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An AP2/EREBP-type transcription-factor gene from rice is cold-inducible and encodes a nuclear-localized protein

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Abstract We cloned an AP2/EREBP gene by dot blotting and named it *OsDREBL*. Analysis of its deduced amino-acid sequence indicated that this protein had a potential nuclear-localization signal, a possible acidic-activation domain and an AP2 DNA binding domain. Northern analysis showed that the transcripts of *OsDREBL* accumulated rapidly (within 30 min) in response to low temperature, but not in response to ABA, NaCl and dehydration treatments. Southern analysis indicated the presence of a single-copy of the *OsDREBL* gene in the *Oryza sativa* genome. Our research also demonstrated that OsDREBL was localized to the nucleus but did not bind effectively to the C-repeat/dehydration responsive element (CRT/DRE). These results suggested that OsDREBL may function as a transcription factor in the cold-stress response, independent of the DREB signal-transduction pathway.

Keywords Transcription factor · AP2/EREBP · Cold-inducible · Rice

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Introduction

Freezing temperatures are major factors limiting the geographical locations suitable for growing crops and horticultural plants, and periodically account for significant losses in plant productivity (Thomashow 1999). Improving the cold hardiness of crop plants is an important goal in agriculture, and thus demands a clear understanding of the cold-stress signal perception and transduction. Toward this goal, many genes induced by cold stress were isolated in plants. Further analysis of these genes revealed that their products either were involved in the biosynthesis of compatible solutes, antioxidants and membrane lipids etc., or functioned as regulatory proteins in the signal-transduction pathway of cold-stress responses (Liu et al. 1998; Karam et al. 2002).

Transcriptional control of the expression of the cold-regulated (COR) genes is a crucial part of the plant response to cold stress. The research carried out in the past few years has been productive in identifying transcription factors that are important for regulating plant responses to cold stress. Alexander et al. (1999) reported that a zinc-finger transcription factor gene *ZPT2-2* of petunia (*Petunia hybrida*) was induced by cold stress, suggesting that the zinc-finger transcription factors may play an active role in the cold signalling. In *Arabidopsis*, three cold-induced zinc finger proteins, *AZF1, AZF3* and *STZ*, have been cloned (Sakamoto et al. 2000). Cold-regulated bZIP proteins have also been identified in *Arabidopsis* (Lu et al. 1996; Choi et al. 2000), rice (Aguan et al. 1993) and maize (Kusano et al. 1995). However, no transgenic plants have been developed to show the role of these bZIP and zinc-finger proteins in cold-responsive gene regulation and cold acclimatization. Recently, a zinc-finger protein, SCOF-1, was isolated from soybean (Kim et al. 2001). The transcription of SCOF-1 is specifically induced by low temperature and abscisic acid (ABA), but not by dehydration or high salinity. Constitutive overexpression of SCOF-1 induced cold-regulated (COR) gene expression and enhanced cold tolerance of non-acclimated transgenic *Arabidopsis* and

tobacco plants. Moreover, SCOF1 enhanced the DNA binding-activity of SGBF1, a bZIP transcription factor, which is cold- and ABA-inducible. Thus SCOF1 interacts with SGBF1 to regulate the cold-responsive gene expression in cold-stress signal transduction (Kim et al. 2001).

Another important insight gained by cold signalling are the AP2/EREBP transcription factors. These transcription factors were characterized by a conserved AP2/EREBP domain of about 60 amino acids present in a large family of plant genes for DNA binding proteins, and are involved in many aspects of development and stress responses. The AP2/EREBP domain was originally identified in *APETALA2* of *Arabidopsis* (Jofuku et al. 1994) and *EREBP1* of tobacco (Ohme-Takagi and Shinshi 1995). One class of such proteins that was proved to regulate expression of the cold-responsive genes is the DREB1/CBF subfamily. Three DREB1/CBF genes, namely CBF1 (DREB1b), CBF2 (DREB1c) and CBF3 (DREB1a), have been isolated from *Arabidopsis* (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998). Their homologous genes have also been found in wheat, *Atriplex* and other plants (Shen et al. 2003a, b). These DREB1/CBFs all contained the AP2/EREBP DNA binding-domain, which can recognize the CRT/DRE (Baker et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994). CRT/DRE elements have a conserved 5-bp core sequence of CCGAC, and are essential for the low-temperature responsiveness of many cold-regulated (COR) genes, including the *Arabidopsis* gene *COR15a* (Baker et al. 1994), the *Brassica napus* gene *BN115* (Jiang et al. 1996) and the wheat gene *WCS120* (Ouellet et al. 1998). Under normal conditions, neither CBF nor COR genes are expressed. However, when treated with cold (4 °C), the expressions of the CBF genes are induced very early, followed by the expression of CBF-regulated target genes. Furthermore, transgenic plants overexpressing CBF1 showed increased tolerance to cold stress without a cold acclimation period (Gilmour et al. 1998; Liu et al. 1998). Recent studies also indicated that the DREB1/CBF cold-signal transduction pathway was conserved in a variety of plant species, including *B. napus*, wheat, rye, tomato (Jaglo et al. 2001) and barley (Choi et al. 2002). However, variation in expression patterns of CBFs in different species was also reported (Xue 2003).

Ethylene-responsive element binding-factors (ERF), another type of AP2/EREBP transcription factor, have been isolated as the GCC box-binding proteins from tobacco (Ohme-Takagi and Shinshi 1995), *Arabidopsis* (Büttner and Singh 1997) and tomato (Zhou et al. 1997). Their involvement in cold signalling was also suggested (Fujimoto et al. 2000).

In the present study, we report a novel cold-inducible and ABA-independent AP2/EREBP transcriptional-factor gene from rice. Its expression pattern was investigated. Its localization and the ability to activate reporter genes containing the DRE/CRT element were also examined.

Materials and methods

Plant materials and treatments

Seeds of rice (*Oryza sativa*) were embedded in water at 37 °C overnight and grown hydroponically at 25 °C with a photoperiod of 12 h for approximately 3 weeks. For dehydration treatment, the plants were carefully pulled out, transferred onto filter paper and allowed to dry. For abscisic acid (ABA) and salt treatments, the plants were transferred to the solution containing 50 μM ABA and 200 mM NaCl, respectively. Cold treatment was conducted by exposure of plants grown at 25 °C to a temperature of 4 °C. In each case, the plants were subjected to the stress treatments for various periods and then frozen in liquid nitrogen. Plant materials were stored at –80 °C prior to RNA extraction.

Screening of genomic clones that contain the AP2-like domain

The AP2 domain (80 amino acids) of the rice *AP2* gene was used as a query to search the Genome Database of Chinese Super-hybrid Rice in Beijing Genomics Institute by using the Blast-analysis program. The sequences that produce significant alignments (E-value $\leq$3e-04) were chosen. The Phrap program (University of Washington) was used to assemble these fragments.

Dot blotting and DNA sequence analysis

Dot-blotting procedures were carried out as described (He et al. 2002). The plasmid DNAs were extracted from each clone, and then dotted on Hybond N^+ nylon filters by hand to construct two identical filters. The two filters were marked as C (control) and T (treatment) respectively. Each clone was dotted two times on the same filter to reduce experimental error. Two rounds of hybridization were performed. In the first round, C and T filters were hybridized with P^{32}-labelled cDNAs that were reverse-transcribed from 10 μg of total RNA isolated from the rice seedlings treated with low temperature(4 °C) for 5 h and control (no-treatment) respectively. In the second round, C and T filters were exchanged.

The hybridization was performed at 65 °C for 16 h. Filters were washed with 2× SSC, 0.1% SDS at 42 °C for 15 min, then washed with 1× SSC, 0.1% SDS at room temperature for 10 min. The hybridization signals were scanned and analyzed with the Phosphoimaging system, and then differentially expressed clones were identified.

DNA sequences were determined using the *Taq* Dye Primer Cycle Sequencing Kit (Amersham) and the ABI 373A automatic sequencer.The nucleotide and amino-acid sequences were compared with those released in the GenBank database by the GAPPED Blast analysis program. The alignment report was produced by software DNASTAR.

Southern and Northern analyses

DNA extraction was performed as described previously (Chen et al. 1991). Genomic DNA (about 8 μg) was fractionated in a 0.8% agrose gel after digestion with *Bam*HI, *Eco*RI and *Kpn*I, and blotted onto the Hybond-N^+ nylon membrane for Southern-blotting analysis. The hybridization and washing procedures followed the standard protocols.

Total RNA was extracted from rice plants as described previously (Zhang et al. 1996). Total RNA (about 30 μg) was fractionated in 1.2% agarose-containing formaldehyde and blotted onto the Hybond-N^+ nylon membrane for Northern analysis. The hybridization was performed at 65 °C for 16 h with the labeled coding region of *OsDREBL* as a probe. The membranes were washed in 2× SSC, 0.1% SDS at 45 °C for 15 min and in 1× SSC, 0.1% SDS at room temperature for 10 min. The membranes were then autoradiographed by using a phosphoimaging system.

Yeast one-hybrid analysis

The entire coding regions of both *OsDREBL* and *AtDREB1* were prepared by PCR and cloned into the *Eco*RI-*Xho*I sites of the GAL4 activation-domain vector (pAD) to generate pAD-*OsDREBL* and pAD-*AtDREB1*, respectively. According to the manufacturer's protocol, the recombinant pAD plasmids were transformed into the yeast strain YM4271 carrying the reporter gene *HIS3* under the control of the 71-bp promoter region of *rd29A* containing the DRE (TACCGACAT) or mutated DRE (TATTTTCAT). The transformed yeast cells were streaked on the plates of SD/-His (without His) in the absence or presence of 10 mM 3-AT (a competitive inhibitor of the *HIS3* product) and their growth was examined.

Subcellular localization of the OsDREBL protein

The termination codon of the *OsDREBL* cDNA was removed by PCR using 5′-ACAGGATCCATGTGCGGGATCAAGCAGGAG-3′ (with the *Bam*HI site) and 5′-ACAGTCCACGTAGCTCCA-GAGTGGGACGTC-3′ (with the *Sal*I site). The resulting product was fused in-frame to the green fluorescent protein (GFP) reporter gene in pUC18 and sequenced. The fusion construct for OsDREBL-GFP and the GFP control plasmid were introduced into the onion epidermis cells by particle bombardment (Bio-Rad). The transformed cells were cultured on the MS medium at 28 °C for 2 days and observed under a confocal microscope (Olympus FV500) (Xie et al. 2003).

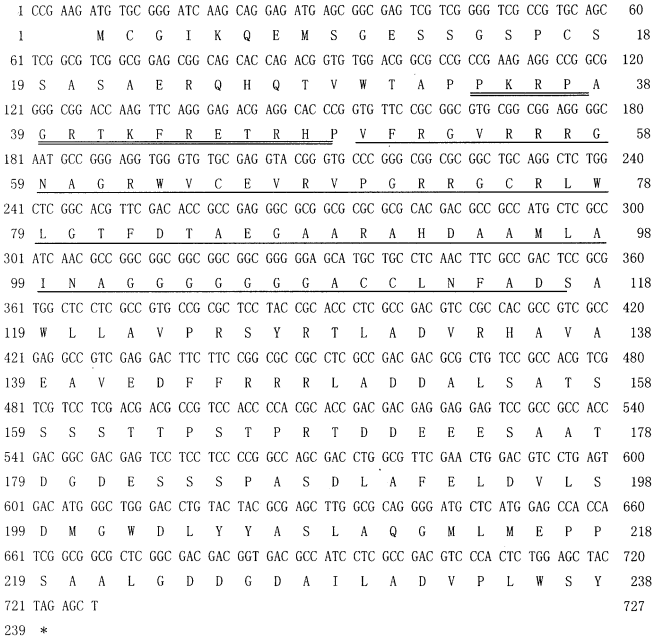

Fig. 1 Nucleotide sequence and deduced amino-acid sequence of the *OsDREBL* gene. The amino acids that comprise a putative nuclear localization signal are shown by a *double underline*. The AP2/EREBP DNA binding domain is indicated by a *single underline*. This sequence has been deposited in the GenBank database under the accession number AF494422

Results and discussion

Isolation and characterization of OsDREBL from rice

It has been estimated that about 140 genes in the *Arabidopsis* genome encoded AP2/EREBP proteins (Riechmann et al. 2000). The completion of the rice-genome sequence allowed researchers to study the AP2/EREBP genes at the full-genome level (Yu et al. 2002). Using the DNA sequence encoding the AP2 domain (80 amino acids) of the rice *AP2* gene as a query, we searched the public database, the Genome Database of Chinese Super hybrid Rice, in The Beijing Genomics Institute. A total of 127,551 sequences were screened and 179 sequences produced significant alignments. These 179 sequences cloned in the *Sma*I-linearized pUC18 plasmid were collected from The Beijing Genomics Institute. Assembly of these sequences by the Phrap program (University of Washington) indicated that they belonged to 32 different contigs.

Then, the dot-blotting method was used to identify the cold-responsive AP2/EREBP genes. In order to reduce false positives caused by experimental errors, two rounds of dot blotting were performed as described in Materials and methods. In the first round, 20 clones were up-regulated by cold treatment. After the second round of dot blotting, 10 clones were removed as false positives. The other 10 clones were still differentially expressed. We then examined these 10 clones and found that they belonged to the same contig. Based on the genomic sequence of the contig, its cDNA was obtained from cold-treated rice seedlings by the RT-PCR method with the forward primer 5′-CCGAAGATGTGCGGGATCAAG-3′ and the reverse primer 5′-AGCTCTAGTAGCTCCA-GAGTG-3′. This cDNA was named as *OsDREBL*, sequenced and compared with its genomic DNA sequence. We found that no introns existed in the coding region.

The *OsDREBL* cDNA (Fig. 1) contained an open reading frame of 714 bp and encoded a putative protein of 238 amino acids with a predicted molecular mass of 25.4 kDa and a pI of 4.97. Analysis of the deduced amino-acid sequence revealed that this protein had an AP2/EREBP DNA binding domain. Furthermore, the OsDREBL protein contains basic residues in its N-terminal region that potentially served as a nuclear localization signal and an acidic C-terminal region that might act as a transcriptional activation domain. These data suggested that the *OsDREBL* cDNA encoded a DNA binding protein that might function as a transcriptional regulator in rice.

The deduced amino-acid sequence of *OsDREBL* was compared with those of other known AP2/EREBP proteins (Fig. 2). It is shown that OsDREBL had homology with many AP2/EREBP proteins in plants, e.g. 47.9% identity to BCBF3 from *Hordeum vulgare* (AF298231), 45.0% identity to DREB2 from *Zea mays* (AF450481), 44.3% identity to CBF1 from *Triticum aestivum* (AF376136) and 32.4% identity to CBF3 from *Arabidopsis* (AF074602). These data suggested that OsDREBL had higher homology with proteins from monocots, but had lower homology with proteins from dicots.

Rice genomic DNA was digested with *Bam*HI, *Eco*RI and *Kpn*I, fractionated on the agarose gel and transferred

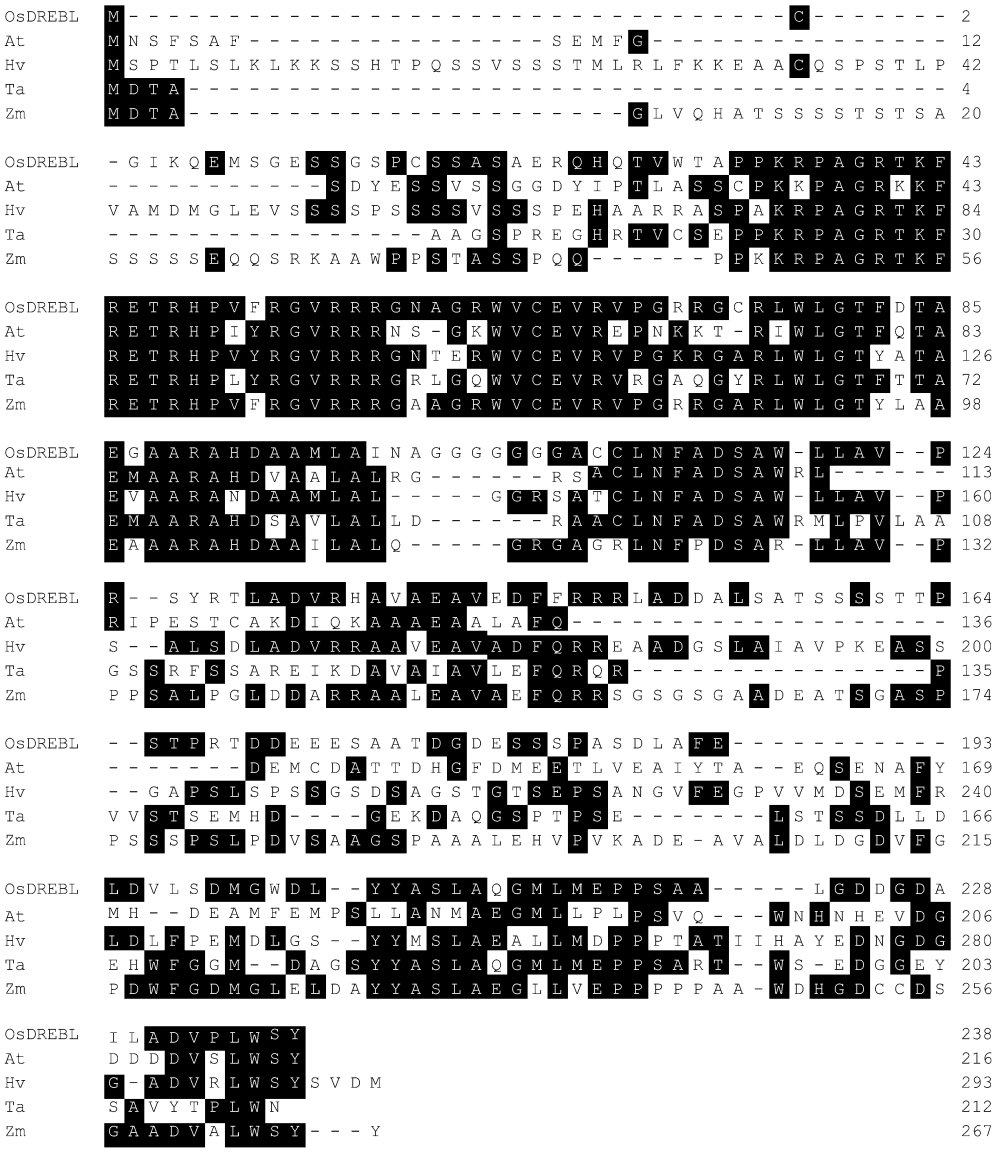

Fig. 2 Alignment of OsDREBL and other AP2/EREBP DNA binding proteins. The amino-acid sequences shown are: *Oryza sativa* OsDREBL (AF494422); At, *Arabidopsis* CBF3 (AF074602); Hv, *Hordeum vulgare* BCBF3 (AF298231); Ta, *Triticum aestivum* CBF1 (AF376136); Zm, *Zea mays* DREB2 (AF450481)

onto the filter. After hybridization with the labeled coding region of *OsDREBL*, the filter was washed under high-stringency conditions. The results showed that one hybridization band was presented in each digestion (data not shown), probably indicating that OsDREBL is a single-copy gene. Blast search also indicated that the *OsDREBL* gene was unique in the Genome Database of Chinese Super-hybrid Rice.

Expression patterns of *OsDREBL*

To elucidate the expression pattern of the *OsDREBL* gene in response to low temperature, total RNA was extracted from rice seedlings exposed to 4 °C and subjected to RNA gel-blot analysis. The results, presented in Fig. 3, showed that the *OsDREBL* transcripts were almost undetectable in the 0-h rice seedlings. Upon transfer to low temperature, the *OsDREBL* transcript levels increased markedly within 30 min and reached a maximum at 5 h. After that, the *OsDREBL* transcript levels began to fall. This expression pattern was similar to those of CBFs in a variety of plants species, including *Arabidopsis*, wheat and rye (Jaglo et al. 2001). However, Unlike CBFs in *Arabdiposis*, wheat and rye, the transcript level of the *OsDREBL* was only slightly higher than those found in 0-h seedlings after 10 h and remained at this low level within 32 h after initiation of the cold treatments.

To determine whether the accumulation of *OsDREBL* transcripts was responsive to other treatments, rice plants were treated with 200 mM NaCl, 50 μM ABA or dehydrated until losing 50% of their initial fresh weight. Total RNA isolated from these plants was used for RNA analysis. Figure 3 showed that *OsDREBL* transcripts did not accumulate in response to drought, salt and ABA treatments, indicating that the expression of *OsDREBL* was specifically regulated by low temperature. This expression pattern was similar to those of DREB1/CBF

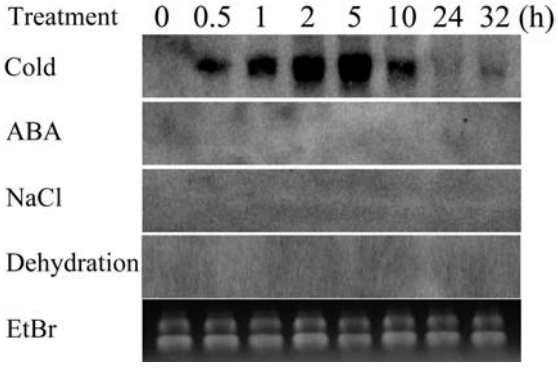

Fig. 3 *OsDREBL* transcripts accumulate in response to low temperature, but not in response to ABA, salt or dehydration stress. RNA-blot hybridizations were performed with total RNA (30 μg per lane) isolated from rice seedlings exposed to 4 °C, immersed with their roots in the 50 μM ABA solution, immersed with their roots in the 200 mM NaCl solution, or dehydrated on filter paper for the indicated time. The probe used was the entire coding sequence of *OsDREBL*

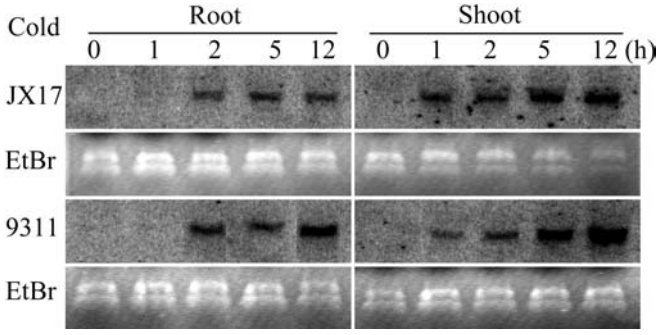

Fig. 4 Expression patterns of *OsDREBL* in two rice subspecies, *japonica* JX17 and *indica* 9311. Seedlings from both rice subspecies were treated with 4 °C for the indicated times. Total RNA from either shoots or roots of plants was isolated and Northern analysis was performed using the labelled coding region of *OsDREBL* as a probe

genes in many plant species including *Arabidopsis* In fact, two AP2/EREBP transcription factors DREB1 and DREB2 separated two cellular signal-transduction pathways at low temperature and drought-responsive gene expression, respectively, in *Arabidopsis* (Liu et al. 1998).

Earlier studies showed that the cold-signalling pathway was ABA-dependent. ABA level increased in many plants in response to low temperature and a set of cold-responsive genes were responsive to exogenous application of ABA. Thus, presumably, cold-induced expression of ABA-responsive genes was due, at least in part, to elevated levels of ABA. Indeed, low-temperature regulation of *Arabidopsis RAB18* is dependent on the action of ABA, as transcripts for this gene did not accumulate in response to low temperature in either the *aba1* (ABA synthesis) or *abi1* (ABA signalling) mutants (Lang and Palva 1992). However, recent effort has proved that cold-induced gene expression can also proceed through at least one ABA-independent pathway (Liu et al. 1998). Like DREB1/CBF in *Arabidopsis*, OsDREBL was not induced by ABA. It is thus possible that the *OsDREBL* cold-signal transduction pathway in rice is ABA-independent.

To further examine the pattern of *OsDREBL* transcript accumulation in different parts of rice and in different subspecies in response to low temperature, seedlings from *japonica* rice JX17 and *indica* rice 9311 were treated with 4 °C for 5 h, and total RNA from either shoots or roots of plants was extracted and subjected to Northern analysis. The results presented in Fig. 4 revealed that the *OsDREBL* expression in both subspecies was up-regulated in both shoots and roots, with a higher expression level in shoots and a lower level in roots. In shoots of both subspecies, the induction occurred earlier than in roots. However, the induction levels in the two subspecies were different. In *indica* rice 9311, the *OsDREBL* transcript level was higher when compared with that of *japonica* rice JX17, indicating that *indica* rice was more responsive to cold stress. The differential expression of the *OsDREBL* gene in the two subspecies may reflect their difference in cold tolerance. Because *indica* rice was generally more sensitive to cold stress, they may need more OsDREBL proteins to resist this unfavorable condition. For *japonica* rice, since they were more tolerant to cold stress, they may not need a strong induction of *OsDREBL* but preferably rely more on a mechanism that already existed under normal conditions.

OsDREBL did not bind to the DRE *cis*-acting element

Since the structure and expression patterns of OsDREBL were similar to those of DREB1/CBF, we used the yeast one-hybrid strategy to test the binding activity of OsDREBL to the CRT/DRE box (Fig. 5A). The recombinant plasmid pAD-*OsDERBL* was transformed into the yeast cells, carrying the reporter gene *HIS3* that had been fused to the 71-bp DNA fragment of the *rd29A* promoter containing the DRE sequence and the mutated DRE, respectively. As shown in Fig. 5B, the resulting yeast-cells harboring pAD-*OsDREBL* plus DRE-*HIS3* and pAD-*OsDREBL* plus mDRE-*HIS3* can grow on media without 3-AT, but cannot grow on media with 10 mM 3-AT. As a positive control, the recombinant plasmid pAD-*AtDREB1* containing the *Arabidopsis* DRE binding protein-1 gene was also transformed into the yeast cells carrying the reporter gene *HIS3* that had been fused to the 71-bp DNA fragment of the *rd29A* promoter containing the DRE sequence and the mutated DRE, respectively. The resulting yeast cells grew very well on media without 3-AT. However, on media with 3-AT, only cells harboring pAD-*AtDREB1* plus DRE-*HIS3* grew well (Fig. 5B). These results indicated that the *Arabidopsis* AtDREB1 can bind to the DRE/CRT element and the activated expression of the reporter gene *HIS3*, whereas the rice OsDREBL may not effectively bind to the CRT/DRE sequence.

In tomato, the interaction of the Pto kinase with Pti4/5/6 (the EREBP-type transcription factors) suggested that

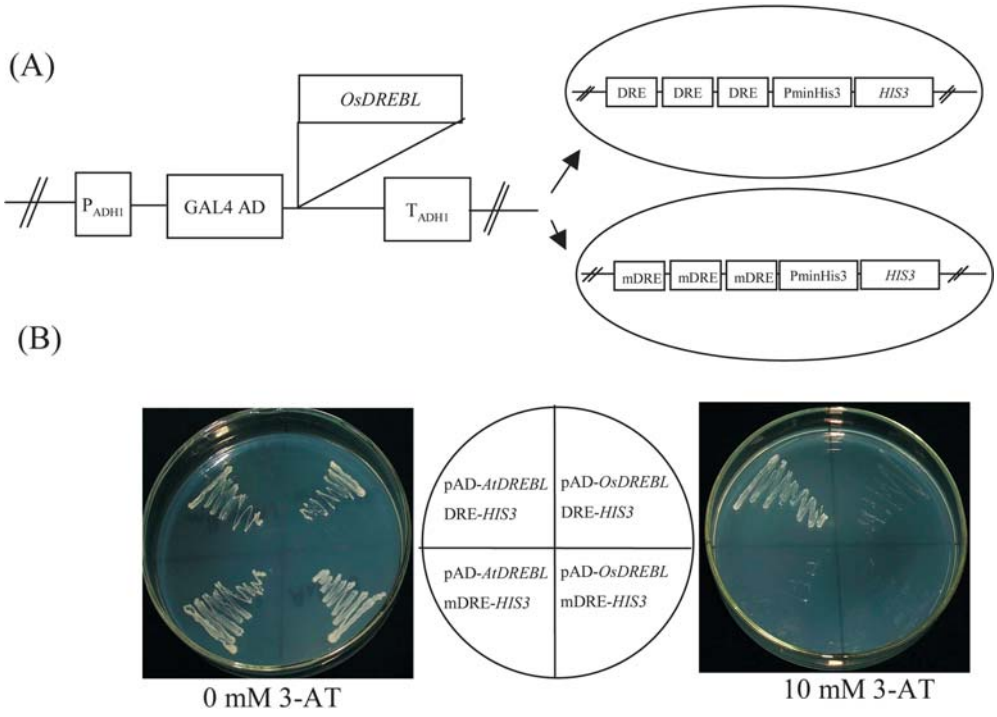

Fig. 5A, B Yeast one-hybrid analysis. (**A**) The entire coding region of *OsDREBL* was fused to the GAL4 activation domain. P_{ADH1} and T_{ADH1} indicated the promoter and terminator of the *ADH1* gene, respectively. DRE, the dehydration responsive element. mDRE, the mutated dehydration responsive element. (**B**) Both pAD-*OsDREBL* and pAD-*AtDREB1* were transformed into yeast cells carrying the reporter gene *HIS3* under the control of the 71-bp promoter region containing the DRE or mutated DRE (mDRE). The transformants were examined for growth in the absence or in the presence of 3-AT

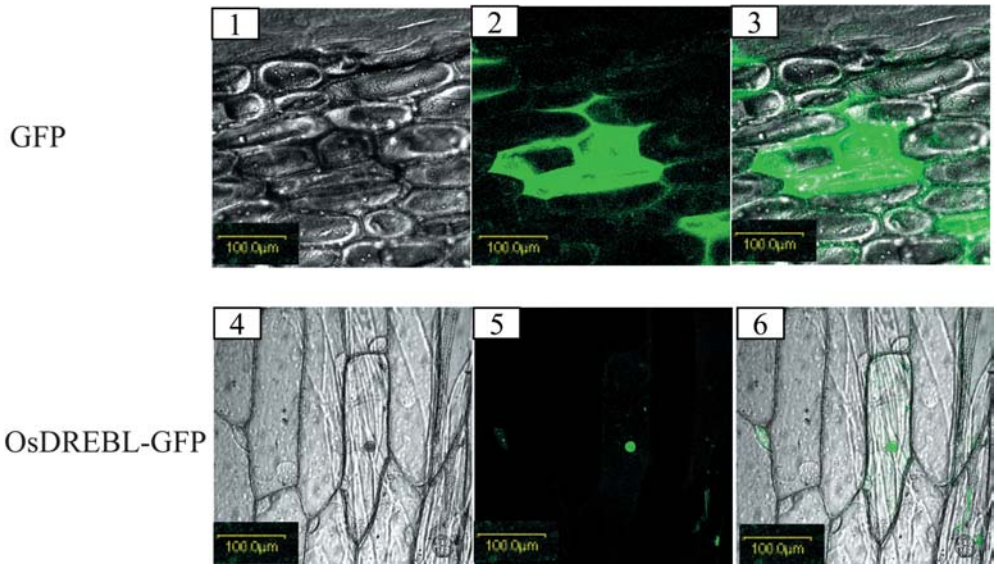

Fig. 6 Subcellular localization of OsDREBL. The fusion construct for OsDREBL-GFP and the GFP control plasmid were introduced into the onion epidermis cells by particle bombardment. The transformed cells were cultured on the MS medium at 28 °C for 2 days and observed under a confocal microscope. The photographs were taken in the bright light for the morphology of the cell (*parts 1, 4*), in the dark field for green fluorescence (*parts 2, 5*), and in combination (*parts 3, 6*)

phosphorylation of Pti4/5/6 and EREBPs may be required for their in vivo activity (Zhou et al. 1997). However, the binding of DREBs and CBF1 to the DRE/CRT box does not appear to require any post-translational modification, as shown by the DNA binding activity of the *Escherichia coli*-expressed proteins (Stockinger et al. 1997; Liu et al. 1998). Therefore, it is likely that the present OsDREBL may preferably bind to other elements such as the ethylene responsive element GCC box (AGCCGCC), rather than the CRT/DREB box (TACCGACAT), since the two boxes were very similar. Further research needs to be done to test such a possibility. Also, OsDREBL may be involved in the cold-signal transduction pathway that was different from the CBF/DREB1 pathway. In *Arabidopsis*, multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold-response pathway (Fowler and Thomashow 2002).

Targeting of *OsDREBL* to the nucleus

The N-terminal region of the putative OsDREBL protein contained a stretch of basic amino acids, PKRPAGR × KF × ETRHP, immediately upstream of the AP2/EREBP

domain. The resemblance of the PKK/RPAGR × KF × ETRHP sequence to nuclear transport signals (Smith and Raikhel 1999) indicated that it might be involved in protein trafficking as previously suggested (Stockinger et al. 1997). To examine if OsDREBL was targeted to the nucleus, we performed an in vivo targeting experiment using a OsDREBL-fused green-fluorescent protein (GFP) as a fluorescent marker. The *OsDREBL* coding region was fused to the GFP gene and the fusion construct was introduced into onion epidermis cells by particle bombardment. As shown in Fig. 6, the fusion protein was localized to the nucleus of onion epidermis cells, whereas the control GFP was uniformly distributed throughout the cells. These results suggest that OsDREBL is a nuclear protein, possibly serving as a transcription factor.

In summary, we have identified a AP2/EREBP-type transcription-factor gene *OsDREBL* from rice. This gene was specifically induced by cold stress but not by other treatments. The encoded protein was localized to the nucleus, but cannot bind to the DRE element. The OsDREBL may bind to other elements and play roles in a new cold-signal transduction pathway.

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