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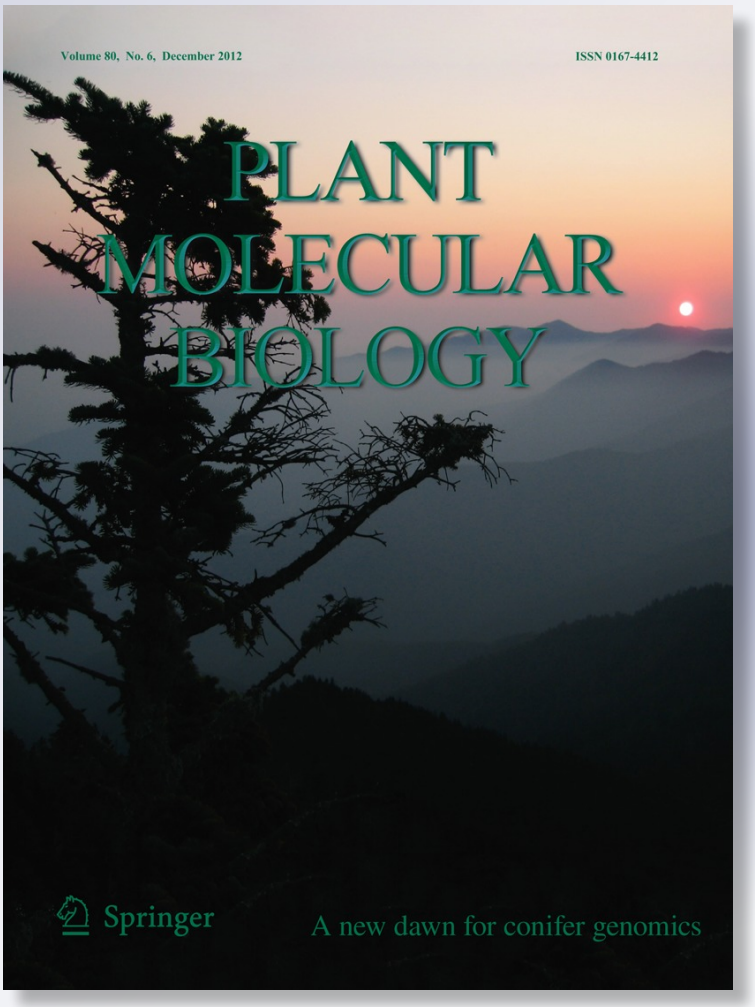

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Function of the HD-Zip I gene *Oshox22* in ABA-mediated drought and salt tolerances in rice

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Abstract Oshox22 belongs to the homeodomain-leucine zipper (HD-Zip) family I of transcription factors, most of which have unknown functions. Here we show that the expression of *Oshox22* is strongly induced by salt stress, abscisic acid (ABA), and polyethylene glycol treatment (PEG), and weakly by cold stress. Trans-activation assays in yeast and transient expression analyses in rice protoplasts demonstrated that Oshox22 is able to bind the CAAT(G/C)ATTG element and acts as a transcriptional activator that requires both the HD and Zip domains. Rice plants homozygous for a T-DNA insertion in the promoter region of *Oshox22* showed reduced *Oshox22* expression and ABA content, decreased sensitivity to ABA, and enhanced tolerance to drought and salt stresses at the seedling stage. In contrast, transgenic rice over-expressing *Oshox22* showed increased sensitivity to ABA, increased ABA content, and decreased drought and salt tolerances. Based on these results, we conclude that Oshox22 affects ABA biosynthesis and regulates drought and salt responses through ABA-mediated signal transduction pathways.

Keywords Rice · Transcription factor · HD-Zip · Drought stress · Regulation · Abiotic stress

Abbreviations

HD-Zip	Homeodomain-leucine zipper
ABA	Abscisic acid
PEG	Polyethylene glycol
GFP	Green fluorescent protein
RT	Reverse transcription
PCR	Polymerase chain reaction
HB	Homeobox

Introduction

Drought and salt are major abiotic stresses that cause tremendous yield losses in crops all over the world. Due to water shortage and less predictable rainfall patterns resulting from global atmospheric changes, the improvement of stress resistance in crops is now of utmost importance. Consequently, the genetic basis of drought and salt resistance is an intensively studied topic. The plant hormone abscisic acid (ABA) plays a central role in drought and salt responses through regulating developmental and physiological processes including stomata closure (Leung and Giraudat 1998; Umezawa et al. 2009;

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S. Zhang · L. Jiang · C.-M. Liu (✉) · P. B. F. Ouwerkerk (✉)
Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
e-mail: p.b.f.ouwerkerk.2@gmail.com

C.-M. Liu
e-mail: cmliu@ibcas.ac.cn

S. Zhang · L. Jiang
Graduate School of Chinese Academy of Sciences, Beijing 100049, China

S. Zhang · A. H. Meijer · P. B. F. Ouwerkerk
Institute of Biology, Leiden University, P.O. BOX 9505, 2300 RA Leiden, The Netherlands

I. Haider · W. Kohlen · H. Bouwmeester
Laboratory of Plant Physiology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

H. Schluepmann
Department of Molecular Plant Physiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Melcher et al. 2010; Huang et al. 2012). Using microarrays, many genes with expression responding to ABA, drought and salt treatments have been identified (Seki et al. 2001, 2002; Rabbani et al. 2003; Bray 2004; Yamaguchi-Shinozaki and Shinozaki 2006). Additional characterization identified key components mediating gene-expression changes in drought responses that include the transcription factors *DREB1A* (Kasuga et al. 2004), *DREB2A* (Sakuma et al. 2006), *SNAC1* (Hu et al. 2006), *OsbZIP23* (Xiang et al. 2008), and *DST* (Huang et al. 2009). Furthermore, a number of transcription factors encoded by homeodomain-leucine zipper (HD-Zip) genes in Arabidopsis, rice and other plants have been implicated in regulating drought tolerance through either ABA-dependent or ABA-independent pathways (e.g. Söderman et al. 1996, 1999; Gago et al. 2002; Himmelbach et al. 2002; Deng et al. 2006; Agalou et al. 2008; Shan et al. 2011). The HD-Zip genes, however, are an abundant group of transcription factors that are exclusively found in plants (Ruberti et al. 1991; Schena and Davis 1992; Aso et al. 1999; Sakakibara et al. 2001; Derelle et al. 2007). HD-Zip proteins, characterized by a DNA-binding HD and a protein–protein interaction Zip domain, have been classified into four families (I–IV) according to their sequence similarities (Ruberti et al. 1991; Morelli and Ruberti 2002). Different members of the HD-Zip families I and II have been implicated in auxin signaling and transport (Morelli and Ruberti 2002; Sawa et al. 2002), vascular development (Scarpella et al. 2000), and light responses including shade avoidance (Steindler et al. 1999; Wang et al. 2003). Several other members, such as *Athb-6*, *Athb-7* and *Athb-12* from Arabidopsis (Söderman et al. 1996, 1999; Lee et al. 2001; Hjellström et al. 2003; Olsson et al. 2004), *Hahb-4* from sunflower (Gago et al. 2002; Dezar et al. 2005a, b; Manavella et al. 2006), and *CpHB2*, *CpHB6* and *CpHB7* from *Craterostigma plantagineum* (Frank et al. 1998; Deng et al. 2002) are induced by drought and ABA, suggesting a function in ABA-mediated adaptation to drought stress. In agreement, inductions of *Athb-6*, *Athb-7* and *Athb-12* are abolished in the ABA-insensitive mutants *abi1* and *abi2* (Himmelbach et al. 2002; Olsson et al. 2004).

Like in dicots, a subset of HD-Zip family I and II genes is regulated by drought in rice (Agalou et al. 2008). Phylogenetic analysis places *Oshox22*, *Oshox24*, *Athb-7* and *Athb-12* in the same subgroup (γ-clade, Henriksson et al. 2005) of the HD-Zip family I, and *Oshox22* is very likely related to *Oshox24* via an ancient chromosomal duplication (Agalou et al. 2008). Our previous work showed that *Oshox22* is strongly induced by drought which spurred our interest for further studies (Agalou et al. 2008). To gain insight into the function of *Oshox22* in drought and salt tolerances, we performed genetic and physiological studies through mutation and over-expression analyses in rice. Our data showed that *Oshox22* regulates drought and salt stress susceptibility through an ABA-mediated signaling pathway.

Materials and methods

Plant materials and stress treatments

Drought-tolerant cultivar IRAT 112 (upland tropical japonica, also named Gajah Mungkur) and drought-sensitive cultivar Nipponbare (lowland japonica) were used for most studies described in this paper. Zhonghua 11 (lowland japonica) was used for transgenic analyses. The T-DNA insertion mutant *oshox22-1* in Dongjin (lowland japonica) background was obtained from the Postech collection in South Korea.

For hormone treatments, 12 day-old Nipponbare seedlings were sprayed with 100 μM ABA, followed by sampling at 0, 1, 3, and 6 h. Alternatively, seedlings at the same stage were irrigated with 10 % PEG 6,000 or 200 mM NaCl followed by sampling at 0, 1, 3, and 6 h. For the cold treatment, seedlings were transferred to 4 °C and sampled after 0, 1, 3, 6, 12, and 24 h. One whole plant was sampled as one replicate and in total four replicates were used in each RNA extraction.

For drought treatments, about 40 plants of the *oshox22-1* mutant and *Oshox22* over-expression lines were grown in square pots (L26 × W12 × H10 cm) filled with a mixture of sand and soil (1:1) together with wild type, respectively. We stopped watering when seedlings were 12 days old, and watering was resumed for 3 days when seedlings were 24 days old, after which the survival rates were calculated. For salt treatments, the same amount of 12 day-old seedlings were irrigated with 150 mM NaCl for 9 days, after which green leaf rates (green leaf area >30 %) and survival rates were determined. To measure the water-loss rate under dehydration conditions, the second leaves from the top of the plant at the tillering stage (plants were 55 days old) were cut and exposed to air at room temperature (approximately 25 °C), and the weight was determined every 30 min. Every treatment was done in triplicate.

Subcellular localization assay

A full-length cDNA of *Oshox22* was amplified from IRAT 112 by RT-PCR using primers Oshox22cdsFW and Oshox22cdsRW (Table S1) based on a full-length cDNA sequence (GenBank accession AY224440) found in a seed-derived cDNA expression library (Cooper et al. 2003). The PCR product was cloned into *pCR2.1-TOPO* (Invitrogen) and sequenced. To create a GFP-tagged construct, the full-length *Oshox22* cDNA was excised by *Eco*RI and ligated into *Eco*RI-digested *pTH2-BN* vector. The cDNA fragment

was inserted to the C-terminus of *GFP* and expressed using the Cauliflower mosaic virus (CaMV) 35S promoter. The construct was transformed into rice protoplasts as previously described (Chen et al. 2006; Osnato et al. 2010). Localization of the GFP-tagged Oshox22 protein was monitored in protoplasts by confocal laser scanning microscopy (Leica SP5) at 24 h after transformation.

Yeast one-hybrid screens

To assay the activation property, the full-length *Oshox22* cDNA PCR product was excised by *Eco*RI and *Bam*HI, then ligated into *Eco*RI/*Bam*HI digested *pAS2-1* (Clontech, GenBank accession U30497), resulting in *pAS2-1-Oshox22*. Partial Oshox22 ORFs were also cloned into *pAS2-1* vector, producing different translational fusions between *Oshox22* and the GAL4 BD. Constructs were sequence-verified before transfer to yeast strain *PJ69-4A* (Table S2) for activation screens. All yeast handling and reporter assays were performed as described before (Meijer et al. 1998, 2000a; Ouwerkerk and Meijer 2001, 2011).

For the DNA binding assay, a full-length cDNA of *Oshox22* was excised from *pAS2-1-Oshox22* with *Eco*RI and *Sal*I and then ligated into *pRED-ATGa* which is replicated via ARS-CEN and maintained via *URA3* selection (unpublished results, P.B.F. Ouwerkerk and A.H. Meijer). The resulting plasmid *pRED-ATG-Oshox22* was assayed in yeast strains *YM4271-4AH1-HIS3* and *YM4271-4AH2-HIS3* which were made using *pINT1* as the integrative vector system (Meijer et al. 1998) which contains the *HIS3* reporter gene preceded by AH1 or AH2 tetramer-binding sites for HD-Zip proteins (Meijer et al. 2000b).

Binary vector constructions and plant transformation

The *Oshox22* over-expression construct was made in the binary vector *pC1300intB-35SnosEX* (Genbank accession AY560325) as following: the full-length cDNA of *Oshox22* was excised from *pAS2-1-Oshox22* and ligated into binary vector *pC1300intB-35SnosEX*, allowing the gene to be expressed under the control of the CaMV 35S promoter. We transformed rice (Zhonghua 11) as previously described (Scarpella et al. 2000) except that the *Agrobacterium tumefaciens* strain used was *LBA4404*. Calli used for transformation were obtained from germinating seeds according to Rueb et al. (1994). Plantlets were maintained in culture on half-strength Murashige Skoog (MS) medium with 10 g/l sucrose until transfer to a greenhouse (28 °C under a 16 h photoperiod and 85 % humidity).

Rice protoplast isolation and transient expression assays

Assays to test DNA binding of Oshox22 involved transient transformations of protoplasts with effector and reporter plasmids. The effector plasmid contained the full-length cDNA of *Oshox22* expressed under the control of the CaMV 35S promoter. The reporter plasmids contained the putative *Oshox22* binding sequences AH1 or AH2 as tetramers upstream of a truncated −90 CaMV 35S promoter directing *GUS* gene expression (Meijer et al. 1997). For protoplast isolation, one hundred rice seeds (Nipponbare) were grown in 10 cm diameter pots (28 °C, 85 % humidity) in the dark for 12–14 days. Stems and leaves were cut into ~0.5 mm pieces and digested with 25 ml enzyme solution containing 1.5 % w/v cellulase (Sigma) and 0.3 % w/v Macerozyme (Sigma) in 50 ml centrifuge tubes. Further preparation of protoplasts and transfection with effector/reporter constructs were as described earlier (Chen et al. 2006; Osnato et al. 2010). Proteins extraction and detection of GUS activity were based on Jefferson et al. (1987). Fluorescence was measured by a Cytofluor 2350 fluorimeter (Millipore).

Northern blot hybridization

Electrophoresis and northern blotting of RNAs were performed as described by Memelink et al. (1994). Baked blots were pre-hybridized in 1 M NaCl, 1 % SDS, 10 % dextrane sulphate and 50 μg/ml denatured herring sperm DNA at 65 °C, washed with 0.1XSSPE, 0.5 % SDS at 42 °C and then autoradiographed. Probes were labeled by random priming with ^{32}P-dCTP. Equal loading of RNA samples was verified on the basis of ethidium bromide staining of ribosomal RNA bands.

Real-time qPCR analysis

Total RNAs from different tissues were pre-treated with RNase-free DNase I (Takara), according to the manufacturer's instruction. Reverse transcription reaction was performed with SuperScript™ III reverse transcriptase (Invitrogen) following the manufacturer's instruction. Primers used for real-time PCR analyses of *Oshox22* were QPCR-22FW and QPCR-22RW (Table S1). qPCR was performed with Rotor-Gene 3000 Real-time PCR System using SYBR1 Green to monitor dsDNA synthesis. Relative expression levels of reporter and target genes were determined by the Two Standard Curves Relative Quantification Method using *ACTIN1* (Table S1) as the internal control.

Determination of ABA content

Five seeds each of the homozygous T-DNA insertion mutant *oshox22-1* and over-expression lines together with their corresponding wild type cultivars (Dongjin and Zhonghua 11 respectively) were surface-sterilized and germinated in half-strength MS salt solution without sucrose and grown at 28 °C with a 16/8 h light/dark photoperiod. To determine the ABA level in response to drought stress, 12 day-old *oshox22-1* and over-expression seedlings were stopped watering and sampled after 10 days dehydration treatment. Quantification of ABA levels was performed using LC–MS/MS with five biological replicates (each 0.2 g of fresh shoot tissue) as described elsewhere, (López-Ráez et al. 2008, 2010). Statistical analyses were performed using Student's *t* test.

Stomata density test

Stomata numbers were counted on the upper epidermis of the leaf blades of mutant and over-expression lines grown under normal conditions. Leaf blades were from flag leaves at the stage that the plants just started to flower. For this method, oval surfaces spanning 1 × 2 cm were painted with clear fingernail polish, while avoiding ribbed veins. After the polish had dried it was peeled off by adding a tape on the polish and transferred to a microscopy slide. Finally, the stomata number was counted using DIC microscopy. Five replicates of every leaf were counted and from every plant line seven plants were used and all numbers were converted to number of stomata per square mm to account for variation in microscopes.

Results

Isolation and sequence analysis of *Oshox22*

Previous studies showed that the expression of *Oshox22* in rice is strongly induced by drought, and that the induction is higher in three drought tolerant upland cultivars compared with three lowland cultivars (Agalou et al. 2008). To elucidate the function of *Oshox22*, the full-length cDNA of *Oshox22* was amplified from the rice cultivar IRAT 112 by RT-PCR. The sequence of the amplified cDNA fragment was identical to that of the cDNA from Nipponbare over the whole length. *Oshox22* (Os04g45810) is located on chromosome 4 and encodes a protein of 262 amino acids (AA) including a 61-AA HD domain for DNA binding and a 43-AA Zip domain for protein–protein interactions.

Expression of *Oshox22* under abiotic stress conditions

First, RNA samples from different rice tissues at several developmental stages were analysed by quantitative RT-PCR (qRT-PCR) in Nipponbare. The results showed that *Oshox22* is ubiquitously expressed, with a lower level in stems and higher in panicles and seeds (Fig. 1a), which is consistent with earlier observations (Agalou et al. 2008). We then monitored the *Oshox22* expression under different abiotic stress conditions. As shown in Fig. 1b, *Oshox22* was rapidly and strongly induced by NaCl, PEG and ABA, and weakly induced by low temperature. These results are in agreement with available microarray data (http://red.dna.affrc.go.jp/RED/).

Subcellular localization and transcriptional activation function of Oshox22

To confirm that Oshox22 is a nuclear protein, a GFP-tagged *Oshox22* construct under the control of the CaMV 35S promoter was made, with GFP fused at the C-terminus of the full-length Oshox22 protein (construct *35S::GFP-Oshox22*). This construct was introduced into rice protoplasts by transient transformation and the fusion protein's subcellular localization was analysed using confocal laser scanning microscopy, where the *35S::GFP* construct served as control. As shown in Fig. 1c, the GFP signal was detected specifically in the nuclei of *35S::GFP-Oshox22* transformed protoplasts, while in the control the GFP signal was located primarily in the cytoplasm. These data suggest that Oshox22 is a nuclear-localized protein. In agreement with this observation, a putative nuclear localization signal sequence (RKRR at the AA 59) was found near its N-terminus, as analyzed by WoLF PSORT (http://wolfpsort.seq.cbrc.jp/).

To test whether Oshox22 has any transcription activation property, Oshox22 was fused to the Gal4p binding domain (*GAL4 BD*) and assayed for the ability to induce expression of a *HIS3* gene preceded by Gal4p binding sites. The result showed that the transformed yeast cells were able to grow on medium lacking histidine, with up to 10 mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3p enzyme activity (Fig. 2a). In contrast, no growth was observed of yeast transformed with a control construct without Oshox22 (*pAS2-1*), indicating that Oshox22 has transcriptional activation activity in yeast. To dissect the activation domain(s), a series of seven truncated Oshox22 constructs with deletions from either the N- and C-termini were tested. No activation activity was observed when either the HD or the Zip domains were deleted (Fig. 2a), suggesting both domains are required for transcriptional activation in yeast.

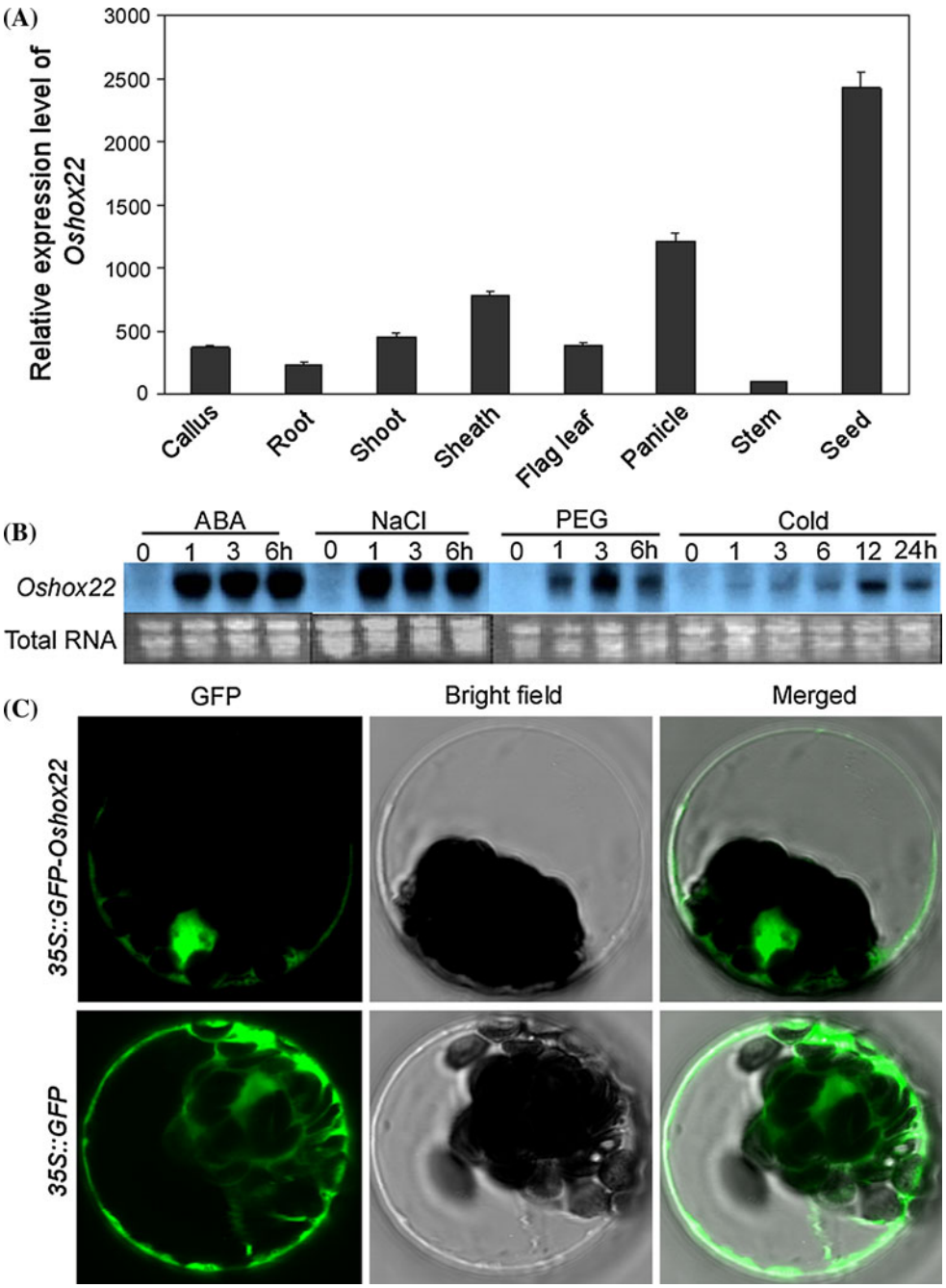

Fig. 1 Expression profiling and subcellular localization of *Oshox22*. **a** qRT-PCR analyses of *Oshox22* expression in scutellum-derived calli, root, shoot, leaf sheath, flag leave, panicle, stem and mature grain from rice. **b** Northern blot analysis of *Oshox22* expressions in response to ABA (100 μM), salt (200 mM NaCl), 10 % PEG 6,000 and low-temperature (4 °C) treatments. **c** Nuclear localization of GFP-tagged Oshox22. The GFP-Oshox22 fusion protein, driven by the CaMV 35S promoter, was transiently expressed in rice protoplasts and visualized by confocal laser scanning microscopy. Construct *pTH2* (Chiu et al. 1996) carrying a *GFP* gene driven by CaMV 35S promoter was used as the negative control

Interactions of Oshox22 in yeast and rice with the CAAT(C/G)ATTG sequence

Previous results showed that HD-Zip family I and II proteins are able to interact with pseudopalindromic AH1 (CAAT(A/T)ATTG) and AH2 (CAAT(C/G)ATTG) sequences, respectively (Sessa et al. 1993; Meijer et al. 1997, 1998, 2000b; Palena et al. 1999; Johannesson et al. 2001). To test whether Oshox22 interacts with either the AH1 or AH2 sequences or both, yeast strains containing a chromosomally integrated *HIS3* reporter gene with upstream AH1 or AH2 tetramers (named *4AH1-HIS3* and *4AH2-HIS3* respectively, Meijer et al. 1998, 2000b) were used. The *Oshox22* ORF was cloned into *pRED-ATGa* (named *pRED-ATGa-Oshox22),* allowing for constitutive expression of full length Oshox22 protein in yeast without fusion to an exogenous activation domain. Construct *pRED-ATGa-Oshox22* was transformed into yeast strains containing constructs *4AH1-HIS3* or *4AH2-HIS3*. The results showed that yeast cells with *4AH2-HIS3*

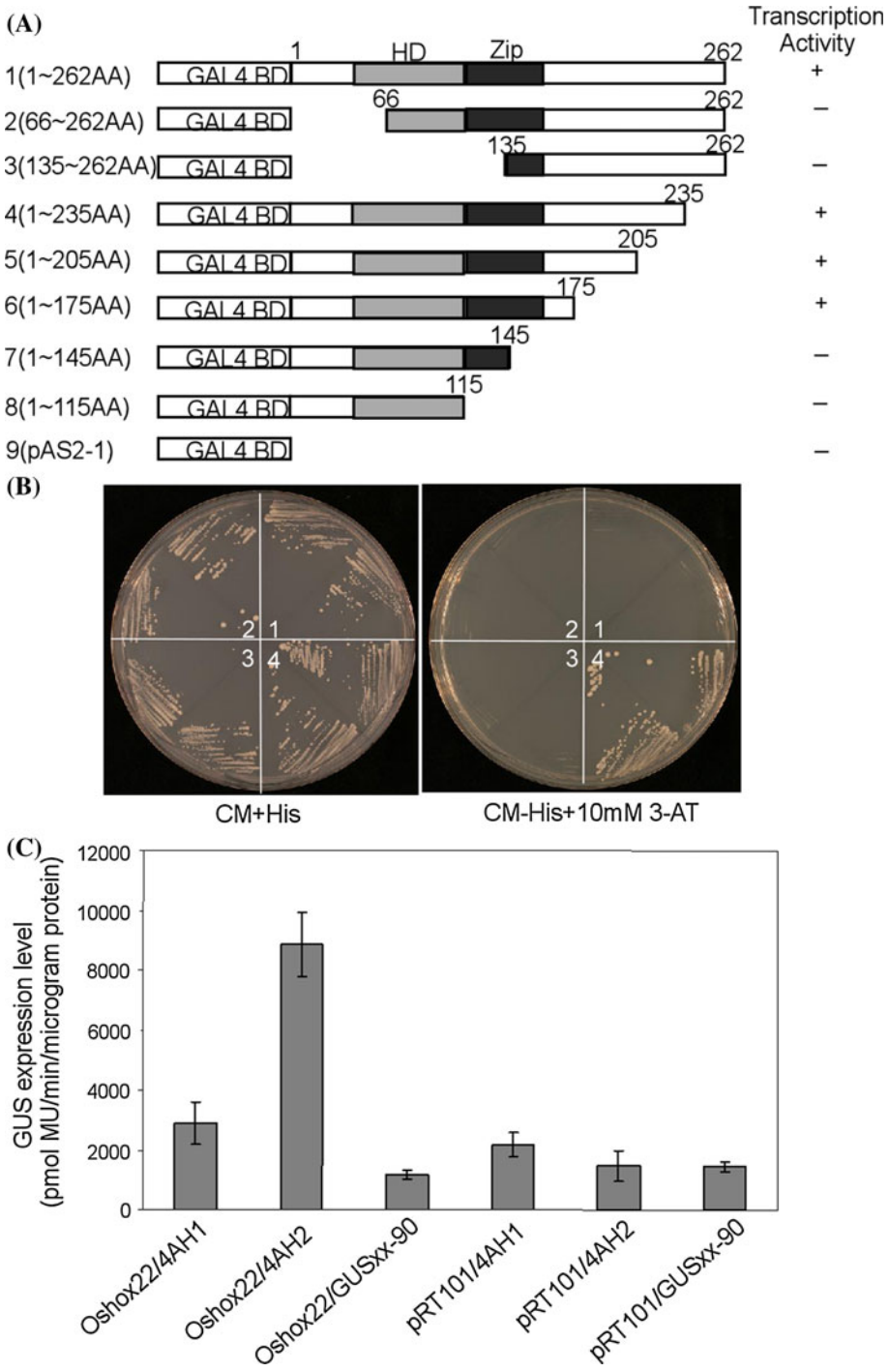

Fig. 2 Trans-activation and DNA-binding specificity of Oshox22. **a** Trans-activation assay in yeast with truncated Oshox22 protein. Fusion proteins of the GAL4 DNA-binding domain and different fragments of Oshox22 were examined for their trans-activation activities in yeast *PJ69-4A*. HD, homeodomain; Zip, leucine zipper. **b** Trans-activation assay of Oshox22 in yeast. *Sections 1* and *2*, negative control; *sections 3* and *4*, Oshox22 fused to GAL4-BD in the vector *pRED-ATGa* was transformed into yeast strains *YM4271-4AH1-HIS3* and *YM4271-4AH2-HIS3*, respectively. **c** Interactions of Oshox22 with the HD-Zip binding site AH2 (CAAT(C/G)ATTG) and activation of reporter gene expression in a transient expression system using rice protoplasts. Transient expression of *Oshox22* was driven by the CaMV 35S promoter. The *Oshox22-OX* construct was co-transformed with the reporter constructs *GUSXX-4AH1* or *GUSXX-4AH2*. The empty vectors *pRT101* and *GUSXX-90* were used as negative controls

transformed with *pRED-ATGa-Oshox22* grew well on a medium lacking histidine but containing up to 10 mM 3-AT (Fig. 2b), whereas no growth was observed in yeast strains with *4AH1-HIS3* or with empty *pRED-ATGa* vector. Thus, in yeast, Oshox22 is able to bind AH2, but not AH1, and can activate reporter gene expression by an intrinsic activation domain.

To confirm binding of Oshox22 protein to the AH2 sequence transient expression assays were carried out with effector and reporter plasmids in rice protoplasts. Two reporter plasmids, *4AH1-90-GUS* and *4AH2-90-GUS* were used, in which the AH1 and AH2 tetramers were fused to a CaMV -90 35S minimal promoter. Construct *Pro35S-Oshox22* with *Oshox22* expressed under control of the

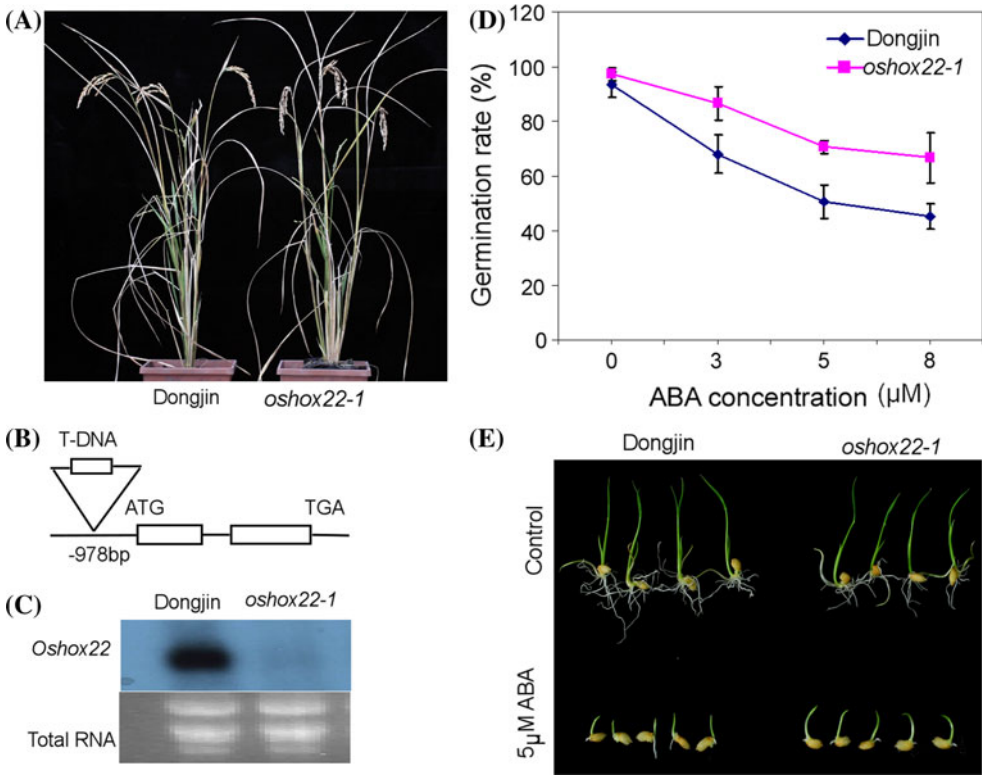

Fig. 3 The phenotype and ABA sensitivity of *oshox22-1*. **a** Morphology of *oshox22-1* at post-anthesis stage. **b** Schematic representation of the exon–intron structure of *Oshox22* gene and the position of the T-DNA insert in *oshox22-1*. **c** *Oshox22* expression was down-regulated in *oshox22-1*. **d** Germination rate of *oshox22-1* on MS medium with 0, 3, 5, and 8 μM ABA for 3 days. **e** Relative growth of *oshox22-1* on medium with 5 μM ABA at the post-germination stage. Dongjin represent wild type plants segregated from a heterozygous *oshox22-1* parent

CaMV 35S promoter was used as an effector. As shown in Fig. 2c, *GUS* expression in protoplasts co-transformed with *Pro35S-Oshox22* and *4AH2-90-GUS* was 3.07 times higher than those co-transformed with *Pro35S-Oshox22* and *4AH1-90-GUS*, and 6.11 times higher than those co-transformed with the empty vector. These data indicate that Oshox22 is capable to activate transcription of the reporter gene when upstream HD-Zip binding sites AH1 or AH2 are present (Fig. 2c). The interaction is less effective at the AH1 site than that at AH2, which is in contrast to earlier observations where HD-Zip I proteins mainly interacted with AH1 and HD-Zip II proteins with AH2 (Sessa et al. 1993; Meijer et al. 1997, 2000b; Palena et al. 1999).

Decreased ABA sensitivity of the *oshox22-1* mutant

We searched the publicly available mutant collections and obtained a putative T-DNA insertion mutant for *Oshox22* in rice (POSTECH_C054121, B-11507) (Jeong et al. 2006) (Fig. 3a). The genomic locus of *Oshox22* in this mutant is shown schematically in Fig. 3b. Alignment of the flanking sequence tag and the genomic sequence showed that the T-DNA insertion is located at 978 bp before the translational start codon of *Oshox22*. To investigate the function of *Oshox22*, an homozygous T-DNA insertion line (named *oshox22-1*) was identified, and Southern blot analysis showed that only one copy of T-DNA was present in *oshox22-1* (Fig. S1). Northern blot analysis showed that in *oshox22-1* the *Oshox22* transcript was below detection level (Fig. 3c). Phenotypic studies showed that *oshox22-1* plants exhibited no obvious morphological difference compared to wild type Dongjin (Fig. 3a). The panicle shape and the grain number remained unchanged. When *oshox22-1* was backcrossed with Dongjin, all F_1 and F_2 plants showed the same plant stature in the greenhouse. In the F_2 population, the T-DNA insert segregated in a 3:1 ratio (n = 326), suggesting no embryo or gamete lethality was involved.

We further analysed the sensitivity of *oshox22-1* to ABA in germination assays. On MS media with 3 and 8 μM ABA, seeds from *oshox22-1* showed significantly higher germination rates (72 and 32 %, respectively) than those from wild type plants segregated from *oshox22-1* backcrosses (40 and 8 % on the medium with 3 and 8 μM ABA, respectively; Fig. 3d). On control media without ABA, the germination rates of *oshox22-1* and wild type seeds showed no significant difference. These results suggest that *Oshox22* may mediate ABA sensitivity. Next, *oshox22-1* seedlings were tested for ABA sensitivity at the post-germination stage using media with different concentrations of ABA. The results showed that shoot development was less inhibited by exogenous ABA in *oshox22-1,* as compared to wild type (Fig. 3e). In summary, down-regulation of *Oshox22* expression in the

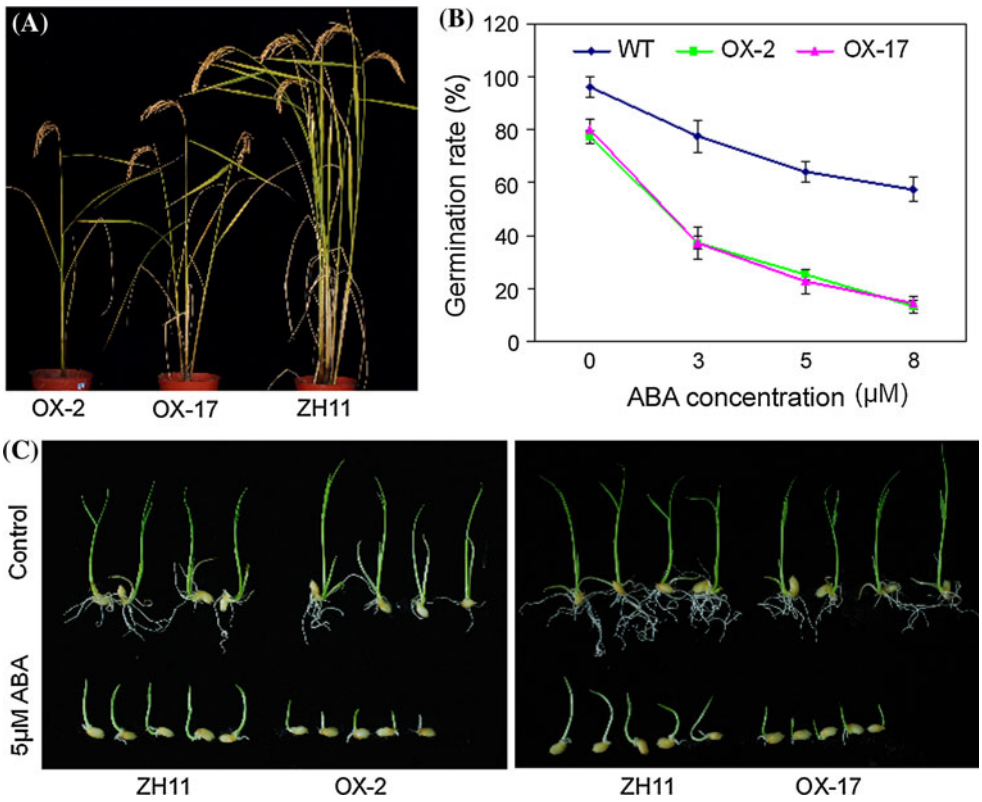

Fig. 4 Phenotype and ABA sensitivity of Oshox22-OX plants. **a** Phenotype of two independent Oshox22-OX lines at mature stage. **b** Germination rate of seeds from Oshox22-OX plants on medium with 0, 3, 5, and 8 μM ABA for 3 days. **c** Shoot and root growth of Oshox22-OX plants on MS medium with 5 μM ABA at post-germination stage. ZH11 (Zhonghua 11), wild type segregated from the T_1 line

T-DNA insertion line led to compromised ABA sensitivity at germination as well as post-germination stages.

Increased ABA sensitivity of transgenic plants over-expressing *Oshox22*

For over-expression analysis we expressed *Oshox22* under the control of the CaMV 35S promoter (construct Oshox22-OX). Northern blot analysis confirmed that *Oshox22* gene expression levels were increased in the transgenics (Fig. S2). Compared to wild type control plants, the Oshox22-OX plants exhibited fewer tillers and decreased height (Fig. 4a). We chose two transgenic lines (Oshox22-OX-2 and Oshox22-OX-17) for further analyses. The seed germination rates were reduced 17 % in these two lines (Fig. 4b). To analyse the sensitivity of the Oshox22-OX plants to ABA, seeds were germinated on solid MS media containing either 0, 3, 5 or 8 μM ABA. As shown in Fig. 4b, germination of seeds from both Oshox22-OX lines was severely inhibited by all concentrations of ABA used and the inhibition was much stronger than that observed for wild type seeds. For instance, the germination rates of the two Oshox22-OX lines plated on medium with 3 μM ABA were 40 %, as compared to 80 % on medium without ABA, whereas in the wild type only a slight reduction (from 98 to 80 %) was observed. The relatively low germination rates observed in Oshox22-OX seeds could be due to an increased ABA sensitivity. We further tested Oshox22-OX seedlings on media containing 0, 3, 5 or 8 μM ABA and found that the growth of Oshox22-OX seedlings was inhibited to a greater extent by 5 μM ABA (Fig. 4c). We therefore concluded that over-expression of *Oshox22* led to increased ABA sensitivity at the germination as well as post-germination stages.

Functions of Oshox22 in regulating ABA biosynthesis

Next, endogenous ABA levels were measured of the Oshox22 mis-expression plants grown under control and drought-stress conditions (Fig. 5). Under normal irrigated conditions, the ABA content of wild type Dongjin seedlings was 9.92 ± 1.06 ng g^{-1} FW, while in *oshox22-1* it was reduced to 3.86 ± 0.86 ng g^{-1} FW, an average reduction of 60 % (Fig. 5a). In wild type Zhonghua 11, the ABA content was 6.38 ± 0.72 ng g^{-1} FW, while in over-expression lines Oshox22-OX-2 and Oshox22-OX-17, the ABA contents were increased to 7.69 ± 0.42 ng g^{-1} FW and 8.35 ± 0.78 ng g^{-1} FW, respectively (Fig. 5a). Thus, down-regulation of *Oshox22* led to a reduced level of ABA and over-expression led to increased ABA levels, therefore we conclude that *Oshox22* functions in regulating biosynthesis or degradation of ABA in rice. The differences in

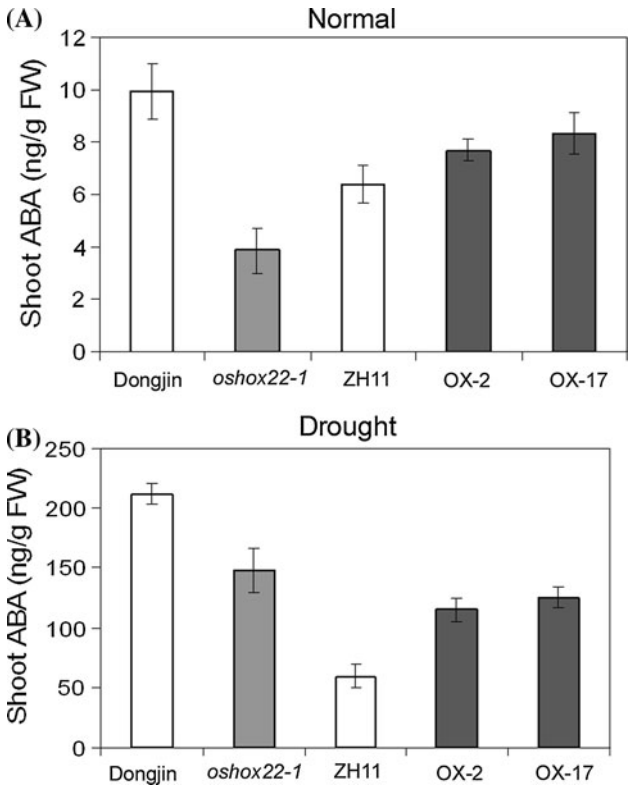

Fig. 5 Effects of *Oshox22* mis-expression on ABA levels. ABA levels in *oshox22-1* and Oshox22-OX plants and wild type cultivars Dongjin and Zhonghua 11. **a** ABA levels under normal condition. **b** ABA levels after drought treatment. After drought treatment, the ABA levels increased with a factor 21.39, 38.10, 9.34, 15.04 and 15.04 for *oshox22-1*, Dongjin, Zhonghua 11, Oshox22-OX-2 and Oshox22-OX-17, respectively

ABA levels are likely causing changes in sensitivity of germinating and developing seedlings towards exogenous applied ABA. Furthermore, we also tested the ABA contents under drought stress conditions. We found that ABA contents were increased in all plants after drought treatment (Fig. 5b), however, there were differences in the levels of induction ranging from 9.34 in wild type Zhonghua 11 to 38.10 in *oshox22-1*. Although the absolute ABA level was lower in the mutant, the level of ABA accumulated 38.31 fold whereas this was 21.39 fold in wild type Dongjin. In the two *Oshox22* overexpression lines the induction level was 15.04 whereas in control Zhonghua 11 plants this was only 9.34, thus not only the absolute ABA levels were higher but also the induced levels were higher.

Drought and salt responses in *oshox22-1* and Oshox22-OX plants

To address the function of *Oshox22* in drought and salt responses, we analysed a homozygous *oshox22-1* line and two Oshox22-OX lines for drought and salt tolerances. When the seedlings were 12 days old (five leaf stage) we stopped watering for 12 days and then resumed watering for 3 days to measure the survival rates. Drought treatment strongly decreased the survival rate of Oshox22-OX plants, while in *oshox22-1* mutants the survival rate was increased, as compared to their corresponding wild type control groups (Fig. 6a, b). As shown in Fig. 6c, d, 92 % of the *oshox22-1* seedlings survived, as compared to 34.7 % in the control group. In contrast, 63 % of the Oshox22-OX seedlings survived, as compared to 87 % in their control group. From these data we conclude that mutation of *Oshox22* led to increased drought tolerance, while over-expression of Oshox22 led to decreased drought tolerance.

Furthermore, we measured water-loss rates in leaves from *oshox22-1* and Oshox22-OX plants during the dehydration process. Leaves from *oshox22-1* had significantly lower rates ($P < 0.05$) of water-loss than control plants (Fig. 6e). In contrast, Oshox22-OX plants showed no significant difference in water-loss rate as compared to the control (Fig. 6f). Because stomata density is a critical factor in drought stress, we determined the stomata density in leaf blades of *oshox22-1* plants at flowering stage, Oshox22-OX, and their corresponding wild type control plants. The *oshox22-1* leaves showed 17.8 % reduction in stomata density, but stomata density remained unchanged in Oshox22-OX, as compared to leaves from wild type plants (Fig. 7). Therefore, the enhanced drought tolerance in *oshox22-1* may partly result from decreased stomata density in leaves.

To evaluate salt tolerance in *oshox22-1* and Oshox22-OX plants, 12-day-old seedlings grown in hydroponic culture were transferred to a 150 mM NaCl solution for 9 days and then the green leaf area was measured. The *oshox22-1* plants had significantly more green leaf area (80 %) than control plants (36 %; Fig. 8). In contrast, Oshox22-OX plants had a reduced green leaf area (65 %) compared with the control (86 %; Fig. 8). These results suggest that in rice, down-regulation of *Oshox22* improved salt tolerance, whilst over-expression of *Oshox22* led to reduced salt tolerance. These data further suggest that *Oshox22* functions as a negative regulator in drought and salt tolerance in rice.

Discussion

In rice and Arabidopsis, about 50 % of all homeobox genes (Chan et al. 1998; Jain et al. 2008; Mukherjee et al. 2009) belong to the HD-Zip family. Although some members have been implicated in the regulation of development and stress responses, the functions of most HD-Zip genes are still unknown. Based on mutant and over-expression analyses, we here propose a function for the rice HD-Zip

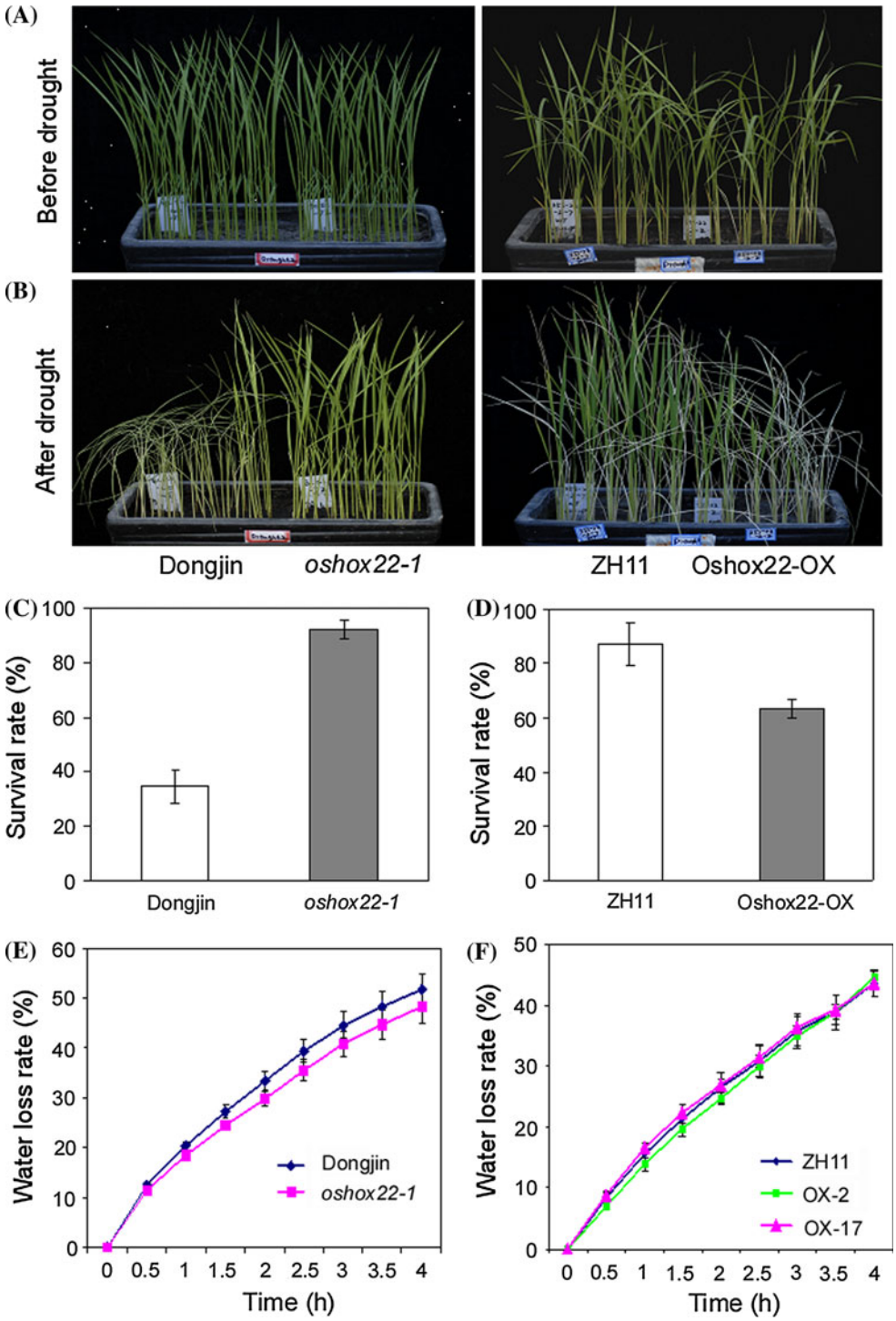

Fig. 6 Drought tolerance of *oshox22-1* and Oshox22-OX plants. **a** Seedlings of *oshox22-1* and Oshox22-OX before applying the drought-stress. **b** After drought stress treatment. **c**, **d**, Survival rates of *oshox22-1* and Oshox22-OX plants after application of drought stresses. **e** Water-loss rates in leaves from *oshox22-1* and corresponding wild type plants. **f** Water-loss rate in leaves from Oshox22-OX and corresponding wild type plants

family I gene *Oshox22* in regulating ABA biosynthesis and ABA-mediated drought and salt tolerances in rice.

In this study, we used a GFP-tagged fusion construct to demonstrate that Oshox22 is a nuclear-localized protein, which is consistent with a function as transcription factor. The DNA-binding and activation properties were analysed in yeast one-hybrid experiments, showing specific binding to a known HD-Zip target sequence and activation of reporter gene expression without the requirement of exogenous activation domains. Further functional analysis in yeast showed that both HD and Zip domains are required for the trans-activation. The function of Oshox22 as a transcriptional activator was further confirmed with transient assays in rice protoplasts using a *GUS* reporter gene. Sessa et al. (1993, 1997) propose that AH1 (CAAT(A/T)ATTG) and AH2 (CAAT(C/G)ATTG) act as consensus binding sites for

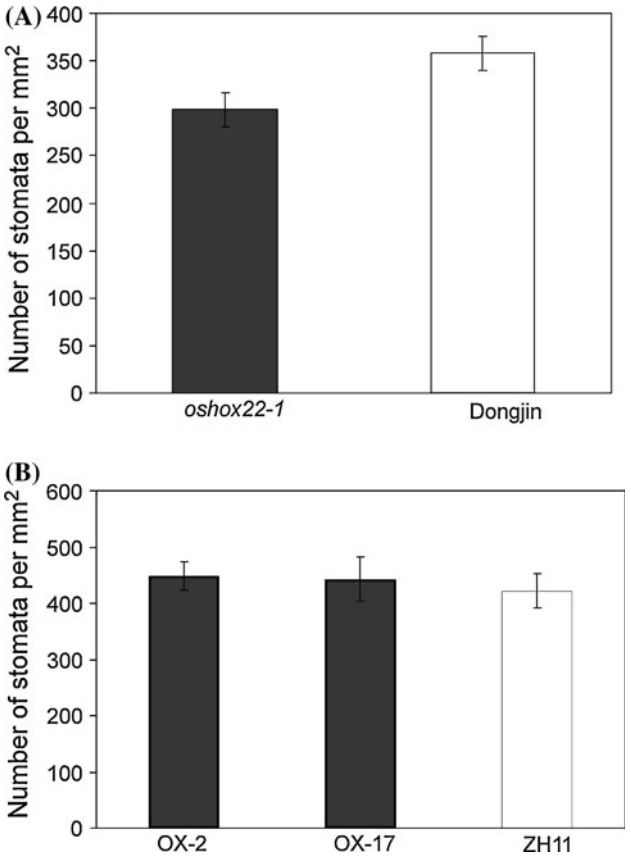

Fig. 7 Stomata counting in leaves from *oshox22-1* and Oshox22-OX plants. **a** The number of stomata on *oshox22-1* leaves compared to wild type ($P < 0.01$, $n = 10$). **b** The number of stomata on two independent Oshox22-OX lines compared to wild type ($P > 0.05$, $n = 10$). Dongjin in this context is the wild type segregated from heterozygous *oshox22-1/Oshox22* line; ZH11 (Zhonghua 11), wild type segregated from an Oshox22-OX T_1 line

HD-Zip I and HD-Zip II proteins, respectively. Apparently, Oshox1 to Oshox7 follow these rules (Meijer et al. 1997, 1998, 2000b). Athb-7 and Athb-12, however, do not seem to bind to either of these consensus sequences and may have totally different binding preferences (Himmelbach et al. 2002; Deng et al. 2006). Our data show that Oshox22 is able to activate gene expression via both AH1 and AH2, but more effectively via AH2 than AH1. Taken together, our data from the yeast and protoplast experiments support the function of Oshox22 as a transcriptional activator, which is typical of HD-Zip I family transcription factors (Ohgishi et al. 2001; Meijer et al. 2000b).

Similar to closely related HD-Zip factors such as *Athb-6*, *Athb-7* and *Athb-12*, our previous (Agalou et al. 2008) and current work shows that expression of *Oshox22* is responsive to drought, salinity and ABA treatments, suggesting its role in regulating stress tolerance. In Arabidopsis, the HD-Zip I protein Athb-6 has been shown to interact with ABI1, a protein phosphatase 2C (Himmelbach et al. 2002). ABI1 is involved in various responses towards ABA including stomata closure, seed dormancy and vegetative growth and thus represents a key component in ABA signaling (Leung et al. 1997; Leung and Giraudat 1998). For *Athb-7* and *Athb-12*, it has been found that their expression is down-regulated in *abi1* mutants (Olsson et al. 2004), which further supports the interaction between ABA signaling and these HD-Zip genes. To perform functional analyses, we generated *Oshox22* over-expression lines and obtained an *oshox22* mutant that contains a T-DNA insertion in the 5′ upstream sequence of *Oshox22* and displays strongly down-regulated expression of this gene. Under normal greenhouse conditions, *oshox22-1* plants did not show any visible difference from wild type. However, the endogenous ABA levels in *oshox22-1* seedlings were 60 % lower than those of the wild type Dongjin plants. When grown on medium with ABA, *oshox22-1* seeds showed a higher germination rate than the wild type. On the other hand, rice plants over-expressing *Oshox22* showed a decreased germination rate on medium supplied with ABA suggesting *Oshox22* is involved in an ABA-regulated seed germination process. It should be noted that the over-expression and mutation phenotypes were analysed in different genetic backgrounds. Zhonghua 11 is an important lowland rice cultivar in Chinese agriculture which is easy to transform using *A. tumefaciens* and therefore this is the preferred model for transgenesis in our laboratory. As a mutant allele of *Oshox22* was not present in the Zhonghua 11 RMD T-DNA collection (Zhang et al. 2006), we analysed a mutant allele of *Oshox22* in Dongjin, which is also a lowland Japonica rice cultivar. Since ABA sensitivity was decreased upon *Oshox22* mutation in Dongjin background and increased upon over-expression in Zhonghua 11 background, our data suggest that the function of *Oshox22* between these cultivars is conserved.

These results of *Oshox22* effects on ABA sensitivity are only partly consistent with those obtained in Arabidopsis with *Athb-7* and *Athb-12,* in spite of the fact that the latter are in the same HD-Zip I subgroup (γ-clade) as *Oshox22* (Olsson et al. 2004; Henriksson et al. 2005; Agalou et al. 2008). Furthermore, over-expression of *CpHB-7* isolated from *C. plantagineum* in Arabidopsis resulted in increased germination on ABA and thus reduced sensitivity towards ABA (Deng et al. 2006). Plants over-expressing *Athb-6* are also less sensitive to ABA on germination (Himmelbach et al. 2002). Thus, different HD-Zip proteins may be involved in different ABA-mediated signaling pathways and differences of closely related genes in the different species suggest that the signaling pathway may have evolved rapidly.

Although *Oshox22* is induced by ABA, constitutive over-expression of *Oshox22* in Zhonghua 11, led to an increased ABA level but compromised drought tolerance.

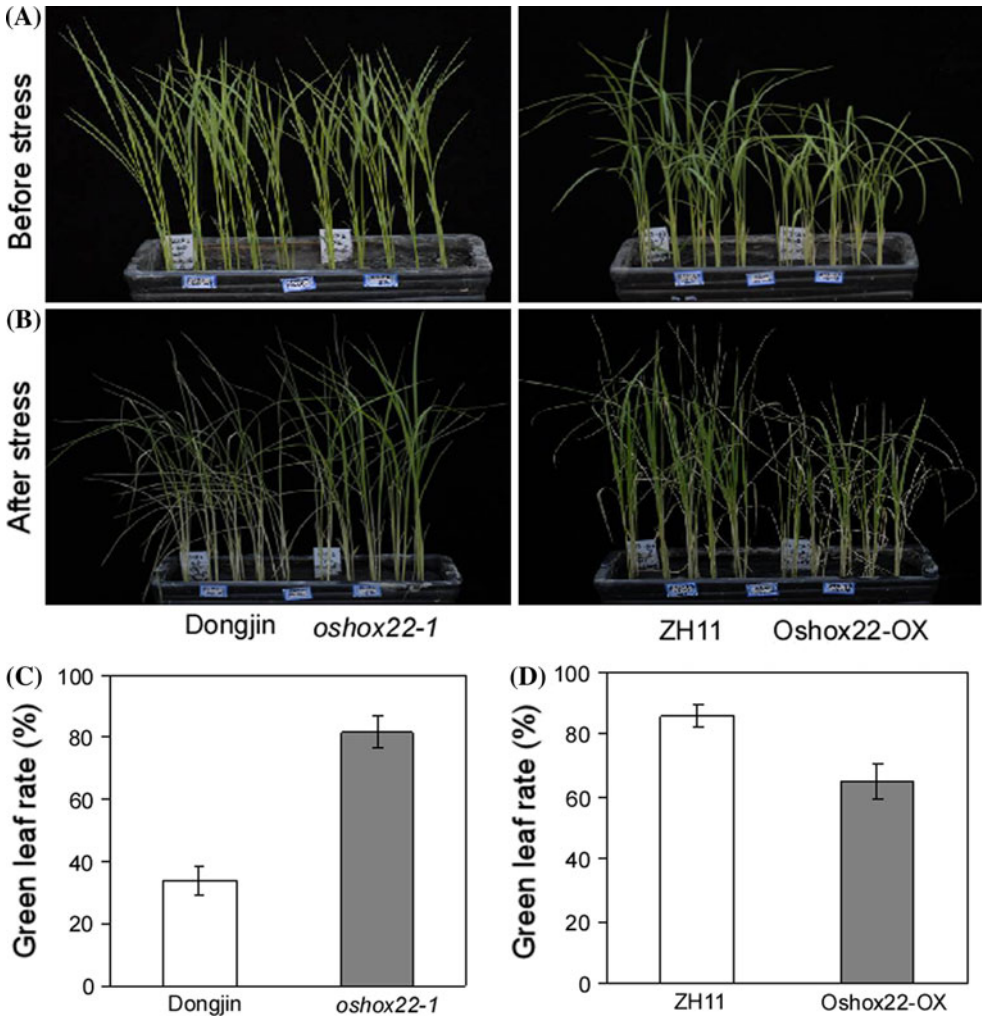

Fig. 8 Examinations of salt tolerance of *oshox22-1* and Oshox22-OX plants. **a** Seedlings of *oshox22-1* and Oshox22-OX before salt treatments. **b** Plants treated with 150 mM NaCl for 9 days. **c**, **d** Green leaf rates for *oshox22-1*, Oshox22-OX and their wild type plants after salt treatments

In addition to decreasing ABA sensitivity, however, the *Oshox22* mutation in Dongjin resulted in an increased tolerance towards drought, which correlated with reduced water-loss efficiencies of mutant seedlings. As numbers of stomata were decreased in *oshox22-1* mutants, regulation of stomata density by *Oshox22* is a possible mechanism underlying enhanced drought tolerance in these mutants. However, a change in stomata density was not found in the *Oshox22-OX* lines although these lines were less drought tolerant. Therefore, most likely stomata density is not the only factor determining drought tolerance under our experimental conditions. We conclude that *Oshox22* negatively regulates drought tolerance in rice. In wheat a similar situation is described where the level of ABA is inversely correlated to the level of drought tolerance. In drought-sensitive cultivars, drought treatment leads to enhanced expression of ABA biosynthesis genes in anthers and ABA accumulation in spikes, while in drought-tolerant wheat the treatment leads to accumulation of lower levels of ABA, which correlates with lower expression of ABA biosynthesis genes and higher level of expression of ABA catabolic genes (e.g. ABA 8′-hydroxylase). Furthermore, wheat *TaABA8′ OH1* deletion lines that accumulate higher levels of ABA in spikes are drought sensitive (Ji et al. 2011). We analysed ABA levels in *oshox22-1* mutants and the two over-expression lines as well as their respective wild type backgrounds. We found that mis-expression of *Oshox22* affected the absolute levels of ABA. However, despite difference in induction levels, in all plants ABA levels increased strongly in response to drought, which is in line with the well-known function of this hormone in responses to stress signals including drought and salinity. Higher levels of ABA are due to induction of ABA biosynthesis genes and in turn ABA reprograms plant cells to withstand and survive adverse environmental conditions (reviewed by Leung and Giraudat 1998; Umezawa et al. 2009; Melcher et al. 2010; Huang et al. 2012). We hypothesize that *Oshox22* may plays a role in ABA biosynthesis or degradation and that a lower endogenous ABA level in *oshox22-1* plants and higher level of ABA, respectively correlate inversely with their drought tolerance. Being a transcription factor, Oshox22 may affect

ABA level by directly regulating genes involved in biosynthesis or degradation of ABA.

We propose that *Oshox22* functions as a negative regulator in drought and salt tolerance similar to *OsABI5*, which is a bZIP transcription factor, and is inducible by ABA and high salinity (Zou et al. 2008). Transgenic rice plants over-expressing *OsABI5* were sensitive to ABA and to high-salinity stress as well as to PEG treatment. Similar as with the *oshox22-1* mutant, down-regulation of *OsABI5* in plants using an RNAi approach exhibited increased tolerance to salt as well as to PEG treatment which is a stress condition we did not check for *Oshox22* (Zou et al. 2007, 2008). It seems that several regulators exist that are able to control both drought and salt tolerances. Other examples are the transcription factors *OsbZIP23* (Xiang et al. 2008) and *DST* (Huang et al. 2009). However, there are also differences with *Oshox22*. Like with *Oshox22*, both factors are nuclear-localized transcriptional activators and down-regulation of *OsbZIP23* expression results in decreased ABA sensitivity but tolerance towards salt and drought stress is decreased and over-expression results in an increase of tolerance which is opposite to the results with *Oshox22*. Microarray experiments with *OsbZIP23* mis-expression plants identified sets of genes regulated by *OsbZIP23* amongst which many genes have known functions in stress tolerance (Xiang et al. 2008). Interestingly, the microarray dataset shows that *Oshox22* as well as *Oshox24* which is on a duplicated chromosome segment (Agalou et al. 2008), are higher expressed in the *OsbZIP23* over-expressor indicating that this gene probably acts upstream of the two HD-Zip I genes. We expect that in turn *Oshox22* and Oshox24 also have similar functions in controlling other sets of stress tolerance genes. Future experiments can involve similar experiments for downstream target genes in order to explain how *Oshox22* regulates drought and salt tolerance and ABA biosynthesis.

Taken together, down-regulation of *Oshox22* expression by T-DNA insertion led to plants with reduced levels of ABA and enhanced tolerance towards drought and salt stresses, while over-expression of the gene increased ABA content and decreased drought and salt tolerances. These results support the conclusion that *Oshox22* acts as a negative regulator in stress responses. Since reporter gene studies in yeast and rice cells suggested that *Oshox22* acts as a transcriptional activator, its function as a negative regulator in stress responses might be explained via activation of other repressors. The fact that *oshox22-1* plants showed no significant reduction in yield makes it a potential candidate for improving stress tolerance in rice. Further research is needed to identify allelic variation related to altered levels of *Oshox22* expression for rice breeding.

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