

Isolation and characterization of a novel AP2/EREBP-type transcription factor OsAP211 in *Oryza sativa*

F. GAO¹, J.-M. CHEN², A.-S. XIONG¹, R.-H. PENG¹, J.-G. LIU¹, B. CAI¹ and Q.-H. YAO^{1*}

Biotechnology Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai, 201106, P.R. China¹
College of Bioscience and Biotechnology, Yangzhou University, Yangzhou, 225009, P.R. China²

Abstract

AP2 (APETALA2)/EREBPs (ethylene responsive element binding proteins) are the primary members of a family of transcription factors and OsAP211 was isolated from *Oryza sativa* L using the yeast one-hybrid system. It can specifically bind to the promoter containing three tandem repeats of DRE core sequence: TACCGACAT and activate the transcription of the downstream *lacZ* gene in the yeast one-hybrid system. *OsAP211* contained a single open reading frame of 225 amino acids and encoded a protein containing a conserved AP2/EREBP domain featuring the DREB family. The semi-quantitative RT-PCR (s-Q RT-PCR) analysis indicated *OsAP211* was strongly induced by low temperatures but not by NaCl and drought. It accumulates primarily in shoot tips during the tillering stage and young spikes during the booting stage. OsAP211 might function as a DRE-binding transcription factor in stress signal transduction pathways in rice.

Additional key words: low temperature, rice, yeast one-hybrid system.

Introduction

In natural conditions, plants are often exposed to various environmental stresses such as drought, high salinity and low-temperature. To understand the molecular and physiological mechanisms of how plants respond to these stressors, a number of stress-related genes and regulatory proteins have been isolated, such as *rd* (responsive to dehydration; Yamaguchi-Shinozaki *et al.* 1992), *cor* (cold-responsive; Hajela *et al.* 1990), *erd* (early responsive to dehydration; Thomashow 1998), protein kinases (Zhang *et al.* 2001) and transcription factors (Chen *et al.* 2002). These genes and regulatory proteins form a complex network to regulate a number of physiological and biochemical processes for tolerance against abiotic and biotic stresses. Transcriptional regulation of these stress-related genes is thought to be important for stress tolerance and response of several kinds of transcription factors such as ethylene-responsive element binding proteins (EREBP) play crucial roles.

The EREBP proteins are characterized by the presence of the AP2/EREBP DNA binding domain (Riechmann *et al.* 1998). This domain was firstly described in *APETALA2* (*AP2*), a transcription factor related to flower and seed development from *Arabidopsis* (Jofuku *et al.*

1994). It was later found to be conserved in EREBPs from tobacco (Ohme-Takagi *et al.* 1995), and involved in the regulation of plant defense systems. The AP2/EREBP domain has been found in different transcription factors from various plant regulatory genes, and shares a highly conserved AP2/EREBP domain of about 58 - 70 amino acids with no apparent similarity except this DNA-binding domain (Okamuro *et al.* 1997). Sakuma *et al.* (2002) characterized the AP2/EREBP gene family in *Arabidopsis thaliana* on the basis of the number of repetitions and amino acid sequence of the AP2/EREBP domain and classified the 144 members into 5 subfamilies based on similarities in their DNA-binding domains: DREB, ERF, AP2, RAV, and others. Furthermore, Nakano *et al.* (2006) identified 122 and 139 ERF subfamily genes in *Arabidopsis* and rice (*Oryza sativa* L), and classified them into 12 and 15 groups, respectively. Their reports revealed the gene structures, phylogeny, conserved motifs and evolution of the ERF subfamily in the AP2/EREBP gene superfamily.

All the DREB proteins have one single AP2/EREBP domain and a conserved WLG motif. The solution structure of AP2/EREBP domain consists of a three-

Received 22 August 2007, accepted 31 March 2008.

Abbreviations: DRE - dehydration-responsive element; DREB - DRE binding factor; X-Gal - 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; RT-PCR - reverse transcription-polymerase chain reaction.

Acknowledgements: The research was supported by 863 Program (2006AA10Z117; 2006AA06Z358; 2008AA10Z401); Shanghai Subject Chief Scientist (06XD14017); Shanghai Key laboratory and basic research project (06DZ19103-07dz22011); Shanghai municipal committee of agriculture (No: 2006-2-1) and Jiangsu province natural science fund (BK2007080).

* Corresponding author: fax: (+86) 21 62205704, e-mail: yaoquanhong_sh@yahoo.com.cn

stranded anti-parallel β -sheet and an α -helix running almost parallel to the β -sheet, in AP2/EREBP domain, and two absolutely conserved amino acids V14 and E19 which play important roles in recognition of the DNA binding sequences (Sakuma *et al.* 2002). Many typical genes encoding DREB-like transcription factors in *Arabidopsis* have already been isolated, including DREB1A, DREB2A (Liu *et al.* 1998), and their homologous genes have also been found in rice (OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D, OsDREB2A, OsDREB2B, RCBF2; Dubouzet *et al.* 2003; Liu *et al.* 2007), barley (HvCBF; Choi *et al.* 2002), wheat (TaDREB1; Shen *et al.* 2003), and other species. They were significantly expressed by exposure to drought, high salinity and cold stress conditions. Even though previous studies have

highlighted the importance of the typical DREB-like transcription factors in *Arabidopsis*, the functions of most rice DREBs were not completely known. Since DREB-like transcription factors in the AP2/EREBP-type family are a crucial part of the plant defense regulation system, it is essential to isolate and characterize some novel DREB transcription factors for describing the whole network of plant stress responses.

In this work, using the yeast one-hybrid system, we isolated a novel cDNA clone encoding DREB-like transcription factors from the rice cDNA library, *OsAP211* (GenBank accession number: AY346094). Homologous analysis revealed that the *OsAP211* gene belongs to the DREB subfamily and we demonstrated that the protein functions as a transcriptional activator in yeast.

Materials and methods

Plants and stress treatments: Rice (*Oryza sativa* L. cv. IR36) seeds were surface sterilized with 5 % (v/v) NaOCl, washed three times for 5 min with sterile water, and germinated at 37 °C for 2 d. Seedlings were grown at 26 °C under cool-white fluorescent lamps (irradiance of 150 - 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 16-h photoperiod) and irrigated with double distilled H₂O. For salinity stress exposure, the roots of the 2-week-old seedlings were immersed in 200 mM NaCl solution for 30 min to 12 h. For the drought treatment, the seedlings were manipulated as previously described (Chen *et al.* 2003). The cold stress treatment was performed by placing the pots with two-week-old seedlings in a cold room at 2 ± 1 °C under irradiance of 25 - 30 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 30 min to 12 h. The roots, shoots, leaves, shoot tips from plants at the tillering stage, young spikes at the booting stage, immature seeds at the flowering stage and mature seeds at the grain filling stage were obtained separately from field-grown plants, quickly frozen in liquid nitrogen and then stored at -70 °C for RNA extraction.

Strains and plasmid: Yeast (*Saccharomyces cerevisiae*) strain EGY48 (*MAT α* , *his3* *trp1* *ura3-52leu::Pleu2-LexAop6*) (Invitrogen, Carlsbad, CA, USA) was used for transformation and screening. *Escherichia coli* strain MC8 (*thi*⁻, *trp*⁻, *ura*⁻, *leu*⁻, *his*⁻) was used for rescuing cDNA library plasmids and electroporation experiments.

To construct the bait plasmid, three tandem copies of the *cis*-element containing a dehydration responsive element (DRE) in the promoter of *rd29A* were synthesized by PCR using two primers with complementary 3' terminals (rd29A1Z:5'-GGTCTAGATCATACCGA CATCAGTATCATACCGACATCAGTATATCATACCG ACATCAGTCACTGACTGTCGTGATGGATCCTG-3'; *cisF*: 5'-CAGGATCCATCACGACAGTCAGTG-3'). The PCR conditions were 10 s at 94 °C, 10 s at 42 °C and 10 s at 72 °C for 30 cycles. After PCR amplification reaction, a fragment was isolated by PAGE and digested with *Xho* I and *Bam* HI restriction enzymes, and then inserted into

pLGA-265UP1 (Guarente 1983) at the corresponding site. This fragment was named pLGA-265UP1 DRE. The *E. coli*-yeast shuttle plasmid pLGA-265UP1 contains an URA-marked 2u, *lacZ* report gene under the control of the iso-1-cytochrome C (*CYC1*) minimal promoter, and acts as bait in the yeast one-hybrid system. The rice cDNA library constructed in pPC86 vector (Chevray *et al.* 1992) was donated by Dr. Zhu Qun (Cold Spring Harbor Laboratory, New York). The *E. coli*-yeast shuttle plasmid pPC86 is a TRP-marked yeast expression plasmid containing a galactose-inducible (GAL4) activating domain under the control of the yeast alcohol dehydrogenase (*ADCI*) promoter.

Yeast-one-hybrid screening of rice cDNA libraries: The bait plasmid pLGA-265UP1 DRE was transferred into yeast EGY48 using the lithium acetate protocol (Gietz and Woods 1998), and plasmid pPC86 was transferred into the positive yeast cells containing bait plasmid pLGA-265UP1 DRE which could grow on the uracil-deficient medium. These yeast cells were then placed on selective yeast media without URA and TRP. About 4×10^6 yeast transformants were overlaid onto media containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) using nitrocellulose filters and the β -galactosidase activity was tested. Plasmid DNA of blue clones were extracted and transferred into *E. coli* strain MC8 by electroporation. Then, plasmid DNA of the positive transformants were prepared and transformed into the yeast strains EGY48 containing bait plasmid pLGA-265UP1 DRE to confirm β -galactosidase activity for true positive clone selection.

Two oligonucleotide primers, (5'-GGATGTTTAAT ACCACT-3') based on the DNA sequence flanking the GAL4 activating domain and (5'-TTGATTGGAGA CTTGACC-3') derived from the ADC1 terminator region in the pPC86 vector were used to amplify the rice cDNA which was inserted into vector pPC86.

DNA sequencing and data analysis: The cDNA fragments were sequenced using the model 373 ABI

automatic sequencer (*Applied Biosystems*, Foster City, CA). The nucleotide and amino acid sequences were compared to the NCBI (National Center for Biotechnology) database using the *BLAST* program with E-value cut-off 0.01. DNA sequence data were analyzed and the alignment was produced by the biosoftware *DNAMAN*. Multiple sequence alignment and phylogenetic tree information were analyzed by *ClustalW/X*. Three-dimensional structure of analysis and molecular modeling was performed using *WISS-MODEL* (<http://swissmodel.expasy.org/>) and the molecular structures were visualized using the *WebLab ViewerPro* software tool.

Reverse transcription-PCR (RT-PCR) analysis: Total RNA was treated with DNase I (*Promega*, Madison, WI, USA) to remove the genomic DNA contamination. The first strand of cDNA was synthesized by using 5 µg total RNA as the template with reverse transcription system (*Promega*) in a 0.02 cm³ reaction volume. To reduce the error of RT-PCR, the specific primers were designed according to the sequence of the *OsAP211* gene for

amplifying the part of the gene. The *OsAP211* gene forward primer (5'-TCGCTCACTCGCTCTCCTCT-3') and antisense primer (5'-GCCGCTTCTTCTTGCCGCG-3') was used to amplify a 186 bp fragment. The PCR conditions were: denature for 5 min at 94 °C, followed by 38 cycles of 40 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C and then extend for 5 min at 72 °C. The rice actin gene (*rac1*, GenBank accession number: X16280) was used as the internal standard with forward primer (5'-AAGATCCTGACGGAGCGTGGTTAC-3') and antisense primer (5'-CTTCCTAATATCCACGTCGCAC TTC-3'). The PCR conditions for amplifying *rac1* gene were carried out for 28 cycles with 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, plus a final extension at 72 °C for 5 min. The PCR products were separated by 1.5 % agarose gel electrophoresis and quantified using *Model Gel Doc 1000* analyzer (*Bio-Rad*, Hercules, CA). DNA ratio of the *OsAP211* gene to *rac1* was analyzed with *ShineTech* gel analyser (*Shanghai Shine Science of Technology Co.*, Shanghai, China) to evaluate the expression pattern of the *OsAP211* gene. The experiments were repeated three times.

Results

Isolation and structural analysis of the OsAP211 cDNA:

To isolate the genes encoding the DRE/C-repeat binding proteins from the rice cDNA library, we used the yeast one-hybrid screening system. This system includes two kinds of plasmids: pPC86 plasmid containing rice cDNA fragments from the expression library, which was used to express the GAL4-cDNA fusion proteins; and pLGA-265UP1 DRE with the *lacZ* reporter gene, which was used as a bait plasmid. The two plasmids were transformed into the same yeast strain EGY48 separately. Eighteen positive clones with deep-blue were obtained by screening 4×10^6 transformants onto media lacking tryptophan and uracil. Some plasmids in the expression library might encode GAL4-cDNA fusion protein which would bind to the upstream of the *lacZ* reporter gene

carrying the DRE/C-repeat sequence. This would activate the transcription of the *lacZ* gene, and result in the formation of blue colonies on X-Gal-treated filters. In order to wipe off pseudo-positive clones, the plasmids of 18 positive clones were recovered and transformed back into yeast EGY48 containing bait plasmid pLGA-265UP1 DRE to confirm their β -galactosidase activity. Nine clones were still deep-blue on X-Gal plates (Fig. 1). The rice cDNA inserts in these positive clones were amplified by PCR, and sequenced by the dideoxy chain termination method. Sequence analysis indicated that cDNAs in these clones were identical except for length differentiation and we assembled a complete cDNA based on the nucleotide sequences. The gene was named as *OsAP211* (GeneBank accession number: AY346094) for further analysis.

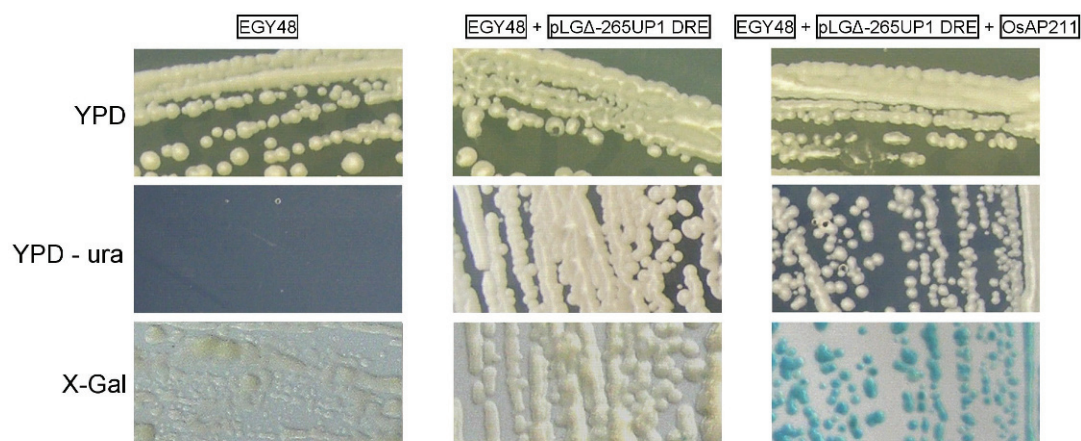


Fig. 1. Identification of positive yeast transformants on plates with X-gal in the yeast one-hybrid screen. *OsAP211* was cloned into the yeast expression vector pPC86 and transformed into the yeast strain EGY48 containing bait plasmid pLGA-265UP1 DRE and report gene *LacZ*. The positive transformants were examined for deep blue on plates with X-gal.

```
CCTCCACCTCACACACACACAGCCACTCGCTCGCTCGCTCGCACAGTCGCTCGCT
CGCCCGCTCTAGCTAGCTCACAGCTCGCTCAGCTCGCTCTCTCTTCCAACCACTCCACC
ACCACCAGTCCACCACAGCGCGCGCCATGACGACTCGTGTTCGGTTCGAGCGGA
1      M D D S S F G S E P
CCACGTCTCTCTGGGCGGGGAGGCGCGGCTCCCGCGCTCCACCGCTGTGTGTCT
11     T T S S S G G E A P A S P P S T A S S S
CAGACGGCGCGCGGCAAGAAGAGCGCGCGCAAGGACGGGCACCAACCGAGTACC
31     S D G A G G K K K R P R K D G H H P T Y
GCGGGGTGCGCATGCGGAGCTGGGGCAAGTGGGTGTCGGAGATCAGGGAGCGCGGA
51     R G V R M R S W G K W V S E I R E P R K
AGTCGCGCATCTGGCTGGGCACCTTCGCCACCGCGAGATGGCGCGCGCGCACGACG
71     K S R I W L G T F A T A E M A A R A H D
TCGCCGCTCGCCATCAAGGGCGCGCGCGCACCTCACTTCGGGACCTCGGCCAGG
91     V A A L A I K G R A A H L N F P D L A H
AGCTGCGCGCGCGGCCACCGCGCGGCCAAGGACGTCCAGGCGCGAGCGCGCTCGCG
111    E L P R P A T A A P K D V Q A A A A L A
CCGCCGCGGACTTCGGGCTCTCCGCCAATGCGGGCGCCAGCAATAACCGGACGGAT
131    A A A D F P A S S A N A G A S N N P D G
CGGACGACGCGAGCGCGGGTCCGCTTCGCGCGCGCGCGCGGACGCGCTGACGACG
151    S D D A S A G S A S P P P P D A A D D
CGCTGTCGACCTCCCGACCTCCTCTCGACCTCAGATACGCGCGCGCGAGCTGGGGC
171    A L F D L P D L L L D L R Y G P P S S G
TCTCGTGCCTGCTCTTGGGAAGACGAAGTGGCTTAATCTCCGCGCGCGGTGCGCG
191    L S C A S S W E D E V G L I S G A G A A
CCGCCGCGGTGTCGCGCTGGAGGAGCCACTTCTGTGGGAGTACTAAAGCCAAAGCGCC
211    A A G V F R L E E P L L W E Y *
GCGGTAAACACTGCTCAGTGCTAAATCGAGCAGCTGCATGTACAACCGTAATTTT
CCACGATGACCAAAAGAAATTCATGTCATGCAAGCCAGCTACGGCTACGGCGACAGAA
CTTGAGCAAAAA
```

Fig. 2. Full-length sequences of *OsAP211* cDNA and deduced amino acid sequences (GenBank accession No. AY346094). The AP2/EREBP domains are *singly underlined*. The putative nuclear localization signal (NLS) is *doubly underlined* and an acidic C-terminal region that might act as a transcriptional activation domain has been *shadowed*.

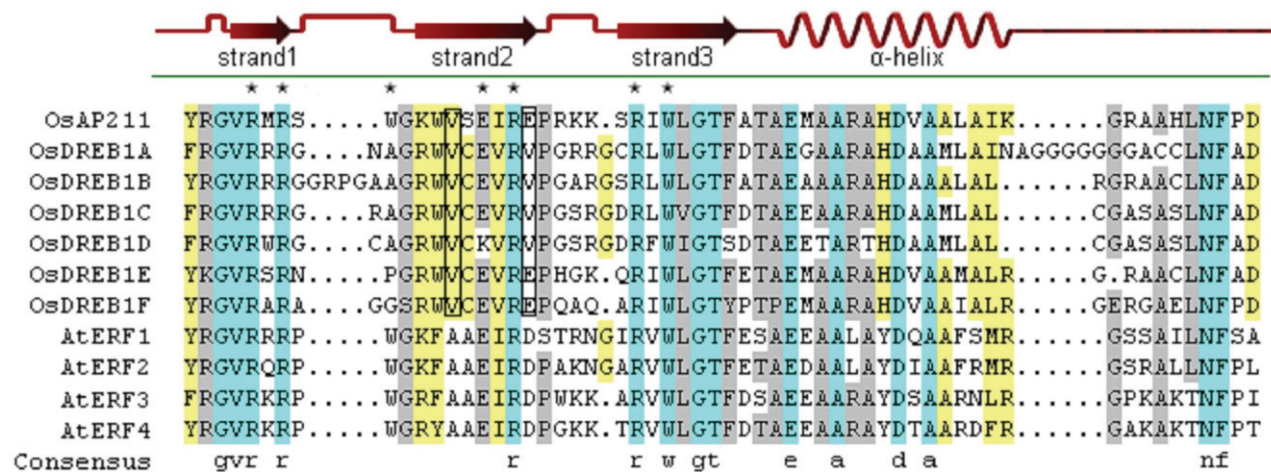


Fig. 3. Alignment of deduced amino acid sequences of the DNA binding domains of *OsAP211* with other AP2/EREBP-like proteins. The amino acid sequences shown are: *Oryza sativa* L. *OsDREB1A* (accession No. AAN02486), *OsDREB1B* (accession No. AAN02488), *OsDREB1C* (accession No. AP001168), *OsDREB1E* (accession No. AAX23722), *OsDREB1F* (accession No. AAX23723), *AtERF1* (accession No. NP_188965), *AtERF2* (accession No. AAM64544), *AtERF3* (accession No. NM_103946), *AtERF4* (accession No. NM_112384). Identical amino acid residues in the alignment are indicated in *light-blue* and conservative amino acid residues are indicated in *grey* and *yellow*. The three β-sheets and the α-helices are marked above the corresponding sequences as a reference (Allen *et al.* 1998). The two residues “V” and “E” which have been identified in *Arabidopsis* are also indicated. *Asterisks* represent amino acid residues that direct make contact with DNA (Allen *et al.* 1998). *Dashes* indicate gaps in the amino acid sequences introduced to optimize alignment.

Nucleotide sequence and predicted amino acid sequence analysis of rice *OsAP211* cDNA: The results of nucleotide sequence analysis revealed that *OsAP211* was involved in a subfamily belonging to AP2/EREBP-type transcription factors (Fig. 2). *OsAP211* cDNA contained a single open reading frame of 225 amino acids encoding a putative protein with a calculated molecular mass of 23.1 kDa and pI of 5.22. Analysis of the amino acid sequence showed that the protein contained a highly conserved AP2/EREBP domain and potential nuclear localization sequence at its N-terminal. The acidic C-terminal region of this protein might act as an activation domain for transcription. The putative amino acid sequences of rice OsAP211 protein was compared with those known AP2/EREBP proteins by the *Clustal W/X* program in the *EMBL* databases and using biosoftware *DNAMAN*. The result showed that these proteins shared very high homology in AP2/EREBP domain (Fig. 3). To further analyze the phylogenetic relationship of OsAP211 and other AP2/EREBP-like proteins from *Arabidopsis* and rice, a phylogenetic tree was constructed based on the sequence identities of the AP2/EREBP domain (Fig. 4). The results showed those known AP2/EREBP-like proteins can be divided into four subgroups. OsAP211 showed higher bootstrap values with the OsDREB1 subfamily but was distant from the *Arabidopsis* ERF subfamily. The high degree of homology shared between OsAP211 and DREB-like proteins indicated that OsAP211 may belong to the DREB subfamily and may function similarly to DREB subfamily members.

Secondary and three-dimensional structural analyses of OsAP211: The DNA-binding domain of OsAP211 was

aligned with AtERF1 whose solution structure of the DNA binding domain contained a three-stranded anti-parallel β -sheet and an α -helix packed approximately parallel to the β -sheet (Fig. 3). The results showed that the two proteins, OsAP211 and AtERF1, have high conservation of the AP2/EREBP domain. The amino acid residues Arg6, Arg8, Trp10, Glu16, Arg18, Arg25 and Trp27 that directly make contact with DNA for DNA-binding activity are completely conserved in the alignment (Allen *et al.* 1998). In β -sheets, some residues have been identified as key residues for DNA-binding activity, such as the conserved V14 and E19 in β -sheet 2. These are crucial residues for recognizing the DNA-binding sequences; however E19 may not be as important as V14 for the recognition of the DNA-binding sequence in the DREB1 proteins (Sakuma *et al.* 2002). We examined the tertiary structure of OsAP211's AP2/EREBP domain in a model of

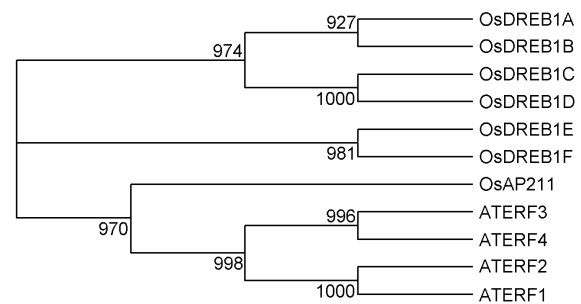


Fig. 4. Phylogenetic analysis of the deduced amino acid sequences of *OsAP211* gene. Phylogenetic tree showing the relationship between OsAP211 and other AP2/EREBP-like proteins from *Arabidopsis* and rice. The bootstrapped tree was produced by CLUSTAL W/X with 1000 repetitions.

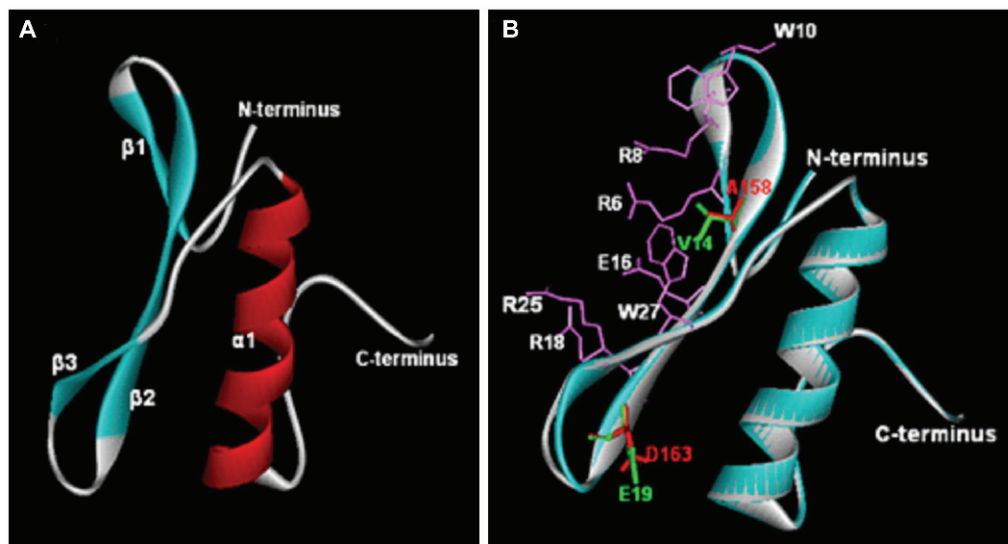


Fig. 5. Molecular modeling of OsAP211 protein using SWISS-MODEL (<http://swissmodel.expasy.org/>). *A* - Molecular modeling of conserved AP2/EREBP domain of AtERF1 derived from *Arabidopsis* (PDB ID: 1gcc). *B* - Molecular modeling of OsAP211 AP2/EREBP domain that we simulated in a model of AtERF1. AP2/EREBP domain of OsAP211 is displayed white and cyan solid ribbon, respectively. The amino acid residues of OsAP211 and AtERF1 that directly contact DNA are displayed purple linear molecule meanwhile conserved amino acid V14 and E19 in the AP2/EREBP domain of OsAP211 and AtERF1 A158 and D163 are displayed green linear molecule and red linear molecule, respectively.

Arabidopsis ERF1 (PDB ID: 1gcc) by the *SWISS-MODEL* database (Fig. 5). The result showed that the embedded DNA-binding domain has a typical feature of the AP2/EREBP domain. It had differences in the second turn and two key residues which may lead to differences in recognizing and binding of some *cis*-acting elements.

Expression pattern of *OsAP211* gene: The semi-quantitative RT-PCR method was used to investigate the expression pattern of the *OsAP211* gene in a variety of rice organs and under various stress treatments. The rice actin gene *Rac1* was used as the internal standard RNA.

To examine the pattern of the expression of *OsAP211* in different tissue organs and at different growth stages, the RNA of the roots, shoots, leaves, shoot tips from rice plants at the tillering stage, young spikes at the booting stage, immature seeds at the flowering stage and mature seeds at the grain filling stage were extracted for RT-PCR analysis (Fig. 6A). Results showed that *OsAP211* was more highly expressed in shoot tips in mature rice and in immature seeds at the booting stage than in other rice organs. Therefore, we speculated that *OsAP211* may play an important role in growth and in the booting stage of rice.

To further examine the pattern of the accumulation of the *OsAP211* transcript under various stress conditions, the two-week-old rice seedlings were treated with 200 mM

NaCl, low temperature at 2 °C, and drought for 30 min at 1, 2, 4, 6, 8, and 12 h (Fig. 6B). *OsAP211* was strongly induced by cold treatment; transcript levels increased significantly within 0.5 and 1 h, and then transcript levels began to fall. The result indicated that *OsAP211* may be involved in stress signal transduction pathways.

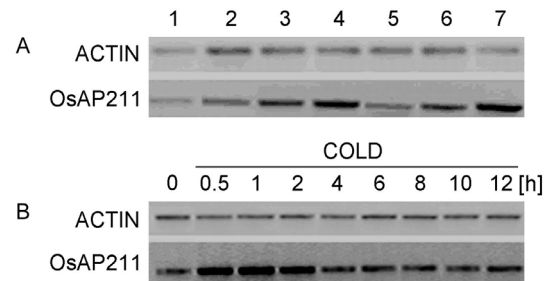


Fig. 6. RT-PCR analysis of expression pattern of *OsAP211* gene. *A* - Expression of *OsAP211* in different organs and at different growth stages. Each lane was loaded with 6 mm³ RT-PCR product of total RNA prepared from roots (lane 1), shoots (lane 2), leaves (lane 3), young spikes at the booting stage (lane 4), mature seeds at the grain filling stage (lane 5), immature seeds at the flowering stage (lane 6) and shoot tips at the tillering stage (lane 7). *B* - Expression of the *OsAP211* in response to cold stress. Each lane was loaded with 0.006 cm³ RT-PCR product. RT-PCR assays were performed as described in Materials and methods.

Discussion

The one yeast-hybrid system has proven to be one of the most efficient techniques in isolating transcription factors and studying interactions between transcription factors and various *cis*-elements. In this research, we isolated a rice cDNA encoding DRE-binding protein, designated *OsAP211*, which can interact specifically with the DRE/CRT, a *cis*-element in the stress inducible gene *rd29A*. *OsAP211* protein belongs to the AP2/EREBP transcription factor family and contains a highly conserved AP2/EREBP domain, a nuclear localization signal, and an activation region.

Sequence alignment of *OsAP211* with other AP2/EREBP-like proteins from various species showed high conservation in the DNA-binding domain. Phylogenetic analysis of the amino acid sequences of *OsAP211* demonstrated that *OsAP211* shared high homology with *OsDREB1*. Although the flexible DREB gene network has been widely developed in *Arabidopsis* and rice (Ito *et al.* 2006, Sakuma *et al.* 2006), little is known about the rice DREB1 proteins except DREB1A, DREB1B and DREB1C (Sakuma *et al.* 2002). Our results indicate that the protein may belong to the DREB subfamily and *OsAP211* may be one functional homolog of DREB1 transcription factors.

Sakuma *et al.* (2002) analyzed the DNA-binding specificity of DREBs and found that two amino acids, valine and glutamic acid were conserved in the AP2/EREBP domains and have important roles in

DNA-binding specificity. We also investigated the structure of *OsAP211*; the consensus AP2/EREBP domain consists of a three-stranded anti-parallel β -sheet and α -helix packed approximately parallel to the β -sheet that interacts with base pairs in the DNA major groove. In the consensus AP2/EREBP domain, valine and glutamic residues in β -sheet 2 can bind specifically to DRE and the amino acid residues Arg6, Arg8, Trp10, Glu16, Arg18, Arg25 and Trp27 that make direct contact with DNA for DNA-binding activity are completely conserved in the alignment (Allen *et al.* 1998). The conservation of these sequences in diverse plant species suggests that they have an important functional role in evolution.

The expression of *OsAP211* was constitutive and affected by some abiotic stress. *OsAP211* was highly expressed in shoot tips in mature rice and in immature seeds at the booting stage. This indicates that *OsAP211* may function in the growth and booting stage of rice. Several DREB genes, such as *OsDREB1A*, *OsDREB1B*, *OsDREB2A* (Dubouzet *et al.* 2003, Sakuma *et al.* 2002, 2006) and *OsDREBL* (Chen *et al.* 2003) have been isolated and characterized. These genes are strongly induced within a short time after exposure to drought, salinity and cold stress. In this research, we also found differences in the expression of the *OsAP211* gene in response to abiotic stresses. *OsAP211* was upregulated by cold stress, indicating that *OsAP211* may be regulated by signaling pathways under cold conditions.

In conclusion, we have cloned and characterized the *OsAP211* gene from rice. The protein encoded by this gene has a highly conserved AP2/EREBP domain and other typical features of transcription factors. The expression patterns of *OsAP211* may reflect its different function and

regulate gene expression in response to abiotic stress in rice. This result may be a significant supplement to the whole network of plant stress responses. For further understanding of the *OsAP211* function, development of a transgenic plant with over-expression is underway.

References

- Allen, M., Yamasaki, K., Ohme-Takagi, M., Tateno, M., Suzuki, M.: A novel mode of DNA recognition by a β -sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. - EMBO J. **17**: 5484-5496, 1998.
- Chen, J., Dong, Y., Wang, Y., Liu, Q., Zhang, J., Chen, S.: An AP2/EREBP-type transcription-factor gene from rice is cold-inducible and encodes a nuclear-localized protein. - Theor. appl. Genet. **107**: 972-979, 2003.
- Chen, W., Provart, N., Glazebrook, J., Katagiri, F., Chang, H., Eulgem, T., Mauch, F., Luan, S., Zou, G.: Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. - Plant Cell **14**: 559-574, 2002.
- Chevray, P., Nathans, D.: Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. - Proc. nat. Acad. Sci. USA. **89**: 5789-5793, 1992.
- Choi, D., Rodriguez, E., Close, T.: Barley Cbf3 gene identification, expression pattern, and map location. - Plant Physiol. **129**: 1781-1787, 2002.
- Dubouzet, J., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E., Miura, S., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K.: OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. - Plant J. **33**: 751-763, 2003.
- Gietz, R., Woods, R.: Transcription of yeast by the lithium acetate/single-stranded carrier DNA/PEG method. - Methods Microbiol. **26**: 53-66, 1998.
- Guarente, L.: Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. - Methods Enzymol. **101**: 181-191, 1983.
- Hajela, R., Horvath, D., Gilmour, S., Thomashow, M.: Molecular cloning and expression of cor (cold-regulated) genes in Arabidopsis thaliana. - Plant Physiol. **93**: 1246-1252, 1990.
- Ito, Y., Katsura, K., Maruyama, K.: Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. - Plant Cell Physiol. **47**: 141-153, 2006.
- Jofuku, K., Boer, B., Montagu, M., Okamura, J.: Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. - Plant Cell **6**: 1211-1225, 1994.
- Liu, J., Zhang, Z., Qin, Q.: Isolated and characterization of a cDNA encoding ethylene-responsive element binding protein(EREBP)/AP2-type protein, RCBF2, in *Oryza sativa* L. - Biotechnol. Lett. **29**: 165-173, 2007.
- Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K.: 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. - Plant Cell **10**: 1391-1406, 1998.
- Nakano, T., Suzuki, K., Fujimura, T., Shinshi, H.: Genome-wide analysis of the ERF gene family in Arabidopsis and rice. - Plant Physiol. **140**: 411-432, 2006.
- Okamura, J., Caster, B., Villarreal, R., Montagu, M., Joffuku, K.: The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. - Proc. nat. Acad. Sci. USA. **94**: 7076-7081, 1997.
- Ohme-Takagi, M., Shinshi, H.: Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. - Plant Cell **7**: 173-182, 1995.
- Riechmann, J., Meyerowitz, E.: The AP2/EREBP family of plant transcription factors. - Biol. Chem. **379**: 633-646, 1998.
- Sakuma, Y., Liu, Q., Dubouzet, J., Abe, H., Shinozaki, K., Yamaguchi-Shinozaki, K.: DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. - Biochem. Biophys. Res. Commun. **290**: 998-1009, 2002.
- Sakuma, Y., Maruyama, K., Osakabe, Y.: Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression. - Plant Cell **18**: 1292-1309, 2006.
- Shen, Y., Zhang, W., He, S., Zhang, J., Liu, Q., Chen, S.: An EREBP/AP2-type protein in Triticum aestivum was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. - Theor. appl. Genet. **106**: 923-930, 2003.
- Thomashow, M.: Role of cold-responsive genes in plant freezing tolerance. - Plant Physiol. **118**: 1-7, 1998.
- Yamaguchi-Shinozaki, K., Koizumi, M., Urao, S., Shinozaki, K.: Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in Arabidopsis thaliana, sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. - Plant Cell Physiol. **33**: 217-224, 1992.
- Zhang, S., Klessig, D.: MAPK cascades in plant defense signaling. - Trends Plant Sci. **6**: 520-527, 2001.