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 Ca²⁺-stimulated protein phosphorylation (Martin and Busconi 2001),...
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/IRGSP/), the Dana-Faber Cancer Institute Plant Gene Index (DFCI) (http://compbio.dfci.harvard.edu/cgi-bin/), 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 ∆N-OsICE1 (323–524 aa) and ∆N-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 BamHI and SalI (OsICE1) or BamHI and XhoI (OsICE2). The resultant OsICE1 and OsICE2 fragments were ligated into BamHI-SalI 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 OD₅₉₅ 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 (H₂N-KMDRASILDAIKYLKELL-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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession No.</th>
<th>Gene Index</th>
<th>Nucleotide Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsICE1</td>
<td>AK109915</td>
<td>Os1g0523700</td>
<td>F: ACCCGATCCGGGAAAGGGGAAGAAGAAGGGGG</td>
</tr>
<tr>
<td>OsICE2</td>
<td>AK102594</td>
<td>Os01g0928000</td>
<td>R: TTTGTCGACCTCTCGTCTGCTGATACATG</td>
</tr>
<tr>
<td>OsDREB1B</td>
<td>AF300972</td>
<td>Os09g0522000</td>
<td>R: ATCCTCGTATTCACCCTCGTCGTCGGGCGGT</td>
</tr>
<tr>
<td>OsHsfA3</td>
<td>AK101934</td>
<td>Os02g0527300</td>
<td>R: ATCCTCGTATTCACCCTCGTCGTCGGGCGGT</td>
</tr>
<tr>
<td>OsTPP1</td>
<td>AB120515</td>
<td>Os02g0661100</td>
<td>F: GACGTTCAAAATCCATCTCGTGGACATG</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>AK243618</td>
<td>Os03g0105600</td>
<td>R: CTGTTTCATGGGCGGGCCTCCTGGGGCCTCCTGGGG</td>
</tr>
</tbody>
</table>

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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
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 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.
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
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 Ca2+-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 SISnRK2C 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-element that can interact with rice DREB/CBFs (Figure 5).

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**References**


negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proc Natl Acad Sci USA* 103: 8281–8286


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