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Characterization of a Cold-Responsive Gene, *OsPTR1*, Isolated from the Screening of β-*Glucuronidase* (*GUS*) Gene-Trapped Rice

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T-DNA gene trap populations have been successfully used to isolate stress responsive genes by probing GUS expression after stress treatment. Here, we have identified cold responsive GUS expression in rice from Line 1C-118-30, where T-DNA was inserted at the 3rd exon of *OsPTR1*. This gene encodes a putative peptide transporter sharing 40% homology with *Arabidopsis* AtPTR2 and barley HvPTR. Northern analysis of *OsPTR1* confirmed that *OsPTR1* is inducible by cold stress, especially in the shoot but not in the root, thereby indicating an organ-specific response. Transcript was also induced by salt, and water stresses. Interestingly, *OsPTR1* mRNA was highly expressed in the *UBI::CBF1/DREB1b* rice, indicating that *OsPTR1* may be regulated by the CBF/DREB stress signaling pathway.

Keywords: cold, gene trap, peptide transporter, rice, stress

Low temperature is a major abiotic stress that limits plant growth and development and crop productivity. Although some species are able to increase their tolerance via cold acclimation (Levitt, 1980; Sakai and Larcher, 1987), others, including rice, tomato, maize, and cucumber, are sensitive to chilling and do not adapt naturally to freezing (de los Reyes and McGrath, 2003). Plants that are able to acclimate to the cold have a mechanism that accompanies not only physiological changes but also much of the biochemical and molecular alterations through modifications in their gene expression (Thomashow, 1999). Although some cold-responsive genes seem to be induced only by low temperature, most are also regulated by abscisic acid (ABA) or water stress (Hughes and Dunn, 1996).

The cellular mechanisms that perceive temperature fluctuations as well as the processes of signal transduction are poorly understood. Expression of stress responsive genes is thought to be regulated by complex signaling, through either an ABA-dependent or an ABA-independent pathway (Ishitani et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). Genetic and biochemical approaches are beginning to be used to elucidate the signaling pathways responsible for activating such genes. For instance, a group of transcription factors belonging to the AP2/EREBP family has been extensively studied (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998). When CBF1/DREB1B is over-expressed, *COR* gene expression can be induced, resulting in increased tolerance to freezing, salt, or drought (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). Mutagenesis of transgenic *Arabidopsis* that expresses the firefly luciferase gene (*LUC*), under control of the *RD29A* promoter, has demonstrated various levels of response by LUC expression mutants (*cos*, *hos*, *los*) to stress and ABA treatment (Ishitani et al., 1997). For example, the *hos1* mutant shows enhanced expression of cold-responsive genes as well as *CBF* genes under cold treatment, indicating that HOS1 is a negative regulator of cold stress signaling (Ishitani et al., 1998; Lee et al., 2001). Expression of *CBF* and the CBF-regulated genes also decreases in the *ice1* mutant, where *ICE1* encodes a MYC-like bHLH transcriptional activator (Chinnusamy et al., 2003).

T-DNA insertional mutagenesis has been established in rice to discover functional genes, using a promoterless *GUS* reporter gene under alternative donor/acceptor sites for splicing (Lee et al., 1999; Jeon et al., 2000; Jeon and An, 2001). When T-DNA is inserted in a suitable orientation, *GUS* and the endogenous plant gene are fused, resulting in a fused transcript. The advantage of the reporter gene trap is that its expression may reflect the time and location of endogenous gene expression. Moreover, the gene trap itself may cause a mutant phenotype that reflects

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the function of the corresponding gene. T-DNA and the Ac/Ds transposon-tagging system have been successfully used to identify genes involved in plant development (reviewed in Sundaresan, 1995; Springer, 2000; Jeong et al., 2002) as well as those regulated by stress (Baxter-Burrell et al., 2003; Lee et al., 2004b).

In this study, we isolated a cold-responsive GUS-positive line from a T-DNA tagged population of rice. The T-DNA flanking sequence was identified by inverse PCR and the tagged gene was characterized in detail.

MATERIALS AND METHODS

Plant Material and Stress Treatments

Rice (*Oryza sativa* cv. Hwayoung) seeds were surface-sterilized, germinated in a growth chamber at 30°C under 16 h of dim illumination, then transplanted to soil. For the mutants, seeds were sown under sterile conditions on MS agar media (Murashige and Skoog, 1962) and then the seedlings were transferred to soil.

To induce various stresses, leaf-blade segments were floated in MS liquid at 5°C (cold treatment), in MS liquid with 100 μM ABA, or in MS liquid containing 300 mM NaCl in the dark. For the drought stress, leaf-blade segments were air-dried to achieve a 30% loss of fresh weight. After each treatment, the tissues were either prepared for *in situ* staining for GUS analysis or frozen directly in liquid N_2 and stored at -70°C.

Screening of T-DNA Tagging Lines for Cold Stress

Forward genetic screening of the T-DNA tagging population (Jeon et al., 2000) followed the procedure of Lee et al. (2004b). Leaves and stems of T-DNA-transformed T_0 plants, grown in a rice paddy field, were cut into 1-cm-long segments, then floated in MS liquid and incubated for 12 h at 5°C. After incubation, the segments were stained with 2.4 mM 5-bromo-4-chloro-3-indoll β-D-glucuronic acid (X-gluc) in GUS staining buffer (100 mM sodium phosphate pH 7.0, 5 mM potassium ferricyanide, 5 mM potassium ferricyanide, 0.5% Triton-X100, 10 mM EDTA pH 8.0, 0.1% X-gluc, 20% MeOH, and 2% DMSO). These samples were then serially decolorized with 70% EtOH and 95% EtOH to remove the chlorophyll. The GUS assay was carried out in a 48-well microtiter plate, according to the method of Dai et al. (1996).

Identification of T-DNA Insertion Sites by Inverse PCR (IPCR)

Inverse PCR was performed as described previously (Triglia et al., 1988; Lee et al., 2004b). Genomic DNA was extracted from mature leaves according to the technique of Chen and Ronald (1999) and digested with *Pst*I. The reaction was stopped by ethanol precipitation, and the DNA was re-suspended in 44.7 μl of water. The DNA solution was ligated in 50 μl final volume using T4 DNA ligase. PCR was performed with 25 μl of a mixture containing 20 ng of plant DNA, 10X ExTaq buffer, 0.25 mM dNTP, 0.5 units of ExTaq polymerase (Takara, Japan), and 1 μM of the primers. Primers for the nested PCR were as follows. Forward primers located at *hygromycin phosphotranferase* gene: 1st, 5′-CCATGTAGTGTATTGACCGATTC-3′; and 2nd, 5′-TCGTCTGGCTAAGATCGGCCGCA-3′. Reverse primers located at RB: 1st, 5′-TTGGGGTTTCTACAGGACGTAA-3′; and 2nd, 5′-CAAGTTAGTCATGTAATTAGCCAC-3′.

The first PCR was performed with an initial 5 min denaturation at 94°C, followed by 35 cycles of 95°C (1 min), 54°C (1 min), and 72°C (2 min), ending with 72°C for 7 min. The second PCR was conducted with an initial 5 min denaturation at 94°C, followed by 35 cycles of 95°C (30 s), 55°C (1 min), and 72°C (2 min), ending with 72°C for 7 min. PCR products were directly sequenced using the second reverse primer. The genomic sequence containing the tagging sequence was retrieved from the Chinese Rice Genome Database (Rice GD, http://btn.genomics.org.cn/rice; Yu et al., 2002), and was annotated using a rice genome automated annotation system developed in the Rice Genome Program (RGP; http://ricegaas.dna.affrc.go.jp).

RT-PCR Analysis

cDNA templates were synthesized using total RNA isolated from the cold-treated seedlings plus Moloney murine leukemia virus reverse transcriptase (New England Biolabs, USA). The cDNA was amplified by PCR in 25 μl of a mixture comprising 250 ng of cDNA, 10X ExTaq buffer, 0.25 mM dNTP, 0.5 units of ExTaq polymerase, and 1 μM of the gene-specific primers. RT-PCR conditions included an initial 5 min denaturation at 94°C, followed by 31 cycles (for leaf tissue) or 25 cycles (stems) of PCR (94°C, 1 min; 62°C, 1 min; 72°C, 1 min 30 s), and a final 10 min at 72°C. The reaction mixtures were separated on an

agarose gel and stained with EtBr. Primers included: OsPTR1-forward, 5'-ATGGCGTACTACGGGATAGC-3'; and OsPTR1-reverse, 5'-GGAGTCCATCCTGAA-CTTGC-3'.

Southern and Northern Blot Analysis

Genomic DNA was extracted from our rice seedlings according to the cetyltrimethylammonium bromide method (Rogers and Bendich, 1988; Lee et al., 1999). Five μg of genomic DNA was digested overnight with *Hin*dIII, *Pst*I, and *Sac*I at 37°C, then separated on a 0.7% agarose gel, and transferred with a TransVac (Hoefer, USA) to a Hybond-N membrane (Amersham, USA). The blots were probed with the [α-^{32}P] dCTP (3000 Ci/mmole)-labeled PCR product of the *GUS* gene, following the random priming method. Total RNAs were isolated from seedlings using Tri-Reagent (MRC, USA). Twenty μg of RNA were fractionated on a 1.3% formamide gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled *OsPTR1* probe. Unincorporated nucleotides were removed via G-50 Sephadex column chromatography. After hybridization, the membrane was washed with 2X SSC, 0.1% SDS at RT for 15 min; 1X SSC, 0.1% SDS at RT for 15 min; and 0.1X SSC, 0.1% SDS at RT for 15 min. Hybridization signals were detected with an image analyzer (BAS-1500, Fuji, Japan) and exposed on Hyperfilm™ MP film (Amersham, USA).

RESULTS

Identification of a Cold-Inducible Line and T-DNA Flanking Sequence Analysis

Screening of the GUS-trapped rice resulted in the identification of Line 1C-118-30, which showed cold-inducible GUS expression (data not shown). The T-DNA flanking sequence of this line was determined by IPCR, using genomic DNA as a PCR template. Sequence analysis demonstrated that the line has a T-DNA insertion in an open reading frame. The deduced amino acid sequence shares 40% homology with the peptide transporter (PTR) from *Arabidopsis* (AtPTR2) and barley (HvPTR1), as well as 20% homology with yeast PTR2p (Song et al., 1996; West et al., 1998). This sequence also shows 33% identity to a rice nitrate transporter, OsNRT1 (Lin et al., 2000), at the amino acid level. Based on homology and the presence of conserved motifs in the putative protein, the gene was named *OsPTR1*. Using Rice GD, a full-length genomic sequence for *OsPTR1* was obtained (contig 15798), and its ORF was deduced with the Rice Genome Annotation system and NCBI BlastP. The primary structure of *OsPTR1* consists of three exons, with the T-DNA being inserted at the 3rd exon (Fig. 1). The *GUS* gene in the T-DNA was in the same orientation as *OsPTR1*. The 3' untranslated region of *OsPTR1* was identified in DDBJ rice EST database (BM419799).

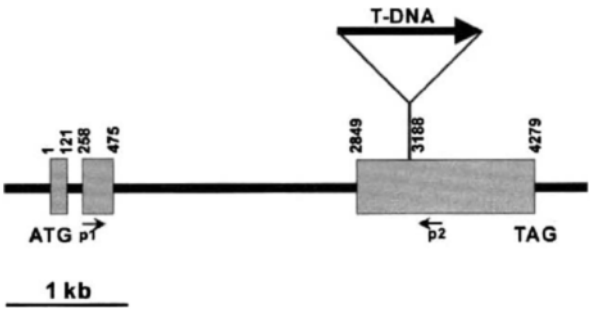

Figure 1. Schematic diagram of *OsPTR1* and position of T-DNA. Filled boxes represent exons; lines between boxes are introns. ATG start codon and TAG stop codons are indicated. Small arrows represent gene-specific primers, p1 and p2, for RT-PCR analysis. Direction of T-DNA, not drawn to scale, is indicated as large arrow.

A full-length cDNA of 1.9 kb was isolated by RT-PCR, and *OsPTR1* encodes a deduced protein of 589 amino acid residues with a molecular mass of 64.8 kD (Fig. 2A). The hydropathy analysis of OsPTR1 predicted that the protein contains 11 putative transmembrane domains (Fig. 2B), which are frequently found in the PTR proteins (Stacey et al., 2002). As shown in Fig. 3, the conserved motifs of the PTR family, ExCERFxYYG, a FING motif, and WQIPQY, are also found here (Steiner et al., 1995; Hauser et al., 2001).

Expression Pattern of *OsPTR1* Gene

Expression patterns for the *OsPTR1* gene were studied by RNA blot, using samples from the calli, seedlings, mature plants, panicles, and developing seeds of wild-type plants. Transcript was detected in the seedling shoots, and was also weakly detected in the flag leaf sheaths, last nodes, and the developing seeds (Fig. 4). This suggests that the gene is expressed in an organ-specific manner in rice.

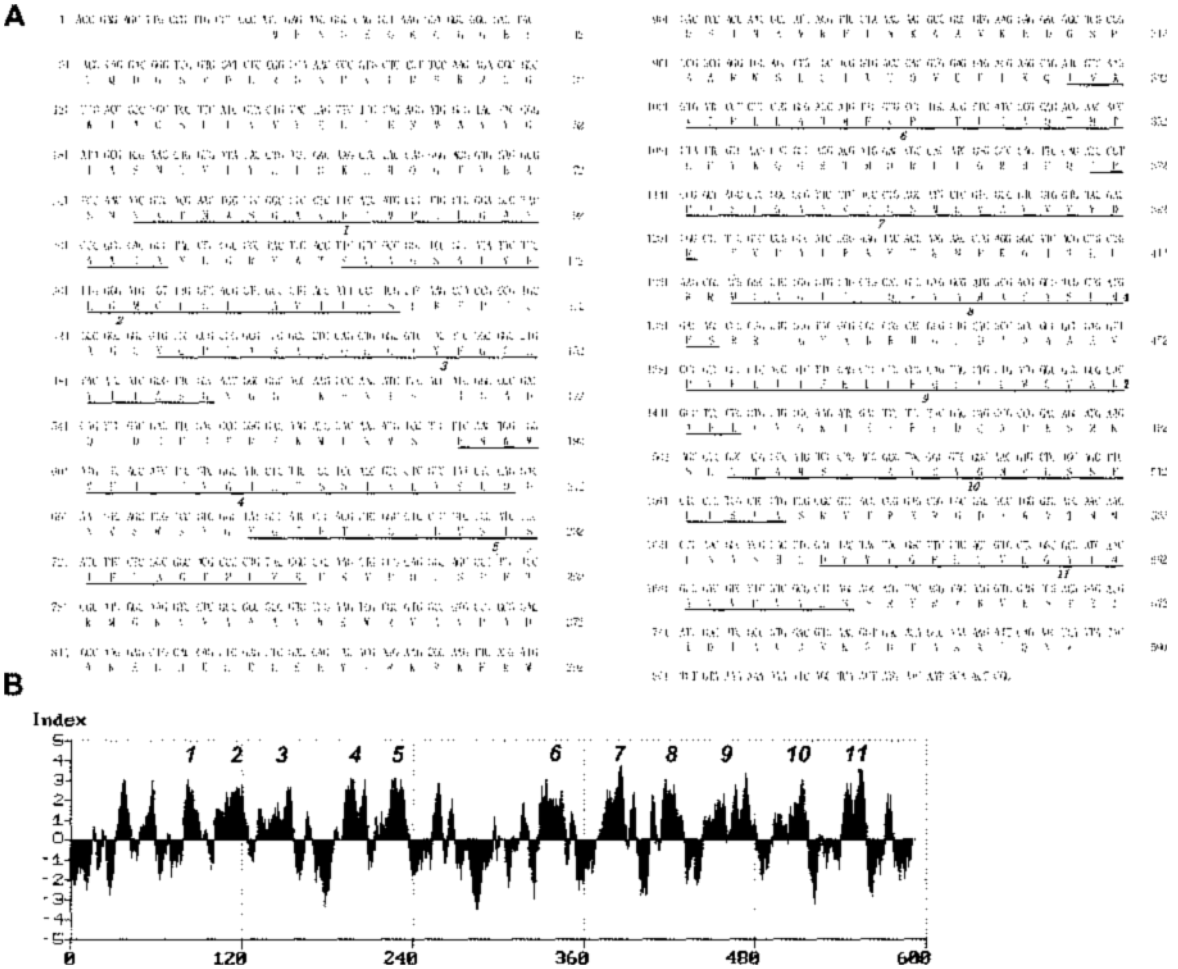

Figure 2. Sequence of *OsPTR1* cDNA and hydropathy profile of OsPTR1. **A**, Nucleotide sequence of *OsPTR1* cDNA and deduced amino acid sequence of OsPTR1 protein. Putative transmembrane regions are underlined and numbered. **B**, Hydropathy profile of OsPTR1 protein was determined by the method of Kyte and Doolittle (1982), using a window of 19 amino acid residues. Putative transmembrane helices, identified using the SOSUI program, are indicated in bold italics.

Effects of Cold, Salt, Water, and ABA Stresses on Expression of *OsPTR1*

We used RT-PCR and northern blot analysis to study the inducibility of the *OsPTR1* gene under various stress conditions (Fig. 5). To do so, gene-specific primers were synthesized from the partial sequence of the 2nd and 3rd exons. PCR products were highly detectable as a result of low-temperature exposure, showing maximum levels in both leaves and stems at 3 h after the stress was imposed (Fig. 5A). Identity of the 741-bp PCR products was confirmed by DNA sequencing. Actin primers served as the control to show that similar amounts of cDNA were used in the reaction. This cold-responsive expression was further examined by RNA blot analysis (Fig. 5B). The size of the hybridizing transcript was about 2.4 kb, indicating that *OsPTR1* cDNA is partial. In the shoots, *OsPTR1* transcripts were most highly accumulated after 3 h of stress, whereas expression was barely detected in the roots at any time point, thereby proving that the cold response was organ-specific. We further investigated whether *OsPTR1* expression was also induced by other types of stress, and found that its transcripts were highly accumulated in the salt- or water-stressed shoots but not in the ABA-treated shoots (Fig. 5C).

Regulation of *OsPTR1* in Transgenic *UBI::CBF1* Rice

CBF genes control the expression of stress-inducible genes that contain the C-repeat/drought response element in their promoters (Taji et al., 2002). Because a CRT/DRE core sequence is located in the putative

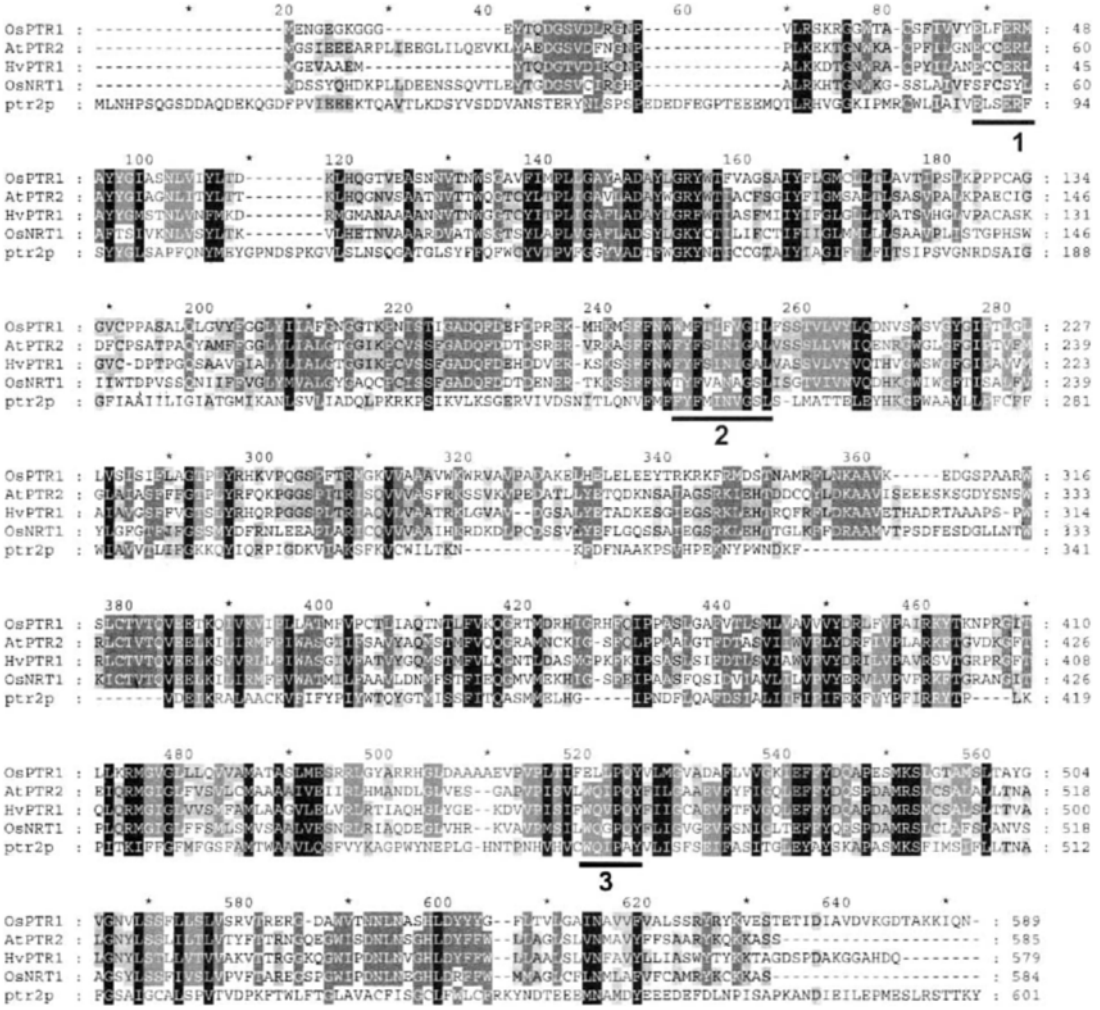

Figure 3. Comparison of OsPTR1 with other PTRs or PTR homolog from *Arabidopsis* AtPTR2 (AAB00858), barley HvPTR1 (T04378), rice OsNRT1 (AAF07875), and yeast PTR2p (NP_013019). Three conserved motifs, ExCERFxYYG (**1**), FING motif (**2**), WQIPQY (**3**), found in PTR family are underlined. Alignment was performed using the ClustalX program with standard settings. White letters in black box indicate 5 out of 5 matches; white letters in gray box indicate 3 or 4 out of 5 matches.

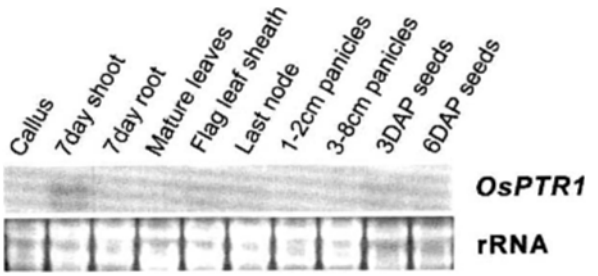

Figure 4. Northern blot analysis of *OsPTR1* expression throughout developmental stages, as isolated from calli, 7-d-old shoots, 7-d-old roots, mature leaves, flag leaf sheaths, highest internodes at pre-heading stage, 1- to 2-cm-long panicles, 3- to 8-cm-long panicles, developing seeds at 3 DAP, and developing seeds at 6 DAP. EtBr-staining rRNA band was used as a control for equal amount of RNA loading.

promoter region of *OsPTR1*, this gene may be controlled by CBF1. To this end, we generated transgenic rice plants that ectopically express *Arabidopsis CBF1* cDNA. RNA gel-blot experiments showed that expression of the *OsPTR1* gene was elevated in those transgenics that strongly expressed the CBF1 transcription factor (Fig. 6).

DISCUSSION

Transporters supply essential nutrients throughout the entire life cycle of the plant. Regulation of transporter production and activity influences plant morphogenesis and development by facilitating coordi-

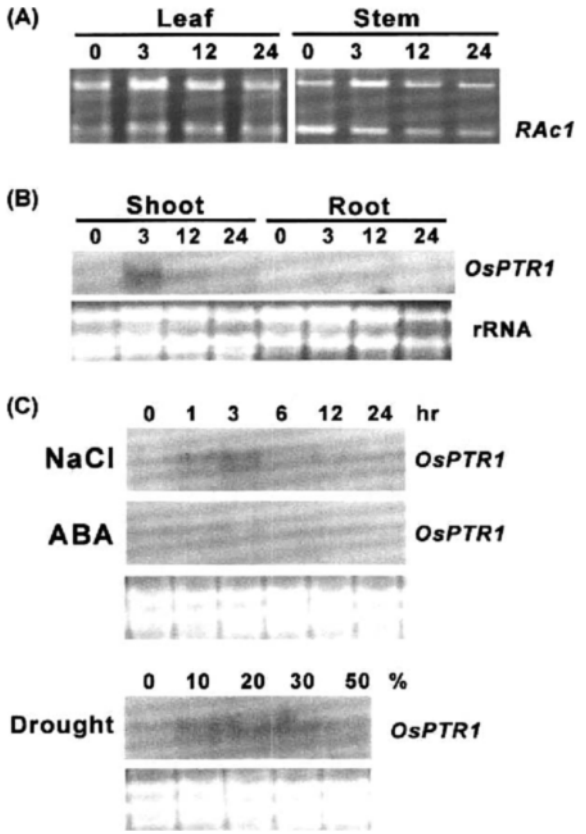

Figure 5. Analysis of *OsPTR1* expression under various abiotic stresses and treatment with ABA. **A**, Semiquantitative RT-PCR analysis of *OsPTR1* after cold stress. RNAs were isolated from plant fragments cold-treated for indicated time. Transcript of rice actin gene *RAc1* served as control for the amount of cDNA used. **B**, Northern analysis of *OsPTR1* after cold stress. RNAs were isolated from seedlings cold-treated for indicated time. **C**, Northern analysis of *OsPTR1* after salt, drought, and ABA stresses. RNAs were isolated from shoot of seedlings cold-treated for indicated time. Each lane was loaded with 30 μg total RNA and membranes were hybridized with [^{32}P]-labeled *OsPTR1* as probe. Ethidium bromide-stained gels are shown at bottom of each figure as a control of equal RNA loadings.

nated exchanges between different cells, tissues, and organs (Delrot et al., 2000). The PTR family is driven by the proton motive force (PMF), transporting di- and tri-peptides as well as single amino acids and nitrate (Williams and Miller, 2001; Stacey et al., 2002). These PTR transporters were first characterized in barley, a plant system where hydrolyzed endosperm proteins are transferred to the embryo via the scutellum (Salmenkallio and Sopanen, 1989). Two *Arabidopsis* PTRs have been cloned (Steiner et al., 1994; Song et al., 1996), and Song et al. (1997) have shown that antisense expression of *AtPTR2-B* delays

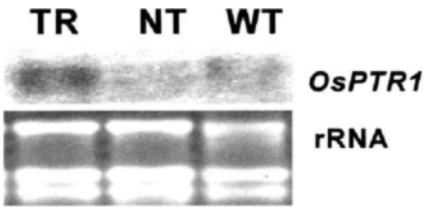

Figure 6. Analysis of *OsPTR1* expression in transgenic rice (TR) expressing *CBF1*. NT, nontransgenic segregant of TR; WT, wild-type. RNA was isolated from transgenic plants grown under normal growing conditions. Ten μg of total RNA was loaded on each lane and membrane was hybridized with [^{32}P]-labeled *OsPTR1* as probe. Ethidium bromide-stained gels are shown at bottom of each figure as a control of equal RNA loadings.

flowering and arrests seed development in *Arabidopsis*.

Although it was known that PTR is related to plant growth and development, the relationship of PTR to abiotic stress had not yet been documented. In this study, we found that *OsPTR1* is responsive to cold as well as to high salt and/or drought conditions. OsPTR1 has three conserved motifs, comprising ExCERFxYYG in the first transmembrane domain, a FING motif (FYxxINxGSL) in the fourth/fifth hydrophobic segment, and WQIPQY in the tenth segment (Steiner et al., 1995; Hauser et al., 2001).

We could not find any mutant pnenotype in the T_2 progenies, although *OsPTR1* knockout has not been retrieved clearly. This may have been due to the functional redundancy of other PTR members. Peptide transporters are encoded by a multigenic family of 51 putative members in the *Arabidopsis* genome (Delrot et al., 2001), while in the rice genome, more than 100 contigs have been retrieved by searching the database.

We estimated the integrated copy number of T-DNA in the genome of Line 1C-118-30 by Southern analysis, using the *GUS* coding region as a probe. Because a few hybridizing bands were detected (data not shown), it was necessary to confirm that the cold-responsive GUS expression was caused by the T-DNA insertion. Three flanking sequences were isolated by IPCR, one encoding *OsPTR1*, another encoding a hypothetical protein, and a third encoding tubulin in the T-DNA vector. In the case of the hypothetical protein gene, T-DNA was inserted at 930 bp upstream of the start ATG codon (data not shown). Via RT-PCR, we determined that the gene encoding a hypothetical protein was not responsive to low temperatures (data not shown). Therefore, we concluded that cold-

responsive GUS expression in this rice line was due to the T-DNA integrated in *OsPTR1*.

We also demonstrated that the expression of *OsPTR1* was regulated by a known stress signaling regulator, CBF1. This regulator was previously reported as a transcription factor related to an ABA-independent stress signaling pathway. CBF1 binds the DRE-core sequence, CCGAC, in the regulatory region of target genes. Under non-stressed conditions, overexpression of *CBF* in transgenic *Arabidopsis* has been shown to cause the overexpression of many stress-inducible genes, e.g., *RD* (responsive to dehydration), *ERD* (early responsive to dehydration), and *COR* (cold regulated) (Kasuga et al., 1999; Gilmour et al., 2000; Seki et al., 2001). Dubouzet et al. (2003) have now characterized rice CBF/DREB1s that are ABA-independent and which can up-regulate some target genes having the DRE-core sequence. In this study, we found a DRE-core sequence present at 1131 bp upstream of the ATG start codon of *OsPTR1*. However, it is still unclear whether that sequence is responsible for CBF1 binding. Some cold-responsive genes, such as *lip5* and *lip9* (Aguan et al., 1991), are also expressed in CBF1 transgenic rice (Lee et al., 2004a). Therefore, these results indicate that CBF1 has a distinct role in regulating specific groups of genes.

It is not well understood how PTR is involved in plant stress responses. To our knowledge, this report is the first to demonstrate that the PTR gene is responsive to cold stress. In *Arabidopsis*, water stress increases the amount of transcripts for two proline transporter genes, *ProT1* and *ProT2*, but decreases transcript levels of the amino acid transporters AAP4 and AAP6 (Rentsch et al., 1996). Whether the increased level of OsPTR1 expression under stress can change the elevated amount of intercellular amino acids remains to be determined.

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