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ORIGINAL PAPER

Acclimation of Antarctic *Chlamydomonas* to the sea-ice environment: a transcriptomic analysis

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Abstract The Antarctic green alga *Chlamydomonas* sp. ICE-L was isolated from sea ice. As a psychrophilic microalga, it can tolerate the environmental stress in the sea-ice brine, such as freezing temperature and high salinity. We performed a transcriptome analysis to identify freezing stress responding genes and explore the extreme environmental acclimation-related strategies. Here, we show that many genes in ICE-L transcriptome that encoding PUFA synthesis enzymes, molecular chaperon proteins, and cell membrane transport proteins have high similarity to the gens from Antarctic bacteria. These ICE-L genes are supposed to be acquired through horizontal gene transfer from its symbiotic microbes in the sea-ice brine. The presence of these genes in both sea-ice microalgae and bacteria indicated the biological processes they involved in are possibly contributing to ICE-L success in sea ice. In addition, the biological pathways were compared between ICE-L and its closely related sister species, *Chlamydomonas reinhardtii* and *Volvox carteri*. In ICE-L transcripome, many sequences homologous to the plant or bacteria proteins in the post-transcriptional, post-translational modification, and signal-transduction KEGG pathways, are absent in the nonpsychrophilic green algae. These complex structural components might imply enhanced stress adaptation capacity. At last, differential gene expression analysis at the transcriptome level of ICE-L indicated that genes that associated with post-translational modification, lipid metabolism, and nitrogen metabolism are responding to the freezing treatment. In conclusion, the transcriptome of *Chlamydomonas* sp. ICE-L is very useful for exploring the mutualistic interaction between microalgae and bacteria in sea ice; and discovering the specific genes and metabolism pathways responding to the freezing acclimation in psychrophilic microalgae.

Keywords Antarctic sea ice · Freezing acclimation · Horizontal gene transfer · Psychrophilic green microalga · Transcriptomic analysis

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Introduction

The frozen polar sea ice is comprised a system of brine channels which characterized by low temperature, high salinity, low light, limited gas exchange, and highly oxic conditions which makes it one of the most extreme environments for the sea-ice living creatures on the earth (Thomas and Dieckmann 2002). A very diverse community of psychrophilic organisms, such as microalgae and bacteria, are inhabited in the semi-enclosed sea ice, and are adapting well to the strong and oscillating environmental conditions (Arrigo et al. 2010).

Several papers have given insights into polar microalgae adaptation strategies at "omics" level to the polar environments. These reports are focused on environmental factors, such as thermal temperature (Hwang et al. 2008), high salinity (Takizawa et al. 2009; Lyon et al. 2011), and ultraviolet radiation (Ryan et al. 2012; Obertegger et al. 2011), and highly oxic conditions (Janknegt et al. 2008). To our knowledge, the cold or freezing acclimation traits of polar sea-ice microalgae have mainly been investigated on diatoms, such as *Fragilariopsis* and *Chaetoceros* (Mock and Junge 2007; Jung et al. 2007). The factors contribute to their success living in the sea ice include increasing concentrations of polyunsaturated fatty acids (PUFAs) of the cell membranes to maintain fluidity under freezing temperatures, promoting the cryospheric enzyme flexibility at low temperatures, producing cellular compatible solutes, cryoprotectants, and extracellular compounds to reduce the intracellular freezing point, as well as changing in photosynthesis and pigment constitutes to adapt to low light (Lyon and Mock 2014). Though there is no enough evidence, horizontal exchange and recombination of genetic material are supposed to promote their acclimation ability of the sea-ice organisms (Lyon and Mock 2014). Sea ice has been proposed as a hotspot for genetic recombination due to its high density of bacteriophage (Wells and Deming 2006). Raymond and Kim (2012) have suggested that the success of diatoms and other algae in sea ice can be at least partly attributed to their acquisition of prokaryotic ice-binding protein (IBP) genes through horizontal gene transfer.

A psychrophilic green microalga *Chlamydomonas* sp. ICE-L was isolated from the floating sea ice with an optimum growth temperature range of 4–10 °C (Liu et al. 2006). Considering the capacity of successful adaptation to the freezing environments, ICE-L would be a valuable resource for the discovery of genes associated with sea-ice environmental stress tolerance. Previous studies in our lab have revealed the induced expression of fatty acid desaturase genes (Zhang et al. 2011; An et al. 2013) and HSP70 (Liu et al. 2010) gene under cold treatment. In this study, we focused on global changes and patterns of expressed genes to provide new insights into the important processes that involved in sea-ice environmental acclimation by exploring the transcriptome data of *Chlamydomonas* sp. ICE-L.

Materials and methods

Stress treatment and RNA extraction

Chlamydomonas sp. ICE-L was isolated from the sample of Antarctic sea ice and monoclonal cultured in our lab (Liu et al. 2006). Cells were grown in Provasoli seawater medium (Provasoli 1968) under a photon flux density of 40 μmol photons m^{-2} s^{-1}, 12L: 12D cycle, and temperature of 7 °C.

For the cDNA library construction and transcriptome analysis, the exponential phase cells were kept in −20 °C refrigerator for 5 h as a freezing treatment. Then, the cells were removed to the 7 °C incubator to melt before centrifugation (4 °C, 5 min, 3,500 rpm). The control samples were kept in 7 °C for the same duration. To avoid the light intensity discrepancy, both the treatment and control samples were shaded in darkness with silver paper. The harvested samples were flash frozen in liquid nitrogen, and stored at −80 °C before RNA extraction. Both the treatment and control samples were replicated for transcriptome sequencing.

RNA was extracted using a modified CTAB (hexadecyltrimethylammonium bromide) method according to Wu et al. (2010). The cDNA libraries were constructed from 100-ng purified mRNA samples using a NEBNext® UltraTM RNA Library Prep Kit for Illumina (NEB, USA). Illumina HiSeq™ 2500 sequencing was performed as a service by Hanyu Bio-tech Cooperation (Shanghai, China).

Sequencing and transcriptomic analysis

High-quality sequences were obtained using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) after the short sequences (<100 bps) removed from the raw data. The sequences were assembled into non-redundant consensus (contigs) using the trinity software (v2013-02-25, default parameters). The protein sequences were predicted with the GetORF software in EMBOSS toolkit (Rice et al. 2000) from the assembled contigs. The putative protein sequences were annotated using the BLASTP algorithm against the non-redundant (nr) database from NCBI with an *E*-value < 1e-5E cutoff. The transcriptomic sequences of ICE-L were compared with the genomic sequences of *C. reinhardtii* and *V. carteri* (downloaded from https://phytozome.jgi.doe.gov/) using the BLASTP algorithm with an *E*-value < 1e-3E cutoff using the Blast-2.2.28 + software package (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein sequences from the genomic data of *C. reinhardtii* and *V. carteri* as well as transcriptomic data of ICE-L were functionally annotated through KAAS [KEGG (Kyoto Encyclopedia of Genes and Genomes) Automatic Annotation Server http://www.genome.jp/tools/kaas/] by GHOST comparisons against the manually curated KEGG GENES database respectively (Moriya et al. 2007).

To identify the differentially expressed genes (DEG) associated with freezing tolerance, the clean reads were mapped against the assembled contigs, the reads number from two compared samples were statistic and transferred

to RPKM (Reads Per Kilo-base per Million reads) (Mortazavi et al. 2008). Differentially expressed contigs in the two samples were calculated with MARS (MA-plot-based method with Random sampling model) in a DEGseq tool kit (Wang et al. 2010). In our study, the false discovery rate (FDR) ≤ 0.001 and the absolute value of the Ratio ≥ 2 (namely absolute value of the log2Ratio ≥ 1) were used as the thresholds to assess the significant difference of gene expression between the two samples.

RT-PCR analysis of selected genes

To confirm the gene expression profiling by the DEG analysis, we conducted the qRT-PCR analysis on several genes under freezing treatment. Five 100-ml cultures were kept in −20 °C for 6, 24, 48, 72, and 120 h, respectively, and then removed to 7 °C to melt until the residual ice less than 1/5 volume in the culture. Another five cultures were kept in 7 °C for the same durations, respectively, as control. Primer pairs were designed using Premier 5.0 (Premierbiosoft, Palo Alto, CA, USA) (Table S1).

For the qRT-PCR analysis, 2 μg of total RNA was used to synthesize the first-strand cDNA with the Maxima first-strand cDNA synthesis kit (Tiangen, China). DNase was supplied in the Kit to remove the residual genomic DNA. The qRT-PCR reaction was performed with an Mx3005P real-time PCR machine (Stratagene, USA) using an SYBR PrimeScript ™ RT-PCR Kit (TaKaRa, China). Forty cycle reactions were conducted (95 °C for 10 s; 60 or 65 °C annealing for 20 s; 72 °C for 20 s). To evaluate the amplification of primers, standard curves of each pair of primers were constructed, and PCR efficiencies were calculated from the slopes of the curves. Relative gene expression determinations were made with the 2(-Delta Delta C_T) method described by Livak and Schmittgen (2001). Results were given as the mean of three biological replicates (three technical replicates for each qRT-PCR reaction). RPL19 and GAPDH were set as reference genes for the qRT-PCR analysis (Liu et al. 2012).

Results and discussion

Sequencing and functional annotation of *Chlamydomonas* sp. ICE-L transcriptome

To understand the freezing stress response of *Chlamydomonas* sp. ICE-L, we performed a replicated sequencing of cDNA libraries of two controls (C1, C2) and two freezing treated samples (F1, F2). A total of 1.027G, 1.317G, 0.827G, and 0.926G clean data were sequenced and mapped to approximately 3.45, 4.23, 2.77, and 3.14 million high-quality, non-redundant reads from the C1, F1, C2, and F2 cDNA library, respectively. The clean reads from the 4 samples were assembled together into 69,430 non-redundant contigs, with an average length of 652 bps and N50 length of 1030 bps. The lengths of 24,602 contigs (35 %) were more than 500 bps.

Of the 69,430 contigs, total 9861 tags can hit the protein sequences from about 430 species in the GenBank. There are 51 species; their genes matched with more than 10 contigs of ICE-L transcriptome (Table S2). About 87.38 % of the 9861-known contigs from ICE-L are homology to the proteins from unicellular green algae. The homology proteins from bacteria, plants, and animals are 4.32, 4.14, and 1.95 %, respectively. There are 1.31 % of the contigs homology to the proteins from the other kind of algae, such as red and brown algae, and 0.90 % to the proteins from moss. The top five organisms and their genes first matched by the contigs of ICE-L are *Volvox carteri f. nagariensis* (3154 contigs), *Chlamydomonas reinhardtii* (2744 contigs), *Coccomyxa subellipsoidea* C-169 (676 contigs), *Chlorella vulgaris* (396 contigs), and *Psychroflexus torquis* ATCC 700755 (253 contigs). Four of them are green algae, while the fifth is a bacterium from the family of Flavobacteriaceae.

Horizontal gene transfer may contribute to the freezing adaptation of ICE-L

P. torquis ATCC 700755 was an extremely psychrophilic bacterial species and originally isolated from algal assemblages in Antarctic multiyear sea ice collected from Prydz Bay. It has several traits linked to sea-ice inhabitation and dependence on algae via epiphytism (Bowman et al. 1998). According to the sequence alignment results, 253 contigs of ICE-L matched with 188 protein sequences of *P. torquis*, and most of them got 99 to 100 % protein sequence identity between the two species. All of these genes are present mainly in bacteria, while not in green algae, such as *C. reinhardtii* and *V. crateri*. Therefore, we supposed that ICE-L acquires these genes from sea-ice bacteria through horizontal gene transfer. The present of 4 transposases genes in the ICE-L transcriptome probably indicated the manners that horizontal gene transfer within the sea-ice communities. The COG classification of the 188 genes was shown in Fig. 1. The functions of 44 genes were unknown, and 22 genes were predicted to be general function only. Total 28 genes were involved in translation, ribosomal structure, and biogenesis category. There were 16 putative HGT genes belonging to the COG category of post-translational modification, protein turnover and chaperones. And 13 genes were involved in the category of amino acid transport and metabolism.

The biological functions of some genes present both in ICE-L and *P. torquis*, such as polyunsaturated fatty acids synthesis, cell membrane transportation, and molecular

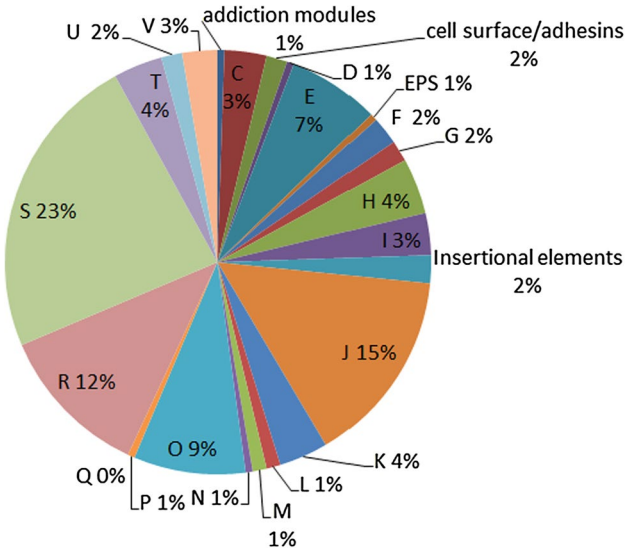

Fig. 1 COG classification of the 188 homologous proteins both in *Chlamydomonas* sp. ICE-L and *Psychroflexus torquis* ATCC 700755. *C* energy production and conversion, *D* cell division and chromosome partitioning, *E* amino acid transport and metabolism, *F* nucleotide transport and metabolism, *G* carbohydrate transport and metabolism, *H* coenzyme metabolism, *I* lipid transport and metabolism, *J* translation, ribosomal structure, and biogenesis, *K* transcription, *L* DNA replication, recombination, and repair, *M* cell envelope biogenesis, outer membrane, *N* cell motility, and secretion, *O* post-translational modification, protein turnover, and chaperones, *P* inorganic ion transport and metabolism, *Q* secondary metabolite biosynthesis, transport, and catabolism, *R* general function prediction only, *S* function unknown, *T* signal-transduction mechanisms, *U* intracellular trafficking and secretion, *V* defense mechanisms

chaperon, have been proved to be important in the bacteria adaptation to various abiotic stress (Table 1). Nine contigs in the transcriptome of ICE-L matched with 7 proteins involved in fatty acids metabolism in *P. torquis* with high sequence identity. Most of them are involved in polyunsaturated fatty acids synthesis process, such as omega-3 polyunsaturated fatty acid synthase gene, long and very long chain acyl-CoA dehydrogenase gene, and delta-9 fatty acid desaturase gene. Increased concentration of polyunsaturated fatty acids (PUFAs) is an important freezing tolerance mechanism, as proved in polar diatoms and green algae (Teoh et al. 2012; Osipova et al. 2009; Chen et al. 2012). These genes in ICE-L got 99 or 100 % sequences identity to that of *P. torquis* and their homologous proteins in the GenBank are mainly from Flavobacteriaceae according to the BLASTP search results. As indicated, some of the special PUFA modification traits of ICE-L might be acquired through HGT from the Flavobacteriaceae.

Several genes encoding cell membrane proteins and transporters also present in the transcriptome of ICE-L with highly similarities to that of *P. torquis*, such as gliding motility associated lipoprotein, outer membrane nutrient binding protein, outer membrane periplasmic chaperone, TonB-dependent biopolymer transport protein and outer membrane receptor channel protein, export-oriented permease, and glycine betaine transporter. Many of them are involved in the cell motility and large molecules (e.g., polysaccharides, proteins, and chelated metallic cations) transport through the outer membrane, which contribute to nutrient acquisition in bacteria (Noinaj et al. 2010). The expression of these proteins on the cell membrane might facilitate the substance exchange between ICE-L and the sea-ice environment, though the functions of these large molecules on the survival of the sea-ice microorganisms are still unclear.

In addition, we identified various molecular chaperone protein genes with 100 % sequence identity to that of *P. torquis* in ICE-L transcriptome, such as cold shock protein *CspD*, heat shock protein *GroEL* as well as *DnaK*, *DnaJ*, *HtpG*, *ClpB*, trigger factor *Tig,* and phage shock protein *A*. These molecular chaperone proteins have been proved to be involved in cold or freezing toleration in bacteria (Kandror and Goldberg 1997; Varin et al. 2012). Furthermore, a proteorhodopsin gene also expressed in the transcriptome of ICE-L that was supposed to be involved in the ability of *P. torquis* responding to changing light and salinity conditions (Feng et al. 2014).

We have also identified 15 contigs in the ICE-L transcriptome that matched with genes from another Flavobacteriaceae bacterium, *Bizionia argentinensis* JUB59, isolated from the surface seawater of Antarctica (Bercovich et al. 2008). Some of the gene encoded proteins are involved in fatty acid desaturase family protein for PUFA synthesis, DNA protection during starvation protein, and DNA topoisomerase I for DNA reparation.

Horizontal gene acquisitions by eukaryotes have been proved to be drivers for their extreme habitats adaptive evolution (Schönknecht et al. 2013). It has been supposed that horizontal gene transfer is a frequent occurrence through the abundant transposase genes within the hydrothermal chimney biofilms on the Mid-Atlantic Ridge, where survival in extreme conditions may require the stable coexistence of multiple phenotypes enabled by horizontal gene transfer (Brazelton and Baross 2009). The adaptation of the thermoacidophilic red alga *Galdieria sulphuraria* to its hot, acid, toxic-metal laden, volcanic environment was facilitated by the horizontally acquisition of at least 5 % of protein-coding genes from extremophile bacteria and archaea (Schönknecht et al. 2014). The HGT between prokaryotes and eukaryotes has been observed in both directions (Andersson 2009). It has been proved that HGT has been occurred much more extensively in *P. torquis* ATCC 700755, compared to its closely related nonpsychrophilic

Table 1 Some of the putative horizontal transfer genes between *Psychroflexus torquis* and *Chlamydomonas* sp. ICE-L

Gene_ID	Contig length	*E*-value	Protein identity (%)	Ref_sequence	Gene function
Insertional elements					
comp543474_c0	418	1.00E−76	99	AFU67320	Transposase, IS1595/ISH4 family
comp661304_c0	438	6.00E−33	80	AFU67150	Transposase, IS4 family
comp626764_c0	222	3.00E−23	49	AFU68597	Transposase, IS66 family, Orf2
comp840999_c0	288	7.00E−42	98	AFU67084	Transposase, IS91 family
Cell surface/transporters					
comp34678_c0	271	6.00E−53	100	AFU67679	Large colossin-like cell-wall protein with CnaB repeat
comp627217_c0	323	7.00E−69	99	AFU67959	Secreted, surface protein containing a fasciclin domain
comp1191365_c0	213	2.00E−41	99	AFU70325	Autotransporter adhesin
comp635955_c0	211	6.00E−45	100	AFU67945	Gliding motility associated lipoprotein GldK
comp279595_c0	661	2.00E−134	100	AFU68245	Outer membrane nutrient binding protein, SusD family
comp472989_c0	362	1.00E−82	100	AFU68245	Outer membrane nutrient binding protein, SusD family
comp419464_c0	354	1.00E−76	100	AFU67995	Outer membrane nutrient binding protein, SusD family
comp571863_c0	556	6.00E−132	99	AFU67995	Outer membrane nutrient binding protein, SusD family
comp153217_c0	884	2.00E−121	100	AFU68544	Outer membrane periplasmic chaperone, OmpH family
comp213109_c0	685	1.00E−131	100	AFU68714	Outer membrane periplasmic chaperone, OmpH family
comp282183_c0	493	6.00E−114	100	AFU68543	Outer membrane periplasmic chaperone, OmpH family
comp30588_c0	835	2.00E−94	99	AFU70050	Outer membrane RND-type efflux membrane fusion protein, NodT family
comp1070481_c0	222	1.00E−41	100	AFU69219	TonB-dependent biopolymer transport ExbD protein
comp430818_c0	218	4.00E−42	100	AFU68246	TonB-dependent outer membrane receptor channel protein, SusC family
comp663761_c0	478	1.00E−104	100	AFU68246	TonB-dependent outer membrane receptor channel protein, SusC family
comp262294_c0	968	0.0	100	AFU68802	TonB-dependent outer membrane receptor protein
comp832725_c0	205	1.00E−39	100	AFU69637	TonB-dependent outer membrane receptor protein
comp536398_c0	286	3.00E−60	99	AFU67994	TonB-dependent outer membrane receptor protein, RagA/SusC family
comp642048_c0	362	2.00E−78	99	AFU67994	TonB-dependent outer membrane receptor protein, RagA/SusC family
comp481890_c0	307	3.00E−55	100	AFU67696	Export-oriented ABC transporter, permease, and ATP binding components
comp982601_c0	250	1.00E−52	100	AFU67696	Export-oriented ABC transporter, permease, and ATP binding components
comp784927_c0	327	2.00E−69	100	AFU70024	Choline/glycine betaine transporter BetT
Lipid transport and metabolism					
comp640178_c0	463	1.00E−51	99	AFU68359	Omega-3 polyunsaturated fatty acid synthase subunit PfaA1
comp772635_c0	202	4.00E−41	100	AFU68359	Omega-3 polyunsaturated fatty acid synthase subunit PfaA1
comp698969_c0	256	1.00E−52	100	AFU69206	Dimethylallyltransferase/farnesyldiphosphate synthase IspA
comp263106_c0	781	2.00E−179	100	AFU70218	Very long chain acyl-CoA dehydrogenase
comp91491_c0	1335	0.0	100	AFU70218	Very long chain acyl-CoA dehydrogenase
comp207721_c0	369	2.00E−83	99	AFU70345	Delta-9 fatty acid desaturase
comp1053737_c0	205	2.00E−42	100	AFU70351	Long-chain acyl-CoA dehydrogenase
comp1046233_c0	253	1.00E−20	100	AFU70501	Enoyl-(acyl-carrier-protein) reductase (NADH) FabI

Table 1 continued

Gene_ID	Contig length	*E*-value	Protein identity (%)	Ref_sequence	Gene function
comp239334_c0	1027	0.0	100	AFU70219	3-Ketoacyl-CoA thiolase/acetyl-CoA acetyltransferase
Molecular chaperone					
comp351019_c0	831	0.0	100	AFU67954	Heat shock protein 60 family chaperone GroEL
comp57097_c0	1118	0.0	100	AFU67954	Heat shock protein 60 family chaperone GroEL
comp173578_c0	1921	0.0	100	AFU67393	Molecular chaperone protein DnaK
comp384360_c0	253	4.00E−52	100	AFU68531	Trigger factor (nascent protein chaperone) Tig
comp216113_c0	210	2.00E−39	100	AFU70350	Cold shock protein CspD
comp278155_c0	752	4.00E−158	99	AFU69336	Chaperone protein DnaJ
comp322985_c0	804	0.0	100	AFU69382	Chaperone protein HtpG
comp1071793_c0	254	2.00E−45	100	AFU70084	ATP-dependent RNA helicase DbpA-like protein
comp421126_c0	446	1.00E−92	96	AFU70461	ATP-dependent chaperone ClpB
comp626716_c0	247	4.00E−48	100	AFU70461	ATP-dependent chaperone ClpB
comp627520_c0	289	9.00E−55	100	AFU70461	ATP-dependent chaperone ClpB
Others					
comp972821_c0	281	1.00E−50	96	AFU68507	DTDP-glucose 4,6-dehydratase RfbB (EPS)
comp982211_c0	244	2.00E−51	100	AFU70112	Glucan endo-1,3-beta-D-glucosidase (laminarinase)
comp103123_c0	1060	6.00E−168	100	AFU67274	Phage shock protein A PspA
comp489615_c0	468	7.00E−103	94	AFU67218	Proteorhodopsin

The gene encoding proteins involved in the biological process, such as insertional elements, cell surface/transporters, lipid transport and metabolism, and molecular chaperone are included. The Gene_ID and Contig length in the transcriptome of ICE-L, the *E*-value and protein identity to the Ref_sequence of *Psychroflexus torquis*, as well as the gene function are shown

sister species. The external origin genes in *P. torquis* were mainly related to the ability of dwelling in sea-ice ecosystem, such as genes for EPS and PUFA biosynthesis, numerous specific modes of nutrient acquisition, and light-sensing (Feng et al. 2014). We suggest that ICE-L achieve some capacities that corresponding to the freezing acclimation from its symbiotic microbes in the polar sea-ice brine channel by transposases.

There are two reasons that we suggest the genes of ICE-L homology to that of *P. torquis* are acquired via horizontal gene transfer, while not from cDNA contamination. The first reason is that we have tried to isolate the symbiotic bacteria in the ICE-L broth. Bacterial strains, such as *Halomonas* sp., *Marinobacter* sp., and several other species except *P. torquis* have been isolated from the marine agar medium (0.5 %w/v proteose peptone, 0.2 % w/v yeast extract) according to the 16S rRNA sequencing results (Fig. S1). However, there was few genes belong to these species present in the transcriptome data of ICE-L. The second reason is the eukaryotic mRNAs were polyadenylated at high efficiency with poly (A) tails of 80–200 nt long, but only a small fraction of prokaryotic mRNAs have much shorter poly (A) tails (Steege 2000). The isolation of mRNA based on poly (A) tails during transcriptomic sequencing would reduce the risk of bacteria mRNA contamination.

Biological pathways comparison among ICE-L, *C. reinhardtii*, and *V. carteri*

C. reinhardtii and *V. carteri* (Volvocales, Chlorophyceae) are closely related species to ICE-L. Both of them have been finished genomic sequencing (Merchant et al. 2007; Prochnik et al. 2010). Totally 14,487 and 15,285 putative expressed protein sequences have been identified from the genomic sequences of *C. reinhardtii* and *V. carteri,* respectively. By performing BLASTP search against the 69,430 transcriptomic contigs of ICE-L, 9757 (67.34 %) protein sequences of *C. reinhardtii* matched with 12505 contigs of ICE-L transcriptome, and 9400 (61.50 %) protein sequences of *V. carteri* matched with 13975 contigs of ICE-L, with an *E*-value of $\leq$ 1.0E–3 as threshold. It means about 81.99 % and 79.87 % of ICE-L transcriptomic contigs could not find homology sequences in the genome of *C. reinhardtii* and *V. carteri*. The strategies of sea-ice environments survival of ICE-L may come from these unmatched special genes.

To investigate the differences of the biological pathways among the three species and to find out the specific pathways that might associated with the psychrophilic character of ICE-L, we have assigned *K* numbers to the sequences in the genome of *C. reinhardtii* and *V. carteri* as well as transcriptomic data of ICE-L to construct KEGG pathways

through KAAS (KEGG Automatic Annotation Server: http://www.genome.jp/kegg/kaas/). Each *K* number represents an orthologous group of genes, and it is directly linked to an object in the KEGG pathway map (Moriya et al. 2007). Results showed that 69,430 contigs of ICE-L were assigned to 3883 *K* numbers belong to 281 KEGG pathways. The 14487 and 15285 putative protein encoding sequences of *C. reinhardtii* and *V. carteri* were assigned to 3682 and 3572 *K* numbers within 273 and 274 KEGG pathways, respectively (Table. S3). The difference of *K* number candidates among ICE-L and the other two species in some KEGG pathways cannot provide us exact information about which pathway is responding to the freezing acclimation of ICE-L, but it might provide us some clues deserved further investigation on the freezing acclimation strategies of sea-ice algae.

In the post-transcriptional modification processes, *K* number candidates of ICE-L are obviously abundant than that of *C. reinhardtii* and *V. carteri*, for instance, *K* number candidates involved in RNA transport are 83, 67, and 72, in RNA degradation are 53, 41, and 48, in mRNA surveillance pathway are 47, 39, and 38 of ICE-L, *C. reinhardtii,* and *V. carteri,* respectively. In eukaryotes, nuclear export of RNA is an integral part of gene regulation in response to abiotic stresses (Chinnusamy et al. 2008). The proteins that constitute the nuclear pore complex are collectively called nucleoporins (NUPs). Recent works have suggested that NUPs are implicated in many aspects of plant life, including the abiotic stress response, by affecting the nuclear import and export (Xu and Meier 2008). We found 20 NUPs in the nuclear pore complex of ICE-L, 18 in *V. carteri*, and only 11 in *C. reinhardtii* (Fig. 2). *Nup133*, *Trp,* and *Nup54* specifically present in ICE-L, and *Nup160* is absent in *C. reinhardtii*. Nup160 and Nup133 interact with Nup98 and play an important role in mRNA export (Vasu et al. 2001). The studies in *Arabidopsis* revealed that Nup160 is critical for mRNA export, cold-responsive gene expression, cold tolerance, as well as plant development at normal temperatures. *AtNup160* mutation impaired the expression of a number of genes involved in plant cold tolerance (Dong et al. 2006). NUP133, characterized from Lotus, showed a participation in host-plant recognition of symbiotic microbes (Kanamori et al. 2006). Post-translational modifications like ubiquitination are also involved in the plant response to abiotic stress. The ubiquitin 26S proteasome system (UPS) has recently emerged as a major player in plant responses to abiotic stresses (Lyzenga and Stone 2012). The proteins involved in the ubiquitin-mediated proteolysis pathway in ICE-L (55) are more abundant than that in *C. reinhardtii* (49) and *V. carteri* (45).

Two-component signal-transduction systems (TCSs) are involved in the environmental stress responses of many bacteria and plants by transcriptional control of gene expression (Stock et al. 2000). In ICE-L transcriptome, 35 contigs were clustered to 20 KO assignments in two-component system, while there are only 13 and 11 *K* number candidates in *C. reinhardtii* and *V. carteri*, respectively (Fig. S2). A *vicRK* signal-transduction system that encodes

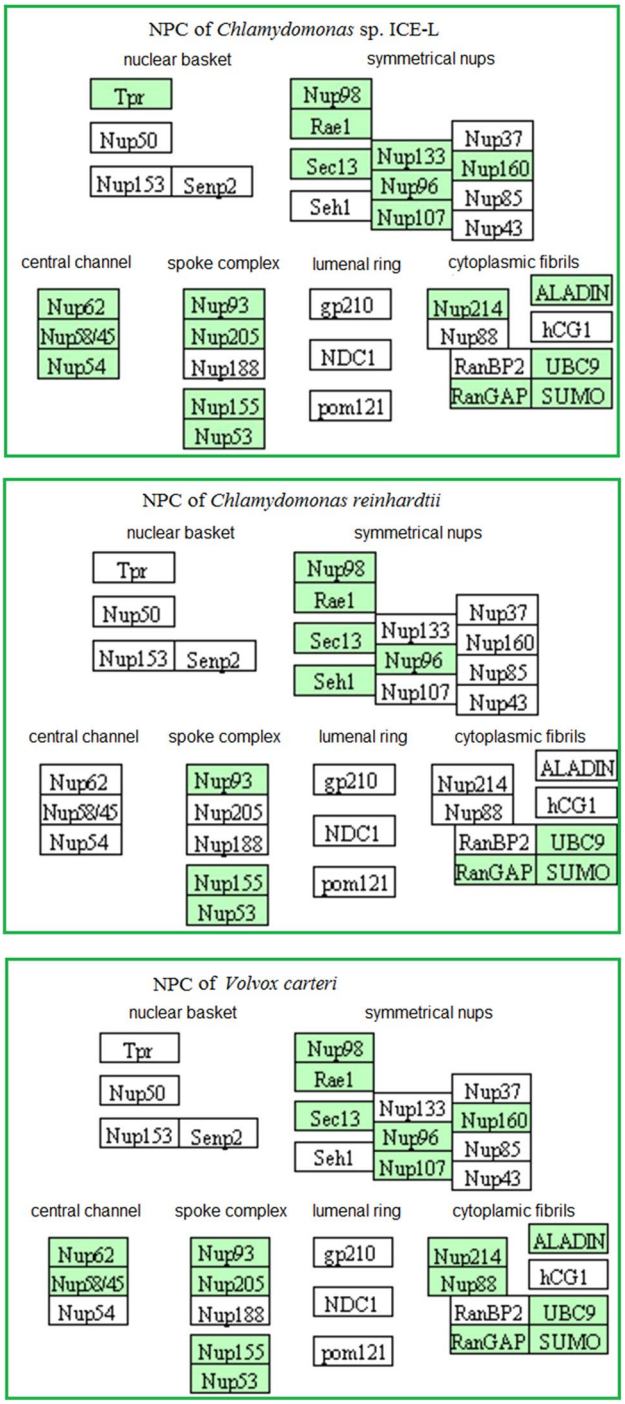

Fig. 2 Nuclear pore complex composition of *Chlamydomonas* sp. ICE-L, *C. reinhardtii,* and *V. carteri* through the process of KEEG pathway mapping of KAAS. The Nups in *green box* are present in the genomic sequences of *C. reinhardtii*, *V. carteri,* and the transcriptomic sequences of ICE-L, and the Nups in the *white box* are absent

a putative histidine kinase (*VicK*) and a response regulator (*VicR*) were specifically present in ICE-L, while not in *C. reinhardtii* and *V. carteri*. The *vicRK* signal-transduction system has been proved to be involved in regulating several important physiological processes and responding to environmental stresses in bacteria (Winkler and Hoch 2008; Liu and Burne 2009).

The *K* number candidates of ICE-L involved in other signal-transduction pathway, such as MAPK signaling pathway (22, 11, 12), cAMP signaling pathway (19, 12, 14), cGMP–PKG signaling pathway (18, 16, 13), and Ras signaling pathway (12, 6, 7) are larger quantities than that of *C. reinhardtii* and *V. carteri*. Some of these signaling systems facilitate adaptation and survival in the face of diverse environmental stresses in plants, suggesting that the signal-transduction pathway might also play critical roles in ICE-L acclimation to the sea-ice environments in polar oceans.

Differentially expressed genes in *Chlamydomonas* sp. ICE-L under freezing treatment

Differentially expressed gene (DEG) analysis was performed to identify genes for freezing stress response in ICE-L by comparing the transcriptomic data of freezing treatment samples and controls. The DEGs in two replication experiments (F1/C1, F2/C2) were analyzed separately (Fig. 3). The DEGs in the two replication groups were shown in the Volcano plot based on the ratio of gene expression fold change and gene RPKM (Fig. 3a). In F1/C1 group, a total of 527 genes were found to be differentially expressed in the freezing treatment sample F1 compared with the control C1, with 381 tags appeared to be up-regulated and 146 tags down-regulated. For the F2/C2 group, among the 395 DEGs between F2 and C2, 278 genes were induced and 117 were repressed expressions. Total 147 up-regulated genes and 73 down-regulated genes were presented in both F1/C1 and F2/C2 groups (Fig. 3b). The heat map of 220 differentially expressed genes indicated their expression profiles in the two treatment samples and two controls are well replicated (Fig. 3b).

We surmise that the reason for the difference of the DEGs number between the two parallel groups might be due to the transcriptome of ICE-l was de novo assembled without a reference genome. Many different expressed partial fragments can not be assembled into the same gene, especially those without homologous protein hit genes (Vijay et al. 2013). In addition, the quantity of the DEGs in response to freezing stress in ICE-L was relatively limited. The explanation could be the psychrophilic microorganisms constitutively synthesize some of the freezing responding proteins aimed at relieving the detrimental effects of possibly forthcoming temperature downshifts. For example, the activities of ice-binding proteins can be detected in our 7 °C cultured alga broth. After the cold shock for 6 h on Antarctic psychrophile *Pseudoalteromonas haloplanktis*, no cold-induced proteins were detected suggesting that there are noticeable differences in the cold shock response between a true psychrophile and mesophiles (Piette et al. 2012).

Some known genes in ICE-L transcriptome that displayed significantly up-regulated expressions under freezing treatment are listed (Table 2). There are genes encoding transporter proteins, proteins involved in protein phosphorylation, carbohydrate metabolism, nitrogen transportation and metabolism, lipid metabolism, and redox equilibrium. Genes encoding a chloride channel, a sodium/phosphate symporter, and a copper ion transporter were up-regulated, indicating the micro molecular transporters participate in the freezing response in ICE-L, possibly by decreasing cellular osmotic potential. The up-regulated expression of genes encoding calmodulin, calmodulin- dependent protein kinase, and serine-threonine protein kinase indicates that protein phosphorylation was induced during freezing exposure in ICE-L. It has been proved that Ca^{2+}- calmodulin signals as well as protein phosphorylation play important roles during the acquisition of freezing tolerance in plants (Storey and Storey 2013).

Moreover, Genes encoding proteins involved in protein translation, transportation, and sorting are up-regulated under freezing treatment in ICE-L, including protein transport protein SEC61, signal recognition particle protein, protein involved in vacuolar protein sorting, and Heat shock protein 70.

In addition, freezing-induced genes also include those encoding peptidase, such as matrix metalloproteinase M11, X-Pro dipeptidyl-peptidase, and protein binding 26S proteasome regulatory complex, which implies that post-translational modification is an important process for ice algae acclimation during the early stage of freezing stress. That might be due to the freezing stress triggered proteins misfolded. The misfolded proteins were transported from the ER-Golgi network to the cytosol through Sec61 pore system and degraded by the 26S proteasome system (Humbert et al. 2012).

Genes encoding nitrate reductase, ferredoxin-nitrite reductase, and nitrate/nitrite transporter were also induced, which implied that the transport and metabolism of nitrogen also related to the freezing acclimation of ICE-L. The high level of nitrate reductase activity and transcription up-regulation of nitrate reductase have been proved in many polar algae species, (Chen et al. 2012; Vona et al. 2004; Di Martino Rigano et al. 2006). Nitrate reductase-mediated early Nitric oxide (NO) burst plays an important role in cold acclimation and freezing tolerance through the modulation of proline accumulation in *Arabidopsis* (Zhao et al.

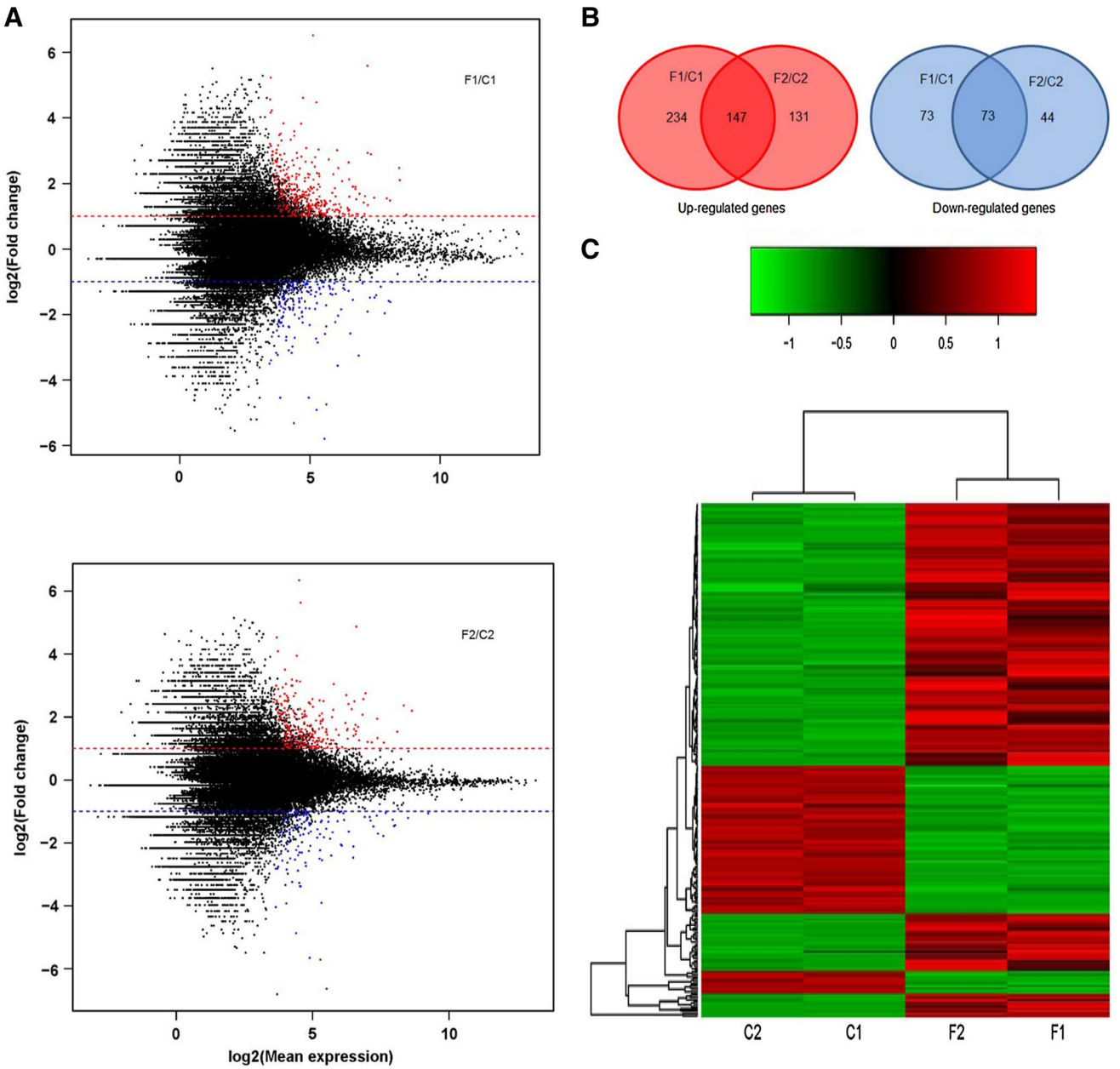

Fig. 3 Transcriptomic analysis of differentially expressed genes (DEGs) within the two replications F1/C1 and F2/C2 of ICE-L under freezing treatment. **a** *Volcano plot* showing DEGs between freezing treatment samples and the controls. *Red plots* represent genes with significantly up-regulated expression; *blue plots* represent those showing significantly down-regulated; and *black plots* represent genes with no significantly expression changes. **b** *Venn diagram* of represent the un-regulated DEG numbers (*red*) and down-regulated DEG numbers (*blue*) and the relationships between the two replications. **c** Heat map of 220 differentially expressed genes. The *color* (from *green* to *red*) represents gene expression level from low to high. *C1* and *C2* represent controls under 7 °C, and *F1* and *F2* represent treatment samples under −20 °C

2009). Nitric oxide (NO) burst phenomena has not been reported in microalgae.

Cellular compatible solutes such as betaine serve as freeze protection molecules by reducing the intracellular freezing point and help maintain enzyme hydration spheres to stabilize catalytic activity (Welsh 2000). The up-regulation of betaine lipid synthase indicates betaine might also play important roles in ICE-L freezing acclimation. A chitinase gene expression of ICE-L was up-regulated under freezing treatment. Chitinase hydrolyse the β-1, 4-linkage of chitin, and also acquire antifreeze activity in some of the overwintering plants during cold stress (Gupta and Deswal 2014). Several chitinase genes showed up-regulation

Table 2 Up-regulated expressed contigs in *Chlamydomonas* sp. ICE-L transcriptome under freezing treatment

Gene_ID	Length	Protein name	Homologous in *C. reinhardtii*	*E*-value	Fold-change	
Post-translational modification, protein transportation, chaperone functions						
comp68121_c0	1378	IQ calmodulin-binding motif	Cre11.g482300	4E−63	1.47	1.07
comp31898_c1	2140	Calmodulin and related proteins	Cre01.g051050	2E−048	1.82	1.51
comp8475_c0	1104	Calmodulin and related proteins	Cre14.g618000	1E−071	1.39	–
comp34858_c1	2103	Calmodulin-dependent protein kinase	Cre16.g661750	3E−038	1.19	1.54
comp13151_c0	1079	Ca^{2+}/calmodulin-dependent protein kinase	g18129	3E−124	1.64	–
comp19667_c0	2073	Serine-threonine protein kinase	Cre14.g612000	2E−018	1.72	1.44
comp8197_c0	2484	Serine-threonine protein kinase	g9960	3E−147	1.47	–
comp64879_c0	1598	Phosphatidylinositol-4-phosphate 5-kinase	Cre16.g688650	4E−051	1.28	–
comp34840_c0	3468	Serine-threonine protein kinase	Cre13.g574350	1E−053	1.18	–
comp55055_c0	1652	Protein phosphatase 1	g17895	2E−070	1.35	–
comp30343_c0	3009	X-Pro dipeptidyl-peptidase (S15 family)	Cre07.g328950	2E−142	1.33	1.15
comp22586_c0	1886	Matrix metalloproteinase Gametolysin peptidase M11	Cre08.g376950	6E−027	2.89	2.86
comp40262_c0	1960	Signal recognition particle protein	Cre16.g683950	0.0	1.88	1.36
comp11297_c0	1531	Protein arginine N-methyltransferase	Cre12.g558100	0.0	1.11	1.83
comp11349_c0	1316	Protein involved in vacuolar protein sorting	Cre13.g577950	5E−069	1.30	1.51
comp13580_c0	1058	Transport protein Sec61, ER-protein translocase.	Cre03.g171350	1E−107	1.28	1.51
comp49492_c0	692	Protein transport protein SEC61 subunit alpha	Cre03.g171350	4E−139	1.36	1.02
comp37292_c0	1700	Heat shock protein 70A	Cre08.g372100	2E−148	1.45	1.68
comp37576_c0	266	Heat shock protein 70A	Cre08.g372100	7E−049	1.61	1.51
Nitrogen transportation and motabolism						
comp34296_c1	3810	Ferredoxin-nitrite reductase	Cre09.g410750	1E−141	2.07	1.10
comp39460_c0	486	Ferredoxin-nitrite reductase	Cre09.g410750	9E−041	2.00	2.28
comp33687_c0	4303	Nitrate reductase	Cre09.g410950	0.0	2.69	2.47
comp34939_c1	2452	Nitrate/nitrite transporter NRT	Cre09.g410800	0.0	2.20	2.10
Transpoter proteins						
comp36606_c0	2863	Bestrophin, chloride channel	Cre16.g662600	2E−113	2.25	2.12
comp34374_c0	3510	Sodium/phosphate symporter	Cre02.g144600	1E−154	1.33	1.34
comp11498_c0	2400	CTR type copper ion transporter	Cre13.g570600	2E−063	1.94	1.71
comp34866_c3	4139	Cation transport ATPase	g16149	6E−057	1.39	–
Carbohydrate metabolism						
comp41458_c0	1283	Beta-1,4-*N*-acetylglucosaminyltransferase	Cre16.g669950	2E−067	2.32	2.28
comp34645_c5	2763	Granule-bound starch synthase I	Cre17.g721500	0.0	1.14	1.12
comp20849_c0	3168	Chitinase, glycosyl hydrolases family 18	Cre07.g317250	2E−010	1.81	1.22
Lipid metabolism						
comp13577_c0	2541	Glycerophosphoryl diester phosphodiesterase	Cre03.g203600	2E−070	1.83	1.71
comp19387_c0	2707	Cytosolic phospholipase A2	Cre12.g534050	4E−096	1.34	–
comp21564_c0	3460	3-Ketoacyl-CoA synthase	Cre17.g722150	0.0	1.03	1.06
comp20469_c1	1407	Stearoyl-CoA desaturase	*Emiliania huxleyi* virus	5E−42	1.01	–
comp30378_c1	1800	Delta 6-fatty acid desaturase	Cre01.g037700	2E−009	1.41	–
comp13297_c0	2625	Betaine lipid synthase	Cre07.g324200	0.0	1.8	–
Photosynthesis						
comp27075_c0	3816	Chloroplast elongation factor G	Cre02.g076250	2E−180	1.11	1.16
comp57517_c0	2034	Chlorophyll a oxygenase	Cre01.g043350	0.0	2.21	1.82
comp38691_c0	1644	Glutamyl-tRNA reductase	Cre07.g342150	0.0	1.74	1.54
comp34282_c1	5376	Magnesium protoporphyrin chelatase subunit H	Cre07.g325500	0.0	1.60	1.36
Others						
comp47229_c0	1186	Carbonic anhydrase	Cre09.g405750	8E−91	2.9	–

Table 2 continued

Gene_ID	Length	Protein name	Homologous in *C. reinhardtii*	*E*-value	Fold-change	
comp26831_c0	2670	Cytochrome P450 reductase	Cre01.g039350	0.0	1.82	1.63
comp32461_c0	830	Thioredoxin-like protein	Cre03.g157800	1E−058	1.89	1.05
comp49872_c0	2487	Type-II calcium-dependent NADH dehydrogenase	g18128	0.0	1.98	1.67
comp64482_c0	753	Low-CO2-inducible protein	g4582	4E−039	2.21	3.02
comp11213_c0	2857	Antifreeze protein, ice-binding protein	*Stigmatella aurantiaca*	1E−25	1.15	–
comp27618_c0	1100	Hydroxyproline-rich glycoprotein component	Cre06.g258800	1E−07	1.20	–
comp36342_c0	2927	Zeaxanthin epoxidase	Cre02.g082550	0.0	1.21	–

The Gene_ID and Contig length in the transcriptome of ICE-L, encoded protein function and its homologous sequence in *C. reinhardtii* or other species, as well as the *E*-value are shown. *Two columns* of fold change showed differential expression results in F1/C1 and F2/C2 groups, respectively. "-" means the gene expression is insignificantly regulated

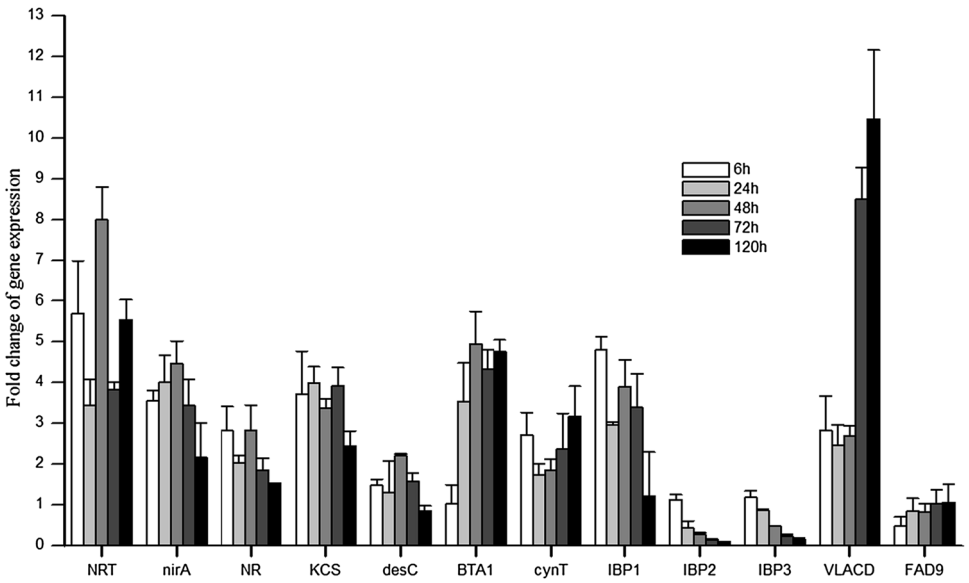

Fig. 4 Expression changes of several genes of *Chlamydomonas* sp. ICE-L under freezing conditions by qRT-PCR. *NRT* (nitrate/nitrite transporter, comp34939_c1), *nirA* (ferredoxin-nitrite reductase, comp34296_c1), *NR* (nitrate reductase comp33687_c0), *KCS* (3-ketoacyl-CoA synthase, comp21564_c0), *desC* (stearoyl-CoA 9-desaturase, comp20469_c1), *BTA1* (betaine lipid synthase, comp13297_c0), *cynT* (carbonic anhydrase, comp47229_c0), *IBP1* (ice-binding protein 1, comp28371_c0), *IBP2* (comp51699_c0), *IBP3* (comp11213_c0), *VLACD* (Very long chain acyl-CoA dehydrogenase, comp91491_c0), *FAD9* (Delta-9 fatty acid desaturase, comp207721_c0)

responses to cold treatment in *Brassica* (Ahmed et al. 2012).

Several differently expressed genes resulted from the DEG analysis were chosen to test their expression patterns during freezing acclimation via qRT-PCR (Fig. 4). The gene transcript levels across the five time points (6, 24, 48, 72, and 120 h) were detected. The expression of nitrite transporter, ferredoxin- nitrite reductase, nitrate reductase, and 3-ketoacyl-CoA synthase genes that induced in both the F1/C1 and F2/C2 groups are still present up-regulation during the freezing treatment based on the qRT-PCR analysis. The most significant changes occurred in nitrate/nitrite transporter gene, with an approximately 8-fold increase after treatment for 48 h, with a fluctuation at 24 and 72 h. Nitrite reductase and nitrate reductase genes also showed up-regulation from 6 to 48 h, reaching maximum at 48 h and decreasing thereafter.

The up-regulated expression of stearoyl-CoA desaturase gene, carbonic anhydrase gene, and betaine lipid synthase gene was only present in a single biological replication of RNA-seq based on their RPKM. The qRT-PCR results also showed the expected tendency, with all having high transcript levels under freezing treatment, except the stearoyl-CoA desaturase gene which showed less than 2.5-fold up-regulation expression at 48 h. The expression level of betaine lipid synthase gene after treated under

low temperature for 6 h was less than two fold, and then induced with 4-to-5-fold change during 24 to 120 h freezing treatment compared to 7 °C controls. The expressions of carbonic anhydrase gene were gradual increase from 24 to 120 h after an abrupt up-regulation at 6 h.

We have identified several ice-binding protein (IBP) genes from ICE-L transcriptome based on DUF3494 domain searching. The expressions of 3 IBP genes have also been detected. According to the DEG results, only IBP3 showed induced express in the F1/C1 group. While according to our qRT-PCR results, IBP1 showed significantly up-regulation tendency in gene expression. The expressions of IBP2 and IBP3 were unchanged in the early stages of freezing treatment and then repressed as freezing treatment time goes on. The expression of IBP genes investigated in the polar sea-ice diatoms *Fragilariopsis* under salinity, and freezing temperature conditions also showed different expression patterns. Some IBPs are up-regulated, while other isoforms stay constant or are strongly down-regulated (Bayer-Giraldi et al. 2010). One explanation could be some isoforms responsible for early response and others for long-term acclimation under freezing conditions. Another possibility is that may be not all of the sequences contain DUF3494 domain responding to the freezing stress as an ice-binding protein. Their ice-binding and thermal hysteresis activities need to be detected through in vitro experiments.

The expressions of a very long chain acyl-CoA dehydrogenase gene (VLACD) and a delta-9 fatty acid desaturase gene (FAD9) of ICE-L that supposed to be horizontally transferred from bacteria have also been detected through qRT-PCR. The results indicated that the expression of VLACD gene show significantly up-regulation especially after a long-time freezing treatment, while the expressions of FAD9 remain stable under freezing treatment compared with the control. An acyl-CoA dehydrogenase has been proved drive heat adaptation of *Caenorhabditis elegans* by sequestering fatty acids (Ma et al. 2015). The up-regulation of VLACD indicated that lipid ß-oxidation process might play an important role in freezing acclimation of ICE-L through modulating the membrane lipid homeostasis.

Conclusions

Studies on mechanisms of life adaptation to the abiotic stress using "omics" technologies have revealed the complex versatile traits in many organisms. However, the acclimation mechanisms of polar psychrophilic eukaryote microalga have been investigated insufficiently, considering their important ecological roles. Annotation of the ICE-L transcriptome provides us some new point on the environmental acclimation strategies of the polar sea-ice organism. Considering the large amounts of identity genes between ICE-L and sea-ice bacteria, we suggested that horizontal gene transfer is one of the most important strategies in the sea-ice organisms acclimating extreme environments, as mentioned in Collins and Deming (2013) that sea ice is a hotspot for HGT. The functions investigation on the mutual genes would be the following work.

Comparing the biological pathways of ICE-L with its sister species *C. reinhardtii* and *V. carteri* revealed that there are much more functional proteins involved in the post-translational and post-transcriptional processes, as well as signal-transduction routes in the psychrophilic species ICE-L than the moderate temperature species. The investigation of these pathways is crucial for the deeper understanding of the molecular mechanisms governing sea-ice algae adaptation to extreme environment. Therefore, we suggest ICE-L could be an excellent model to study the evolutionary process of abiotic stress adaptation.

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