OsRMC, a negative regulator of salt stress response in rice, is regulated by two AP2/ERF transcription factors

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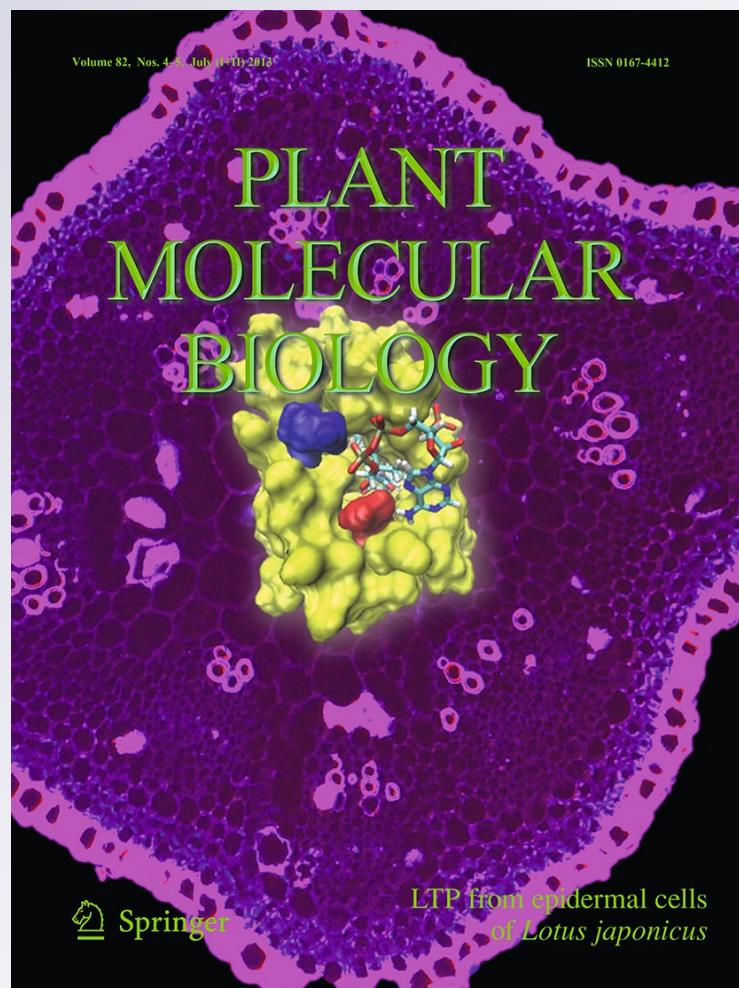# OsRMC, a negative regulator of salt stress response in rice, is regulated by two AP2/ERF transcription factors

Tânia S. Serra · Duarte D. Figueiredo · André M. Cordeiro · Diego M. Almeida · Tiago Lourenço · Isabel A. Abreu · Alvaro Sebastián · Lisete Fernandes · Bruno Contreras-Moreira · M. Margarida Oliveira · Nelson J. M. Saibo

Abstract High salinity causes remarkable losses in rice productivity worldwide mainly because it inhibits growth and reduces grain yield. To cope with environmental changes, plants evolved several adaptive mechanisms, which involve the regulation of many stress-responsive genes. Among these, we have chosen *OsRMC* to study its transcriptional regulation in rice seedlings subjected to high salinity. Its transcription was highly induced by salt treatment and showed a stress-dose-dependent pattern. *OsRMC* encodes a receptor-like kinase described as a negative regulator of salt stress responses in rice. To investigate how *OsRMC* is regulated in response to high salinity, a salt-induced rice cDNA expression library was constructed and subsequently screened using the yeast one-hybrid system and the *OsRMC* promoter as bait. Thereby, two transcription factors (TFs), OsEREBP1 and OsEREBP2, belonging to the AP2/ERF family were identified. Both TFs were shown to bind to the same GCC-like DNA motif in *OsRMC* promoter and to negatively regulate its gene expression. The identified TFs were characterized regarding their gene expression under different abiotic stress conditions. This study revealed that *OsEREBP1* transcript level is not significantly affected by salt, ABA or severe cold (5 °C) and is only slightly regulated by drought and moderate cold. On the other hand, the *OsEREBP2* transcript level increased after cold, ABA, drought and high salinity treatments, indicating that OsEREBP2 may play a central role mediating the response to different abiotic stresses. Gene expression analysis in rice varieties with contrasting salt tolerance further suggests that *OsEREBP2* is involved in salt stress response in rice.

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Introduction

Plants undergo several types of abiotic stress conditions, such as drought, cold and high salinity, throughout their life cycle. Soil salinity causes high losses in crop productivity worldwide mainly because it inhibits growth and grain yield (Hasegawa et al. 2000; Pardo 2010; Wankhade et al. 2010). To minimize the deleterious effects of salt stress, plants need to coordinate several adaptation mechanisms that occur at the cellular and molecular levels (Munns and Tester 2008). Cellular adaptations include the vacuolar compartmentation of Na^+ through vacuolar Na^+/H^+ transporters, such as OsNHX1 (Tester and Davenport 2003). The exclusion of excessive Na^+ from the cytosol allows the maintenance of ion homeostasis, preventing Na^+-specific damage in plant tissues leading to, among others, photosynthesis inhibition (Hauser and Horie 2010). Na^+ transport to the vacuole is accompanied by adaptive changes at the molecular level, such as the synthesis and accumulation of osmoprotectants in the cytosol (Hasegawa et al. 2000; Tester and Davenport 2003; Xiong et al. 2002). Osmoprotectans, such as proline and trehalose, are involved in the maintenance of the cell osmotic balance but also in the scavenging of reactive oxygen species and protection of cellular structures (Sahi et al. 2006; Tester and Davenport 2003).

Adaptation mechanisms rely on the perception and transduction of stress signals from the environment. Stress signals can be initially perceived by apoplast molecules, such as peptides and enzymes, which will lead to the activation of specific signaling pathways in the cell (Sattelmacher 2001; Zhang et al. 2009a). The rice *root meander curling* (*OsRMC*) gene was described as an apoplast protein belonging to the DUF26 group of receptor-like kinases (RLKs) (Jiang et al. 2007; Zhang et al. 2009a). This protein was predicted to have a receptor domain containing a cysteine-rich repeat (CRR), but no kinase or transmembrane domain, suggesting that this protein is secreted (Chen 2001; Jiang et al. 2007). Some secreted proteins have been described as putative ligands for receptor-like kinases in Arabidopsis (Rojo et al. 2002), Brassicaceae (Dixit et al. 2000) and tomato (Tang et al. 2002). The CLAVATA pathway in Arabidopsis is one of the best characterized signaling pathways involving the binding of a ligand (CLV3) to a receptor complex (CLV1/CLV2) (Jeong et al. 1999; Rojo et al. 2002). The interaction between CLV3 and CLV1/CLV2 is required for the formation of the active receptor complex involved in the regulation of meristem development. In Brassicaceae, a cysteine-rich protein, SCR, was shown to be a ligand for the stigma-expressed S-locus receptor kinase SRK involved in the self-incompatibility response (Dixit et al. 2000; Kachroo et al. 2002). Although the cysteine-rich region of SCR and SRK is structurally distinct from the CRR domain present in OsRMC, this apoplastic

protein may be a ligand for CRR receptor-like kinases (CRKs) regulating abiotic stress responses (Chen 2001). The role of OsRMC in the salt stress response was previously described using rice knockdown lines. These lines showed improved salt tolerance associated with induction of stress-responsive genes, indicating that OsRMC is a negative regulator of rice salt stress response (Zhang et al. 2009a).

The regulation of abiotic stress responses relies on the ability of transcription factors (TFs) to coordinate the expression of stress-responsive genes (Agarwal and Jha 2010; Saibo et al. 2009). The NAC (NAM, ATAF1,2 and CUC2), ABA Responsive Element Binding (AREB) protein and APETALA2/Ethylene Response Factor (AP2/ERF) families of transcription factors were shown to be highly involved in the salt stress tolerance mechanisms (Roy-Choudhury et al. 2008; Takasaki et al. 2010; Wu et al. 2007; Negrão et al. 2011). The AP2/ERF protein family is characterized by the presence of the AP2 DNA binding domain (Dietz et al. 2010; Okamuro et al. 1997). The subfamily AP2 contains two AP2 domains and functions mainly in the regulation of plant development (Okamuro et al. 1997; Zhao et al. 2006). On the other hand, the subfamilies Dehydration-Responsive Element-Binding protein (DREB) and Ethylene-Responsive Factor (ERF) have only a single DNA binding domain and are involved in biotic and abiotic stress responses (Dietz et al. 2010; Gutterson and Reuber 2004). Over-expression of genes from these subfamilies, such as *OsBIERF3* and *OsDREB1D*, in tobacco and Arabidopsis, respectively, were shown to highly increase plant salt tolerance (Cao et al. 2005; Zhang et al. 2009b).

Rice is among the most important food crops worldwide and its productivity is highly affected by high salinity, which causes reduced growth and grain yield. This crop shows higher sensitivity to increases in soil salinity compared to others, such as durum bread wheat or barley (Munns and Tester 2008). Understanding the molecular mechanisms underlying salt stress response in rice may provide useful tools to improve its tolerance. In this work, we investigated the transcriptional regulation of *OsRMC*, a rice gene highly induced by salt stress. We identified and characterized the function of two transcription factors that bind to the *OsRMC* promoter and may be associated with its salt stress response.

Materials and methods

Plant material and stress treatments

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were submerged for 30 min at 50 °C in 0.1 % Benlate, washed with sterile water and disinfected with 70 % ethanol for 1 min

and with a solution of 2 % sodium hypochlorite containing 0.02 % Tween 20 for 30 min. After washing six times with sterile water, the seeds were germinated in the darkness for 3 days at 28 °C in Petri dishes containing 3MM paper pieces soaked in sterile water. Germinated seeds were transferred to glass tubes containing Yoshida's medium (Yoshida et al. 1976) and grown under a 12 h photoperiod (500 μEm^{-2}s^{-1}) regime. The cDNA expression library and the *OsRMC* expression analysis (in whole seedling) was performed with RNA extracted from 14-day-old rice seedlings transferred to Yoshida's medium supplemented with 100 or 200 mM NaCl. Samples were collected after 0, 2, 5, 12 and 24 h of treatment, frozen in liquid nitrogen and kept at −80 °C until RNA extraction. The shoot and root expression pattern of *OsRMC* and the novel TF genes identified was performed using 14-day-old seedlings subjected to drought, cold (5 and 10 °C), salt or ABA treatments. For the drought treatment, seedlings were left to dry on paper towels in the flow chamber. The salt and ABA treatments were performed using Yoshida's medium supplemented with 200 mM NaCl or 100 μM ABA, respectively. Shoot and root samples were separated prior to freezing in liquid nitrogen and storage at −80 °C. The transcriptional studies in Nipponbare, IR29 and Pokkali rice varieties were performed with seeds supplied by International Rice Research Institute (IRRI, Manila, Philippines). Seeds were heat-treated for 5 days at 50 °C and afterwards germinated in the darkness for 3 days at 28 °C in Petri dishes containing 3MM paper pieces soaked in sterile water. Germinated seeds were transferred to float tanks containing Yoshida's medium (Yoshida et al. 1976) and grown for 11 days under a 12 h photoperiod (500 μEm^{-2}s^{-1}) regime. The salt treatment was performed transferring these seedlings to Yoshida's medium supplemented with 120 mM NaCl. Shoot and root were sampled separately frozen in liquid nitrogen and kept at −80 °C.

Reverse transcription-PCR (RT-PCR) analysis

OsRMC gene expression analysis in whole seedlings was performed using the total RNA purified to construct the cDNA expression library (see below). Reverse transcription was performed with 1 μg total RNA using an oligo-(dT)$_{12–18}$ primer and the Superscript II reverse transcriptase (Invitrogen, CA, USA) following the manufacturer's instructions. The shoot and root gene expression profile of *OsRMC*, *OsEREBP1* and *OsEREBP2* was obtained using total RNA extracted from rice seedlings (cv. Nipponbare), using the RNeasy Plant Mini kit (Qiagen, Courtaboeuf, France). The cDNA first strand was synthesized from 1 μg total RNA using an anchored-oligo(dT)$_{18}$ primer and according to the instructions from the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). PCR reactions

were performed using the gene specific primers OsEREBP1-Fw/Rv and OsEREBP2-Fw/Rv described in Supplementary Table S1. The *OsACT1* and *OsUBC* genes were used as internal controls. The results shown are representative of at least two biological replicates.

Real-time quantitative PCR (qPCR) analysis

Quantitative PCR was performed using the LightCycler 480 system (Roche, Basel, Switzerland) and the SYBR Green I Master mix (Roche, Basel, Switzerland). The PCR running conditions were as follows: one cycle at 95 °C for 5 min and 45 cycles of amplification at 95 °C for 10 s, 58–60 °C for 10 s and 72 °C for 10 s. The CT values were calculated from means of three technical replicates and the relative expression of transcripts (to 25S rRNA) was calculated using the method: relative quantification with kinetic PCR efficiency correction. The rice 25S rRNA gene was used as internal control. The qPCR reactions were carried out with the qOsEREBP1-Fw/Rv and qOsEREBP2-Fw/Rv primers described in Supplementary Table S1. The gene expression profile of the novel TFs identified, observed under abiotic stress conditions, was validated by qPCR using cDNA prepared as previously described. The TFs transcriptional study in Nipponbare, Pokkali and IR29 rice varieties was carried out with cDNA prepared with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland) from 2 μg total RNA isolated with the RNeasy Plant Mini kit (Qiagen, Courtaboeuf, France) and using a random hexamer primer.

cDNA expression library construction

Total RNA was isolated from a pool of five rice seedlings (cv. Nipponbare) subjected to 2, 5, 12 and 24 h of treatment with 100 and 200 mM NaCl, using the TRIzol method described by the manufacturer (Invitrogen, CA, USA). After mRNA purification with the PolyATtract mRNA Isolation System III (Promega, Wisconsin, USA), cDNA was synthesized according to the HybriZAP-2.1XR cDNA synthesis kit manual (Stratagene, CA, USA). The cDNA expression library was constructed into the HybriZAP-2.1 vector according to the manufacturer's instructions. To verify the average size of the cDNA inserts, PCR amplification was performed from 20 individual plaques using the HybriZAP primers described in Supplementary Table S1. After in vivo excision and amplification of the excised phagemid, the cDNA library was used to transform the yeast bait strains.

Construction of yeast bait strains

Yeast strain Y187 (Clontech, USA) was used to generate different strains containing fragments of the *OsRMC*

promoter as bait. The *OsRMC* promoter region was defined as the 2,120 bp sequence upstream of the start codon. The promoter was divided in six overlapping fragments with 300–500 bp, which were inserted in the yeast integrative vector pINT1-HIS3 (Ouwerkerk and Meijer 2001) as a *Not*I-*Xba*I or *Xba*I-*Spe*I fragment. The *Nco*I-*Sac*I fragment of this construct, containing the *PDC6* sequences necessary for homologous recombination, was used to transform Y187 cells. The *APT1* marker gene and the *OsRMC* promoter fragments are upstream of the *HIS3* reporter gene. The obtained yeast bait strains were selected on YAPD medium containing 0.2 mM G418 (Duchefa Biochemie B.V., Netherlands). Because some DNA promoter sequences allow leaky expression of the *HIS3* reporter gene, leading to some background growth in medium lacking histidine, a 3-amino-1,2,4-triazole (3-AT) titration was performed. 3-AT is a competitive inhibitor of the yeast His3 enzyme and will suppress its activity (Ouwerkerk and Meijer 2001). To assess the putative *HIS3* leaky expression, the bait strains were titrated in complete minimal medium (CM) lacking histidine and containing up to 25 mM 3-AT. Yeast transformations were performed by the lithium acetate polyethylene glycol method as described by Ouwerkerk and Meijer 2001.

Yeast one-hybrid screening and validation

Yeast bait strains were transformed with 1 μg of cDNA library. Transformation efficiency was assessed on CM medium without leucine, using 100- and 1,000-fold dilutions of transformation reactions. Putative positive clones were selected on CM medium containing 5 mM 3-AT and lacking leucine and histidine. The identified clones were re-streaked on selective medium to confirm growth. Direct PCR on the yeast colonies was performed to amplify the cDNA insert, using specific primers for the library plasmid. Library plasmid DNA was recovered from these colonies and amplified in *Escherichia coli*. To validate the DNA–protein interaction in yeast, the plasmid DNA was re-transformed into the yeast bait strain. To know whether the isolated clones encode transcription factors, the plasmid DNA was sequenced and the results were analyzed with BLAST programs.

Phylogenetic analysis

The amino acid sequences were aligned using the ClustalW program and the Genedoc software. The sequence alignment used for the phylogeny is provided as Supplemental Fig. S4. The phylogenetic analysis was conducted with the MEGA version 4 (Tamura et al. 2007) using the neighbor-joining algorithm and the obtained alignment. One thousand bootstrapped replicates were performed.

Identification of TF-binding sites in *OsRMC* gene promoter

In order to identify *cis*-elements related to abiotic stress responses, we have used the PlantPAN database. This analysis was performed for the *OsRMC* promoter region ranging from −1,260 to −613 bp, which includes the fragment 3 (−1,160 to −613 bp) (Supplementary Fig. S3). Additionally, the footprintDB and 3D-footprint databases (http://floresta. eead.csic.es/footprintdb and http://floresta.eead.csic.es/3d footprint) were scanned with OsEREBP1 and OsEREBP2 protein sequences to identify DNA-binding proteins that bind to similar DNA motifs and to define the interface amino acid residues that putatively interact with DNA (Contreras-Moreira 2010; Steffens et al. 2004). Two such proteins were selected, ERF-4 and AtERF1A (http://floresta.eead.csic.es/ 3dfootprint/complexes/1gcc_A.html) and their corresponding position weight matrices (PWMs), which capture their binding specificity, scanned along the *OsRMC* promoter. The search for significant matches within the promoter sequence was performed with the software *matrix-scan*, with default parameters (Turatsinze et al. 2008). Several putative OsEREBP1 and OsEREBP2 *cis*-elements were identified in fragment 3.

Construction of plasmids

For the transient expression assays in Arabidopsis protoplasts, we have used the reporter plasmid pLUCm35GUS-pRMC, which contains the fragment 3 of the *OsRMC* promoter driving the *GUS* gene (Supplementary Fig. S3). To construct this plasmid, we have amplified fragment 3 with the primers pRMCF3-Fw and -Rv (Supplementary Table S1) and cloned it into the *Sal*I-*Pst*I restriction sites of the pLUCm35GUS (Figueiredo et al. 2012). To construct the effector plasmids over-expressing *OsEREBP1* and *OsEREBP2*, the respective coding sequences were PCR amplified from cDNA, using gene specific primers containing Gateway adapters, GW-OsE-REBP1-Fw/-Rv and GW-OsEREBP2-Fw/-Rv (Supplementary Table S1) and the Phusion™ high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland). To amplify the *OsEREBP2* sequence, the reaction needed to be supplemented with 1 M betaine and 2.5 % DMSO. The coding sequences amplified were cloned into the entry vector pDONR221 with the BP recombinase, according to the manufacturer's instructions (Invitrogen, CA, USA), to obtain pENTRY-OsEREBP1 and pENTRY-OsEREBP2. The *OsEREBP1* fragment was afterwards transferred to the binary vector pH7WGF2 through LR recombination. The region containing the 35S::GFP:: OsEREBP1 fragment was removed from the binary vector through *Xba*I-*Hin*dIII restriction and cloned into the pGREEN0029 vector to obtain the 35S::OsEREBP1 plasmid.

The 35S::OsEREBP2 effector plasmid was obtained by LR recombination of the pENTRY-OsEREBP2 with the pEarleyGate201 vector following the manufacturer's instructions.

For recombinant protein expression in *E. coli*, OsEREBP1, OsEREBP2 and OsBWMK1 coding sequences were amplified with the GX-OsEREBP1-Fw/-Rv, GX-OsEREBP2-Fw/-Rv or PET-OsBWMK1-Fw/-Rv primers (Supplementary Table S1), respectively, and the Phusion™ high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland) according to the manufacturer's instructions. The *OsEREBP1* and *OsEREBP2* fragments were cloned in frame with the GST tag as an *Eco*RI-*Xho*I fragment into the pGEX-4T-1 vector. The *OsBWMK1* fragment was cloned in frame with the His tag in the pET200/D-TOPO vector according to the manufacturer's instructions.

Production of recombinant proteins

The *E. coli* Rosetta strain was transformed with the pGEX-4T-1 vector alone or pGEX-4T-1 containing the OsEREBP1 and OsEREBP2 coding sequences. Cells were grown in TB broth until the OD_{600} reached ≈ 0.5. Afterwards, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.4 mM, and the culture was further incubated for 1–2 h at 28 °C. Cells were harvested by centrifugation at $3,500 \times g$ for 20 min at 4 °C and lysed by passing 2 times through a French Press at 900 psi. Cell debris was removed by sedimentation at $16,600 \times g$ for 45 min at 4 °C. The GST-fusion proteins were purified in a 1 ml GSTrap HP (GE Healthcare, UK) with a flow rate of 1 ml/min according to the manufacturer's instruction.

For OsBWMK1 over-expression, Rosetta strain was transformed with the pET200/D-TOPO vector containing the coding sequence and grown in TB broth. When OD_{600} reached ≈ 0.5, protein production was induced with IPTG 0.4 mM and the culture was grown for 2 h at 28 °C. Cells were harvested through centrifugation as described above and lysed in 20 mM phosphate buffer pH7.5, containing 0.5 M NaCl, 10 mM imidazole, 50 μg/ml lysozyme, 100 mM PMSF, 5 μg/mL DNase and 10 μM $MgCl_2$. Cell debris was removed by centrifugation at $\approx 27,000 \times g$ for 40 min at 4 °C and the obtained supernatant was filtrated with a 0.45 μm Millex^R GV low protein binding filter (Millipore, Billerica, USA). The filtered lysate was loaded at 5 ml/min onto a 5 ml Ni containing HiTrap IMAC HP (GE Healthcare, UK) column, pre-equilibrated with 20 mM phosphate buffer pH7.5, containing 0.5 M NaCl and 10 mM imidazole. The His-tagged protein was eluted with the same buffer, but containing 0.25 M of imidazole. The OsBWMK1 tag protein was further purified in a gel-filtration column (Hiprep 26/60 Sephacryl S-200 HR, GE Healthcare, UK) with 20 mM phosphate pH7.5, 200 mM NaCl, 5 % glycerol, 1 mM DTT, and 1 mM EDTA.

Protein-containing fractions were pooled and protein was concentrated in an Amicon ultracell 10,000 MWCO (Millipore, Billerica, USA).

For immunoblots and Coomassie Brilliant Blue staining, 3–30 μg of purified GST-fusion proteins or 1.7 μg His-OsBWMK1 tag protein and Rosetta total protein extract were resolved on SDS-PAGE gels containing 10 % polyacrylamide. Proteins were stained with Coomassie Brilliant Blue or transferred to PVDF membranes. The membranes were probed with anti-GST (abm, NBS Biologicals, UK) or anti-HIS (GE Healthcare, UK) mouse monoclonal antibody and an anti-mouse horseradish peroxidase-conjugated antibody according to the manufacturer's instructions.

Measurement of OsBWMK1 phosphorylation activity

The reactions were conducted at ≈ 22 °C for 20 min in 20 μL reaction volume containing 20 mM Tris–HCl pH 8, 1 mM DTT, 10 mM $MgCl_2$, 0.1 mM ATP, 5 μCi [γ-^{32}P]ATP and 1.7 or 2.5 μg of purified OsBWMK1. To analyze OsEREBP1 phosphorylation, the reaction was performed with approximately 3 μg of purified TF. The reactions were terminated by boiling in SDS sample buffer and resolved in SDS-PAGE gels containing 10 % acrylamide. Gels were dried on Whatman 3MM paper and exposed to X-ray film (Amersham, GE Healthcare, UK).

Electrophoretic mobility shift assay (EMSA)

DNA probes were generated by annealing oligonucleotide pairs, S1-Fw/-Rv, S1 M-Fw/-Rv, S2-Fw/-Rv, S2 M-Fw/-Rv or GCC-Fw/-Rv (Supplementary Table S1), in a PCR machine with the advanced protocol described in Pierce Tech Tip #45 ("Anneal complementary pairs of oligonucleotides", www.piercenet.com) and the annealing temperatures 52, 46, 58 and 54 °C, respectively. The reactions were performed in the presence of 10 mM Tris–HCl pH 8, 50 mM NaCl and 1 mM Na_2EDTA pH8. Probes were labeled with [α-^{32}P]ATP using the DNA polymerase I/Klenow fragment (3′->5′ exo-) (NEB, USA) following the manufacturer's instructions. The DNA-binding reaction was performed in a volume of 10 μL, which contained 50 fmol of labeled probe, 0.002 μM poly(dI/dC), binding buffer (25 mM HEPES pH 7.5, 40 mM KCl and 0.1 mM EDTA) and 3 μg of purified GST-OsEREBP1, 6 μg GST-OsEREBP2, 30 μg GST tag or 300 μg untransformed Rosetta total protein extract. In some experiments, 1.7 μg of purified OsBWMK1 was added together with 100 μM ATP. Competition reactions were performed with 500-fold molar excess of unlabeled probe. The reactions were incubated for 30 min in a water bath at ≈ 22 °C and loaded

onto native 5 % polyacrylamide gel (37.5:1). Electrophoresis was run at 200 V for 2 h with $0.5 \times$ TBE (50 mM Tris, 50 mM boric acid, 1 mM Na_2EDTA) at approximately 22 °C. Gels were dried on Whatman 3MM paper and exposed to X-ray film (Amersham, GE Healthcare, UK).

Transient expression assays in Arabidopsis protoplasts

Arabidopsis protoplasts were isolated as previously described (Anthony et al. 2004). Approximately 3×10^5 protoplasts were transfected with 3 μg of reporter plasmid and 6 or 8 μg of effector plasmid 35S::OsEREBP1 or 35S::OsEREBP2, respectively, to obtain a molar ratio 1:3. The transfections were performed in triplicate and cells were incubated 48 h at 22 °C in the darkness. After harvesting, the cells were lysed through ressuspension in 100 mM K_2PO_4, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1 % Triton X-100 and 10 % glycerol, and two freeze–thaw cycles (−20, 22 °C). The cell debris was sedimented at $17,000 \times g$ for 2 min and the cleared lysate was stored at −80 °C. For the GUS quantification assay, 20 μL of cleared lysate was incubated with 0.5 μL 50 mM MUG (4-methylumbelliferyl-β-D-glucuronide) in triplicate at 37 °C in the darkness for 1 h. The reaction was stopped by adding 180 μL 200 mM Na_2CO_3 and the fluorescence signal was detected using a spectrofluorimeter (Fluoromax-4 with Micromax plate reader, Horiba, Portugal) with excitation at 365 nm, emission at 455 nm and a slit of 1.5 nm. The luciferase assay was performed through addition of 150 μL of LUC reagent (20 mM tricine pH 7.8, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA pH8, 3.3 mM DTT and 2 mM ATP) and 75 μL of 1.5 mM luciferine to 20 μL of cleared lysate. The light intensity was measured for 10 s in a luminometer (Berthold MicroLumatPlus LB96 V, Germany). Transcriptional activity was calculated as a GUS/LUC ratio.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers, *OsRMC* (BAF16049.1), *OsACT1* (BAF13000.1), *OsUBC* (BAF09 429.1), 25S rRNA (AK119809), ERF-4 (At3g15210), AtERF1A (At4g17500), *OsEREBP1* (BAF10233.1), *OsE-REBP2* (BAF06832.1), OsBWMK1 (AAD52659.1), OsERF1 (BAF15388.1), OsERF3 (BAB16083.1), AtERF2 (At5g47220), AtERF3 (At1g50640), AtERF4 (At3g15210), AtERF5 (At5g47 230), AtERF6 (At4g17490), AtRAP2-2 (At3g14230), AtRAP2-6 (At1g43160), ABR1 (At5g64750), JERF3 (AAQ91334.1), NtERF2 (BAA07324.1), NtERF5 (AAU81956.1), Pti (ACF57 857.1), ZmERF1 (AAT75013.1), AtDREB1A (At4g25480), AtDREB1B (At4g25490), AtDREB1C (At4g25470), AtDREB 2A (At5g05410), AtDREB2B (At3g11020), OsDREB1A

(BAF25626.1), OsDREB1B (AAP83888.1), OsDREB1C (BAF18575.1), OsDREB1D (BAF18823.1), OsDREB2 (BAF0 4021.1), ZmDREB2A (BAE96012.1), AtAPETALA2 (At4g 36920), OsAP2-1 (BAE78578.1).

Results

Expression analysis of the salt stress responsive gene *OsRMC*

The expression of the salt-responsive gene *OsRMC* was analyzed through RT-PCR in rice seedlings subjected to high salinity treatments: 100 and 200 mM NaCl (Fig. 1). In the presence of 100 mM NaCl, *OsRMC* gene expression was detected 2 h after treatment, and the transcript level increased thereafter up to 24 h. When the seedlings were subjected to 200 mM NaCl, transcript accumulation followed the same trend but was higher, showing a clear stress-dose-dependent response. *OsRMC* gene expression was also analyzed separately in shoots and roots of seedlings subjected to 200 mM NaCl (Supplementary Fig. S1). In roots, we observed that *OsRMC* transcript level gradually increased after 2 h exposure to stress, reached the maximum at 10 h treatment, and the high levels were maintained up to 24 h. In leaves, it followed the same pattern as in roots, but the transcript levels decreased after reaching the maximum level at 10 h of stress.

Identification of transcription factors binding to the *OsRMC* gene promoter

In order to identify the transcription factors binding to the *OsRMC* promoter, we have used the yeast one-hybrid (Y1H) system. Initially, it was necessary to construct both the library and the yeast bait strains. To have enrichment in salt stress responsive genes, the cDNA expression library was prepared from rice seedlings subjected to salt stress.
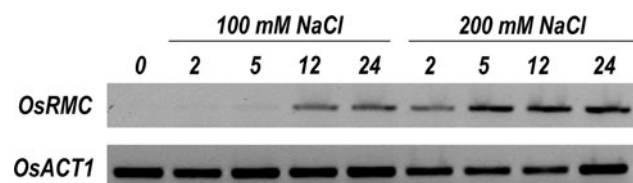

Fig. 1 Expression analysis of *OsRMC* in response to high salinity conditions. RT-PCR reactions were performed with cDNA prepared from 1 μg of total RNA isolated from 14-day-old rice seedlings (cv. Nipponbare) subjected to high salinity (100 or 200 mM NaCl) during 0, 2, 5, 12 or 24 h. *OsRMC* was amplified with 25 cycles. *OsACT1* was used as internal control and amplified using 25 cycles

After constructing this library and to assess the average length of the cDNA inserts, PCR amplification was performed using 20 individual phage plaques (Supplementary Fig. S2). The size range of the inserts was 750–1,500 bp and the average length was close to 1,000 bp. Regarding the yeast bait strains, we have constructed six baits carrying the *OsRMC* promoter fragments upstream the *HIS3* reporter as illustrated in Supplementary Fig. S3.

Using the Y1H system, 3–7 million clones from the salt-induced cDNA expression library were screened for each of the six yeast bait strains, resulting in a total of 115 positive clones identified. The putative positive clones were re-streaked in selective medium, to validate growth, and then analyzed by direct PCR. Twenty-five clones did not grow after re-streak and from the clones with high to moderate growth on selective medium, 42 did not amplify a cDNA insert. Library plasmid DNA was isolated only from the yeast clones with high growth on selective medium and from which a cDNA insert could be amplified. After plasmid amplification in bacteria, the inserts were sequenced and a BLAST analysis was performed to identify the genes. Six clones were confirmed to be false positives containing, for example, an insert encoding a ribosomal protein and an endopeptidase. The analyses of the remaining 42 clones, led to the identification of two TFs belonging to the AP2/ERF family and interacting with fragment 3 of the *OsRMC* promoter: *OsEREBP1* and *OsEREBP2*. *OsEREBP1* gene was found in 38 clones while *OsEREBP2* was only found in four. To further validate the DNA–protein interaction, the plasmid DNA was retransformed into the respective yeast bait strain. High growth on selective medium was observed for the yeast bait strain transformed with each of the identified TFs.

Protein and phylogenetic analysis of OsEREBP1 and OsEREBP2

The nucleotide sequence analysis revealed that *OsEREBP1* contained an open reading frame of 1,098 bp and encoded a protein of 365 amino acids with a predicted molecular mass of 40.1 kDa and pI of 4.79. Analysis of deduced amino acid sequence showed that the protein contained a single AP2 DNA-binding domain (120–175 aa) and a potential nuclear localization sequence (NLS) (Supplementary Fig. S4). The *OsEREBP2* gene sequence contains an open reading frame of 1,056 bp encoding a 351 amino acids long protein. The deduced protein has a predicted molecular mass of 36.3 kDa and pI of 6.18. Similarly to OsEREBP1, the amino acid sequence contained one AP2 domain (130–185 aa) and a NLS (Supplementary Fig. S4). The predicted amino acid sequences of OsEREBP1 and OsEREBP2 were aligned with known members of the AP2/ERF family of TFs (Supplementary Fig. S4). The results revealed that these proteins

were significantly similar in the AP2 DNA-binding domain to other family members from rice, Arabidopsis, maize, tomato, potato and tobacco. To further analyze the phylogenetic relationship of OsEREBP1 and OsEREBP2 with AP2/ERF proteins, a phylogenetic tree was constructed based on their predicted amino acid sequences (Fig. 2). This analysis revealed that both TFs are grouping in the ERF subfamily of TFs and are distant from the DREB subfamily. Interestingly, OsEREBP1 and OsEREBP2 are located in different subgroups inside the ERF family.

OsEREBP1 and OsEREBP2 bind to a GCC-like motif in *OsRMC* promoter

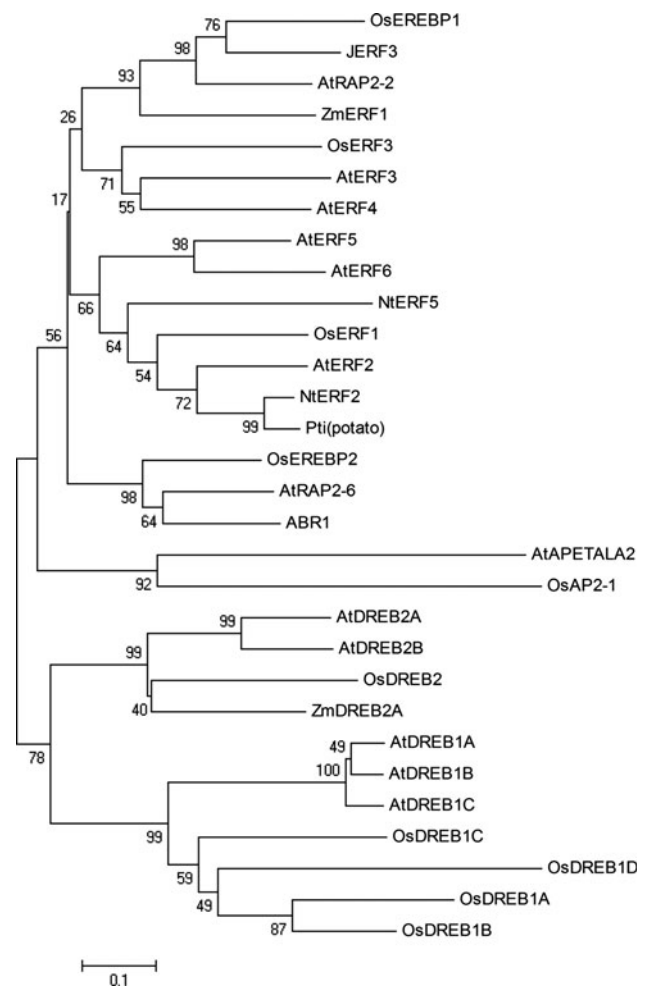In order to determine where OsEREBP1 and OsEREBP2 bind to the *OsRMC* gene promoter, we have used the

Fig. 2 Phylogenetic analysis of the deduced amino acid sequences of OsEREBP1, OsEREBP2 and the AP2/ERF proteins described in Supplementary Fig. S4. The amino acid sequences were aligned by ClustalW and then used in the MEGA program. Numbers along branches are bootstrap percentage values

PlantPAN database to identify *cis*-elements related to abiotic stress responses present within the region ranging from −1,260 to −613 bp (Supplementary Fig. S5). This promoter region corresponds to fragment 3 (−1,160 to −613 bp), which was used as bait for the Y1H screening. Within this promoter region, we have identified four ABA Responsive Element (ABRE) sequences with the core motif ACGT, one Dehydration-Responsive Element (DRE) and one Low-Temperature-Responsive Element (LTRE). DREs and LTREs are usually recognized by Dehydration-Responsive Element Binding (DREB) proteins, which are well known regulators of abiotic stress responses (Yamaguchi-Shinozaki and Shinozaki 2005). In addition, we found three MYB-recognition motifs. Several MYB proteins have also been shown to function in abiotic stress responses (Dubos et al. 2010). However, given that no GCC box motifs (known ERF binding sites) were predicted with this initial analysis, a more detailed bioinformatics approach was performed to identify OsE-REBP1 and OsEREBP2 DNA-binding sites in the *OsRMC* gene promoter. Initially, the footprintDB database was interrogated to identify similar DNA-binding proteins that bind to known regulatory motifs and to define the amino acid residues that shape the DNA-binding interface. This search revealed significant sequence similarity with ERF proteins for the AP2 domain (Fig. 3a), confirming that both proteins belong to the ERF subfamily of TFs. It also showed that the interface amino acid residues involved in DNA-binding interface were conserved with other ERF proteins, suggesting that OsEREBP1 and OsEREBP2 recognize similar DNA motifs (Fig. 3a). Position weight matrices (PWMs) of proteins ERF-4 and AtERF1A were used to predict putative *cis*-elements within the *OsRMC* promoter. Two of these sites were predicted by both PWMs (S1 and S2, with *P* values of 2.9×10^{-5} and 1.5×10^{-3}, respectively) and were selected for further analysis (Fig. 3b).

Electrophoretic mobility shift assays (EMSAs) were performed to check whether OsEREBP1 and OsEREBP2 proteins interact with the predicted DNA-binding sites S1 and S2, and also with a synthetic probe containing the GCC box motif (Cheong et al. 2003) (Fig. 3b). Both TFs were produced as GST-fusion proteins and purified by affinity chromatography. In addition to the predicted bands for our proteins, other bands were detected and assumed to be products of degradation of the fusion proteins or eventually contaminant proteins from bacteria (data not shown). To confirm that the DNA-binding activity detected in the EMSA assays was not related to the interaction of contaminant proteins with the DNA fragments, we included a control reaction with the total protein extract from bacteria. Figure 4 shows that both OsEREBP1 and OsEREBP2 interact with the probe S1 and the TF-DNA complexes

formed migrate similarly. No DNA-binding activity was detected for the GST protein and only unspecific binding was detected with the total protein extract from bacteria. When the specificity of the TFs-DNA interaction was analyzed through competition experiments, using 500-fold excess of unlabeled S1, an efficient challenge with the cold-probe was observed for both TFs. In contrast, when the competition was performed with the mutated probe S1M (Fig. 4), the specific binding was enhanced, probably due to higher unspecific binding to unlabeled S1M. The DNA-binding activity of both TFs was also assessed for the probe S2, but no signal was detected (data not shown). In addition, a competition reaction was performed, using the labeled probe S1 together with 500-fold excess of unlabeled probe S2 and the TFs' binding to S1 was not affected by S2 presence (data not shown).

OsEREBP1 was previously reported to be phosphorylated by the OsBWMK1 MAPK and this modification enhanced OsEREBP1 DNA-binding activity to the GCC box motif (Cheong et al. 2003). Thereby, OsBWMK1 was also produced and its activity was confirmed by an in vitro kinase assay (Supplementary Fig. S6). No significant difference in OsEREBP1 DNA-binding activity was detected when the TF was incubated with OsBWMK1 and the probe S1 (Fig. 4). EMSAs were also performed with a sequence containing two tandem repeats of the GCC box motif (Cheong et al. 2003). Figure 5 shows that OsEREBP1 and OsEREBP2 bind to the synthetic probe and the interaction specificity was confirmed through competition with 500-fold excess of unlabeled GCC probe. However, only a weak enhancement in OsEREBP1-DNA binding activity was detected when the protein was incubated with OsBWMK1 and ATP. Curiously, the migration of OsEREBP1-DNA complex seems to be slower than that of the OsEREBP2-DNA, opposite to what was observed when the TFs interact with the S1 probe (Fig. 4). The putative phosphorylation of OsEREBP2 by OsBWMK1 was not assessed since no MAPK phosphorylation sites were predicted in OsEREBP2 protein sequence.

OsEREBP1 and OsEREBP2 act as transcriptional repressors

To assess the TFs transcriptional activity, transient expression assays were performed in Arabidopsis protoplasts. These protoplasts were transfected with the reporter plasmid pLUCm35GUS-pRMC alone or in combination with the effector plasmids 35S::OsEREBP1 or 35S::OsE-REBP2. As shown in Fig. 6, the expression of the *GUS* reporter gene was repressed when each effector plasmid was co-transfected with the reporter plasmid, thus revealing that OsEREBP1 and OsEREBP2 negatively regulate *OsRMC* gene expression.
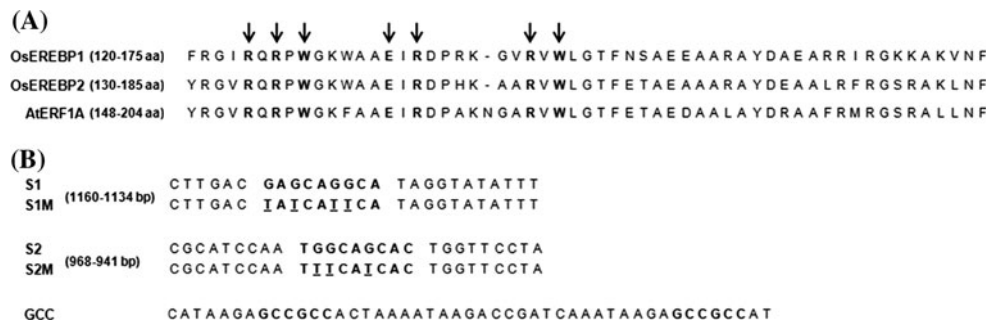
(A)

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                          ↓ ↓ ↓        ↓ ↓            ↓ ↓
OsEREBP1 (120-175aa)   F R G I R Q R P W G K W A A E I R D P R K - G V R V W L G T F N S A E E A A R A Y D A E A R R I R G K K A K V N F
OsEREBP2 (130-185aa)   Y R G V R Q R P W G K W A A E I R D P H K - A A R V W L G T F E T A E A A A R A Y D E A A L R F R G S R A K L N F
AtERF1A (148-204aa)    Y R G V R Q R P W G K F A A E I R D P A K N G A R V W L G T F E T A E D A A L A Y D R A A F R M R G S R A L L N F
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(B)

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S1                     C T T G A C  G A G C A G G C A  T A G G T A T A T T T
S1M   (1160-1134 bp)   C T T G A C  I A I C A I I C A  T A G G T A T A T T T

S2                     C G C A T C C A A  T G G C A G C A C  T G G T T C C T A
S2M   (968-941 bp)     C G C A T C C A A  T I I C A I C A C  T G G T T C C T A

GCC                    C A T A A G A G C C G C C A C T A A A A T A A G A C C G A T C A A A T A A G A G C C G C C A T
```

Fig. 3 Analysis of the OsEREBP1 and OsEREBP2 AP2 binding domains and the GCC-like boxes in the *OsRMC* promoter. **a** Alignment of OsEREBP1 and OsEREBP2 with AtERF1A (At4g17500) protein sequence, obtained after querying the footprintDB database, to spot the amino acid residues putatively involved in DNA binding interface. The *arrows* indicate the conserved amino acid residues involved in DNA-binding interface, inferred from the Protein Data Bank entry 1GCC (Allen et al. 1998). The *numbers* represent the residues position in the protein sequence. **b** DNA sequences used in the EMSA assays. In *bold* are the predicted OsEREBP1 and OsEREBP2 DNA-binding sites (S1 and S2) identified as GCC-like boxes in fragment 3 of the *OsRMC* promoter. S1M and S2M are mutated forms of site S1 and S2, respectively. Point mutations are *underlined*. The sequence containing the GCC box motif in tandem was described by Cheong et al. (2003). The *numbers* represent the position in the promoter upstream the ATG start codon of the *OsRMC* gene

Fig. 4 Analysis of the DNA-binding specificity of OsEREBP1 and OsEREBP2 to the S1 sequence present in the *OsRMC* gene promoter. EMSA was carried out with ^{32}P-labeled S1 probe incubated with GST, bacteria total protein extract (Cell Extract) and purified proteins GST-OsEREBP1 and GST-OsEREBP2. Binding reactions contained no (−) or a 500-fold molar excess (+) of unlabeled S1 or S1M probe. For OsEREBP1, OsBWMK1 and 100 μM ATP reactions were also tested. The *black* and *white arrows* indicate a specific band shift and non-specific signal, respectively. Details of the EMSAs are described in "Materials and methods"
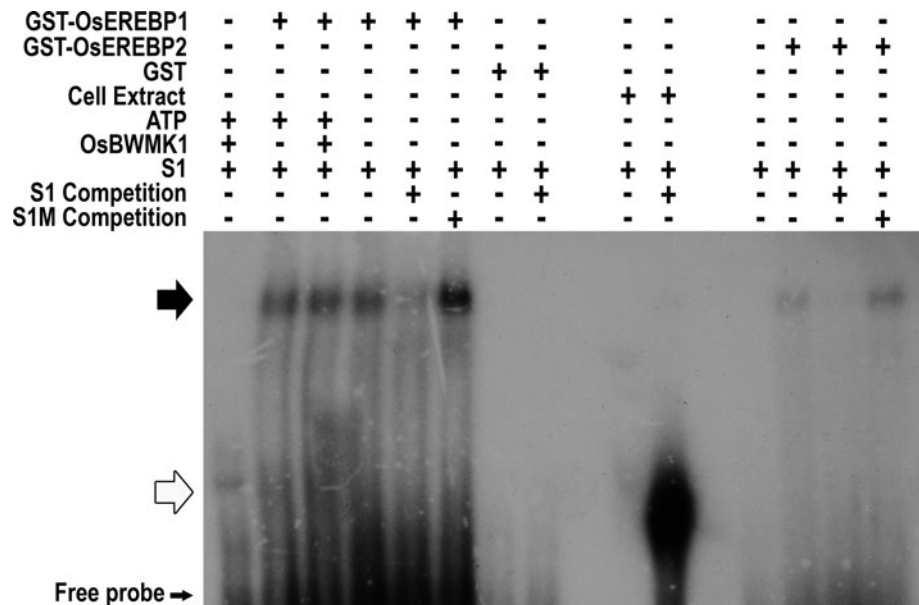
OsEREBP1 and OsEREBP2 gene expression under different abiotic stresses

To investigate whether OsEREBP1 and OsEREBP2 were regulated at transcriptional level by abiotic stress conditions, a gene expression analysis was performed in rice seedlings subjected to different treatments (salt, drought, cold and ABA). Figure 7 shows that *OsEREBP1* gene expression was not significantly and reproducibly altered under control conditions in both shoots and roots during the analyzed period. Similarly, no significant changes in the transcript accumulation were observed in roots and shoots of seedlings treated with salt, severe cold (5 °C) and ABA. In roots, *OsEREBP1* transcripts accumulated in the late time points of the moderate cold (10 °C) treatment, whereas under drought stress the transcript level tends to decrease after 10 min. The *OsEREBP1* gene expression pattern in response to moderate cold and drought stress was confirmed by qPCR (Supplementary Fig. S7).

OsEREBP2 gene expression analysis revealed that its transcription is significantly induced in response to salt, drought, ABA and both cold treatments (Fig. 7). In roots, the transcript accumulation in response to salt was visible within 20 min, reaching the maximum at 1–2 h treatment and then decreasing to a level slightly higher than the observed for the untreated plants. A similar pattern was observed in shoots. Drought stress enhanced *OsEREBP2* transcript accumulation also after 20 min exposure to stress in both shoots and roots. This increase in transcript abundance was observed up to 40 min treatment and was
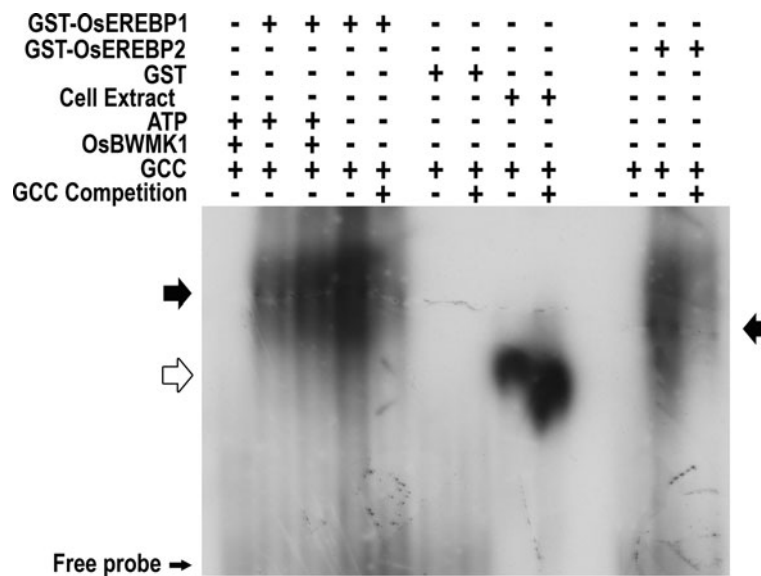
Fig. 5 Analysis of the DNA-binding specificity of OsEREBP1 and OsEREBP2 to the GCC probe described by Cheong et al. (2003). EMSA was performed with ^{32}P-labeled GCC probe incubated with GST, bacteria total protein extract (Cell Extract) and purified proteins GST-OsEREBP1 and GST-OsEREBP2. An assay control was performed with a 500-fold molar excess of unlabeled GCC probe. OsBWMK1 and 100 μM ATP were also tested for OsEREBP1. The *black* and *white arrows* indicate a specific band shift and non-specific signal, respectively. Details of the EMSAs are described in "Materials and methods"

followed by a gradual decrease in gene expression. Under cold stress, the transcript level of *OsEREBP2* increased in shoots and roots at both temperatures tested. In both shoots and roots, the increase in *OsEREBP2* expression occurred earlier at 10 °C (within 1–2 h) than at 5 °C (only after 5 h). In addition, at 10 °C the maximum transcript level was reached within 5–10 h treatment, while at 5 °C it required at least 24 h. Upon ABA treatment, the TF transcript accumulation was visible in both shoots and roots within 1 h, rapidly decreasing thereafter to the level of the untreated plants. The *OsEREBP2* transcript level in rice seedlings subjected to ABA treatment was confirmed by qPCR (Supplementary Fig. S7).

OsEREBP1 and OsEREBP2 gene expression under salt stress in different rice varieties

In order to better understand the function of OsEREBP1 and OsEREBP2, we have analyzed, under salt stress, their transcription expression in rice varieties with contrasting tolerance to high salinity (Nipponbare, IR29 and Pokkali). We observed that *OsEREBP1* gene expression was not significantly affected by salt treatment in the different varieties analyzed (data not shown), whereas *OsEREBP2* showed different gene expression patterns among varieties (Fig. 8). In the shoots of Nipponbare seedlings, *OsEREBP2* transcripts accumulated gradually after 15 and 30 min of salt stress and then decreased to the level of untreated plants. This transcript accumulation was not observed in either IR29 or Pokkali shoots, in which no striking

differences were observed. In Nipponbare roots, high salinity gradually enhanced *OsEREBP2* transcription until 30 min exposure and the high level of transcript was maintained during the 12 h of treatment. In IR29 roots, *OsEREBP2* transcript also reached a peak at 30 min exposure, but after 8 h treatment the transcript level was similar to the untreated plants. In Pokkali roots, only a slight induction of the *OsEREBP2* transcript level was observed between 15 min and 4 h after treatment. When the *OsEREBP2* transcript level was analyzed in rice seedlings under control conditions, we observed that it was similar in the shoots of the three varieties, whereas in the roots, Pokkali showed lower levels than Nipponbare and IR29 (Supplementary Fig. S8).

Discussion

High salinity induces changes in the expression of several genes being estimated that the transcription level of approx. 8 % of all genes is affected by this abiotic stress (Chao et al. 2005; Rabbani et al. 2003; Tester and Davenport 2003). To study the transcription regulation of *OsRMC*, an initial gene expression analysis was performed on rice seedlings subjected to salt stress, revealing a stress-dose-dependent response. The observed transcript accumulation is well correlated with the described increase in the protein level in plants treated with 200 mM NaCl (Zhang et al. 2009a). *OsRMC* encodes an apoplast protein described as a negative regulator of salt stress response
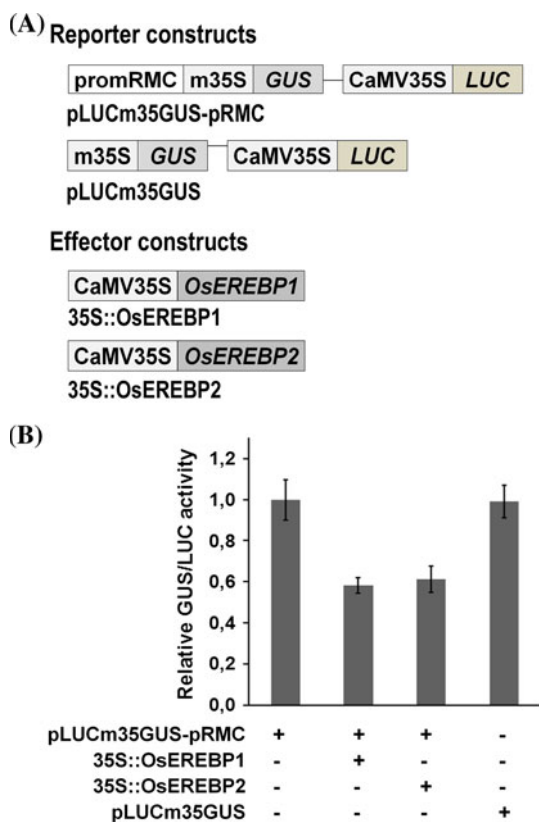
Fig. 6 Transcriptional activity of OsEREBP1 and OsEREBP2 proteins. **a** Illustrated plasmids used for the transient expression assays. The pLUCm35GUS-pRMC plasmid contains the *GUS* reporter gene driven by the fragment 3 of the *OsRMC* promoter and the minimal CaMV35S promoter. **b** GUS and LUC activities were measured in Arabidopsis protoplasts transfected with the reporter plasmid, pLUCm35GUS-pRMC or pLUCm35GUS and the effector plasmid, 35S::OsEREBP1 or 35S::OsEREBP2 in a molar ratio 1:3. Transcriptional activity was calculated as a GUS/LUC ratio. *Bars* indicate GUS/LUC ratio ± SD after 48 h protoplast incubation. Data are mean ± SD of three replicates

(Zhang et al. 2009a). In Arabidopsis, other genes induced by high salinity have been described as negative regulators of salt stress response (Tran et al. 2007). For instance, *AHK2*, which is induced by drought and salt stress, encodes a receptor histidine kinase characterized as a negative regulator. *ahk2* mutants showed enhanced tolerance to drought and salt stress associated with the accumulation of several stress-responsive genes. Stress responses involve the induction of several genes whose products, when present in high amounts, can have adverse effects on plant development. Therefore, plants need to have mechanisms to finely regulate the burst of expression occurring upon environmental changes (Kazan 2006; Mito et al. 2011; Tieman et al. 2000). OsRMC may act as a fine tuner of salt stress responses by repressing the induction of salt-responsive genes whose products can induce damages to cellular components. Its high responsiveness to high salinity and the reduced knowledge available about the

function of salt stress negative regulators lead us to further analyze OsRMC involvement in this abiotic stress condition through a detailed study of its transcriptional regulation.

The classification of OsEREBP1 and OsEREBP2 as ERF transcription factors was previously reported based on a bioinformatics analysis (Nakano et al. 2006). OsEREBP1 was shown to belong to the CMVII group which includes proteins involved in abiotic stress response, like the JERF3 whose over-expression enhanced salt stress tolerance in tomato (Wang et al. 2004). OsEREBP2 was predicted to belong to the group Xa due to the presence of a CMX-1-like motif in the amino acid sequence. The ABR1 protein from Arabidopsis also belongs to the Xa group and was shown to be involved in the ABA-dependent osmotic stress response. In agreement with this classification, the phylogenetic analysis showed that OsEREBP1 groups with JERF3 and OsEREBP2 is a close relative to ABR1. Together, these data show that, although OsEREBP1 and OsEREBP2 belong to the same ERF subfamily of TFs, they are phylogenetically distant from each other and may have different roles mediating *OsRMC* gene expression levels.

Several ERF proteins were shown to interact with the *cis*-element ERE (Ethylene-Responsive Element), which contains the core sequence designated GCC box (AGCCGCC) (Mizoi et al. 2012). This sequence was shown to be the target *cis*-element for several ERFs; however some TF proteins display binding flexibility and are able to interact with variants of this sequence (Fujimoto et al. 2000). EMSA experiments revealed that both OsEREBP1 and OsEREBP2 bind to either the GCC-like motif S1 or the canonical GCC box (Cheong et al. 2003), but none of them binds to the GCC-like motif S2. In addition, the OsEREBP1—GCC box complex displayed a slower migration than the OsEREBP2—GCC box complex. This difference was not detected when the TFs were incubated with S1, in which the TF-DNA complexes migrate similarly. This result suggests that two OsEREBP1 molecules bind to the GCC box tandem repeats as a dimer or the TF binding may induce the recruitment of other TF molecule to adjacent sites (Garvie and Wolberger 2001). OsEREBP1 cooperative binding was not detected in the EMSAs with S1, probably because this sequence only contains one GCC-like motif. Cooperative binding has been described for other regulatory proteins. The Arabidopsis *XERO2* dehydrin gene expression was shown to be regulated by ABA and cold through combinatorial TF binding (Chung and Parish 2008). Two Dehydration-Responsive Elements/C-Repeat elements (DRE/CRT) which are binding sites for DREB proteins were shown to be required for *XERO2* gene expression induced by cold stress. In addition, the regulation of *PDF1.2* gene expression by jasmonate and ethylene was reported to depend on the activity of two GCC box
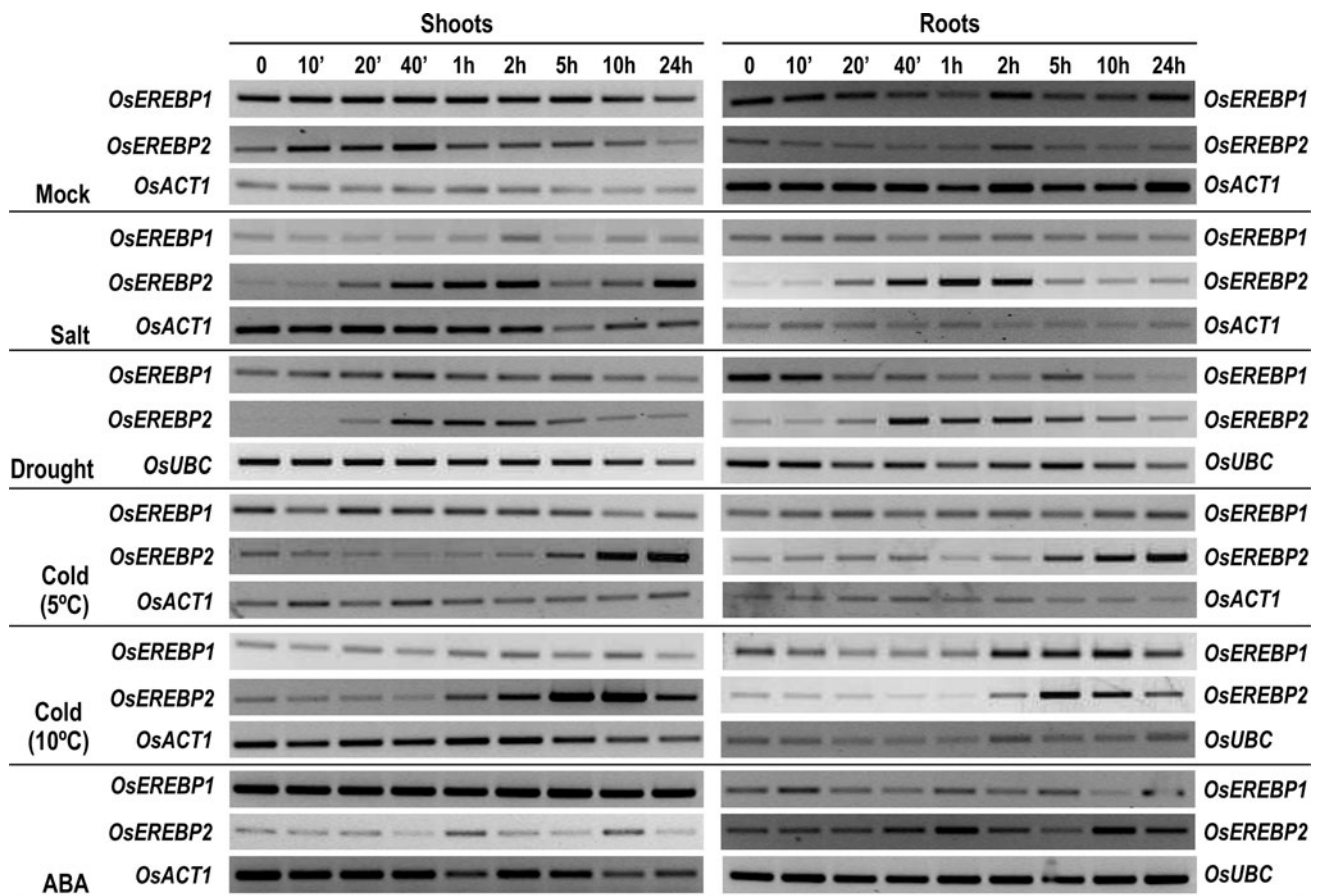
Fig. 7 Analysis of *OsEREBP1* and *OsEREBP2* gene expression in response to different abiotic stress conditions. RT-PCR reactions were performed with cDNA prepared from total RNA extracted from shoots and roots of 14-day-old rice seedlings (cv. Nipponbare) subjected (or not) to salt (200 mM), drought, cold (5, 10 °C) and ABA (100 μM). *OsEREBP1* was amplified with 25 cycles, except for shoot samples under control, 5 °C and ABA treatments (20 cycles). *OsEREBP2* was amplified with 30 cycles, except for shoot samples under drought stress (25 cycles). Internal controls: *OsACT1* and *OsUBC* (ubiquitin-conjugase). *OsACT1* was amplified using 25 cycles or 20 cycles for the root samples under salt and 5 °C. *OsUBC* reactions were performed with 23, 25 or 30 cycles for root samples under 10 °C, ABA and drought, respectively

motifs (Zarei et al. 2011). Transient expression assays revealed that the AP2/ERF protein ORA59 displays enhanced transactivation activity when both GCC box motifs are present. In tobacco the AP2/ERF protein Tsi1 recruits and interacts with the Zn-finger protein Tsip1 to cooperatively promote the transcriptional activation of stress-responsive genes (Ham et al. 2006). The formation of AP2/ERF dimers was reported for CRF proteins but their biological function is still unknown (Cutcliffe et al. 2011).

OsEREBP1 and OsEREBP2 transcriptional activity suggests that OsEREBP1 and OsEREBP2 negatively regulate *OsRMC* gene expression. Opposite results were obtained by Cheong et al. (2003), who reported that OsEREBP1 functions as a transcriptional activator when it interacts with GCC box sequences. Previous studies suggest that TF proteins can function as both activators and repressors (Bossi et al. 2009; Mena et al. 2002). The barley *BPBF* gene encodes a DOF TF that induces gene expression in developing endosperm and has

a repressor activity upon germination (Mena et al. 2002). During germination the TF was proposed to compete with other transcriptional activators for DNA-binding or to interact with other TFs that regulate the same target gene. *ABI4* encodes an AP2/ERF protein with a dual function in sugar signaling (Bossi et al. 2009). The authors suggest that the TF transcriptional activity is dependent on the sequences that surround the ABI4-binding site or on the binding of other factors. OsEREBP1 may also display different transcriptional activity depending on the DNA-binding motifs present in the target gene promoter sequence. The cooperative binding effect suggested by the EMSA results also indicates that the presence of additional DNA-binding motifs may interfere with the TF function.

The AP2/ERF family of TFs has been shown to play a role in the response to several abiotic stress conditions (Agarwal and Jha 2010; Dietz et al. 2010; Nakano et al. 2006). To assess the transcriptional regulation of *OsEREBP1* and *OsEREBP2* by abiotic stress conditions, the expression of these genes was
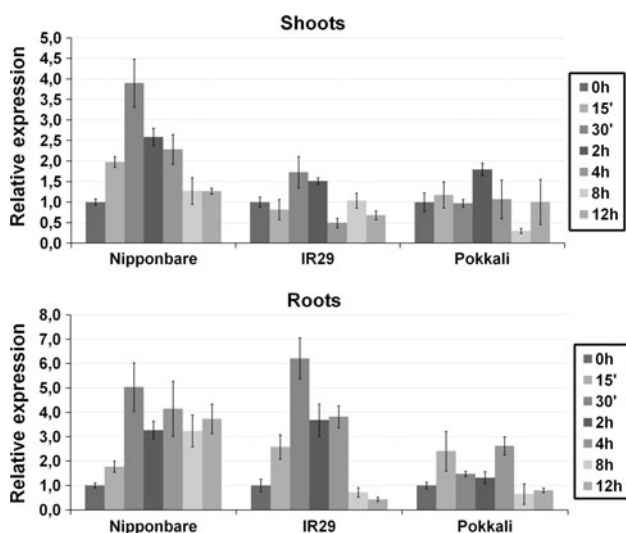
Fig. 8 *OsEREBP2* gene expression analysis in different rice varieties subjected to salt stress. Quantitative PCR reactions were performed with cDNA prepared from total RNA extracted from shoots and roots of 11-day-old rice seedlings (different varieties: Nipponbare, IR29 and Pokkali) subjected to high salinity (120 mM) during 0, 15, 30 min, 2, 4, 8 or 12 h. The data was normalized to the internal control 25S rRNA. *Error bars* represent standard deviation

analyzed in rice seedlings subjected to salt, drought, cold and ABA treatments. OsEREBP1 gene expression was not significantly affected under control, high salinity, severe cold (5 °C) and ABA. An increased transcript accumulation was detected in roots under moderate cold (10 °C), whereas no significant difference was observed in shoots. The *OsEREBP1* gene expression in the japonica cultivar CT6748-8-CA-17 was previously shown to be induced upon cold exposure (10 °C) (Cheng et al. 2007). The response to drought stress was also tissue specific, being only observed in the roots. Several genes have shown this pattern of expression (Wang et al. 2011).

OsEREBP2 gene expression was shown to be induced in response to salt, drought, ABA and cold stress treatments in both shoots and roots. Curiously, *OsEREBP2* response to moderate cold (10 °C) occurred earlier than the response to severe cold (5 °C). Previous studies suggested that rice response to moderate and severe cold involves two different signaling pathways (Wen et al. 2002). Thus, OsEREBP2 may be involved in both pathways and the induction of its gene expression depends on the severity of the stress. *OsEREBP2* transcript accumulation by salt and drought was shown to occur earlier than the induction by cold. Being rapidly induced by these stresses, *OsEREBP2* may be involved in the early responses to osmotic stress. Moreover, given that induction of *OsEREBP2* only occurs 1 h after ABA treatment, we suggest that the TF regulation by osmotic stress is not ABA dependent. ERF proteins with a putative role in the early responses of the osmotic stress were also identified in tomato (Huang et al. 2004), wheat

(Xu et al. 2007) and tobacco (Park et al. 2001) and its over-expression enhanced tolerance to this abiotic stress. In rice, *OsBIERF1* and *OsBIERF4* were also shown to be rapidly induced (within 1 h) by salt and drought treatments (Cao et al. 2006). Our results indicate that OsEREBP2 can be considered as a multifunctional TF putatively involved in several stress responses in rice. Several TFs belonging to the AP2/ERF family have been reported as such (Hu et al. 2010; Shao et al. 2007). In tomato, *JERF1* gene expression was shown to be induced in response to salt, ABA and ethylene (Zhang et al. 2004). Over-expression of this ERF protein in tobacco enhanced the plant tolerance to high salinity and low temperature, indicating an important role in both abiotic stress conditions (Wu et al. 2007).

The expression pattern observed for *OsRMC* gene expression cannot be directly correlated with *OsEREBP1* transcript level, which was not affected by salt stress, but can be correlated with *OsEREBP2*. This TF is quickly induced by salt stress (within 20 min), reaching a peak and then starting to decrease, suggesting that *OsRMC* induction 2 h after salt stress is, at least partially, due to the decreased levels of its repressor OsEREBP2. We cannot however rule out the action of other TFs interacting either with the *OsRMC* promoter or with any of the TFs, as well as the putative post-translational modifications of the identified TFs. Each factor may contribute to the fine tuning of the *OsRMC* transcript level. OsRMC has been described as a negative regulator of rice salt stress response (Zhang et al. 2009a), with a putative role in the mechanisms that lead to the attenuation of the strong plant response to high salinity. Therefore, it is expected that, during the initial stress response, *OsRMC* gene expression is maintained at a low level, to allow the activation of the stress tolerance mechanisms and OsEREBP2 may play an important role in this regulation.

To further characterize OsEREBP1 and OsEREBP2 function in rice salt stress response, we investigated their gene expression in Nipponbare and two rice varieties with contrasting salt stress tolerance: IR29 and Pokkali. Nevertheless, only *OsEREBP2* showed to be regulated by high salinity at the transcriptional level. IR29 and Pokkali are *indica* (Walia et al. 2005), whereas Nipponbare is a *japonica* variety (Liu et al. 2010). Pokkali is highly salt-tolerant and its tolerance was proposed to be related to the reduced levels of Na^+ in roots and shoots under salt stress, as compared to salt-sensitive varieties (Kader et al. 2006). This lower Na^+ level has been associated with efficient retrieval of Na^+ from the xylem (Cotsaftis et al. 2012) and reduced influx of this toxic ion into the roots (Kader and Lindberg 2005). IR29 and Nipponbare have been described as salt-sensitive varieties and shown to accumulate high levels of Na^+ in shoots (Cotsaftis et al. 2012). In agreement with previous studies showing that differences in salt

stress responses are related to transcriptome modulation (Cotsaftis et al. 2011; Kumari et al. 2009; Walia et al. 2005), we observed that *OsEREBP2* was differently regulated by salt stress in the different rice varieties analyzed. A transient *OsEREBP2* transcript accumulation was detected in Nipponbare shoots, whereas no significant changes were detected in IR29 and Pokkali. Comparative studies have revealed that, in general, salt tolerance in the *indica* varieties is higher than in *japonica* (Lee et al. 2003). The *indica* varieties under salt stress had a lower shoot weight reduction associated with lower Na^+ shoot concentration and, therefore, lower Na^+ toxicity at the cellular level. Negrão et al. (2013) also showed that, under salt stress, Nipponbare has higher Na^+ concentration (Negrão et al. 2013). In addition, it has been suggested that transcriptome responses are highly correlated with the shoot Na^+ concentration (Walia et al. 2005, 2007). Therefore, *OsEREBP2* response in Nipponbare shoots may be due to a higher Na^+ accumulation, as compared to IR29 and Pokkali shoots.

In roots subjected to salt stress, the *OsEREBP2* transcript level was much less induced in Pokkali than in Nipponbare or IR29. It has been suggested that salt tolerance in Pokkali is at least partially due to the constitutive expression of specific genes (TFs, signaling-related and ion transporters) that are induced by stress in a salt-sensitive variety (Kumari et al. 2009). This response strategy may allow a faster adaptation to salt stress with only minor transcriptional changes. If the *OsEREBP2* transcript level in Pokkali under control conditions was higher than in the sensitive varieties, this would explain the results observed in roots. However, a lower level of *OsEREBP2* transcript was detected in Pokkali roots under control conditions, as compared to Nipponbare and IR29. This might be due to the fact that OsEREBP2 acts as a repressor and, therefore, it is maintained low in control conditions and is less induced in a salt-tolerant variety like Pokkali. In Nipponbare and IR29 roots, *OsEREBP2* transcript level was highly up regulated by salt stress, suggesting that these varieties have common salt stress response mechanisms. Root performance (root weight) of salt-sensitive *japonica* and *indica* varieties, subjected to salt stress, was shown to be similar (Lee et al. 2003), indicating that similar mechanisms are involved in their response. We suggest that OsEREBP2 plays an important role in salt stress responses in rice, particularly in salt-sensitive varieties, and is involved in specific salt response mechanisms.

In this work, we have shown that *OsRMC* gene expression is highly induced by high salinity conditions and this regulation is stress-dose-dependent. In addition, we identified two transcription factors, OsEREBP1 and OsEREBP2, which bind to the same GCC-like motif in the promoter of *OsRMC* and function as transcriptional repressors. The analysis of gene expression in response to

abiotic stress conditions revealed that *OsEREBP1* is not significantly regulated at transcriptional level under salt, ABA and severe cold (5 °C), but seems to be induced or repressed in roots exposed to moderate cold (10 °C) and drought, respectively. On the other hand, *OsEREBP2* gene expression was modulated by salt, drought, ABA and both cold treatments. This indicates that OsEREBP2 may play an important role in the transcriptional network controlling rice responses to different abiotic stresses, possibly through the regulation of *OsRMC* gene expression but also other stress responsive genes. Further characterization of these TFs, namely using their mutants or silencing lines, will provide new insights on their role in the salt stress response in rice.

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