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A Study on Cloning and Expression of Stress Induced DREB Transcription Factor Gene from Rice (*Oryza sativa*)

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The transcription factors DREBs/CBFs specifically interact with the dehydration-responsive element/C-repeat (DRE/CRT) cis-acting element (core motif: G/ACCGAC) and control the expression of many stress inducible genes in Arabidopsis. In rice, we isolated five cDNAs for DREB homologs: OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D, and OsDREB2A. Expression of OsDREB1A and OsDREB1B was induced by cold, whereas expression of OsDREB2A was induced by dehydration and high-salt stresses. The OsDREB1A and OsDREB2A proteins specifically bound to DRE and activated the transcription of the GUS reporter gene driven by DRE in rice protoplasts. Over-expression of OsDREB1A in transgenic Arabidopsis induced overexpression of target stress-inducible genes of Arabidopsis DREB1A resulting in plants with higher tolerance to drought, high-salt, and freezing stresses. This indicated that OsDREB1A has functional similarity to DREB1A. However, in microarray and RNA blot analyses, some stress-inducible target genes of the DREB1A proteins that have only ACCGAC as DRE were not over-expressed in the OsDREB1A transgenic Arabidopsis. The OsDREB1A protein bound to GCCGAC more preferentially than to ACCGAC whereas the DREB1A proteins bound to both GCCGAC and ACCGAC efficiently. The structures of DREB1-type ERF/AP2 domains in monocots are closely related to each other as compared with that in the dicots. OsDREB1A is potentially useful for producing transgenic monocots that are tolerant to drought, high-salt, and/or cold stresses.

Introduction

Plants are always exposed to multiple stresses such as biotic and abiotic stresses during their life cycle. Being sessile in the environment, plants have adapted to environmental stresses via morphological and physiological changes. These include the efficiency with which the plant draws water from surrounding soil, the water retaining capacity within plant tissues or cells, control of water loss from transpiration through stomatal pores, and developmental adaptations to avoid seasonal water shortage during flowering (Zhu *et al.,* 2002; Shinozaki *et al.,* 2003). Agricultural production and quality are adversely affected by a broad range of abiotic stresses including drought, salinity, heat, and cold. Especially when these stresses occur in combination, it can have devastating effects on plant growth and productivity. It is estimated that more than 50% of worldwide yield loss for major crop are caused by abiotic stresses (Shao *et al.,* 2009; Ahuja *et al.,* 2010; Lobel *et al.,* 2011). Many researchers worldwide have reached a consensus that breeding stress-tolerant crops with higher yields and improved qualities against multiple environmental stresses is an effective strategy, as well as one of the greatest challenges faced by modern agriculture (Takeda and Matsuoka, 2008; Newton *et al.,* 2011; Liu *et al.,* 2004).Two main approaches have been employed to this process. One is traditional breeding methods which often bring about unpredictable results. Another is modern transgenic technology by introducing novel exogenous genes or altering the expression levels of endogenous genes to improve stress tolerance. Since conventional breeding approaches have marginal success due to the complexity of stress tolerance traits, the transgenic approach is now being popularly used to develop transgenic crops tolerant to abiotic stresses (Yamaguchi-Shinozaki and Shinozaki, 2006). Therefore, deciphering the molecular mechanisms by which plants perceive and transduce stress signals to cellular machinery to initiate adaptive responses is an essential prerequisite for identification of the key genes and pathways to engineer stress-tolerant crop plants (Sanchez-Rodriguez *et al.,* 2011). Transcription factors are master regulators of gene expression and a single transcription factor can control the expression of many target genes by specifically binding to cis-acting elements in the promoter regions of specific target genes (Nakashima *et al.,* 2009)

Rice (*Oryza sativa.* L) is the staple food for half of the world's population. Rice production was dramatically affected by severe drought occurred every year in rainfed rice-growing areas worldwide (Pandey, 2009). In rice DREB1A/CBF2 DREB1B/CBF1, DREB1C/ CBF3, and DREB2A, DREB2B, are the six major DREB transcription factors, which can be classified into two groups namely DREB1 group functioning in cold and high salinity and DREB2 group showing gene expression in response to drought. Overexpression of OsDREB1A could enhance abiotic stress tolerance in Arabidopsis (Sakuma *et al.,* 2002). OsDREB1F is highly induced by drought stress and exogenous ABA application and overexpression of OsDREB1F is reportedly to lead to enhanced tolerance to salt, drought, and low temperature in both rice and Arabidopsis. In addition, higher expression of OsDREB1G in transgenic rice plants could significantly improve their tolerance to water deficit stress (Lim *et al.,* 2007). Similarly, DREB2-type TFs are involved in a conserved regulatory mechanism in several crop plants in response to drought, salinity, and heat stresses (Lata and Prasad, 2011; Nakashima *et al.,* 2012).

With this viewpoint, the present study was undertaken on cloning and expression analysis of DREB transcription factor from rice (*Oryza sativa*) under abiotic stress conditions.

Materials and Methods

Chemicals

The materials, media, reagents used for this study were procured from Sigma-Aldrich, SRL and Hi-Media, India.

Isolation and Amplification of DREB Gene by PCR

Plant Germination and Stress Treatment in Rice

The rice seeds were germinated at 25°C in the dark and the 15-day-old etiolated seedlings were then placed in a 48°C chamber for 24 h (cold), exposed to air drying for 10 h (dry), or dipped in 250 mM NaCl for 10 h (NaCl). For time course analysis of OsDREB expression, rice seeds were germinated and grown in distilled water at 25°C for 17 days in a growth chamber set at 16/8 h light/dark cycle. The seedlings were then exposed for 0, 10, 20, 40, 60 min, 2, 5, 10, and 24 h to the following treatments: dry, air-drying on filter paper in a room; cold, chilling at 48°C in a growth chamber; NaCl, saline solutions containing 250 mM NaCl; Abscisic acid (ABA), solutions containing 100 mM ABA, or water, transferred to trays containing fresh, distilled water. For the wounding treatment, the seedling leaves were pruned to a height of 8-10 cm. After stress treatment for 24 hours' control and stress exposed tissue were harvested and should be stored at particular manner.

cDNA cloning of OsDREB

The total RNA was extracted from collected samples using the standard TRIzol protocol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was then synthesized using the PrimeScript™ RT reagent (Takara, Shiga, Japan) and a poly(T)-adaptor primer. An alignment of the DREB genes of model plants such as *Arabidopsis thaliana*, *Brachypodium distachyon*, revealed the AP2/ERF domain was relative conservative. Accordingly, based on the conserved AP2/ERF sequences, a cDNA fragment of OsDREB from the *A. sativa* cultivar was then PCR-amplified using the cDNA as template, and a degenerate primer pair (Forward primer - 50-GGGGAAT TCATGGAGCGGGGGGAGGGGAG-30; Reverse primers - 50-GGGCTCGAGAT CCTTCTCATCAGAAGGTA-30) was used to amplify the first fragment of the target gene from the cold-treated, dry and salt stress leaf samples. The PCR conditions were as follows: pre-heating at 94°C for 3 min; 30 cycles at 94°C for 30s, 50°C for 30s and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were separated by agarose gel electrophoresis, and the target DNA fragment was recovered using a gel extraction kit (Axygen, Corning, NY, USA). The recovered DNA fragments were then ligated into a pMD19-T vector (Takara). DNA fragments were sequenced. The full-length cDNA sequence was isolated using RACE-PCR. The 3'- and 5'- ends of the gene were amplified by using the 3'-Full RACE Core Set Ver.2.0 kit (Takara) and the 5'-Full RACE kit (Takara). For 3'-RACE PCR, the reaction conditions were as follows: preheating at 94°C for 3 min; 30 cycles at 94°C for 30 s, 50°C for 30s, and 72°C for 1 min; and a final extension at 72°C for 10 min. For 5'-RACE PCR, the reaction conditions were as follows: pre-heating at 94°C for 3 min, 30 cycles at 94°C for 30s, 50°C for 30s, and 72°C for 1 min; and a final extension at 72°C for 10 min. DNA recovery and ligation were the same as described above.

Preparation of Fusion Proteins and Gel Mobility Shift Assay

A 477-bp OsDREB1A was amplified by PCR from the OsDREB1A cDNA. A 439-bp fragment in OsDREB2A was amplified by

PCR and cloned into the EcoRI and XhoI sites of the pGEX-4T-1 vector.

Escherichia coli XL-1 Blue was transformed with these plasmid constructs. The glutathione S-transferase fusion proteins were produced, purified, probe labeled, and assayed by gel mobility.

Transactivation Experiments with Rice Protoplast

The ORFs for the DREB and OsDREB cDNAs were inserted into the plant expression vector p35S-shD-stop; this plasmid has a CaMV 35S promoter, internally deleted first intron of Sh1, multiple cloning sites, stop codons in three frames, and a nopaline synthase terminator. An ubiquitin–luciferase plasmid served as an internal control to normalize GUS values.

We inserted two contiguous copies of the 75-bp promoter region of rd29A containing the DRE/CRT motif just before the minimal TATA box of the pIG46 GUS reporter construct reported by Hobo *et al.,* (Hobo *et al.,* 1999).

Suspension culture of a rice cell line (Oc), protoplast isolation, and electroporation were performed according to Hattori *et al.,* (Hattori *et al.,* 1999). The transactivation data is based on two independent transformations conducted at a 1-week interval.

Transgenic Plants Overexpressing the OsDREB cDNAs

Plasmids used in the transformation of Arabidopsis were constructed with the full-length OsDREB1A or OsDREB2A cDNAs cloned into a polylinker site of a binary vector pBE2113 as described by Liu *et al.,* (Liu *et al.,* 2014). The constructs were introduced into *Agrobacterium tumefaciens* C58. Plants were transformed by the vacuum infiltration method.

Arabidopsis Full-length cDNA Microarray Analysis

Total RNA was isolated using TRISOL reagent (Gibco BRL, Maryland, USA). mRNA was prepared using MACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Glabach, Germany).

Preparation of fluorescent probes, microarray hybridization, and scanning. Lambda control template DNA fragment (Takara, Kyoto, Japan) was used as an external control to equalize hybridization signals generated from different samples. To assess the reproducibility of microarray analysis, each experiment was repeated three times.

Freezing and High-salt Stress Tolerance of Transgenic Plants

Plants were grown in9-cm pots filled with a 1:1 mixture of perlite and vermiculite. They were grown under continuous illumination of approximately 2500 Lux at 28°C.

Freezing stress was conducted by transferring the 4-week-old plants into a chamber at 28°C, decreasing temperature in the chamber at 68°C, and exposing the plants to 68°C for 30 h, then returning to 28°C for 5 days.

High salt stress was created by soaking 3-week-old plants grown on agar plates and gently pulled out of the growing medium in 600 mM NaCl solution for 2 h. The plants were then transferred to pots under normal growing conditions for 3 weeks. The numbers of plants that survived and continued to grow were counted. Then repeated experiments on salt- and freezing stress tolerance for five and three times, respectively.

Results and Discussion

Identification of DREB1A and DREB2A Homologs in Rice

When compared the amino acid sequences of six Arabidopsis DREB1-related proteins and found high homology in the bipartite nuclear localization signal at their N-terminal regions and in some parts of their C-terminal regions as well as their ERF/AP2 DNA-binding domains. The DREB type proteins of rice are defined as those that show high homology to Arabidopsis DREB1A proteins in these conserved regions. OsDREB1C and OsDREB1D contained an open reading frame of 253 and 214 amino acids and encoded a putative protein with a predicted molecular mass of 27.7and23.1 kDa, respectively. The region most similar to DREB1A of OsDREB1C was used to probe cDNA libraries prepared from cold and salt-stressed rice seedlings, and then identified a different DREB1-type sequence that we subsequently labeled OsDREB1A. Then compared the amino acid sequence of the OsDREB1 proteins with that of several DREB1-related proteins below Figure1a. These proteins shared extensive homology even outside the ERF/AP2 DNA-binding domain. By founding homology in the nuclear localization signal at their N-terminal region and in some parts of their C-terminal region such as a characteristic LWSY motif. Then compared the amino acid sequence of the OsDREB2A protein with those of several DREB2-related proteins. These proteins shared high sequence homology not only in the DNA-binding domain but also in the N-terminal region show in below figure1b.

Expression and Identification of the OsDREB Genes by RNA Gel Blotting

The expression patterns of the OsDREB genes were analyzed by RNA-gel blot hybridization. By prepared the probes consisting of the 30-terminal region of the OsDREB genes genomic DNA was digested with BglII, EcoRI, EcoRV, SacII, XbaI, or XhoI and hybridized using the 30-terminal regions of the OsDREB genes as probes. The OsDREB1A probe generated single hybridization signals under the high stringency condition. The corresponding probe from OsDREB1B also showed single fragments.

The OsDREB1C probe generated a very weak band from the BglII digest and only smears were detected in the rest of the lanes containing digested DNA. These results indicate that the probes of OsDREB1A and OsDREB1B can detect a single gene but the probes of OsDREB1C and OsDREB1D are insufficient for analyzing the expression of a single gene. The 30 probe of the OsDREB2A gene hybridized to distinct single bands in the genomic DNA gel blots, under both low and high stringency conditions. This indicates that, unlike the DREB2 genes of Arabidopsis, OsDREB2A does not have a closely related homolog in the rice genome and the OsDREB2A probe can be used to detect a single gene. Den by monitored the transcription of OsDREB1A, OsDREB1B, OsDREB1C, and OsDREB1D in response to various stress factors by RNA-gel blot hybridization (Figure 2).

The OsDREB1A and OsDREB1B genes were not responsive to exposure to ABA but they were induced within 40 min after exposure to cold stress. OsDREB1A was also induced within 5 h after salt treatment and was transiently expressed in wounded seedlings. The probe of OsDREB1C hybridized to all blots, indicating constitutive expression of this gene. To confirm this result, we carried out RT-PCR analysis using a specific primer of the OsDREB1C gene. The responses of OsDREB2A to various stress treatments were analyzed using its 30terminal region as a probe. The expression of OsDREB2A is

faintly detectable in control plants and those exposed to cold stress whereas OsDREB2A was gradually induced within 24 h after dehydration and salt treatments. Like DREB2A, OsDREB2A is faintly detectable in plants transferred to ABA solutions or to distilled water (Nakashima *et al.,* 2000).

Overexpression of OsDREB1 and OsDREB2 in Arabidopsis

To compare the function of OsDREB1A and OsDREB2A with that of DREB1A and DREB2A, by generated transgenic plants in which the OsDREB1A or OsDREB2A cDNA was over expressed (35S:OsDREB1A and 35S:OsDREB2A). Transgenic plants of the T2 generation were used for further analyses. By carried out RNA-gel blot analysis to check the overexpression of OsDREB1A and OsDREB2A in the transgenic Arabidopsis. The OsDREB1A gene was over-expressed in five of the six 35S:OsDREB1A transgenic plants and expression of the OsDREB2A gene was detected in 13 of the 14 35S:OsDREB2A transgenic plants. These lines were used for further analyses. Growth of the 35S:OsDREB1A transgenic plants was compared with that of control plants carrying pBI121 vector (wild type) and the 35S:DREB1A plants over-expressing Arabidopsis DREB1A at 33 days after sowing. Previously, we reported that the 35S:DREB1A transgenic Arabidopsis showed various levels of growth retardation. Some of the transformants showed severe growth inhibition and were classified as 35S:DREB1Aa plants. Transformants exhibiting weaker growth retardation were classified as either 35S:DREB1Ab (moderate phenotypic changes) or 35S:DREB1Ac (mild phenotypic changes) plants. Although three plants expressed the OsDREB1A gene strongly and two plants expressed the OsDREB1A gene weakly, all the five 35S:OsDREB1A plants growing in pots showed mild growth retardation, especially in bolting timing like that of the 35S:DREB1Ac plants (Figure 5). By contrast, the 35S: OsDREB2A plants showed little phenotypic change like 35S:DREB2A plant salt though OsDREB2A was over-expressed strongly in the plants.

Analysis of Effect of Over-expression of OsDREB1A on the Expression of the Target Genes of DREB1A in Transgenic Arabidopsis

To analyze whether over-production of the OsDREB1A protein caused the expression of the target genes of DREB1A in plants without stress treatment, used a full-length cDNA microarray containing 7000 Arabidopsis full-length cDNAs (Seki *et al.,* 2002). mRNAs prepared from the 35S:OsDREB1A and control plants were used for the generation of Cy3-labeled and Cy5-labeled cDNA probes, respectively. These cDNA probes were hybridized with the cDNA microarray, and the expression profiles of the 7000 genes were analyzed. By chose genes with an expression ratio greater than 2.5 times in the 35S: OsDREB1A transgenic plants than in wild-type control plants as candidates for the OsDREB1A target genes. Six candidate genes were identified by the cDNA microarray analysis (Table 1).

Then performed RNA-gel blot analysis to confirm the results obtained by the cDNA microarray. analyzed the expression of six genes identified by the cDNA microarray and that of the other four target genes of DREB1A in the transgenic and the wild-type plants. Also found that all six identified genes were clearly overexpressed in the 35S:OsDREB1A plants (Figure 4). Some of the DREB target genes such as kin1, kin2, and erd10, containing ACCGAC but not GCCGAC as the DRE core motifs in their promoter, were not over-expressed in the 35S:OsDREB1A plants

(Figure 4), although these genes were over-expressed strongly in the 35S:DREB1A plants (Kasuga *et al.*,). The rd29B, rd22, and erd1 genes that do not contain typical DRE in their promoters were not over expressed in the 35S:OsDREB1Aor35S:DREB1A transgenic plants. These results indicate that the OsDREB1A protein binds to GCCGAC more preferentially than to ACCGAC in the promoter region of stress-inducible genes and activates their expression in the transgenic plants.

Competitive DNA-binding Assay of OsDREB1A and DREB1A

By performed a gel mobility shift assay with DNA fragments containing the GCCGAC or ACCGAC sequences as competitors to analyze the difference in the binding affinity between OsDREB1A and DREB1A. The extent of binding of OsDREB1A was reduced more effectively by the addition of the unlabeled DNA fragment containing GCCGAC than by the addition of the unlabeled DNA fragment containing ACCGAC in the Figure 5. By contrast, in DREB1A, the competition by the DNA fragment containing GCCGAC was almost the same as that by the DNA fragment containing ACCGAC (Figure 5). OsDREB1A binds preferentially to GCCGAC rather than to ACCGAC, whereas DREB1A binds to both GCCGAC and ACCGAC efficiently.

Tolerance of the 35S OsDREB1A Transgenic Plants to Freezing and High-Salt Stress

The tolerance to freezing and high-salt stresses of the 35S:OsDREB1A plants was compared with those of the wild-type and 35S:DREB1Ac plants (Figure 6). We have shown that the 35S:DREB1Ac plants had the lowest level of stress tolerance among the 35S:DREB1A transgenic Arabidopsis plants. The plants were grown in pots at 228C for 4 weeks, exposed to a temperature of 68°Cfor30 h, and returned to 22°C for 5 days. While 17% of the wild-type plants survived this treatment, the 35S:OsDREB plants were highly tolerant to the freezing stress (45 or 28% survived) like the 35S:DREB1Ac plants (62% survived; Figure 6). Plants grown on agar plates were removed from the plates, soaked in 600 mM NaCl solution for 2 h and grown in pots for 3 weeks to examine the tolerance of the transgenic plants to high-salt stress (Figure 6). While 26% of the wild-type plants survived, 72 and 66% of the 35S:OsDREB1Aa and 35S:OsDREB1Ab plants survived, respectively. The 35S:DREB1Ac plants showed high-salt stress tolerance similar to that of the 35S:OsDREB1Aa plants. These results indicate that the 35S:OsDREB1A plants had higher tolerance to freezing and high-salt stresses.

In this study isolated five rice DREB homologs, OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D, and OsDREB2A, to analyze the DREB/DRE regulon in monocots. The DREB1-type ERF/AP2 domains in these proteins in the monocots were closely related to each other but distant from those in the dicots (Figure 1a, 1b). The gel mobility shift assay revealed that the OsDREB1A and OsDREB2A proteins bound to the DRE sequence in the promoter region of rd29A specifically. However, the OsDREB1A protein bound to GCCGAC more efficiently than to ACCGAC as the DRE-related core motif (Figure 5). This observation was also supported by the analysis of over-expression of the target stress-inducible genes of DREB1A in the transgenic Arabidopsis over-expressing the OsDREB1A cDNA. Seven stress-inducible genes containing GCCGAC as the DRE related core motif in their promoter regions are overexpressed in the 35S:OsDREB1A transgenic plants (Table 1; Figure 4). By contrast, three stress-inducible

genes containing only ACCGAC as the DRE-related core motif in their promoter were not over-expressed. The Arabidopsis DREB1A protein binds to both ACCGAC and GCCGAC at the same efficiency, but the OsDREB1A protein prefers GCCGAC to ACCGAC as its binding site (Figure 5) (Sakuma *et al.,* 2002).

Previously, it was reported in the literature that two amino acids, the 14th valine and the 19th glutamic acid conserved in the ERF/AP2domains of the DREB proteins have important roles in the determination of DNA-binding specificity (Sakuma *et al.,* 2002; Liu *et al.,* 1998). The OsDREB1 proteins have a conservedvaline in the V14 position but do not have glutamicacid in the E19 position except OsDREB1C (Figure 1b). Valine is conserved in the E19 position of these OsDREB proteins. The difference in the DNA-binding specificitybetween the DREB1A and OsDREB1A proteins may bedue to this conversion of the amino acid (from glutamicacid to valine) in the DNA-binding domains. The other DREB1-type proteins in the monocots (barley, wheat, and rye) also have a conserved valine in the E19 position (Figure 1b). The DNA-binding specificity of these DREB1-type proteins may be similar to that of OsDREB1A. On the other hand, the OsDREB2A protein binds to both ACCGAC and GCCGAC at the same efficiency as DREB2A (Sakuma *et al.,* 2002).

The DREB1-type NLS consensus is PKRPAGRTKFRETRHP, and the presence of this sequence clearly distinguishes these DREB1-type proteins from the rest of the proteins that have the ERF/AP2 domain. Most of these DREB1-type proteins also display a well-conserved sequence (DSAW) at the end of their ERF/AP2 domains. The DSAW motif is not found in other types of proteins with the ERF/AP2 domains and its function is unknown. The LWSY motif is conserved at the end of the C-terminal of most of the DREB1-type proteins (Figure 1b). The DREB2-type proteins also share extensive homologous regions beyond the ERF/AP2 domain especially in the N-terminal region. These conserved regions probably play important roles in the function of these DREB1-type and DREB2-type proteins.

All OsDREB1 proteins except OsDREB1D in Figure 1b have short (4–5 aa residues) acidic sites in their C-terminal but the distribution pattern/location is not well conserved. The acidic sites in the C-terminal region may correspond to activation domains (Liu *et al.,* 1998; Stockinger *et al.,* 1997). The distribution of acidic sites in the C-terminals of OsDREB1A and OsDREB1B is similar to that of their counterparts in Arabidopsis. Like OsDREB1A and OsDREB1B, OsDREB1C has an acidic site just before the LWSY motif at its C-terminal. Only OsDREB1D has no distinct acidic site in its C-terminal region.

Expression of the OsDREB1A and OsDREB1B genes is induced by cold stress but not by exogenous ABA or drought stress (Figure 2). OsDREB1A was induced by high-salt stress and transiently induced by wounding stress (Figure 2). Arabidopsis DREB1C is also sensitive to wounding stress (Nakashima *et al.,* 2000). The responsiveness of OsDREB1A to NaCl and wounding stresses, in addition to its sensitivity to cold stress, imply that its role in the transduction of abiotic stress signals in rice is more similar to DREB1C rather than DREB1A in Arabidopsis. On the other hand, OsDREB1C was constitutively expressed in rice plants, which has a unique expression profile (Figure 2). We have reported expression of six DREB1-type genes in Arabidopsis and none of them is expressed constitutively (Sakuma *et al.,* 2002). OsDREB1D was used to probe the same RNA panels but no hybridization signal was detected (data not shown). Recently, 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 17-day-old seedlings. The OsDREB2A gene is weakly expressed in control plants and is induced by dehydration and high-salt stresses (Figure 2). As the OsDREB2A gene is not strongly upregulated by exposure to ABA, it probably participates in the ABA-independent stress signal transduction pathway as well as DREB2A in Arabidopsis as described in Liu *et al.,* (Liu *et al.,* 1998). This similarity in response to stress between the OsDREB2A and Arabidopsis DREB2 genes is altogether expected in view of their high level of sequence homology in their N-terminals and ERF/AP2 domains. Over-expression of the DREB1A cDNA in transgenic Arabidopsis plants activated the expression of many stress inducible genes and resulted in higher tolerance to drought, high salt, and freezing (Liu *et al.,* 1998; Kasuga *et al.,* 1999). Over-expressions of the OsDREB1A cDNA in transgenic Arabidopsis also induced expression of some stress-inducible genes such as rd29A, cor15a, and rd17 that have GCCGAC as the DRE core motif in their promoter regions (Figure 6). As the expression level of the OsDREB1A target genes was not so high in the 35S:OsDREB1A plants, only six genes were identified by cDNA microarray analysis. More target genes of OsDREB1A can be detected when the expression ratio is decreased. The 35S:OsDREB1A transgenic Arabidopsis revealed tolerance to freezing and high-salt stresses as well as growth retardation (Figures 3 and 6). The level of stress tolerance and growth retardation in the 35S:OsDREB1A plants was relatively lower than that in the 35S:DREB1A plants. This may be due to the difference in the number of the target stress-inducible genes between the3 5S:OsDREB1A and 35S:DREB1A plants. In addition, the relative expression levels of OsDREB1A did not always correlate with expression of theDREB1A target genes in the 35S:DREB1A plants, which may be caused by the overexpression of rice DREB homolog in transgenic Arabidopsis. We also generated transgenic Arabidopsis plants overexpressing the OsDREB2A cDNA (data not shown). However, over-expression of the OsDREB2A protein was not sufficient for the induction of the target stress-inducible genes. The OsDREB2A protein probably requires modification such as phosphorylation for its function like the Arabidopsis DREB2A protein (Liu *et al.,* 1998). A stress signal may be necessary for the modification of both DREB2A and OsDREB2A to their active forms in expression of the target stress-inducible genes. Both proteins contain a serine- and threonine-rich conserved region following the ERF/AP2 motif. On the other hand, both DREB2A and OsDREB2A could activate the GUS reporter gene driven by DRE in rice protoplasts. Removal of the cell wall is an extremely stressful treatment for plant cells, which activates many stress-responsive signal pathways. One of these pathways may have been induced during protoplast preparation leading to the activation of DREB2A and OsDREB2A. Transcription factors are powerful tools for genetic engineering because over-expression of a transcription factor can lead to the upregulation of the whole array of genes under its control. BLAST search has revealed several putative rice genes with probable DREB recognition sites in their promoter regions. On the other hand, we carried out rice cDNA microarray analysis to identify genes induced in response to drought, high-salt, and cold stresses in rice. Several known and well-characterized stress-inducible rice genes were identified by the cDNA microarray analysis.

Table.1 Significantly Upregulated Transcripts In 35s:Osdreb1a Plants[a]

Gene[b]	MIPS ID[c]	Ratio[d]	DRE(ACCGA[e]	DRE(GCCGA[e]	Annotation[f]	Norther[g]
cor15A	At2g4254	5.00	-417 to -422	-360 to -355	Cold-inducible protein	++
FL05-21-F13	At1g1685 0	3.91	-225 to -230	-70 to -65	Late embryogenesis abundant protein	+
			-133 to -138			
Rd29A	At5g5231	3.29	-275 to -270	-131 to -126	Hydrophilic protein	+
			-225 to -220		Drought-responsive	
			-168 to -163			
rd17	At1g2044	3.10	-995 to -990	-966 to -961	Dehydrin	+
			-161 to -156			
AtGolS3	At1g0935 0	2.83	-815 to -820	-391 to -396	Glycosyl transferase family 8	+
			-787 to -792			
FL05-20-N18	At2g4253 0	2.63	-748 to -753	-194 to -189	Cold-inducible protein, cor15b	+
kin1	At5g1596	1.77	-119 to -114		Cold-inducible protein	-
FL06-16-B22	At2g1597 0	1.54	-414 to -409	-805 to -800	Cold-acclimation protein	+
Kin2	At5g1597 0	1.43	-131 to -126		Cold-inducible protein, cor6.6	-
erd10	At1g2045	1.18	-965 to -960		Dehydrin	-
			-172 to -167			
			-168 to -163			
			-164 to -159			

[a]mRNAs from 35S:OSDREB1A and pbi121 plants were used for preparation of Cy3-labeled and Cy5-labeled cdna probes. These cdna probes were mixed and hybridized with the cdna microarray. In this study, we used the lambda DNA as an internal control because its flourescence level is almost the same in both the plants.
[b]Gene names are full-length cdna clones (Seki et al., 2002).
[c]MIPS ID for cDNAs used in this study.
[d](Flourescence intensity of each cdna of 35S:OsDREB1a/flourescence intensity of each cdna of pbi121) (Flourescence intensity of lambda DNA of 35S: OsDREB1a/flourescence intensity lambda DNA of pbi121).
[e]DRE sequence (ACCGAC or GCCGAC) observed in 1000 nucleotides existing upstream of the 50-termini of the longest cDNA clones isolated are listed. Numbers indicate the numbers of nucleotides beginning at the 50-termini of the longest cDNA clones isolated. Minus sign means that the nucleotide exists upstream of the 50-termini of the putative transcription site.
[f]Annotations are listed based on results of searches with protein sequences against Pfam library. [g]Signs of plus indicate that accumulation of transcripts was certified by northern hybridization. Signs of minus indicate that we cannot certify accumulation of transcripts by Northern hybridization.

Fig.1a Alignment Of Amino Acid Sequences Of The Osdreb1- And Dreb1-Related Proteins. The Yellow Box Indicates Erf/Ap2 Domains And Asterisks Indicate The Well-Conserved Valine And Glutamic Acid Among The Dreb-Related Proteins.

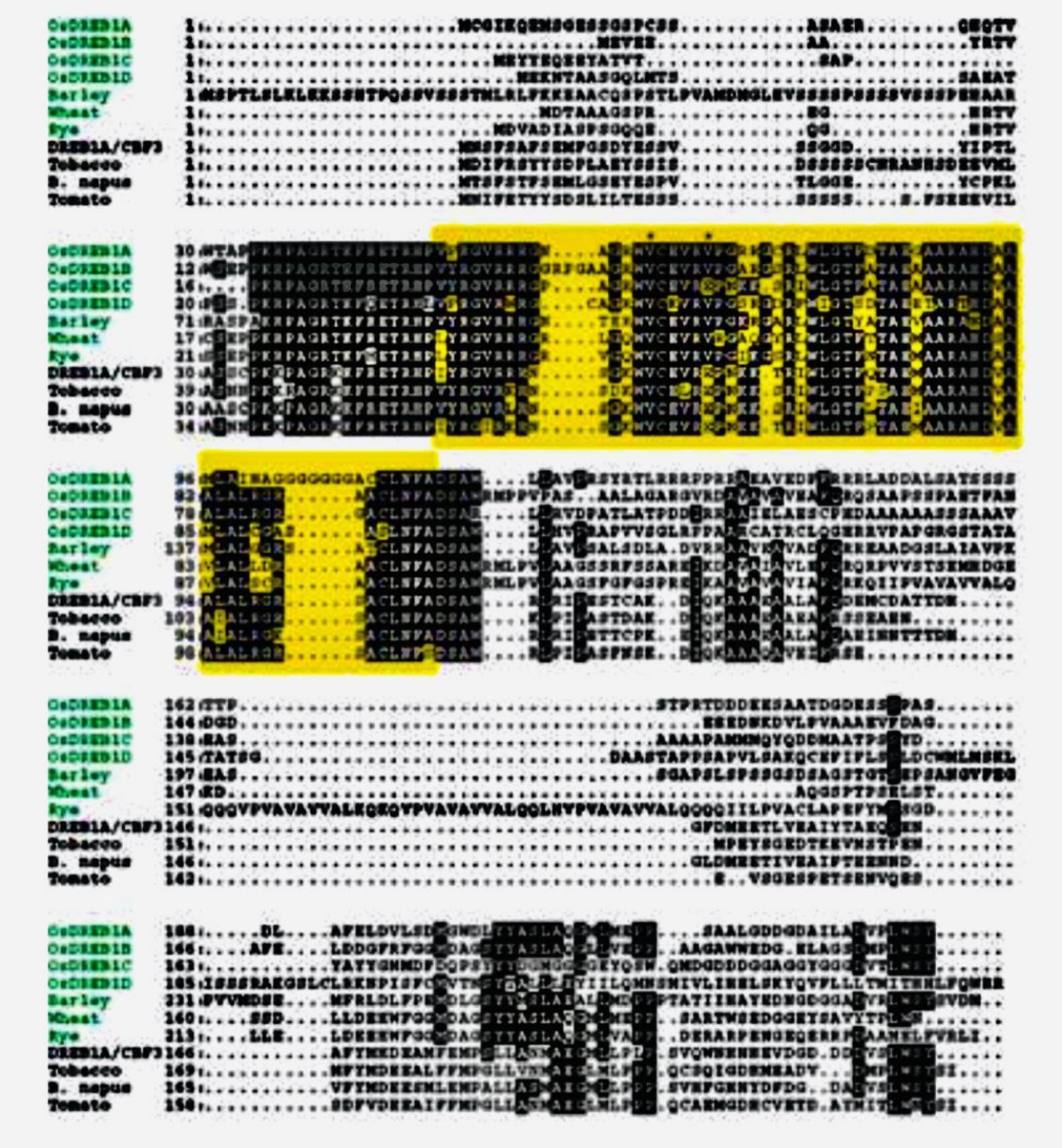


Fig.1b Alignment Of Amino Acid Sequences Of The Osdreb2- And Dreb2-Related Proteins. The Yellow Box Indicates Erf/Ap2 Domains And Asterisks Indicate The Well-Conserved Sequences, Valine And Glutamic Acid, Among The Dreb-Related Proteins.

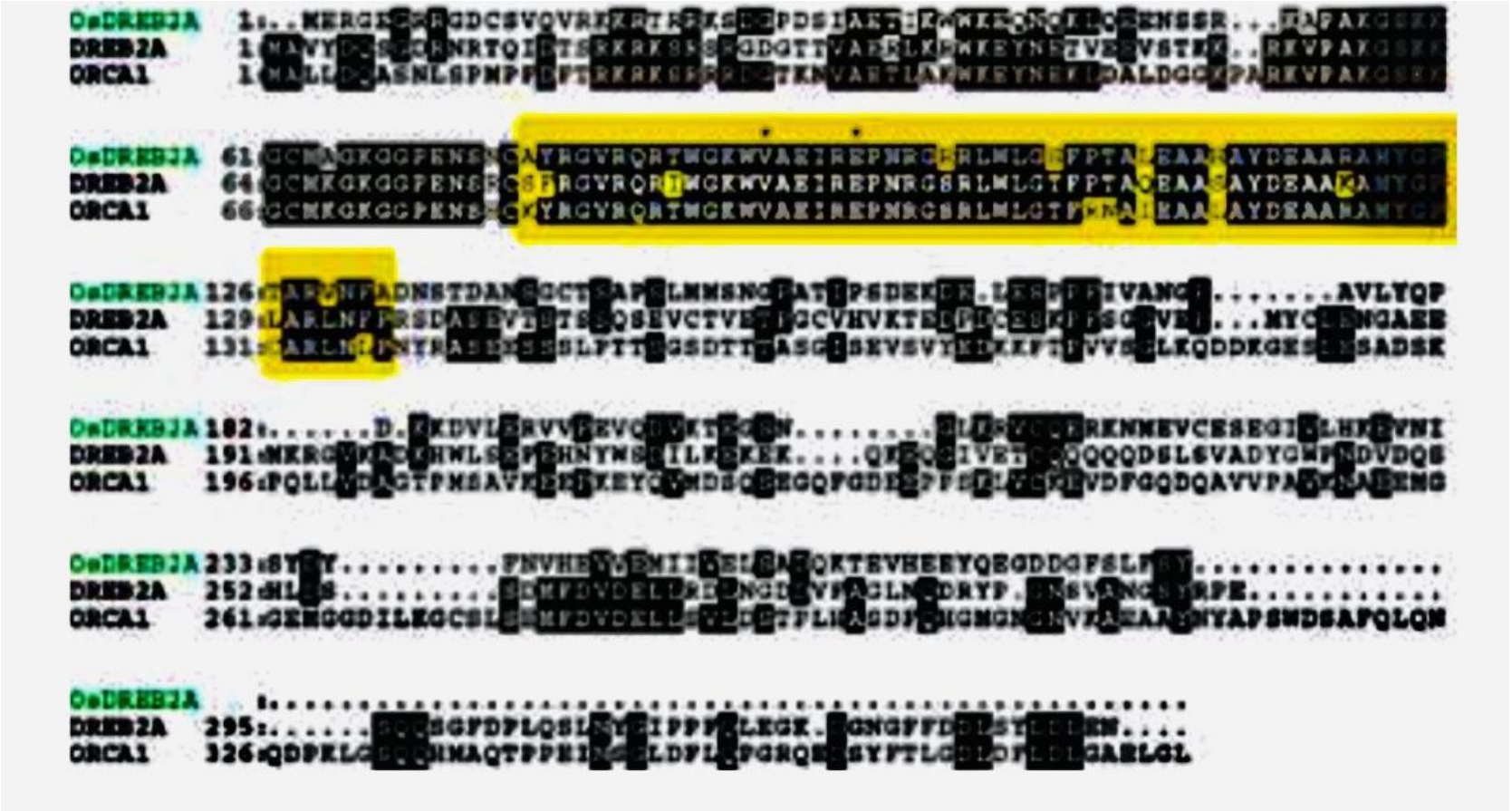


Fig.2 Rna-Gel Blot Analysis Of The Osdreb Transcripts Under Various Stress Conditions. Seventeen-Day-Old Rice Seedlings That Had Been Dehydrated (Drought), Transferred To 48c (Cold), Transferred For Hydroponic Growth In 250 Mm Nacl (Nacl), Transferred To 100 Mm Aba (Aba), Transferred To Water (H2o), Or Wounded Were Used To Prepare Total Rnas, And 30mg Of Each Total Rna Was Fractionated By Electrophoresis In A Formaldehyde Agarose Gel. Rna Gel Blots Were Hybridized To The 30-Terminal Regions Of The Osdreb1a, Osdreb1b, Osdreb1c, And Osdreb2a Genes As Probes.

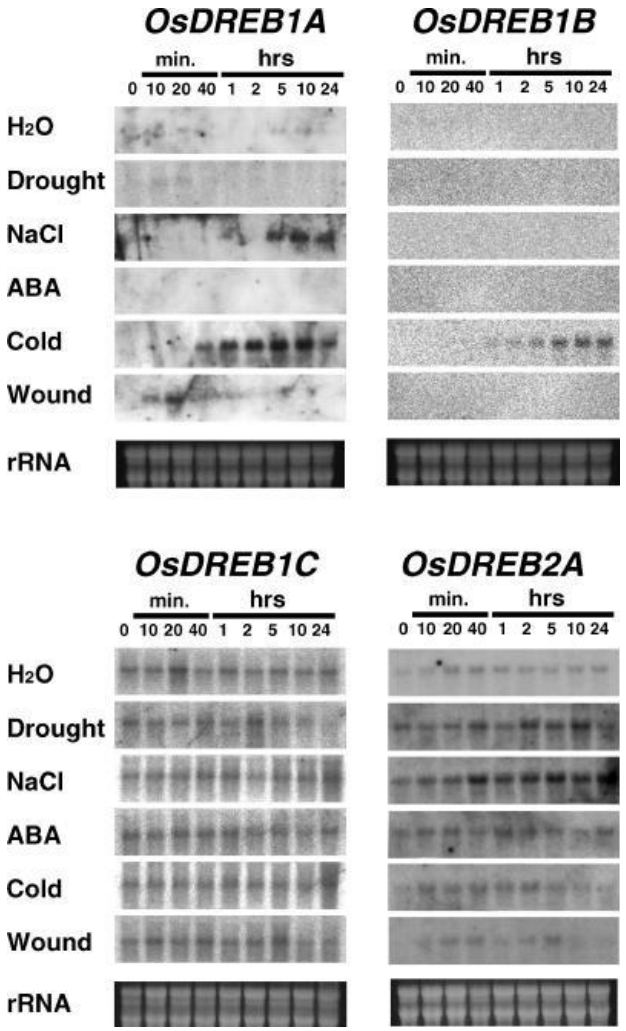


Fig.3 Comparison Of Growth Retardation Among The 35s:Osdreb1ac, 35s:Osdreb1ad, 35s:Dreb1ac, And Wild-Type (Pbi121) Plants. Plants Were Grown For 33 Days

Fig.4 Expression Of The Dreb1a Target Genes In 35s:Osdreb1a Transgenic Plants And In The Wild-Type Plants. Each Lane Was Loaded With 10 Mg Of Total Rna Prepared From Arabidopsis Plants That Had Been Dehydratedfor5 H(Dry), And Treated At 48c For 5 H(Cold), Or Left Untreated (Control).

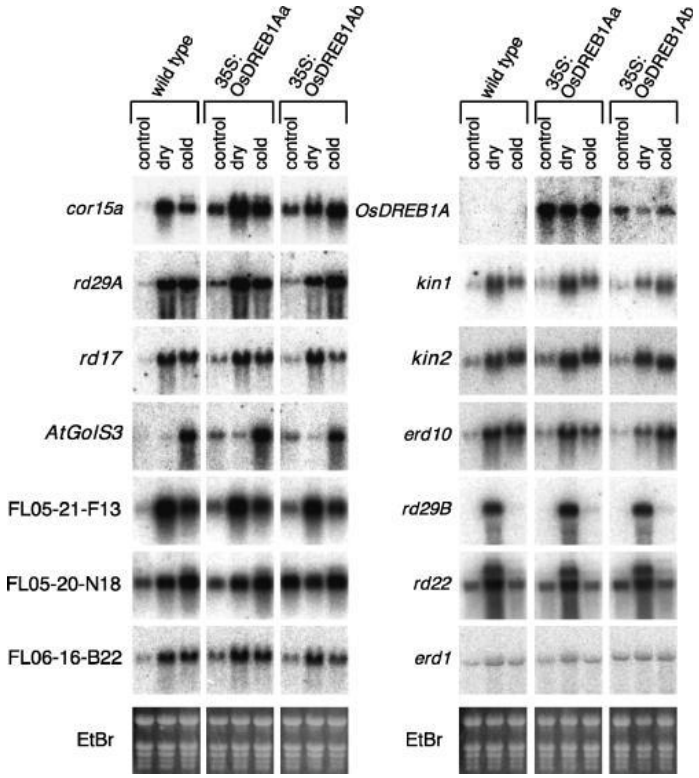
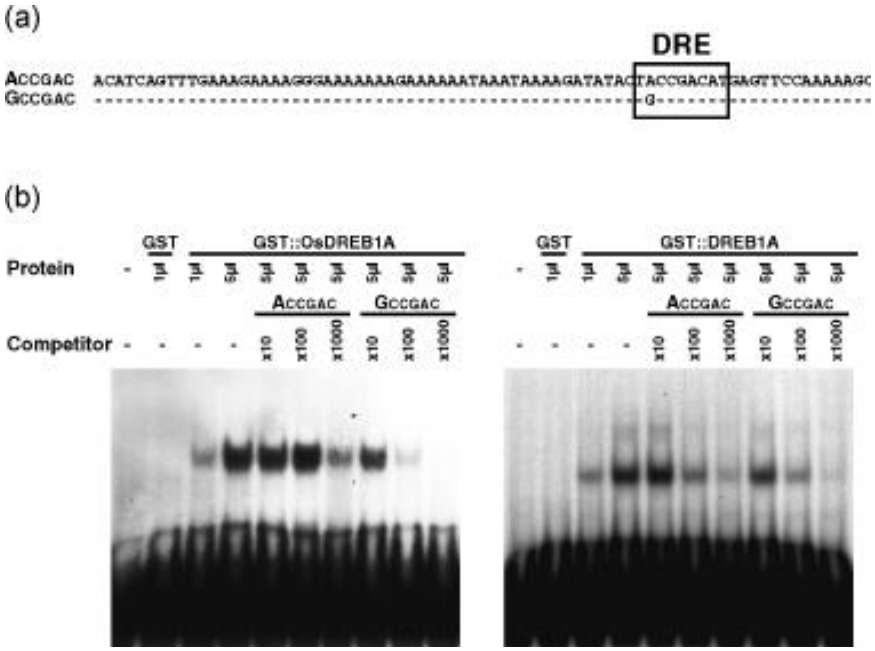
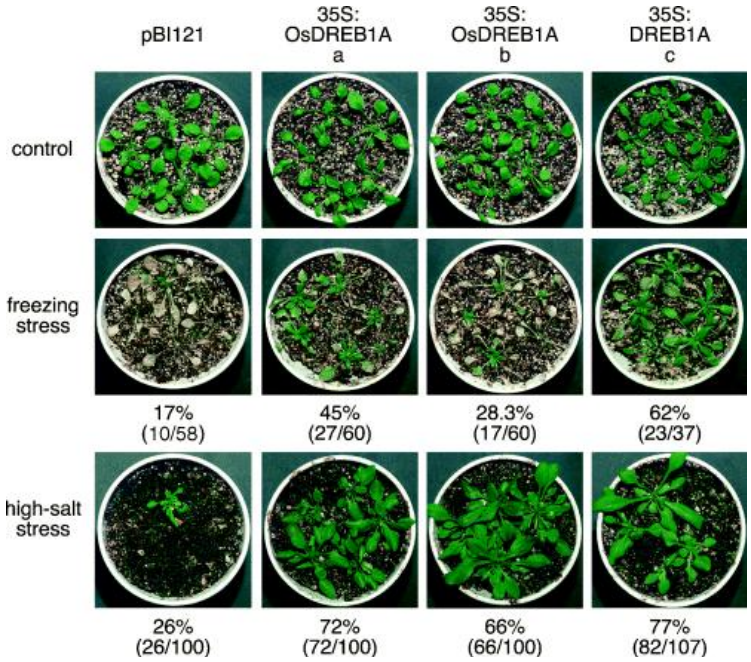


Fig.5 Characterization Of The Dna-Binding Affinities Of The Recombinant Osdreb1a And Dreb1a Proteins



(a) Sequences of the 75-bp fragment of the wild-type rd29A promoter (ACCGAC) and the mutated fragment (GCCGAC) used as competitors. The ACCGAC fragment was also used as a probe.
(b) Gel mobility shift assay showing a different binding affinity between the recombinant OsDREB1A and DREB1A proteins to the ACCGAC or GCCGAC sequences. GST (1 ml) or recombinant protein solutions (1 or 5 ml) were pre-incubated with or without competitors at 25°C for 5 min. Then the ^{32}P labeled probe (50 pg for OsDREB1A and 500 pg for DREB1A) was added and the mixture incubated at 25°Cfor 30 min. Ascompetitors,10-fold (10), 100-fold (100), or 1000-fold (1000) excess amounts of the unlabeled ACCGAC or GCCGAC fragments were used. One microliter of the GST, the recombinant OsDREB1A and DREB1A protein solutions contained 1.0, 0.2, and 0.4 mg corresponding proteins respectively.

Fig.6 Freezing And High-Salt Stress Tolerance Of The 35s:Osdreb1aa, 35s:Osdreb1ab, And 35s:Dreb1ac Transgenic Plants



In addition, several novel stress-inducible genes were also identified that respond to drought, cold, and high-salt stresses. We searched the rice genomic database and found that some of these stress inducible genes contain the GCCGAC sequence in the promoter regions. The OsDREB proteins probably bind to the sequence and activate expression of these genes in rice. These rice genes are expected to be upregulated by overexpression of the OsDREB proteins. Therefore, we are now generating rice transgenic plants over-expressing various OsDREB proteins in combination with several promoters. We think that the DREB/DRE regulon can be used to produce transgenic dicots and monocots with higher tolerance to drought, high-salt, and/or cold stresses, and especially OsDREB1A is useful for the monocots. We also think that Arabidopsis is a good system to analyze functions of rice homologs of the DREB proteins, which are thought to be master switches in drought-, cold-, and high-salt-responsive gene expression in plants.

Transcription factors are powerful tools for genetic engineering because over-expression of a transcription factor can lead to the upregulation of the whole array of genes under its control. On the other hand, they were carried out rice cDNA microarray analysis to identify genes induced in response to drought, high-salt, and cold stresses in rice. Several known and well-characterized stress-inducible rice genes were identified by the cDNA microarray analysis. In addition, several novel stress-inducible genes were also identified that respond to drought, cold, and high-salt stresses. When searched the rice genomic database and found that some of these stress inducible genes contain the GCCGAC sequence in the promoter regions. These rice genes are expected to be up regulated by over expression of the OsDREB proteins. Therefore, now generating rice transgenic plants over-expressing various OsDREB proteins in combination with several promoters. We think that the DREB/DRE regulon can be used to produce transgenic dicots and monocots with higher tolerance to drought, high-salt, and/or cold stresses, and especially OsDREB1A is useful for the monocots, and also think that Arabidopsis is a good system to analyze functions of rice homologs of the DREB proteins, which are thought to be master switches in drought-, cold-, and high-salt-responsive gene expression in plants.

References

Ahuja I, de, Vos, R. C., Bones, A. M., Hall, R. D., 2010. Plant molecular stress responses face climate change. Trends in plant science. 15(12):664-74.

Hattori, T., Terada, T. and Hamasuna, S., 1995. Regulation of the Osem gene by abscisic acid and the transcriptional activator VP1: analysis of cis-acting promoter elements required for regulation by abscisic acid and VP1. The Plant Journal. 7(6), 913-925.

Hobo, T., Kowyama, Y. and Hattori, T., 1999. A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. Proceedings of the national academy of sciences. 96(26), 15348-15353.

Lata, C. and Prasad, M., 2011. Role of DREBs in regulation of abiotic stress responses in plants. Journal of experimental botany. 62(14), 4731-4748.

Lim, C.J., Hwang, J.E., Chen, H., Hong, J.K., Yang, K.A., Choi, M.S., Lee, K.O., Chung, W.S., Lee, S.Y. and Lim, C.O., 2007. Over-expression of the Arabidopsis DRE/CRT-binding transcription factor DREB2C enhances thermotolerance. Biochemical and biophysical research communications. 362(2), 431-436.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K., 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in Arabidopsis. The Plant Cell. 10(8), 1391-1406.

Liu, T., Song, X., Duan, W., Huang, Z., Liu, G., Li, Y. and Hou, X., 2014. Genome-wide analysis and expression patterns of NAC transcription factor family under different developmental stages and abiotic stresses in Chinese cabbage. Plant Molecular Biology Reporter, 32(5). 1041-1056.

Lobell, D. B., Schlenker, W. and Costa-Roberts, J., 2011. Climate trends and global crop production since 1980. Science, 333(6042). 616-620.

Nakashima, K., Ito, Y. and Yamaguchi-Shinozaki, K., 2009. Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. Plant physiology. 149(1), 88-95.

Nakashima, K., Shinwari, Z. K., Sakuma, Y., Seki, M., Miura, S., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2000. Organization and expression of two Arabidopsis DREB2 genes encoding DRE-binding proteins involved in dehydration-and high-salinity-responsive gene expression. Plant molecular biology. 42(4), 657-665.

Nakashima, K., Takasaki, H., Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2012. NAC transcription factors in plant abiotic stress responses. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms. 1819(2), 97-103.

Newton, A. C., Johnson, S. N. and Gregory, P. J., 2011. Implications of climate change for diseases, crop yields and food security. Euphytica. 179(1), 3-18.

Pandey, M. M., 2009. Indian agriculture–An introduction. Submission to the Fourth Session of the Technical Committee of APCAEM. 2009:10-2.

Sakuma, Y., Liu, Q., Dubouzet, J. G., Abe, H., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2002. DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration-and cold-inducible gene expression. Biochemical and biophysical research communications. 290(3), 998-1009.

Sanchez-Rodriguez, E., Moreno, D.A., Ferreres, F., del Mar Rubio-Wilhelmi, M. and Ruiz, J.M., 2011. Differential responses of five cherry tomato varieties to water stress: changes on phenolic metabolites and related enzymes. Phytochemistry. 72(8), 723-729.

Shao, H. B., Chu, L. Y., Jaleel, C. A., Manivannan, P., Panneerselvam, R. and Shao, M. A., 2009. Understanding water deficit stress-induced changes in the basic metabolism of higher plants–biotechnologically and sustainably improving agriculture and the ecoenvironment in arid regions of the globe. Critical reviews in biotechnology. 29(2), 131-151.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M., 2003. Regulatory network of gene expression in the drought and cold stress responses. Current opinion in plant biology. 6(5), 410-417.

Stockinger, E. J., Gilmour, S. J. and Thomashow, M. F., 1997. Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA

regulatory element that stimulates transcription in response to low temperature and water deficit. Proceedings of the National Academy of Sciences. 94(3), 1035-1040.

Takeda, S. and Matsuoka, M., 2008. Genetic approaches to crop improvement: responding to environmental and population changes. Nature Reviews Genetics. 9(6), 444-457.

Yamaguchi-Shinozaki, K. and Shinozaki, K., 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu. Rev. Plant Biol. 57, 781-803.

Zhu, J. K., 2002. Salt and drought stress signal transduction in plants. Annual review of plant biology, 53(1), 247-273.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K.,1999. Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat. Biotechnol. 17:287–291.

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