RESEARCH LETTER

Characterization of the tandem-arrayed *hiC6* genes in Antarctic and temperate strains of *Chlorella vulgaris*

Yali Wang, Xiaoxiang Liu, Hong Gao & Xudong Xu

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China

Correspondence: Xudong Xu, The State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei 430072, China. Tel.: +86 27 68780659; fax: +86 27 68780123; e-mail: xux@ihb.ac.cn

Received 14 March 2011; revised 13 September 2011; accepted 14 September 2011.
Final version published online 3 October 2011.

DOI: 10.1111/j.1574-6968.2011.02420.x

Editor: Claire Remacle

Keywords
Chlorella vulgaris; Antarctic strain; temperate strain; *hiC6*; tandem array.

Abstract

HIC6 is a group-3 late embryogenesis abundant protein found in *Chlorella vulgaris*. In the Antarctic strain NJ-7 of this unicellular green alga, it is encoded by a tandem array of five *hiC6* genes (designated as *NJ7hiC6*-1, -2, -3, -4 and -5); in the temperate strain UTEX259, it is encoded by four *hiC6* genes in tandem (designated as *259hiC6*-1, -2, -3 and -4). Except for *NJ7hiC6*-3 and -4, the encoding regions of all other *hiC6* genes differ from each other by 2–19 bp in each strain. Based on RT-PCR and sequencing of total *hiC6* cDNA clones, the relative transcript abundance of each *hiC6* gene was evaluated. *NJ7hiC6*-2 and *259hiC6*-2 were not expressed or expressed at low levels, whereas *259hiC6*-1 and *NJ7hiC6*-3/4 exhibited the highest *hiC6* transcript levels in the respective strains. *In vitro* assays showed that different isoforms of HIC6 provided almost identical cryoprotection of lactate dehydrogenase. Our studies suggest that the formation of the tandem arrays of *hiC6* in *Chlorella* is a process of gene duplications accompanied by gene expression divergence.

Introduction

Chlorella vulgaris is a unicellular green alga often used as the eukaryotic model in studies of stress responses. Using *C. vulgaris* strain C-27, acquisition of freezing tolerance by cold-hardening has been extensively studied (Hatano *et al.*, 1976; Honjoh *et al.*, 1995, 1999, 2000, 2001; Machida *et al.*, 2008). More recently, *C. vulgaris* NJ-7, a strain isolated from the Antarctic, was used to investigate the adaptation of eukaryotic microbes to permanently cold environments (Hu *et al.*, 2008; Li *et al.*, 2009). The strain NJ-7 possesses the same 18S rRNA gene sequence as that of UTEX259, a strain isolated from the temperate region, but shows a significantly intensified freezing tolerance (5- to 1000-fold higher viability) than the temperate strain. Comparative studies of the two *C. vulgaris* strains provide opportunities to understand how intra-species evolution is undertaken in eukaryotic microbes to adapt to the Antarctic or other extreme environments.

HIC6 is a group-3 late embryogenesis abundant (LEA) protein found in *C. vulgaris*. Together with HIC12, it was first identified by 2D-HPLC and SDS-PAGE to be hardening (cold treatment)-induced in the strain C-27 (Honjoh *et al.*, 1995). Its cDNA was also identified by differential screening of a cDNA library (Joh *et al.*, 1995) or suppression subtractive hybridization (Machida *et al.*, 2008). LEA proteins were initially found at the late stage of embryogenesis in cotton (Galau *et al.*, 1986) and were subsequently found in algae (such as *C. vulgaris*), cyanobacteria (Close & Lammers, 1993), nematodes (Browne *et al.*, 2002) and fungi (Abba' *et al.*, 2006). The proteins can be divided into different groups on the basis of similarities in amino acid sequences (Colmenero-Flores *et al.*, 1997; Cuming, 2005; Battaglia *et al.*, 2008). Like many other LEA proteins, HIC6 remained soluble under boiling conditions and showed *in vitro* cryoprotective activities on lactate dehydrogenase (LDH) (Honjoh *et al.*, 2000). Overexpression of HIC6 in plant or yeast could enhance their freezing tolerance (Honjoh *et al.*, 1999, 2001), and in the transgenic plant, HIC6 was localized to mitochondria (Honjoh *et al.*, 2001). In strains NJ-7 and UTEX259, the encoding gene *hiC6* was also induced upon exposure to cold, and the expression was intensified in strain NJ-7 in comparison with UTEX259 (Li *et al.*, 2009). These results suggest that the enhanced expression of *hiC6* is probably involved in the development of freezing tolerance in *C. vulgaris*.

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The intensified expression of *hiC6* in NJ-7 could be due to gene duplication, increased transcription or post-transcriptional regulation. In our previous study, only one *hiC6* gene was identified in each of the two *Chlorella* strains, NJ-7 and UTEX259 (Li *et al.*, 2009). In the present study, however, sequencing of that chromosomal region revealed that multiple *hiC6* genes are organized in tandem in both strains. The tandem-arrayed genes encode different HIC6 isoforms and are differentially expressed.

Materials and methods

Strains and culture conditions

Chlorella vulgaris strains were grown in BG11 (Stanier *et al.*, 1971) in the light of 50 μE m^{-2} s^{-1} at 20 °C with aeration. Cells grown at 20 °C were cooled to 4 °C in a water bath and transferred to a 4 °C refrigerator with aeration and illumination (50 μE m^{-2} s^{-1}) for different periods of time.

Sequencing of the tandem arrays of *hiC6* genes

Clones carrying *hiC6* were identified from cosmid libraries of NJ-7 and UTEX259 (Wang & Xu, 2011) by PCR screening. For each strain, one cosmid carrying *hiC6* was analyzed by physical mapping and sequencing. For construction of physical maps, cosmids were digested by single or double restriction enzymes, and the sizes of restricted fragments were calculated based on their migration distances in agarose gel electrophoresis. *hiC6* genes were localized to restriction fragments by PCR. For sequencing of the *hiC6* region in the NJ-7 cosmid, a library of 2–4 kb *Sau*3AI DNA fragments (partial digestion) was constructed by insertion into the BamHI site of pUC19. *hiC6*-containing subclones were selected by PCR screening and sequenced. The sequence of the NJ-7 *hiC6* region was assembled from overlapping subclone sequences. With the reference of the NJ-7 sequence, PCR fragments were generated for the *hiC6* region of UTEX259 and sequenced. In addition, restriction fragments of this region in the UTEX259 cosmid were cloned and sequenced. The whole sequence of the UTEX259 *hiC6* region was assembled from those of PCR and restriction fragments. In each case, the sequence was confirmed by a series of PCRs using genomic DNA as the template. DNA sequences were deposited in the NCBI GenBank under accession numbers JF333588 (NJ-7 *hiC6* genes) and JF333589 (UTEX259 *hiC6* genes).

Southern blot analysis

Genomic DNA of *C. vulgaris* was extracted using the cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson, 1980). A 10-μg aliquot of DNA was digested completely with one or two restriction enzymes. Separation of digested DNA with 0.7% agarose electrophoresis and capillary transfer of the separated DNA fragments onto Immobilon-Ny^+ membrane (Millipore) were performed as standard methods (Sambrook *et al.*, 1989). The digoxigenin (DIG)-labeled *hiC6* probe for hybridization was prepared by PCR using hiC6-5 and hiC6-6 as primers and genomic DNA of NJ-7 as the template. Labeling, hybridization and detection were performed with DIG High Prime DNA Labeling and Detection Starter kit I (Roche) according to the manufacturer's recommendations.

Northern blot analysis

Total RNA was extracted using Trizol reagent (Invitrogen) from *C. vulgaris* strains according to manufacturer's instructions, separated by agarose/formaldehyde gel electrophoresis and blotted onto Immobilon-Ny^+ membranes by capillary transfer. The *hiC6* transcripts were probed by a PCR-generated 322-bp fragment overlapping the 3′-end of *hiC6*-3/4 cDNA (nt.380-701) of NJ-7.

Evaluation of relative transcript abundance of each *hiC6* gene

NJ-7 and UTEX259 were grown at 20 °C for 7 days and exposed to 4 °C for 24 h. Total RNA extracted from the algal cells with or without exposure to 4 °C was treated with RNase-free DNase I to remove residual DNA until no DNA could be detected by PCR, and then converted into cDNA using M-MLV reverse transcriptase (Promega). The transcription of each *hiC6* gene was shown with RT-PCR with gene-specific primers listed in Supporting Information, Table S1. Transcripts of *NJ7hiC6*-1, -2, -2/-3/-4/-5 were detected using primer pairs NJ7hiC6-1a/NJ7hiC6-1b, hiC6rt-2/NJ7hiC6-1b, NJ7hiC6-3a/NJ7hiC6-3b and NJ7hiC6-5a/NJ7hiC6-5b, respectively. Transcripts of *259hiC6*-1, -2, -3, -4 were detected using 259hiC6-1a/259hiC6-1b, 259hiC6-2a2/259hiC6-2b, 259hiC6-3a/259hiC6-3b and 259hiC6-4a/259hiC6-4b, respectively.

The transcription of each *hiC6* gene was also quantitatively evaluated by calculating the percentage of its cDNA clones in clones of total *hiC6* cDNA. DNA fragments of *hiC6* coding regions were generated by PCR using cDNA as the template and cloned into the T-vector pMD18-T (Takara). For NJ-7, primers hiC6rt-3 and hiC6rt-6 (Table S1) were used; for UTEX259, primers hiC6rt-3 and hiC6rt-4 (Table S1) were used. Clones of *hiC6* RT-PCR fragments were sequenced, and different *hiC6* clones were counted and used for calculation of their percentages of the total *hiC6* clones.

Expression of HIC6 isoforms in *Escherichia coli* and purification

Using total cDNA of *C. vulgaris* NJ-7 as the template, a PCR fragment containing the encoding region of *NJ7hiC6*-3 was generated using primers hiC6pcc-1 and hiC6pcc-2. The PCR fragment containing *259hiC6*-1 was generated using UTEX259 cDNA and a pair of primers hiC6pcc-2 and hiC6pcc-3. For *259hiC6*-3 and *259hiC6*-4, the primers hiC6pcc-1/hiC6pcc-2 were used. The PCR fragments were cloned into pMD18-T and sequenced, and clones of *259hiC6*-3 and *259hiC6*-4 were identified after sequencing. Using clones carrying different *hiC6* genes as the templates and hiC6his-4/hiC6his-2 as the primers, PCR fragments for expressing mature HIC6 isoforms in *E. coli* were generated, cloned into pMD18-T and confirmed by sequencing. The inserts in these plasmids were excised with NdeI and HindIII and cloned into pET21b (Novagen) for expression in *E. coli* BL21 (DE3).

Expression of the HIC6 isoforms in *E. coli* was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3–4 h at 37 °C. Cells broken by sonication were centrifuged at 13 000 ***g*** for 10 min to remove cell debris, and total soluble proteins were boiled for 10 min, followed by centrifugation at 13 000 ***g*** for 20 min. The recombinant HIC6 isoforms were purified from the supernatant using His·Bind resin (Novagen) under nondenaturing conditions according to the manufacturer's recommendations. The eluted proteins were desalted using Microcon YM-10 (Millipore) centrifugal filters and diluted 25-fold with potassium phosphate buffer (pH 7.5). Protein concentrations were determined by Bradford's method (Kruger, 2002) and confirmed by SDS-PAGE (Sambrook *et al.*, 1989).

Cryoprotective activity assays

The cryoprotective activities of HIC6 isoforms were assayed as described (Honjoh *et al.*, 2000) with modifications. The freeze-labile enzyme LDH (Fluka) was diluted to 3 μg mL^{-1} with 10 mM sodium phosphate buffer (pH 7.5). Cryoprotectant solutions were prepared with potassium phosphate buffer (pH 7.5) and diluted to the indicated concentrations. A 500-μL aliquot of LDH was mixed with an equal volume of cryoprotectant solution and frozen at −20 °C for 24 h and thawed at 20 °C for 20 min. LDH activities were measured using a spectrophotometer (Shimadzu).

Results

Tandem-arrayed *hiC6* genes in strains NJ-7 and UTEX259

In a previous study (Li *et al.*, 2009) we identified one *hiC6* gene in each of the two *C. vulgaris* strains by PCR. In this study, we performed a more extensive PCR screening of the cosmid libraries of both strains and obtained the *hiC6*-containing cosmids for each strain. A physical map of a NJ-7 cosmid was constructed, and the restriction fragments containing *hiC6* were identified by PCR. A 13 503-bp region of the cosmid was sequenced, in which five tandem-arrayed *hiC6* genes were identified. Figure 1a shows the structure of the NJ-7 cosmid. The

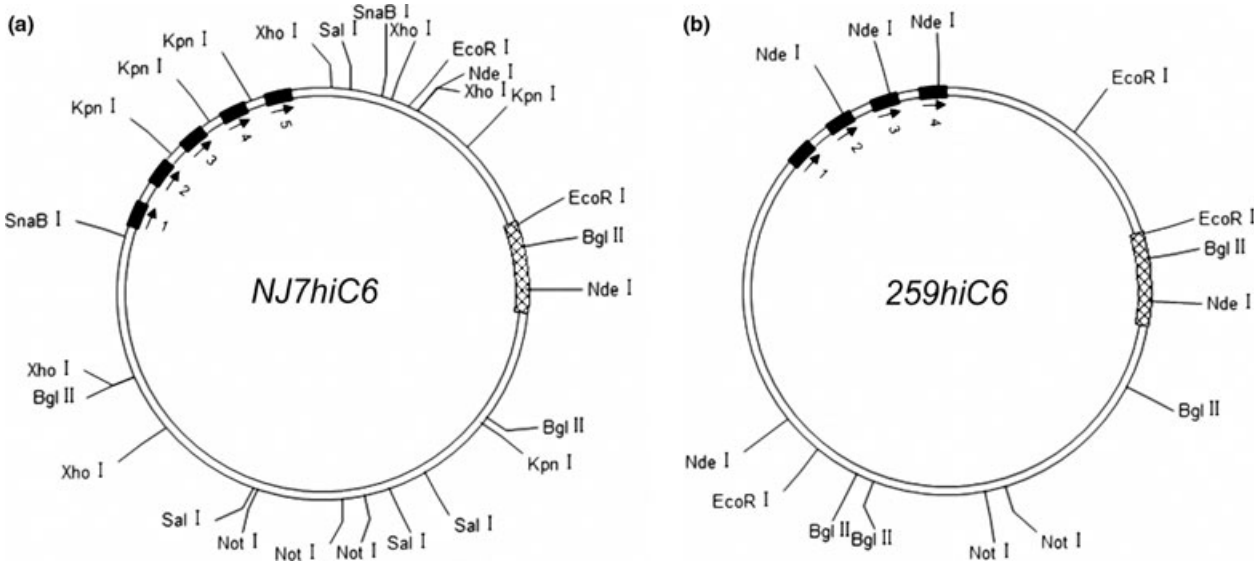

Fig. 1. Cosmids carrying the tandem array of *hiC6* genes in *Chlorella vulgaris* NJ-7 (a, *NJ7hiC6*) or UTEX259 (b, *259hiC6*). Black boxes stand for *NJ7hiC6*-1, -2, -3, -4 and -5 or *259hiC6*-1, -2, -3 and -4; arrows indicate the orientation of genes; hatched boxes stand for the cosmid vector.

structure of the tandem array of *hiC6* genes was confirmed by a series of PCR detections of chromosomal DNA using gene-specific primers (data not shown). The physical map of an UTEX259 cosmid was also constructed, and an 8210-bp region of the cosmid was sequenced, in which four tandem-arrayed *hiC6* genes were identified. Figure 1b shows the structure of the UTEX259 cosmid. The *hiC6* genes in NJ-7 are designated as *NJ7hiC6*-1, -2, -3, -4 and -5, and those in UTEX259 as *259hiC6*-1, -2, -3 and -4.

Each *hiC6* gene in the two strains possesses four exons and three introns. The alignments of cDNAs of five *NJ7hiC6* genes and four *259hiC6* genes are shown in Fig. 2a and b. *NJ7hiC6*-3 and -4 are identical to each other, whereas all other *hiC6* genes have 2–19 bp that differ from each other. *NJ7hiC6*-3, -4 and -5 encode identical HIC6 protein, whereas other copies in the two strains are predicted to encode HIC6 isoforms of 1–10 amino acid substitutions (Fig. 2c). Introns show higher degrees of divergence between the *hiC6* genes compared with exons. As shown in Table S2, in both strains, the intron sequences of *hiC6*-1 (*NJ7hiC6*-1, *259hiC6*-1) as a whole are 84–89% identical to those of other *hiC6* genes, whereas the other sequences are 97–99% identical compared to each other. Apparently, *NJ7hiC6*-1 and *259hiC6*-1 are more distantly related to other *hiC6* genes in phylogeny.

To find out whether there was only one tandem array of *hiC6* genes in each strain, we performed Southern blot hybridizations. Restriction enzymes were chosen according to their sequences. As shown in Fig. 3, there was only one region of *hiC6* genes in the genome of NJ-7 or UTEX259. Due to the presence of an NheI site in the tandem array, digestion of NJ-7 genomic DNA with NheI + DraI resulted in two hybridization bands, whereas digestion with other restriction enzymes all resulted in a single band.

Differential expression of *hiC6* genes

In a previous report (Li *et al.*, 2009), we showed that the transcription of *hiC6* was increased in NJ-7 and UTEX259 after transfer from 20 to 4 °C, and that at 20 °C, *hiC6* was expressed at a much higher level in NJ-7 than in UTEX259. In this study, we further examined the abundance of total *hiC6* transcripts at different time points after transfer to the low temperature. Consistently, at 20 °C, *NJ7hiC6* genes showed much stronger expression than *259hiC6* genes. *259hiC6* transcripts were detected after 6 h at 4 °C, whereas *NJ7hiC6* transcripts showed almost no up-regulation except a slight increase at 6 h (Fig. 4a). After 72 h, *hiC6* transcripts became undetectable in both strains.

As multiple copies of *hiC6* were detected in both *C. vulgaris* strains, we investigated whether tandem-arrayed genes were differentially regulated. Due to the substitutions in cDNA sequences, we were able to evaluate the transcript abundance of most *hiC6* genes by RT-PCR using gene-specific primers. One or two-base substitutions at the 3′ end of a primer can distinguish a gene from others. Figure 4b shows the result of RT-PCR detection of different *hiC6* transcripts in cells at 20 °C or exposed to 4 °C for 24 h. In NJ-7, no primers could distinguish *NJ7hiC6*-3 or -4 from *NJ7hiC6*-2. The relative transcript abundance of each gene appeared to be similar at different temperatures. *NJ7hiC6*-2 and *259hiC6*-2 were both expressed at very low levels, whereas *259hiC6*-1 contributed to a larger proportion of total *hiC6* transcripts in UTEX259 than *NJ7hiC6*-1 in NJ-7. Two independent experiments showed similar results.

We also quantified the relative transcript abundance of each *hiC6* gene based on the sequences of total *hiC6* cDNA clones. Using primers (hiC6rt-3/hiC6rt-6 for NJ-7, hiC6rt-3/hiC6rt-4 for UTEX259; Table S1) matching all *hiC6* cDNAs in NJ-7 or UTEX259, RT-PCR products were generated and cloned into a T-vector. In each experiment, 114–176 *hiC6* cDNA clones of each strain were sequenced, and the percentages of different *hiC6* genes were calculated (Table 1). The relative transcript abundance of *hiC6* genes was consistent with the result of RT-PCR detection shown in Fig. 4, but *NJ7hiC6*-3 and -4, which are identical to each other, could be distinguished from *NJ7hiC6*-2 using the sequences. *NJ7hiC6*-2 and *259hiC6*-2 showed no or almost no transcription, whereas *259hiC6*-1 in UTEX259 and *NJ7hiC6*-3/4 in NJ-7 produced the largest proportion of *hiC6* transcripts.

The difference of transcript abundance could be due to divergence of regulatory regions. Figure S1 shows alignments of upstream sequences of *hiC6* genes. Compared to *hiC6*-3 and -4, *hiC6*-2 shows no or a very low level of expression in both strains. Accordingly, *hiC6*-2 has many insertions/deletions/substitutions (> 58.9%) in a ~230-bp region that is ~290-bp upstream of the transcriptional start point (tsp), whereas *hiC6*-3 and -4 from the same strain show little difference from each other. *NJ7hiC6*-5 has an upstream sequence identical to that of *NJ7hiC6*-4. Relative to the intron sequences, the 230-bp upstream region of *hiC6*-2 has significantly higher percentages of sequences different from that of *hiC6*-3 and -4. *NJ7hiC6*-1 and *259hiC6*-1 show very different expression from each other. Accordingly, they have 56-bp differences in upstream sequences. In a 28- to 38-bp region which is ~415-bp upstream of the tsp, *NJ7hiC6*-1, -3 and -4 have 13- to 27-bp deletions compared with their counterparts in UTEX259. Whether these variations are related to their expression modes could be investigated by transient expression analyses (Chen *et al.*, 2001) in future.

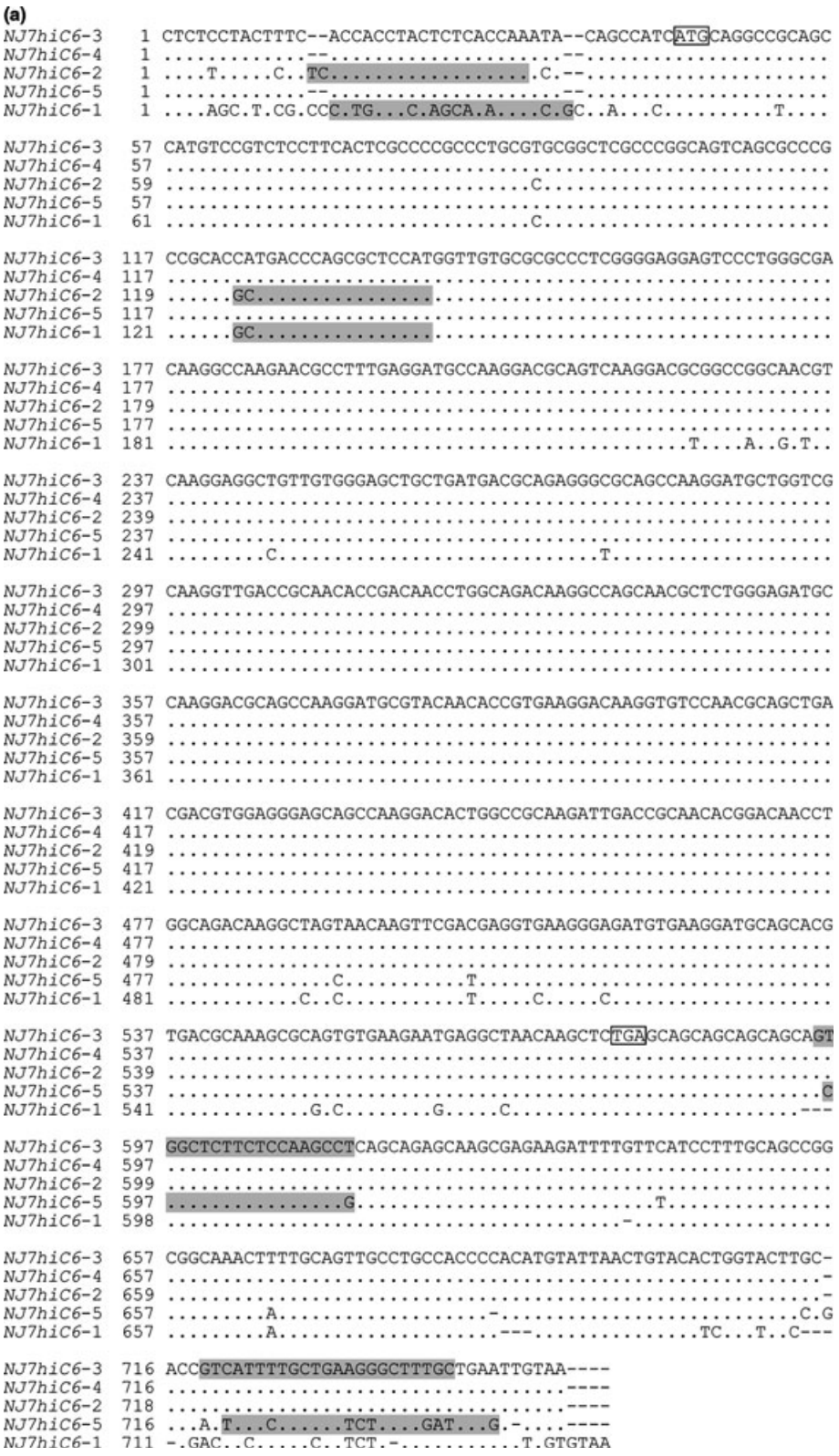

Fig. 2. Alignments of the full length cDNA of *hiC6* genes and amino acid sequences of HIC6 isoforms. The translational start and stop codons are boxed. Regions of the gene-specific primers (see Materials and methods and Table S1) are marked in gray. (a) *hiC6* genes in NJ-7. (b) *hiC6* genes in UTEX259. (c) HIC6 isoforms of NJ-7 and UTEX259.

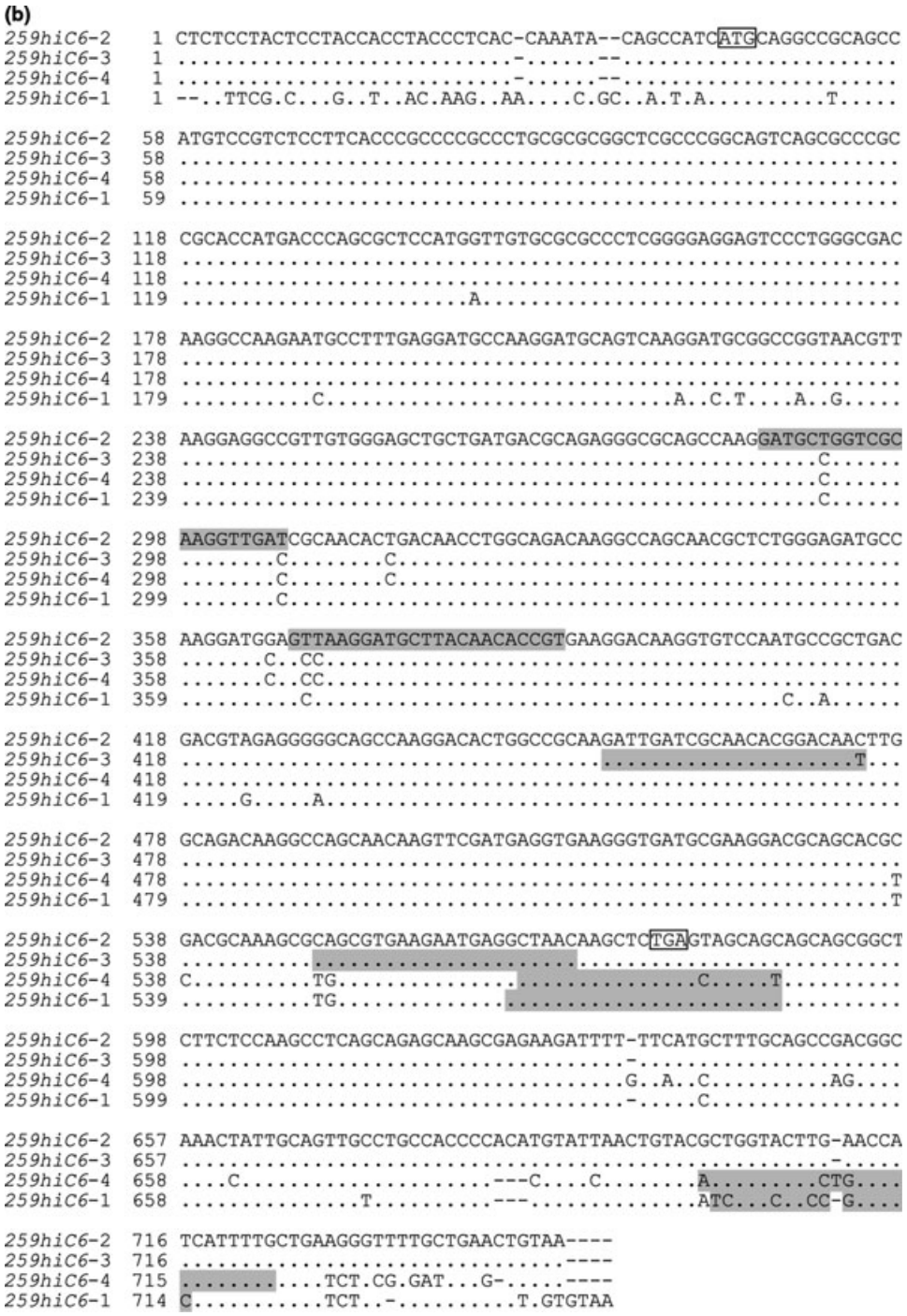

Fig. 2. Continued.

Cryoprotective activities of different isoforms of HIC6

Besides the enhanced expression of cold adaptation genes, accumulation of point mutations that enhance the activities of proteins at low temperatures could be an alternative strategy for adaptation to permanently cold environments. Given that *hiC6* genes were differentially expressed in the two strains at 20 and 4 °C, we wondered whether the expressed isoforms of HIC6 have different cryoprotective activities. To answer this question, we cloned the encoding regions of *NJ7hiC6*-3 (*NJ7hiC6*-4

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(c)
259HiC6-2    1 MQAAAMSVSFTRPALRAARPAVSARRTMTQRSMVVRALGEESLGDKAKNAFEDAKDAVKD
259HiC6-3    1 ............................................................
NJ7HiC6-2    1 ........................L...................................
NJ7HiC6-3    1 ............................................................
259HiC6-4    1 ............................................................
NJ7HiC6-1    1 ...S........................L...................................
259HiC6-1    1 ...S.........................I...................................

259HiC6-2   61 AAGNVKEAVVGAADDAEGAAKDAGRKVDRNTDNLADKASNALGDAKDGVKDAYNTVKDKV
259HiC6-3   61 ..............................................AA..........
NJ7HiC6-2   61 ..............................................AA..........
NJ7HiC6-3   61 ..............................................AA..........
259HiC6-4   61 ..............................................AA..........
NJ7HiC6-1   61 V.SD...........................................AA..........
259HiC6-1   61 V.SD...........................................A...........

259HiC6-2  121 SNAADDVEGAAKDTGRKIDRNTDNLADKASNKFDEVKGDAKDAARDAKRSVKNEANKL
259HiC6-3  121 ............................................................
NJ7HiC6-2  121 ..................................V.........................
NJ7HiC6-3  121 ..................................V.........................
259HiC6-4  121 ..............................................H...G........
NJ7HiC6-1  121 ..................................V.........G..K.....
259HiC6-1  121 ..............................................G........
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Fig. 2. Continued.

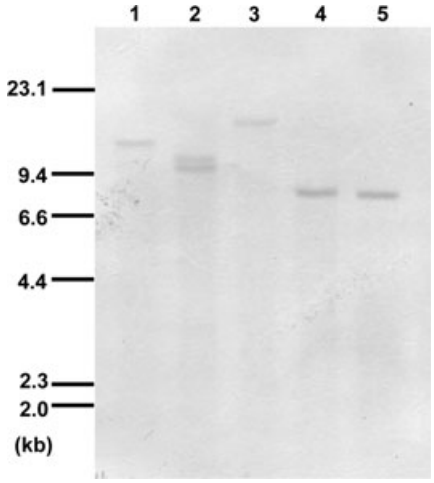

Fig. 3. Southern blot analyses of the tandem-arrayed *hiC6* genes in *Chlorella vulgaris* strains NJ-7 and UTEX259. A PCR fragment internal to *hiC6* gene(s) from NJ-7 was used as the probe. (1) NJ-7 DNA + StuI; (2), NJ-7 DNA + NheI + DraI; (3), NJ-7 DNA + SacI + SalI; (4) UTEX259 DNA + StuI; (5) UTEX259 DNA + SphI + SacI.

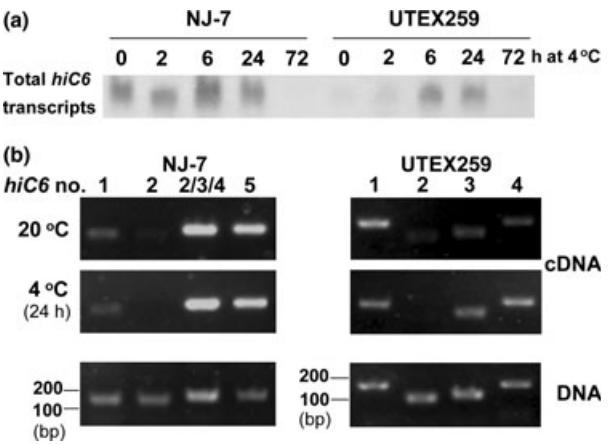

Fig. 4. Differential expression of *hiC6* genes in *Chorella vulgaris*. (a) Northern blot analysis of total *hiC6* transcripts in strains NJ-7 and UTEX259 transferred from 20 to 4 °C. The same batch of mRNA samples was used to detect the transcription of another LEA-like protein gene, *Ccor1*, with 18S rRNA gene as the internal control, in the paper by Liu *et al.* (2011). (b) Differential expression of *hiC6* genes as shown with RT-PCR using gene-specific primers. The third lane of NJ-7 (denoted as 2/3/4) is a mixture of PCR products of *NJ7hiC6*-2, -3, and -4. The primers are listed in Table S1 and are described in Materials and methods. In contrast to the RT-PCR products (using cDNA as the template), the PCR products generated using genomic DNA as the template showed comparable brightness, with the exception of the mixture of *NJ7hiC6*-2, -3 and -4.

and -5 encode the same protein) and *259hiC6*-1, -3 and -4, and expressed them as fusion proteins with 6His·tag in *E. coli*. In the fusion proteins, the N-terminal 36-amino acid transit signal of HIC6 (Joh *et al.*, 1995; Honjoh *et al.*, 1995) was deleted.

The cryoprotection of LDH was assayed with different concentrations of HIC6 isoforms. Bovine serum albumin was used as the positive control as in other reports (Honjoh *et al.*, 2000; Griffith *et al.*, 2005). The cyanobacterial RNA-binding protein 1 (Rbp1), which has a very slight protective effect on LDH, was used as the negative control. As seen with the LDH residual activities after a freeze–thaw cycle, the cryoprotective activities of all four isoforms of HIC6 showed no differences from each other (Fig. 5). This result suggested that the amino acid

Table 1. The relative transcript abundance (%)* of each *hiC6* gene at 20 or 4 °C in two independent experiments

	20 °C		4 °C (24 h)	
Genes	Experiment 1	Experiment 2	Experiment 1	Experiment 2
NJ-7				
NJ7hiC6-1	5.7	3.2	2.6	3.1
NJ7hiC6-2	4.0	0.5	0	0.6
NJ7hiC6-3/4	72.2	80.1	71.5	77.6
NJ7hiC6-5	18.2	16.1	25.8	18.6
UTEX259				
259hiC6-1	51.8	68.5	56.6	37.6
259hiC6-2	0	0	0	0.7
259hiC6-3	18.4	15.3	17.2	22.0
259hiC6-4	29.8	16.2	26.2	39.7

*In each experiment, percentages of different *hiC6* transcripts at a certain temperature were calculated based on the sequences of 114–176 *hiC6* cDNA clones of each strain.

substitutions in HIC6 made no or only a very slight contribution to the increased freezing tolerance of the Antarctic strain.

Discussion

HIC6 and HIC12 are two cold-inducible LEA proteins found in *Chlorella*, both possessing cryoprotective activities. HIC6 has been shown to enhance the freezing tolerance in transgenic plants (Honjoh *et al.*, 2001). Initially identified in C-27 of *C. vulgaris* (Joh *et al.*, 1995; Honjoh *et al.*, 1995), their encoding genes were also found in the temperate strain UTEX259 and the Antarctic strain NJ-7 of *C. vulgaris* (Li *et al.*, 2009). In this study, we identified a tandem array of five *hiC6* genes in NJ-7 and a tandem array of four *hiC6* genes in UTEX259 and investigated the differential expression of these genes. Unlike *hiC6*, *hiC12* is present as a single gene in the two *Chlorella* strains (Y. Wang and X. Xu, unpublished).

In C-27 and UTEX259, the expression of *hiC6* can be detected at very low levels at 20–25 °C but was greatly induced after exposure at 3–4 °C (Joh *et al.*, 1995; Li *et al.*, 2009). In the Antarctic strain NJ-7, however, *hiC6* genes can be expressed at a relatively high level even without cold induction, and the expression appeared to be less dependent on temperature. At the other extremity of temperature adaptation, the chilling-sensitive strain C-102 of *C. vulgaris* has no *hiC6* (Joh *et al.*, 1995). The induced expression of *hiC6* probably reflects the seasonal changes of temperature in temperate regions. However, in the permanently cold environments of Antarctica the induction of *hiC6* genes in response to cold stress might have been unnecessary and, consequently, *hiC6* genes in *C. vulgaris* evolved towards constitutive expression.

In notothenioid fish, gene duplications that enhance gene expression play an important role in adaptation to the Antarctic environments (Chen *et al.*, 2008). As we found a slightly higher copy number of *hiC6* in NJ-7 than UTEX259, it would be interesting to isolate more *C. vulgaris* strains with a lower freezing tolerance and determine whether the copy number of *hiC6* would decrease to one to about three accordingly. We also wondered whether all copies of *hiC6* in the tandem array were identically expressed. Because *hiC6* genes have almost identical mRNA sequences, their expression can barely be distinguished by Northern blot hybridization. We employed gene-specific primers to perform RT-PCR detections and, in addition, calculated the relative transcript abundance based on sequences of total *hiC6* cDNAs. Our results showed that the tandem-arrayed genes were differentially expressed in both strains. In NJ-7, almost all *hiC6* transcripts were expressed from *NJ7hiC6*-3, -4 and -5, whereas in UTEX259, *hiC6* transcripts were essentially expressed

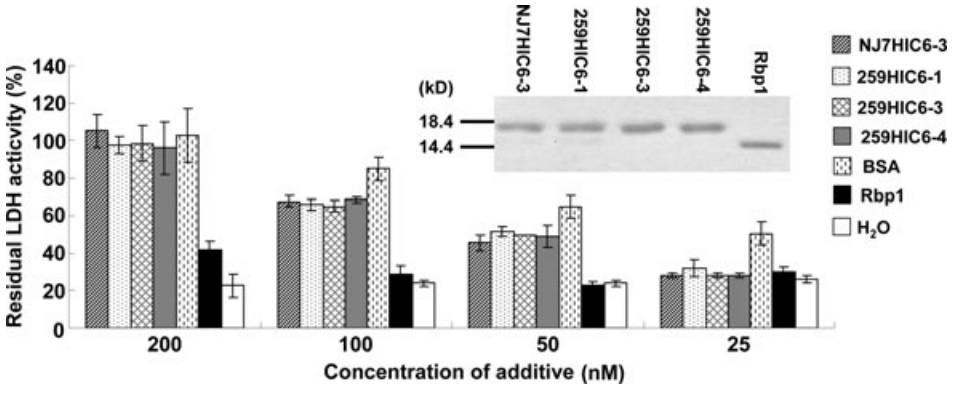

Fig. 5. Cryoprotective activities of HIC6 isoforms as shown with residual LDH activities. The LDH solution was mixed with the solution of one of the additives and subjected to a freeze–thaw cycle before measurement of its residual activity. The inset is a portion of SDS-PAGE showing that the four HIC6 isoforms were used in equal amounts. Rbp1, RNA binding protein 1 of *Synechocystis* 6803.

from *259hiC6*-1, -3 and -4. Therefore, the formation of the tandem array of *hiC6* does not appear to be a simple process of gene duplications but takes place in combination with gene expression divergence.

In an Antarctic green alga species, the nitrate reductase showed a lower maximal temperature compared to that of a temperate species (di Rigano *et al.*, 2006). This finding suggests that proteins can be evolved to promote the adaptation to Antarctic environments. We wondered whether amino acid substitutions within HIC6 can enhance the freezing tolerance of *C. vulgaris*. *In vitro* assays showed that different HIC6 isoforms provided similar protection of LDH from inactivation by freeze and thaw. Therefore, compared to changes in gene expression level, accumulation of substitutions to enhance the cryoprotective activities of LEA proteins is probably a much slower process for adaptation to the Antarctic environments.

In addition to HIC6 and HIC12, we have very recently identified two novel cold-inducible LEA proteins, Ccor1 and Ccor2, in NJ-7 (Liu *et al.*, 2011). Probably, more LEA proteins remain to be identified. These proteins may exert cumulative effects on the freezing tolerance of *Chlorella*. Alternatively, they may be involved in protection of enzymes or membranes of different cellular structures and play independent roles in freezing tolerance. For example, HIC6 seemed to be localized to mitochondria in transgenic plants (Honjoh *et al.*, 2001). Further analyses of these LEA proteins and their encoding genes should be very useful for an in-depth understanding of the development of freezing tolerance in the Antarctic *Chlorella*.

Acknowledgements

This research was supported by the Key Projects KSCX2-YW-G-060 and KSCX2-SW-332 of Knowledge Innovation Program of Chinese Academy of Sciences.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignments of upstream non-coding sequences of *hiC6* genes in the two strains.
Table S1. Primers used in this study.
Table S2. Identities between introns of *hiC6* genes in *C. vulgaris* NJ-7 and UTEX259.

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