Plant Science 175 (2008) 238–246

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Isolation of cDNAs for hardening-induced genes from *Chlorella vulgaris* by suppression subtractive hybridization

Takeshi Machida [a], Hisako Murase [a], Eri Kato [a], Ken-ichi Honjoh [b,*], Kiyoshi Matsumoto [b], Takahisa Miyamoto [b], Masayoshi Iio [b]

[a] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan
[b] Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka 812-8581, Japan

ARTICLE INFO

Article history:
Received 21 February 2008
Received in revised form 8 April 2008
Accepted 9 April 2008
Available online 22 April 2008

Keywords:
cDNA subtraction
Chlorella vulgaris
Freezing tolerance
Hardening-induced genes
qPCR

ABSTRACT

Several organisms acquire freezing tolerance by altering intracellular reactions under sublethal low temperatures. Although many researchers have reported that organisms change their intracellular functions, such as transcript level and enzymatic activity, under stress conditions, acquisition mechanisms of freezing tolerance are still poorly clarified. In this study, we attempted to identify novel hardening-induced genes from *Chlorella vulgaris* C-27, a frost-hardy strain. A PCR-based suppression subtractive hybridization library was generated and the corresponding cDNA clones were isolated. A total of 263 unique cDNA clones were obtained and sequenced. Homology analysis showed that 64 distinct known proteins were encoded by the respective clones. The expression patterns of 29 of the genes were analyzed by using qPCR. Especially, six genes, which respectively encode two late embryogenesis abundant (LEA) proteins (HIC6 and HIC12), NADPH thioredoxin reductase (NTR), chlorophyll *a*/*b*-binding protein (Cab), ζ-carotene desaturase, and Nip7, showed remarkable increase in transcripts over 100 times greater than those of unhardened cells. We discuss the possible contribution of the genes, which showed remarkable transcriptional increases, in acquisition of freezing tolerance of *Chlorella*.

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1. Introduction

Freezing injury is one of the most severe constraints that limits crop productivity [1], and leads to intracellular harmful effects such as denaturation of proteins and precipitation of various molecules [2,3]. On the other hand, cold acclimation results in alteration of gene expression, increased synthesis of proteins, and accumulation of soluble sugars in plants, which then improve freezing tolerance [4–7]. In order to investigate the acquisition of freezing tolerance, several researchers have performed comprehensive analyses of the transcripts of low-temperature-inducible genes by using microarray analysis [8,9]. After the genome project for *Arabidopsis* was completed, transcriptome analysis led to the understanding of various physiological events in plants, and many genes have been reported to be involved in the acquisition of freezing tolerance of plants [10].

We have studied the acquisition of freezing tolerance in plants, using *Chlorella vulgaris* C-27 as an eukaryotic model. Cells of *C. vulgaris* C-27 hardened at 3 °C can survive even after exposure to slow freezing down to −196 °C [11]. Several factors, such as accumulation of HIC6 and HIC12 proteins [12] and expression of two low-temperature-inducible fatty acid desaturases [13], are involved in the acquisition of freezing tolerance of *Chlorella*. In particular, HIC6 and HIC12 proteins were homologous to group 3 late embryogenesis abundant proteins and showed cryoprotective activity, suggesting that the proteins are important for the acquisition of freezing tolerance of *Chlorella*. Although overexpression of *hiC6* gene led to improvement in freezing tolerance of transgenic tobacco [14] and *Saccharomyces cerevisiae* [15], the levels of freezing tolerance were not substantial enough to be applied to agricultural products. The mechanisms for the acquisition of freezing tolerance of *Chlorella* remain to be fully understood. Cold hardening has been reported to result in many different intracellular changes to withstand the many environmental stresses, such as cellular dehydration, temperature stress, drought stress, and salt stress, which are accompanied by the freezing–thawing process [16]. It is important to first obtain information on

* Corresponding author. Tel.: +81 92 642 3024; fax: +81 92 642 3025.
E-mail address: honjoh@agr.kyushu-u.ac.jp (K. Honjoh).

Abbreviations: EST, expressed sequence tag; RT-PCR, reverse transcription-polymerase chain reaction; qPCR, quantitative real-time RT-PCR.

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doi:10.1016/j.plantsci.2008.04.002

hardening-induced genes which play an important role in the acquisition of freezing tolerance of *Chlorella*.

In order to search for novel genes involved in the acquisition of freezing tolerance, an EST library of genes specifically or preferentially expressed in *Chlorella* during hardening was generated by using a PCR-based suppression subtractive hybridization (SSH) technique between mRNAs of unhardened cells and 24-h hardened cells. The isolated EST clones were sequenced and databases were searched for homologous genes. The expression patterns of functionally identified ESTs were analyzed by qPCR and the relationships between the genes and freezing tolerance are discussed.

2. Materials and methods

2.1. Plant materials

Cells of *C. vulgaris* Beijerinck IAM C-27 were synchronously grown in MC medium, and L_2 stage (an intermediate stage during the ripening phase of the cell cycle) cells were used for experiments as described previously [11].

2.2. Hardening

Cold-hardening treatment was done using L_2 stage cells of *C. vulgaris* C-27 at 3 °C as described previously [4]. Portions of cell culture were withdrawn at intervals and centrifuged at 2500 × *g* for 5 min at 4 °C. After washing twice with sterilized water, cells were suspended in extraction buffer (0.1 M Tris–HCl, pH 9.0, 0.1 M NaCl, 10 mM EDTA, and 1% SDS) at a concentration of approximately 1.5×10^9 cells/ml. The suspension of cells was frozen in liquid nitrogen and stored at −80 °C until further use.

2.3. Isolation of poly(A)$^+$RNA

The frozen suspension of cells was thawed at 4 °C and an equal volume of a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1, v/v/v, pH 5.2) and 0.2 volumes of 2-mercaptoethanol were added. The mixture was homogenized with 0.5 mm diameter glass beads on a reciprocal shaker (Vibrogen-Zellmühle; Edmund Bühler Co., Tübingen, Germany), operated at 4500 rpm at 4 °C for 20 min. The homogenate was freed from the beads by passage through a sintered-glass funnel and then centrifuged at 2300 × *g* for 10 min at 4 °C. The upper aqueous phase containing total nucleic acids was washed twice with an equal volume of the mixture of phenol, chloroform and isoamyl alcohol for complete removal of proteins and cell debris. Total nucleic acids were precipitated by the addition of 0.05 volumes of 5 M NaCl and 2.5 volumes of ethanol and were incubated for 1 h at −20 °C. After centrifugation at 5800 × *g* for 20 min at 4 °C, nucleic acids were washed twice with ice-cold 70% ethanol and then dissolved in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). Total RNA was precipitated overnight at 4 °C after the addition of 5 M LiCl to a final concentration of 2 M. The RNA was pelleted by centrifugation at 20,000 × *g* for 30 min at 4 °C, washed twice with ice-cold 70% ethanol and then dissolved in TE buffer. For removal of genomic DNA, the RNA solution was treated with deoxyribonuclease (RT-Grade; Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instruction. Poly(A)$^+$RNAs were purified by the use of *Oligotex*™-MAG mRNA Purification Kit (TaKaRa, Kyoto, Japan) according to the manufacturer's instruction.

2.4. cDNA subtraction

In order to isolate ESTs corresponding to hardening-induced genes from *Chlorella*, a PCR-based cDNA subtraction was performed using BD Clontech PCR-Select™ cDNA Subtraction Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instruction. Tester cDNA was prepared from 2 μg of poly(A)$^+$RNA derived from 24-h hardened *Chlorella* cells. Driver cDNA was synthesized from poly(A)$^+$RNA derived from unhardened *Chlorella* cells. At the end of the protocol, the PCR products were subcloned into a pGEM-T easy vector (Promega, Madison, WI, USA) for sequencing.

2.5. DNA sequencing and homology analysis

Nucleotide sequences of the subcloned cDNAs were determined by using a Pharmacia ALFexpress DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden), a Gene Rapid sequencer (Amersham), or an Applied Biosystems 3730 sequencer (Perkin-Elmer, Wallesley, MA, USA). DNA sequences were analyzed in both directions. The obtained nucleotide sequences were identified by comparison with those of nr and dBEST databases using the tBlastX programs [17] on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST) at a threshold *e*-value of 10^{-5} or better. The ESTs were grouped into functional categories using the classification method of Bevan et al. [18].

2.6. Primer design and qPCR

Primer pairs for qPCR were designed using Primer 3 Software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers were used for RT-PCR performed with *Ex-Taq* DNA polymerase (TaKaRa). Gene-specific primers were designed so that the resulting PCR products were 70–300 bp in size. The specificity of the gene-specific primer pairs was checked by PCR, using cDNA corresponding to mRNA from 24-h hardened *Chlorella* cells. The quality of PCR products was visually inspected by agarose gel electrophoresis, and the appearance of only one band of the expected size was taken as a criterion for specificity. Confirmation of the PCR products was performed by subcloning each product into a pGEM-T easy vector (Promega), followed by sequencing of the inserts.

In order to ensure the transcriptional up-regulation of the genes for the subtracted EST clones, qPCR analyses were performed. Poly(A)$^+$RNAs were prepared from 0-, 3-, 6-, 9-, 12- and 24-h hardened *Chlorella* cells according to the method described above. Two micrograms of each poly(A)$^+$RNA were reverse-transcribed for 60 min at 42 °C, using ReverScript I (Wako Pure Chemical Industries) and oligo(dT)$_{18}$ primer. The synthesized cDNAs were used as templates for qPCR. Quantification of transcripts corresponding to the obtained EST clones was performed on Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA, USA) with the *SYBR® Premix Ex Taq*™ (Perfect Real Time) (TaKaRa) according to the manufacturer's instruction. Each reaction was performed using 2 μl of 1:10 (v/v) dilution of the first-stranded cDNA with 0.2 μM of each primer in a total reaction of 25 μl. The qPCR was performed as follows: 40 cycles of 10 s at 95 °C, 15 s at 55 °C, and 20 s at 72 °C. The specificity of the PCR amplification procedures was checked with a heat dissociation protocol (from 55 to 95 °C) after the final cycle of the PCR. Each reaction was done three times and the corresponding threshold cycle values were determined. Actin (Genbank accession no. AB080313) gene from *C. vulgaris* was used as an internal standard to normalize differences in template amounts.

Statistical analysis was performed by using Excel 2004 (Microsoft, USA) with the add-in software Statcel 2 [19].

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