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Ectopic Expression of a Cold-Responsive *OsAsr1* cDNA Gives Enhanced Cold Tolerance in Transgenic Rice Plants

Soo-Jin Kim[1], Sang-Choon Lee[1], Soon Kwan Hong[1,3], Kyungsook An[2], Gynheung An[2], and Seong-Ryong Kim[1,*]

The *OsAsr1* cDNA clone was isolated from a cDNA library prepared from developing seed coats of rice (*Oryza sativa* L.). Low-temperature stress increased mRNA levels of *OsAsr1* in both vegetative and reproductive organs. *In situ* analysis showed that *OsAsr1* transcript was preferentially accumulated in the leaf mesophyll tissues and parenchyma cells of the palea and lemma. For transgenic rice plants that over-expressed full-length *OsAsr1* cDNA in the sense orientation, the Fv/Fm values for photosynthetic efficiency were about 2-fold higher than those of wild type-segregating plants after a 24-h cold treatment. Seedlings exposed to prolonged low temperatures were more tolerant of cold stress, as demonstrated during wilting and regrowth tests. Interestingly, *OsAsr1* was highly expressed in transgenic rice plants expressing the C-repeat/dehyhdration responsive element binding factor 1 (*CBF1*), suggesting the regulation of *OsAsr1* by CBF1. Taken together, we suggest that *OsAsr1* gene play an important role during temperature stress, and that this gene can be used for generating plants with enhanced cold tolerance.

INTRODUCTION

As sessile organisms, plants must cope with environmental stresses, e.g., low temperatures and drought. After such exposures, a series of changes occurs in their patterns of gene expression (Chinnusamy et al., 2007; Guy, 1999), a response that affects growth rates, productivity, and species distribution. Rice, a cereal crop of tropical or subtropical origin, frequently suffers from chilling injury, and plants exhibit various symptoms such as chlorosis, necrosis, or growth retardation (De Datta, 1981). In temperate regions, rice frequently confronts chilling at the seedling stage as well as cool-summer damage during flowering.

To understand the cold-response mechanism, researchers have isolated a number of genes that encode cold-inducible proteins in several plant species (Guy, 1999; Thomashow, 1999). The genes induced under low temperatures are thought to function in protecting cells by producing various gene products. For example, *Arabidopsis* MAP kinases (ATMPK4 and ATMPK6) and C-repeat/DRE binding factor (CBF/DREB) transcription factors have roles in cold-stress signal transduction (Chinnusamy et al., 2007; Mizoguchi et al., 1993; Stockinger et al., 1997), while lipid desaturase is involved in membrane modifications during chilling (Gibson et al., 1994). Cold-induced pathogenesis-related proteins, e.g., chitinase-like and thaumatin-like proteins, function as antifreeze proteins (Hiilovaara-Teijo et al., 1999). Moreover, chaperones, late embryogenesis abundant (LEA) proteins, calmodulin-related proteins, and 14-3-3 proteins might contribute to enhanced freezing tolerance (Thomashow, 1999). Finally, the enzymes required for biosynthesis of various osmoprotectants, such as sugars, prolines, and betaines are also important to a plant's osmotic adjustment after exposure to cold (Igarashi et al., 1997; Kishor et al., 1995; MaNeil et al., 1999).

The *Asr* genes, which are plant-specific and responsive to ABA, osmotic stress, and ripening, have been identified in various species, including tomato, potato, grape, loblolly pine, lily, maize and rice (Cakir et al., 2003; Chang et al., 1996; Iusem et al., 1993; Jeanneau et al., 2002; Silhavy et al., 1995; Vaidyanathan et al., 1999; Wang et al., 1998). High levels of the tomato *Asr1* mRNA can be detected in ripe fruit and in leaves subjected to water stress (Hagit et al., 1995). The *Asr* gene family is widespread in spermatophyta and seems to be subject to concerted evolution in same species (Frankel et al., 2006).

The level of lily ASR proteins is also increased through desiccation during pollen maturation (Wang et al., 1998). The physiological roles of ASR proteins are unclear yet. The localization of tomato ASR1 proteins in nucleus proposed the nature of tomato ASR proteins as nonhistone chromosomal proteins (Rossi and Iusem, 1994). Also, the structural and functional similarity of some ASR proteins with LEA or dehydrin proteins suggests a possible role of ASR in the seed development (Maskin et al., 2001; Silhavy et al., 1995). Many known ASR proteins contain two conserved regions of a putative Zn-binding site at the N-terminal region and a putative nuclear localization sequence (NLS) at the C-terminal region of ~70 amino acids

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[1]Department of Life Science, Sogang University, Seoul 121-742, Korea, [2]National Research Laboratory of Plant Functional Genomics, Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea, [3]Present address: Department of Plant Biotechnology, Kangwon National University, Chuncheon 200-701, Korea
*Correspondence: sungkim@sogang.ac.kr

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(Cakir et al., 2003; Silhavy et al., 1995). These facts as well as the binding of grape ASR (VvMSA) to the promoter sequence of a monosaccharide transporter gene suggested that VvMSA is a component of the transcription-regulating complex involved in sugar and ABA signaling (Cakir et al., 2003). Recently, it has been shown that desiccation induces changes of unfolded form of tomato ASR1 to folded form with presence of zinc (Goldgur et al., 2007). *In vitro* assay confirmed that tomato ASR1 homodimerizes and bind DNA in a zinc-dependent manner (Maskin et al., 2007). Furthermore, tomato ASR1 binds coupling element 1 (CE1) of ABI4 promoter competing with ABI4, which produces ABA insensitive phenotype such as NaCl insensitivity during seed germination (Shkolnik and Bar-Zvi, 2008).

Vaidyanathan et al. (1999) have identified an *ASR* cDNA, *OsAsr1*, from 'Pokkali' rice, where *OsAsr1* was both ABA- and osmotic (NaCl) stress-inducible. In the study presented here, we examined the relationship of the *OsAsr1* gene to cold stress.

MATERIALS AND METHODS

Plant samples and bacterial strains

'Dongjin', a cultivar of japonica rice (*Oryza sativa*), was used for constructing our seed-coat cDNA library and for generating transgenic plants. The other cultivars or wild types tested for cold tolerance are listed in Table 1. Seeds were surface-sterilized and then germinated either in water or on an MS agar medium (Murashige and Skoog, 1962). The seedlings were transferred to soil and kept in a growth chamber at 30°C under continuous light (intensity of 60 to 70 μmol m^{-2}s^{-1}). For the cold treatment, 11-d-old seedlings exposed to 4°C or 12°C in the dark were harvested. For the ABA, 5-d-old seedlings transferred to an MS liquid medium containing 10 μM ABA were harvested. To study the effect of cold stress at the reproductive stage, field-grown rice plants at ca. The 4 days before heading were transferred to the greenhouse (30°C, 14-h light/10-h dark) for 2 d of adapting. Afterward, they were treated for 4 d at 12°C under a 14-h photoperiod (ca. 300 μmol m^{-2}s^{-1}). All tissue samples were immediately frozen with liquid nitrogen and stored at -70°C. The *E. coli* strain, XL-1 Blue MRF' {Δ(mcrA)183Δ (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F′ proAB, lacI^{q}ZΔM15 Tn10 (Tet^r)]} was used as a host for molecular cloning.

Construction of cDNA library and EST analysis

The coats from 4- to 8-d-old seeds were manually dissected under a microscope. A cDNA library was then constructed from poly (A) RNA prepared from the seed coats. cDNA clones were randomly selected from the library, and their 5′ ends were sequenced. The DNA preparation, sequencing, and computer analysis were preformed as described by Hong et al. (1998). Briefly, template DNAs were prepared by the alkaline lysis method, and the inserts were sequenced with an ABI PRISM™ BigDye™ Terminator Cycle Sequencing kit (Amersham). Computer software, such as DNAsis, Prosis (Hitachi), ClustalX, ClustalW, and GeneDoc (Nicholas and Nicholas, 1997; Thompson et al., 1994), were used for our sequence analysis. The Genbank, EMBL, and Swiss-Prot databases were then searched for amino-acid sequence homology using the BLASTX algorithm (Altschul et al., 1997).

DNA and RNA gel-blot analysis

DNA gel-blot analyses were conducted on three japonica cultivars - 'Namyang 21', 'Dongjin', and 'Odae'. Namyang 21 is cold-sensitive (the cold tolerance index, CTI = 7), Odae is cold-tolerant (CTI = 3), and Dongjin is intermediate (CTI = 5) (Lim, 1998). In the CTI value given between 0 to 9, 0 is most tolerant, 9 most sensitive. Genomic DNA was extracted from their seedlings according to the cetyltrimethylammonium bromide method (Roger and Bendich, 1988). Ten micrograms of DNA, digested with restriction enzymes for 6 h at 37°C, was separated on a 0.8% agarose gel, then transferred to a Hybond-N membrane (Amersham) using a vacuum transfer system (Hoefer). For RNA gel-blot analyses, 10 μg of total RNA was resolved on a 1.3% formalehyde agarose gel and blotted onto a nylon membrane. The DNA and RNA blot analyses were performed using the radiolabeled *OsAsr1* probe. To prepare this probe, *OsAsr1* cDNA fragments were radioactively labeled with 〔α-^{32}P〕 dCTP (3000 ci mmol^{-1}), following the random priming method. Unincorporated nucleotides were removed via G-50 Sephadex column chromatography. After hybridization, the membrane was washed with 2× SSC, 0.1% SDS at RT for 15 min; 1× SSC, 0.1% SDS at RT for 15 min; and 0.1× SSC, 0.1% SDS at RT for 15 min. Hybridization signals were detected with an image analyzer (BAS-1500, Fuji) and exposed on Hyperfilm™ MP film (Amersham).

RNA *in situ* hybridization

Rice flowers and leaves were fixed overnight at 4°C in 2% (wt/vol) paraformaldehyde plus 2.5% (vol/vol) glutaraldehyde in a 50-mM PIPES buffer (pH 7.2). The fixed tissues were dehydrated by graded concentrations of ethanol, then embedded in a paraplast medium (Oxford). The embedded tissues were sliced into 7-μm sections with a rotary microtome (Leica), and each section was attached to a silanized glass slide (Matsunami). Paraffin was removed through a graded series of ethanol concentrations, and the samples were dried for 1 h. Dioxygenin-labeled sense or antisense RNA probes were prepared from the linearized pBluscript carrying the *OsAsr1* cDNA, using either T3 or T7 RNA polymerase. The sections were hybridized with the probes at 48°C for 16 h in a hybridization solution, then washed in a solution containing 2× SSC, 1× SSC, and 0.1 × SSC for 15 min at 50°C. The hybridizing probe was detected colorimetrically using an anti-DIG conjugated alkaline phosphatase (Boehringer Mannheim). Photographs were taken under bright-field microscopy (Nikon Eclipse 600).

Production and analysis of transgenic rice plants

Full-length *OsAsr1* cDNA was introduced into the binary vector pGA1611 (An et al., 1988; Kim et al., 2003), in the sense and antisense orientations downstream of the maize *ubiquitin* promoter (Christensen et al., 1992). Rice transformation was performed via the *Agrobacterium*-mediated cocultivation method (Jeon et al.,1999; Lee et al.,1999). All transgenic rice plants were generated on a 40-mg L^{-1} hygromycin B-containing medium, and were transferred to the greenhouse after regeneration. PCR analysis to verify those transgenic plants was performed using the forward primer (5′-CAC CCT GTT GTT TGG TG-3′) and the reverse primer (5′-GCG GGA CTC TAA TCA TAA AAA CC-3′). PCR conditions included 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, through 30 cycles.

Determination of chlorophyll fluorescence

About 5-cm-long segments of the extended leaves from mature plants were floated on MS liquid media at 4°C under white fluorescent light (260 μmol m^{-2}s^{-1}) for 0, 6, 12, or 24 h. After 30 min of dark-adaptation, chlorophyll fluorescence signals were measured with a Plant Efficiency Analyzer (Hansatech). All experiments were repeated four times.

Determination of cold stress tolerance

Rates of survival were calculated for plants exposed to cold stress by assessing either their degree of wilting (Saijo et al., 2000) or the amount of their regrowth (Lee et al., 1993). Ten day-old seedlings (at the three-leaf stage), that had been raised in a growth chamber (16-h light/8-h dark; light intensity of 60 μmol $m^{-2}s^{-1}$; 30°C), were exposed to 4°C for 3, 4, 5, 6, 7, 10, or 12 d under continuous light (60 μmol $m^{-2}s^{-1}$). Afterward, they were returned to standard growth-chamber conditions for 10 d to allow for recovery. The wilting ratio was based on the level of prominent chlorosis and withering of the leaves. To analyze regrowth, seedlings that had previously been exposed to 4°C for 4 d were returned to the growth chamber for 13 d of recovery. Regrowth was defined as the production of new, fourth leaves on the seedlings.

RESULTS

Identification of *OsAsr1* cDNA from the rice seed-coat library

The cDNA clone isolated from our developing seed coats is highly homologous with *OsAsr1*, found previously by Vaidyanathan et al. (1999). This clone is 838 bp long and comprises a 66-bp, 5′ untranslated region (UTR), a 417-bp open reading frame, a 325-bp 3′ UTR, and a poly (A)-tail (data not shown). It is 4 bp shorter at the 5′ end than the previously reported clone, but its nucleotide sequence exactly matches *OsAsr1* except for a T-to-C change at the 3′ UTR (304 bp downstream of the termination codon). Because the previously isolated cDNA was from ‘Pokkali’, an indica rice, and the clone identified here is from the japonica type ‘Dongjin’, we propose that this one-nucleotide variation at the 3′ UTR is due to the difference in cultivars.

An *OsAsr1* genomic sequence was retrieved from the indica rice WGS genomic database (Yu et al., 2002). *In-silico* analysis of the clone predicted that core sequences of the ABA response element (ABRE), ACGT, are present at positions -261, -610 and -616 from the first ATG codon. An important cold-responsive *cis* element, C-repeat/dehydration responsive element (CRT/DRE) sequence, GCCGAC, is also found at -714. Putative Myb (-143, -149, and -698), Myc (-71, -512, and -694) and bZIP (-202 and -711) binding sites are also found. A putative Zn-binding His-residue and the putative NLS domain are present in the N-terminal and the C-terminal region of OsAsr1 protein, respectively (Fig. 1). A comparison of the cDNA and the genomic sequences revealed a 119-bp-long intron in the *OsAsr1* gene. TBLASTN searches (Altschul et al., 1997), using the OsAsr1 protein sequence (accession no. AAB96681) as the query, were able to retrieve five homologues in the scaffolds (002913, 037286, 026604, 023736, and 081294). Their phylogenetic relationship presented as a dendrogram indicates that OsAsr1 has the highest homology with ZmAsr1 of maize (Fig. 2). ASR proteins had been categorized into 4 main groups (Hong et al., 2002) and OsAsr1 belongs to group I related to ZmAsr1. More than 100 rice *OsAsr1* cDNA sequences are registered in the GenBank database, indicating that the gene belongs to an abundantly expressed gene family. Moreover, the *OsAsr1* homologues have been found only in plant species.

DNA gel-blot analysis of *OsAsr1*

The results of our DNA gel-blot analyses on three japonica cultivars showed only one copy of the *OsAsr1* gene (Fig. 3). This observation matches that previously made with ‘Pokkali’ (Vaidyanathan et al., 1999). No significant polymorphism was found with regard to restriction-fragment lengths for these three cultivars of different cold tolerance.

Induction of *OsAsr1* by cold stress

We used RNA gel-blot analyses to examine gene expression at different tissues. The *OsAsr1* transcript was detectable in all the tissues and organs except in calluses (Fig. 4A). The transcript size was about 0.9 kb, indicating that the *OsAsr1* cDNA clone is nearly full-length. The transcript was present at high levels in the shoots and roots of seedlings, sheath of flag leaves, and most abundantly in the internodes between node I and II, demonstrating organ-preferential expression.

OsAsr1 was expressed at the basal level in the leaves of mature plants, but more abundantly in mature flowers. Interestingly, low temperatures elevated overall transcript levels in both organ types (Fig. 4B). Cold treatment increased the transcript level also at the seedling stage, but more significantly at 12°C instead of 4°C (Fig. 4C). The cold-responsive *OsAsr1* accumulation was restricted to shoots, demonstrating the organ-specific stress response of the gene expression (data not shown).

Because *OsAsr1* is ABA-inducible (Vaidyanathan et al., 1999), we compared the induction kinetics of cold stress versus that of ABA. Whereas transcript levels reached their maximum after 3 h of cold stress, ABA treatment caused levels to increase more slowly, achieving their highest point only after 6 h (Fig. 4C). The expression of *OsAsr1* in the physiological range of growth temperature (12, 20, and 30°C) was compared between the cold-tolerant cultivar Odae and the cold-sensitive cultivar Namyang 21. However, we did not observe any significant difference of the gene expression between the cultivars (data not shown).

We performed RNA *in-situ* hybridization to further elucidate the cold-induced expression pattern of the *OsAsr1* gene at the tissue level. In the leaves, induction was confined to the mesophyll tissues, and was not present in the epidermal or vascular tissues (Figs. 5E and 5G). Because the flower is a primary target organ of cold stress, hybridization was also performed for the flowers. Immature flowers at 4 d before heading were cold-treated at 12°C for 4 d. Afterward, *OsAsr1* transcript was detectable in the parenchyma cells of the palea and lemma from those stressed flowers (Figs. 5A and 5C). In the control flowers that had not been cold-stressed, the *OsAsr1* expression pattern was similar to that of the cold-stressed flowers although the expression level was much lower (data not shown).

Expression of *OsAsr1* cDNA in transgenic plants

We constructed binary vectors containing *OsAsr1* cDNA, in the sense (pSK167) or antisense (pSK168) orientation under the maize *ubiquitin* promoter (Fig. 6A), to determine the function of *OsAsr1*. Twenty transgenic plants were generated via the *Agrobacterium* co-cultivation method, and integration of the transgene into the rice genome was examined by DNA gel-blot analyses. The copy number was usually one to two, although three or more copies of the introduced genes were detected (Fig. 6B). Transgenic plants showed no significant morphological changes in their T1 and T2 generations.

To examine whether the changed expression of *OsAsr1* has a role in the cold tolerance of the transgenic rice, RNA gel-blot analyses were conducted (Fig. 6C). Overall, the *OsAsr1* transcript level was higher in the transgenic plants compared with the wild-type segregants. Size of the hybridized transcript in the transgenic plants was slightly bigger than those of wild type. Two strong over-expressers, S2 and S15, were selected for further analyses.

Chlorophyll fluorescence of transgenic plants under cold stress

We measured chlorophyll fluorescence as an indicator of chill-

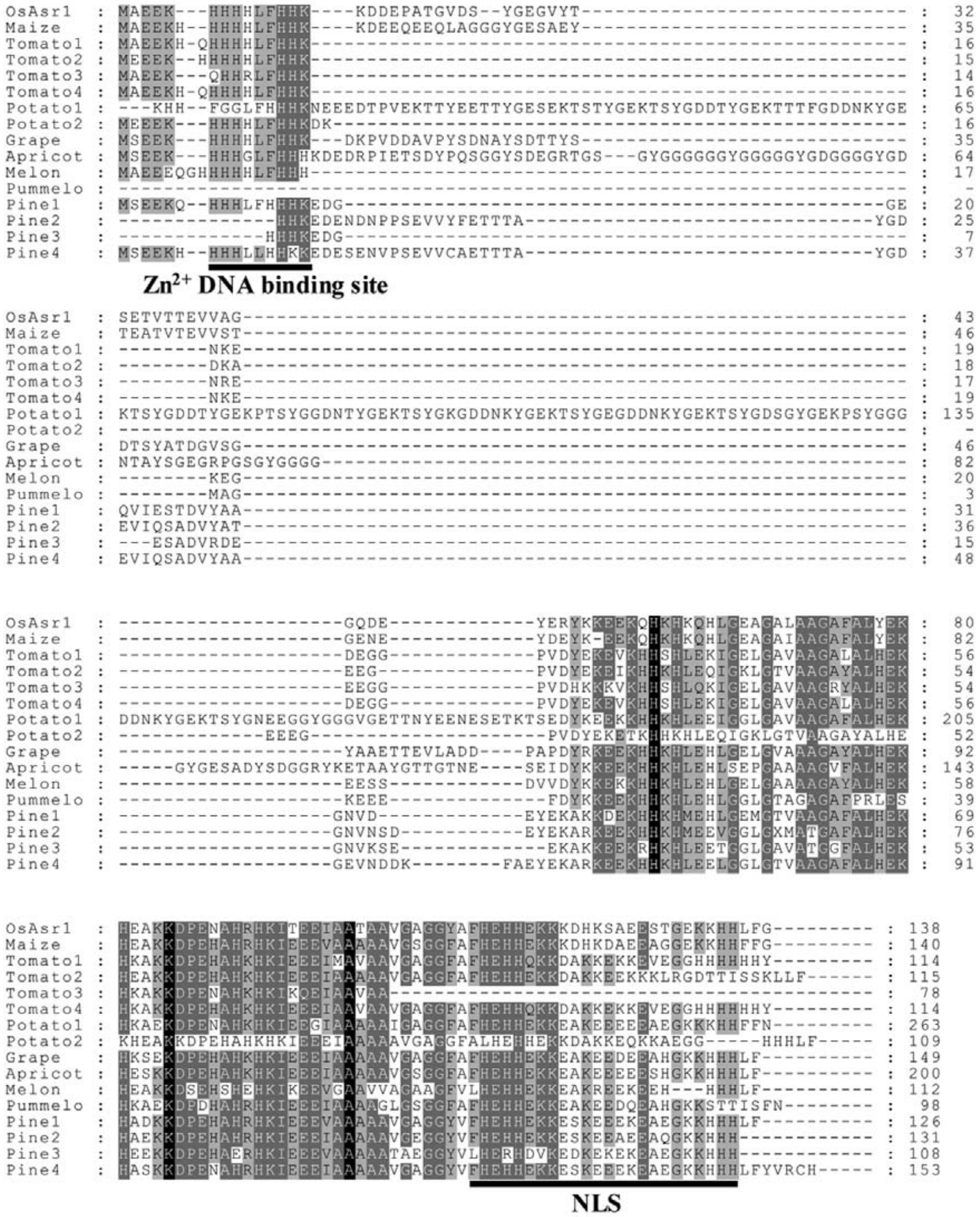

Fig. 1. Comparison of amino acid sequence of the deduced OsAsr1 protein with other known Asr proteins performed with the CLUSTALW program. Putative Zn^{2+} DNA binding site and nuclear localization signal (NLS) were underlined. White letters in a black box indicate 16 out of 16 matches.

ing tolerance after cold treatment (4°C). The ratio of Fv to Fm, which represents the activity of Photosystem II, is used to assess functional damage in plants (Genty et al., 1989). For our wild-type segregants, Fv/Fm progressively decreased following chilling. This decline illustrates the extent of photoinhibition caused by cold stress (Krause, 1994). Values for Fv/Fm had been 0.84 ± 0.01 before stress was induced. Following the 6-h cold treatment, Fv/Fm decreased slightly, and no significant difference in values was found between the transgenic and the wild-type segregants. After 24 h of treatment, however, the Fv/Fm values for the wild-type were reduced significantly, to 0.25 ± 0.021 (Fig. 7). In contrast, the Fv/Fm values for the over-expressers S2 and S15 were 0.53 ± 0.049 and 0.60 ± 0.067, respectively. These ratios were about two-fold higher those

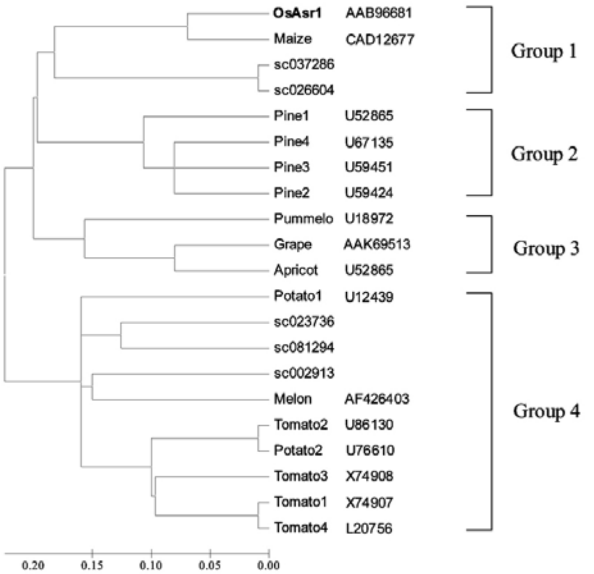

Fig. 2. A phylogenetic analysis of various Asr proteins with rice homologues. The rice homologues were retrieved from TBLASTN search against the indica rice WGS contigs. The dendrogram was constructed using the ClustalX and Mega2 program. The respective accession number is described next to the plant name. The lengths of horizontal lines reflect the evolutionary distance.

calculated for the wild-type (Fig. 7), which indicates that the transgenic plants had a higher degree of cold tolerance.

Survival test

The rate of survival for transgenic plants at the seedling stage was measured in wilting and regrowth tests. The critical length of the cold treatment was first determined by assessing the amount of wilting (i.e., the ratio of the number of wilted seedlings to the total number of cold-treated seedlings) after stress.

The cold-tolerant cultivars, ‘Odae’ and ‘Stejaree 45’, had wilting ratios of 10/18 (55.6%) and 8/20 (40%), respectively, after 5 d of exposure to low temperatures (Table 1). This wilting frequency increased to about 75% after 6 d of stress. In contrast, the parental line of our *OsAsr1* transformation, ‘Dongjin’, exhibited wilting symptoms in 12 of 17 plants (70.6%) after 5 days of stress. The wilting frequency increased to about 94% after 6 days of stress. A cold-sensitive indica x japonica cultivar, ‘Milyang 23’, showed 100% wilting after only 4 d. Therefore, we concluded that the critical length of time for tolerating low-temperature stress by ‘Dongjin’ was about 5 to 6 days. Based on that assumption; we chose to cold-treat our T2 transgenic plants for 6 d. As shown in Table 1, of the 34 seedlings obtained from the S2 plants, 23 (68%) showed wilting after chilling while 10 (67%) of 15 seedlings from the S15 plants exhibited those symptoms. In contrast, 63 (96%) of 66 seedlings derived from the wild-type segregating plants had wilted. The antisense transgenic plants were examined as well, with the occurrence of wilting in A3, A12, and A14 being 9/9 (100%), 9/11 (82%), and 10/11 (91%), respectively.

The regrowth test, conducted in the T3 generation, involved analyzing homozygous lines for the transgene after hygromycin selection (Table 1). Of the 35 seedlings from S15 plants, 14 (40%) showed active development of a fourth leaf. In contrast, the S18 plants, which are from a weaker *OsAsr1*-expressing line, presented only 7 of 35 plants (20%) with signs of regrowth.

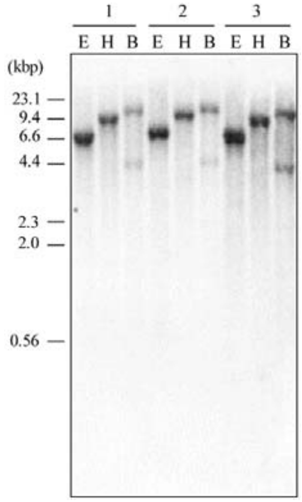

Fig. 3. Southern blot analysis of *OsAsr1*. Twenty μg of genomic DNA from three japonica cultivars, Namyang 21 (1), Odae (2), Dongjin (3), was cut with *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B), and hybridized with radiolabeled *OsAsr1* probe. Positions and sizes in kb of *Hin*dIII-digested lambda DNA are indicated.

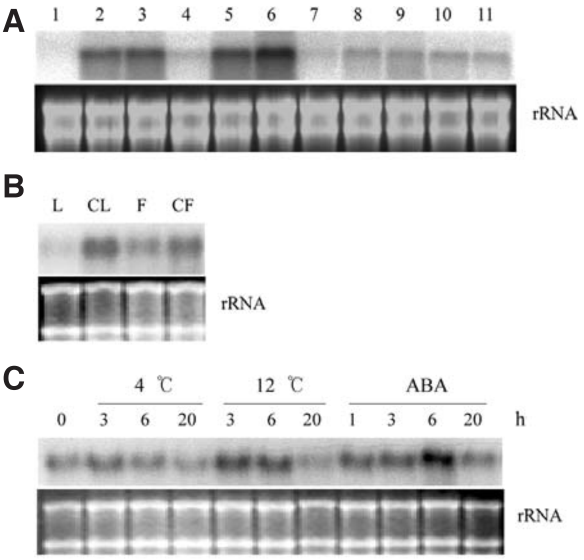

Fig. 4. Northern blot analysis of *OsAsr1*. Thirty (A) or 10 μg (B and C) of total RNA was separated, blotted, and hybridized with radiolabeled *OsAsr1* probe. (A) *OsAsr1* expression in the various organs or tissues. Lanes: 1, callus; 2, shoot of seedling; 3, root of seedling; 4, mature leaf; 5, leaf sheath of flag leaves; 6, highest internode (between node I and II) at prehead stage; 7, 1-2 cm panicle; 8, 3-8 cm panicle; 9, mature panicle prior to anthesis, 10, developing seeds of 3 days after pollination (DAP); 11, developing seeds of 6 DAP. (B) The cold inducible expression of *OsAsr1.* Mature plants were treated at 12°C for 4 d. L, leaves; CL, cold-treated leaves; F, florets; CF, cold-treated florets. (C) Expression of *OsAsr1* by different temperature and ABA. Seedlings grown at 30°C were treated with 4°C, 12°C, or 10 μM ABA for 3, 6, and 20 h.

Nevertheless, the wild type-segregating control plants had even lower rates of regrowth, i.e., 9%, or 9 out of 105 plants. Similar results were seen from the A3 plants, in which only 7 of

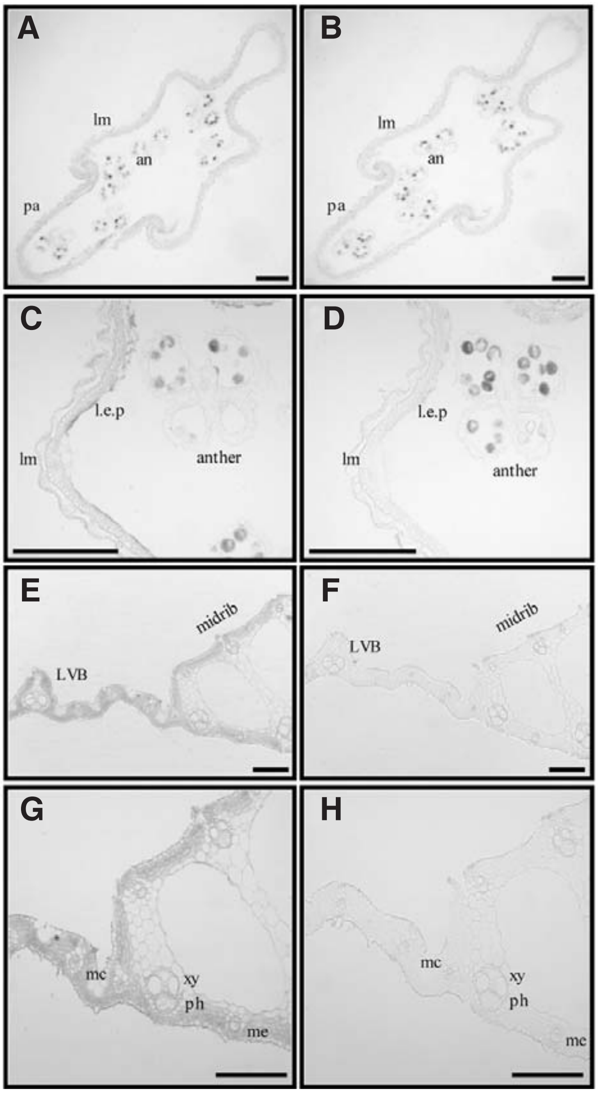

Fig. 5. *In situ* localization of *OsAsr1* mRNA. Cross sections of the rice flowers of 4 d before heading and leaves exposed to 12°C for 4 d were hybridized with digoxygenin-labeled antisense (A, C, E, and G) or sense (B, D, F, and H) *OsAsr1* probes. Higher magnification of the cross section is shown (C, D, G, and H). an, anther; lm, lemma; pa, palea; l.e.p, lower epidermis of palea; LVB, large vascular bundle; mc, motor cell; xy, xylem; ph, phloem; me, mesophyll. Bar = 0.3 mm

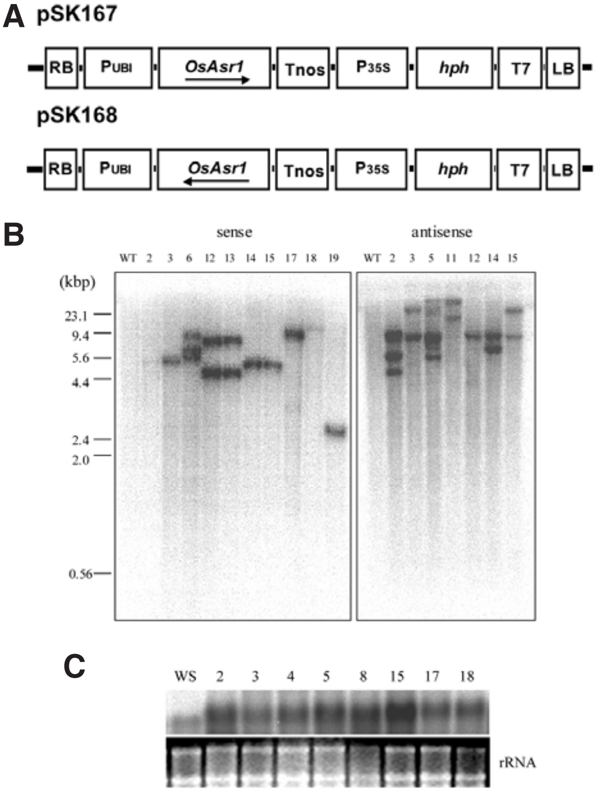

Fig. 6. Analysis of transgenic plants expressing *OsAsr1* in sense and antisense oritentation. (A) Construction of *OsAsr1* sense (pSK167) and antisense (pSK168) expression vector for rice transformation. P_{UBI}, maize ubiqutin promoter; P_{35S}, CaMV 35S promoter; Tnos, terminator sequence of the nopaline synthase gene; T7, terminator sequence of the transcripts 7; *hph*, the hygromycin phophotransferase gene for selection of transgenic rice callus; RB and LB, the right and left border sequences, respectively, of the Ti plasmid from *Agrobacterium tumefaciens*. (B) Southern blot analysis of transgene. Southern blot was conducted using 10 μg of genomic DNA cut with *Hin*dIII from transgenic plants. The ubiquitin promoter fragment in the transformation vector was used as a probe. Positions and sizes in kb of *Hin*dIII-digested lambda DNA are indicated. (C) Expression of *OsAsr1* in the sense transgenic rice (*Ubiquitin::OsAsr1*). Leaf total RNA from wild type segregant (WS) and sense transgenic plants under normal growth condition was separated, blotted, and hybridized with radiolabeled *OsAsr1* probe. Number represents each transgenic line. EtBr-stained rRNA bands indicate an equal amount of loading.

70 (10%) showed any response. Taken together, these results indicate that the transgenic plants over-expressing the *OsAsr1* transcript gained increased tolerance to cold stress. However, the level of tolerance did not change noticeably when *OsAsr1* expression was suppressed in the antisense plants.

Expression of *OsAsr1* cDNA in transgenic rice plants expressing Arabidopsis *CBF1*

Since a CRT/DRE core sequence is located in the putative promoter region of *OsAsr1*, the gene may be controlled by CBF1. To this end, we have generated transgenic rice plants that ectopically expressed *Arabidopsis CBF1* cDNA. RNA gel-blot experiments showed that expression of the *OsAsr1* gene was elevated in transgenic plants (18-2 and 18-3) that strongly expressed the CBF1 transcription factor (Fig. 8). Expression of *OsAsr1* in the transgenic plants was further increased by cold stress.

DISCUSSION

We have isolated the *OsAsr1* cDNA clone from a rice seed-coat cDNA library. The seed-coat tissue protects the embryo from adverse environmental conditions, such as drought and low temperature. *OsAsr1* was expressed at the basal level in the seedlings, and at a much lower degree in mature leaves. We also found that *OsAsr1* transcripts were induced by cold stress. Because *OsAsr1* was also rapidly induced by ABA, reaching a maximum level after 6 h of treatment (Fig. 4C), we

Table 1. Cold stress tolerance of rice cultivars and transgenic *OsAsr1* rice

Cultivar or Line	Cold treatment period (days)								Regrowth
	0	3	4	5	6	7	10	12	
Odae	0/18[1] (0[2])	2/20 (10)	5/20 (25)	10/18 (56)	13/17 (76)	17/18 (94)	19/19 (100)	19/19 (100)	
Stejaree 45	0/18 (0)	0/20 (0)	6/20 (30)	8/20 (40)	15/20 (75)	18/19 (95)	20/20 (100)	19/19 (100)	
Hapchunaengmi	0/18 (0)	0/20 (0)	4/20 (20)	8/20 (40)	10/20 (50)	18/19 (95)	20/20 (100)	19/19 (100)	
Chuchung	0/18 (0)	0/19 (0)	5/20 (25)	9/20 (45)	18/19 (95)	19/19 (100)	20/20 (100)	20/20 (100)	
Dongjin	0/20 (0)	6/20 (30)	6/20 (30)	12/17 (71)	17/18 (94)	17/18 (94)	20/20 (100)	19/19 (100)	
Namyang 21	0/19 (0)	10/16 (63)	12/20 (60)	16/18 (89)	18/18 (100)	19/19 (100)	19/19 (100)	20/20 (100)	
Milyang 23	0/19 (0)	13/18 (72)	17/17 (100)	17/17 (100)	17/17 (100)	19/19 (100)	19/19 (100)	20/20 (100)	
S2					23/34 (68)				
S15					10/15 (67)				14/35 (40)
S18									7/35 (20)
Wild type segregant					63/66 (95)				9/105 (9)
A3					9/9 (100)				7/70 (10)
A12					9/11 (82)				
A14					10/11 (91)				

[1] no. of wilted seedlings / no. of cold treated seedlings

[2] Percentage of wilted seedling to total cold treated seedlings

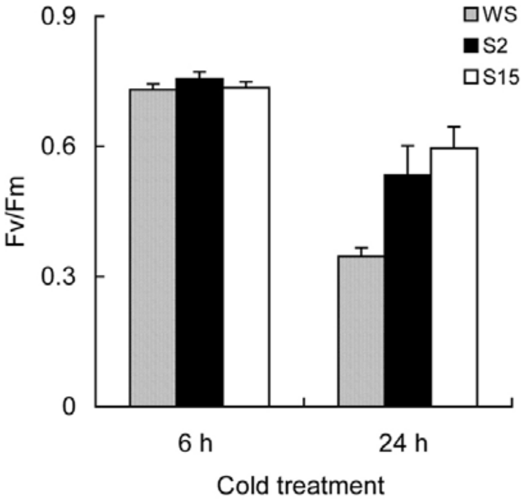

Fig. 7. Stress tolerance of *Ubiquitin::OsAsr1* plants as judged by the measurement of chlorophyll fluorescence. Changes in the chlorophyll fluorescence of the extended leaves under cold stress was measured. Functional damage to photosynthesis was estimated by measuring the mean and standard error of Fv/Fm values. (▨), wild type segregant (WS); (■), S2 ; (□), S15.

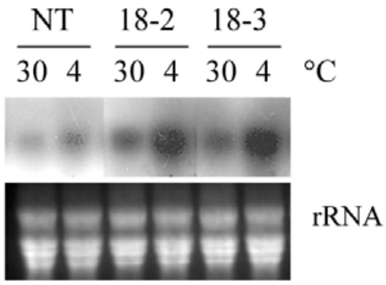

Fig. 8. Expression of *OsAsr1* in *CBF1* transgenic rice. Total RNA from mature leaves of two strong *CBF1*-expressors (18-2 and 18-3) and the wild type non-transgenic line (NT) under control (30°C) or cold (4°C) treated condition was hybridized with radiolabeled *OsAsr1* probe. EtBr-stained rRNA bands indicate an equal amount of loading.

postulate that the cold induction is ABA-dependent. This is consistent with observations that many genes responsive to drought and/or cold stresses are also inducible by the exogenous application of ABA (Chandler and Robertson, 1994).

In cold-stressed mature leaves, *OsAsr1* expression was found only in the mesophyll cells. Using immunohistochemistry, Nylander et al. (2001) also have shown that low temperature-induced *LTI29* and ABA-induced *RAB18* are located in both the vascular tissue and the mesophyll cells of cold-treated plants. Many stress-responsive genes, such as salt-inducible *SalT* homologs and ABA- and drought-inducible glycine-rich protein genes, are expressed specifically in the bundle sheath cells, including *Bss1*, a maize *Asr* gene (Furumoto et al., 2000). Therefore, the cold-induced expression of *OsAsr1* in the mesophyll cells is characteristic of this gene. Likewise, for panicles containing young florets, our cold treatment induced *OsAsr1* expression in the parenchyma cells of the palea and lemma. Exposing rice to cold temperatures at the flowering stage is also known to cause severe sterility by delaying heading time and inducing malformations in the reproductive organs (Nishiyama, 1984).

Hydrophilicity seems to be a common feature among many low temperature-induced proteins, perhaps ameliorating the injurious effects that are commonly associated with cellular dehydration after either chilling or desiccation stress (Boothe et al., 1997; Steponkus, 1984). For example, the low temperature-induced group 2 LEA proteins exist primarily as hydrophilic random coils, and are expressed during periods of seed desiccation (Dure, 1993; Gilmour et al., 1992). Similar hydrophilic proteins, such as CI17 in potato, *Arabidopsis* Kin1 and CORs, alfalfa Cas15, and wheat Wcs120, are involved in enhanced freezing tolerance (Houde et al., 1992; Joachim et al., 1994; Kirsten et al., 1998; Kurkela and Borg-Franck, 1992; Lin and Thomashow, 1992; Monroy et al., 1993). Although Vaidyanathan et al. (1999) have predicted that the OsAsr1 protein has a hydrophilic α-helical structure, no evidence has been found for that. Instead, a high proportion of charged amino acids and small amino acids may make the OsAsr1 protein a flexible structure similar to a molten globule.

Ectopic expression of tomato *Asr1* gene in *Arabidopsis* enhanced salt tolerance during seed germination (Shkolnik and Bar-Zvi, 2008). Our transgenic plants over-expressing the *OsAsr1* transcript showed increased tolerance to cold stress. The optimal quantum yield of PSII was measured by the chlorophyll fluorescence method. This fast, non-destructive technique allows one to distinguish degrees of stress, and is widely used for studying physiological stress (Schreiber et al., 1994). The Fv/Fm values for the transgenic plants were about two-fold higher than those for the wild type segregating controls. After prolonged cold treatment, our regrowth and wilting tests showed that the seedlings of transgenic plants were more tolerant of low temperatures. Although the survival rate for the sense transgenic plants was higher than for the wild type-segregating control, antisense plants were no less tolerant than the control, perhaps because *OsAsr1* is a member of a multigene family. Therefore, elaborate field-testing of those transgenic plants is necessary to estimate chilling tolerance under more agronomical conditions.

Due to the presence of the putative CRT/DRE sequence in the *OsAsr1* promoter region and the high expression of *OsAsr1* in the *Ubi::CBF1* plants, it can be postulated that *OsAsr1* might be a target of the rice orthologue of *CBF1*. Interestingly, *OsAsr1* expression was further induced by cold stress in the *Ubi::CBF1* plants. Similarly, expression of *galactinol synthase* gene (*AtGol3*), in which the promoter contains DRE motifs, was upregulated more significantly in the cold-stressed *35S::DREB1A* plants than in the non-stressed plants (Taji et al., 2002). Microarray analysis of the *35S::DREB1A Arabidopsis* plants coupled with RNA gel-blot experiments identified many stress-inducible genes such as *RD*, *COR*, and *KIN,* which were upregulated under unstressed conditions (Gilmour et al., 2000; Kasuga et al., 1999; Seki et al., 2001). However, an *OsAsr1* homologue had not been found.

Recently, it has been shown that a grape Asr, VvMSR, might be a transcription factor containing Zn^{2+} dependent DNA binding site and NLS (Cakir et al., 2003). Since OsAsr1 protein also have a predicted Zn^{2+} dependent DNA binding site and NLS, it would be interesting to know the cellular location of the protein. Microarray analysis of the *OsAsr1* overexpressing lines would help identifying downstream genes.

The phylogenetic dendrogram indicated that plant ASR can be divided into four groups and OsAsr1 is categorized into Group 1 with ZmAsr1. It has been shown that *ZmAsr1* gene is co-localized with two drought-related QTLs, Leaf Senescence and Anther-Silking Interval (ASI), on chromosome 10. Computer analysis of the *OsAsr1* flanking sequence revealed that *OsAsr1* is also co-localized with Leaf Senescence on chromosome 11. *ZmAsr1* overexpressing maize lines clearly presented an increase in the drought tolerlance. Similarily, it could be expected that our *OsAsr1* overexpressor has enhanced drought tolerance.

Rice is the primary food staple for about half the world's population. As the population increases, we need new means for improving productivity, such as by developing crops that are more tolerant to abiotic stress, e.g., drought or low temperature. Elucidation of stress-tolerant tissues and the tolerance mechanism would greatly improve the agronomic traits of stress-sensitive rice and maize, which share genomic synteny.

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