Isolation and characterization of a OsRap2.4A transcription factor and its expression in *Arabidopsis* for enhancing high salt and drought tolerance

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Dehydration responsive element/C-repeat (DRE/CRT) is a *cis*-acting element involved in the regulation of abiotic stress-responsive gene expression in higher plants. Using a 50-nucleotide bait containing DRE *cis*-acting element localized on the downstream mini TATA box of glutamate dehydrogenase-like protein (*JRC2606*) promoter in yeast one-hybrid screening, we have identified two transcription factors belonging to A6 subgroup of DREB subfamily – OsRap2.4A and OsRap2.4B. Expression of *OsRap2.4A* was induced by drought, high salt and temperature stress conditions, and also by abscisic acid treatment. Binding assay showed that OsRap2.4A bound specifically to DRE sequence in both *in vivo* and *in vitro* experiments. Transient expression of *OsRap2.4A* in protoplasts revealed that OsRap2.4A functioned as a transcriptional activator and upregulated expression of the GUS reporter gene. Transgenic *Arabidopsis* plants expressing *OsRap2.4A* at low level showed no significant growth retardation under normal condition, whereas at high level expression of *OsRap2.4A* caused an obvious retardation. Expression of OsRap2.4A showed the tolerance against drought and salt stresses in comparison with control. These results suggested that *OsRap2.4A* gene may be involved in a new regulatory pathway in plant responses to abiotic stresses and it is potentially useful for the transformation into crop plants to improve tolerance against drought and high salt stresses.

Keywords: Abiotic stress, AP2 domain, *Arabidopsis* plants, DRE element, transcriptional repressor.

PLANTS are immobile in nature and thus must respond and adapt to abiotic stress such as drought, high salt, heat and cold in order to survive. Under these stresses, plants induce various biochemical and physiological changes in the process of acquiring stress tolerance. A number of genes responsible for stress tolerance have been discovered, many of them are transcription factors (TFs). TFs play a central role in gene expression by regulating expression of downstream genes as *trans*-acting elements via specific binding to *cis*-acting elements in the promoters of target genes. From analyses of stress-responsive promoters, *cis*- and *trans*-acting elements involved in transcriptional responses of stress-responsive genes have been identified[1].

In the plant kingdom, the AP2/ERF family is a specific and large group of transcription factors containing AP2/ERF-type DNA binding domain, and these family members are encoded by 145 and 163 genes in *Arabidopsis* and rice respectively[2,3]. This domain was first found in the *Arabidopsis* homeotic protein APETALA2, and a similar domain was found in tobacco ethylene-responsive element-binding protein (ERFBP)[4,5]. These domains consist of 50–60 amino acids, which are closely related[6].

In *Arabidopsis*, ERFBP/AP2 family consists of 145 members divided into three subgroups based on the presence of number of ERFBP/AP2 domains in each molecule that are related to the AP2 and ERFBP subgroups. The AP2 subgroup includes of 14 members, each of which contains two ERFBP/AP2 domains. The RAV subgroup includes six members that conserve two different DNA-binding domains, ERFBP/AP2 and B3. The ERFBP subgroup consists of 125 members, with only one ERFBP/AP2 domain. Of these, 121 proteins contain a conserved WLG motif in the middle of their ERFBP/AP2 domain[2]. The members of the ERFBP subgroup are further divided into two subfamilies: DREB (dehydration responsive element binding) subfamily and DREB-like protein, based on the similarity of the amino acid sequence of the DNA-binding domain. DREB subfamily consists of 56 members in *Arabidopsis* and all of them contain one ERFBP/AP2 domain considered to play a crucial role in the process of response to environmental stresses. DREB is divided into six small groups based on similarities in the binding domain. The first and second small groups (A1, A2) include the DREB1/CBF 4 and DREB2 families respectively. The third small group (A3) has only ABI4. The fourth small group (A4) contains 16 members, including TINY protein. The fifth small group (A5) consists of 16 genes, including RAP2.1, RAP2.9 and RAP2.10. The sixth small group (A6) consists of nine genes, including RAP2.4 protein[2].

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Among the characterized stress responsive transcription factors, DREB proteins are widely validated in several plant systems to reveal their role in stress adaptation[7,8]. Members of the DREB subfamily play an important role in the resistance of plants to abiotic stress by recognizing and binding to the dehydration responsive element (DRE) or DRE-like *cis*-acting element, which has a core sequence of A/GCCGAC frequently existing in promoters of plant genes induced by dehydration, high salt, heat and cold stresses[8]. DRE-like *cis*-acting elements, such as C-repeat (CRT) and low-temperature-responsive element (LTRE) possess a CCGAC core motif which plays a role in the regulation of low-temperature inducible promoters[9,10]. Three DREB1 proteins, DREB1A, DREB1B and DREB1C, members of the A-1 DREB group interact with DRE, present in the promoters of the target genes and enhance plant freezing tolerance[11]. Similar results have been reported for constitutive active form of DREB2 proteins, the A-2 DREB group, under dehydration and high salinity stress conditions[7,12]. TINY, a member of the A-4 DREB group, can activate the expression of both DRE- and ERE-regulated genes. In this way, TINY plays a role in the cross-talk between abiotic and biotic stress-responsive gene expression pathways by connecting the DRE- and ERE-mediated signaling pathways[13]. Rap2.4, a member of the A6 group, functions as a transactivator of DRE- and ERE-mediated genes that are responsive to light, ethylene and drought, suggesting that Rap2.4 acts in the crosstalk between the light and ethylene signalling pathways[14]. Transcription factors of the DREB subfamily have been identified in various plant species, including *Arabidopsis*, rice, cotton and asparagus[7,15–19]. The role of DREB transcription factors in plant response to abiotic stress has also been characterized in detail[7,11,20,21]. Significantly, the introduction of genes encoding DREB transcription factors via gene transfer into model plants and some other crop plants resulted in improved stress tolerance[1,3,7,22–24].

Screening of stress-inducible genes from rice database, Rabbani *et al.*[25] identified an unreported gene named as *JRC2606* (accession number: NM_001059992.2) encoding glutamate dehydrogenase-like protein[24]. Interestingly, promoter sequence of *JRC2606* gene revealed a putative *cis*-acting DRE motif localized downstream of the TATA box, suggesting a new regulatory pathway in plant response to abiotic stresses. Here, we report the identification of a novel DREB-type transcriptional factor named OsRap2.4A that binds to the DRE sequence downstream of the TATA box of *JRC2606* promoter and activates transcription process.

Materials and methods

Plant materials and treatments

Seeds of rice Nipponbare (*Oryza sativa* L. ssp. *japonica*) were embedded in water at 37°C for 2 days and the seedlings were then hydroponically grown in MS solution at 28°C for 3 weeks. The three-leaf-stage plants were subjected to different treatments as described previously[26]. The plants were transferred from the basal nutrient solution to nutrient solution containing 250 mM NaCl (salt treatment), 20% polyethylene glycol (PEG–drought treatment) and 100 μM abscisic acid (ABA treatment). For cold and heat treatments, plants were subjected to 4°C and 42°C respectively. For imposing dehydration stress, plants were exposed to air on tissue papers placed in a box hood. The stress-imposed plants were harvested at different time points as shown in Figure 1 and frozen immediately in liquid nitrogen. Total RNA was isolated from stress-treated and untreated control plants using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol.

Stress-treated cDNA library construction

mRNA was purified from total RNA by biotinylated-oligo(dT) probe and streptavidin paramagnetic particles (Promega) according to the manufacturer's instructions. Five microgram of mixed poly $(A)^+$ RNA population from a variety of conditions, including treatment with 250 mM NaCl, 20% PEG, cold (4°C) and heat (42°C) was used for the preparation of HybriZAP®-2.1 cDNA libraries (Stratagene) according to the supplied manual, with a small modification. Instead of the suggested sepharose CL-2B column which demands the radioactive materials, sephadex 400 column was used for cDNA size fractionation. Aliquots of the amplified HybriZAP®-2.1 libraries were stored in 7% (v/v) DMSO at –80°C until use.

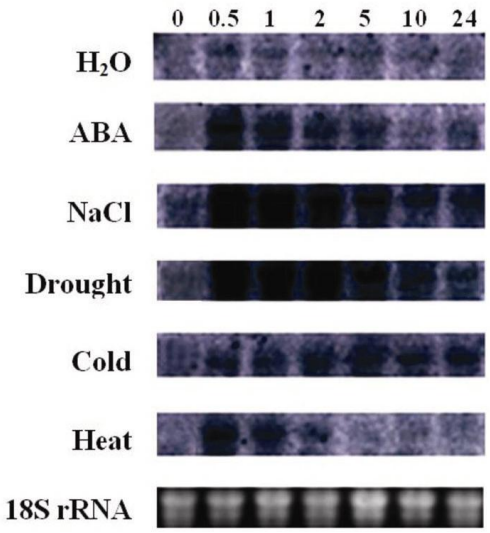

Figure 1. Expression of OsRap2.4A detected using RNA-gel blot assay after the following treatments of dehydration (air-dried), ABA, salt (200 mM NaCl), drought (20% PEG), cold (4°C) and heat (42°C) for 0 h (control), 0.5, 1, 2, 5, 10 and 24 h.

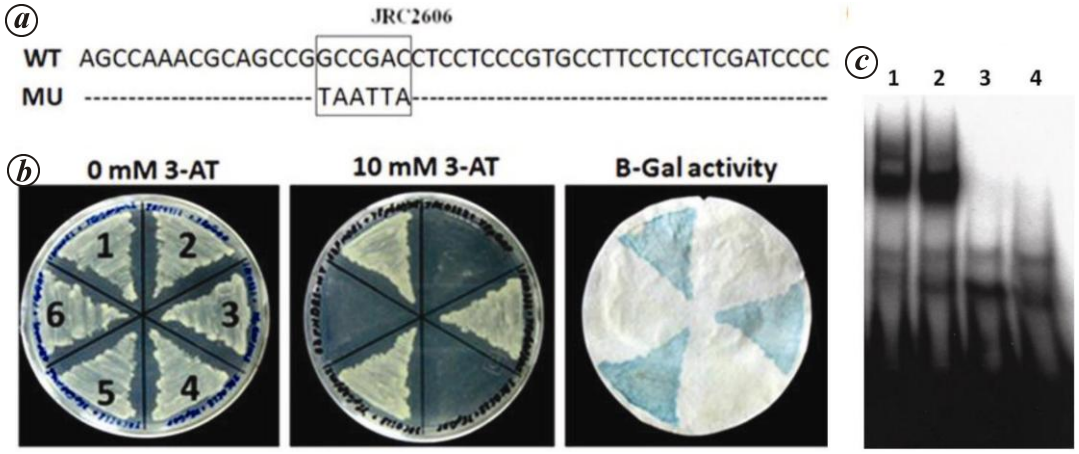

Figure 2. *In vivo* and *in vitro* specific binding of Rap2.4A protein to DRE motif. ***a***, Sequences of wild-type DNA target fragment *JRC2606* containing DRE motif (WT) and mutant fragment in which DRE motif was replaced by TAATTA motif. ***b***, Two cDNA fragments encoding Rap2.4A and Rap2.4B and positive control sequence (DREB1A) were fused to GAL4 acting domain in the constitutively expressed pAD-GAL4 vector. The resulting plasmids were introduced into the YM4271 reporter yeast strain carrying the reporter genes driven by the promoter constructed with four tandemly repeated wild-type DNA fragments of *JRC2606* (1, 3 and 5) or mutant DNA fragments of *JRC2606* (2, 4 and 6). All the transformants carrying wild-type target sequence were able to transactivate the expression of both *HIS3* and l*acZ* reporter gene. The yeast cells transformed reporter plasmid carrying mutant target sequence in promoter region did not show the similar expression of two reporter genes. ***c***, Lanes 1, 2 and 4, *In vitro* DNA-binding reactions performed with the wild-type 50 bp fragment *JRC2606* containing DRE motif and lane 3, the base-substituted 50 bp fragment in which the DRE motif has been replaced by the TAATTA sequence. The radioactive wild-type and base-substituted fragments were incubated with the OsDREB1A–GST fusion protein (as a positive control; lane 1), OsRap2.4A–GST fusion protein (lanes 2 and 3) and the GST protein (as a negative control; lane 4).

Generation of yeast reporter strains

The target-reporter constructs were prepared by the cloning of tandems containing four repeats of a 50-bp DRE fragment of the cold-inducible promoter *JRC2606* (Figure 2) into pHISi-1 and pLacZi vector (Clontech, Palo Alto, CA). Integration of the reporter constructs into yeast (YM4271) genome was performed as described in Yeast Protocol Handbook (Clontech). The background expression of reporter genes in yeast with integrated target-pLacZi and pHISi-1 constructs was tested according to the supplied MATCHMAKER One-Hybrid System User Manual (Clontech). Yeast strains with the lower background level of *HIS3* and *lacZ* were used in the one-hybrid cDNA library screening.

Screening of stress-treated rice cDNA libraries

Approximately 5.5×10^7 yeast transformants were screened using 20 μg of AD-cDNA libraries as described in MATCHMAKER One-Hybrid System User Manual (Clontech). The cDNA isolation, subcloning and sequencing of the positive clones were performed as described previously[7]. Positive cDNA clones were isolated using PCR with forward primer 5′-GCACAGTTGAAGTGA-ACTTGC-3′ and reverse primer 5′-AGGGATGTTTA-ATACCACTAC-3′ and then cloned in pGEM-T Easy vector (Promega). Nucleotide sequence identity was analysed using BLAST (GenBank, NCBI).

Northern blot analysis

RNA gel-blot analyses were carried out as described by Nakashima and Yamaguchi-Shinozaki[27]. Specific DNA fragments of full-length *OsRap2.4A* cDNA and 18S rRNA (as a control) were labelled with [α^{32}P]-dCTP and used as probes for hybridization. Total RNA was separated on 1.2% formaldehyde-MOPS agarose gels and blotted onto Hybond-N^+ membranes (Amersham Biosciences). After hybridization, blots were washed twice in 2X SSC and 0.1% SDS for 20 min at 65°C and once in 1X SSC and 0.1% SDS. Hybridized nucleic acids were recorded on X-ray film. 18S rRNA was used as a control.

DNA-binding activation analysis in yeast

The DNA fragments containing full open reading frames (ORFs) of *OsRap2.4A* and *OsRap2.4B* were amplified from the cDNA library with the specific primers (for *OsRap2.4A:* forward 5′-GGATCCATGGCAGCTGCTA-TAGAAGGA-3′ and reverse 5′-GGATCCCTAGTTA-TTGTTGTTGAGCAG-3′; for *OsRap2.4B:* 5′-GGAT-CCATGGCCGCAGCAATAGACATG-3′ and reverse 5′-GGATCCTTATGAGAGGATTGAGTCCCA-3′; *Bam*HI sites are underlined). The amplified fragments were first cloned into pGEM-T Easy vector (Promega) and verified by sequencing. These fragments were subsequently released by digestion with *Bam*HI and fused in frame with GAL4 DNA acting domain in pAD-GAL4 2.1 vector

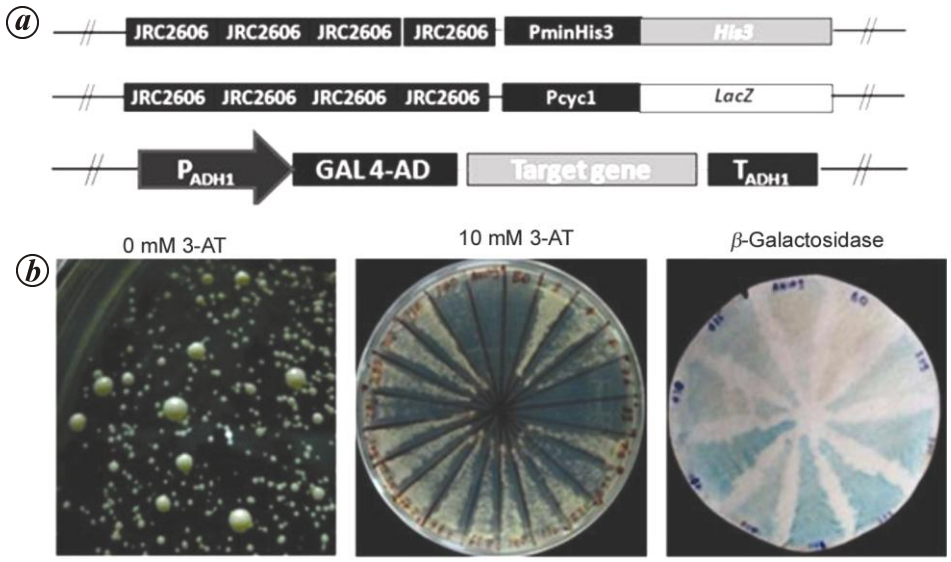

Figure 3. Isolation of cDNAs encoding proteins binding DRE contained in *JRC2606* promoter, using yeast one-hybrid system. ***a***, Illustration of two reporter vectors containing four times tandemly repeated 50 bp DNA fragment of the *JRC2606* promoter and hybrid pAD-GAL4 vector containing protein coding sequences fused to the end of the GAL4 activation domain (GAL4-AD). ***b***, cDNA fragments fused to GAL4 acting domain in pAD-GAL4 vector and then introduced into yeast reporter strain carrying the reporter genes driven by four tandemly repeated fragments of *JRC2606* containing DRE motif (GCCGAC). Yeast cells which grew on histidine-lacking medium were second screened on this medium in the presence of 10 mM 3-Amino-1,2,4-triazole (3-AT) and then continuously applied for *β*-galactosidase assay. There were eight transformants which were able to transactivate the expression of both *HIS3* and *lacZ* reporter genes.

(Figure 3). The constructs was then transformed into yeast reporter strain YM4271 integrated target-pLacZi and pHISi-1 reporter plasmids. These plasmids were previously fused with four tandem copies of 50-bp wild-type or MU fragments containing either wild-type or mutant DRE elements (Figure 2). *OsDREB1A* cDNA was used as a positive control[16]. The DNA-binding activity of the candidate was evaluated based on the expression of reporter genes *HIS3* and *lacZ* as described in the Yeast Protocol Handbook (Clontech).

Preparation of glutathione S-transferase fusion proteins and gel mobility shift assay

The ORF of OsRap2.4A was cloned in *Eco*RI site of pGEX-4T-1 vector (Pharmacia) to generate pGEX : GST–OsRap2.4A plasmid. The plasmid was then transformed into the bacterial strain BL21(DE3) for production and purification of the GST fusion proteins, as described by Urao *et al.*[28]. The cells were induced with 0.1 mM IPTG (isopropyl-1-thio-b-D-galactopyranoside) for 5 h at 28°C and harvested by centrifugation. The cell pellet was re-suspended in 1X PBS, 1 mM PMSF, 1 mM benzamidine buffer and lysed using a sonicator. The cell lysate was applied on the glutathione sepharose 4B column (GE Healthcare Bio-Sciences Corp, NJ) and the column was washed with 1X PBS. The bound proteins were eluted with an elution buffer (50 mM Tri-HCl, pH 8.0, 10 mM glutathione).

Gel electromobility shift assays were conducted according to the methods described by Sakuma *et al.*[2] and Chinnusamy *et al.*[29]. The 50-bp probe fragments containing DRE from the *JRC2606* promoter with (WT) or without (MU) a base substitution were synthesized as duplexes, with the sequences shown in Figure 2 *a*. The nucleotides were labelled by Klenow fill-in reaction in the presence of [α^{32}P]-dATP. The binding reactions (20 µl) were performed with the following binding buffer: 25 mM HEPES, pH 7.5, 5% glycerol, 40 mM KCl, 1 mM DTT, 0.5 mM EDTA. Binding reactions were incubated at room temperature for 20 min and analysed by electrophoresis on 6% non-denaturing polyacrylamide gels in TBE buffer. The recombinant protein OsDREB1A was used in parallel as a positive control.

Transactivation experiments with rice protoplasts

To construct the effector plasmids, cDNA fragment encoding OsRap2.4A was digested from pGEM-T and inserted at the *Bam*HI site downstream of maize *Ubiquitin* constitutive promoter in plant expression pBI-Ubi (Figure 4 *a*) derived from the binary pBI101 vector (Clontech)[30]. For reporter plasmid, four tandem copies of the 50-bp fragment of the *JRC2606* promoter (Figure 2 *a*, see Figure S1 in Supplementary Information online) containing DRE element were inserted into the upstream site of the *JRC2606* minimal TATA sequence, by which CaMV35S promoter in pBI121 vector was replaced

(Figure 4 *a*)[30]. Reporter and effector plasmids were co-transformed into rice protoplasts and the transient expression assay was conducted according to the method described by Dubouzet *et al.*[16] and Kosugi *et al.*[31]. pBI-Ubi containing *OsDREB1A* ORF was used as a positive control in transient expression assay. A ubiquitin–luciferase construct was used as an internal control to normalize GUS values[32]. LUC activity of rice protoplasts was assayed with the Pica Gene luciferase assay kit (Toyo Ink, Tokyo, Japan), according to the manufacturer's protocol. The transactivation data are based upon three independent transformations.

Construction of plant transformation vector and generation of transgenic plants

To generate the pBI121 : OsRap2.4A plant expression vector, pGEM : OsRAP2.4A plasmid was digested with *Bam*HI and 1.3 kb *OsRap2.4A* cDNA was cloned under the control of 35S promoter at *Bam*HI site in pBI121 binary vector (Clontech). The resulting construct was electroporated into *Agrobacterium tumefaciens* EHA105 for further transformation of *Arabidopsis* by the floral dip method[33]. The T1 seedlings were confirmed by PCR analysis using 35S promoter primer (5′-CCCACTATCCTTCGCAA-3′) and the *OsRap2.4A* primer (5′-GGATCCCTAGTTATTGTTGTTGAGCAG-3′) and by Northern blot analysis. T2 seeds of the resulting T1 lines were germinated in a 1/2 MS medium containing 30 mg/l hygromycin and positive T2 plants were used for additional experiments.

High salt and drought tolerance assay

Hygromycin-resistant T2 transgenic *Arabidopsis* lines were utilized for further functional analysis. Analyses of stress tolerance were followed using the methods of Tran *et al.*[34] and Dubouzet *et al.*[16]. In brief, plants were grown in petri dishes containing selective agar germination

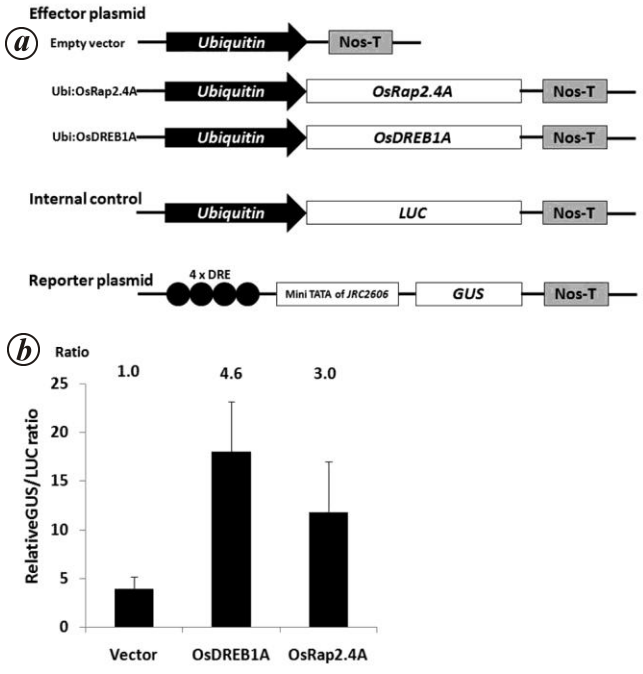

Figure 4. Transactivation of the *JRC2606*-GUS reporter gene by the OsDREB1A and OsRap2.4A proteins using rice protoplasts. ***a***, Schematic diagram of the effecter and reporter constructs used in the transactivation experiments. The reporter plasmid was constructed using four tandemly repeated 50-bp DNA fragments of the *JRC2606* promoter containing the DRE sequence. The promoter was fused to the *JRC2606* minimal TATA promoter – GUS construct. ***b***, Transactivation of the GUS reporter gene by the OsDREB1A or OsRap2.4A proteins. The cauliflower mosaic virus 35S promoter-LUC plasmid was cotransfected in each experiment to normalize for transfection efficiency. Transactivation activity of each protein was determined by the value of GUS/LUC, and the relative transactivation activity was calculated by defining the GUS/LUC value of vector control as 1.00.

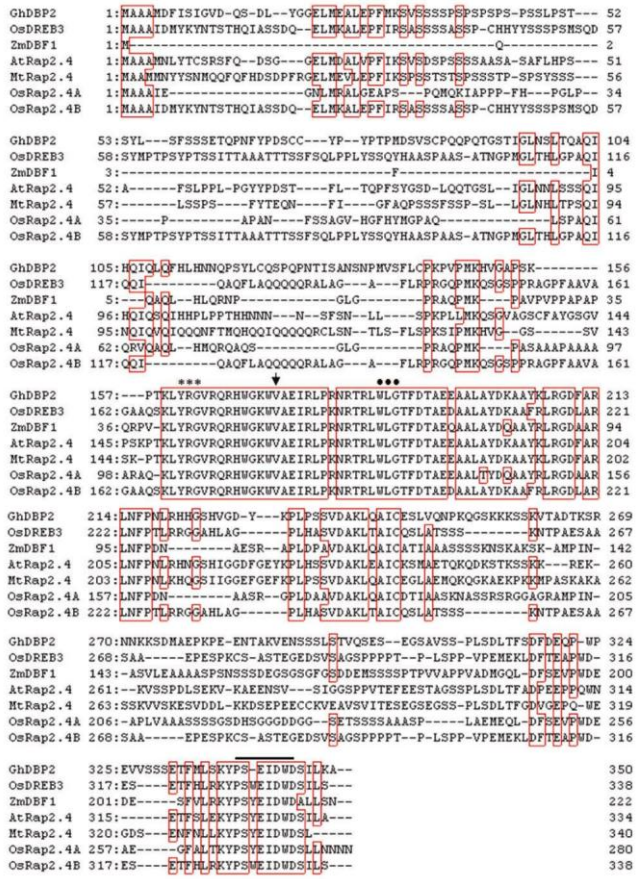

Figure 5. Deduced amino acid sequence alignment of OsRap2.4A and OsRap2.4B 4B with other known A6-subgroup DREB proteins. AtRAP2.4 (NP177931) from *Arabidopsis*, ZmDBF1 (AAM80466) from *Zea mays*, GhDBP2 (AAT39542.1) is from *Gossypium hirsutum* and MtRap2.4 (XP_003638785.1) is from *Medicago truncatula*. Sequences were aligned using Genetyx 6.0 with default parameters. Identical and similar amino acid residues are shown in red box. Gaps required for optimal alignment are indicated by dashes. The DNA binding-related motifs YRG and WLG are indicated by black stars and circles. Val-14 (marked by a black arrow) is highly conserved in AP2 domains of group A-6. The conserved motif PSxEIDW is marked by a line on the top.

medium for 3 weeks, then transferred to 8 cm pots filled with a 1 : 1 mixture of perlite and vermiculite, and grown for one more week. Four-week-old transgenic and control plants with similar phenotype were subjected to salt and drought stress treatments. High salt stress was created by dipping roots of 4-week-old plants in 600 mM NaCl solution for 2 h. The plants were then transferred to pots under normal conditions (no stress) for 2 weeks. For drought treatment, these plants were dehydrated for 2 weeks. The dehydrated plants were rewatered for 3 days. After these stress treatments, the numbers of plants that survived and continued to grow was counted.

Results

Isolation of two novel genes OsRap2.4 encoding DRE-binding proteins

In this study, we carried yeast one hybrid screening to isolate a DRE-binding protein, using a 50-bp DNA fragment of cold-inducible *JRC2606* promoter containing a DRE motif at the centre as a bait (Figure 2). The yeast reporter strain was generated by co-transformation of two plasmids pHISi-1 and pLacZi containing reporter gene *HIS3* and *lacZ* respectively, fused into a four-time tandemly target *JRC2606* sequence (Figure 3 *a*). This reporter strain showed the background expression of both *HIS3* and *lacZ* genes at the basal level, which was confirmed by the fact that it could not grow on SD/–His medium containing 10 mM 3-AT and formed white colonies on filter paper incubated in X-Gal solution for 60 min in β-galactosidase assay. We transformed pAD-GAL4 cDNA library constructed from the mixture of cDNA fragments of stress-treated rice mRNAs into the reporter strain for screening experiments. Approximately 10^7 transformants were screened and eight yeast clones which were resistant to 10 mM 3-AT and formed blue colonies in β-galactosidase assay were isolated (Figure 3 *b*). All the positive cDNA clones were sub-cloned into pGEM-T vector and confirmed by sequencing. Based on the sequencing data, these clones were separated into three groups. The first and second groups possessed four and two positive clones respectively, which were homologous each other. However, the third group differed from the other two sequences. The first and second cDNA clones contained an ORF of 843 and 1020 nucleotides, respectively, called *OsRap2.4A* and *OsRap2.4B* based on their sequence similarity with *DREB* subfamily genes. Further analyses of their deduced amino acid sequences using SMART (http://smart.embl-heidelberg.de) revealed that both proteins contained a conserved DNA-binding domain AP2 of 59 amino acids that might act as a transcriptional regulatory domain. When aligned with several closely related and well-characterized AP2 domain-containing transcription factors, the AP2 domain of OsRap2.4A and OsRap2.4B showed high similarity with those of these proteins (Figure 5). All proteins have a identical WLG motif in the middle and an extremely conserved YRG motif in front of the AP2 domain. The alignment result also showed that their AP2 domain contain the conserved valine (V) in the 14th position and lack the conserved glutamine (E) in the 19th position. However, only OsRap2.4A was different from the others because of its LTYD instead of LAYD motif. The alignment results suggested that OsRap2.4(s) may function as a transcriptional activator in plants as a novel member of the ERF/AP2 proteins.

In order to clarify the relationship of OsRap2.4B in the superfamily of ERF/AP2 transcription factor in plants, we conducted systematic phylogenic analyses of OsRap2.4A and OsRap2.4B proteins, based on the classification of ERF/AP2 transcription factors in *Arabidopsis* and rice[2]. AP2/EREBP domain proteins were classified into four subfamilies, DREB, ERF, AP2, and RAV, and the DREB subfamily was further subdivided into six groups, A1–A6. As a result, both the proteins were classified to the A6 subgroup of the DREB subfamily with AtRap2.4 from *Arabidopsis*, ZmDBF1 from maize, AoDREB from *Asparagus officinalis* L. and so on, which are close in evolution (Figure 6).

Expression profile of OsRap2.4A gene

The expression pattern of *OsRap2.4A* gene was analysed by Northern hybridization. We performed a time-course experiment using a variety of stress and hormone treatments. Four-week-old rice seedlings were subjected to 42°C (heat) and 4°C (cold), transferred to hydroponic

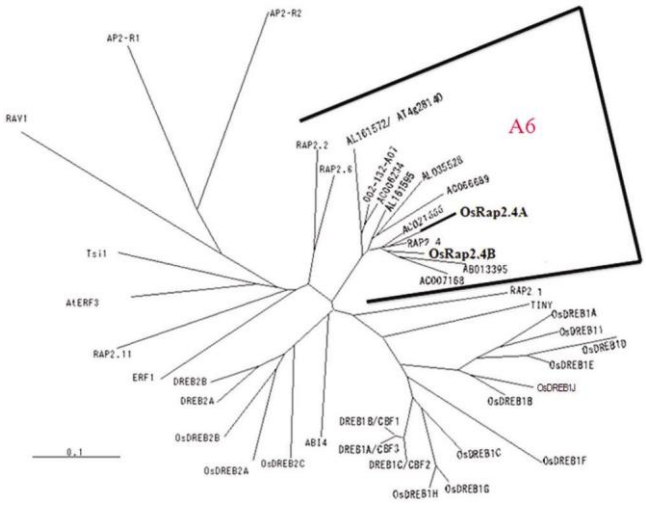

Figure 6. Phylogenetic analysis of OsRap2.4A and OsRap2.4B (bold) and other AP2 domain-containing transcription factors that have been either well characterized or are closely related. The phylogenic tree was constructed using Cluster. Both proteins OsRap2.4A and OsRap2.4B encoded by two isolated cDNAs belong to A6 subgroup of DREB subfamily and have a homology with the rest in AP2 domain.

growth in 250 mM NaCl (salt), 20% PEG (drought) and 100 μM ABA (hormone treatment). As shown in Figure 1, *OsRap2.4A* transcript was strongly induced within 30 min of high salt and drought treatments. Whereas the amount of *OsRap2.4A* mRNA slightly increased in ABA-treated plants and decreased after 1 h, the high accumulation of *OsRap2.4A* mRNA in plants subjected to NaCl and PEG treatments remained unchanged until 5 h and decreased after 10 h. As many A6-subgroup *DREB* genes showed the ABA-uninduced expression profile, expression pattern of *OsRap2.4A* gene in ABA hormone-treated experiment suggested that *OsRap2.4A* is involved in the regulation of stress-responsive genes via an ABA-independent pathway[14,35]. In addition, Northern blot analysis also revealed that when treated with cold stress, *OsRap2.4A* was weakly induced. Under heat treatment, the highest expression of *OsRap2.4A* was achieved early at 0.5 h but quickly decreased to basal line after only 2 h of treatment. When the plants were dehydrated, no increased expression of target gene was detected during the whole experimental period. These results demonstrated that *OsRap2.4A* was involved in the responses of rice to almost all abiotic stresses and significantly upregulated by exposure to drought and high salt conditions.

OsRap2.4A specifically binds to the DRE motif

Majority of the AP2 domain-containing proteins have been identified to bind DRE element[36]. The DNA-binding specificity of OsRap2.4A protein was examined by both gel mobility shift assay and yeast hybrid assay. To directly test the DNA specific-binding activity of OsRAP2.4, GST fusion protein was expressed in *Escherichia coli* Rosetta (DE3) and purified recombinant protein was then used for electrophoretic gel mobility shift assay (EMSA). The EMSA revealed that the GST–OsRap2.4A fusion protein was seen to find to wild-type 50-bp fragment, but not the mutant fragment. However, no retardation band was detected when the pure GST protein was tested as the negative control (Figure 2 *c*). For *in vivo* DNA binding analysis, the full length ORFs of *OsRap2.4A* and *OsRap2.4B* (as another test) were fused to AD domain pAD-GAL4 plasmid and then transformed into a yeast strain carrying the two reporter genes fused to four tandemly repeated copies of the mutated 50 bp fragment in which the DRE motif was replaced with the sequence TAATTA (MU; Figure 2 *a*). cDNA fragment of *OsDREB1A* which was previously identified as it bound specifically to DRE motif, was used as a positive control[16]. The recombinant yeast strains introduced reporter plasmids carrying wild-type DRE motif (WT), but not mutant core sequence (MU), either grew on medium lacking histidine in the presence of 3-AT or induced *lacZ* activity. These *in vivo* data suggested that the both OsRap2.4A and OsRap2.4B proteins specifically interact with the DRE motif, and not with mutant sequence TAATTAT (Figure 2 *b*). These results suggest that OsRap2.4A could bind specifically to DRE motif GCCGAC both *in vivo* and *in vitro.*

OsRap2.4A protein acts as a transactivator in rice protoplast

In previous studies, several DREB proteins classified to A6 subgroup have been reported as transcriptional activators. The multiple sequence alignment showed that the PSxEIDW motif is conserved in all identified A6 subgroup members (data not shown); it is reasonable to assume that they possess not only structural similarity but also conserved functions[19]. To determine whether OsRap2.4A protein is capable of activating DRE-mediated transcription in plant cells, we performed transactivation assays using protoplasts prepared from cultured rice cells. The *GUS* reporter gene was fused to four tandemly repeated 50-bp DNA fragments containing DRE sequence and a minimal TATA sequence of *JRC2606* promoter which used as a reporter plasmid. The effector plasmid consisted of maize *ubiquitin1* promoter fused to the coding region of either *OsRap2.4A* or *OsDREB1A* cDNAs (Figure 4 *a*). The construct *ubiquitin1–LUC* (luciferase) was co-transfected with both reporter and effector plasmids in order to control the transfection efficiency. We quantified LUC activity and used it as an internal control to normalize the transfection efficiency. As shown in Figure 4 *b*, the relative GUS/LUC activity was distinctly upregulated in the presence of either OsRap2.4A or OsDREB1A compared with the vector control in rice protoplasts. Although *OsRap2.4A* exhibited slightly lower transactivation activity compared to *OsDREB1A* (positive control) in rice protoplast, our results revealed that the transactivation activity of OsRap2.4A is higher than the vector control, with about a three-fold higher GUS/LUC ratio. Together with yeast assay in which OsRap2.4A showed specific binding to DRE element in *JRC2606* promoter region, these results demonstrate that OsRap2.4A protein indeed functions as a transcriptional activator in rice cells.

Overexpression of OsRap2.4A gene in transgenic Arabidopsis plants

To test *in vivo* function of OsRap2.4A protein, transgenic *Arabidopsis* expressing *OsRap2.4A* was generated by *Agrobacterium*-mediated transformation. The *OsRap2.4A* cDNA was placed under control of the maize *ubiquitin1* promoter in the binary vector pBI121. The plants were selected by growing on hygromycin MS medium. The hygromycin-resistant plants were screened for the presence of transgene using PCR. Then Northern blot analysis was employed to screen the expression levels of

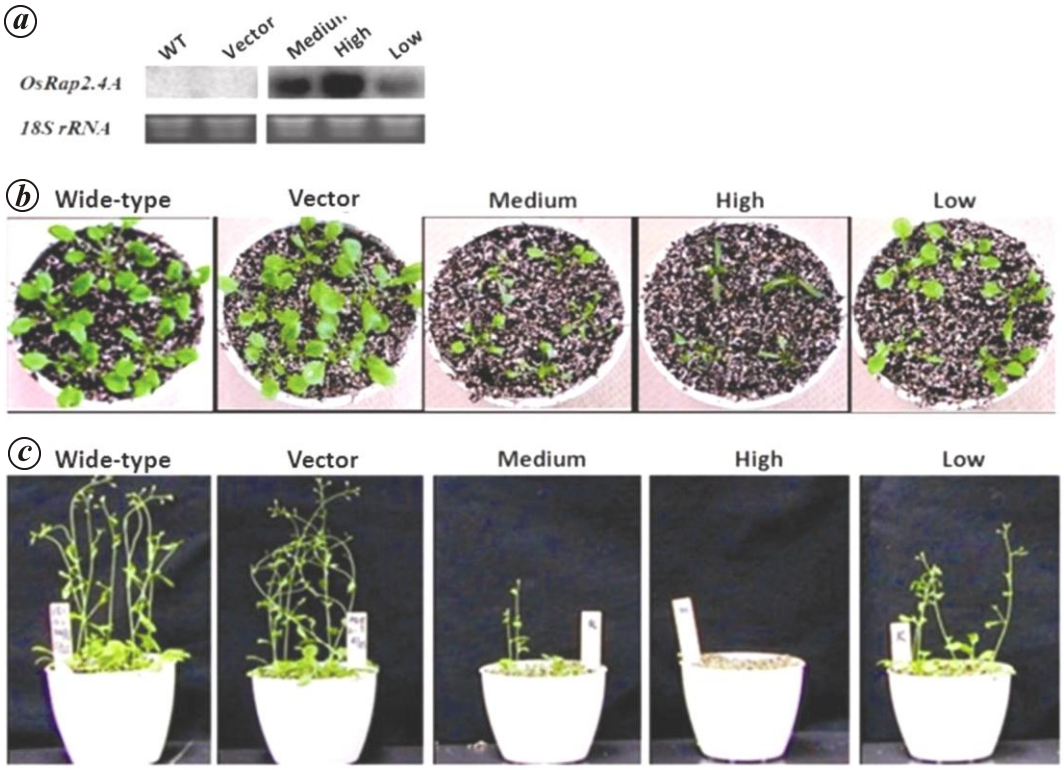

Figure 7. ***a***, Different *OsRap2.4A* expression levels resulted in different effects on growth of transgenic lines under normal condition. ***b***, Wild-type plants (WT), control transgenic plants transformed empty vector pBI121 (Vector) and three 35S : OsRap2.4A plants in which *OsRap2.4A* expressed at low, medium and high levels respectively germinated on selective medium and grown for 4 weeks. ***c***, Four-week-old plants transferred to pots and grown for two additional weeks.

OsRap2.4A in 22 T1 plants. As the controls, transgenic plants introduced with empty expression construct (control transgenic plants) were analysed together with wild-type and *OsRap2.4A* transgenic plants. As a result, eight plants (L1, L4, L5, L8, L9, L12, L14, L15) expressed the transgene at an undetectable level, showing no clearly different phenotype compared with the wild-type plants and transgenic control plants, and 14 plants (64%) expressed *OsRap2.4A* at various level (see Figure S2 in Supplementary Information online). Among *OsRap2.4A*-expressed plants, two plants expressed *OsRap2.4A* at high level (L11, L16) showing severe growth retardation and significantly delayed bolting time; seven plants (L2, L3, L10, L14, L17, L19, L21) expressed the transgene at medium level displaying dwarfed shape and delayed bolting time; and the remaining five plants (L6, L7, L13, L18, L20) expressed the gene at a low level exhibiting slightly different phenotype compared with wild-type and control transgenic plants (Figure 7 *b* and *c*). These results indicate that the degree of growth retardation and dwarfed phenotype of these plants correlated with the overexpression level of the *OsRap2.4A* gene and the higher level of *OsRap2.4A* transcription caused more dwarf phenotypes of the plants. L18 transgenic line showing morphological similarities to wild-type plants was chosen as a representative of transgenic plants for further investigation of stress tolerance. Interestingly, all four-week-old T2 plants generated from this line showed no obviously different phenotype from the wild-type plants under unstressed conditions (Figure 8). Notably, high salt and drought tolerance analysis indicated that the survival rates of L18 transgenic plants were significantly higher than the wild-type plants (Figure 8). After soaking in 600 mM NaCl solution for 2 h and re-growing in normal condition for 2 weeks, the survival percentage of wild-type plants was 21 (8/38), whereas that of transgenic plants was more than 72 (27/37). Similar results were observed in drought tolerance experiment in which all the wild-type plants died (0/36), while 22% (8/36) of the *35S : OsRap2.4A* transgenics survived. These results suggest that the overexpression of *OsRap2.4A* may confer high salt and drought tolerance to the transgenic *Arabidopsis* plants.

Discussion

Many transcription factors, including DREB which play a critical role in improving the abiotic stress tolerance of plants have been characterized and their regulatory mechanisms are relatively well studied. These proteins are able to activate and/or repress transcription of functional protein-encoding genes by interacting with DRE/

CRT *cis*-elements present in the promoter region of various abiotic stressresponsive genes[36]. Using cDNA microarray and RNA gel-blot analyses, Rabbani *et al.*[25] identified 73 rice stress-inducible genes, including glutamate dehydrogenase-like protein-encoded *JRC2606*. We analysed promoter sequence of all these genes for the presence of stress-responsive elements. Interestingly, unlike other plant stress-inducible promoters which contain DRE *cis*-acting element located upstream of the TATA box, the *JRC2606* promoter possessed DRE element downstream of the TATA box, which seems to be play a role of *cis*-acting element involved in an unknown stress response pathway[7,9,34,37–39]. In this study, we have identified two novel transcription factors, OsRap2.4A and OsRap2.4B from rice cDNA library using a target 50-bp DRE motif of *JRC2606* promoter as bait for yeast one-hybrid screening. The deduced amino acid sequence of these two proteins had a similar AP2 domain which is typical for AP2/ERF proteins (Figure 5). Both proteins were classified into the A-6 subgroup of DREB subfamily, based on amino acid sequence alignment (Figure 6). Structural analyses of these proteins showed that they have two conserved regions of AP2-domain-containing proteins like YRG and RYD/LAYD/RAHD[8,40]. The 20 amino-acids-long YRG region possessed two conserved motifs, including YRG and WLG (Figure 5), in which Arg and Trp residues were identified, which have role in interaction with DNA because of their basic characteristics[36]. Previous studies have reported that AP2 domain of DRE-binding proteins harboured specific residues such as Val14 and Glu19 which play an important role in determining the DNA-binding specificity[14,40]. However, analyses of the deduced amino acid sequences of OsRap2.4A and OsRap2.4 showed that the protein contains conserved Val14, but lacks Glu19 in AP2 domain (Figure 5). *In vitro* experiments showed that OsRap2.4A recombinant protein could bind specifically to DRE *cis*-acting element (Figure 2 *c*). We also identified that proteins OsRap2.4A and OsRap2.4B fused to GAL4-acting domain activated the expression of reporter genes *His3* and *LacZ* controlled by four tandem copies of 50-bp DNA sequence containing DRE *cis*-acting elements in yeast cells (Figure 2 *b*). These *in vivo* and *in vitro* experiments indicated that the two proteins, or at least OsRap2.4A, have DRE binding activity despite the absence of Glu19 in AP2 domain. This characteristic is similar to all A-6 subgroup proteins and several A-5 subgroup proteins[2]. Additionally, when the AP2 domains of OsRap2.4 proteins were analysed, we found a LAYD motif in second C-terminal 40-amino-acid region of the domains (Figure 5), which is also found in several other AP2/ERF proteins and is proposed to mediate protein–protein interaction or to have an alternative role in DNA binding[8,36]. However, this motif was only present in amino acid sequence of OsRap2.4A but not OsRap2.4B, in which it was replaced by LTYD motif (Figure 5). This is the main reason why we chose OsRap2.4A for further studies.

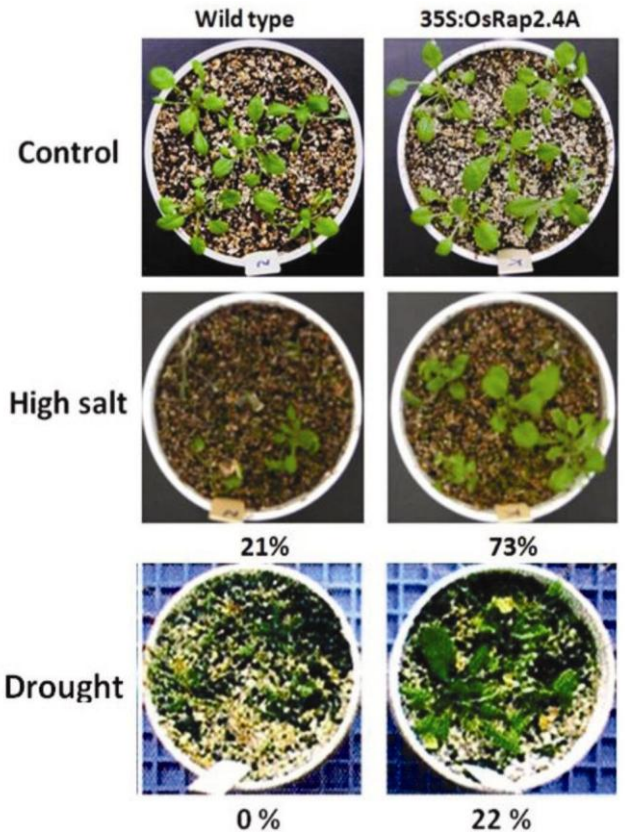

Figure 8. High salt and drought tolerance phenotype of wild-type and 35S : OsRap2.4A plants. Control, four-week-old plants grown under normal condition. High salt stress, Wild-type and transgenic *Arabidopsis* plants were dipped in 600 mM NaCl solution for 2 h and then transferred to normal growing conditions for 2 weeks. Drought stress, Water withheld from plants for 2 weeks; then rewatering for 3 days. Percentage of surviving plants is indicated below.

Almost all previously identified A-6 subgroup AP2/ERF transcription factors have been shown to function as transcriptional activators which could activate a variety of downstream stress-related genes[14,19,35,41–43]. Although downstream regulated genes were not found out in this study, transactivation experiment of the OsRap2.4A protein in rice protoplasts implied that this protein might induce the expression of genes driven by promoter containing DRE motif (Figure 4). This finding was supported by yeast experiments in which the expression level of reporter genes *His3* and *LacZ* was also repressed by the expression of OsRap2.4A in YM4271 yeast cells co-transformed reporter vectors containing four tandem copies of 50-bp DNA fragment of *JRC2606* in promoter regions and pBD-GAL4 vector containing full-length ORF of *OsRap2.4A* inserted downstream into site of GAL4 DNA-binding domain (data not shown). These observations indicate that OsRap2.4A likely acts as a transcriptional activator of DRE-containing gene expression in plant cells.

Previous studies have shown that many DREBs from different plants, though all classified into the A6 subgroup, respond differently to stresses[3,19,44,45]. *ZmDBF1* shows a response to dehydration and salt[35]; *AhDREB1* plays a role in the salt- and drought-responsive pathways[43]; the transcripts of *GmDREBb* are induced by low temperature as well as salinity and drought[42]; *GhDBP2* is greatly induced by drought, NaCl and low temperature[41]; the expression of the *Rap2.4* gene in *Arabidopsis* is upregulated by salt and drought stresses, but downregulated by light[14]. In this study, we found that the expression of OsRap2.4A in rice was upregulated by multiple stress signals, including salt and drought (Figure 1). These observations suggest that DREB proteins sharing high structural similarity might play different roles in response to stresses and might be regulated by different upstream genes responsive to different stresses[19]. Additionally, although all reported abiotic stress-related DREB transcription factors, including A6 subgroup DREB protein mentioned above and the OsRap2.4A protein, show structural and functional similarity (Figure 3), their expression patterns in response to ABA are significantly different, which indicates that there are at least two basic regulatory pathways – ABA-dependent and ABA-independent[3,46]. Li *et al.*[42] identified three AP2 proteins (GmDREBa, GmDREBb and GmDREBc) in soybean; *GmDREBc* and *GmDREBa* genes were strongly induced by ABA, whereas *GmDREBb* gene, which has now been classified into A6 subgroup was greatly induced by salt, cold and drought stresses through an ABA-independent regulatory pathway. In contrast, expression pattern of another A6 subgroup gene *MtWXP1* in *Medicago truncatula* showed that it was clearly upregulated during ABA treatment[24]. Our Northern analysis revealed that *OsRap2.4A* was induced during high salinity and drought stress, but showed no obvious response to the low temperature or exogenous ABA treatment (Figure 1); thus, it implies that OsRap2.4A probably participates in the ABA-independent stress signal transduction pathway as well as Rap2.4 in *Arabidopsis* or several other A6 subgroup DREB proteins[14,19,24]. This finding supports the previous knowledge that there is variation of transcriptional regulation mechanism for AP2 transcription factors, which demands further studies for better understanding.

Proteins with similar domains usually have the same or similar biological function; therefore, members belonging to the group of AP2/ERF proteins are likely to play similar roles in stress tolerance of plants. OsRap2.4A had the closest relationship genetically with *Arabidopsis* Rap2.4 transcription factors whose overexpressions enhanced drought tolerance in transgenic plants[14]. In order to verify the functional role of *OsRap2.4A* in abiotic stress tolerance, we analysed transgenic *Arabidopsis* plants over expressing *OsRap2.4A* controlled by constitutive promoter *35S*. Our experimental results show that overexpression of *OsRap2.4A* causes obvious growth retardation of *35S : OsRap2.4A* transgenic plants in which the decrease in growth rate is correlated to transcript accumulation level of *OsRap2.4A* gene (Figure 7, also see Figure S2 in Supplemental Information online). The overexpression of some identified stress-responsive genes in transgenic plants, such as *OsNAC6/SNAC2*, *OsDREB1A*, *OsDREB1B*, *AtDREB1A* and *AtDREB1B*, led to growth retardation under normal condition, which may finally cause significant reduction of potential yield[17,47,48]. In particular, constitutive over expression of such ABA signalling-related genes might significantly retard growth, since ABA plays a pivotal role in plant development and growth regulation[49]. Maruyama *et al.*[50] the mechanism of growth retardation of *35S : DREB1A* transgenic plants and found that transgene upregulated some transcription factors whose expression represses photosynthesis and carbohydrate metabolism in transgenic plants.

In our study we noted that salt tolerance of transgenic lines correlated to expression level of *OsRap2.4A* in stress tolerance assay. In almost all previous studies, overexpression of identified stress-responsive transcription factors induced either increased or decreased stress tolerance of transgenic plants. However, several reports suggest that changes in the expression level of a transcription factor may lead to various degrees of sensitivity to different stresses, such as *XERICO* and *ABR1*[51,52]. Our experiments indicated that the low expression level of *OsRap2.4A* leads to significantly increased drought and salt tolerance in transgenic *Arabidopsis* plants (Figure 8). Although further studies are needed to understand the regulatory functional mechanism of OsRap2.4A in stress responses of plant, our findings suggest that it seems to be a novel transcriptional activator that may regulate plant stress tolerance via an ABA-independent pathway.

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