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Chloroplastic Glucose 6–Phosphate Dehydrogenase from *Chlorella vulgaris* Alleviates Freezing and Menadione–Induced Oxidative Stresses in *Saccharomyces cerevisiae*

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Enhanced glucose 6–phosphate dehydrogenase (E.C.1.1.1.49, G6PDH) activity has been identified as a hardening–induced intracellular change of *Chlorella vulgaris*, which acquires freezing tolerance during hardening. In the present study, a full–length cDNA clone corresponding to a gene encoding a chloroplastic isoform of G6PDH, designated CvchG6PDH, was isolated from *C. vulgaris* C–27. By comparing the deduced amino acid sequence of *CvchG6PDH* with the N–terminal amino acid sequence of mature $G6PDH_2$ protein isolated previously, a DNA region encoding mature CvchG6PDH was determined and designated *mCvchG6PDH*. The deduced amino acid sequence of *CvchG6PDH* showed higher homology to those of plant plastidic *G6PDH* genes than those of cytosolic ones. A recombinant mCvchG6PDH protein expressed in *Escherichia coli* showed similar enzymatic properties to previously–isolated *Chlorella* $G6PDH_2$, suggesting that the gene encoded plastidic $G6PDH_2$ protein. Expression of *CvchG6PDH* was induced transcriptionally throughout 24–h hardening, while the translation was induced up to 9–h hardening and then decreased, and the change did not reflect the enhanced G6PDH activity during hardening. Furthermore, the mCvchG6PDH alleviated both freezing and menadione–induced oxidative stresses in yeast. We showed the contribution of *CvchG6PDH* in menadione stress tolerance as one of its functions in the acquisition of freezing tolerance of *Chlorella*.

INTRODUCTION

Freezing injury is one of the most severe constraints limiting plant growth and it has been a major preoccupation in northern latitude (Levitt, 1980). It could be said that to investigate the developing mechanisms of plants against freezing is very important for the purpose of stable food production and establishment of the method for long–term food storage. Some plants acquire freezing tolerance when exposed to non–freezing low temperatures, and their physiological strategies used to tolerate freezing stress vary (Sadakane *et al*., 1980; Sarhan and Perras, 1987; Thomashow, 1990; Uemura *et al*., 1997).

We have studied the acquisition of freezing tolerance of plants, using *Chlorella vulgaris* C–27 as a convenient eukaryotic model. Hardened cells of *C. vulgaris* C–27 can survive after freezing treatment even at –196 °C (Hatano *et al*., 1976). In our previous studies, we have found an increase in the activity of glucose 6–phosphate dehydrogenase (G6PDH) as an intracellular change of *Chlorella* during hardening (Sadakane *et al*., 1980). The enzyme, G6PDH (EC 1.1.1.49), catalyses the rate–limiting step of the oxidative pentose phosphate pathway (OPPP), an important catabolic route for the provision of NADPH and sugar phosphates (Wendt *et al*., 2000). In plants, G6PDH isoenzymes reside in two compartments, cytosol and plastids (Schnarrenberger *et al*., 1973). The plastidic G6PDH is known to be reductively inactivated, achieved via reversible dithiol–disulphide interchange of two highly conserved regulatory cysteine residues specific to the plastidic one (Wenderoth *et al*., 1997). The main role of G6PDH in plastids is the supply of reducing equivalents in the form of NADPH required for various reductive biosyntheses (e.g. amino acids, fatty acids) and to provide sugar–phosphate intermediates that serve as precursors of nucleotides and secondary plant products. In plants, substantial evidences show that G6PDH might be involved in responses to stresses, such as oxidative stress (Hauschild and von Schaewen, 2003; Debnam *et al*., 2004) and salt stress (Nemoto and Sasakuma, 2000).

A number of factors have appeared to be involved in freezing injury of plants, including water deficit due to ice formation, lipid phase separation, and inactivation of ATPase in plasma membrane (Tao *et al*., 1998). In addition, production of reactive oxygen species (ROS) during freezing–thawing may be involved. In research on coniferous plant, increases in superoxide dismutase activity and ascorbate content have been found when suffering photooxidative damage in fall and winter (Tao *et al*., 1990; 1992). Many researchers have studied the relationship between ROS–scavengers and freezing tolerance, and clarified the importance and the role in the acquisition of freezing tolerance (Seppanen and Fagerstedt, 2000; Kocsy *et al*., 2001; Yang *et al*., 2007). Those reports suggest that protection system against ROS–mediating oxidative damage plays an important role in improvement of freezing tolerance of plants.

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We have previously purified and characterized two isoforms of G6PDHs from *Chlorella*. The two G6PDHs appeared to be the cytosolic and the chloroplastic enzymes based on their sensitivities to DTT (Honjoh *et al*., 2003). Furthermore, we have isolated and characterized a cDNA encoding cytosolic G6PDH, and investigated the involvement in the acquisition of freezing tolerance using transformed yeast (Honjoh *et al*., 2007). Although the gene encoding cytosolic G6PDH, designated *CvcG6PDH*, was induced at a transcriptional level during hardening, leading to the change in the activity, it did not reflect the increase in the activity of G6PDH during hardening. The previous study also showed that the freeze–thaw stress tolerance of the transformed yeast expressing *CvcG6PDH* was not improved sufficiently in spite of the 8.7–fold high activity of G6PDH compared to that of the control strain. Thus, involvement of other isoform in freezing tolerance should be investigated.

In the present study, we have isolated and characterized a cDNA for chloroplastic *G6PDH* gene from *C. vulgaris* C–27. The effect of low temperatures, responsible for the freezing tolerance of *Chlorella*, on the expression of the gene was investigated at transcriptional and post–transcriptional levels. Furthermore, the involvements of two isotypes of *Chlorella* G6PDH in the acquisition of stress tolerance were investigated using transformed yeast expressing the gene.

MATERIALS AND METHODS

Strains and conditions

Chlorella vulgaris Beijerinck IAM C–27 was obtained from the Algal Culture Collection of the Institute of Applied Microbiology, Tokyo University, Japan. Cells of *C. vulgaris* C–27 were cultured at 25 °C synchronously and hardened at 3 °C as described previously (Honjoh *et al*., 2007).

Escherichia coli BL21(DE3)pLysS (Novagen, Madison, WI, USA) was used for production of a large amount of recombinant mCvchG6PDH protein in *E. coli*.

Saccharomyces cerevisiae INVSc2 (*Mat α*, *his3 Δ200*, *ura3–167*; Invitrogen, Carlsbad, CA, USA) was used for expression of *mCvchG6PDH* in yeast.

Isolation of a full–length cDNA clone encoding CvchG6PDH and sequence analysis

Hardened cells of *Chlorella* were harvested, washed twice with sterilized water, and suspended in an extraction buffer, which contained 0.1 M Tris–HCl (pH 7.0), 0.1 M NaCl, 10 mM EDTA, and 0.1% SDS, dissolved in diethylpyrocarbonate–treated water. Purification of poly(A) + RNA and cDNA synthesis were performed based on the method of Machida *et al*. (2009). A partial *CvchG6PDH* cDNA fragment was amplified using two degenerated primers, 5'– GAT GCT TTT A(C/T)C ATT C(C/G/T)G GTC AGT A(C/T)A A –3' and 5'– CTC (C/T) TT (A/G)CC CA(A/G) GTA (A/G)TG GTC (A/G)AT –3', designed based on conserved regions of G6PDHs. DNA regions that extended 5'– and 3'–ends of the full–length *CvchG6PDH* cDNA were amplified using rapid amplification of cDNA ends (RACE) method. Full–length *CvchG6PDH* cDNA was amplified with two primers, 5'– GGC CCT TTT CGT TGT TGA GCC TG –3' and 5'– CCA GGC AAC AGC AAT TAC AGG GGA –3'. Amplified fragments were subcloned into a pGEM–T easy vector (Promega, Madison, WI, USA), and nucleotide sequences of the fragments were determined based on the method of Sanger *et al*. (1977).

Homologous genes to the *CvchG6PDH* were searched for using the tBlastX program (Altschul *et al*., 1990) on the National Center of Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/), and the identity (%) was calculated using the clustal W program (http://align.genome.jp/). Phylogenetic tree was constructed with the Neighbor–Joining method based on the method of Wendt *et al*. (1999). Subcellular localization of the protein encoded by the cloned cDNA was predicted using the WoLF PSORT program (Houton *et al*., 2006).

Northern blot analysis

Total RNAs from unhardened, 3, 9, and 24–h hardened cells were isolated as described above. Ten micrograms of total RNA were separated on a 1.0% agarose gel containing 1.8% formaldehyde, and blotted onto a Hybond–N^+ nylon membrane (GE healthcare, Heidelberg, Germany). The membrane was subjected to Northern blot analysis using a DIG High Prime DNA Labeling and Detection Starter Kit Ⅱ (Roche, Basel, Switzerland). The full–length *CvchG6PDH* cDNA was labeled by the random–labeling method with DIG–dUTP, and then used as a probe. The hybridized bands were detected using a chemiluminescent substrate, [3–(5–Chloro–4'–methoxyspiro[adamantane–2,3'–[1,2]dioxetan]–4'–yl) phenyl] phosphate phosphoric acid 3–[4'–methoxy–5–chlorospiro [tricyclo[3.3.1.1^{3,7}]decane–2,3'–[1,2]dioxetane] –4'–yl]phenyl ester, supplied in the kit. The loading amount of total RNA was standardized by comparison with the electrophoretic band intensity of rRNA.

Expression of mCvchG6PDH in *E. coli*

A gene encoding a mature form of CvchG6PDH, designated *mCvchG6PDH*, was amplified by PCR using the full–length cDNA as a template. To amplify *mCvchG6PDH*, two primers with the recognition sites of *Nhe*I and *Xho*I were designed (5'– TAT GCT AGC GGG TTG CAA GAG GAG AAC TGG –3' and 5'– GGC CTC GAG TCA CTC GTC GTC AGC G –3'; recognition sites of *Nhe*I and *Xho*I are underlined). The amplified DNA fragment was digested with the corresponding enzymes and ligated into the *Nhe*I–*Xho*I site of a pET–17xb vector (Novagen). *E. coli* BL21(DE3)pLysS was transformed with the plasmid by the electroporation method according to the manufacturer's instructions (BTX Division of Genetronics, Inc., Holliston, MA, USA). The transformants were selected by cultivation on LB/Amp/Cam agar containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C for 16 h.

The *mCvchG6PDH* was separately amplified by PCR to be ligated into a pET–32a(+) vector (Novagen) with two primers, 5'– TAT GGA TCC GGG TTG CAA GAG GAG

AAC TGG –3' and 5'– CGG CGT CGA CTC ACT CGT CGT CAG CG –3'containing *Bam*HI and *Sal*I recognition sites (underlined). The amplified DNA fragment was digested and incorporated into the *Bam*HI–*Sal*I site of the vector. The plasmid generated was introduced into *E. coli* BL21(DE3)pLysS by the electroporation method. The transformants were selected by cultivation on LB/Amp/Cam agar plates at 37 °C for 16 h.

Expression of *mCvchG6PDH* in *E. coli* and purification of His–tagged mCvchG6PDH protein

The *E. coli* transformants were cultured in LB/Amp/Cam medium at 37 °C with shaking. When the OD_{600} reached 0.5, isopropyl–β–D–thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 1 mM. After 6 h of cultivation at 25 °C, the cells were collected by centrifugation, then resuspended in 50 mM potassium–phosphate buffer (pH 7.5; K–Pi) containing 1 mM phenylmethylsulfofluoride (PMSF), and disrupted at 4 °C by sonication using a Tomy Ultrasonic Disrupter UP–201 (Tomy Seiko, Tokyo, Japan) for 10 min at 48 W with 0.5–s pulses at 0.5–s intervals. The suspension was centrifuged at 20,000 × *g* for 10 min at 4 °C and the resulting supernatant was used for further experiments. His–tagged mCvchG6PDH was purified by Ni^{2+}–affinity chromatography using a His・Bind resin (Novagen) according to the manufacturer's instructions. Expression and purification of the proteins were checked by SDS–PAGE on a 10% polyacrylamide gel by the method of Laemmli (1970).

Preparative SDS–PAGE and N–terminal amino acid sequencing

The partially–purified His–tagged mCvchG6PDH protein was treated with enterokinase at 23 °C for 16 h or thrombin (Novagen) at 25 °C for 16 h to remove tagging peptides. And then, the digested proteins were purified additionally by preparative electrophoresis. The purified mCvchG6PDH was subjected to SDS–PAGE and electroblotted onto a polyvinylidene difluoride membrane (Towbin *et al.*, 1970). The N–terminal sequence of the detected protein was analyzed with a gas–phase protein sequence analyzer (model PPSQ–21; Shimadzu, Kyoto, Japan). Rabbit anti–mCvchG6PDH polyclonal antibodies were raised using the purified mCvchG6PDH as an antigen.

Enzyme assays

G6PDH activity was assayed in a double–beam mode using a Shimadzu UV–visible recording spectrophortometer (Shimadzu) as described previously (Honjoh *et al.*, 2003). The assay mixture contained 55 mM Tris–HCl (pH 7.8), 3.3 mM $MgCl_2$, 0.2 mM $NADP^+$, 3.3 mM glucose 6–phosphate, and protein extract. One unit of activity was defined as the amount of enzyme forming 1.0 μmol of NADPH per minute at 25 °C.

Dithiothreitol (DTT)–sensitivity test was performed as follows. Protein samples were incubated on ice for 3 h in the presence of 100 mM DTT. Residual activity (%) was expressed as the percentage of specific activity of treated sample against that of untreated sample.

Protein extraction from *Chlorella*

Chlorella cells were harvested, washed twice, and suspended in 50 mM K–Pi containing 1 mM PMSF. The suspension was frozen in liquid nitrogen and stored at –80 °C until use. The frozen suspension of cells was thawed at 4 °C and homogenized with 0.5 mm diameter glass beads on a reciprocal shaker (Vibrogen–Zellmühle, Edmund Bühler Co., Tübingen, Germany), operated at 4,500 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 20,000 × *g* for 30 min at 4 °C. The upper phase was recovered and centrifuged again at 20,000 × *g* for 60 min at 4 °C. The resulting upper phase was used in further experiments as a *Chlorella* cell extract.

Western blot analysis

Protein extracts of unhardened, 3, 9, and 24–h hardened *Chlorella* cells were separated by SDS–PAGE, and then electroblotted onto a nitrocellulose membrane. The membrane was blocked with 3% skimmed milk in TBS containing 0.1% Tween–20 (TTBS) for 1 h, incubated with antibodies for CvchG6PDH at a 1:500 (v/v) dilution in TTBS containing 3% skimmed milk for 1 h, washed four times with TTBS, and then incubated with anti–rabbit IgG (whole molecule)–peroxidase (Sigma–aldrich, St. Louis, MO, USA) at a 1:500 (v/v) dilution in TTBS containing 3% skimmed milk for 1 h. After washing the membrane four times with TTBS, bands were detected with 3,3'–diaminobenzidine tetrahydrochloride and hydrogen peroxide. All procedures in this section were performed at room temperature.

Quantification of band intensity

The bands shown in the results of Northern blot and Western blot were quantified using the Yab's Gel Image software (http://homepage.mac.com/yabyab/rb/gelimage–j.html).

Plasmid construction and transformation of yeast

To express the *mCvchG6PDH* genes in *S. cerevisiae*, the *mCvchG6PDH* was amplified by PCR using Phusion DNA polymerase (Finnzymes, Keilaranta, Finland). To amplify *mCvchG6PDH*, two oligonucleotides with the recognition sites of *Bgl*Ⅱ and *Eco*RI were designed (5'–TAT AGA TCT ATG GGG TTG CAA GAG GAG AAC –3' and 5'– CGG CGA ATT CTC ACT CGT CGT CAG CGG –3'; recognition sites of *Bgl*Ⅱ and *Eco*RI are underlined). The amplified DNA fragment was digested with the corresponding enzymes and introduced into the *Bgl*Ⅱ–*Eco*RI site of a pTG887 vector (Transgene S. A., France), downstream of the *PGK* promoter to allow a high level of expression of introduced genes. The plasmid generated was designated pTG887/*mCvchG6PDH*.

S. cerevisiae INVSc2 was transformed with the plasmids by the lithium acetate method (Ito *et al.*, 1983). Transformants were screened onto an uracil–deficient synthetic raffinose (SR–ura) agar plate, which contained

0.67% yeast nitrogen base, 2% raffinose, 0.2% complete supplement mixture without uracil (CSM–URA, BIO 101, Vista, CA, USA), and 2% agar, at 30 °C for 48 h.

Expression of the introduced genes in yeast

The transformed yeast cells were cultured overnight in SR–ura medium with shaking at 30 °C. The overnight cultures were inoculated into SR–ura containing 2% glucose to express the introduced genes. After 24 h of cultivation at 30°C, the yeast cells were harvested by centrifugation at 1,000 × *g* for 10 min, and suspended in 10 mM Tris–HCl (pH 7.5) containing 1 mM PMSF. The cell suspension was mixed with an equal volume of 0.5 mm diameter glass beads and homogenized with a vortex mixer. The homogenates were centrifuged at 20,000 × *g* at 4 °C for 10 min. G6PDH activity in yeast was determined using the soluble lysate by the method described above.

Freezing tolerance test

Yeast cells expressing *mCvchG6PDH* were pre–cultured in 2 ml of SR–ura medium with shaking at 30 °C for 48 h. Five microliters of pre–cultured cells were inoculated into 5 ml of SR–ura medium containing 2% glucose. When the OD_{600} reached 1.0, the yeast cells were harvested by centrifugation at 560 × *g* for 5 min at room temperature, and resuspended in a 0.9% NaCl solution at a cell concentration of 2–3 × 10^6 cells/ml (OD_{600} = 0.1). One hundred microliters of the cell suspensions were transferred to 1.5–ml microcentrifuge tubes and pre–chilled in a deep–freezer at –80 °C for 2 min for ice formation. Then, the samples were subjected to freezing treatment in a program freezer at –20 °C for 24 h. After freezing treatment, the samples were thawed at 25 °C, serially diluted with 0.9% NaCl, and immediately plated onto YPD agar plates, containing 1% yeast extract, 2% polypeptone, 2% glucose, and 1.5% agar. After 2 d of incubation at 30 °C, colonies were counted. Viability (%) was calculated by dividing the counts of frozen sample by those of unfrozen one. Yeast cells carrying pTG887 and pTG887/*CvcG6PDH* (Honjoh *et al.*, 2007) were also tested.

Oxidative stress tolerance test

The pre–cultured transformants were inoculated into 5 ml of SR–ura medium containing 2% glucose, and cultured at 30 °C with shaking until OD_{600} reached 0.5. Then, the cells were harvested by centrifugation at 560 × *g* for 5 min at room temperature, washed twice with 100 mM sodium–phosphate buffer (pH 7.0, Na–Pi), and resuspended in Na–Pi at a cell concentration of 2–3 × 10^6 cells/ml (OD_{600} = 0.1). One milliliter of the cell suspensions was mixed with menadione, which induces production of superoxide anion inside the cells, at a final concentration of 200 μM and then incubated at 30 °C for 1 h with shaking. After stress treatment, the samples were serially diluted with Na–Pi and immediately plated onto YPD agar plates. After 2 d of incubation at 30 °C, the colonies were counted. Viability (%) was calculated by dividing the counts of frozen sample by those of unfrozen one. Yeast cells carrying pTG887 and pTG887/*CvcG6PDH* were also tested.

Statistical analysis

Statistical analysis was performed using Excel 2004 (Microsoft, USA) with the add–in software Statcel 2 (Yanai, 2002). The Tukey–Kramer test was used to evaluate significance among the groups by one–way ANOVA.

RESULTS

Isolation of full–length cDNA encoding CvchG6PDH

A 309 bp *CvchG6PDH* DNA fragment was amplified by PCR using degenerated primers. Nucleotide sequence of a full–length cDNA clone of *CvchG6PDH* gene was determined using 5'– and 3'–RACE procedures based on the sequence of the 309–bp fragment. The full–length cDNA fragment was amplfied by PCR, subcloned into a pGEM–T easy vector, and sequenced. The length of *CvchG6PDH* was 2,397 bp in length and contained an open reading frame encoding 598 amino acid residues. A putative active region (–RIDHYLGKE–) and two coenzyme–binding sites (–GXXGXXG/A–) (Cosgrove *et al.*, 1998) were found in the deduced amino acid sequence of *CvchG6PDH* (Fig. 1).

A computer search of the databases using the Clustal W program revealed that the deduced amino acid sequence of the obtained cDNA exhibited 58–59% identity to those of the plastidic G6PDHs from higher plants and 41–44% identity to those of the cytosolic G6PDHs (Table 1). Phylogenetic analysis was performed to estimate the subcellular localization of *CvchG6PDH* based on the deduced amino acid sequence (Fig. 2). Moreover, the result of analysis using the WoLF PSORT program also indicated that the encoded protein is targeted or localized in the chloroplast. The N–terminal (NH_2–GLQEENWEKAALSIV–) and internal (–LVIRIQPNEGIYLKVNNKVP–) amino acid sequences of chloroplastic G6PDH protein ($G6PDH_2$, Honjoh *et al.*, 2003) were found in the deduced amino acid sequence

Table 1. Sequence homology between the deduced amino acid sequence of *CvchG6PDH* and those of other cytosolic and plastidic *G6PDH* genes as given by Clustal W program

Origin	Identity (%)	Accession number
Plastidic G6PDH		
Solanum tuberosum	58.9	AJ010712
Dunaliella bioculata	58.6	AJ132346
Arabidopsis thaliana	58.5	AJ001359
Nicotiana tabacum	58.5	AJ001772
Spinacia oleacea	57.6	AJ000182
Petroselinum crispum	56.5	AF012861
Cytosolic G6PDH		
A. thaliana	43.9	AJ010971
S. tuberosum	43.4	X74421
N. tabacum	43.4	AJ001770
Oryza sativa	42.8	AY078072
Chlorella vulgaris	40.9	AB085846

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   1 GGCCCTTTTCGTTGTTGAGCCTGCTTTTCATTCTTGCGCTTTGTCAACCCAGCCACCAGGGGTGTTGTCACTATCAGCGGGCGCACGTTC

  91 TTGAGCAGCGATCGGGGCGCGCAGCAGGCCGACCCGTGATGCCCGCTCTGCACAGCATGAGGCTCGTTGCACAAGTTCTGACCCTCAGCG
                                          M  P  A  L  H  S  M  R  L  V  A  Q  V  L  T  L  S
 181 GCAAGGTGGCGCAACGCCATGCGGCTGACCATCAACCCCAGCGAGCTGTCTGTCGCCAGCCTGCCCGACAGCACCACAGGCTGCGAGCAC
      G  K  V  A  Q  R  H  A  A  D  H  Q  P  Q  R  A  V  C  R  Q  P  A  R  Q  H  H  R  L  R  A
 271 TGACAGCACAAAGCAGTGCGCCATCGGCATCAACCACTGGCAGCGGCGCAGCGACCACTCAGAGTCCAGAGATTGAAGCAGCGGAGGTGG
      L  T  A  Q  S  S  A  P  S  A  S  T  T  G  S  G  A  A  T  T  Q  S  P  E  I  E  A  A  E  V
 361 AGGCAGCAGCAGCAGACGCACGTCAAAATGGCTCGCCTTCTTTCTCAAATATGAAGGTGTATGGGTTGCAAGAGGAGAACTGGGAGAAGG
      E  A  A  A  A  A  D  A  R  Q  N  G  S  P  S  F  S  N  M  K  V  Y  G  L  Q  E  E  N  W  E  K
 451 CTGCACTGAGCATCGTGGTGGTGGGCGCGTCTGGTGACCTGGCCAAGAAGAAAATCTTCCCCTCCCTCTTTGCGCTGTACTATGAAAACA
      A  A  L  S  I  V  V  V  G  A  S  G  D  L  A  K  K  K  I  F  P  S  L  F  A  L  Y  Y  E  N
 541 TGCTTCCGCAGAACTTCAAGGTGTATGGCTACGCCCGCAGCAAGATGAACGACGAGGAGTTCCGAGACCTGATTGCCGGCAGCCTGACCT
      M  L  P  Q  N  F  K  V  Y  G  Y  A  R  S  K  M  N  D  E  E  F  R  D  L  I  A  G  S  L  T
 631 GCAGGCTGAACGACGCTGGTGACTGCGGCAAGAAGATGGACGAGTTCCTGGAGCGCTGCTTCTACCAGGCGGGCCAGTACGCATCTGAGG
      C  R  L  N  D  A  G  D  C  G  K  K  M  D  E  F  L  E  R  C  F  Y  Q  A  G  Q  Y  A  S  E
 721 ACGACTTTGCGGCGCTGGACAAGCGCATGGCTGACGGGGAGAGCGAGCAGTCCTGCGCAGACAGAATGTTCTACCTCTCGATCCCGCCAA
      D  D  F  A  A  L  D  K  R  M  A  D  G  E  S  E  Q  S  C  A  D  R  M  F  Y  L  S  I  P  P
 811 ACATCTTCACCACGGTGGCAGCCTGCGCCTCCAAGGCAGCATCCAGCAAGTGTGGCTGGACGCGCATGATTGTTGAGAAGCCGTTTGGCA
      N  I  F  T  T  V  A  A  C  A  S  K  A  A  S  S  K  C  G  W  T  R  M  I  V  E  K  P  L  G
 901 AGGACAGCGAGTCGTTCCAGGAGTTGAGCGCTGCGCTGTACGAGCACCTTCGAGAGGACCAGATATACCGCATTGACCACTACCTGGGCA
      K  D  S  E  S  F  Q  E  L  S  A  A  L  Y  E  H  L  R  E  D  Q  I  Y  R  I  D  H  Y  L  G
 991 AGGAGCTGATCGAGAACCTGACGGTGCTGCGCTTTGCAAACCTGGTGTTTGAGCCGCTGTGGAGCAGGCAGTACATCAGAAACGTGCAGG
      K  E  L  I  E  N  L  T  V  L  R  F  A  N  L  V  F  E  P  L  W  S  R  Q  Y  I  R  N  V  Q
1081 TCATCTTCAGCGAGAACTTTGGGACAGAGGGCCGAGGGGGCTACTTTGACCAGTACGGCATTGTGAGGGACGTCATCCAAAACCACCTGC
      V  I  F  S  E  N  F  G  T  E  G  R  G  G  Y  F  D  Q  Y  G  I  V  R  D  V  I  Q  N  H  L
1171 TCCAAATACTGGCCCTCTTCGCCATGGAGCAGCCGGCCAGCCTGGATGCCGAGGACATTCGTAACGAGAAGGTCAAGGTGCTGAAGAGCA
      L  Q  I  L  A  L  F  A  M  E  Q  P  A  S  L  D  A  E  D  I  R  N  E  K  V  K  V  L  K  S
1261 TGGGCCAGGTGCGGCTGGAGGACATGGTGTTGGGGCAGTACCGCTCACGCACCACACGCGGGACCACGCTGCCAGGATACCTGGACGACG
      M  G  Q  V  R  L  E  D  M  V  L  G  Q  Y  R  S  R  T  T  R  G  T  T  L  P  G  Y  L  D  D
1351 ACACGGTACCGCCAAACAGCATCACCCCCACCTTTGCCGCCTGCTCCGTGTTCATCAACAATGCCAGGTGGGACGGCGTGCCCTTCCTGC
      D  T  V  P  P  N  S  I  T  P  T  F  A  A  C  S  V  F  I  N  N  A  R  W  D  G  V  P  F  L
1441 TCAAGGCGGGCAAGGCGCTGGCCAACAAGGCGGCCGAGATCCGGGTGCAGTTCCGGCATGTGCCGGGCAACCTGTACCGCAACAAACTGG
      L  K  A  G  K  A  L  A  N  K  A  A  E  I  R  V  Q  F  R  H  V  P  G  N  L  Y  R  N  K  L
1531 GGCTGGACCTGGACAAGGCCACCAACGAGCTGGTCATCCGCGTCCAGCCCAACGAGGGCATCTACCTGAAAGTGAACAACAAGGTGCCGG
      G  L  D  L  D  K  A  T  N  K  L  V  I  R  V  Q  P  N  E  G  I  Y  L  K  V  N  N  K  V  P
1621 GGCTGGGCCTGCGCATAGACACAACGCGCCTCGACCTCACCTACAAGTCCAAGTACCAGGCAACACTGCCAGATGCATATGAGCGGCTGA
      G  L  G  L  R  I  D  T  T  R  L  D  L  T  Y  K  S  K  Y  Q  A  T  L  P  D  A  Y  E  R  L
1711 TCCTGGACTGCATCAACGGCGACCGCCGCCTGTTCATCCGCAACGACGAGCTGGAGGTGGCCTGGGAGAAGTTCACTCCGGTGCTCAAGG
      I  L  D  C  I  N  G  D  R  R  L  F  I  R  N  D  E  L  E  V  A  W  E  K  F  T  P  V  L  K
1801 AGATTGAGGATCGGGGTGTGCAGCCGGAGCTGTACCCCTACGGCAGCCGCGGCCCCGTTGGCGCGCACTACCTGGCAGCCAAGCACGGCG
      E  I  E  D  R  G  V  Q  P  E  L  Y  P  Y  G  S  R  G  P  V  G  A  H  Y  L  A  A  K  H  G
1891 TGCGGTGGGGGGACCTGGCCGCTGACGACGAGTGAGCCGCCCGCCTGGCTGGCAACACAAGCTGCCTGCCAGTCCATTGTCCACAAACCC
      V  R  W  G  D  L  A  A  D  D  E  *
1981 CGTCCCTCCTCCTCCAACAACTTGACCTCTACGCCTCCGGCTGGCATGCCACGCAACTTCATTTCGCAGCCGCCCTTTGAACGACGCCCC

2071 ATCCAAGGCATAAACTGTCACGGCTATCGCCACACTGCTGGCCGGATGCCCCGTCAGCGCCCGGCTGCCGCAGCGGTTGGCACACCCACG

2161 TCCTGTACACCACACTACACATACCTCCTCTGCCTTCCTTCACCCTGTTTAGCTGCTGTTTAGGTGCTGCTGCTGCACTTTTGGTTGCCC

2251 CCACTGCGCATGGTGCAGCGGGTCACCAGCACAGGCCACACACACCCTGTGACCTTGTCACCGCTGTTCTGTCCCTCTCTCGCCCCTAGC

2341 TGACCCTAGCTGACACCATGTCACCAACCCATGTCCCCTGTAATTGCTGTTGCCTGG
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Fig. 1. Nucleotide and deduced amino acid sequences of the cDNA of *CvchG6PDH* are shown. The putative active site (–RIDHYLGKE–) of G6PDH is double–underlined and two NADP–binding sites (–GXXGXXG/A–) are boxed. The two regions that were identical to the internal amino acid sequences of purified $G6PDH_2$ (Honjoh *et al*., 2003) are shown in bold letters. Two conserved cysteine residues found in common plastidic G6PDH are circled.

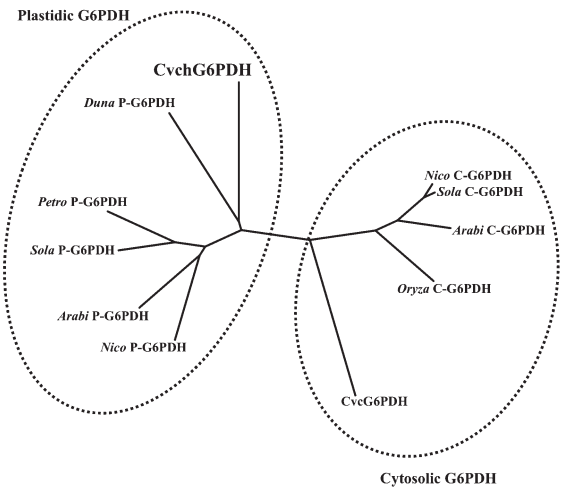

Fig. 2. Phylogenetic tree of G6PDHs was constructed with the Neighbor–Joining method using the clustal W program. Accession numbers for the sequences are shown in parentheses as follows: *Duna* P–G6PDH: *Dunaliella bioclat*a plastidic G6PDH (AJ132346), *Petro* P–G6PDH: *Petroselinum crispum* plastidic G6PDH (AF012861), Sola P–G6PDH: *Solanim tuberosum* plastidic G6PDH (AJ010712), *Arabi* P–G6PDH: *Arabidopsis thalian*a plastidic G6PDH (AJ001770), *Nico* C–G6PDH: *Nicotiana tabacum* cytosolic G6PDH (AJ001770), *Sola* C–G6PDH: *S. tuberosum* cytosolic G6PDH (X74421), *Arabi* C–G6PDH: *Arabidopsis thaliana* cytosolic G6PDH (AJ010971), CvcG6PDH: *Chlorella vulgaris* C–27 cytosolic G6PDH (AB085846).

of *CvchG6PDH* cDNA, and thus the DNA region encoding mature CvchG6PDH (*mCvchG6PDH*) was determined. The length of *mCvchG6PDH* was 1,500 bp in length and it encoded 500 amino acid residues with a predicted molecular mass of about 56.6 kDa and an isoelectric point of 5.35. The region containing 98 amino acids from N–terminus of CvchG6PDH was determined as a transit peptide region. Based on the results, *CvchG6PDH* was suggested to encode the chloroplastic isoform of the enzyme. The nucleotide sequence of the cloned *CvchG6PDH* cDNA was deposited at DDBJ/EMBL/GenBank under accession number AB331729.

Expression of *mCvchG6PDH* in *E. coli* and G6PDH assays of the expressed protein

To confirm that the protein encoded by *mCvchG6PDH* is functionally active, the gene was introduced into *E. coli* using a pET–17xb vector. According to the result of SDS–PAGE, the mCvchG6PDH (56.9 kDa) protein was expressed as a soluble protein when treated with IPTG at 25 °C (Fig. 3A). G6PDH activity and DTT–sensitivity of the mCvchG6PDH was examined. An extract of *E. coli* expressing *CvcG6PDH* (Honjoh *et al*., 2007) was also prepared to compare DTT–sensitivity of CvcG6PDH with that of mCvchG6PDH. The mCvchG6PDH protein showed remarkably high G6PDH activity (Table 2), and its activity was inhibited in the presence of DTT, while the activity of CvcG6PDH was not inhibited with DTT (Fig. 3B).

Expression and purification of His–tagged mCvchG6PDH

To produce anti–mCvchG6PDH antibodies, *mCvchG6PDH* gene was expressed in *E. coli* as a His–tagged protein. *E. coli* cells expressing the gene were cultured at 25 °C for 6 h after addition of 1 mM IPTG. Expression, purification, and removal of His–tag region of His–tagged mCvchG6PDH protein were confirmed by SDS–PAGE

Table 2. Specific activity of enzyme extracts of *E. coli* transformed with pET–17xb/*CvcG6PDH* and pET–17xb/*mCvchG6PDH*

Plasmid	Specific activity (U/mg protein)
pET–17xb	Not detected
pET–17xb/*CvcG6PDH*	19.9 ± 2.7
pET–17xb/*mCvchG6PDH*	51.0 ± 2.5

Values are means ± SD obtained from three independent experiments.

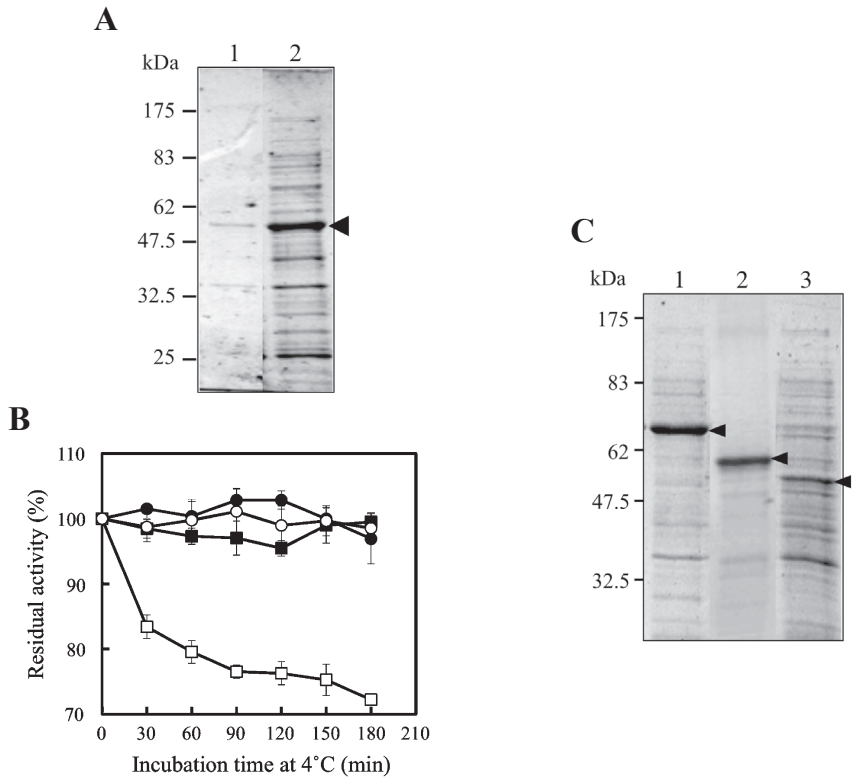

Fig. 3. (A) The *mCvchG6PDH* was expressed in *E. coli* with a pET–17xb vector. Lane 1, insoluble protein; lane 2, soluble protein from the transformed *E. coli*. (B) DTT sensitivity of mCvchG6PDH was examined using *E. coli* crude extract. Closed symbols: incubation without DTT; (●) CvcG6PDH; (■) mCvchG6PDH. Open symbols: incubation with 100 mM DTT; (○) CvcG6PDH; (□) mCvchG6PDH. All values are means ± SD obtained from three independent experiments. (C) The *mCvchG6PDH* was expressed in *E. coli* as a His–tagged protein with a pET–32a(+) vector to purify the expressed protein. Lane 1, purified His–mCvchG6PDH by Ni^{2+}–affinity chromatography; lane 2, His–mCvchG6PDH digested with thrombin; His–mCvchG6PDH digested with enterokinase. The arrowheads indicate the bands of the target proteins in each figure.

(Fig. 3C). The N–terminal sequence of the purified protein was determined as NH_2–AMADIGSGLQEENWEK (tagging peptide region was underlined), and the sequence without an underline was identical to the N–terminal sequence of $G6PDH_2$ identified previously (Honjoh *et al.*, 2003). Then, antibodies against mCvchG6PDH were raised using the purified protein as an antigen.

Change in the expression pattern of *CvchG6PDH* during hardening

Northern blot analysis of *CvchG6PDH* gene was performed using total RNAs isolated from 0, 3, 9, and 24–h hardened *Chlorella* cells. The transcription of *CvchG6PDH* increased up to 3–h hardening and the high level of transcription continued thereafter (Fig. 4A).

Western blot analysis was performed using protein extracts of cells hardened for 0, 3, 9, and 24 h to investigate change in the CvchG6PDH protein amount in cells during hardening. CvchG6PDH protein was detected with anti–mCvchG6PDH antibodies. The protein amount of CvchG6PDH decreased at the early phase of hardening and then increased, but it did not show dramatic change in the amount (Fig. 4B).

Expression of the *mCvchG6PDH* in yeast

The *mCvchG6PDH* was expressed in yeast with a pTG887 vector. Expression of the genes was confirmed by enzyme assays (Table 3). Specific activity of the extract of INVSc2/pTG887/*mCvchG6PDH* (4.58±0.37 U/mg protein) was remarkably higher than those of control yeast (INVSc2/pTG887; 0.28±0.01 U/mg protein) and INVSc2/pTG887/*CvcG6PDH* (1.01±0.09 U/mg protein).

Table 3. Specific activity of enzyme extracts of yeast transformants expressing *CvcG6PDH* and *mCvchG6PDH*

Plasmid	Specific activity (U/mg protein)
pTG887 (control)	0.28 ± 0.01
pTG887/*CvcG6PDH*	1.01 ± 0.09*
pTG887/*mCvchG6PDH*	4.58 ± 0.37**

Values are means ± SD obtained from three independent experiments. Significance against the value of control yeast is indicated as: *$p<0.05$; **$p<0.01$.

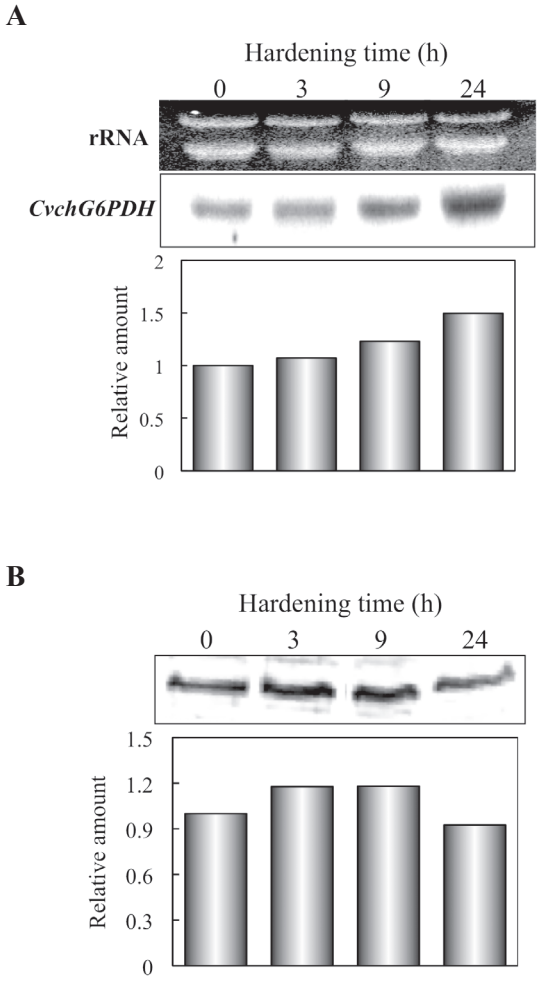

Fig. 4. Hardening-induced change in the expression level of *CvchG6PDH* in *Chlorella* cells was investigated at (A) the transcriptional and (B) the translational levels.

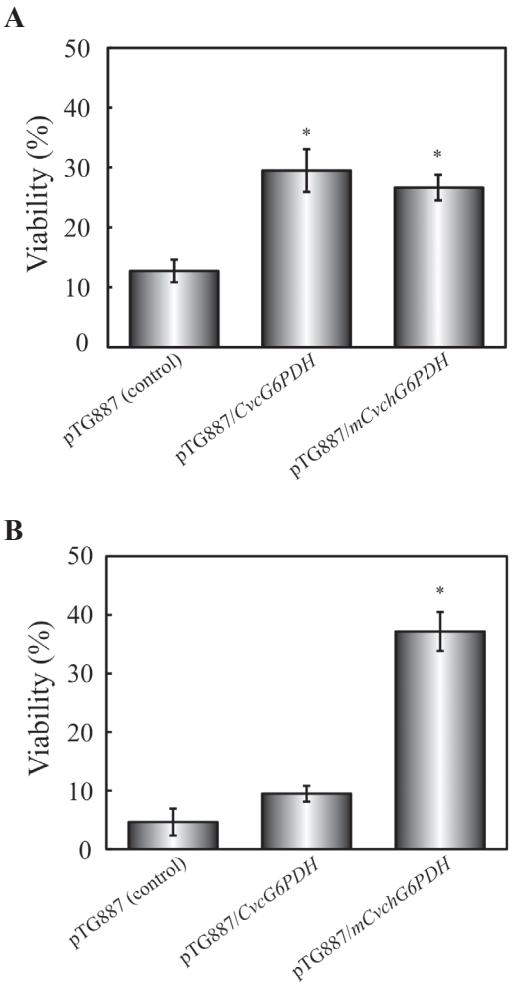

Fig. 5. (A) Freezing and (B) menadione-induced oxidative stress tolerances of yeast expressing *Chlorella G6PDH* genes. Values are means ± SD obtained from three independent experiments. Significance against the value of control yeast is indicated as: *$p<0.01$.

Stress tolerances of the transformed yeast

Effects of mCvchG6PDH on freezing and oxidative stress tolerances were investigated using *S. cerevisiae* INVSc2 as a model eukaryote. As shown in Fig. 5A, the viability of INVSc2/pTG887/*mCvchG6PDH* (26.7±2.2%) after freezing and thawing was significantly higher than those of control yeast (12.7±1.9%), while it was slightly lower than that of INVSc2/pTG887/*CvcG6PDH* (29.5±3.6%).

Oxidative stress treatment was performed using menadione as an intracellular ROS generator. As shown in Fig. 5B, the viability of INVSc2/pTG887/*mCvchG6PDH* (37.17±3.33%) after 1–h incubation with 200 μM menadione was significantly higher than those of control yeast (4.64±2.30%) and INVSc2/pTG887/*CvcG6PDH* (9.47±1.35%).

DISCUSSION

G6PDH is a key–enzyme of the pentose phosphate pathway (Wendt *et al*., 2000). *C. vulgaris* C–27 cells acquire freezing tolerance after cold–hardening, at which the G6PDH activity increased approximately 1.5–fold (Sadakane *et al*., 1980). Two types of G6PDH isozymes, which were predicted as cytosolic and chloroplastic G6PDHs, were identified in *C. vulgaris* C–27 and characterized previously (Honjoh *et al*., 2003). In the present study, we isolated a full–length cDNA clone encoding the chloroplastic isoform, and investigated its involvement and function in freezing tolerance.

A characteristic feature of chloroplastic G6PDH is considered as inactivation by covalent redox modification mediated by the ferredoxin–thioredoxin system (Scheibe, 1990; Buchanan, 1991) or DTT (Honjoh *et al*., 2003), and this inactivation is due to the reduction of two cysteine residues specific to chloroplastic G6PDH (Wendt *et al*., 1999). The cysteine residues were also observed in the deduced amino acid sequence of *CvchG6PDH* as shown in Fig. 1. G6PDH activity in crude extract of *E. coli* expressing *mCvchG6PDH* was suppressed when the extract was treated with DTT, while that of *E. coli* expressing *CvcG6PDH* did not show DTT–sensitivity (Fig. 3B). Our results suggested that the *CvchG6PDH* cDNA isolated in the present study encoded chloroplastic G6PDH in *C. vulgaris* C–27.

The CvchG6PDH protein amount in *C. vulgaris* C–27 did not show similar change to the up–regulation of the total G6PDH activity during hardening (Fig. 4B), although the transcript level of *CvchG6PDH* increased after 3–h hardening and was kept at a high level throughout 24–h hardening (Fig. 4A). Our previous study showed the change in CvcG6PDH activity during hardening did not reflect the hardening–induced increase in the total G6PDH activity (Honjoh *et al*., 2007). Those results on expression changes in CvcG6PDH and CvchG6PDH were not sufficient to explain the increase in G6PDH activity of *C. vulgaris* C–27 during hardening. Several higher plants have several genes encoding G6PDH, for example, *Arabidopsis* has two genes encoding cytosolic G6PDH and four genes encoding plastidic G6PDH (Wakao and Benning, 2005), and tobacco has two genes encoding cytosolic G6PDH and two genes encoding plastidic G6PDH (Wendt *et al*., 1999). To clarify the involvement of G6PDH in the acquisition of freezing tolerance of *Chlorella*, further isotypes of G6PDH should be searched for and isolated.

The mCvchG6PDH improved freezing tolerance of yeast significantly, and the improvement of freezing tolerance was also observed in yeast expressing *CvcG6PDH* (Fig. 5A). Since there was no significant difference between freezing tolerances of *CvcG6PDH*– and *mCvchG6PDH*–expressing yeasts, the observed effect was considered as common effect of G6PDH isozymes. One of the roles of G6PDH in the acquisition of freezing tolerance is perhaps due to the production of a reducing equivalent NADPH, which serves as an activator of several antioxidant enzymes, such as glutathione reductase, thioredoxin reductase (Carmel–Harel and Storz, 2000), monodehydroascorbate reductase (Foyer *et al*., 1994), and catalase (Salvemini *et al*., 1999). Those reports suggest that G6PDH is involved in the acquisition of freezing tolerance by activating antioxidant enzymes that can play a role in the acquisition of freezing tolerance. Freezing–thawing process can generate oxidative stress such as production of reactive oxygen species (O_2^-, H_2O_2, $OH^\bullet$), reactive nitrogen species (NO, $ONOO^-$), and lipid peroxides (Carmel–Harel and Storz, 2000; Xin and Browse, 2000; Chatterjee and Gagnon, 2001; Mittler, 2002; Neil *et al*., 2002). Since the target peroxides of the antioxidant enzymes vary, the activities of such enzymes and the resulting products should be investigated to clarify the function of G6PDH in the acquisition of freezing tolerance of *Chlorella*.

The mCvchG6PDH also improved menadione–induced oxidative stress tolerance of yeast, while the improvement was not significant in CvcG6PDH–expressing yeast (Fig. 5B). McAims *et al*. (2003) have demonstrated that menadione inactivated G6PDH activity when tested using bovine endothelial cells, causing decrease in cellular antioxidant capacity. Thus, the intrinsic G6PDH activity of yeast might be suppressed due to such function of menadione in our studies, and the introduced *Chlorella* G6PDHs may enhance G6PDH activity in yeast cells to maintain cellular reactions under such unfavorable condition in which G6PDH activity was inhibited. Based on the consideration, the difference between the viabilities of the *CvcG6PDH*– and *mCvchG6PDH*–expressing strains (Fig. 5B) is perhaps due to the difference between their G6PDH activities (Table 3). Although we could not find any specific function of CvchG6PDH under stress condition, the contribution of enhanced G6PDH activity to alleviation of cellular damages induced by menadione was first identified in the present study. The effect would be important to survive under freezing–thawing stress since the stress is accompanied by several types of oxidative stress as mentioned above. Our data suggest that the one of the functions of G6PDH in the acquisition of freezing tolerance of *Chlorella* is to alleviate oxidative stress generated under low–temperature and freezing–thawing processes.

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