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Enhanced expression of antifreeze protein genes drives the development of freeze tolerance in an Antarctica isolate of *Chlorella vulgaris*

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Abstract

In adaptation to new environments, organisms may accumulate mutations within encoding sequences to modify protein characteristics or acquire mutations within regulatory sequences to alter gene expression levels. With the development of antifreeze capability as the example, this study presents the evidence that change in gene expression level is probably the most important mechanism for adaptive evolution in a green alga *Chlorella vulgaris*. *C. vulgaris* NJ-7, an isolate from Antarctica, possesses an 18S rRNA sequence identical to that of a temperate isolate, SAG211-11b/UTEX259, but shows much higher freeze tolerance than the later isolate. The chromosomal DNA/cDNA of four antifreeze genes, namely *hiC6*, *hiC12*, *rpl10a* and *hsp70*, from the two isolates of *C. vulgaris* were cloned and sequenced, and very few variations of deduced amino acid sequences were found. In contrast, the transcription of *hiC6*, *hiC12* and *rpl10a* was greatly intensified in NJ-7 compared to that in UTEX259, which is correlated to the significantly enhanced freeze tolerance of the Antarctica isolate.

Keywords: *Chlorella vulgaris*; Antarctica isolate; Temperate isolate; Freeze tolerance; Gene expression

1. Introduction

Chlorella is a genus of unicellular green algae distributed all over the world. It initially included nine species and six varieties based on morphological characters. More recently, molecular data, in combination with other lines of evidence, showed that only four species, *Chlorella vulgaris*, *Chlorella lobophora*, *Chlorella sorokiniana* and *Chlorella kessleri*, were 'true' *Chlorella* [1]. *Chlorella* species are of simple cellular structure, similar to higher plants in certain physiological activities, and often used as the research model for plant life sciences. As an example, like higher plants, *C. vulgaris* shows hardening-induced antifreeze capability [2]. Upon induction by a low temperature at 4 °C, many genes are up-regulated in *C. vulgaris*, including antifreeze protein genes *hiC6* and *hiC12* [3–5]. The regulation of these genes or syntheses of new proteins are probably directly involved in the enhancement of freeze tolerance of the green alga.

Antarctica is the coldest region on the globe. How microalgae adapt to the extremely low temperature of this region awaits extensive investigation. Previously, our laboratory isolated two strains of *Chlorella* sp. from Antarctica [6]. One of the isolates was *C. vulgaris* NJ-7. Its 18S rRNA sequence was identical to that of SAG211-11b/UTEX259 isolated from the Netherlands, but the antifreeze mode has changed. Tests of the viability of frozen NJ-7 cells by plating showed a hardening-independent high freeze tolerance [6], which may reflect the enhancement of antifreeze

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capability after its emigration into Antarctica. Finding out the genetic differences of strains NJ-7 and UTEX259 may provide a unique opportunity for the study of the molecular basis of adaptive evolution.

Random mutations driving the adaptive evolution of organisms may occur in encoding regions of genes, altering protein characteristics, regulatory sequences, or gene expression levels. In this research, Antarctica and temperate ecotypes of *C. vulgaris* were compared, and mutations altering gene expression levels were found to be more important in adaptive evolution.

2. Materials and methods

2.1. Algal strains, culture conditions and assays of freeze tolerance

Chlorella vulgaris NJ-7 was isolated from near the Zhongshan station of Antarctica [6]; strain UTEX259 was purchased from the Algal Culture Collection of the University of Texas and confirmed by sequencing of 18S rDNA. Algal cells were grown in BG11 [7] under the light of 30 $\mu E\ m^{-2}\ s^{-1}$ at different temperatures without shaking or with aeration. To assay freeze tolerance, cells were spun round and subjected to freeze at −20 °C for 8 days. Frozen cells were resuspended in BG11, diluted and spread on BG11 plates to allow photoautotrophic growth at 15 °C, and colony-forming units (CFU) per OD_{730} milliliter was calculated.

2.2. Determination of full-length cDNA sequences

Total RNA was extracted with Trizol RNA Extraction Kit (Invitrogen) from *C. vulgaris* NJ-7 grown at 4 °C. mRNA was isolated using the PolyA Tract mRNA Isolation System (Promega). Complementary DNA templates were synthesized using the SMART RACE cDNA Amplification Kit (Clontech). Primers designed on the basis of partial cDNA fragments cloned by suppression subtractive hybridization (Li, unpublished), paired with the end primers included in the kit, were used in polymerase chain reactions (PCR) to amplify 5′- and 3′-portions of cDNA. Full-length cDNA sequences were obtained by juxtaposing sequences of the two portions. cDNA sequences and NCBI GenBank accession numbers are listed in Table 1.

2.3. Determination of chromosomal DNA sequences of genes

Primers designed based on cDNA sequences were used in screening with PCR to identify genomic DNA clones from a cosmid library of NJ-7 (Liu, unpublished). Chromosomal DNA sequences were obtained by sequencing target DNA portions of cosmid clones. Sequences of UTEX259 genes were obtained by PCR using primers designed on the basis of NJ-7 sequences. Chromosomal DNA sequences and GenBank accession numbers are listed in Table 1.

2.4. Northern blot analysis

Total RNA was isolated from *C. vulgaris* NJ-7 and UTEX259 grown at 20 °C with aeration and from cells exposed to 4 °C for 24 h. The RNA was separated and blotted onto nylon filters (Amersham) according to standard protocols [8] and probed with cloned partial cDNA of NJ-7 for *hiC6*, *hiC12*, *rpl10a* or *hsp70*. The probe sequences were 92–100% identical to that of UTEX259. Probe labeling and Northern blot hybridization were performed using DIG High Prime Labeling & Detection Starter Kit I (Roche) and following the procedure provided by the manufacturer.

3. Results and discussion

3.1. Differences of freeze tolerance between C. vulgaris strains NJ-7 and UTEX259

Strains NJ-7 isolated from Antarctica and UTEX259 isolated from the Netherlands have identical 18S rRNA sequences and both belong to *C. vulgaris*. At temperatures of 15–20 °C, the two isolates cultivated without shaking showed very close growth rates (~0.22 doublings d^{-1}); at 4 °C, NJ-7 was able to grow at 0.17 doublings d^{-1}, while UTEX259 showed almost no growth (0.057 doublings d^{-1}). Cells grown at 20 or 4 °C were frozen at −20 °C for 8 days and assayed for viability. The results indicated that the freeze tolerance of NJ-7 was much higher than that of UTEX259 in either case. The viability of frozen NJ-7 cultivated at 20 °C and that cultivated at 4 °C were $5.83 \pm 1.47 \times 10^6$ and $7.23 \pm 1.44 \times 10^6$ CFU $OD_{730}{}^{-1}\ mL^{-1}$, respectively, while UTEX259 treated in parallel showed inconsistent results

Table 1
Comparisons of four genes in *C. vulgaris* strains NJ-7 and UTEX259.

Gene	NCBI GenBank No.			Nucleotide substitutions and insertions/deletions (%)	
	NJ-7		UTEX259	Introns	Exons
	cDNA	Genomic sequence	Genomic sequence		
hiC6	DQ415645	EF411204	EF411211	14.88	4.84
hiC12	DQ415646	EF411208	EF411206	16.22	2.54
hsp70	EF411215	EF411209	EF411212	4.63	0.83
rpl10a	EF411216	EF411210	EF411214	7.02	0.46

in the range from $6.33 \pm 4.8 \times 10^3$ to $1.17 \pm 0.09 \times 10^6$ CFU OD_{730}^{-1} mL^{-1}.

3.2. Comparisons of antifreeze genes in NJ-7 and UTEX259

Strains NJ-7 and UTEX259 were compared for differences in sequences or expression levels of the genes that may confer antifreeze activities. Such comparisons may help to reveal the molecular basis of the enhanced freeze tolerance in NJ-7. Many cold-inducible genes have been identified in *C. vulgaris* IAM C-27 by using suppression subtractive hybridization, including antifreeze protein genes *hiC6* and *hiC12*, ribosome 60S subunit protein gene *rpl10a*, and chaperonin protein gene *hsp70* [5]. Because the antifreeze capability of *C. vulgaris* was significantly promoted by low-temperature pretreatment, these cold-induced genes could be involved in the development of freeze tolerance in this alga. Use of suppression subtractive hybridization to search for cold-induced genes in NJ-7 also targeted these four genes and many others (Li, unpublished).

Based on the partial cDNA sequences which resulted from the suppression subtractive hybridization, full-length cDNA and chromosomal DNA sequences of *hiC6*, *hiC12*, *rpl10a* and *hsp70* from NJ-7 were generated by PCR and sequenced. Exons and introns were determined by comparing sequences of cDNA and chromosomal DNA of the same genes. A similarity search in the NCBI GenBank showed that the predicted proteins HiC6 (E value = 2e−51), HiC12 (E value = 2e−89), Rpl10a (E value = 3e−81) and Hsp70 (E value = 0.0) in NJ-7 were highly similar to HiC6 and HiC12 in *C. vulgaris* C-27, Rpl10a in *Ostreococcus lucimarinus* and Hsp70 in *Chlorella pyrenoidosa*, respectively.

The corresponding genes in UTEX259 were also cloned and sequenced by PCR. Fig. 1 shows the structure of these genes in *C. vulgaris* strains. Except for a single amino acid substitution within HiC6 and HiC12, these four predicted proteins showed almost no variations at the amino acid level in NJ-7 and UTEX259. Nucleotide sequences of the genes, however, showed obvious differences between the two strains. More differences were found in introns than in exons, suggesting that mutations in exons were subject to higher selection pressure (Table 1).

Although NJ-7 and UTEX259 have shown remarkable differences in antifreeze characteristics, examinations of the four cold-inducible genes indicated that the enhancement of freeze tolerance was not due to mutations of the encoding sequences of antifreeze genes. In contrast, these genes are highly conserved at the amino acid level.

3.3. Transcriptional expression of antifreeze genes in NJ-7 and UTEX259

Two *Chlorella* strains were cultivated at 20 °C, transferred to 4 °C for 24 h. Northern blot hybridization was performed to analyze the transcription of *hiC6*, *hiC12*, *rpl10a*, *hsp70* and *cab-1* in cells treated at different temperatures. *cab-1*, a chlorophyll a/b-binding protein gene, was down-regulated at 4 °C compared to 20 °C and used as a control of down-regulation (Li, unpublished), and 18S rRNA was used as the control of constitutive transcription.

The result of Northern blot analysis is shown in Fig. 2. Genes *hiC6*, *hiC12*, *rpl10a* and *hsp70* were up-regulated upon cold induction in both strains, and *cab-1* was down-regulated under the same conditions. While 18S rRNA, *hsp70* and *cab-1* showed comparable expression lev-

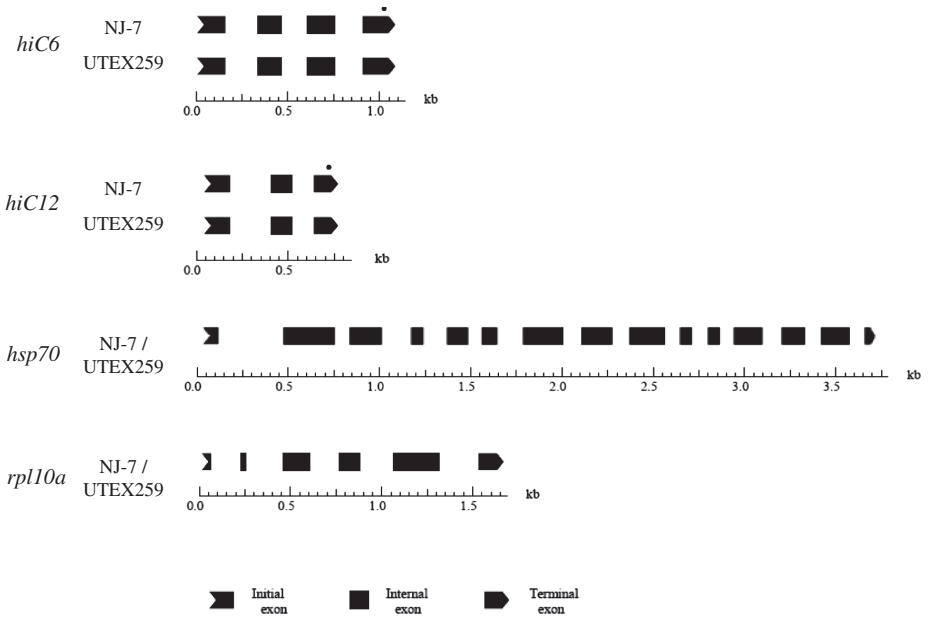

Fig. 1. The structure of four cold-responsive genes in *C. vulgaris* strains NJ-7 and UTEX259. The dots above encoding regions of *hiC6* and *hiC12* indicate the predicted single amino acid substitutions.

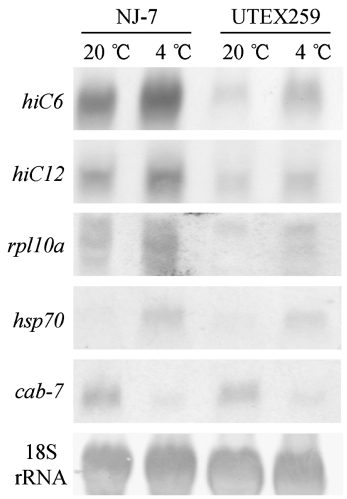

Fig. 2. Comparisons of transcriptional expression levels of five genes in *C. vulgaris* strains NJ-7 and UTEX259 at 20 and 4 °C.

els in the two strains, *hiC6* and *hiC12* showed much higher expression levels in NJ-7 than in UTEX259. Gene *rpl10a*, as indicated by its multiple hybridization bands, also showed significantly increased expression in NJ-7 relative to UTEX259.

The significant difference of freeze tolerance between the two strains was correlated with the expression levels of *hiC6*, *hiC12* and *rpl10a*. *hiC6* and *hiC12* encode 14.4 and 10.8 kDa antifreeze proteins, respectively. After a freeze–thaw cycle, these two proteins showed cryoprotective activities on lactate dehydrogenase [9]; overexpression of *hiC6* in yeast or tobacco plant could significantly enhance their freeze tolerance [10,11]. In NJ-7, *hiC6* and *hiC20* showed high expression levels at 20 °C, and further elevated expression after exposure to 4 °C. Accordingly, NJ-7 showed high freeze tolerance at 20 °C and a slight increase of freeze tolerance after treatment at 4 °C. Gene *rpl10a* may improve stress tolerance in yeast [12], but its role in antifreeze remains to be tested in *Chlorella*.

In summary, cold-inducible genes showed almost identical deduced amino acid sequences in a temperate and an Antarctic strain of *C. vulgaris*. However, among these genes, *hiC6*, *hiC12* and *rpl10a* in the two strains showed significantly different expression levels that were correlated to their antifreeze capability. Our research provides an example that organisms in a new extreme environment may increase the expression of multiple related genes to drive a rapid adaptive evolution.

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