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Expression of dehydrin gene from Arctic *Cerastium arcticum* increases abiotic stress tolerance and enhances the fermentation capacity of a genetically engineered *Saccharomyces cerevisiae* laboratory strain

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Abstract We investigated Arctic plants to determine if they have a specific mechanism enabling them to adapt to extreme environments because they are subject to such conditions throughout their life cycles. Among the cell defense systems of the Arctic mouse-ear chickweed *Cerastium arcticum*, we identified a stress-responsive dehydrin gene *CaDHN* that belongs to the SK_5 subclass and contains conserved regions with one S segment at the N-terminus and five K segments from the N-terminus to the C-terminus. To investigate the molecular properties of CaDHN, the yeast *Saccharomyces* was transformed with *CaDHN*. *CaDHN*-expressing transgenic yeast (TG) cells recovered more rapidly from challenge with exogenous stimuli, including oxidants (hydrogen peroxide, menadione, and *tert*-butyl hydroperoxide), high salinity, freezing and thawing, and metal (Zn^{2+}), than wild-type (WT) cells. TG cells were sensitive to copper, cobalt, and sodium dodecyl sulfate. In addition, the cell survival of TG cells was higher than that of WT cells when cells at the mid-log and stationary stages were exposed to increased ethanol concentrations. There was a significant difference in cultures that have an ethanol content >16 %. During glucose-based batch fermentation at generally used (30 °C) and low (18 °C) temperatures, TG cells produced a higher alcohol concentration through improved cell survival. Specifically, the final alcohol concentrations were 13.3 and 13.2 % in TG cells during fermentation at 30 and 18 °C, respectively, whereas they were 10.2 and 9.4 %, respectively, in WT cells under the same fermentation conditions. An in vitro assay revealed that purified CaDHN acted as a reactive oxygen species scavenger by neutralizing H_2O_2 and a chaperone by preventing high temperature-mediated catalase inactivation. Taken together, our results show that *CaDHN* expression in transgenic yeast confers tolerance to various abiotic stresses by improving redox homeostasis and enhances fermentation capacity, especially at low temperatures (18 °C).

Keywords *DHN* gene · *Cerastium arcticum* · Stress tolerance · Fermentation capacity · *Saccharomyces cerevisiae*

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Introduction

Throughout their life cycle, Arctic plants are subjected to abiotic stresses, including extreme temperature, varying oxygen concentrations, drought, nutrient depletion, very short growing seasons, common summer frosts, strong winds, low

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light quality, and changes in photoperiod (Bokhorst et al. 2010; Hagen et al. 2001). Accordingly, Arctic plants have a greater variety of morphological and physiological specializations than their temperate relatives (Archambault and Strömvik 2011). To grow at freezing temperatures, Arctic plants have long life cycles, extended primordium development of leaves and flowers, well-developed root systems, and efficient photosynthetic and respiratory systems at 10 °C or lower (Archambault and Strömvik 2011; Chew et al. 2012). Although scientific interest in Arctic plants has been increasing, most studies on these plants have focused on their genetic diversity, ecology, and phylogeny rather than on their adaptation mechanisms.

Low temperature, which affects plant growth, is one of the most common environmental stresses that Arctic plants face (Chew et al. 2012). To protect themselves from cold stress, Arctic plants have developed a wide range of cell rescue systems. Among cell defense systems, a subclass of dehydrins (DHNs) belongs to the late embryogenesis abundant group 2 proteins. Typically, DHNs are highly hydrophilic; contain a high proportion of charged and polar amino acids and a low fraction of non-polar, hydrophobic residues, and are frequently deficient in tryptophan and cysteine residues (Battaglia et al. 2008). A distinct feature of DHNs is a conserved Lys-rich K-segment domain composed of 15 amino acid residues (EKKGIMDKIKEKLPG) that usually localizes to the C-terminal region. Additional domains found in DHNs include the Y segment ([T/V]D[E/Q]YGNP) in the N-terminus of the proteins, the S segment (Ser-rich tract), and the less conserved Ø-segment motifs, which are abundant in polar amino acids (Battaglia et al. 2008). Based on the presence and arrangement of these different domains in a single polypeptide, DHNs are classified into several subgroups that have a wide range of molecular masses, ranging from 9 to 200 kDa (Allagulova et al. 2003).

DHNs are distributed in a wide range of organisms, including higher plants, algae, and cyanobacteria (Rorat 2006). Most DHNs induced by abiotic stresses, including dehydration, accumulate in the cytoplasm, although some also localize to the nucleus, plasma membrane, mitochondria, vacuole, and endoplasmic reticulum (ER) (Battaglia et al. 2008). Although accumulation of DHNs is one of the most common responses to unfavorable environmental conditions (Rorat 2006), the relationship between DHNs and the stress response is not completely understood. Some studies on plants have reported DHN overexpression leading to increased stress tolerance. For example, overexpression of various *Arabidopsis* DHN-like proteins, ERD10, RAB18, COR47, and LTI30, confer increased freezing tolerance and improve survival under chilling stress. Moreover, ectopic expression of wheat *DHN-5* in transgenic *Arabidopsis* plants enhances stress tolerance to high salinity and water deprivation (Battaglia et al. 2008). In the presence of compatible solutes, DHN proteins from *Hordeum vulgare* (barley) and *Rhododendron catawbiense* showed cryoprotective activity during desiccation and low temperature in in vitro assays (Peng et al. 2008; Reyes et al. 2008). However, little is known about *DHN* genes in Arctic plants other than the *DHN* (*DaDHN*) gene from the Arctic monocot *Deschampsia antarctica* (Chew et al. 2012; Gidekel et al. 2003). Furthermore, the functional role of *DaDHN* in the stress response has not yet been elucidated. Accordingly, elucidating the molecular properties of the *DHN* gene involved in adaptations in Arctic plants is of fundamental scientific importance and economic interest.

Saccharomyces cerevisiae is an important industrial microorganism that has long been used to raise dough; produce alcoholic beverages and pharmaceuticals such as insulin; and ferment sugars derived from rice, wheat, barley, corn, and grape juice (Belloch et al. 2008; Pizarro et al. 2008). This yeast strain has specifically been used as an agent in bioethanol production (Ding et al. 2009; Ma and Liu 2010; Stanley et al. 2010). During fermentation processes, yeast cells are affected by osmotic and oxidative stress due to the high sugar concentration and the dissolved oxygen. As fermentation progresses, other stresses, including high concentrations of ethanol and nutrient depletion, become relevant (Belloch et al. 2008). Increased ethanol concentration is one of the most common stresses that yeast cells encounter during fermentation. In addition, temperature is a major control parameter in the fermentation process. Temperatures that are suboptimal for *S. cerevisiae* growth (10–18 °C) are routinely used in the production of white wine to minimize the loss of aromatic volatiles, while red wine fermentation is conducted at higher temperatures (18–30 °C) to enhance the quality of anthocyanin pigments (Belloch et al. 2008; Pizarro et al. 2008). Similarly, beer fermentation occurs at different temperatures (8–23 °C) (Belloch et al. 2008). Temperature shifts and increased ethanol concentration affect a variety of cellular characteristics, including protein translation rate, cell membrane fluidity, RNA stability, and the enzyme activity of cell rescue proteins, which ultimately affects cell growth and eventually decreases fermentation yield and by-product quality (Pizarro et al. 2008). These problems can be resolved through the development of low temperature- and ethanol-tolerant yeast strains by using effective genetic engineering.

The present study was conducted to investigate the molecular properties of dehydrin from the Arctic mouse-ear chickweed *Cerastium arcticum* (CaDHN), which has evolved to survive intense and combined stresses in the Arctic region, and to determine it is a potential for use in the development of genetically modified yeast with greater adaptability to the environmental conditions of industrial fermentation. To accomplish this, we constructed *CaDHN*-expressing transgenic *S. cerevisiae* cells of a haploid laboratory strain and evaluated their cellular responses to abiotic stresses. We also performed glucose-based batch fermentation using the transgenic *S. cerevisiae* to compare ethanol

production at generally used (30 °C) and low (18 °C) temperatures applied during alcohol fermentation. We found that heterologous expression of *CaDHN*, which has reactive oxygen species (ROS) scavenger and chaperone-like activity, confers tolerance to abiotic stresses in transgenic yeast and improves fermentation capacity, especially at low temperatures (18 °C).

Materials and methods

Construction of CaDHN-expressing recombinant yeast and *E. coli*

C. arcticum L. (*Caryophyllaceae*) was collected from Brogger-Halvoya, Svalbard (N75 55″, E11 54″) at the Dasan Korean Arctic Research Station. Total RNA was isolated from the leaves of *C. arcticum* using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany). The complementary DNA (cDNA) probe was synthesized using an reverse transcription PCR premix kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The *CaDHN*-coding region was amplified from cDNA by PCR using *ExTaq* polymerase (Takara Bio Inc., Tokyo, Japan) with 5′-ACGGCTAGCATGTCGAACTTACACCCAACC-3′ and 5′-AGCGGATCCTTAGTAACCCTTGTGAGCTTTATC-3′ as the sense and antisense primers, respectively. The underlined sequences indicate the *Nhe*I and *Bam*HI restriction enzyme sites. The PCR reaction conditions were as follows: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min; and final extension at 72 °C for 7 min. The PCR product was purified using a Gel Extraction kit (Nucleogen, Siheung, Korea) and then inserted into the TOPO TA cloning vector (Invitrogen, Carlsbad, USA). The cloned plasmid was sequenced using the T7 primer set to confirm that no PCR-induced mutations had been introduced, after which it was digested with the *Nhe*I and *Bam*HI restriction enzymes. The digested plasmid DNA fragment was ligated into the *Escherichia coli* expression vector pKM260 (Euroscarf, Frankfurt, Germany), and the resulting plasmid was named pKM260::CaDHN. In addition, the TOPO TA cloning vector harboring the *CaDHN* gene was digested with the *Eco*RI restriction enzyme, after which the plasmid DNA fragment was ligated into the yeast expression vector p426GPD (Euroscarf, Frankfurt, Germany). The cloned plasmid was then sequenced using the PF primer (5′-TGTTTTCTTCACCAA TCAG-3′), which anneals to the glyceraldehyde-3-phosphate dehydrogenase (*GPD1*) promoter region, to confirm proper gene ligation and gene direction. The resulting plasmid was named p426GPD::CaDHN. Competent *E. coli* DH5α cells (Takara Bio Inc., Tokyo, Japan) were used to amplify each plasmid DNA in Luria–Bertani (LB) broth containing ampicillin (50 $\mu g mL^{-1}$). Each plasmid DNA was then isolated with a plasmid isolation kit (Nucleogen, Siheung, Korea) according to the manufacturer's protocols, after which the resulting plasmids were used to transform *S. cerevisiae* and *E. coli* (Table 1). *S. cerevisiae* BY4741 cells were grown overnight in YPD broth (1 % yeast extract, 2 % peptone, and 2 % glucose), inoculated into fresh YPD broth, and then incubated for 4 h at 30 °C with shaking (160 rpm) until the optical density of the cultures at 600 nm (A_{600}) reached approximately 1.0 (1×10^7 cells mL^{-1}). The resulting yeast cells were then transformed with the p426GPD::CaDHN plasmid by using the PEG/LiCl method (Gietz and Woods 2001). The transformed cells were plated on yeast synthetic dropout agar medium containing 0.67 % yeast nitrogen base without amino acids and with ammonium sulfate, 0.192 % yeast synthetic dropout medium supplement without uracil (Sigma, St. Louis, USA), 2 % glucose, and 2 % agar. The samples were incubated at 30 °C for 3 days. Positive colonies were grown in medium lacking uracil. The transformed strains (p426GPD or p426GPD::CaDHN) were used for subsequent experiments. *E. coli* BL21 (DE3; Invitrogen, Carlsbad, USA) cells were transformed with the pKM260::CaDHN plasmid by the $CaCl_2$-mediated method (Kim et al. 2012). Positive transformants were grown on LB agar plates supplemented with 50 μg mL^{-1} of ampicillin for 24 h at 37 °C. The strains containing the transformed plasmid (pKM260 or pKM260::CaDHN) were used for subsequent experiments.

Western blot analysis

E. coli and yeast cells were cultured in LB and YPD media, respectively. Crude proteins were extracted from yeast cells in the mid-log phase (A_{600}=4.0; OD=0.6) or over time using glass beads in a lysis buffer containing 20 mM HEPES, pH 7.4; 5 % glycerol; 1 mM dithiothreitol (DTT); 1 mM phenylmethylsulfonyl fluoride (PMSF); and ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktails (Roche Applied Science, Indianapolis, USA). After the protein extracts were vigorously vortexed four times for 1 min each with 2-min intervals on ice, they were cleared by centrifugation at 13,000 rpm for 20 min at 4 °C. In *E. coli*, mid-log phase cells (A_{660}=0.4) grown in LB broth in the presence of ampicillin (50 $\mu g mL^{-1}$) were treated with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then incubated for another 3 h at 37 °C. Crude extracts were subsequently prepared by sonication in lysis buffer containing 50 mM sodium phosphate, pH 8.0; 0.3 M NaCl; 10 mM imidazole; 1 mM PMSF; and an EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA). Each crude extract was collected by centrifugation at 13,000 rpm for 30 min at 4 °C. The protein concentration of yeast and *E. coli* crude extracts was then determined using Protein Dye Reagent (Bio-Rad,

Table 1 Strains used in this study

Strain	Genotype	Source	Accession number
E. coli			
BL21 (DE3)	*F⁻dcm ompT hsdS*($r_B^-m_B^-$) *gal* λ(DE3)	KCTC	
WT	*F⁻dcm ompT hsdS*($r_B^-m_B^-$) *gal* λ(*DE3*), *pKM260*	This study	
TF	*F⁻dcm ompT hsdS*($r_B^-m_B^-$) *gal* λ(*DE3*), *pKM260*::*CaDHN*	This study	
S. cerevisiae			
BY4741	*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*	EUROSCARF	Y00000
WT	*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *p426GPD*	This study	
TG	*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *p426GPD*::*CaDHN*	This study	

EUROSCARF European *Saccharomyces cerevisiae* Archive for Functional Analysis, *KCTC* Korean Collection for Type Cultures

Hercules, USA), after which 20 μg of protein was subjected to 15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were electrophoretically transferred to polyvinyl difluoride membranes (Bio-Rad, Hercules, USA) at 180 mA for 3 h at 4 °C. The membranes were subsequently incubated in blocking buffer containing 5 % skim milk and 0.02 % sodium azide in TBST (25 mM Tris–HCl, pH 7.4; 150 mM NaCl; and 0.05 % Tween-20) for 1 h at room temperature and then incubated overnight at 4 °C with anti-His tag (Invitrogen, Carlsbad, USA) and anti-DHN antibodies diluted 1:1,000 with blocking buffer. Antirabbit DHN antibody was prepared as described in the Supplementary Material. The blots were then washed with TBST for 40 min (four washes, 10 min each), after which they were incubated with antimouse and antirabbit (Santa Cruz Biotechnology, Santa Cruz, USA) antibodies conjugated with horseradish peroxidase diluted to 1:2,000–1:4,000 with blocking buffer without sodium azide for 1.5 h at room temperature. After washing with TBST for 40 min, antibody binding was visualized using enhanced chemiluminescence Western blotting detection reagent (GE Healthcare, Piscataway, USA). Anti-*S. cerevisiae* tubulin and anti-*E. coli* DnaJ (Santa Cruz Biotechnology, Santa Cruz, USA) antibodies were used as loading controls for yeast and *E. coli*, respectively.

Cellular response to abiotic stresses

Yeast cells grown in YPD broth until the mid-log phase (A_{600}=4.0) were exposed to 25 mM hydrogen peroxide (H_2O_2), 0.2 mM menadione (MD, water-insoluble form), 10 mM *tert*-butyl hydroperoxide (*t*-BOOH), 5 M sodium chloride (NaCl), 0.1 M zinc chloride ($ZnCl_2$), 10 mM copper chloride ($CuCl_2$), 0.2 % SDS, 50 mM cobalt chloride ($CoCl_2$), and 0.4 M lactic acid for 1 h with shaking (160 rpm). They were then serially diluted 10-fold with fresh YPD broth. Five microliters of diluted solution was then loaded onto YPD agar (YPD plus 2 % agar) plates, which were incubated for 2–3 days at 30 °C and then photographed. For freezing and thawing, cultures (1.0 mL) of yeast cells were frozen at −70 °C for 1 h and then thawed at room temperature for 1 h. This process was repeated three times. To monitor the stress response to ethanol, mid-log-phase (A_{600}=4.0) and stationary-phase (A_{600}=8.0) yeast cells were exposed to various concentrations of ethanol (0, 12, 16, and 20 %) for 1 h with shaking, diluted serially with YPD broth, and then loaded onto YPD agar plates. All agar plates were incubated for 3 days at 30 °C.

Analysis of morphological change and redox state

The morphological changes in the mid-log phase (A_{600}=4.0) cells exposed to 25 mM H_2O_2 for 1 h were visualized using a fluorescence microscope (Nikon, Tokyo, Japan) at ×400 magnification. To measure the cytosolic ROS, mid-log phase cells were incubated for 20 min at 30 °C with 50 μM 2′,7′-dichlorofluorescein diacetate (DCFHDA; Invitrogen, Carlsbad, USA), exposed to 25 mM H_2O_2 for 1 h, washed twice with phosphate-buffered saline (PBS, pH 7.3 to pH 7.5), and then resuspended in the same PBS buffer. Cells loaded with fluorescent probes were subsequently visualized with a fluorescence microscope (excitation wavelength, 488 nm; emission wavelength, 525 nm).

Laboratory-scale batch fermentation

To evaluate fermentation ability, yeast cells were grown under aerobic fermentation conditions on a shaker (160 rpm) for 84 h at 30 °C or for 180 h at 18 °C in GY medium containing 20 % glucose and 1 % yeast extract. The alcohol concentrations were then determined based on the percentage (*v*/*v*) of alcohol in the distillate after fermentation, which was measured using an alcohol hydrometer (REF 503; Korins, Incheon, Korea). Residual total sugar concentrations were measured using an N1 Hand-Held Refractometer (Atago, Tokyo, Japan). Alcohol and residual glucose concentrations were obtained after removing cell debris by centrifugation at 3,000 rpm for 5 min at room temperature. To measure cell

survival during the fermentation process at 30 °C, the cells were harvested at various time points (24 and 72 h) and then serially diluted to 10^{-9} with YPD broth, after which 5 μL of the diluted solution was loaded onto YPD agar plates. The cells were incubated for 3 days at 30 °C and photographed. For low-temperature fermentation (18 °C), cell survival was evaluated using a streaking assay. Briefly, cells were sampled at 24 and 180 h, streaked onto YPD agar plates, and then incubated for 2–3 days at 30 °C.

In vitro assay of ROS scavenger and molecular chaperone-like activity

For CaDHN protein purification, *E. coli* BL21 (DE3) cells harboring the pKM260::CaDHN plasmid were grown in LB medium supplemented with 50 $\mu g mL^{-1}$ of ampicillin with vigorous shaking (200 rpm) at 37 °C. When the culture reached the mid-log phase (A_{660}=0.4), IPTG was added at a final concentration of 0.5 mM, and the culture was incubated for another 3 h at 37 °C. The cells were then harvested by centrifugation at 5,000 rpm for 10 min at 4 °C and washed once with cold PBS. The resulting cell pellet was resuspended in a lysis buffer containing 50 mM sodium phosphate, pH 7.5; 0.3 M NaCl; 10 mM imidazole; 1 mM PMSF; an EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA); and lysozyme (0.3 $mg mL^{-1}$; Sigma, St. Louis, USA). The suspension was incubated on ice for 30 min and then sonicated to disrupt the cells. The homogenate was subsequently centrifuged at 15,000 rpm for 30 min at 4 °C to remove cell debris, after which the cleared supernatant containing soluble protein was purified by immobilized metal affinity chromatography (IMAC) at 4 °C. All subsequent processes were performed at 4 °C. The clear supernatant was loaded by gravity onto a column containing Ni-NTA affinity resin (Qiagen, Hilden, Germany) that had been pre-equilibrated with lysis buffer without lysozyme. The column was then washed twice with wash buffer (50 mM sodium phosphate, pH 7.5; 0.3 M NaCl; and 50 mM imidazole), after which CaDHN protein bound to Ni-NTA resin was eluted with elution buffer (50 mM phosphate, pH 7.5; 0.3 M NaCl; and 0.25 M imidazole). The eluted CaDHN protein was then filtered by a size-separation membrane. CaDHN protein bound to the membrane was resuspended at 4 °C in cold 20 mM potassium phosphate, pH 7.2, containing 1 mM PMSF and a protease inhibitor cocktail. The resulting purified CaDHN protein (2 μg) was loaded onto 15 % SDS-PAGE, run at 50 V, stained with CBB R-250, and then destained. ROS scavenging activity was measured using ferrous oxidation of xylenol-orange (FOX) reagent containing 100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM sulfuric acid (Kim et al. 2011). Five micrograms each of CaDHN and catalase (Sigma, Hercules, USA) was added to the 17 μM H_2O_2 solution, after which the solutions were incubated at 25 °C for 15 min. Next, 50 μL of the reaction mixture was added to 950 μL of FOX reagent. The mixtures were then incubated at room temperature for 1 h, after which they were centrifuged to remove any flocculants before measuring the absorbance at 560 nm. ROS scavenging activity was considered 100 % in the 17 μM H_2O_2 solutions without enzymes. Chaperone-like activity was also measured to investigate the molecular chaperone activity of CaDHN. Catalase (1 μg; CAT; Sigma, Hercules, USA) and CaDHN solutions (1 μg) were added to 50 mM potassium phosphate (pH 7.5) and heated at 50 °C for 15 min. Glucose-6-phosphate dehydrogenase (G6PDH, 1 μg; Sigma, Hercules, USA) and human heat shock protein 90 (Hsp90; 1 μg; Sigma, Hercules, USA) solutions containing catalase (1 μg) were used as negative and positive controls, respectively. Each sample was taken at the indicated time, and the catalase activity was measured based on the absorbance at 240 nm for 1 min. The enzyme activity in the solution with catalase alone was set at 100 %.

Multiple sequence alignment of DHN amino acids

CaDHN was aligned with known DHN sequences using the DNA Data Bank of Japan (DDBJ) ClustalW software (http://clustalw.ddbj.nig.ac.jp/). The amino acid sequences used were as follows: *C. arcticum* (CaDHN; accession number ADM14335), *Oryza sativa* (OsDHN; accession number ABS44866), *Phaseolus vulgaris* (PvDHN; accession number AAB00554), *Citrus* × *paradisi* (CpDHN; accession number AAN78125), *Arabidopsis thaliana* (AtDHN; accession number CAA62449), *Citrus unshiu* (CuDHN; accession number BAA74736), *H. vulgare* (HvDHN; accession number AAF01696), *Zea mays* (ZmDHN; accession number AAA33480), *Glycine max* (GmDHN; accession number AAB71225), and *Nicotiana tabacum* (NtDHN; accession number BAD13498).

Statistical analysis

All experiments were independently repeated at least three times, and the results were expressed as mean and standard deviation (SD). The results of the spotting and streaking assays, redox state, and fermentation ability were representative of at least two independent experiments conducted under identical conditions.

Results

Molecular properties of *CaDHN*

The dehydrin gene (*CaDHN*) was identified by GeneFishing and genome walking analyses of leaf tissues from *C. arcticum* collected at the Dasan Korean Arctic Research Station

(data not shown). The *CaDHN* gene contains 813 nucleotides and encodes 270 amino acids. The gene and protein are registered as HQ123366 and ADM14335 in the National Center for Biotechnology Information nucleotide and protein databases, respectively. The predicted molecular weight of the CaDHN enzyme is approximately 30 kDa. To examine the molecular properties of CaDHN, multiple sequence alignment (MSA) was performed on CaDHN using the known OsDHN, CpDHN, AtDHN, CuDHN, HvDHN, ZmDHN, GmDHN, NtDHN, and PvDHN sequences. Pairwise alignment of CaDHN and the other DHNs was conducted using the DDBJ ClustalW software. MSA revealed five K segment-like regions and one segment-like region (Fig. 1a, b and Fig. S1, Supplementary Material). Three of the K segments were highly conserved relative to the other DHNs (Fig. 1a). CaDHN showed 43, 36, 40, 32, 46, 63, 47, 63, and 37 % homology with OsDHN, CpDHN, AtDHN, CuDHN, HvDHN, ZmDHN, GmDHN, NtDHN, and PvDHN, respectively (Fig. S1, Supplementary Material). These results indicate that conserved and non-conserved domains will be present in CaDHN.

Construction of *CaDHN*-expressing recombinant yeast

The product of *CaDHN* showed structural differences at the molecular level when compared to products of other *DHN* genes (Fig. 1). Based on these results, we examined whether heterologous *CaDHN* expression affected acquired tolerance against abiotic stresses in yeast cells. To accomplish this, cDNA (accession number HQ123366) containing the open reading frame (ORF) of the *CaDHN* gene was cloned into yeast vector p426GPD, which allows constitutive expression of a target gene under control of the yeast *GPD1* promoter (Mumberg et al. 1995). Upon digestion with the *Eco*RI restriction enzyme, the inserted DNA fragment was found to be approximately 840 bp. The construct was then sequenced to confirm that the ORF orientation was correct, after which it was introduced into *S. cerevisiae* BY4741 strain (Table 1). Effective expression of the *CaDHN* gene was assessed by Western blot. As shown in Fig. 2a (left panel), immunoblotting analysis using the anti-CaDHN antibody revealed the presence of a single band produced by *CaDHN*-expressing transgenic yeast (TG) cells grown to the mid-log phase (Fig. S2, Supplementary Material) under normal conditions. No signal was detected in wild-type cells containing an empty vector (WT) under the same conditions. In addition, the expression of *CaDHN* was highest at 6 h (A_{600}=3.5–4.0) of culture, after which gene expression was saturated (Fig. 2a, right panel). Based on these results, yeast cells corresponding to A_{600}=4.0 were used for subsequent experiments.

Cellular response of genetically modified *CaDHN*-expressing yeast cells to abiotic stresses

To determine if *CaDHN* expression enhances cellular responses to environmental stresses, cell survival was evaluated in yeast cells (TG and WT) transformed with the p426GPD plasmid containing the *CaDHN* gene or with the vector alone. The stress response was measured based on a spotting assay. TG cells recovered more rapidly than WT cells in the presence of exogenous stimuli, including H_2O_2, MD, *t*-BOOH, NaCl, $ZnCl_2$, and freezing and thawing, but were more sensitive to $CuCl_2$, SDS, and $CoCl_2$ (Fig. 2b). However, there was no difference between TG and WT cells under normal conditions (Fig. S2, Supplementary Material).

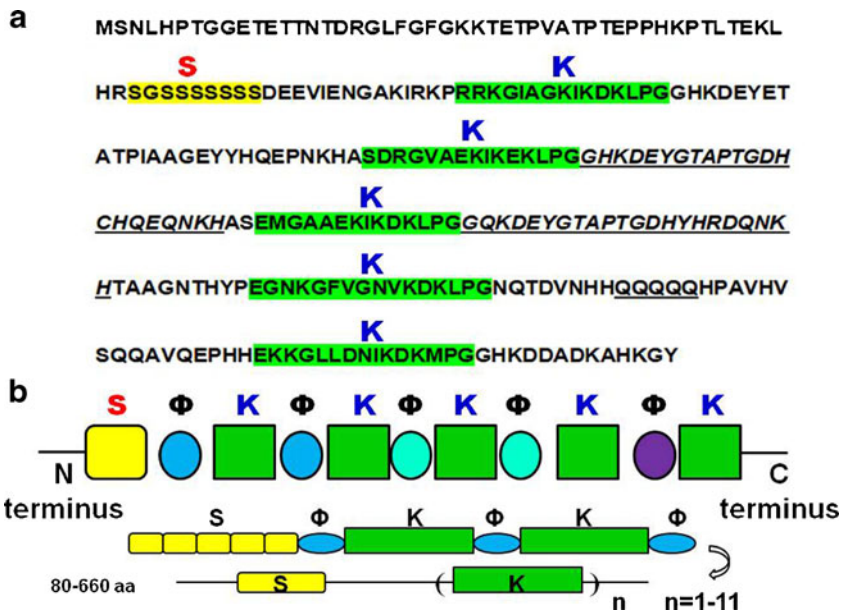

Fig. 1 Sequence analysis and characterization of CaDHN. **a** Predicted amino acid analysis of CaDHN protein. The conserved S segment with eight serine residues is boxed in yellow. The five K segments are *boxed in green*. **b** Schematic diagram of the conserved regions in CaDHN (*upper panel*) and SK_n-type DHNs (*lower panel*). *K* K segment, *S* S segment with a track of serine residues, *Φ* Φ segment, *n* number of K segments

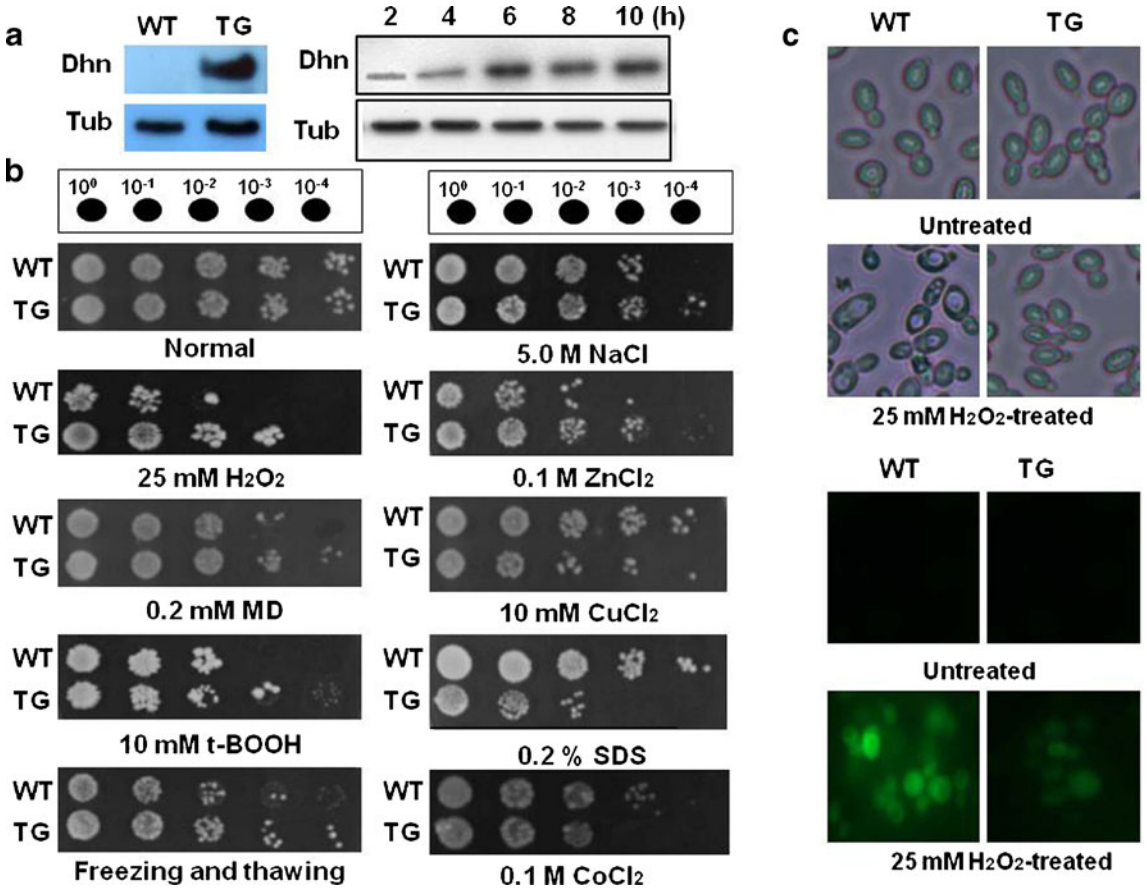

Fig. 2 Heterologous expression of the *CaDHN* gene and the stress response in transgenic yeast cells. **a** *CaDHN* expression after 6 h of culture (*left panel*) and over time (*right panel*) as detected by Western blot analysis using anti-Dhn antibody. Tub antibody from *S. cerevisiae* was used as a loading control. **b** *CaDHN*-mediated stress response in the presence of exogenous stimuli. Yeast cells were exposed to the indicated stressors for 1 h with shaking, serially diluted to 10^{-4}, and then spotted onto YPD agar plates. **c** Cellular morphology (*upper panel*) and redox state analyses using the oxidant-sensitive probe DCFHDA (*lower panel*), in the absence and presence of 25 mM H_2O_2 for 1 h. *WT* yeast cells with an empty vector, *TG CaDHN*-expressing yeast cells

To support these results, growth kinetics were followed up. TG cells had a better adaptation than WT cells in the presence of H_2O_2, MD, *t*-MD, and *t*-BOOH (Fig. S3, Supplementary Material). We also determined whether stress tolerance conferred by *CaDHN* expression in TG yeast cells led to improved redox homeostasis. The redox state was analyzed by determining the cellular hydroperoxide level using an assay based on the cytosolic oxidant-sensitive probe DCFHDA. The oxidative conversion of DCFHDA to the highly fluorescent compound dichlorofluorescein (DCF) was measured in the absence and presence of 25 mM H_2O_2. DCF fluorescence increased in both WT and TG cells upon 25 mM H_2O_2 for 1 h, but the intensity of intracellular hydroperoxides was greater in WT cells than in TG cells (Fig. 2c). The alleviation of the redox state was inversely proportional to cell recovery under oxidative stress conditions generated by H_2O_2. In addition, the cells without staining were imaged using a fluorescence microscope for comparison with fluorescence staining. In general morphological analysis, a moderate morphological difference was observed between WT and TG cells under oxidative conditions, but not under normal conditions (Fig. S4a and b, Supplementary Material). To investigate the differences of oxidized cells, scanning electron microscope (SEM) and transmission electron microscope (TEM) analyses were carried out. Before H_2O_2 challenge, the shape of WT and TG cells was not affected, but the shape of WT cells was significantly deformed 60 min after H_2O_2 treatment compared to that of TG cells. TEM analysis revealed that the cellular damage to WT cells was higher than that to TG cells in the presence of H_2O_2 (Fig. S4c and d, Supplementary Material). There was no difference between WT and TG cells under normal conditions. Therefore, our results show that heterologous *CaDHN* expression in transgenic yeast cells confers acquired tolerance to abiotic stresses by maintaining intracellular redox homeostasis and morphological features through a reduction in the hydroperoxide levels generated by H_2O_2.

Effect of *CaDHN* expression under batch fermentation conditions

We first investigated the response to various concentrations of ethanol. Yeast cells were grown to the log phase (A_{600}= 4.0) or stationary phase (A_{600}=8.0). They were then exposed to 12, 16, and 20 % ethanol for 1 h with shaking, serially diluted, and spotted onto YPD agar plates. The acquired response of TG yeast cells exposed to different concentrations of ethanol was higher than that of WT cells in both the log and stationary phases, and the response occurred in a dose- and phase-dependent manner (Fig. 3a). In particular, the adaptation to ethanol in TG cells was visible in the log phase rather than in the stationary phase. Since TG cells expressing *CaDHN* were more resistant to high ethanol concentrations and ROS-induced oxidative stress than WT cells, glucose-based laboratory-scale batch fermentation in GY medium using TG or WT yeast cells was performed at 30 and 18 °C under aerobic conditions. A significant difference in alcohol yield and residual glucose content was observed between the TG and WT cells at 30 °C, which is the temperature generally used for industrial fermentation. During fermentation for 84 h at 30 °C, the alcohol yield of TG cells was approximately 23 % higher than that of WT cells. The final alcohol concentration was approximately 13.3 % (0.63±0.03 gg^{-1}) and 10.2 % (0.48±0.04 gg^{-1}) following fermentation with TG and WT cells, respectively. The residual glucose concentration was inversely proportional to the alcohol concentration during fermentation (Fig. 3b). In addition, a distinct difference in cell survival was observed between WT and TG cells during the fermentation period. Specifically, the survival of TG cells was significantly higher than that of WT cells during fermentation for 84 h in GY medium, although cell viability decreased with time (Fig. 3c). Similar results were observed in both WT and TG cells during fermentation at low temperature (18 °C). The alcohol concentration and residual glucose content differed between WT and TG cells when

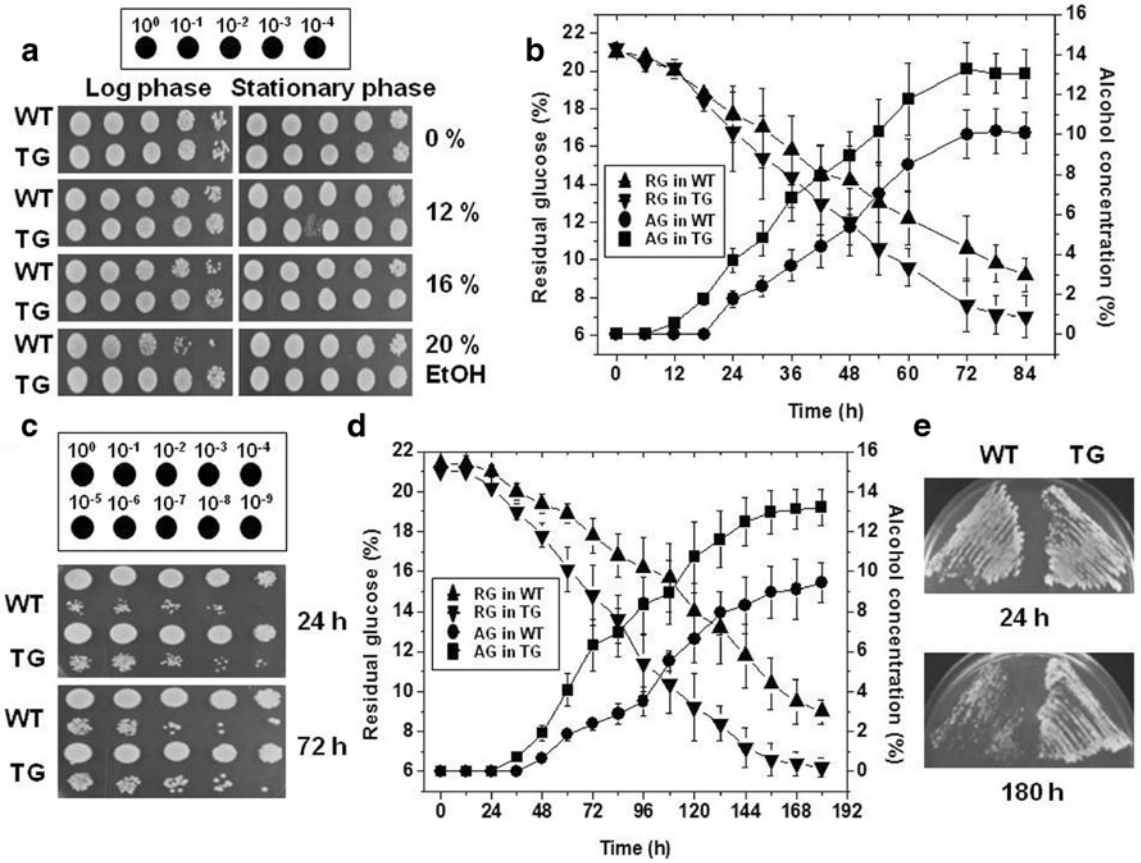

Fig. 3 Fermentation ability and cell survival in *CaDHN*-expressing yeast cells during the batch fermentation process. **a** To investigate the stress response to ethanol, log- and stationary-phase yeast cells were challenged with various concentrations of ethanol (0, 12, 16, and 20 %) for 1 h, serially diluted to 10^{-4}, and then spotted onto YPD agar plates. **b** Fermentation capacity in YG medium for 84 h at 30 °C. **c** Cell viability during fermentation at 30 °C, assessed by a spotting assay. Cells were harvested at 24 h (*upper panel*) and 72 h (*lower panel*) after initiating fermentation and serially diluted to 10^{-9}. Five microliters of diluted solutions was spotted on YPD agar plates. **d** Fermentation yield in YG medium for 180 h at 18 °C. Fermentation ability was analyzed by measuring the alcohol (*AC*) and residual glucose (*RG*) concentrations after incubation. **e** Cell survival at initial (24 h; *upper panel*) and final (180 h; *lower panel*) stages during fermentation at 18 °C, assessed by a streaking assay. *WT* yeast cells with empty vector, *TG* yeast cells with *CaDHN* gene

the same fermentation medium was used. Specifically, the alcohol yield produced by TG cells was approximately 30 % higher than that produced by WT cells. The final alcohol concentrations were approximately 13.2 % (0.62±0.04 g g^{-1}) and 9.4 % (0.44±0.02 gg^{-1}) in TG and WT cells, respectively, following fermentation for 180 h at 18 °C (Fig. 3d). In addition to fermentation yield, TG cells were more tolerant than WT cells under the same fermentation conditions (Fig. 3e). Therefore, our findings indicate that *CaDHN* expression enhances fermentation capacity at moderate and low temperatures, which is an important factor for the increased alcohol yield of TG cells during fermentation.

ROS scavenging and molecular chaperone-like activity of CaDHN in vitro

To further explore the molecular properties of CaDHN, a recombinant His-CaDHN fusion construct was developed and transformed into *E. coli* BL21 (DE3) cells. Its expression was confirmed by Western blot using anti-His tag and anti-Dhn antibodies. A single signal was detected in *CaDHN*-expressing *E. coli* (TF) cells, while no signal was observed in control wild-type cells containing an empty vector (pKM260) (WT) under the same conditions (Fig. 4a). CaDHN protein was purified via the IMAC method using Ni-NTA resin. The protein showed the same molecular weight in the absence and presence of DTT (Fig. 4b) and thus existed in monomer form. To determine the in vitro ability of CaDHN to act as an ROS scavenger by converting H_2O_2 to H_2O, the hydroperoxide level was spectrophotometrically evaluated using a FOX assay, which is based on color production following the oxidation of ferrous to ferric ion by hydroperoxide under acidic conditions. CaDHN's activity was assessed relative to that of catalase (CAT). As shown in Fig. 4c, the absorbance of the CaDHN solution (1 μg) containing 17 μM H_2O_2 was 4.5-fold lower than that of the solution containing only 17 μM H_2O_2, although the absorbance of the CaDHN solution was 2-fold higher than that of the CAT solution. The absorbance of the CAT solution (1 μg) containing 17 μM H_2O_2 was similar to the blank value obtained with 20 mM potassium phosphate (pH 7.2) alone. These results show that CaDHN can convert H_2O_2 to H_2O. We also determined whether CaDHN has the molecular chaperone-like ability to prevent protein inactivation in the presence of high temperature (50 °C). The activity of CAT in a solution containing CaDHN was approximately 3-fold higher than that of CAT in a solution without CaDHN, although CAT activity decreased in a time-dependent manner in both solutions. Unlike Hsp90, which was used as a positive control for molecular chaperone-like activity, CaDHN stabilized CAT by buffering the rate constant of thermal inactivation. In contrast, the activity of CAT in a solution with G6PDH, which was used as a negative control, was similar to the activity of CAT in a solution without any other protein (Fig. 4d). The thermoprotective effect of CaDHN was due to transient interactions between CaDHN and CAT conformers, which were native-like or could refold during the activity assay. Therefore, our results suggest that CaDHN could play an important role as multifunctional cell defense system by acting, for example, as a ROS scavengers to neutralize H_2O_2, and as a molecular chaperone to allay heat-induced protein inactivation.

Discussion

MSA revealed that CaDHN belongs to protein subclass SK_5 of dehydrins, comprising proteins that contain five K segments, one S segment, and five Ø segments. A distinct feature of DHN proteins is a conserved Lys-rich K-segment domain localized at the C-terminus region and Y segment in the N-terminus region (Battaglia et al. 2008). However, there was no Y segment in CaDHN (Fig. 1 and Fig. S1, Supplementary Material). Unlike other known DHNs, the K segments in CaDHN were abundant and distributed from the N-terminus to the C-terminus (Fig. 1b). In addition, there were two uncharacterized conserved domains (underlined region, Fig. 1a) between the second K segment and the Q-rich region. The generally accepted classification of dehydrins is based on their structural features, such as the presence of conserved sequences, designated Y, S, and K segments. The K segment is predicted to form an amphipathic α-helix, an important structural property of DHNs (Hanin et al. 2011). The S segment contains a phosphorylable serine-rich tract. Phosphorylation of S segment may be related to the nuclear transport of DHNs. The other conserved segment, the Y segment, which is missing in CaDHN, has significant amino acid sequence similarity to a portion of a nucleotide binding site found in plant and bacterial chaperones (Sun and Lin 2010). Based on defining structural features, DHNs are stress-related proteins that usually accumulate under unfavorable conditions in plants (Hanin et al. 2011). For more than 20 years, they have been thought to play an important protective role during cellular dehydration, but their precise function remains unclear (Hanin et al. 2011; Zai et al. 2011). The unusual features of CaDHN indicate that this protein could have a specific function not present in other DHNs. The results of this study help progress toward elucidating the structural, physical, and functional features of *CaDHN* and determining how these features could be exploited to improve stress tolerance in yeast.

To understand the functional role of CaDHN, a *CaDHN*-expressing TG strain was created (Fig. 2a). *CaDHN* expression in TG cells increased acquired tolerance to ROS-induced abiotic stresses, including H_2O_2, MD, *t*-BOOH,

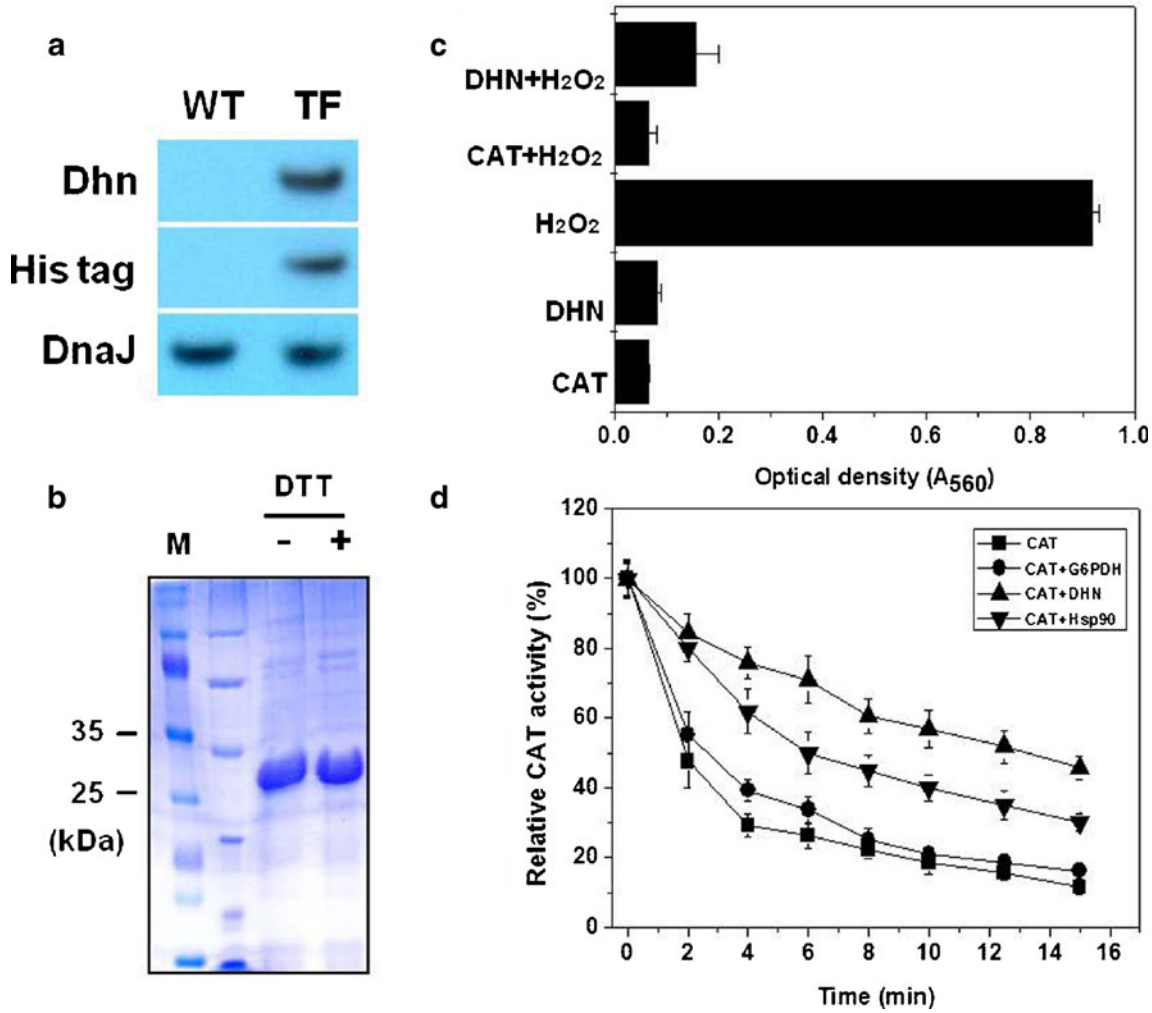

Fig. 4 In vitro assay of the ROS scavenging and molecular chaperone-like activities of CaDHN. **a** CaDHN expression detected by immunoblotting analysis using anti-Dhn and anti-His tag antibodies. DnaJ from *E. coli* was used as a loading control. *WT E. coli* cells with an empty vector, *TF E. coli* cells expressing CaDHN. **b** CaDHN protein purification via the IMAC method. Purification was confirmed by analyzing 1 μg of protein by 15 % SDS-PAGE in the absence (−) and presence (+) of DTT. **c** ROS-scavenging ability of CaDHN. The assay was carried out spectrophotometrically using the FOX reagent. Catalase (1 μg) was used as a positive control. The absorbance of sample solution with only H_2O_2 (17 μM) was set as 100 %. **d** Chaperone-like activity of CaDHN. To observe the chaperone-like activity, sample solutions in the absence (CAT) and presence (CAT + CaDHN) of CaDHN (1 μg) were collected within the specified time at 50 °C, after which the catalase activity was measured. G6PDH (1 μg; CAT + G6PDH) and Hsp90 (1 μg; CAT + Hsp90) solutions containing catalase were used as negative and positive controls, respectively. The enzyme activity was calculated as 100 % in the catalase solution alone (1 μg) without heat treatment

high salinity, zinc, and freezing and thawing (Fig. 2b), particularly by improving redox homeostasis under H_2O_2-induced oxidative conditions (Fig. 2c). Similar results were also observed in *CaDHN*-expressing transgenic *E. coli* (TF) cells under the control of the T_7 promoter. Heterologous *CaDHN* overexpression in TF cells conferred improved tolerance to exogenous stimuli, including oxidants (H_2O_2, MD, and *t*-BOOH), metals (Li, Al, Ca, Zn, Cd, and Mn), high and low temperatures, salt, and freezing and thawing (Fig. S5, Supplementary Material). Many studies have reported a positive correlation between the accumulation of DHNs and the response to abiotic stresses in crop plants. For example, overexpression of *A. thaliana DHN* genes such as *LTI29* (*ERD10*, SK_3-type) and *LTI30* (K_6) increased tolerance to freezing, dehydration, and chilling stress (Hanin et al. 2011; Kovacs et al. 2008; Puhakainen et al. 2004). Additionally, overexpression of the *C. unshiu COR19* (*CuCOR19*; K_3S) gene in transgenic tobacco decreased ion leakage and lipid oxidation under low temperature and freezing conditions, leading to increased cold stress tolerance (Hara et al. 2003; Rorat 2006). Wheat *DHN-5* (YSK_2) in *Arabidopsis* led to an increase in salt and osmotic stress tolerance (Brini et al. 2007). *Brassica juncea DHN2* and *DHN3* expression in transgenic tobacco enhanced tolerance

to heavy metals (Cd^{2+} and Zn^{2+}) by attenuating lipid peroxidation and protecting cellular membranes following lower electrolyte leakage (Xu et al. 2008). In contrast, *CaDHN*-expressing transgenic yeast and *E. coli* cells were more sensitive to Cu, Co, and SDS, compared to control WT cells containing an empty vector (Fig. 2b and Fig. S5, Supplementary Material). Similarly, homologous *RAB18* overexpression in *Arabidopsis* failed to improve freezing and drought tolerance (Lång and Palva 1992). Decreased DHN hydration status (loss of water molecules in their ambient microenvironments) or the addition of high amounts of compatible solutes (e.g., glycerol) or detergents (e.g., SDS) into a DHN aqueous solution led to conformational changes because hydrophobic amino acids (aa) localized to the surface of the protein, while hydrophilic aa were on the interface. An irreversible change in DHN conformation resulted in changes in protein function, such as enzyme inactivation and protein denaturation (Hanin et al. 2011; Mouillon et al. 2008). Conversely, overexpression of *Arabidopsis ERD10* (SK_3 type) and rice *PR46* (SK_3-type) in *E. coli* inhibited bacterial growth, which was linked to the binding of K segments to host transmembranes (Campos et al. 2006; Zhai et al. 2011); however, this phenomenon did not occur in *CaDHN*-expressing *E. coli* cells under normal conditions (Fig. S5, Supplementary Material). It should be noted that *CaDHN*-overexpressing *E. coli* cells are more tolerant to abiotic stresses and to growth inhibition under normal conditions. A stress-responsive function of *DHN* in yeast and Arctic plants has not yet been identified. However, our results indicate that *CaDHN* improves tolerance to abiotic stresses when heterologously expressed in transgenic yeast and *E. coli* by functioning as an antioxidant, ion sequester, or ion transporter, thus indicating a potential role of *CaDHN* in stress tolerance.

The SK_5-type CaDHN showed cryoprotective and metal-chelating ability in vivo (Fig. 2 and Fig. S5, Supplementary Material), as indicated by *CaDHN*-expressing transgenic yeast and *E. coli* cells that showed increased intrinsic tolerance to freezing and thawing and metal stresses. Moreover, these cells showed an increased ROS-scavenging capacity, as indicated by the reduction of hydrogen peroxide in vitro (Fig. 4). Many recent in vitro studies have reported that K_nS-type DHNs exhibit distinct functions, including protein- and lipid-binding ability, radical-scavenging ability, metal-binding ability, cryoprotective activity, and contribution to stress tolerance, even though the precise function of DHNs has not yet been completely established (Hanin et al. 2011; Hara et al. 2005; Rorat 2006). For example, the K_3S-type CuCOR19 protein shows cryoprotective activity by protecting catalase and lactate dehydrogenase from protein inactivation caused by freezing (Rorat 2006), and it prevents in vitro liposome oxidation by scavenging peroxyl and hydroxyl radicals generated by a metal/H_2O_2 system during cellular stress, resulting in cold tolerance in transgenic tobacco (Hara et al. 2003). The presence of K segments in CaDHN is essential for DHN cryoprotective activity (Reyes et al. 2008). IMAC analysis has shown that the KS-type CuCOR15 has metal-binding activity. Ni^{2+}, Cu^{2+}, and Zn^{2+} bind to CuCOR15 protein, but Mg^{2+}, Ca^{2+}, and Mn^{2+} do not (Hara et al. 2005). ITP dehydrin from castor bean binds Fe^{2+} (Krüger et al. 2002), and *Arabidopsis* ERD14 (Alsheikh et al. 2003) and celery VBA45 dehydrin (Heyen et al. 2002) bind Ca^{2+}. As seen in K_nS-type DHNs, the SK_5-type CaDHN bound to various metal ions (Li^{2+}, Ca^{2+}, Cd^{2+}, Mn^{2+}, and Zn^{2+}), leading to stress tolerance in yeast and *E. coli* (Fig. 2 and Fig. S2, Supplementary Material), while the protein did not bind to Co^{2+} and Cu^{2+}, despite conservation of the histidine (H) residues involved in Cu^{2+}-DHN binding (Hara et al. 2005). CaDHN also showed molecular chaperone-like activity by preventing thermal aggregation or heat inactivation of catalase (Fig. 4). Wheat DHN-5 improved the activity and thermostability of fungal β-glucosidase and glucose oxidase enzymes in vitro (Hanin et al. 2011). *A. thaliana* ERD10 (SK_3) and ERD14 (SK_2) prevented thermal aggregation of citrate synthase and luciferase, inactivation of lysozyme, and thermal inactivation of alcohol dehydrogenase (Hanin et al. 2011; Kovacs et al. 2008). Therefore, it is possible that CaDHN can act as a chaperone for other proteins to facilitate proper folding and/or prevent their aggregation under stress conditions such as heat. Considering these facts, our results suggest that *CaDHN* expression led to multiple activities, including metal chelation, cryoprotection, ROS scavenging, and chaperone-like activity in TG cells, indicating that CaDHN could increase acquired resistance by improving redox homeostasis, metal homeostasis, and proteostasis in the presence of abiotic stresses. Accordingly, CaDHN might be useful as a new SK_n-type dehydrin that responds to abiotic stresses in a direct and/or indirect manner.

To further elucidate the functions of CaDHN, we compared glucose-based batch fermentation capacities at 30 and 18 °C because the most important physical factor influencing the life of yeast during alcoholic fermentation is temperature. During fermentation at 30 and 18 °C, the alcohol concentrations were 13.3 % (0.63±0.03 $g\,g^{-1}$) and 13.2 % (0.62±0.04 $g\,g^{-1}$), respectively, in TG cells, while the concentrations were 10.2 % (0.48±0.04 $g\,g^{-1}$) and 9.4 % (0.44±0.02 $g\,g^{-1}$), respectively, in WT cells under the same conditions. At 30 and 18 °C, the alcohol concentration in the TG cells was 23 % (0.15 $g\,g^{-1}$) (Fig. 3b) and 30 % (0.19 $g\,g^{-1}$) (Fig. 3d) higher, respectively, than the concentration in WT cells. In general, the theoretical yield of ethanol from sugar is 0.50–0.55 $g\,g^{-1}$ when *S. cerevisiae* is used for fermentation (Zhao and Bai 2009). Interestingly, *CaDHN*-expressing TG cells could ferment sugar at 18 °C to produce alcohol at concentrations very similar to those produced at

30 °C, although fermentation at the lower temperature required more time. Cell survival was also higher in TG cells than in WT cells during fermentation at 30 °C (Fig. 3c) and 18 °C (Fig. 3e). Grapes used in wine fermentation are typically fully matured, resulting in considerable concentration of sugar. The high levels of sugar lead to uniformly high concentrations of ethanol, sometimes >15 %. The ethanol concentrations obtained here were lower, as expected, because we used 20 % glucose in the medium and also because the yeast laboratory strain BY4741 is normally unable to complete fermentation, i.e., to consume all the glucose for transformation to ethanol (Gomes et al. 2012). In beer fermentation, the ethanol content ranges from 4 to 9 % (Belloch et al. 2008). One of the most common stresses that yeast cells encounter during fermentation is increased ethanol concentration, which causes a reduction in cell viability, growth rate, and fermentation rate. Although many studies have reported the development of effective mechanisms for withstanding and/or preventing various types of damage caused by increased ethanol concentration during alcoholic fermentation, the results of most of these studies indicated that yeast is limited by different metabolic environments, such as high ethanol concentrations, and osmotic and oxidative stresses during fermentation (Belloch et al. 2008; Cebollero et al. 2007; Ding et al. 2009; Gibson et al. 2007; Ma and Liu 2010; Orozco et al. 2012; Stanley et al. 2010). In addition to ethanol toxicity, organisms involved in alcoholic fermentation are subjected to controlled temperatures. In general, grape fermentation is carried out between 18 and 25 °C. Growth at low temperatures (e.g., 16–23 °C) alters cellular factors involved in the biomass composition, leading to decreased translation rate, sugar uptake, and levels of storage carbohydrates such as trehalose, which results in reduced biomass and product yields (Pizarro et al. 2008). To obtain a high yield of ethanol, fermentation processes need to use *S. cerevisiae* strains tolerant of ethanol and low temperatures (Salvadó et al. 2011), such as the *CaDHN*-expressing transgenic yeast developed herein (Fig. 3). In our laboratory strain, our results show that metabolic engineering of genetically modified yeast has the potential to improve the fermentation industry. Furthermore, our results suggest that adaptation of *CaDHN* for enabling growth at extreme temperatures could also be a very important factor in the molecular engineering of also industrial *Saccharomyces* strains. To validate the hypothesis that a higher ethanol tolerance in industrial fermentations can be achieved with TG cells, further studies need to be performed with *CaDHN*-transformed industrial yeast strains to test their behavior at higher ethanol concentrations.

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