Expression of a rice DREB1 gene, OsDREB1D, enhances cold and high-salt tolerance in transgenic Arabidopsis

Yang Zhang1,2,*, Chen Chen1,2,*, Xiao-Fen Jin2, Ai-Sheng Xiong2, Ri-He Peng2, Yi-Huan Hong1, Quan-Hong Yao2,*, & Jian-Min Chen1,*

1College of Bioscience and Biotechnology, Yangzhou University, 88 Daxue Road, Yangzhou 225009, 2Biotechnology Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai, 201106, China

OsDREB1D, a special DRE (dehydration responsive element binding protein) homologous gene, whose transcripts cannot be detected in rice (Oryza sativa L.) either with or without stress treatments, was amplified from the rice genome DNA. The yeast one-hybrid assay revealed that OsDREB1D was able to form a complex with the dehydration responsive element/C-repeat motif. It can also bind with a sequence of LTRE (low temperature responsive element). To analyze the function of OsDREB1D, the gene was transformed and over-expressed in Arabidopsis thaliana cv. Columbia. Results indicated that the over-expression of OsDREB1D conferred cold and high-salt tolerance in transgenic plants, and that transgenic plants were also insensitive to ABA (abscisic acid). From these data, we deduced that this OsDREB1D gene functions similarly as other DREB transcription factors. The expression of OsDREB1D in rice may be controlled by a special mechanism for the redundancy of function. [BMB reports 2009; 42(8): 486-492]

INTRODUCTION

Land plants are greatly affected by environmental stresses such as drought, high-salt, and extreme temperature. These stresses induce various biochemical and physiological responses in plants. Expression of a variety of genes has been demonstrated to be induced by these stresses in a variety of plants, especially the DREB transcription factors. These proteins bind to a dehydration-responsive element (DRE) with the core sequence A/GCCGAC identified as a cis-acting promoter element, and regulate downstream gene expressions in response to drought, high-salt, and cold stresses (1).

Many DRE-binding transcription factors have been identified in kinds of plants, such as eucalyptus (Eucalyptus globules), sweetpotato (Ipomoea batatas) and tall fescue (Festuca arundinacea) (2-4). The function of these transcription factors were further studied, especially in Arabidopsis and rice. Strong tolerance to freezing stress was observed in transgenic Arabidopsis plants that over-express CBF1 (DREB1B) cDNA under control of the cauliflower mosaic virus (CaMV) 35S promoter (5). The over-expression of the CBF3 (DREB1A) also resulted in higher tolerance to drought, high-salt, and freezing stress (6-8). Novillo reported that CBF2/DREB1C negatively regulates the expression of CBF1/DREB1B and CBF3/DREB1A, which guarantees the proper induction of downstream genes and the accurate development of Arabidopsis tolerance to freezing and related stresses (9). In contrast to the three identified CBF/DREB1 homologs, which were induced under cold stress, but not under ABA treatment, CBF4 gene expression was up-regulated by drought stress and ABA treatment, but not by low temperature. Over-expression of CBF4 in transgenic Arabidopsis plants improved tolerance to freezing and drought stress (10). In rice, the OsDREB1-type proteins were defined as those that show high homology to Arabidopsis DREB1A proteins (11). The expression of both OsDREB1A and OsDREB1B was induced by cold. Transgenic rice plants over-expressing the OsDREB1A genes showed growth retardation under normal growth conditions and improved tolerance to drought, high-salt and low-temperature stresses like the transgenic Arabidopsis plants over-expressing OsDREB1A (11, 12). The over-expression of these DREB-type transcription factors in transgenic Arabidopsis plants induced expression of Arabidopsis CBF-targeted genes involved in cold acclimation and drought adaptation (10).

In this study, we report the OsDREB1D, a gene encoding a protein that is the closest homolog of CBF/DREB1 in rice. However, the expression of OsDREB1D is not detected in rice plants, either with or without stress treatment. The over-expression of OsDREB1D under the CaMV 35S promoter resulted in the tolerance to freezing and high-salt stress in transgenic plants. The transgenic plants are insensitive to ABA.

Keywords: Abiotic stress, Arabidopsis, DREB-type transcription factors, Rice, Transgenic plants

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*Corresponding author. Jian-Min Chen, Tel: 86-514-87979286; Fax: 86-514-87979286; E-mail: jinch@yzu.edu.cn, Quan-Hong Yao, Tel: 86-21-62203180; Fax: 86-21-62205704; E-mail: yaoquanhong@saas.sh.cn
#These authors contributed equally to this work.
RESULTS

Identification and phylogenetic analysis of the OsDREB1D

Dubouzet searched the rice genome database with amino acid sequence of the DREB1A protein, and found a genomic clone showed high homology in the conserved region (accession No. AP023482, nucleotide 1489-2250). The gene was named as OsDREB1D. Of interest, that OsDREB1D mRNA was not detected in rice plants either with, or without stress treatments (11). We amplified it by PCR, and cloned it from rice genome DNA. OsDREB1D contained an open reading frame of 253 amino acids.

We were unable to detect the OsDREB1D mRNA in any organs including roots, leaves, shoots, growing points, spike at late bolting stage and seeds of rice (Oryza sativa L.) with, or without stress treatments. To predict the function of OsDREB1D, phylogenetic analysis was carried out based on the similarities of AP2 domains in AP2/EREBP proteins from rice and Arabidopsis (Supplementary Fig. 1). The OsDREB1D was closest to OsDREB1A on the phylogenetic tree. These two genes show 70% identity at the amino acid level over the entire ORFs (data not shown), and they are the highest homologs in the genes included in the phylogenetic tree. It seems likely that OsDREB1D and OsDREB1A may be orthologous and play similar roles under stress conditions.

Over-expression of OsDREB1D gene improved stress tolerance in transgenic Arabidopsis

The high degree of sequence similarity between OsDREB1D and OsDREB1A suggested that the proteins were probably functional homologs. To test this hypothesis, the OsDREB1D gene was constitutively over-expressed under the control of the CaMV 35S promoter in transgenic Arabidopsis plants. Three independent homozygous T3 transgenic lines, named as 8217-1, 8217-2, 8217-4, were chosen to undergo physiological experiments. Although the expression levels of OsDREB1D were different in each independent line, it was readily detectable (Fig. 1A). We treated them with several stresses including cold, heat, high-salt, and drought. The transgenic plants and wild-type plants showed no difference when treated by heat stress or drought stress (data not shown). For high-salt tolerance analysis, seeds of the transgenic lines and wild-type plants were germinated on MS media containing 0, 75 and 100 mM NaCl, respectively, at 22°C. The germination rates of the transgenic and the wild-type lines were compared. Seeds were considered to have germinated when the seed coat was broken. After 5 d in 75 mM NaCl, 65% to 80% seeds of three selected transgenic lines germinated, compared to 40% of wild-type seeds. In medium containing 100 mM NaCl, 40% to 50% seeds of transgenic lines germinated in contrast to only 20% of wild-type seeds. High-salt stress inhibited germination in the wild-type
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seeds more severely than in all transgenic plants. The germination rates of transgenic plants on the plates without salt were almost the same as those of the wild-type plants (Fig. 1B-D). These results indicated that the transgenic lines displayed strong tolerance against high-salt stress.

Three-week-old plants were used to compare the degree of cold tolerance between transgenic and wild-type lines. After cold acclimation, the plants were placed under normal growth conditions for one week. The constitutive over-expression of the OsDREB1D gene resulted in an increase in cold tolerance (Fig. 2). The degree of cold tolerance was correlated with the level of OsDREB1D expression, in which the 8217-2 and 8217-4 transgenic lines exhibited higher level of expression and greater cold tolerance than the 8217-1 plants. These results indicate that the OsDREB1D protein also plays an important role in cold tolerance.

DNA-binding activity analysis of OsDREB1D using yeast one-hybrid assays
As OsDREB1D belonged to the DREB subgroup, we tested the DRE-binding activity using yeast one-hybrid assays. A similar motif was identified as C-repeat (CRT) and low temperature-responsive element (LTRE) in cold-inducible genes (13, 14). For the 35S:OsDREB1D enhances the cold tolerance, the binding activity between OsDREB1D and the cis-element LTRE was also tested. The yeast containing only bait plasmid pG221 did not display blue while the yeast containing bait plasmid pG221 and the expression plasmid display blue (Fig. 3A). This result demonstrated that OsDREB1D can bind with both DRE and LTRE.

OsDREB1D activates the expression of cold-responsive genes
To elucidate the molecular mechanism of OsDREB1D in the cold response, we monitored the expression of cold-responsive genes identified in the regulated pathways by real-time PCR analysis. Under 4°C cold treatment for 24 h, the expression of the tested marker genes, including RD29A, COR15A, KIN1, showed significant induction in both wild-type plants and transgenic plants under cold-stress conditions, consistent with

![Fig. 2. Cold tolerance analysis of 35S:OsDREB1D transgenic Arabidopsis plants. Control: three-week-old plants grew under normal conditions; Cold stress: the wild-type and the transgenic plants were placed under –20°C for 30 min, and then recovered under normal conditions for 10 d. The survival rates of the wild-type plants and the transgenic plants were shown under the Fig.](image)

![Fig. 3. Analysis of DNA-binding activity and transcriptional activation activity of OsDREB1D protein. (A) Yeast one-hybrid analysis for DNA-binding activity analysis of OsDREB1D. The yeast strain EGY48 was transformed with the constructs indicated (left). The expression of β-galactosidase was determined using filter-lift assay (right). (B-D) To investigate the transcriptional activation activity of OsDREB1D protein, expression patterns of stress responsive genes were carried out using real-time PCR. Total RNA was extracted from two-week-old plants grown under cold treatment for 24 h. Transcript levels of RD29A (B) KIN1 (C) and COR15A (D) under 4°C for 24 h were measured by real-time RT-PCR (black bars). Actin was used as an internal control. Data represent means and SES of three replicates.](image)
previous studies (1, 15-18). The expression of these three genes in OsDREB1D-overexpressed transgenic plants was substantially higher than that in wild-type plants (Fig. 3B-D). The variations in the gene expressions among the lines are consistent with the different expression of the target genes. As the three cold-inducible genes contain the DRE element in their promoter regions and have been identified as downstream genes of AtCBF3 in Arabidopsis (19), over-expression of OsDREB1D increases expression of RD29A, COR15A, KIN1, all of which are involved in plant tolerance.

**ABA insensitive of transgenic plants**

ABA plays an important role in the tolerance response of plants to drought and high salinity. Exogenous application of ABA also induces a number of genes that respond to dehydration and cold stress (20, 21). However, the role of ABA in cold stress-responsive gene expression is not clear. To explore whether OsDREB1D responds to exogenous ABA, we analyzed the sensitivity of the transgenic plants to ABA. First we analyzed the germination rates of the transgenic and wild-type plants on the MS plates containing 0, 1.2, 2.0 μM ABA, respectively. ABA inhibited the germination in the wild-type plants more severely than in the transgenic plants (Fig. 4A). After three weeks, the effect of ABA on the growth of these transgenic plants compared with that of the wild-type plants was also observed. The roots of the transgenic plants were much longer than those of the wild-type plants (Fig. 4B). These results indicate that the OsDREB1D protein does respond to exogenous ABA and may be involved in ABA-dependent pathway.

**DISCUSSION**

The over-expression of OsDREB1D conferred cold and high-salt tolerance and ABA insensitiveness in transgenic Arabidopsis

In this study, we reported a new DREB-type transcription factor in rice, OsDREB1D. To study its function, the OsDREB1D gene was over-expressed in Arabidopsis. The transgenic plants increased the cold and high-salt tolerance. The similar phenotypes have been observed for the constitutive over-expression of CBF1 (5), CBF3 (6-8) and OsDREB1A (11, 12), indicating that OsDREB1D is, in fact, a new member of the CBF/DREB1 family. Interestingly, the level of cold tolerance that can be achieved by OsDREB1D over-expression is very similar to that of the OsDREB1A gene. And the over-expression of OsDREB1D resulted in constitutive expression of COR15A, RD29A and KIN1, which also happened in over-expression of OsDREB1A (11), suggesting that these two genes may have redundancy in function.

Most CBF/DREB1-type transcription factors have been reported not to be induced by ABA treatment and they are involved in the ABA-independent pathway (15, 22-24). However, recent studies revealed that ABA-dependent pathway can also involve the CRT/DRE elements and AP2-type transcription factors (25, 10). In the ABA treatment assay, we found that the transgenic plants over-expressing the OsDREB1D were less sensitive to ABA than the wild-type plants. The result demon-
strates again that the CRT/DRE elements are also involved in ABA signal transduction.

**Why the transcripts of OsDREB1D cannot be detected in rice?**

As mentioned above, the mRNA of OsDREB1D cannot be detected in any organs of rice. However, an independent group submitted the cDNA sequence for OsDREB1D to GenBank (AF243384.1). Therefore, it is possible that OsDREB1D is expressed in a different specific growth stage or organ other than those found in our experiment. We attempted to over-express OsDREB1D in rice by Agrobacterium-mediated transformation. The transgenic rice plants were confirmed by expression of the GUS gene via histochemical staining, which is an adjacent reporter gene with the cloned OsDREB1D within the T-DNA region. However, we were unable to detect any transcripts of OsDREB1D from transgenic plants (data not shown). On the one hand, the transgenic plants are likely false positive. On the other hand, there may be a special mechanism to control the expression of OsDREB1D. It is likely that there was a certain miRNA which degraded the mRNA once the OsDREB1D was transcribed. However, we did not find any possible miRNA from the DNA sequence of OsDREB1D. In plants, the DNA methylation of promoter regions usually inhibits transcription. In some cases, DNA methylation controls the overall level of expression from a family of repeated genes (26). For example, the expression of tryptophan biosynthesis genes in the Phosphoribosylanthranilate isomerases (PAI) family can be modulated by DNA methylation. In some A. thaliana accessions, a PAI1-PAI4 inverted repeat triggers the silencing of the homologous PAI2 gene (27). If the expression of OsDREB1D is really silenced, it may be related with DNA methylation. What’s more, phylogenetic analysis revealed that OsDREB1D and OsDREB1A are the closest genes on the phylogenetic trees. And the over-expression of OsDREB1D and OsDREB1A in Arabidopsis showed that the function of these two genes was very similar. We predicted that the mRNA of OsDREB1D was likely to be degraded in particular developmental stage for its redundant function.

**MATERIALS AND METHODS**

**Phylogenetic analysis**

The nucleotide and amino-acid sequences were compared with those released in GeneBank databases by using the BLAST program, and associated molecular information were analyzing by BioEdit and Clustal W.

**Generation of transgenic Arabidopsis**

The incubation and growth conditions of A. thaliana were the same as described by Zhang et al. (28). The full length OsDREB1D DNA was amplified from rice genome DNA. Reactions were performed using the PCR primers 5’-CCACAGAACAGTACCGTAC TCC-3’; 5’-GGAAAAAGTACTCCCTCCAGAGCC-3’. The binary vector pYF7716 derived from pCAMBIA-1201, in which there are two SAR regions at each side of the expression unit and an Intron-kanamycin resistance gene (29) as marker gene under control of CaMV35S and TMV Omega leader sequence, was used in transformation of Arabidopsis. Subsequently, the OsDREB1D DNA digested with Sac I and Bam HI was cloned into the binary vector pYF7716 digested with Sac I and Bam HI at the MCS site under the control of enhanced double CaMV 35S promoter (30), and the tobacco mosaic virus (TMV) Ω sequence (31) was inserted upstream of the DNA to increase the translation level, to form the transformed plasmid. The constructs were introduced into A. tumefaciens GV3101 by electroporation. A. thaliana cv. Columbia was transformed by floral dip method as described previously (28).

**Stress tolerance analysis and germination assay of transgenic Arabidopsis plants**

Cold treatment was performed by transferring the seedlings grown at 22°C to a temperature of −4°C ± 1 for 24 h for cold acclimation, then the seedlings were transferred to a temperature of −20°C for 30 min. The plants were recovered under normal condition for 10 d. To detect the expression of downstream genes, the two-week-old transgenic plants and wild-type plants were treated under 4°C for 24 h. For germination assay, the sensitivity of seed germination to ABA and NaCl was assayed on MS agar plates saturated with ABA and NaCl solution (32). Seeds were considered germinated when radicles completely penetrated the seed coat. Germination was scored daily up to 10 d after seeds were placed at room temperature. We repeated cold-tolerance experiments and germination assays three times.

**Semi-quantitative RT-PCR and quantitative real-time PCR**

A part of OsDREB1D gene with 264 bp fragment was amplified using two specific primers (5’-CGGCGGGGTAAAGAAGTG TGTTC-3’; 5’-CATGAGTCTCCGGGAGGAAGTTG-3’) according to the sequence of OsDREB1D gene. Semi-quantitative RT-PCR was assayed as described previously (33). For real-time PCR, triplicate quantitative assays were performed on 1 μl of each cDNA dilution with the SYBG GreenMaster mix and an ABI 7,900 sequence detection system according to the manufacture’s protocol (Biorad). The relative quantification method (Delta-Delta CT) was used to evaluate quantitative variation between the replicates examined. The amplification of Actin was used as an internal control to normalize all data. Gene-specific primers for RD29A, 5’-AAGGAAAAGCAGGACAAGAGA-3’; 5’- CCACACCCCAACCAGCCAGA-3’; for COR15A, 5’-TAAAGCA GGAGTAGCTAAGGA-3’; 5’-AGAATGACGTTAGCAGTCTTG A-3’; for KIN1, 5’-TGCCCTTCAAAGGTCCTGA-3’; 5’-AGGC CGGTCTTTGCCATC-3’. The bait plasmid pG221 and the expression plasmid pPC86 were reconstructed as described previously (34). The plasmid pG221 was first transformed into yeast EGY48 using lithium
acetate protocol as described by Gietz and Woods (35), and then the plasmid pPC86 was transformed into the yeast containing bait plasmid pG221. Then transformed yeast was overlaid onto media containing X-gal (5-bromo-4-chloro-3-indolyl b-D-galactoside) using nitrocellulose filters and tested the β-galactosidase activity.

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