the highest identity with *Chlamydomonas reinhardtii.* We also successfully made ICE-LGCL protein express in *E. coli* BL21. The optimum expression conditions are the induced reagent IPTG of 0.2 mmol/L, temperature of 37 °C, and the induced time of 4 h. The expression patterns of *ICE-LGCL* in mRNA by real-time PCR analysis showed that *ICE-LGCL* expressed under the different temperature and salinity challenges. Low temperature and low salinities stimulated the accumulation of ICE-LGCL mRNA in ICE-L cells. These results indicate that GCL might play an important role in Antarctic *Chlamydomonas* sp. ICE-L.

Key words: *Chlamydomonas* sp. ICE-L, glutamate cysteine ligase (GCL), temperature, salinity, expression analysis, prokaryotic expression

1. Introduction

Glutathione (GSH) is an important low molecular weight intracellular tripeptide thiol composed of glutamate, cysteine, and glycine. Synthesis of GSH is a two-step process catalyzed by glutamate-cysteine ligase (GCL) and glutathione synthetase (GS), respectively. GCL is the rate-limiting enzyme (also known as γ-glutamylcysteine synthetase) as it is subject to feedback inhibition by the overproduct of GSH. In mammals, GCL is heterodimeric protein consisting of catalytic and regulatory subunits (Griffith, 1999), but it is a single polypeptide in many lower organisms. The catalytic mechanism of GCL has been disclosed to be the initial activation of the γ-carboxyl group of L-Glu by ATP-phosphorylation to form a γ-glutamylphosphate intermediate, followed by reacting with L-Cys to generate a tetrahedral transition state. Then the phosphate is eliminated to yield γ-glutamylcysteine, which is ligated with glycine by GS in the presence of ATP. As a key enzyme of GSH synthesis, the genomic genes or cDNAs encoding GCL have been determined in many organisms including *Saccharomyces cerevisiae* (Ohtake and Yabuuchi, 1991), *Plasmodium berghei* (Sharma and Banyal, 2009), mouse (Reid et al., 1997), annelid polychaete (Sandrini et al., 2006), *Chorispora bungeana* (Wu et al., 2009), *Anabaena* sp. PCC 7120 (Hiroyuki et al., 2005), and so on.

Glutathione peroxidase (GPx) is an important antioxidative enzyme, and can reduce H_2O_2 produced intracellularly as a byproduct of oxidative metabolism. However, the capacity of GPx to reduce H_2O_2 is highly dependent upon the availability of its antioxidant cofactor GSH. Detoxification of peroxides by GPx can result in the depletion of GSH through its conversion to glutathione disulfide (GSSG). Thus the pool of free GSH can be diminished by the acute peroxidative challenge, leading to a variety of detrimental effects including oxidation of protein and DNA, DNA strand breaks, and so on (Reed and Fariss, 1984).

In plants, GSH has been found to have multiple functions during growth and development, such as the regulation of cell division, tracheary element differentiation, anthocyanin accumulation, and the

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regulation of sugar metabolism. It also plays an important role in the adaption of plants to biotic and abiotic stresses (Anderson et al., 1985; May et al., 1998a, 1998b). Low temperature, salt stress, heavy metal exposure, and the other stresses can lead GSH to accumulate, which can help cells to challenge the stresses (Kocsy et al., 1996, 1997, 2000; Ruiz and Blumwald, 2002; Mittova et al., 2003).

Organisms can protect against oxidative stresses by inducing the expression of antioxidative enzymes or the related transcription factors, for example via the activation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (*Nrf* 2) (Eggler et al., 2008). *Chlamydomonas* sp. ICE-L is an ice alga living in Antarctica, which has freezing, intense ultraviolet radiation, and unstable salinity. Our previous study showed that the GSH-related enzymes (glutathione *S*-transferase, glutathione reductase, and glutathione peroxidase) played important roles in *Chlamydomonas* sp. ICE-L to adapt to the Antarctic environment (Ding et al., 2005, 2007, 2012). The increasing expression of *GCL* contributes to organisms' antienvironmental stresses (Noctor et al., 1998). For example, the levels of GCL mRNA increased in *Brassica juncea* when it was exposed to heavy metals (Schäfer et al., 1997). Hyperthermic stress induced increase in the expression of *GCL* and GSH levels in the symbiotic sea anemone *Aiptasia pallida* (Sunagawa et al., 2008). Overexpression of *E. coli GCL* in plants improved the tolerance to cadmium and arsenic (Zhu et al., 1999, Dhankher et al., 2002), and Indian Mustard overexpressing GCL exhibited higher Cd tolerance and GSH accumulation (Reisinger et al., 2008). When *Chorispora bungeana* was exposed to −4 °C, the transcript levels of *GCL* increased rapidly (Wu et al., 2009). Here, to understand further the intrinsic adaptation mechanism of Antarctic sea ice alga to the environmental stimulations and to evaluate the role of GCL in the cellular responses against the environmental stimuli in *Chlamydomonas* sp. ICE-L, the full-length cDNA sequence of GCL in *Chlamydomonas* sp. ICE-L was cloned and characterized by RT-PCR and RACE technique, and *GCL* gene expression level was also analyzed under different temperature and salinity stresses in the present study. ICE-LGCL protein was successfully expressed in *E. coli* BL21 (DE3) as well.

2. Materials and methods

2.1. Chemicals and other experiment materials

Common laboratory reagents used for cloning and real-time PCR were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). 5′/3′ RACE Kit, 2nd Generation was from Roche (Germany). TRIZOL Reagent was from Invitrogen (Carlsbad, CA, USA). *DNase*I was from NEB (USA). DAB was from Boster (Wuhan, China). Anti-His antibody and horseradish peroxidase were from Tiangen Biotech Co. Ltd. (Beijing, China). NC membrane was from PALL.

E. coli DH5α, pET-28a(+), and *E. coli* BL21 (DE3) were conserved by Guangdong Provincial Key Lab of Pathogenic Biology and Epidemiology for Aquatic Economic Animals.

2.2. Algae and culture condition

Chlamydomonas sp. ICE-L was obtained from the Antarctic pole region (68°30′E, 65°00′S; 71°45′E, 66°15′S) by Key Laboratory of Marine Bioactive Substance, the First Institute of Oceanography, State Oceanic Administration, China. Algae were cultured in Provasoli medium (Provasoli, 1968) in flasks, with temperature of 6–8 °C, light intensity of 40 μmol photons m^{-2} s^{-1}, and 12 h light/12 h dark in the refrigerator, and the flask was shaken 4–5 times a day. Seawater used for the culture medium was taken from the coast of Zhanjiang City, China, and the salinity was about 33. Algae were transferred to the fresh medium with 20% inoculation to maintain cells in logarithmic growth. Under the temperature treatment, algae were cultured in 0 °C and 14 °C, while 8 °C was used as the control. As to the salinity stress treatment, water or NaCl was added to Provasoli medium (salinity 33) to get the final culture salinity of 11, 22, 66, 99. Then 1 mL of ice algal cells was collected at 0, 6, 12, 24, 36, 48, and 72 h to investigate the effects of the different stresses on the expression of *ICE-LGCL*. The algal cultures were centrifuged at 12,000 rpm for 5 min at 4 °C with a 1.5-mL RNase-free tube, and the medium was discarded.

2.3. RNA extraction and amplification of ICE-LGCL fragment

First 1-mL ice algal cultures were centrifuged with a 1.5-mL RNase-free tube like above. Then the total RNA was isolated directly from the harvested fresh ICE-L using TRIZOL Reagent following the manufacturer's recommendation and verified by running samples on 1.0% (*w*/*v*) agarose gels. The template of cDNA was synthesized by Reverse Transcriptase M-MLV (*RNase* H^-) and 3′adapter primer (Table). We designed the degenerated primers of F1 and R1 (Table) according to the published *GCL* sequences of *Chlamydomonas reinhardtii* (XM_001701595), *Volvox carteri* (XP_002949540), *Lotus japonicus* (AY204516), *Arabidopsis thaliana* (DQ993178), *Brassica juncea* (AJ005587), and *Medicago truncatula* (XM003611038). Then the cDNA was used as a template and the F1/R1 as primers programmed in 35 cycles of PCR (94 °C for 30 s, 53 °C for 30 s, 72 °C for 60 s) to amplify partial ICE-LGCL cDNA. PCR products were separated through electrophoresis on 1% agarose gel.

2.4. Rapid amplification of 3′-cDNA and 5′-cDNA ends of ICE-LGCL

PCR amplification of the 3'-regions of ICE-LGCL cDNA was carried out in 35 cycles of PCR with annealing temperature of 59 °C with the gene specific primer GCL and the 3′anchor primer (Table).

Table. List of primers used for amplification of DNAs and cDNAs in the present paper.

PCR	Primer name	Oligonucleotide sequence (5′–3′)
cDNA and fragment PCR	3′adapter	AAGCAGTGGTATCAACGCAGAGTACT (30) VN
	R1	ACATWCCNATVATGCCMAAGG
	F1	CRTCCGCVCCBCGCATYTCCA
cDNA and 5’ RACE	GCL1	GGAAGATGGTTGTCAAGTGGT
	GCL2	TCCACGACTGTCCCAGGGCATT
	GCL3	ACACGAAAGGCAGACCACCACA
	OligodT-anchor	GACCACGCGTATCGATGTCGACT(16)
	5′anchor primer	GACCACGCGTATCGATGTCGAC
3’ RACE	GCL	GTGGTGGTCTGCCTTTCGTGTTT
	3′anchor primer	AAGCAGTGGTATCAACGCAGAGT
Real-time PCR	β-actin	GCTTTGCTATGTGGCACTTGACTT
		GGTTGGAACAGGACCTCGGG
	ICE-LGCL	GGTGGTCTGCCTTTCGTGT
		CCTCAGGGAAGATGGTTGC

Note A/C, W; A/T/G/C, N; G/A/C, V; A/C, M; A/G, R; G/T/C, B; C/T, Y

The first-strand cDNA used for 5’-regions amplification was synthesized according to the manufacturer’s guidelines of the 5’/3’ RACE Kit, 2nd Generation, with the gene-specific primer GCL1 (Table). Then a homopolymeric A-tail was added to the 3’end of first-stand cDNA using the recombinant terminal transferase and dATP. The first PCR was carried out by denaturing cDNA at 94 °C for 3 min followed by 35 cycles of amplification (94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s) and a final extension at 72 °C for 5 min with the oligo-dT-anchor primer and GCL2 (Table). The nested PCR was then performed with the internal primers of 5′anchor primer and GCL3 (Table) with annealing temperature of 55 °C.

2.5. Cloning and sequencing of PCR products

The *ICE-LGCL* middle fragment, 3’ RACE and 5’ RACE purified PCR fragments from agarose gels were purified using the agarose gel DNA purification kit Ver.2.0. The purified products were ligated into the T/A cloning vector pMD18-T (TaKaRa) and transformed into the competent *E. coli* DH5α. Positive clones were isolated by Amp^+/LB plates that contained IPTG/X-Gal, and then were sent to Sangon Biotech Co., Ltd. (Shanghai) for DNA sequencing.

2.6. Bioinformatics analysis

GENETYX 7.0 and ORF finder were used to detect the amino acid sequence, molecular weight, and isoelectric point. The signal peptide, transmembranous region, N-Glycosylation sites, phosphorylation sites, and subcellular localization were calculated by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), TargetP1.1 (http://www.cbs.dtu.dk/services/TargetP/), and WoLF PSORT (http://wolfpsort.org/) respectively. Phylogenetic analysis was performed using ClustalX2 and MEGA 5.0.

2.7. Relative expression of ICE-LGCL exposed to the different temperatures and salinities by real-time PCR

To investigate the accumulation of ICE-LGCL mRNA under different stresses, we extracted the total RNA from *Chlamydomonas* sp. ICE-L cultured under different temperatures and salinities like above. The total RNA was treated with *Dnase* I before being used in cDNA synthesis as the templates to investigate the mRNA level of *ICE-LGCL* by real-time PCR (Zhang et al., 2010). The real-time PCR was performed with Bio-Rad IQ5 using Hot Start Fluorescent PCR Core Reagent kits (SYBR Green I), with a first step (95 °C for 5 min) followed by 40 cycles (95 °C 10 s, 60 °C 10 s, 72 °C 10 s) with ICE-LGCL primers. To evaluate the amount of template RNA in each real-time PCR reaction, gene fragments of β-actin (primers refers to Table) was also amplified. Relative mRNA levels were calculated with reference to the ddCt algorithm. All experiments were performed in three independent repetitions.

2.8. The prokaryotic expression of ICE-LGCL in *E. coli* BL21 and western blot analysis

The open reading frame of ICE-LGCL was amplified by PCR using cDNA as template and 5’-CGCGGATCCATGTATCTATCTAGTAGGAT-3’

(*BamH* I site is underlined), 5'-CCGCTCGAGGAAC TCCTTGTAGAGGGGGT-3' as primers (*Xho* I site is underlined) with annealing temperature of 61 °C. The PCR product was purified with agarose gel DNA purification Kit Ver.2.0. The purified product and pET-28a (+) were digested with *BamH* I and *Xho* I for 3 h at 37 °C, respectively. The digestion products were linked by T_4 DNA ligase to construct the recombinant expression plasmid (named pET-*ICE-LGCL*). pET-*ICE-LGCL* was then transformed into the competent *E. coli* BL21. The positive clones were identified by PCR and digested with restrictive endonuclease and visualized by the electrophoresis on 1% agarose gel prior to DNA sequencing.

The positive clone was cultured until $OD_{600\ nm}$ 0.6–0.8 at 37 °C in Luria-Bertani (LB) medium containing 50 mg/mL kanamycin, and induced with 0.2 mM IPTG for 4 h to express the ICE-LGCL protein. The proteins were collected by centrifugation and resuspended in 1×PBS (KH_2PO_4 1.48 mM $Na_2HPO_4 \cdot 12H_2O$ 7.9 mM, NaCl 137.0 mM, KCl 2.7 mM, pH 7.4). The proteins were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after being treated with 2×SDS loading buffer and were transferred to a nitrocellulose (NC) membrane. The membrane was then treated with the blocking solution (5% nonfat milk) overnight. The membrane was then washed three times with TBST (NaCl 544.0 mM, KCl 10.7 mM, Tris-base 99.1 mM, Tween 20 0.1%, pH 7.4) and incubated with 1:3000 anti-His antibody for 2 h at 37 °C. The membrane was washed three times with TBST and incubated with horseradish peroxidase conjugated goat anti-mouse antibody (1:5000) for 2 h at 37 °C. Lastly, the membrane was washed three times with TBST prior to coloration using DAB and the reaction ended with ddH_2O (Wang et al., 2017).

3. Results

3.1. RNA extraction, cDNA cloning, and sequencing of ICE-LGCL gene

High-quality total RNA was obtained using TRIZOL reagent and the first strain of cDNA was successfully synthesized as well. A cDNA fragment of ICE-LGCL about 600 bp was obtained by PCR with a pair of degenerate primers. Based on the fragment, we designed a gene-specific primer for 3' RACE PCR and three primers for 5' RACE PCR. Then fragments of about 1200 bp and 1100 bp nucleotides were obtained, respectively. The sequences of the middle region product, 3' RACE, and 5' RACE were aligned and spliced by the ContigExpress software. A putative GCL cDNA sequence was identified on the basis of the consensus pattern and sequence homology to the published GCLs. The *ICE-LGCL* complete sequence is composed of 2199 bp nucleotides with an open reading frame of 1452 bp encoding a protein of 483 amino acids. 5′ and 3′ untranslated regions are 36 bp and 711 bp, respectively (data not shown). The sequence was submitted to GenBank with an accession number JN792440.

3.2. Bioinformatics analysis of ICE-LGCL

According to the deduced amino acid sequence, the molecular weight of ICE-LGCL was 55.17 kD with an estimated isoelectric point (pI) of 5.97. Subcellular localization predicts that ICE-LGCL exists in the chloroplast in *Chlamydomonas* sp. ICE-L. There is a structure domain of GCL family and 27 phosphorylation sites, but no signal peptide, trans-membranous region, or N-Glycosylation sites are found. The sequence was submitted to a BLASTP search at the NCBI site (http://www.ncbi.nlm.nih.gov/). ICE-LGCL shared 51%–70% amino acids sequence identity with GCLs of the other plants, of which the highest was with *Chlamydomonas reinhardtii* (70%), followed by *Oryza sativa* (62%), *Chorispora bungeana* (61%), *Zinnia elegans* (61%), *Allium cepa* (61%), *Arabidopsis thaliana* (61%), and *Lotus japonicus* (60%).

The deduced amino acid sequence of GCLs was aligned using DNAStar software (Figure 1), and a molecular phylogenetic tree (Figure 2) was constructed by ClustalX2 and MEGA5.0 software. The results also showed that ICE-LGCL keeps a closer relativity with that of *Chlamydomonas reinhardtii*.

3.3. Gene expression analysis of ICE-LGCL in response to the different treatments

Figure 3 showed that *ICE-LGCL* expressed under different temperatures. In the control group, the accumulation of ICE-LGCL mRNA stayed at a proportionate level during 72 h ($P > 0.05$). However, at 0 °C, ICE-LGCL mRNA accumulation kept increasing in the first 24 h until it reached twice as much as the control at 24 h ($P < 0.05$) and then it decreased in the following time. At 14 °C, the accumulation of ICE-LGCL mRNA decreased in the first 6 h and then kept a lower level about half as much as the control.

Figure 4 shows that the low salinities stimulated the gene expression of *ICE-LGCL*, but the accumulation of ICE-LGCL mRNA was less than the control when treated with high salinities. In low salinity groups (11 and 22), a successive increase in the expression level of *ICE-LGCL* was observed during 48 h and reached the highest value about more than two times as much as the control ($P < 0.01$). In high salinity groups (66 and 99), the expression level of *ICE-LGCL* decreased in the first 6 h and kept a stable level in the following experimental period about one third of the control.

3.4. Prokaryotic expression of ICE-LGCL and western blot analysis

Figure 5 shows that the recombinant plasmid was successfully constructed as well as the sequencing results.

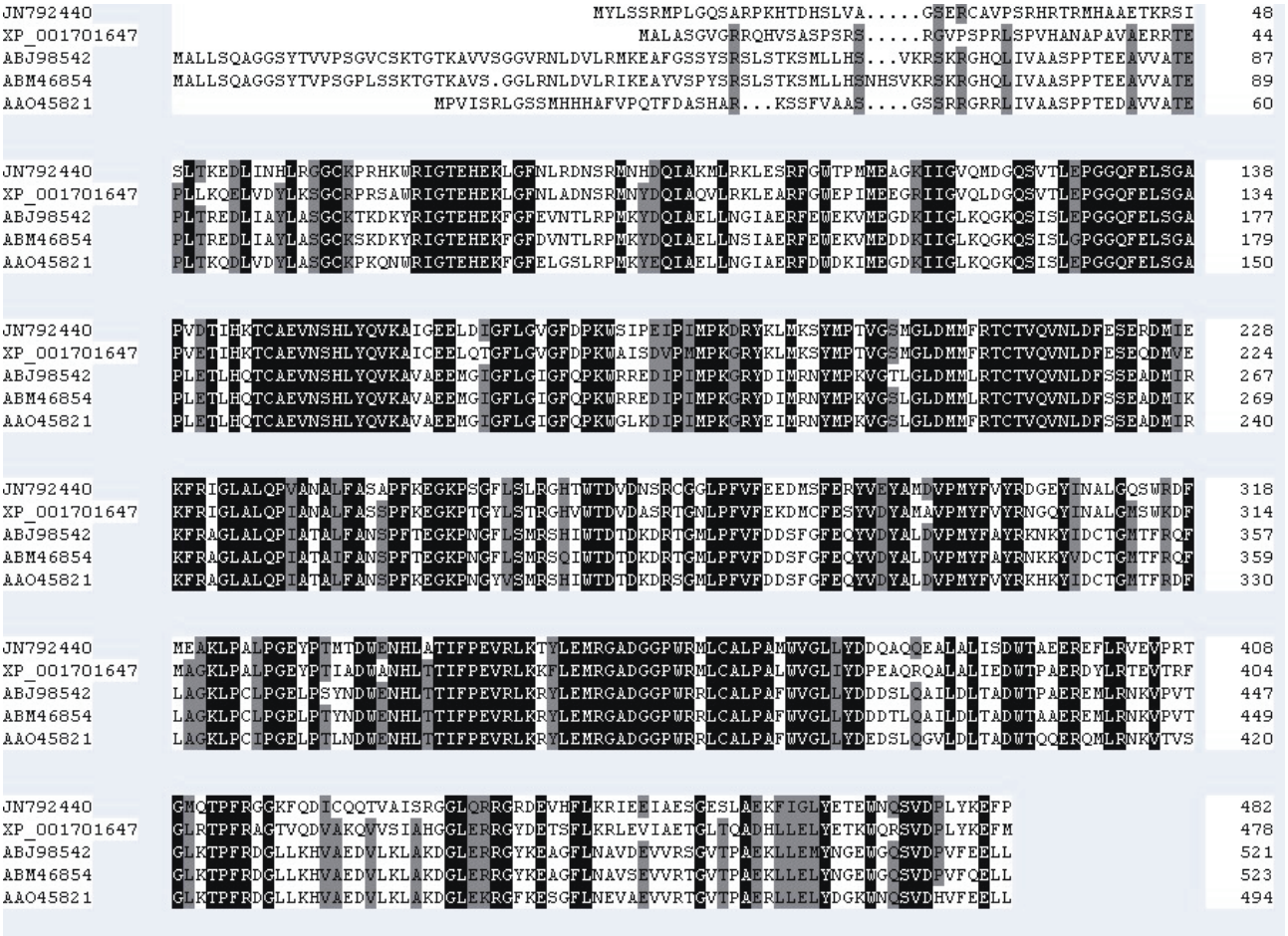

Figure 1. Alignment and comparison of the deduced amino acid sequence of ICE-LGCL with different species. *Note* Alignment of GCL amino acid sequences from *Chlamydomonas* sp. ICE-L (JN792440), *Chlamydomonas reinhardtii* (XP_001701647), *Arabidopsis thaliana* (ABJ98542), *Chorispora bungeana* (ABM46854), and *Lotus japonicus* (AAO45821). GenBank IDs (Accession numbers) are shown in figure and parentheses in this note.

The optimum expression conditions of pET-*ICE-LGCL* in *E. coli* BL21 are 0.2 mmol/L IPTG, induced temperature of 37 °C, and induced time of 4 h. ICE-LGCL expressed mainly in the form of inclusion body in *E. coli* (Figure 6). The SDS-PAGE showed that the recombinant ICE-LGCL protein was about 58 kD (Figure 7A), consistent with the western blot analysis (Figure 7B). These revealed that the expressed protein was ICE-LGCL.

4. Discussion

GSH is an important molecule involved in plant growth and development and stress responses (Ogawa, 2005). The *GCL* genes, encoding a rate-limiting enzyme in GSH synthesis, have been cloned and characterized from various species. Bioinformatic analysis of these sequences suggested that GCLs can be grouped into three families, sequences primarily from gamma-proteobacteria, nonplant eukaryotes, and alpha-proteobacteria and plants (Copley and Dhillon, 2002). In the present study, we obtained the *ICE-LGCL* gene from *Chlamydomonas* sp. ICE-L with a full length of 2 199 bp encoding a protein of 453 amino acids and a theoretical MW of 55.17 kD. The data about MW agreed with the SDS-PAGE result of ICE-LGCL protein (Figure 7). The subcellular localization analysis showed that ICE-LGCL may be localized in the chloroplast, which was similar to the result of GSH biosynthesis in the chloroplast and the cytosol (Hell and Bergmann, 1990). Identity analysis of the deduced amino acid sequences showed that ICE-LGCL was similar to GCLs from algae and plants, especially with *Chlamydomonas reinhardtii* (70%). The phylogenetic analysis result showed that ICE-LGCL evolved in parallel with that of *Chlamydomonas reinhardtii*, which confirmed that the cDNA sequence cloned from *Chlamydomonas* sp. ICE-L was the *GCL* gene. The results indicated that the *ICE-LGCL* gene was grouped into the alpha-proteobacteria and plants family.

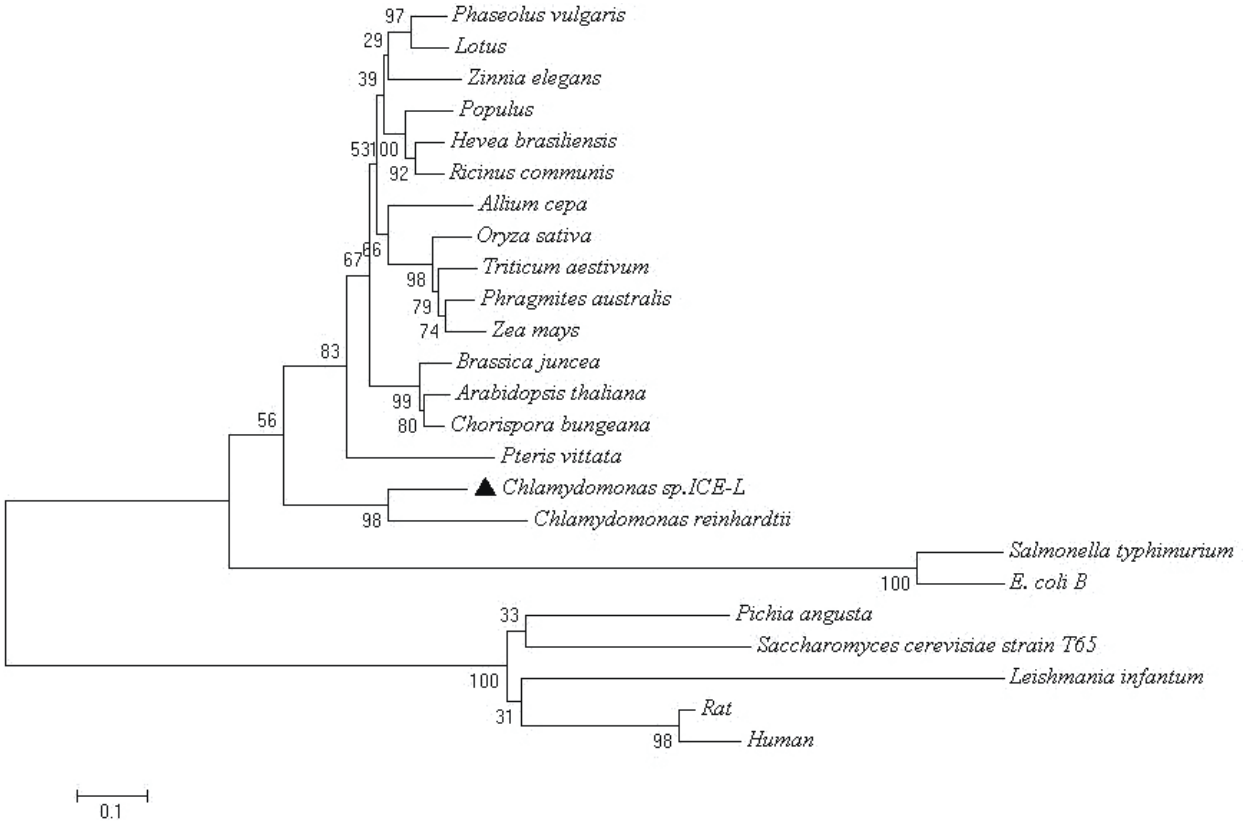

Figure 2. Phylogenetic tree of GCL family members constructed with the neighbor-joining method. *Note* Tree construction of GCL amino acid sequences from *Chlamydomonas reinhardtii* (XM_001701595), *Oryza sativa* (AJ508916), *Zea mays* (NM_001111672), *Zinnia elegans* (AB158510), *Phaseolus vulgaris* (AF128454), *Allium cepa* (AF401621), *Lotus* (AY204516), *Brassica juncea* (AJ005587), *Arabidopsis thaliana* (DQ993178), *Chorispora bungeana* (EF137428), *Hevea brasiliensis* (GU997638), *Triticum aestivum* (AY864064), *Phragmites australis* (FJ463872), *Ricinus communis* (XM_002509754), *Populus* (HQ658456), *Pichia angusta* (AF435121), *Leishmania infantum* (XM_001464941), *Salmonella typhimurium* (AF055352), rat (J05181), *Saccharomyces cerevisiae* strain T65 (JF701606), *Pteris vittata* (AB553577), *E. coli* B (X03954), and human (M90656). GenBank IDs (Accession numbers) are shown in parentheses in this note. The reliability of the branching was tested by bootstrap re-sampling (1000 pseudo-replicates).

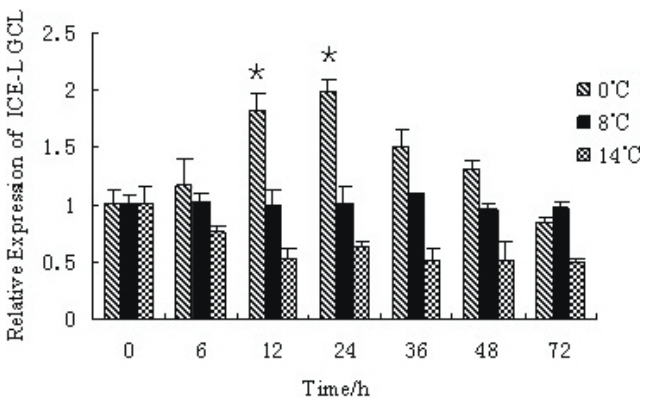

Figure 3. The real-time PCR analysis of the expression level of ICE-LGCL at different temperatures. *Note* Expression levels of ICE-LGCL mRNA were analyzed from control and temperature stressed algae (temperature 0 °C, 8 °C, and 14 °C) at 0, 6, 12, 24, 36, 48, and 72 h; 8 °C was used as control. Relative mRNA levels were calculated with reference to the ddCt method using iQTM 5 Optical System Software. All experiments were carried out in three independent repetitions. Results were expressed as mean ± standard error (SE). Standard error bars are shown. Significance was concluded at a significance level of $P < 0.05$. Statistical analysis was performed using SPSS 11.0 for Windows. Data differing significantly ($P < 0.05$) from those in control 8 °C are marked with asterisks.

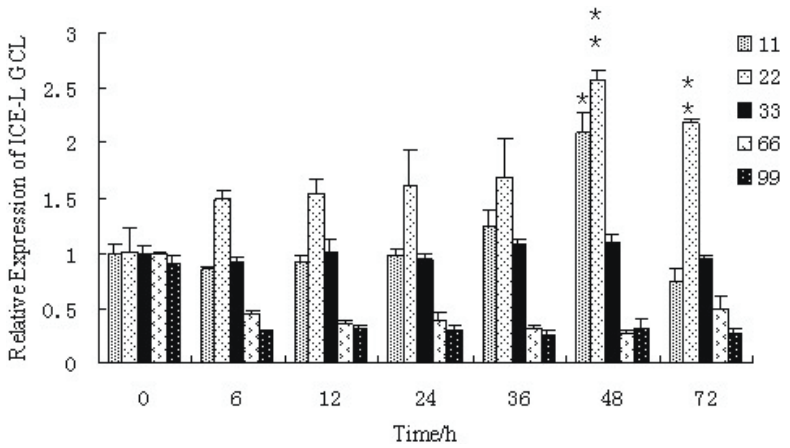

Figure 4. The real-time PCR analysis of the expression level of ICE-LGCL under different salinities. *Note* Expression levels of ICE-LGCL mRNA were analyzed from control and salt stressed algae (Salinity 11, 22, 33, 66, and 99) at 0, 6, 12, 24, 36, 48, and 72 h. Salinity 33 was used as control. Relative mRNA levels were calculated with reference to the ddCt method using iQTM 5 Optical System Software. All experiments were carried out in three independent repetitions. Results were expressed as mean ± standard error (SE). Standard error bars are shown. Significance was concluded at a significance level of $P < 0.05$. Statistical analysis was performed using SPSS 11.0 for Windows. Data differing significantly ($P < 0.05$) from those in control salinity 33 are marked with asterisks.

It is well known that environmental stresses can induce the formation of reactive oxygen species (ROS) and consequently activate the expression of some genes encoding antioxidative enzymes (Kaminaka et al., 1998). Under the natural environment, *Chlamydomonas* sp. ICE-L lives in Antarctic Ocean, which provides a range of habitats with low temperature, unstable salinity, high ultraviolet radiation and dissolved gases, and so on (Thomas and Dieckmann, 2002; Iwamoto and Shiraiwa, 2005; Mock and Thomas, 2005). GSH is a central factor of the antioxidant defense system in organisms (Arteel and Sies, 2001; Dickinson and Forman, 2002). Resistance to GSH depletion by GPx depends on the cysteine availability and GCL activity. To date, some studies revealed that GSH synthesis is regulated by GCL activity and the feedback inhibition of GSH (Griffith, 1999; Lu, 2000), and GSH level is related to the tolerance to stress. For example, increase in the GCL activity of corn elevated its tolerance to chilling (Kocsy et al., 1996), but inhibition of GSH synthesis decreased chilling tolerance in *C. bungeana* callus (Wu et al., 2008). What should be emphasized is that, in most cases, the elevation of GSH level is due principally to de novo synthesis as a consequence of the increased accumulation of GCL mRNA (Dickinson et al., 2004). However, the concentration of GSH was regulated not only by the internal matters but also the environmental factors. GSH plays an important role in response to the various environmental stresses and keeps the tolerance to stresses in plants. Our results showed that the accumulation of ICE-LGCL mRNA stayed at a proportionate level during 72 h ($P > 0.05$) in the control. However, it increased and reached a peak at 24 h ($P < 0.05$), followed by decreasing until to the normal level under 0 °C (Figure 3). Overaccumulation of GSH may partially inhibit its synthesis (Noctor et al., 1998). Thus *Chlamydomonas* sp. ICE-L was able to maintain higher ICE-LGCL mRNA and GSH levels in cells under low temperature. In our previous studies, the results showed that GSH is positively correlated to low temperature, and GSH concentration was higher significantly at 0 °C and –10 °C (Ding et al., 2005,

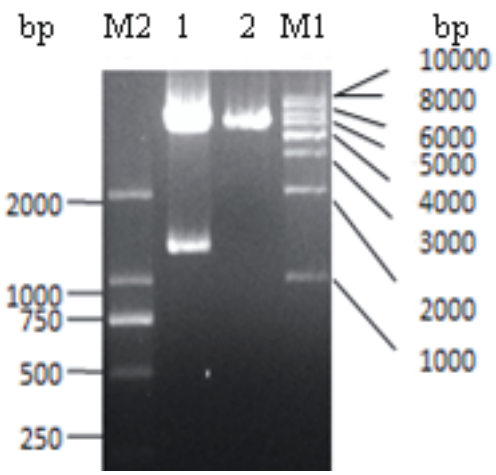

Figure 5. Agarose gel electropherogram of the recombinant plasmid pET-ICE-LGCL digested by *BamH* I and *Xho* I. *Note* Lane 1, Digestion of pET-ICE-LGCL with *BamH* I and *Xho* I; Lane 2, plasmid pET-28a (+) control; Lane M1, 1 kD ladder DNA marker; Lane M2, DL2000 DNA marker.

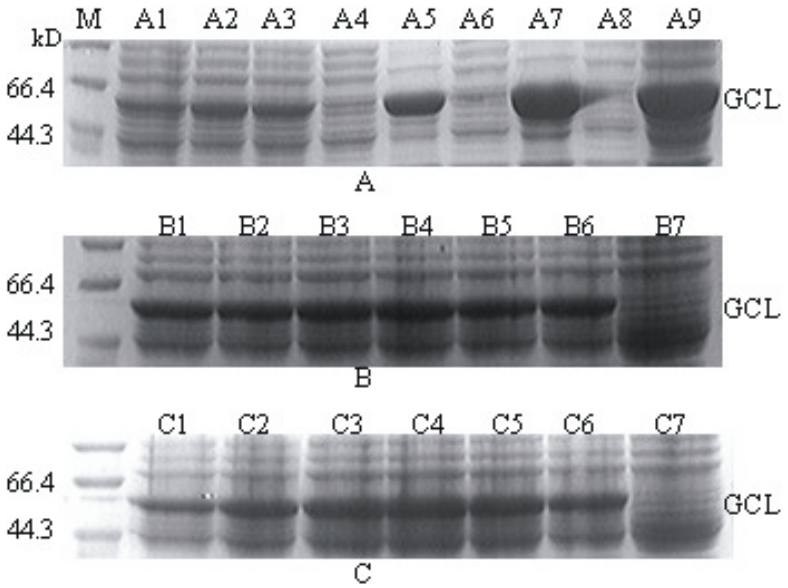

Figure 6. The optimal conditions of prokaryotic expression of pET-ICE-LGCL. *Note* A: SDS-PAGE analysis of recombinant protein ICE-LGCL expressing at different temperature. Lane M, Standard protein marker; Lane A1, A4, A5; A2, A6, A7; A3, A8, A9, Total protein, supernatant and precipitation of pET-ICE-LGCL recombinant bacteria induced at 18, 28, and 37 °C, respectively. B: SDS-PAGE analysis of pET-ICE-LGCL expression induced by different concentration of IPTG. Lane B1–B6, pET-ICE-LGCL was induced with IPTG at a final concentration of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mmoL/L at 37 °C for 4 h, respectively, Lane B7 is the control. C: SDS-PAGE analysis of pET-ICE-LGCL expression with different induced time. Lane C1-C6, pET-ICE-LGCL induced by 0.2 mM concentration of IPTG for 1, 2, 3, 4, 5, and 6 h, respectively, Lane C7 is the control.

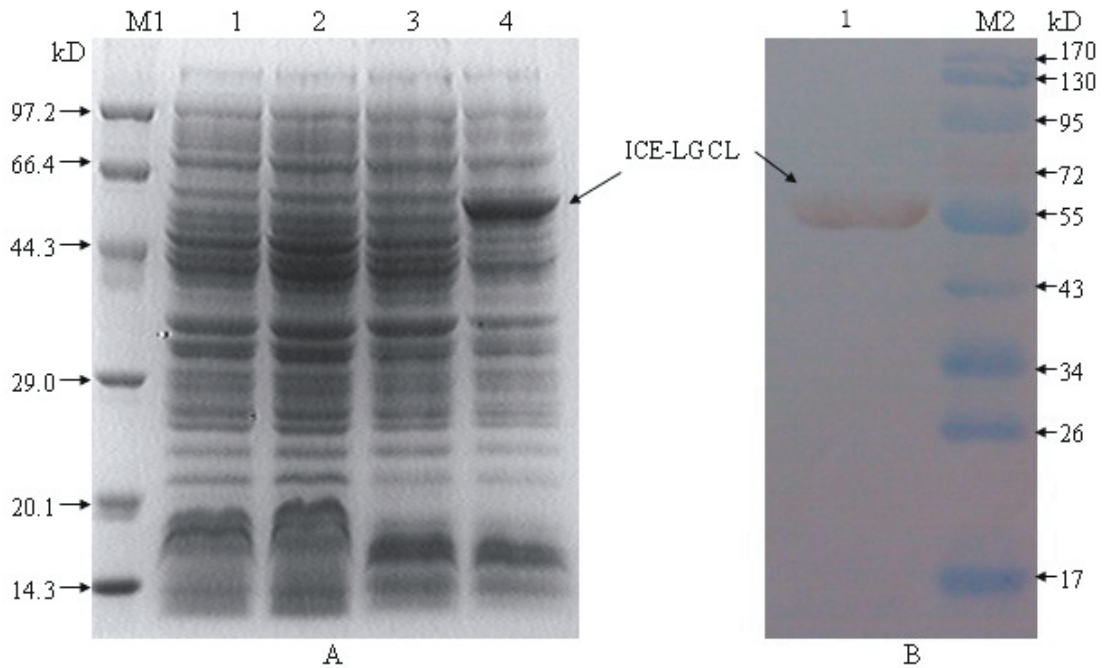

Figure 7. SDS-PAGE analysis and western blot analysis of expressive product of pET-ICE-LGCL in *E. coli* BL21. *Note* A: SDS-PAGE analysis. Lane M1, Protein molecular weight marker (Low); Lane 1, pET-28a (+) without IPTG induction; Lane 2, pET-28a (+) with IPTG induction; Lane 3, pET-ICE-LGCL without IPTG induction; Lane 4, pET-ICE-LGCL with IPTG induction. B: Western blot analysis using the His·Tag monoclonal antibody. Lane M2, Prestained protein ladder, Lane 1, product of pET-ICE-LGCL

2007). Increased *GCL* transcription in maize leaves by short-term chilling has also been found (Gomez et al., 2004). As to 14 °C, the accumulation of ICE-LGCL mRNA decreased at first. Other enzymes and mechanisms may take a part in protecting algae from injury under high temperature.

In the natural environment, *Chlamydomonas* sp. ICE-L grows in salinity about 30, but the salt concentration of seawater in Antarctica will change because of the seawater freezing and thawing in different seasons. Zheng et al. (2010) found that the ICE-L could survive salinity of 132 under laboratory conditions. Our result showed that ICE-L can survive salinity as high as 99 (Figure 4). In the experiment of real-time PCR, the accumulation of ICE-LGCL mRNA stayed at a normal level in the control. However, it increased in the first 48 h and came to the highest level ($P < 0.01$) in the low salinity groups (11, 22), and kept only about one third of the normal level in the high salinity groups (66, 99). Mechanisms of salinity tolerance in plants include the common gene products and the other regulatory pathways (Thomashow, 2001). As a gene product, GCL may play a great role in regulating the concentration of GSH to adaption to low salinity. However, the other enzymes and mechanisms may take a part in protecting algae from injury under high salinity. Ding et al. (2012) found that the activity of glutathione reductase increased in the first 12 h after being treated with salinity of 66 and 99. The role of glutathione reductase may be more important than GCL when ICE-L adapts to high salinity. It has also been shown that the changes in some proteins might be correlated with the resistance for *Chlamydomonas* sp. ICE-L to high salinity (Zheng et al., 2010). Kan et al. (2006) also found that the contents of SOD, MDA, and proline and the cell membrane permeability can indicate the salt resistance of *Chlamydomonas* sp. ICE-L.

It has been proven that the bacterial expression system is optimal for expressing heterologous proteins (Yin et al., 2007). ICE-LGCL protein was successfully induced to express in *E. coli* BL21 in the present paper. The maximum expression level was obtained when BL21 was induced with 0.2 mmol/L IPTG at 37 °C for 4 h (Figure 6). In order to certify the expressed protein in *E. coli* BL21 was ICE-LGCL protein, western blot analysis showed the anti-His monoclonal antibody recognized a 58 kD band, which was similar to the size of ICE-LGCL recombinant fusion protein containing His·Tag. This result also indicated that ICE-LGCL was successfully expressed in BL21. The prokaryotic express of ICE-LGCL would help us to study further on the character and catalysis mechanism of the enzyme.

In the present study, a *GCL* gene in *Chlamydomonas* sp. ICE-L was successfully characterized, and its expression analysis was performed under the different temperatures and salinities in our study. ICE-LGCL evolved in parallel with that of *Chlamydomonas reinhardtii*, and ICE-LGCL plays a great role in regulating the concentration of GSH for *Chlamydomonas* sp. ICE-L to adapt to low temperature and low salinity. Our present results can further clarify the mechanism of Antarctic ice microalgae in acclimating to the Antarctic environment.

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