Identification of a Novel LEA Protein Involved in Freezing Tolerance in Wheat

Kentaro Sasaki1,6, Nikolai Kirilov Christov1,2,6, Sakae Tsuda3,4 and Ryozo Imai1,5,*

1Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Hitsujigaoka 1, Toyohira-ku, Sapporo, 062-8555 Japan
2AgroBioInstitute, Dragan Tsankov 8, Sofia 1164, Bulgaria
3Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Toyohira-ku, Sapporo, 062-8517 Japan
4Graduate School of Life Science, Hokkaido University, Kita-ku, Sapporo, 060-0810 Japan
5Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo, 060-8589 Japan
6These authors contributed equally to this work.

*Corresponding author: E-mail, rzi@affrc.go.jp; Fax, +81-11-857-9382.

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Late embryogenesis abundant (LEA) proteins are a family of hyper-hydrophilic proteins that accumulate in response to cellular dehydration. Originally identified as plant proteins associated with seed desiccation tolerance, LEA proteins have been identified in a wide range of organisms such as invertebrates and microorganisms. LEA proteins are thought to protect proteins and biomembranes under water-deficit conditions. Here, we characterized WCI16, a wheat (Triticum aestivum) protein that belongs to a class of plant proteins of unknown function, and provide evidence that WCI16 shares common features with LEA proteins. WCI16 was induced during cold acclimation in winter wheat. Based on its amino acid sequence, WCI16 is highly hydrophilic, like LEA proteins, despite having no significant sequence similarity to any of the known classes of LEA proteins. Recombinant WCI16 protein was soluble after boiling, and 1H-nuclear magnetic resonance (NMR) spectroscopy revealed that the structure of WCI16 is random and has no hydrophobic regions. WCI16 exhibited in vitro cryoprotection of the freeze-labile enzyme L-lactate dehydrogenase as well as double-stranded DNA binding activity, suggesting that WCI16 may protect both proteins and DNA during environmental stresses. The biological relevance of these activities was supported by the subcellular localization of a green fluorescent protein (GFP)-fused WCI16 protein in the nucleus and cytoplasm. Heterologous expression of WCI16 in Arabidopsis (Arabidopsis thaliana) plants conferred enhanced freezing tolerance. Taken together, our results indicate that WCI16 represents a novel class of LEA proteins and is involved in freezing tolerance.

Keywords: Cold acclimation • Freezing tolerance • LEA protein.

Abbreviations: BSA, bovine serum albumin; CA, cold acclimation; DA, de-acclimation; dsDNA, double-stranded DNA; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; GFP, green fluorescent protein; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; LDH, L-lactate dehydrogenase; LEA, late embryogenesis abundant; NLS, nuclear localization signal; NMR, nuclear magnetic resonance; ORF, open reading frame; ssDNA, single-stranded DNA; WCI16, Wheat Cold Induced 16.

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Introduction

Low temperature is a major factor that limits plant growth and productivity. In overwintering plants, freezing tolerance is increased after a period of exposure to low, but non-freezing, temperatures (Levitt 1980, Sakai and Larcher 1987, Guy 1990). This process, known as cold acclimation (CA), allows plants to develop tolerance essential for winter survival through alteration of gene expression (Guy 1990, Pearce 1999). CA-induced genes have been identified from many plant species including Arabidopsis (Thomashow 1999) and winter wheat (Pearce 1999), and the level of freezing tolerance has been reported to correlate positively with the expression of cold-induced genes (Monroy et al. 1993, Houde et al. 1995). Functions in freezing tolerance have been suggested for several of these cold-induced genes (Uemura et al. 1996, Keresztessy and Hughes 1998).

Late embryogenesis abundant (LEA) proteins are a group of CA-induced genes that encode extremely hydrophilic polypeptides (Danyluk et al. 1994, Artus et al. 1996). LEA proteins accumulate to high levels in seeds during the late stage of embryogenesis and in vegetative tissues under dehydration stress conditions such as cold, drought and high salinity.
(Ingram and Bartels 1996, Bray 1997, Thomashow 1999). LEA proteins are hyper-hydrophilic and intrinsically disordered, and therefore can remain soluble after boiling and freeze–thaw treatments (Dure 1993b). These characteristics have led to the suggestion that LEA proteins have a protective role during dehydration (Baker et al. 1988, Bray 1993, Dure 1993b). LEA proteins have been classified into various groups on the basis of amino acid sequence similarity and the presence of specific motifs (Bray 1993, Dure 1993a, Wise 2003). For example, group 1 LEA proteins typically contain a hydrophilic 20-mer motif (TRKEQ[L/M][T/E]EGY[Q/K]EMGRKGG[L/E]) (Espelund et al. 1992, Galau et al. 1992), and group 2 LEA proteins (dehydrins) are characterized by a K-segment (EKKGIME/[T/E]EGY[Q/K]EMGRKGG[L/E]) (Espelund et al. 1992, Galau et al. 1992). LEA proteins of each group have been suggested to have specific functions during dehydration (Dure et al. 1989, Bray 1993, Dure 1993a).

Functional analyses of LEA proteins have been carried out both in vitro and in vivo. Ectopic overexpression of genes encoding LEA proteins can enhance stress tolerance under water-deficit conditions. LEA25, a group 4 LEA protein from tomato (Solanum lycopersicum), can improve tolerance against high salinity and freezing when expressed in Saccharomyces cerevisiae (Imai et al. 1996). Overexpression of barley (Hordeum vulgare) HVA1 in wheat (Triticum aestivum) and rice (Oryza sativa) confers enhanced drought tolerance (Xu et al. 1996, Sivamani et al. 2000). In addition, freezing tolerance of Arabidopsis is increased by overexpression of wheat WCS19 or Arabidopsis Car15A (Artus et al. 1996, NDong et al. 2002). On the other hand, overexpression of individual genes encoding LEA proteins from spinach (Spinacia oleracea) and Craterostigma plantagineum in tobacco (Nicotiana tabacum) does not lead to any significant changes in freezing or drought tolerance (Iturriaga et al. 1992, Kaye et al. 1998). These results may indicate either that not all LEA proteins have significant roles in stress tolerance or that they need other factors for activation (Houde et al. 2004, Hundertmark and Hincha 2008). Biochemical analyses showed that several LEA proteins behave as cryoprotectants in vitro (Houde et al. 1995, Hundertmark and Hincha 2008). LEA proteins are not restricted to plants; LEA-like proteins, otherwise termed hydrophilins, exist in a range of organisms including Escherichia coli, yeast and invertebrates (Garay-Arroyo et al. 2000, Browne et al. 2002, Gal et al. 2004, Kikawada et al. 2006). Reductions in CelEA1, encoding an LEA-like protein from Caenorhabditis elegans, results in reduced survival after desiccation, osmotic and heat stresses (Gal et al. 2004). Recently, it has been demonstrated that hydrophilins are required for desiccation and freezing tolerance in yeast (Dang and Hincha 2011, Lopez-Martinez et al. 2012, Rodriguez-Porrata et al. 2012). Therefore, the function of LEA proteins in stress tolerance appears to be conserved in a variety of organisms.

In the present work, we characterize a cold-induced protein from wheat, WCI16, and show that it functions in freezing tolerance. Our data thus identify a group of proteins of unknown function as a novel class of LEA proteins.

## Results

### cDNA isolation and sequence analysis

We previously performed a macroarray-based screen for genes that are induced during CA in wheat (Christova et al. 2006). The screening identified 69 clones that were up-regulated during CA. Here, we characterized a clone that contained a 477 bp open reading frame (ORF) encoding a putative 16.3 kDa protein, and named the corresponding gene *Wheat Cold Induced 16* (WCI16) (DDBJ accession No. AB830333). The WCI16 protein is rich in alanine (16.46%), lysine (14.56%), glycine (12.03%) and glutamic acid (12.03%), but lacks cysteine, tryptophan, arginine and histidine residues ([Supplementary Fig. S1](#)). WCI16 possesses two alternating lysine and glutamic acid residue-rich regions (EKKSVEGIDKEEKKSGGEEKEEK) ([Supplementary Fig. S1](#)). Hydrophathy plots predicted that WCI16 is predominantly hydrophilic, with few hydrophobic amino acid residues ([Fig. 1B](#)). This degree of hydrophilicity is reminiscent of that of LEA proteins, although WCI16 did not show significant sequence similarity to any known classes of LEA proteins. Homology searches indicated that the amino acid sequence of WCI16 was similar to those of a group of plant proteins with unknown function, including rice Os10g0330000 and Arabidopsis At1g13930 and At2g03440 (Fu et al. 2010) ([Supplementary Fig. S2](#)). At2g03440 was previously named AtNRP1 ([for Arabidopsis thaliana] Nodulin-related protein 1) and shows partial similarity to the alfalfa (*Medicago sativa*) early nodulin 12B precursor (Fu et al. 2010). WCI16 showed 57.1, 42.1 and 42.1% identity to Os10g0330000, At1g13930 and AtNRP1, respectively. A phylogenetic tree generated with the sequences of WCI16 homologs from several plant species that are available in the GenBank database showed that there are at least three groups within the plant WCI16 homologs ([Fig. 1A](#)). WCI16 homologs were identified in monocots and dicots, but not in ferns, mosses, green algae and other non-plant species (1e⁻⁸⁰ < E-value < 1e⁻² in BLAST searches).

### Expression of WCI16 in response to cold treatment

As the WCI16 cDNA was isolated by a differential screening for cold-induced genes, its expression during the course of CA was examined by RNA gel blot analysis. Accumulation of the WCI16 mRNA in crown tissue reached its maximum after 1 d of CA and declined somewhat thereafter, but maintained relatively high levels during 14 d of CA ([Fig. 2A](#)). In contrast, WCI16 mRNA decreased immediately after 1 d of de-acclimation (DA).
The short-term response of WCI16 to cold was determined using young seedlings. RNA gel blot analysis indicated that the expression of WCI16 mRNA steadily increased up to 24 h of cold treatment and slightly declined thereafter (Fig. 2B).

**Purification and heat stability of intact WCI16 protein**

To explore the possible functions and activity of WCI16, a glutathione S-transferase (GST)-fused form of WCI16 was produced...
in *E. coli* and purified as an untagged form after on-column cleavage with PreScission™ protease. Although the calculated molecular mass of WCI16 is 16.3 kDa, the purified WCI16 migrated as a larger protein on SDS–polyacrylamide gels (Fig. 3, lane 4). Similar phenomena have been observed for many LEA proteins (Kovacs et al. 2008, Hara et al. 2009, Koag et al. 2009); because of their amino acid composition, intrinsically unstructured proteins bind less SDS than other proteins do and their apparent molecular mass is often higher than that calculated from sequence data (Tompa 2002). Recombinant GST alone was also purified (Fig. 3, lane 1) and was used as a negative control in the following experiments.

We first examined the boiling solubility of WCI16. WCI16 and GST were boiled for 20 min, separated into soluble and insoluble fractions, and analyzed by SDS–PAGE. After boiling, GST was not detected in the soluble fraction but was present in the insoluble fraction (Fig. 3, lanes 2 and 3), reflecting the heat-labile nature of the protein. In contrast, the WCI16 protein band was detected in the soluble fraction after boiling (Fig. 3, lane 5) and was not found in the insoluble fraction (Fig. 3, lane 6). These data indicate that WCI16 is a boiling-soluble protein that shares characteristics with LEA proteins.

**WCI16 protects lactate dehydrogenase from freeze inactivation**

We next evaluated the cryoprotective activity of the purified WCI16 protein by testing its ability to protect L-lactate dehydrogenase (LDH), a freeze-labile enzyme, from freeze inactivation. Equivalent concentrations of bovine serum albumin (BSA) and sucrose were used as positive and negative controls, respectively. Measurements of residual LDH activity indicated that WCI16 had cryoprotection activity comparable with that of BSA (Fig. 4). In contrast, sucrose failed to protect LDH in an identical freeze–thaw cycle. These data strongly support the hypothesis that WCI16 protects cellular enzymes from freeze-induced inactivation.

**Structural analysis of WCI16**

One of characteristic features of LEA proteins is unstructured folding due to amino acid compositions biased toward hydrophilic residues (Dure 1993b). We tested whether WCI16 shares this characteristic, using 1H-nuclear magnetic resonance (NMR) analysis of purified recombinant WCI16 dissolved in 10% D₂O. The resultant 1H-NMR spectrum was analyzed with empirical rules that correlate 1H-NMR spectra with protein structural composition (Wishart et al. 1991, Wishart et al. 1992). As shown in Fig. 5, no significant secondary shifts were observed in the high-field-shifted methyl region (0–1.0 p.p.m.), suggesting that hydrophobic side chains including aromatic rings do not form any clustered regions in WCI16. In addition, there were no shifts in the down-field-shifted CαH region (5.0–6.0 p.p.m.), indicating that WCI16 does not form definitive secondary structure, such as an α-helix or β-strand. Further, no secondary shift was found in the extremely low-field-shifted NH-proton region (9.0–12.0 p.p.m.), revealing that there is no significant specific three-dimensional structure in WCI16. The resonances observed between 8.2 and 9.0 p.p.m. are attributable to random-coiled NH groups. For WCI16, the total intensity of this spectral region was small compared with that of similar sized globular proteins [e.g. hen egg lysozyme (129 residues)], suggesting that most of the amide group of WCI16 is easily exchanged with solvent water. It is therefore highly likely that WCI16 does not form any rigid structure but favors a random state at 4°C.
Subcellular localization of WCI16

To elucidate further the function of WCI16 in plant cells, we transiently expressed a green fluorescent protein (GFP)-fused WCI16 protein. Wheat leaf sheath epidermal cells were transformed with the fusion gene by particle bombardment. Fluorescence of WCI16::GFP was detected in cytosolic and nuclear regions of wheat cells (Fig. 6). The GFP-only positive control also showed a cytoplasmic and nuclear localization (Fig. 6). From these data, we conclude that WCI16 localizes to the cytosol and nucleus in wheat cells.

Nucleic acid binding activity of WCI16

Recent bioinformatics analysis predicted that LEA proteins have DNA binding activity (Wise and Tunnacliffe 2004). In order to explore the possible function of WCI16 in the nucleus, the nucleic acid binding activity of WCI16 was tested. Recombinant GST::WCI16 protein was purified and gel-shift assays were performed utilizing single- or double-stranded DNAs (ssDNA or dsDNA) as substrates. As shown in Fig. 7, shifts of the only dsDNA band were detected when GST::WCI16 was added. In contrast, shifts of both ssDNA and dsDNA bands were detected when 700 pmol GST::WCSP1 (Karlson et al. 2002) was added to the reaction. When GST was added as a negative control, no shift was detected for either ssDNA or dsDNA substrates. RNA binding assays were performed using in vitro transcribed luciferase (luc) mRNA as a substrate. As shown in Fig. 7, no shift was detected with GST::WCI16 or GST, whereas a clear shift was observed with GST::WCSP1. These data indicate that WCI16 specifically binds to dsDNA.

Overexpression of WCI16 confers freezing tolerance

To test the effects of WCI16 expression on freezing tolerance, we generated transgenic Arabidopsis lines overexpressing WCI16. We analyzed two homozygous 35S::WCI16 transgenic lines (WCI16-15 and -30) (Fig. 8A). The 35S::WCI16 plants showed no difference in phenotype compared with the wild type under normal growth conditions (Fig. 8B). The 35S::WCI16 and wild-type plants were then exposed to freezing temperatures under non-cold-acclimated conditions. As shown in Fig. 8C and D, 35S::WCI16 transgenic plants displayed higher survival rates than did wild-type plants at $-10^\circ$C. The WCI16-15 line had a survival rate of 95.8%, while the survival of wild-type plants was 72.2%. Similar results were also observed with the WCI16-30 line, with 87.5% survival vs. 65.3% for the wild-type plants in these experiments (Fig. 8D). Freezing tolerance was further examined by electrolyte leakage assay. Rosette leaves of 35S::WCI16 lines exhibited lower levels of leakage.
In the present study, we isolated the *WCI16* gene encoding a hyper-hydrophilic protein from cold-acclimated wheat. The *WCI16* protein belongs to a group of plant proteins whose function is unknown. Hyper-hydrophilicity is one of the characteristics of LEA proteins; however, the amino acid sequence of *WCI16* showed no significant similarity to any known classes of LEA proteins. LEA proteins generally share several features (Baker et al. 1988, Dure 1993b): (i) hyper-hydrophilicity; (ii) boiling solubility; (iii) stress inducibility; and (iv) stress-protecting activity. Several lines of evidence in this study demonstrate that *WCI16* represents a novel class of LEA proteins and is involved in freezing tolerance.

As shown in Fig. 3, purified *WCI16* remained soluble after boiling for 20 min. Hydropathy plots indicated that the overall protein lacks hydrophobic regions (Fig. 1B). Our $^1$H-NMR structure analysis revealed that *WCI16* is unstructured in solution (Fig. 5). It is likely that the boiling-soluble nature of *WCI16* is due to a lack of hydrophobic interaction and folded structures.

*WCI16* mRNA expression was strongly induced in crown tissue during CA (Fig. 2A). Crown tissue is important for winter survival and develops the highest tolerance against freezing (Chen et al. 1983, Perras and Sarhan 1989, Houde et al. 1995). In addition, *WCI16* transcript was induced by cold treatment in shoot tissues (Fig. 2B). In contrast, the expression of *WCI16* was not induced by other dehydration-related stresses such as drought and salt, or by treatment of seedlings with the dehydration-related hormone ABA (data not shown). These data therefore suggest that *WCI16* function is mainly associated with freezing tolerance. This hypothesis is further supported by the fact that *WCI16* protects LDH from freeze–thaw inactivation (Fig. 4). Functions in protection of LDH during freezing have been demonstrated for LEA proteins from several plant species (Wisniewski et al. 1999, Honjoh et al. 2000, Hara et al. 2001, Bravo et al. 2003, Nakayama et al. 2007). The cryoprotective activity of *WCI16* protein was comparable with that reported for other LEA proteins, such as PCA60 (Wisniewski et al. 1999) and Cor15am (Nakayama et al. 2007).

We found that *WCI16* protein has dsDNA binding activity (Fig. 7). Recently, it was demonstrated that the lysine and glutamic acid cluster in the dehydrin CuCOR15 plays an important role in DNA binding (Hara et al. 2009). *WCI16* also has lysine and glutamic acid clusters, namely EKKSVEGIDKEK and KKGEEEKE (Supplementary Fig. S1), which might contribute to the DNA binding activity of *WCI16*. An increase in reactive oxygen species production due to environmental stress can cause oxidative modifications to proteins, lipids and DNA (Apel and Hirt 2004, Bartoli et al. 2004). Dps, a non-specific DNA binding protein in *E. coli*, protects DNA from genotoxic stress caused by oxidative stress (Martinez and Kolter 1997). *WCI16* may play a similar role in DNA protection by interacting with DNA under stress conditions.

*WCI16::GFP* showed a nucleo-cytoplasmic localization in wheat cells (Fig. 6). This nuclear and cytoplasmic localization is consistent with roles for *WCI16* in protecting proteins and DNA from freezing stress. Sequence analysis of *WCI16* did not reveal any known nuclear localization signal (NLS), and it also lacks the S-segment that is believed to be involved in nuclear localization (Jensen et al. 1998). The wheat LEA protein WCS120 localizes to the nucleus and cytoplasm, but is similarly devoid of an NLS or S-segment (Houde et al. 1995). It is possible that an unknown nuclear localization mechanism, independent of NLSs and S-segments (Rorat 2006), delivers *WCI16* to the nucleus.

Transgenic Arabidopsis plants overexpressing *WCI16* were generated in order to test the contribution of *WCI16* to freezing tolerance in planta (Fig. 8A). The *WCI16*-overexpressing plants displayed no morphological or developmental defects, suggesting no negative effect of *WCI16* overexpression on growth under non-stress conditions (Fig. 8B). Overexpression of *WCI16* conferred improved freezing tolerance to non-acclimated plants in both whole plant survival and leaf electrolyte.
leakage (Fig. 8C–E). Although there is a discrepancy between the temperature ranges of tolerance in the two experiments, the difference can be explained by the effect of sucrose added to the agar medium, which benefits clear identification of survivors, on lowering the killing temperatures (K. Sasaki, unpublished observation). LEA proteins are highly divergent, and the contribution of each protein to overall stress tolerance is considered to be rather small. However, several reports have shown that overexpression of single LEA proteins can confer stress tolerance. Electrolyte leakage tests revealed that the

Fig. 8 Effect of WCI16 overexpression on freezing tolerance. (A) RNA gel blot analysis of 10-day-old seedlings in two independent transgenic Arabidopsis lines (WCI16-15 and -30). (B) Phenotype of 3-week-old wild-type and WCI16-overexpressing plants. (C, D) Survival of WCI16 overexpressors (WCI16-15 and -30) and wild-type plants after freezing stress. Ten-day-old plants grown on MS medium containing 2% sucrose were frozen with ice chips and cooled (−1°C h⁻¹) until the designated temperatures were reached. Plates were then maintained in the dark at 4°C for overnight and transferred to a growth chamber (16 h light/8 h dark) at 22°C for survival rate determination. The photographs were taken after 6 d of recovery from freezing treatments (C). Experiments were performed in quadruplicate, and the percentage of plants surviving was calculated (n = 18 plants used in each treatment). Data represent means ± SE of quadruplicate experiments. * and ** indicate statistically significant differences from the wild type at P < 0.05 and P < 0.01 (Student’s t-test), respectively (D). (E) Electrolyte leakage of 4-week-old wild-type and 35S::WCI16 plants after exposure to freezing temperatures (−5°C to −8°C). One detached rosette leaf per plant was placed in a tube containing 200 µl of ice-containing distilled water for ice nucleation at −2°C. The temperature was lowered at a rate of −1°C h⁻¹. The tubes were removed from the freezer at the designated temperatures and shaken at 25°C for 4 h before electrical conductivity measurements.
freezing tolerance of Arabidopsis is increased by ectopic expression of the wheat LEA gene WCS19 or the Rhododendron catawbiense LEA gene RcDhn5 (NDong et al. 2002, Peng et al. 2008). Recently, it was demonstrated that overexpression of OpsDHNL, an LEA gene from Opuntia streptacantha, increases the survival rate of Arabidopsis at the whole plant level after freezing treatment (Ochoa-Alfaro et al. 2012).

A knock-out line of At1g13930, a WCI16 homolog in Arabidopsis, is hypersensitive to salt in terms of germination, although the expression of the At1g13930 gene is not induced by salt treatment (Du et al. 2008). According to data from the public microarray database (BAR; http://www.bar.utoronto.ca/), the At1g13930 transcript is 5.2-fold up-regulated in shoots and 3.6-fold up-regulated in roots by 24 h of cold treatment. At1g13930 could be involved in CA and the acquisition of dehydration stress tolerance in Arabidopsis. The expression of another WCI16-like gene, AtNRP1, is also up-regulated by low temperature (Fu et al. 2010). One possible mechanism for the increased freezing tolerance of Arabidopsis plants overexpressing wheat WCI16 is that the overproduction and nucleocytoplasmic localization of the WCI16 protein protects endogenous proteins and DNA from lethal freezing damage.

Our current data, together with the recent findings in Arabidopsis, support a functional role for WCI16 family proteins in stress tolerance. Together, this work shows that WCI16 family proteins exhibit the hyper-hydrophilicity, boiling solubility, stress-inducing activity and stress-protecting activity shared by LEA proteins, suggesting that WCI16 is a novel LEA protein that is induced during CA.

Materials and Methods

Plant materials

Surface-sterilized seeds of winter wheat (T. aestivum L. cv. Chihoku) were germinated on wet paper and planted on commercial soil mix. Plants were grown at 22°C/18°C (16 h light/8 h dark) in a growth chamber. Seeds of Arabidopsis [Arabidopsis thaliana ecotype Columbia (Col-0)] were surface sterilized and planted on MS medium (Murashige and Skoog 1962) containing 2% sucrose. Plates were maintained in the dark at 4°C for 2 d and were transferred to a growth chamber (16 h light/8 h dark) at 22°C.

Stress treatments

Plants of winter wheat were grown in pots (pot size: 60 mm × 155 mm × 100 mm; four wheat plants per pot) for 14 d. CA was performed at 6°C/2°C (8 h light/16 h dark at 211 µmol m⁻² s⁻¹) for an additional 14 d. Subsequently, plants were subjected to DA treatment at 22°C/18°C (16 h light/8 h dark at 211 µmol m⁻² s⁻¹) for 3 d. Crown tissue was harvested before acclimation (NaCl day 0), after 1, 3, 7, 10 and 14 d of CA, and after 1 and 3 d of DA. Collected plant materials were frozen in liquid nitrogen and stored at −80°C until processed for RNA extraction. For short-term cold treatment, germinated seeds of winter wheat were cultivated hydroponically in water on plastic mesh grids that were suspended just above the water line in plastic containers. The container was maintained in a growth chamber at 22°C under continuous illumination. After growing for 7 d, seedlings were subjected to cold treatment by transferring them into plastic containers with cold water (4°C). At each designated time period, shoot tissue was collected, frozen with liquid nitrogen, and stored at −80°C until processed for RNA extraction.

cDNA isolation and sequence analyses

Screening of cold-regulated cDNA clones in a cDNA library was performed using a macroarray-based differential screening method (Christova et al. 2006). Isolated cDNA clones were sequenced with a 373A DNA sequencer (Applied Biosystems) using a Big Dye Terminator Cycle Sequencing Kit v 1.1 (Applied Biosystems). Multiple amino acid alignments were performed using the online ClustalW alignment program at a website maintained by the DDBJ (http://clustalw.ddbj.nig.ac.jp/top-j.html). Protein hydropathy was predicted by Kyte–Doolittle hydropathy plot analysis (http://fasta.bioch.virginia.edu/fasta www2/fasta www.cgi?rm=misc1). A phylogenetic tree was created using Geneious Pro 5.3.4 software (Biomatters Ltd.).

Total RNA extraction and RNA gel blot analyses

Total RNA was isolated from wheat shoot and crown tissues using TRIzol reagent (Invitrogen). Total RNA was separated on 1.2% agarose gel containing formaldehyde and transferred to Hybond N+ membrane (GE Healthcare). Hybridization was performed with a full-length WCI16 cDNA probe that was labeled with the ECL Kit (GE Healthcare) to eliminate the ECL Kit (GE Healthcare) according to the manufacturer’s instructions. Detection and quantification of the signals were carried out using a LAS3000 image analyzer (Fuji Film).

Recombinant protein production and purification

The ORF of WCI16 was amplified with 5'-GGATCCATGGAGGG TGAGAAGAAC-3' and 5'-GTCGACCTACTTACGAAACCCCT GA-3' (underlining denotes restriction sites) and cloned into the BamH1–Sall site of pGEX6P-3 (GE Healthcare) to produce a GST-fused protein (pGEX-WC16). Construction of pGEX-WCSP1 was described previously (Karlson et al. 2002). Escherichia coli cells containing pGEX-WC16, pGEX-WCSP1 or pGEX6P-3 were cultured in LB medium until the OD₆₀₀ reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was subsequently added to a final concentration of 1 mM and recombinant proteins were induced for 3 h. The bacterial cultures were centrifuged and the resuspended pellets were disrupted with sonication. The cell lysate was centrifuged and the total soluble fraction (supernatant) was affinity purified with a glutathione–Sepharose 4B column (GE Healthcare) according to the manufacturer’s instructions. Subsequent to washing, bound GST-fused proteins were eluted or further purified by on-column cleavage with PreScission® protease (GE Healthcare) to eliminate GST according to the manufacturer’s
instructions. Eluted proteins were washed and concentrated with buffer (10 mM Tris–HCl, pH 7.5). Protein concentration was determined with the BioRad Protein Assay Kit (Bio-Rad Laboratories) using IgG as a standard.

Boiling treatment

The protein samples were heated to 100°C for 20 min and subsequently cooled on ice. The samples were centrifuged at 12,000 × g for 10 min at 4°C in order to separate the soluble and insoluble fractions. The soluble and insoluble fractions were suspended with SDS sample buffer, separated by SDS–PAGE and stained with Coomassie Brilliant Blue according to standard protocols.

Cryoprotection assay

The cryoprotective activities of purified intact recombinant WC16 were assayed as described previously (Lin and Thomashow 1992). Briefly, LDH from Lactobacillus casei (Nacalai tesque) was diluted to 12.5 μg ml−1 in 10 mM KPO4, pH 7.5. A 50 μl aliquot of this solution was mixed with an equal volume of the tested cryoprotectant and frozen at −30°C for 24 h. After thawing at room temperature, 20 μl of sample was added to 1 ml of substrate solution containing 80 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM pyruvic acid and 0.3 mM NADH. The absorbance decrease at 340 nm was measured using a spectrophotometer (Implen). The rate of decrease in absorbance during the first 3 min of the reaction was used to calculate LDH activity.

1H-NMR structure analysis

The ORF of WC16 was amplified with 5′-GGATCCATGGAGGG TGAGAAGAAC-3′ and 5′-GGATCCATGGAGGGTGAGAAGAACCTTGACCCCT GA-3′ (underlining denotes restriction sites) and cloned into the BamHI–SalI site of pQE30 (Qiagen) to produce a His-tagged fusion protein (pQE30-WC16). Escherichia coli cells containing pQE30-WC16 were cultured until the OD600 reached 0.6. IPTG was subsequently added to a final concentration of 1 mM and production of recombinant proteins was induced for 3 h. The bacterial cultures were centrifuged and the pellets were disrupted with sonication. The cell lysate was centrifuged and the total soluble fraction (supernatant) was affinity purified with Ni-NTA agarose (Qiagen) according to the manufacturer’s instructions. Subsequent to washing, bound His-fused proteins were eluted. Eluted proteins were washed and concentrated with Milli-Q water. The protein concentration was determined with the BioRad Protein Assay Kit (Bio-Rad Laboratories) using IgG as a standard. For NMR sample preparation, His-WC16 was dissolved at 10 mg ml−1 in 10 mM Tris–HCl, pH 7.5 containing 10% D2O for spectrometer locking. The 1H-NMR measurement was performed using a Varian Unity INOVA 500 MHz spectrometer (Varian) at 4°C. The spectrum was acquired with the 1H-carrier centered at the water frequency (5.01 p.p.m.), and water suppression was achieved by a low-power (20 Hz) water pre-saturation pulse of 1.0–1.5 s duration. The 1H chemical shifts were referenced to the trimethylsilyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) at 0 p.p.m., which was added as an internal standard.

Subcellular localization of GFP-fused protein

A GFP gene, sGFP (S65T), was used as a reporter for an in-frame C-terminal fusion to WC16 (Niwa et al. 1999). To generate the GFP fusion construct, the ORF of WC16 was amplified with primers 5′-GGATCCATGGAGGGTGAGAAGAACCTTGACCCCT GA-3′ and 5′-GGATCCATGGAGGGTGAGAAGAACCTTGACCCCT GA-3′ (underlining denotes restriction sites) that removed the native stop codon of the ORF and added BamHI and NcoI sites for subsequent cloning. The resulting PCR fragment was digested with BamHI and NcoI and ligated into BglII–NcoI sites of the sGFP (S65T) vector (Niwa et al. 1999). WC16::GFP plasmid was bound to 1.0 μm gold particles (Bio-Rad Laboratories) according to the manufacturer’s instructions. Particles were bombarded into wheat leaf sheath cells with a PDS-1000 bio-listic delivery system (Bio-Rad Laboratories) with a rupture setting of 1,100 p.s.i. After bombardment, the tissues were maintained in the dark for 16 h at 25°C and viewed on glass slides with a FW4000 fluorescence imaging workstation (Leica). Deconvolution images were obtained with Leica DEBLUR software (Leica).

Nucleic acid binding analysis

Gel shift assay with ds/ssDNA substrates was performed as previously described (Nakaminami et al. 2005). ssDNA (M13mp8) or dsDNA (M13mp8 RFI) (Nippon Gene) was incubated with GST–WC16, GST–WCP1 or GST in 15 μl of binding buffer (10 mM Tris–HCl, pH 7.5) and was kept on ice for 15 min. The samples were then separated on 1% agarose gels and stained with ethidium bromide for visualization of gel shifts. Gel shift assays with mRNA substrates were performed as previously described (Nakaminami et al. 2005). Luciferase mRNA was in vitro transcribed with the RiboMAX kit (Promega).

Overexpression of WC16 in Arabidopsis

To generate the construct for overexpression of WC16 under the control of the Cauliflower mosaic virus 35S promoter in Arabidopsis, the ORF of WC16 was amplified by PCR with primers 5′-GGATCCATGGAGGGTGAGAAGAACCTTGACCCCT GA-3′ and 5′-GGATCCATGGAGGGTGAGAAGAACCTTGACCCCT GA-3′ (underlining denotes restriction sites) and ligated into the sGFP (S65T) vector (Niwa et al. 1999). WCI16::GFP plasmid was bound to 1.0 μm gold particles (Bio-Rad Laboratories) according to the manufacturer’s instructions. Particles were bombarded into wheat leaf sheath cells with a PDS-1000 bio-listic delivery system (Bio-Rad Laboratories) with a rupture setting of 1,100 p.s.i. After bombardment, the tissues were maintained in the dark for 16 h at 25°C and viewed on glass slides with a FW4000 fluorescence imaging workstation (Leica). Deconvolution images were obtained with Leica DEBLUR software (Leica).
high humidity and placed in the dark for 12–20 h. Plants were grown under normal growth conditions (16 h light/8 h dark at 22°C) and seeds were harvested. For selection of transformants, T1 seeds were plated on MS medium containing 2% sucrose and 50 μg ml⁻¹ kanamycin. Expression of WCI16 in independent transgenic lines was examined by RNA gel blot analysis using the ORF region as a probe. Homozygous T4 plants were used for further analysis.

**Freezing stress tolerance analysis**

Whole-plant freezing tolerance was assayed as described previously (Chinnusamy et al. 2003), with some modifications. Non-acclimated 10-day-old seedlings were transferred to a programmed freezer LU-112 (ESPEC) set at −1°C. After 1–2 h, the plates were sprinkled with ice chips and maintained at −1°C for at least 16 h. The temperature was lowered at a rate of −1°C h⁻¹ and plates were removed from the freezer at specific temperatures. Plates were thawed overnight at 4°C and the tube was maintained in the programmed freezer with the temperature set at −1°C/C0. After adding 800 μl of distilled water, the tubes were shaken at 25°C/C14 for 1 h. After adding 800 μl of distilled water, the tubes were shaken at 25°C/C14 for 4 h. The electrolyte leakage was measured using a compact conductivity meter C-172 (HORIBA). The tubes were frozen at −80°C/C0 for 16 h and then shaken at 25°C/C14 for 4 h to obtain a value for 100% electrolyte leakage for each sample.

**Supplementary data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

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