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Short Communication

Rice homologs of inducer of *CBF* expression (OsICE) are involved in cold acclimation

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Abstract Cold stress on crops results in severe yield losses through growth retardation and irreversible damage. Recently, Inducer of CBF Expression 1 (ICE1) was identified as the master regulator inducing dehydration responsive element binding protein/C-repeat binding factor (DREB/CBF)-type transcriptional factors involved in the signaling of cold and osmotic stress in *Arabidopsis*. To examine whether rice ICE homologs function in cold acclimation via regulation of rice *DREB* homologs in response to cold stress, we assessed a polypeptide epitope containing an ICE-specific motif. Chilling stress on rice seedlings induced two ICE-related proteins with molecular masses of approximately 55 and 40 kDa. These sizes are consistent with those predicted for OsICE1 and OsICE2, respectively. In contrast to the proteins, cold stress had little or no effect on the expression of *OsICE1* and *OsICE2*. Semi-quantitative RT-PCR indicated that both were constantly expressed, but that cold stress sequentially upregulated *OsDREB1B*, *rice heat shock factor A3* (*OsHsfA3*), and *trehalose-6-phosphate phosphatase* (*OsTPP1*). Trehalose treatment enhanced the cold tolerance of seedlings. These results suggest that OsICE homologs function in transcriptional regulation at upstream of a cold-stress-induced transcription factor cascade involving *OsDREB1B* and *OsHsfA3*, leading to cold acclimation, possibly involving trehalose synthesis.

Key words: Cold stress, DREB, ICE, rice, trehalose.

Cold stress is one of the most important environmental stresses limiting plant growth and crop yield (Hayashi et al. 2009). It induces a set of cold-regulated genes and the synthesis of osmolytes (galactinol and trehalose) and lipid desaturases, leading to the acquisition of cold tolerance (Oono et al. 2006; Phan et al. 2010; Suwabe and Yano 2008). Among cold-stress-induced genes, dehydration-responsive-element–binding protein (DREB)/C-repeat binding factor (CBF) genes appear to encode key transcription factors in the major transcription cascade that responds to cold and drought (Shinozaki and Yamaguchi-Shinozaki 2000; Zhang et al. 2004). Although different sets of transcriptional factors, including *DREB*/*CBF*, *bZIP*, *MYC*, *MYB*, and *Hsf* (*heat shock factor*), appear to be induced in different profiles under drought, salinity, and cold stresses, details of the networks and relationships among them remain unclear. Recent studies of *Arabidopsis* have revealed that a MYC-like basic helix-loop-helix (bHLH) domain, Inducer of CBF Expression 1 (ICE1), enhances the expression of *DREB*/*CBF* genes by binding to their promoter regions (Toledo-Ortiz et al. 2003; Zarka et al. 2003; Zhu et al. 2007). *ICE1* mRNA is expressed at a constant level under various conditions. In contrast, ICE1 protein is complexly regulated by post-translational modification, including phosphorylation (Chinnusamy et al. 2003), ubiquitination (Dong et al. 2006; Miura and Hasegawa 2010), and sumoylation (Miura et al. 2007). Both monocots and dicots possess *ICE*-related genes (Badawi et al. 2008). Interestingly, monocots have two *ICE* homologs encoding closely related proteins with molecular masses of about 40 and 55 kDa. In contrast, dicots have a single *ICE* gene (Wang et al. 2005; Zarka et al. 2003). Several studies in *Arabidopsis* have clarified various post-translational modifications and protein profiles of ectopically expressed ICE1 under cold and salt stress, but there is little information about whether the endogenous ICE1 proteins in rice are regulated in the same way.

The mechanisms involved in the improvement of cold tolerance in rice appear to involve Ca^{2+}-stimulated protein phosphorylation (Martin and Busconi 2001),

Abbreviations: DREB, dehydration-responsive-element–binding protein; HSF, heat shock factor; ICE, Inducer of CBF Expression; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse-transcription polymerase chain reaction; TPP, trehalose-6-phosphate phosphatase

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trehalose synthesis (Pramanik and Imai 2005), and the induction of *DREB/CBF* (Dubouzet et al. 2003; Gutha and Reddy 2008) and *HsfA3* (Liu et al. 2010). ICE homologs could serve as master regulators of *DREB* and *HsfA3*, and thus of cold acclimatization. Here, we report the immunological detection of rice ICE-related proteins and their possible involvement in cold acclimation via the regulation of *OsDREB1B*, *OsHsfA3*, and *trehalose-6-phosphate phosphatase* (*OsTPP1*).

Two-week-old rice (*Oryza sativa* L. cv. Nipponbare) seedlings raised in distilled water at 22°C were incubated at 4 or 42°C or in solution of 0.2 M NaCl and sampled at 0, 1, 3, 8, and 24 h.

Candidates for *ICE* gene homologs in dicots and monocots were identified by BLAST searches with the nucleotide sequence of *Arabidopsis ICE1* in the Rice Annotation Project Data Base (RAP-DB) (http://ftp.staff.or.jp/J/IRGSP/), the Dana-Faber Cancer Institute Plant Gene Index (DFCI) (http://compbio.dfci.harvard.edu/tgi/), and Phytozome v. 6.0 (http://www.phytozome.net/). The deduced amino acid sequences were aligned by CLUSTALW (http://align.genome.jp/), and a phylogenetic tree was built. A set of specific primers for semi-quantitative RT-PCR was designed from *OsICE1*, *OsICE2*, *OsDREB1B*, *OsHsfA3*, and *OsTPP1* (Table 1). Rice *β-tubulin* was used as a positive control. RT-PCR was performed with total RNA from seedlings by using ReverTra Ace reverse transcriptase (TOYOBO, Tokyo, Japan) and GoTaq Green Master Mix (Promega, Tokyo, Japan) according to the manufacturer's manuals with the gene-specific primers shown in Table 1. PCR was performed on a PC-816 thermal cycler (ASTEC Co., Fukuoka, Japan) in a 20 μl reaction mixture containing 1 μl cDNA sample, 200 μM dNTPs, 400 nM F (Forward)-and R (Reverse)-primers, and GoTaq Green Master Mix in PCR reaction buffer under the following thermal cycle conditions: an initial 94°C for 2 min; 25 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s; and a final 72°C for 5 min. The numbers of cycles were 36 for *OsICE1* and *OsICE2*, 30 for *OsDREB1B* and *OsHsfA3*, and 27 for *OsTPP1* and *β-tubulin*. After electrophoresis in 1.5% agarose gels and staining with ethidium bromide (Nang et al. 2008), the amplified products were visualized by FluorChem imager (Alpha Innotech, San Leandro, CA, USA).

To prepare glutathione S-transferase (GST)-fused OsICE1 and OsICE2, we constructed pGEX-*OsICE1* and -*OsICE2*. PCR fragments encoding ΔN-OsICE1 (323–524 aa) and ΔN-OsICE2 (158–381 aa) were amplified with KOD Plus DNA polymerase (TOYOBO, Tokyo, Japan), rice cDNA, and gene-specific primer sets (Table 1), and then digested with *BamH*I and *Sal*I (*OsICE1*) or *BamH*I and *Xho*I (*OsICE2*). The resultant *OsICE1*and *OsICE2* fragments were ligated into *BamH*I-*Sal*I sites of pGEX4T-1 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) by DNA Ligation Kit v. 2 (TaKaRa Bio Inc., Tokyo, Japan). The cloned *OsICE1* and *OsICE2* cDNAs were confirmed by sequencing on an ABI Prism 310 DNA sequencer with a Big Dye Terminator Cycle Sequencing Kit v. 1.1 (Applied Biosystems, Foster City, CA, USA). Recombinant proteins of GST-fused OsICE1 and OsICE2 were induced in *E. coli* in the presence of 0.5 mM IPTG for 2 h at 37°C after growing in LB/Amp medium over night at 37°C.

Protein extract was prepared from 2-week-old stress-treated seedlings (~0.1 g) by homogenization in liquid nitrogen and mixed with 500 μl lysis buffer containing 1×TBS, 10 mM EDTA, 5% glycerol, 0.2% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor), 10 μg ml^{-1} leupeptin (a cysteine protease inhibitor), and 1 mM benzamidine, with or without 1% Triton X-100. The resultant extracts were centrifuged at 10,000×*g* for 5 min at 4°C. Protein concentrations were determined by measuring OD_{595} with a Bio Rad protein assay kit (Bio Rad, Hercules, CA, USA), using 1 mg ml^{-1} bovine serum albumin as a standard.

An anti-ICE peptide-specific polyclonal antibody was raised in a rabbit with a synthetic oligopeptide antigen (H_2N-KMDRASILGDAIKYLKELL-COOH) which appeared to be a highly conserved amino acid motif present in the carboxyl-half region of the bHLH domain

Table 1. Gene specific oligo DNA primers used for RT-PCR and cloning

Gene name	Accession No.	Gene Index	Nucleotide Sequences
OsICE1	AK109915	Os11g0523700	F: ACCGGATCCGGGAAGGGGAAGAAGAAGGGG R: TTTGGTCGACCTCTCCTGTCCTAGATCATG
OsICE2	AK102594	Os01g0928000	F: ATGGGATCCTCGGCCTCGCTGGAGAATGCC R: AAACTCGAGTGCATTGGTCTTGACTGCTTT
OsDREB1B	AF300972	Os09g0522000	F: GCCAACGATGGCGACGAAGAAGAA R: GGAGCCGGCGAGCTCGCCGTCCTC
OsHsfA3	AK101934	Os02g0527300	F: GATGGATCCGAAGCACCAGACGACATCGGG R: TCCCATGGTGCGTTTGATCGACGAATTCAT
OsTPP1	AB120515	Os02g0661100	F: GAGGTTACCAAGTCCCTCTTGCAA R: CTACAGTTATTTCTGCCGGAGTAG
β-tubulin	AK243618	Os03g0105600	F: ACTTCTTCATGGTCGGCTTCGCCCCGCT R: ATCCTCGTATTCACCCTCGTCGTCGGGCGGT

among ICE homologs (Sigma-Aldrich Co., St. Louis, USA) (Figure 1). For immunoblot, polypeptides that had been separated by SDS-PAGE in 10% acrylamide gel were electro-transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) in blotting buffer containing 25 mM Tris-base, 0.05% SDS, and 20% methanol at 10 V cm^{-1} for 2 h. The membranes were then incubated in blocking buffer containing 1×TBS and 2.5% skim milk for 1 h, and then in blocking buffer supplemented with anti-ICE homolog common peptides primary antibody (1/1000 dilution) and 0.05% Tween 20 for 2 h at 4°C. After washing in 1×TBS containing 0.05% Tween 20, the membrane was incubated in blocking buffer supplemented with horseradish peroxidase–labeled antibody (1/5000 dilution, v/v; GE Healthcare Bio-Sciences) for 1 h. Immunoreactive signals were visualized by an ECL Plus kit (GE Healthcare Bio-Sciences) and FluorChem.

To observe any effect of trehalose treatment, we grew rice seedlings on 1.5% agarose medium until they were about 2 cm tall, and then poured trehalose (0.1 M) or distilled water (DW) on the medium. After the seedlings spent 2 weeks at 4°C in a growth chamber, we photographed them to identify any change in color and length.

A phylogenetic tree of ICE homologs in dicots and monocots, using *Arabidopsis* PIF3 and human c-myc as outgroups, showed that *ICE*-related genes can be classified into monocot and dicot subfamilies (Figure 1A). The monocots (all cereals) have two *ICE*-related

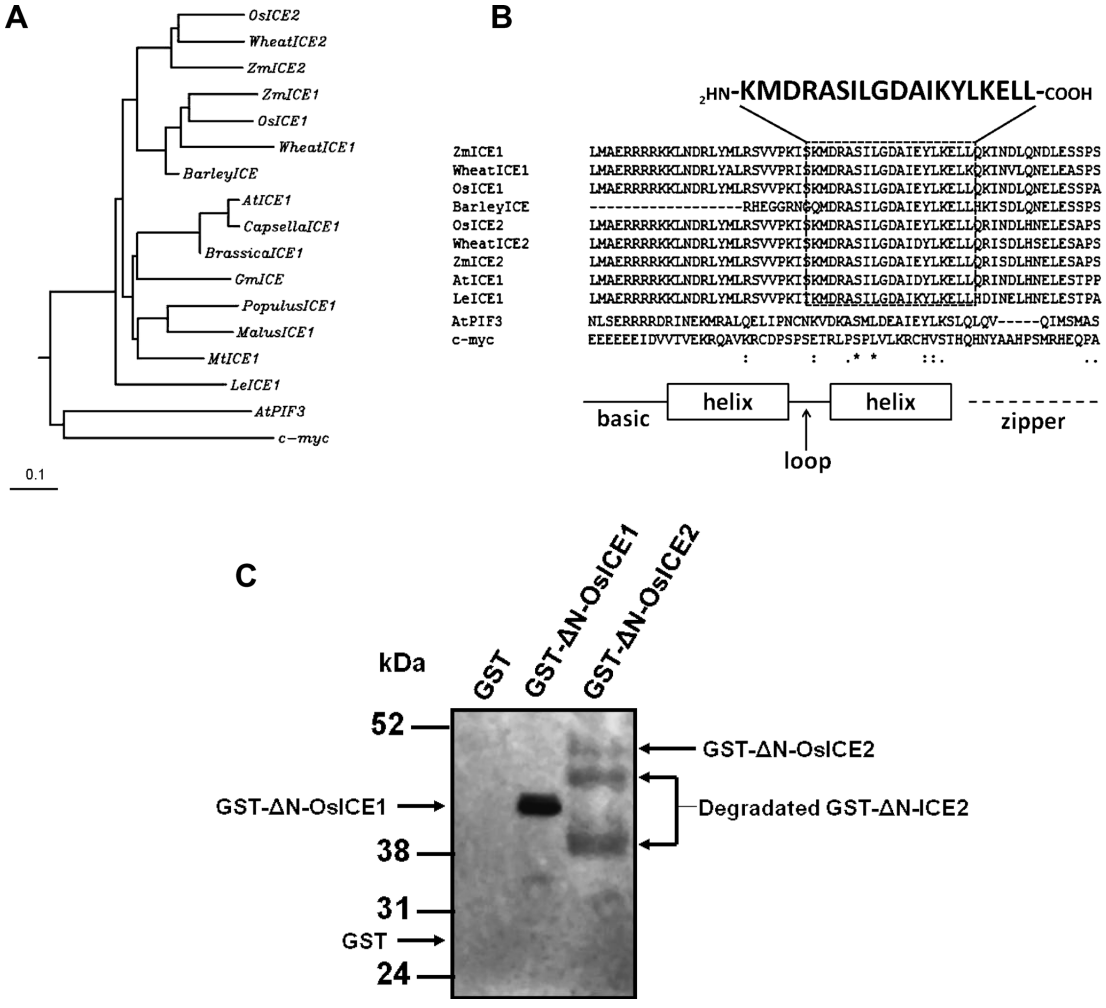

Figure 1. Phylogenic tree of ICE (Inducer of CBF Expression) homologs. (A) ICE homolog sub-families are apparently classified into two groups of monocots (rice, wheat, and maize) and dicots. The monocot ICE sub-family are divided into two clades, ICE1 and ICE2. Plant ICE homologs have weak similarity in the bHLH domain to those of human c-myc and *Arabidopsis* PIF3. (B) Alignments of deduced amino acid sequences of ICE homologs. A highly conserved amino acid motif (KMDRASILGDAIKYLKELL) present in the carboxyl-half region of the bHLH domain was used as an epitope for raising anti-ICE-specific antibody. Os, *Oryza sativa*; Wheat, *Triticum aestivum*; Zm, *Zea mays*; Barley, *Hordeum vulgare*; At, *Arabidopsis thaliana*; Capsella, *Capsella bursa-pastoris*; Brassica, *Brassica napus*; Gm, *Glycine max*; Populus, *Populus trichocarpa*; Malus, *Malus ×domestica*; Mt, *Medicago truncatula*; Le: *Lycopersicon esculentum* (*Solanum lycopersicum*). (C) Anti-ICE specific antibody cross-reacted with recombinant proteins of GST-OsICE1 and OsICE2, but not GST nor endogenous *E.coli* proteins. Immunoblot was conducted with crude extracts (10 mg protein per lane) of *E. coli* containing pGEX4T-1 (GST), pGEX-DN-*OsICE1* (GST-OsICE1) and pGEX-DN-*OsICE2* (GST-OsICE2).

genes encoding polypeptides of about 40 and 55 kDa, whereas the dicots have a single *ICE*-related gene (Figure 1A), as reported by Badawi et al. (2008). OsICE1 and OsICE2 share very similar sets of motifs, notably an acidic domain, a Ser-rich domain, a bHLH domain, and a possible zipper region, with *Arabidopsis* ICE1, although OsICE2 has a shorter junction region between the acidic region and the Ser-rich region in the amino terminus than OsICE1 and Arabidopsis ICE1. Although OsICE1 and OsICE2 have different predicted molecular masses, they share 48% similarity at the amino acid level. They also have similarities of 39% and 45%, respectively, to *Arabidopsis* ICE1. Alignments of the bHLH domains among various ICE-related homologs revealed a highly conserved motif of 19 amino acids (KMDRASILGDAI(D/E)YLKELL) that is specific to ICEs but not to other MYC-like proteins (Figure 1B), as reported by Toledo-Ortiz et al. (2003).

Crude extracts (10 μg protein per lane) of *E.coli* cells expressing recombinant GST-OsICE1 and GST-OsICE2 were subjected to SDS-PAGE (10% acrylamide) and immunoblot with anti-ICE antibody (Figure 1C). Immunoreactive signals were detected with GST-OsICE1 and GST-OsICE2, but not with GST, nor endogenous *E. coli* polypeptides. Anti-ICE antibody also cross-reacted with a recombinant tomato ICE1 and with endogenous ICE1 polypeptides in tomato and *Arabidopsis* (Yuasa, in preparation). Those results indicate that the antibody specifically cross-reacted with ICE-related protein in higher plants.

To examine expression profiles of *OsICE1, OsICE2* and cold stress-inducible genes, semi-quantitative RT-PCR was conducted. The expression of both *OsICE1* and *OsICE2* remained constant (Figure 2). In contrast, *OsDREB1B* was greatly induced between 1 and 8 h (Figure 2). This observation suggests that cold stress has a marginal effect on the expression of *OsICE1* and *OsICE2*, but it induced *OsDREB1B* as expected. *Arabidopsis ICE1* appeared to be induced at both the mRNA and protein levels under cold stress (Chinnusamy et al. 2010). Thus, the transcription of *ICE* homologs could be regulated differently between rice and *Arabidopsis*.

Expression profiles of rice *HsfA3* homologs under cold and heat stresses were examined. *Arabidopsis HsfA3* is regulated downstream of DREB cascades and is induced in response to heat stress (Sakuma et al. 2006). In contrast, *OsHsfA3* is induced under cold stress (Figure 2). Yet despite this difference, OsHsfA3 has 31% similarity at the amino acid level to *Arabidopsis* HsfA3 (Baniwal et al. 2004; Liu et al. 2010). Expression profiles of *OsDREB1B*, *OsHsfA3*, and *OsTPP1* were compared using rice seedlings which were subjected to cold stress. *OsHsfA3* mRNA increased greatly from 3 to 8 h (Figure 2). *OsTPP1*, which has been reported as a

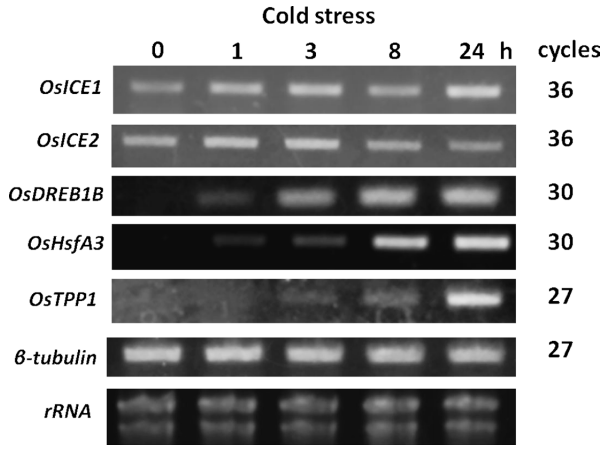

Figure 2. Cold stress induced cold-related genes in rice seedlings. Expression of *OsICE*-homolog and cold stress-related genes was analyzed by semi-quantitative RT-PCR. Two-week-old rice seedlings grown at 22°C were subjected to cold stress (4°C) and harvested at the indicated times for preparation of total RNA.

cold-inducible gene involved in cold acclimation (Pramanik and Imai 2005), increased from 8 to 24 h. While expression of *OsICE1* and *OsICE2* remained constant, the induction of *OsDREB1B*, *OsHsfA3*, and *OsTPP1* began at 3, 8, and 24 h, respectively, in an apparently sequential manner after cold stress treatment (Figure 2). In contrast to cold stress, heat had little or no effect on the expression of *OsDREB1B* or *OsHsfA3* (data not shown). This result suggests that rice *HsfA3* is regulated differently from *Arabidopsis HsfA3* and other *Hsf* genes.

Immunoblot was conducted to determine whether the anti-ICE antibody cross-reacted specifically with endogenous ICE-related polypeptides in rice seedlings subjected to cold, heat, or salt stress (Figure 3). Two immunoreactive signals with molecular masses of about 40 and 55 kDa appeared after cold stress, weakly at 1 h and strongly at 3 h, when protein extracts were prepared with a lysis buffer containing Triton X-100 (Figure 3A, right), but only the 40-kDa signal appeared (again weakly at 1 h and strongly at 3 h) in the absence of Triton X-100 (Figure 3A, left). In contrast to cold stress, heat stress had no effect on immunoreactive signals (Figure 3A, right). It can be assumed that the 55 and 40 kDa polypeptides were derived from the proteins coded by *OsICE1* and *OsICE2*, respectively. Both proteins appeared to be induced by cold stress even though their mRNAs did not. The induction of the endogenous OsICE1 protein (55 kDa) under cold stress is consistent with previous reports that cold stress upregulated an epitope-tagged *Arabidopsis* ICE1 (Dong et al. 2006; Miura et al. 2007). We suspect that the difference in immunoreactive signals of OsICE1 between the presence and absence of Triton X-100 in the lysis buffer depends on its hydrophobicity or nuclear localization status.

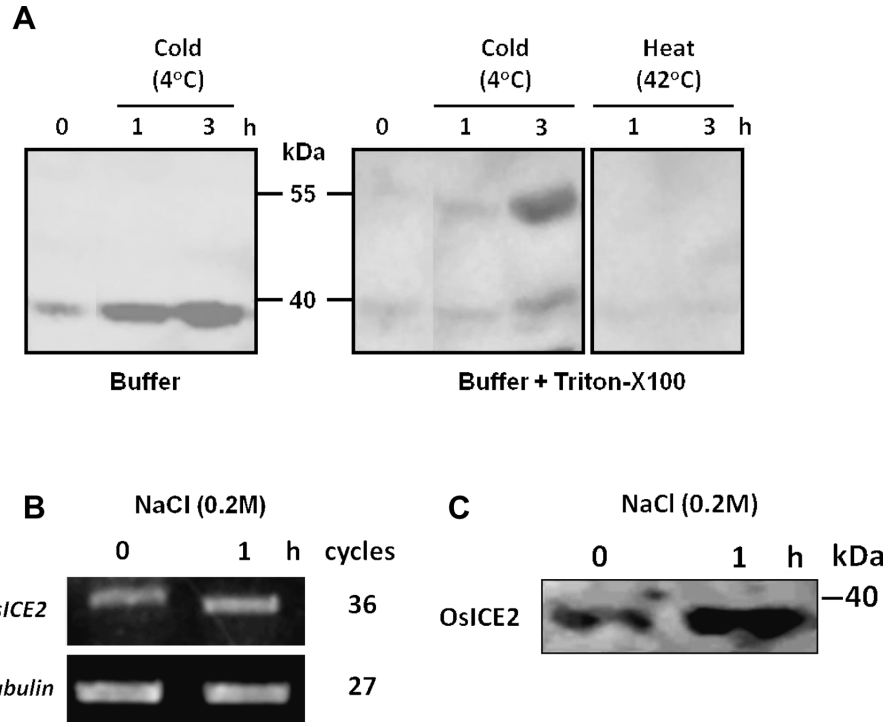

Figure 3. Cold and salt stresses upregulated rice ICE homologs at the protein level. (A) ICE-related polypeptides in rice were detected by immunoblot with the anti-ICE specific antibody. The predicted molecular masses of OsICE1 and OsICE2 are 57.6 kDa and 39.5 kDa, respectively. Proteins were extracted with lysis buffer (B) Expression of *OsICE*-homolog was analyzed by semi-quantitative RT-PCR. Two-week-old rice seedlings grown in distilled water 22°C were subjected to salt stress (0.2 M NaCl) and harvested at the indicated times for preparation of total RNA. (C) Immunoblot of protein extracts (prepared by lysis buffer without Triton X-100) from 0.2 M NaCl treatment.

Thus, we presume that OsICE1 is a membrane associated transcriptional factor, as like NTL6 and NTL8 (Seo et al. 2008) or tightly associates to nuclear matrix.

In addition to cold stress, salt stress enhances *DREB*/*CBF* transcription via induction of ICE1 protein in *Arabidopsis* (Chinnusamy et al. 2003). Salt stress upregulated levels of OsICE2 protein (Figure 3C) but had no effect on *OsICE2* mRNA levels (Figure 3B). These data suggest that rice ICE homologs are induced at the protein level in response to cold and salt stresses, but not to heat stress.

It has been reported that the accumulation of trehalose enhances cold acclimation in rice (Pramanik and Imai 2005), according to induction of *OsTPP1* under cold stress (Figure 2). Since cold-stressed plants incubated at normal temperature in the light showed irreversible breakdown of chlorophyll due to damage to photosystem I in chloroplasts (Kudoh and Sonoike 2002), we evaluated the effect of trehalose treatment on cold-induced damage in cold-stressed seedlings subsequently incubated at 25°C for 5 days under continuous light. The whole culms in the control treatment bleached from green to yellow (Figure 4A, left), whereas the lower parts of the culms in 0.1 M trehalose treatment stayed green (Figure 4A, right). In particular, a significant difference of the chlorophyll colors was observed in the outermost part of the sheaths within 2 cm of the base (Figure 4B). In contrast to the leaf color, the leaf and stem length showed little or no difference between treatments (Figure 4C). Because trehalose appeared to improve cold tolerance of rice, it is reasonable to assume that the cold-induced transcriptional factors discussed above are implicated in cold acclimation *via* trehalose synthesis, which results from the induction of *OsTPP1*.

Our present data with immunoblot and RT-PCR revealed that cold stress increased the levels of OsICE1 and OsICE2 proteins but did not enhance the expression of their genes. These results suggest that OsICE1 and OsICE2 are regulated mainly by post-translational mechanisms, in a manner similar to that of *Arabidopsis* ICE1. Furthermore, the induction of the proteins under cold stress was followed by the sequential upregulation of *OsDREB1B*, *OsHsfA3*, and *OsTPP1*. Originally, *Arabidopsis* ICE1 was identified as being responsible for the induction of *DREB*/*CBF* in response to cold stress and to bind specifically to *cis*-elements in the promoter region of *DREB*/*CBF* (Chinnusamy et al. 2003; Zarka et al. 2003). Thus, it is reasonable to assume that the rice ICE homologs induce rice *DREB*/*CBF* and a set of genes related to cold acclimation, on account of the similarity of the biochemical properties between *Arabidopsis* ICE1 and the OsICEs and the expression profiles of the cold-inducible genes *OsDREB1B*, *OsHsfA3*, and *OsTPP1* (Figure 5). Increasing numbers of molecular biological

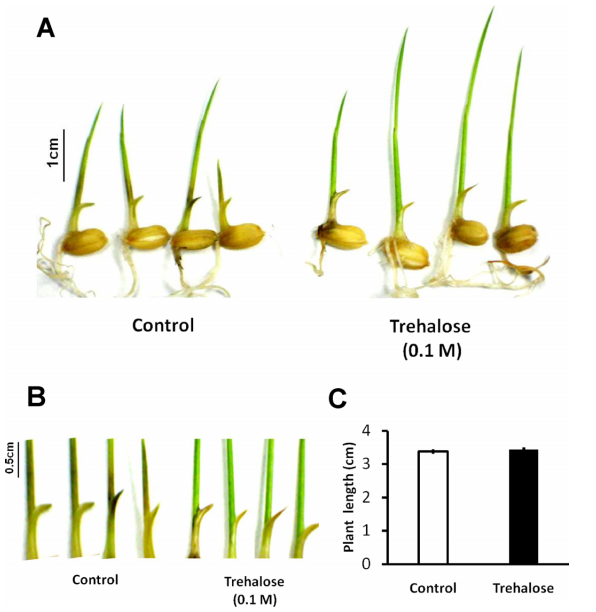

Figure 4. Effect of trehalose treatment on damage of rice under cold stress. (A) Rice seedlings were treated with distilled water (control) or 0.1 M trehalose. (B) Enlarged photographs of lower sheaths of rice seedlings. (C) Average plant length (n=4). Control, distilled water; Trehalose, 0.1 M trehalose solution.

and biochemical studies of *Arabidopsis* ICE1 indicate that E3 ligases, HOS1-dependent ubiquitination, and SIZ1-dependent sumoylation play pivotal roles in the degradation and regulation of ICE1 proteins in response to cold stress (Miura et al. 2007). The rice SIZ1 homologs, OsSIZ1 and OsSIZ2, have sumoylation activity, but it remains unclear whether they are involved in the regulation of OsICE proteins (Park et al. 2010). Although phosphorylation of a Ser-rich region in ICE1 is also involved in cold stress signaling, protein kinases or secondary messengers connecting the two events are yet to be clarified. Several lines of evidence have revealed that cold stress enhances expression of genes for Ca^{2+}-dependent protein kinases (OsCDPKs) in rice (Wan et al. 2007) and enzymatic activities of CDPK in rice (Martin and Busconi 2001), AtMPK4 in Arabidopsis (Ichimura et al. 2000), SAPK in rice (Kobayashi et al. 2005) and SlSnRK2C in tomato (Yuasa et al. 2007). Thus, it is reasonable to assume that the cold-stress-induced protein kinases are involved in phosphorylation of rice ICE proteins, leading to cold-inducible gene expression downstream through the regulation of ubiquitination or sumoylation of the ICEs. Arabidopsis DREB2A is involved in the expression of *HsfA3* under heat stress (Sakuma et al. 2006; Schramm et al. 2008). In contrast, rice *HsfA3* is induced under cold stress (Figure 2) but not under heat stress, as described by Liu et al. (2010). Thus, rice *HsfA3* may be regulated by rice DREB/CBFs that is induced under cold stress, if the promoter region of the rice *HsfA3* has C-repeat-like motif in its *cis*-elements that can interact with rice DREB/CBFs (Figure 5).

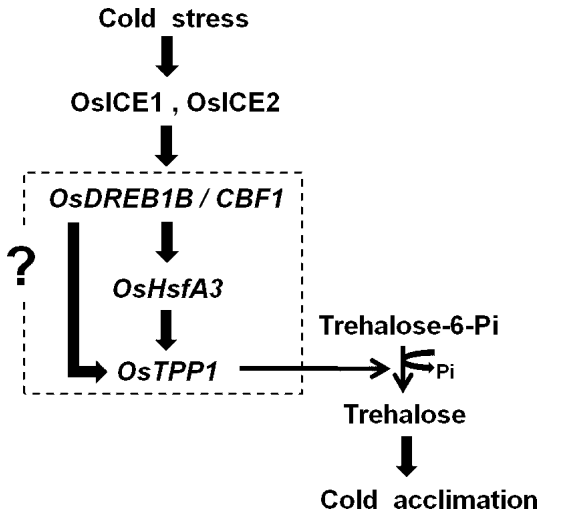

Figure 5. Schematic representation of proposed cold acclimation mechanism in rice seedlings. The cold stress is transduced by inducing a signal cascade composing of ICE, DREB/CBF and HsfA3. The ICE–*DREB*/*CBF*–*Hsf* cascade possibly controls expression of OsTPP1, which converts trehalose-6-phosphate (trehalose-6-Pi) to trehalose. "?": Question mark is an unknown pathway in rice seedlings.

It still remains uncertain whether OsDREB1B is involved directly or indirectly in the induction of *OsHsfA3* or *OsTPP1*, leading to cold acclimation via trehalose synthesis. To investigate this issue, it will be necessary to analyze the interactions between OsICE proteins and *cis*-element of *OsDREB1B* and to investigate a potent transcriptional cascade composing of *OsDREB1B*, *OsHsfA3*, *OsTPP1* by gel-shift assays and chromatin-immunoprecipitation assays and in transgenic rice plants.

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