



OsERF101, an ERF family transcription factor, regulates drought stress response in reproductive tissues

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Abstract

Key message An ERF transcription factor *OsERF101* is predominantly expressed in rice reproductive tissues and plays an important role in improving rice seed setting rate under drought stress.

Abstract Drought reduces grain yield due to the cumulative damage effects to plant vegetative and reproductive developmental processes. However, the genes involved in these processes are still not completely understood. In this study, we identified a gene named *OsERF101* as an important positive regulator in the adaptive responses to dehydration stress during the reproductive and vegetative stages. This gene encodes a member of APETALA2/Ethylene-Responsive Element Binding Protein (AP2/EREBP) family. *OsERF101* was predominantly expressed in flowers, particularly in the tapetum and microspores under normal growth conditions. It was induced by drought, PEG6000 and abscisic acid (ABA) in leaves. During the vegetative stage, *OsERF101*-overexpression plants were more resistant to osmotic stress caused by PEG6000 compared to the control plants. They also had higher survival and seed setting rates than wild type when subjected to reproductive-stage drought stress. Further physiological analysis revealed that the pollen fertility was improved in the overexpression lines, while the knockout mutant and RNAi lines showed reduced pollen fertility and compromised drought tolerance during the reproductive stage. The increased proline content and peroxidase activity in *OsERF101*-overexpression plants might contribute to the improved drought-tolerance of plants. In addition, *OsERF101*-overexpression plants displayed ABA susceptible phenotype, in which the expression levels of ABA-responsive genes *RD22*, *LEA3*, and *PODs* were up-regulated. Taken together, our results indicate that *OsERF101* is a gene that regulates dehydration responses during the vegetative and reproductive stages.

Keywords *Oryza sativa* · *OsERF101* · Drought · Reproductive development

Accession numbers Sequence data of genes reported in this paper can be found in RAP-DB (<http://rapdb.dna.affrc.go.jp/>) in the following numbers: *OsERF101* and *OsRAP2.6* (Os04g0398000), *ABA8OX3* (Os09g0457100), *ABA2* (Os04g0448900), *GAMYB* (Os01g0812000), *DREB2* (Os01g0165000), *LEA3* (Os05g0542500), *ERF34* (Os04g0550200), *POD1* (Os03g0369000), *POD2* (Os01g0326300), *RD22* (Os01g0733500), *OsP5CS1* (Os05g0455500), *OsP5CDH* (Os05g0536400), *OsOAT* (Os03g643300), and in TAIR (<http://www.arabidopsis.org>): *AtRAP2.6* (At1g43160) and *AtRAP2.6L* (At5g13330).

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Introduction

Drought limits plant growth, development and productivity (Bartels and Sunkar 2005). Due to the frequent occurrence of dry spell, it is important to develop crop cultivars that have better performance under drought conditions for maintaining the annual crop yield. To date, many genes have been identified to regulate water stress responses at the vegetative stage in rice and other cereals. However, whether they are also capable of improving the reproductive development and stabilizing grain yield under drought stress are often unknown. In our studies, we aim to find genes that can improve drought tolerance in terms of improving seed setting rate, and use these genes for developing drought-tolerant rice varieties.

Drought signaling pathways are usually classified into ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki 2007; Shinozaki et al.

2003), within which transcription factors play important roles in controlling gene expression. For example, DREB and NAC transcription factors regulate gene expression in ABA-independent pathway, while AREB, MYC and MYB function in ABA-dependent pathway (Shinozaki and Yamaguchi-Shinozaki 2007; Shinozaki et al. 2003). These transcription factors coordinate to regulate plant adaptation to various stresses, and disruption of them often cause altered stress response phenotypes. Their downstream target genes include a large array of drought-responsive genes, such as *Responsive to Dehydration 22 (RD22)* (Iwasaki et al. 1995; Yamaguchi-Shinozaki and Shinozaki 1993), *Late Embryogenesis Abundant 3 (LEA3)* (Hundertmark and Hinch 2008; Xiao et al. 2007), *Stress-Induced type 2c Protein Phosphatase (SIPP2C1)* (Li et al. 2013b), *Drought-hypersensitive Mutant 2 (DSM2)* (Du et al. 2010) and *H-type Thioredoxins (OsTRXh1)* (Zhang et al. 2011) in ABA-dependent pathway, and *Early Responsive to Dehydration1 (ERD1)* and *Responsive to Dehydration 29A (RD29A)* (Kiyosue et al. 1993; Yamaguchi-Shinozaki and Shinozaki 1994) in ABA-independent pathway. These genes participate in regulating different physiological processes of drought response. For example, *OsTRXh1* is involved in maintaining the balance between reactive oxygen species (ROS) production and scavenging (Du et al. 2010; Zhang et al. 2011). Overexpression of *OsTRXh1* enhances abiotic stress tolerance by eliminating the excessive ROS (Mittler 2002).

Among the stress-related transcription factors, AP2/EREBP (*APETALA2/Ethylene Responsive Element Binding Protein*) genes constitute a large super-family in plants. They are classified into four subfamilies based on the number of AP2/ERF domains and the phylogenetic relationships: AP2, RAV (related to ABI3/VP1), dehydration-responsive element-binding protein (DREB) and ethylene-responsive factor (ERF) (Sakuma et al. 2002; Sharoni et al. 2011). Among them, DREB and ERF subfamilies contain a single AP2/ERF domain that can bind to DNA cis-regulatory elements directly (Nakano et al. 2006). Members from these two subfamilies are shown to be involved in diverse abiotic and biotic stress responses. ERF subfamily mainly regulate the expression of biotic stress-related genes through interaction with GCC box present in their promoters (Chakravarthy et al. 2003; Ohme-Takagi and Shinshi 1995; Wang et al. 2004), while the DREB subfamily modulate the expression of abiotic stress responsive genes by binding to C-repeat or dehydration response element (DRE) (Chen et al. 2008; Gilmour et al. 1998; Stockinger et al. 1997). In the last few years, the involvement of ERF subfamily in abiotic stress responses has also been reported. For example, tobacco stress-induced gene 1 (Tsi1) and tomato stress-responsive factor 1 (TSRF1), can bind to both GCC box and DRE sequences and promote salt or drought tolerance in transgenic plants (Park et al. 2001; Quan et al. 2010; Zhang

et al. 2004). Rice ERF subfamily protein, submergence 1A (SUB1A), is important for plant survival from rapid dehydration following de-submergence, in addition to submergence and drought tolerance (Fukao et al. 2011). Another two ERF subfamily proteins OsERF922 and OsDERF1, negatively modulate rice salt and drought tolerance, respectively at the vegetative stage (Liu et al. 2012a; Wan et al. 2011).

There are 163 AP2/EREBP family genes in rice and 77 of them belong to ERF subfamily (Sharoni et al. 2011). Few of them have been characterized. In our previous studies, we analyzed the transcriptomic changes of rice flowers in response to drought to seek candidate genes that can promote the drought tolerance of reproductive tissues (Jin et al. 2013). Among the identified genes, *OsERF101* gene was found to be induced by drought and expressed predominantly in flowers. Here we report the functional characterization of *OsERF101* in rice. The results revealed that this gene plays an important role in regulating rice drought tolerance during both the vegetative and reproductive stages.

Materials and methods

Plant materials and growth conditions

Oryza sativa L. cv. Nipponbare (background for overexpression and RNAi lines) or *O. sativa* L. cv. Dongjin (background for T-DNA insertion mutant) were used for study in this work. Rice seeds were sterilized by 3% H₂O₂ for half an hour and then imbibed at 37 °C for 2 days in the dark. After imbibition, the germinating seeds were grown on floating plates in Yoshida nutrition solution (Yoshida et al. 1976) or grown in soil pots at 28 °C under a photoperiod of 12-h light/12-h dark cycle with the light intensity of approximately 375 μmol m⁻² s⁻¹ and the humidity around 50%.

Transgenic line construction and identification

In our previous studies on floral transcriptomic changes under drought stress, different sizes of rice florets (2–3, 3–4, 4–5 and 5–7 mm) were harvested to perform microarray analysis. The *OsERF101* gene showed strong induced expression by drought stress in flowers. The full-length coding sequence of *OsERF101* was amplified by reverse transcription (RT)-PCR using the RNA isolated from the flowers of drought-stressed rice plants. The *OsERF101* overexpression construct was generated by inserting the open reading frame of *OsERF101* into the pCAMBIA1301U vector, driven by the maize *Ubiquitin* gene promoter. The RNAi construct was generated by cloning an *OsERF101* sequence-specific fragment (371 bp) into the pGEM-RNAi vector in inverted directions, then integrating the hairpin fragment into pCAMBIA1301U vector. The GUS reporter

plasmid was constructed by fusing the *OsERF101* promoter fragment (3080 bp upstream of the start codon) with the β -glucuronidase (*GUS*) reporter gene in the pCAMBIA1301 vector with ClonExpress™ One Step Cloning Kit (Vazyme Biotech, China).

The *oserf101* knockout mutant and its parent wild type seeds (Dongjin) were ordered from Rice T-DNA Insertion Sequence Database (http://cbi.khu.ac.kr/RISD_DB.html) (Jeon et al. 2000; Jeong et al. 2006). The mutant was verified by genomic DNA PCR and RT-PCR.

All primers used for vector construction and mutant identification are listed in the Supplementary Table 1. All constructs were introduced into rice by *Agrobacterium*-mediated transformation method (Ozawa 2009).

Phylogenetic analysis

Similar sequences (E-value = 10^{-4}) of OsERF101 were searched through BLAST in the phytozome database (<http://www.phytozome.net/>), and the results were inspected manually. Sequences were aligned with MUSCLE (MEGA 5.0), followed by manual alignment. Phylogenetic tree was constructed using an alignment of full-length protein sequences in neighbor-joining algorithm with MEGA 5.0. To test inferred phylogeny, we used bootstraps with 1,000 bootstrap replicates (Tamura et al. 2011).

PCR and quantitative RT-PCR (qRT-PCR) analyses

Genomic DNA was extracted based on the previously described method (Walbot 1988). The *HPT* (*Hygromycin Phosphotransferase*) gene was amplified for identifying the positive transgenic plants. Total RNA were isolated from flowers or leaves with TRIzol Reagent (TaKaRa, Japan), and then reverse-transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan). Gene-specific primers were designed using Primer Express 3.0 or Primer Premier 5.0 software. The rice *Ubiquitin* gene was used as an internal control. For analyzing the expression of *OsERF101* and other marker genes in plants, qRT-PCR was performed with PrimeScript™ RT Master Mix (TaKaRa, Japan) using the CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). Thermal cycling conditions were: 95 °C 1 min, 40 cycles of 95 °C 10 s, 60 °C 30 s, followed by dissociation curve analysis. Relative expression was normalized to the reference gene *Ubiquitin*. Mean and standard deviation values were calculated from three biological replicates.

GUS staining and RNA in situ hybridization

GUS staining was used to investigate the *OsERF101* expression in T₂ generation of *OsERF101* promoter-GUS transgenic rice based on the methods described before (Jefferson

et al. 1987). After staining and clearing, tissues were photographed using the stereo microscope S8APO (Leica).

For RNA in situ hybridization, wild-type flowers at various developmental stages were fixed in Histochoice MB Tissue Fixative reagent (Amresco, USA) for 16 h. After dehydration through an ethanol series and clearing through Histo-clear (Amresco, USA), tissues were embedded in Paraplast Plus (Sigma, USA) and sectioned at 3–6 mm thickness using an RM2245 Microtome (Leica Microsystems, Germany). For generating the RNA hybridization probe, a 389 bp *OsERF101*-specific cDNA fragment was amplified and cloned into pGEM-T vector (Promega, USA). The RNA probe was then transcribed in vitro using T7 or SP6 RNA polymerases provided in the DIG RNA labeling kit (Roche, Switzerland). RNA hybridization and immunological detection were performed following the protocol described previously (Yong et al. 2003). Tissues were photographed using a transmitted light microscopy (Carl Zeiss, Germany).

Trans-activation activity assay of OsERF101

The open reading frame of *OsERF101* was cloned into the yeast vector pDEST-GBKT7, which was then transformed into AH109 host strain based on the described method (Gietz and Woods 2002). Yeasts were plated onto SD/-Trp dropout media for selection of the positive transformants. The positive colonies were then spotted onto SD/-Trp media with X- α -Gal for the reporter protein MEL1 activity assay. The blue colonies grown on SD/-Trp media indicated that OsERF101 activates the *GAL4* reporter gene expression.

Abiotic stress treatments during vegetative and reproductive stages

The homozygous T3 generation of transgenic rice plants were used for phenotypic observation. For studying transcriptional responses to abiotic stresses, rice seedlings were grown in 1/8 Yoshida nutrition solution (Yoshida et al. 1976) for 14 days in a growth chamber under standard growth conditions described above, then NaCl, ABA or PEG6000 was added into the culture solution at a final concentration of 200 mM, 50 μ M and 20%, respectively. Rice leaves were sampled at 0 h, 1 h, 3 h 7.5 h and 24 h after each treatment. *OsERF101* expression level was then analyzed by qRT-PCR. For osmotic stress at the vegetative stage, rice seedlings were grown in 1/8 Yoshida nutrition media until five leaf-stage, then treated by 20% PEG6000 in the media. Three days later, the seedlings were analyzed and photographed.

For drought stress treatment at the reproductive stage, wild-type, overexpression, RNAi and knockout mutant were grown in soil pots in a greenhouse under standard growth conditions. To minimize experimental errors, each pot was filled with the same amount of soil and supplied with the

same volume of water. After grown for about 2 months, rice plants started to switch into reproductive stage marked by the appearance of inflorescence primordia. Water was then drained off the pot until the soil water content dropped to 25%, and this level was maintained as described previously (Guo et al. 2016; Jin et al. 2013; Yao et al. 2018) for 1 week for Nipponbare genetic background plants, and for 10 days for Dongjin genetic background plants, due to its stronger drought tolerance. Control plants were irrigated normally all the time. After drought stress, watering was recovered till grain ripening. At least 40 plants for each line were analyzed, and the experiments were repeated for three times.

Physiological measurements of rice plants

After drought stress at the reproductive stage, the flag leaves were harvested for relative water content, chlorophyll content, proline content, superoxide dismutase (SOD) and peroxidase (POD) activity determination. Leaf relative water content as a percentage of (fresh weight – dry weight)/fresh weight was calculated (Kahn et al. 1993). Chlorophyll was extracted with 80% acetone; absorption of the extracts at 646.6 nm and 663.6 nm were measured and the total chlorophyll contents were calculated according to the following equation: $Chl_{total} [\mu g ml^{-1}] = 17.76 \times A_{646.6} + 7.34 \times A_{663.6}$ (Porra et al. 1989). Proline content was determined following the sulphosalicylic acid method (Troll and Lindsley 1955). SOD activity was measured based on its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride (Beauchamp and Fridovich 1971), and POD activity was decided by its H_2O_2 -dependent oxidation activity for benzidine (Giannopolitis and Ries 1977). Rice pollen was stained by Alexander red to show the viability (Peterson et al. 2010). In addition, the survival rate and 1000-grain weight of the stressed plants were also surveyed for evaluating their drought tolerance trait.

Statistical analysis

All experiments were repeated three times unless otherwise stated. Significance of difference between samples and controls was performed using Student's t-test.

Results

OsERF101 has trans-activation activity

Our previous comparative transcriptome analysis with rice flowers from normal and drought conditions has identified the gene of Os04g0398000 as a drought-inducible gene in flowers (Jin et al. 2013). Sequence analysis showed that this gene encodes an AP2/ERF transcription factor and

was designated as *OsERF101* in a genome-wide analysis of rice ERF family proteins (Nakano et al. 2006). A previous study has reported its function in regulating rice innate immunity and named it as *OsRAP2.6* according to the sequence homology of its AP2/ERF domain to Arabidopsis *RAP2.6* (Wamaitha et al. 2012). However, from the sequence analysis results, this gene has the highest similarity to *AtRAP2.6L* (*RAP2.6*-Like gene) (*At5g13330*) (identity 38.4%) instead of *AtRAP2.6* (*At1g43160*) (identity 30.6%) in the full-length of protein sequence (Supplementary Fig. S1). We constructed the phylogenetic tree of OsERF101 homologous proteins using the DREB subfamily as the out-group for rooting (Nakano et al. 2006). The phylogenetic analysis also showed that OsERF101 has closer relationship with *AtRAP2.6L* than *AtRAP2.6*. ERF family members from eudicots and monocots grouped respectively, indicating that the functional diversification within this branch predated the eudicot/monocot divergence (Fig. 1, Supplementary Fig. S1).

It has been reported that *OsRAP2.6* is located in both nucleus and cytoplasm (Wamaitha et al. 2012). Our subcellular localization results using tobacco leaves also confirmed this result (Supplementary Fig. S2). So far, it is not known whether OsERF101 has transcription activation activity. To test this, we cloned *OsERF101* into pDEST-GBKT7 vector, and transformed it into the yeast cell. Yeast cells carrying BD empty vector failed to turn blue on SD-/Trp supplemented with the chromogenic substrate X- α -Gal, while those containing BD-*OsERF101* vector can activate the expression of reporter gene *MEL1* and turned blue (Fig. 2), indicating that OsERF101 has transcriptional activation activity.

OsERF101 was preferentially expressed in the tapetum and microspores in flowers and induced by abiotic stress

To study the tissue-specific distribution of *OsERF101* in rice, we used p*OsERF101*: *GUS* reporter lines to observe the *GUS* activity driven by *OsERF101* promoter (Fig. 3a). *GUS* signal was not detected in root, callus, young leaf (leaves at five-leaf stage) and old leaf (leaves at three-tiller stage), but was slightly induced in young leaf and strongly induced in old leaf, especially after drought stress (Fig. 3a). *GUS* activity was strong in reproductive tissues including inflorescence primordia, lemma/palea of young flowers, anther and pistil.

To further examine the *OsERF101* expression pattern in anther, we performed RNA in situ hybridization using the wild-type floral sections. The *OsERF101* transcript was detected in the tapetum and microspores, especially at anther developmental stage 4, 5 and 8 (Fig. 3b). From the stage 10 on, the *OsERF101* expression signal became weaker. Taken together, *OsERF101* was expressed in developing

Fig. 1 Phylogenetic analysis of the ERF subfamily members in various species. Bootstrap values were indicated on each branch point of the tree. Root was placed using an outgroup of the closest subfamily, DREB protein. Gm: *Glycine max*, Pt: *Populus trichocarpa*, At: *Arabidopsis thaliana*, Zm: *Zea mays*, Si: *Setaria italica*, Os: *Oryza sativa*, Bd: *Brachypodium distachyon*, Sb: *Sorghum bicolor*. Black diamonds show proteins from *Arabidopsis*; black triangles show proteins from rice

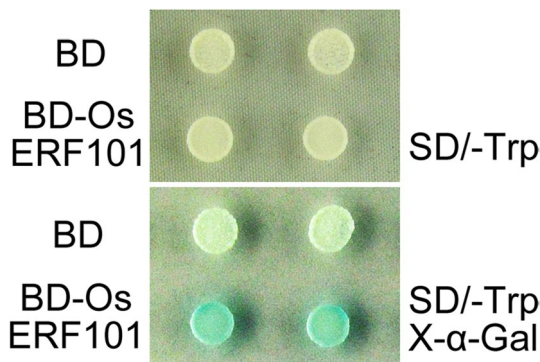
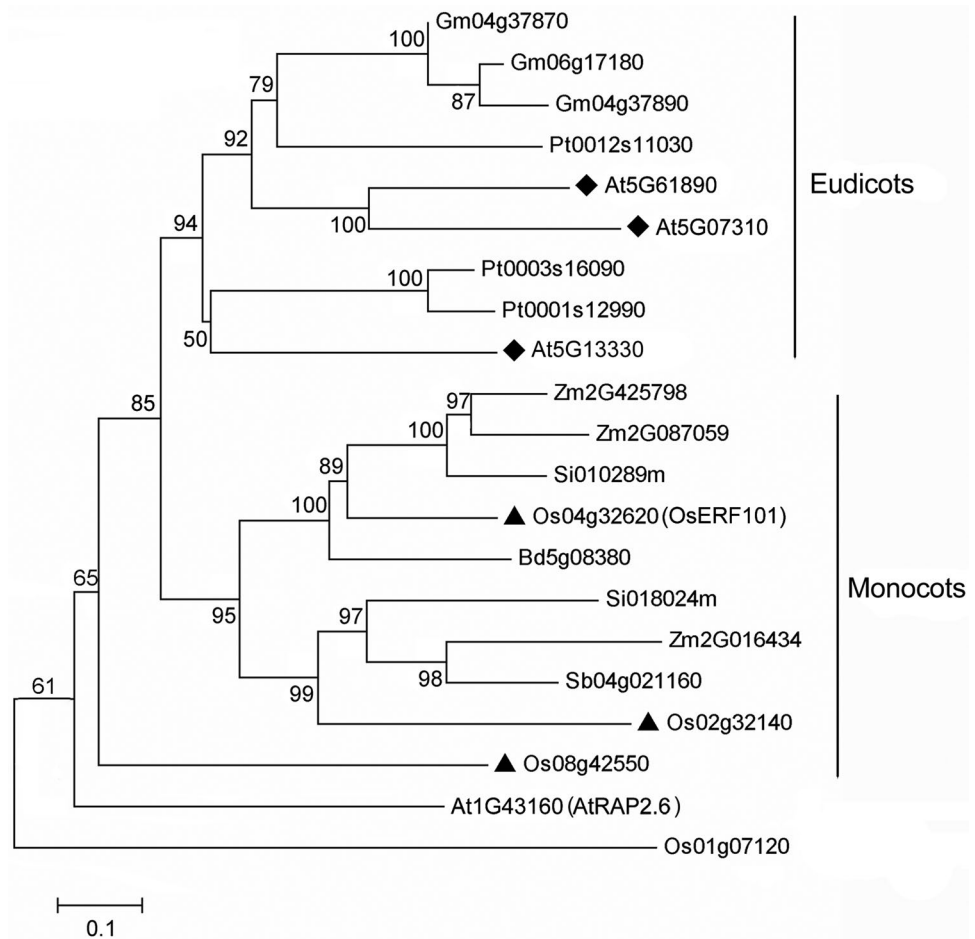


Fig. 2 OsERF101 had transcriptional activation activity. Yeast transformed with the BD empty vector was used as control. The blue colonies indicated the GAL4-dependent activation of the reporter gene *MEL1*

microspores and tapetum, which suggests that it might play a role in the anther development.

qRT-PCR method was employed to examine the transcriptional responsiveness of *OsERF101* to abiotic stresses. The result showed that *OsERF101* was induced by 20% PEG6000 and ABA, but not by NaCl (Fig. 3c), indicating

that *OsERF101* is an osmotic stress- and ABA-responsive gene.

Overexpression of *OsERF101* gene in rice plants enhanced dehydration tolerance at both vegetative and reproductive stages

To study *OsERF101* function, we generated the overexpression and RNA interference (RNAi) transgenic plants of *OsERF101* (Supplementary Fig. S3a). Meanwhile, we also ordered the *osertif101* T-DNA insertion mutant from the seed stock at Kyung Hee University (RISD). PCR results showed that the T-DNA fragment was inserted in the second exon of *OsERF101* and the genotypic identification showed that *osertif101* is a null mutant (Supplementary Fig. S3b). Based on the RT-PCR and qRT-PCR results, representative overexpression lines and RNAi lines (Supplementary Fig. S3c, d) were chosen for further assessment for their tolerance to drought. Among them, OX6 has nearly the same *OsERF101* expression level as the wild type and was used as a vector control (VC) (Supplementary Fig. S3d).

To assess the effects of altered *OsERF101* expression level on dehydration/drought resistance, positive

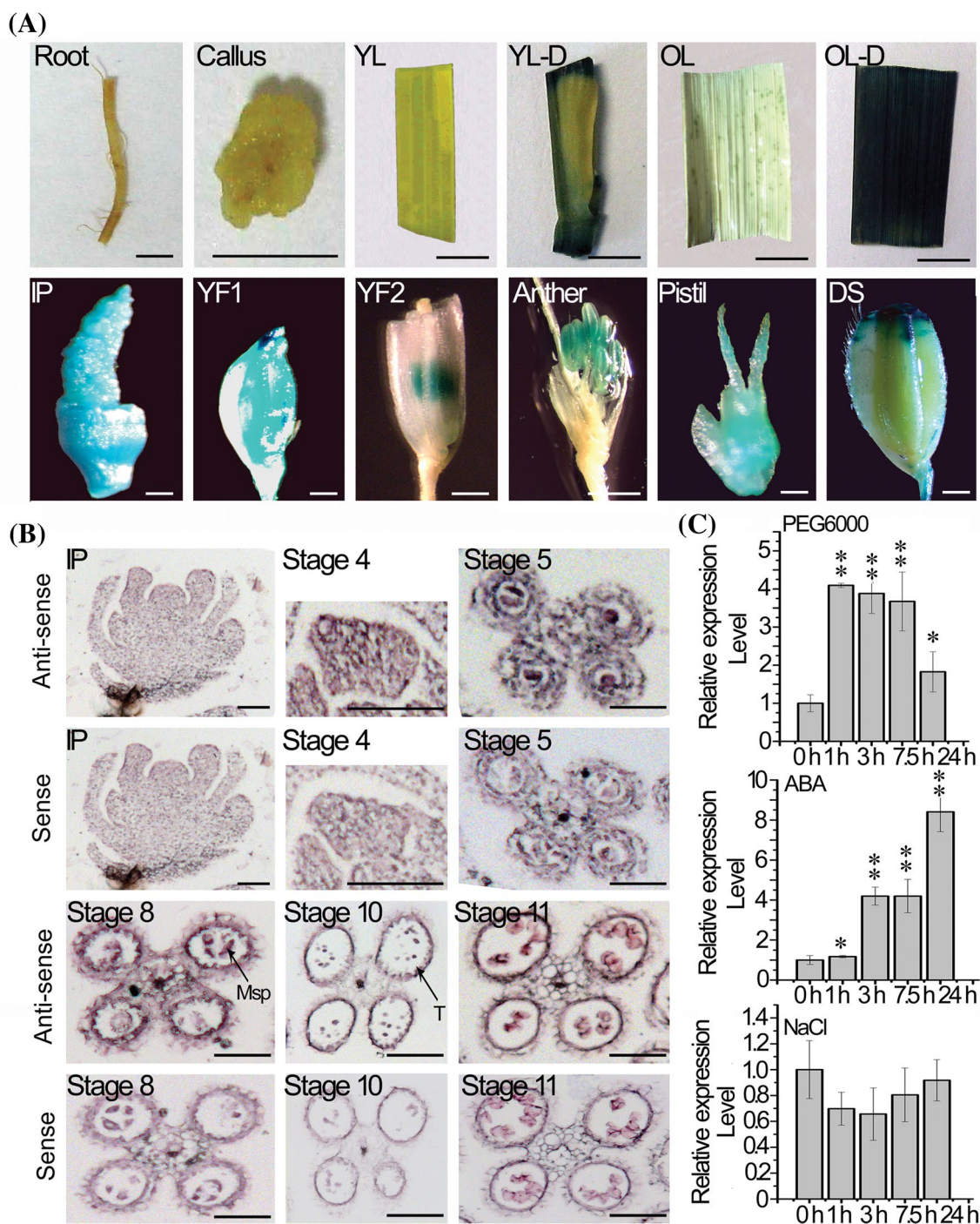


Fig. 3 Expressional studies of *OsERF101* in rice. **a** GUS staining pattern of the *pOsERF101::GUS* transgenic lines grown under normal growth conditions. *YL* young leaf at five-leaf stage, *YL-D* drought stressed young leaf at five-leaf stage, *OL* old leaf at three-tiller stage, *OL-D* drought stressed old leaf at three-tiller stage, *IP* inflorescence primordia, *YF1* young flower at stage 4 or 5, *YF2* young flower at stage 7 or 8, *DS* developing seeds on the fifth day post fertilization. Scale bars: 1 cm in root, callus, *YL*, *YL-D*, *OL* and *OL-D*; 0.2 cm in *IP*; 0.5 mm in *YF1* and pistil; 1 mm in *YF2*, anther and *DS*. **b** RNA in situ hybridization analyses of the *OsERF101* expression in

the anthers under the normal conditions. *IP* inflorescence primordia; brown signal indicates the expression of *OsERF101*. Sense probe was used as the hybridization control. *T* tapetum, *Msp* microspore. Scale bars: 50 μ m. **c** Time-dependent expression analyses of *OsERF101* by qRT-PCR under different treatments. Two-week old seedlings were treated by PEG6000, ABA or NaCl in the culture media. PEG6000, 20%; ABA, 50 μ M; NaCl, 200 mM. Values indicated means \pm SD from three biological replicates. Significance of difference between 0 h and other time points was analyzed by Student's t-test, * $P < 0.05$; ** $P < 0.01$

transgenic plants were grown in 1/8 Yoshida nutrition solution till five-leaf stage, then treated by 20% PEG6000. Three days after treatment, most of the leaves in control plants rolled severely while those of the *OsERF101*-overexpression lines only curled mildly (Fig. 4b, d). We also imposed drought stress on the plants grown in soil pots upon the onset of reproductive growth. The overexpression plants displayed stronger drought-resistance phenotype than control plants (Fig. 4f, h), while no difference between them was observed under normal growth conditions (Fig. 4a, c, e, g). After recovery for 3d, only 38.3% of the control plants survived, whereas up to 98.2% of the *OsERF101*-overexpression plants survived (Fig. 4i). The transgenic plants with higher *OsERF101* expression level displayed a higher survival rate. In addition, the leaves of *OsERF101*-overexpression plants also lost water less quickly than WT control under drought stress (Fig. 4j). Photosynthetic capacity can be used as a reliable indicator to evaluate the energetic/metabolic imbalance of photosynthesis and yield performance under water deficit condition (Araus et al. 1998; Guo et al. 2008). Therefore, we examined the chlorophyll contents of the plants under normal and drought conditions, respectively. While drought stress reduced the chlorophyll contents of all plants, the chlorophyll content of plants with the highest level of *OsERF101* (OX9) was still higher than that of the control plants (Fig. 4k). These results suggested that higher *OsERF101* expression level could enhance plant tolerance to drought stress.

Proline accumulation is protective for plants to cope with water stress (Zhou et al. 2009). We measured the proline content in wild type and transgenic plants before and after drought stress. The proline content of *OsERF101*-overexpression lines ($62.5\text{--}65.6\ \mu\text{g g}^{-1}$) were higher than that of control plants ($47.7\ \mu\text{g g}^{-1}$) after drought stress (Fig. 4l). No consistent difference of proline content was detected among transgenic and control plants before drought stress (Fig. 4l). The results suggested that overexpression of *OsERF101* enhanced the accumulation of free proline in rice to improve drought tolerance. Drought stress also activates the generation of reactive oxygen species (ROS), which causes multiple levels of damages to plants (Xia et al. 2009). We therefore examined the ROS-scavenging activities in both wild-type and transgenic plants. The results showed that under drought stress, overexpression of *OsERF101* led to increased POD activity ($207.4\text{--}216.5\ \text{U g}^{-1}\ \text{min}^{-1}\ \text{FW}$) compared to wild-type plants ($173.6\ \text{U g}^{-1}\ \text{min}^{-1}\ \text{FW}$) (Fig. 4m), whereas no obvious difference was observed for SOD activity between transgenic and control plants under both normal and drought conditions (Fig. 4n). These results suggested that the increased drought tolerance of *OsERF101*-overexpression plants is at least partially due to increased proline accumulation and ROS clearing activities in plants.

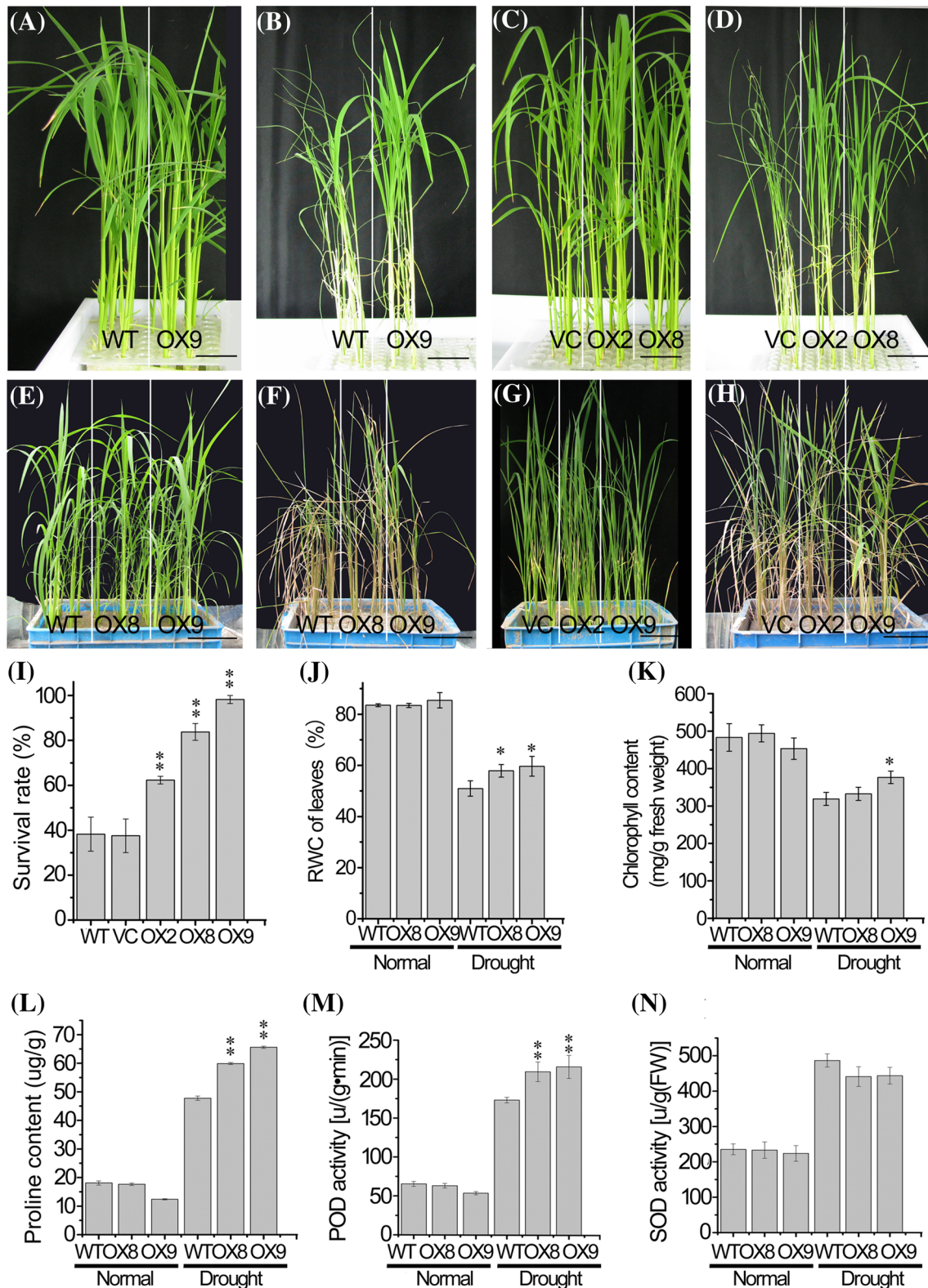
Disruption of *OsERF101* in rice reduced drought tolerance during the reproductive stage

In addition to the overexpression lines, we also observed the phenotype of the *OsERF101*-RNAi plants and the *oserf101* mutant (KO) under osmotic stress (Supplementary Fig. S4). The RNAi plants and KO mutant showed no growth phenotype distinct from the wild-type plants under normal conditions (Supplementary Fig. S4 a, c) and 20% PEG6000 treatment (Supplementary Fig. S4 b, d). However, when treated by drought at reproductive stage, *OsERF101*-RNAi lines showed more sensitive phenotype to drought stress than the control plants (Fig. 5a). Meanwhile, the KO mutant was also more susceptible to drought than its wild type control Dongjin (DJ) (Fig. 5a). Since DJ genetic background plants had stronger drought tolerance than Nipponbare plants, DJ plants (DJ wild type and KO mutant) were stressed three more days than Nipponbare rice (Nipponbare wild type and RNAi lines). About 32.3–35.7% of *OsERF101*-RNAi transgenic plants were recovered, compared with 58.1% of the wild type control plants after drought stress (Fig. 5b), and the KO mutant also had a survival rate at approximately 60% of the DJ control plants. The leaves of *OsERF101* RNAi lines and KO mutant also lost water more quickly than their wild type siblings under drought (Fig. 5c). The chlorophyll content of RNAi and knockout plants were not significantly different from that of the control plants, although the drought stress seriously reduced the chlorophyll content of all plants (Fig. 5d).

We further examined the proline content, POD and SOD activities in the wild type, RNAi and KO plants during the reproductive stage. Results showed that after water stress, the proline content, POD and SOD activities were all reduced in the RNAi plants as well as in the KO mutant (Fig. 5e–g). However, before drought stress, most of them had no significant differences except POD. The POD activity in the *oserf101* mutant was lower than that of wild-type plants even without stress, suggesting a possible ecotype influence on the gene function.

Overexpression of *OsERF101* improved pollen fertility and seed setting rate under drought stress

OsERF101 is drought-responsive and mainly expressed in flowers (Fig. 3), suggesting that it could be involved in drought-adaptive response in reproductive tissues. Yield stability is an important crop trait for agricultural production and largely determined by the reproductive growth conditions of plants. Therefore, we analyzed the effects of *OsERF101* expression on pollen development and gain yield under drought stress. The *OsERF101*-overexpression lines had significantly more viable pollen grains under drought stress (73.5%) than the control plants (62.3%) and



OsERF101-RNAi lines (41.5%) (Fig. 6a, b). Meanwhile, the *ERF101* overexpression plants also had higher seed setting rate (62.6%) (Fig. 6c). In the RNAi plants, the viable pollen grains are less than wild type both before and after drought

stress, indicating that disruption of *OsERF101* may affect pollen development. No significant difference in 1000-kernal weight was observed between transgenic plants and control plants, although the 1000-kernal weight of all plants

Fig. 4 Phenotype of *OsERF101* overexpression plants at the vegetative and reproductive stages under osmotic and drought stresses. **a–d** Phenotype of the *OsERF101* overexpression seedlings under osmotic stress caused by 20% PEG6000. **a, c** Plants before stress; **b, d** plants after stress. A transgenic line with *OsERF101* expression level similar to wild type was used as a vector control (VC). OX2, 8, 9, overexpression lines. Scale bars, 2 cm. **e–h** Phenotype of the *OsERF101* overexpression plants at the reproductive stage under drought stress. **e, g** Before drought treatment; **f, h** after drought treatment. Scale bars, 10 cm. **i** The survival rates of different plants under drought stress at the reproductive stage. **j** The relative water contents (RWC) of leaves at the reproductive stage. **k** The chlorophyll contents of leaves at the reproductive stage. **l** The proline contents of leaves at the reproductive stage. **m** The POD activities of leaves at the reproductive stage. **n** The SOD activities of leaves at the reproductive stage. Drought stress experiments were repeated three times with similar results. Values were shown as means \pm SD ($n \geq 40$) in **i–n**. Significance of difference between WT control and the transgenic lines under the same conditions was analyzed by Student's *t*-test, * $P \leq 0.05$, ** $P \leq 0.01$

decreased after drought stress (Fig. 6d). These results indicated that *OsERF101* can improve pollen fertility and thus enhance seed setting rate under drought stress.

Expression of ABA-responsive genes was increased in overexpression lines

To seek the molecular cues underlying the phenotype of *OsERF101* overexpression and mutant plants, we compared the expression levels of stress-responsive genes in flowers from the overexpression, KO mutant and the wild-type control plants, under normal and drought conditions, respectively. The results showed that many drought-responsive genes such as *ABA8OX3*, *ABA2*, *DREB2*, *LEA3*, *P5CS1*, *OAT* and *PODs* genes were strikingly up-regulated in the overexpression lines (OX8 and OX9) after drought stress, but down-regulated in the KO mutant for most of them. ABA degradation gene *ABA8OX3* and ABA synthesis gene *ABA2* were both up-regulated in the overexpression plants compared to wild type after drought stress (Fig. 7a), suggesting a possible feedback regulation mechanism of ABA on ABA homeostasis. In order to track the time-dependent expression level changes of ABA-responsive genes under osmotic stress, we selected three marker genes, *POD2*, *LEA3* and *RD22*, for further analysis. 20% PEG6000 was used to simulate the drought stress. Results showed that *POD2* expression level increased all the time whereas *LEA3* and *RD22* firstly increased and then dropped under osmotic stress, and for most of the time, these genes were expressed at a higher level in overexpression lines than that in the wild-type plants (Fig. 7b). *OsERF101* probably improves rice drought tolerance by indirectly or directly up-regulating the expressions of these drought-responsive genes. Correspondingly, *LEA3* and *RD22* genes were expressed at relatively lower levels in the knockout mutant than wild type. Since *LEA3* and *RD22* are the typical ABA-responsive genes (Hundertmark

and Hinch 2008; Iwasaki et al. 1995; Xiao et al. 2007; Yamaguchi-Shinozaki and Shinozaki 1993), to further explore the possibility that *OsERF101* enhanced drought tolerance through ABA signaling pathway, we treated the three-leaf-stage rice with 50 μ M ABA. Results showed that *OsERF101* overexpression plants grew more slowly than the control plants (Fig. 7c, d), indicating that overexpression of *OsERF101* confers sensitivity to ABA treatment and *OsERF101* is involved in ABA signaling pathway.

Discussion

Sessile plants have evolved distinct mechanisms to respond and adapt to drought stress. We found that *OsERF101* plays a role in this process. Previous work reported that in *Arabidopsis*, the ERF transcription factor AtRAP2.6 is involved in JA-regulated plant disease resistance (Ali et al. 2013; He et al. 2004). Overexpression of AtRAP2.6 resulted in a dwarf phenotype with extensive secondary branching and small siliques, indicating a development role of AtRAP2.6 as well (Krishnaswamy et al. 2011). *OsERF101* was named as *OsRAP2.6* since it has an AP2/ERF domain with 94% identity to that of AtRAP2.6 and also functions in suppressing blast fungus growth in rice (Wamaitha et al. 2012). However, we found that this gene is more similar to AtRAP2.6L in the full-length sequence and phylogenetic relationship. *Arabidopsis* RAP2.6L is reported to play a role in abiotic stress tolerance such as salt, drought and water logging stress (Krishnaswamy et al. 2011; Liu et al. 2012b), but not in biotic stress response. Our results also showed that rice *OsERF101* was induced by ABA and osmotic stress, and overexpression of *OsERF101* improves plant survival and pollen fertility under the drought stress, supporting a role of this gene in abiotic stress response. Therefore, *OsERF101* might encode an intermediate phylogenetic type of ERF protein in evolution and functions in both biotic and abiotic stress responses.

Drought stress can lead to excessive ROS generation, and cause damages and even cell death in plants (Li et al. 2013a). ROS scavenging enzymes, such as SOD and POD, are important for plants to deal with oxidative stress induced by environmental stresses. Under drought stress, the activity of PODs in *OsERF101* overexpression plants was increased while it was reduced in the RNAi lines and knockout mutant (Figs. 4, 5). Consistent with this, two genes encoding PODs (*POD1* and *POD2*) were up-regulated in the *OsERF101* overexpression lines shown by qRT-PCR. SOD activity displayed no significant changes in *OsERF101* overexpression plants but was slightly decreased in the RNAi and mutant plants (Figs. 4, 5). On the other hand, overproduction of proline in plants may lead to increased tolerance against abiotic stresses through osmotic adjustment or membrane

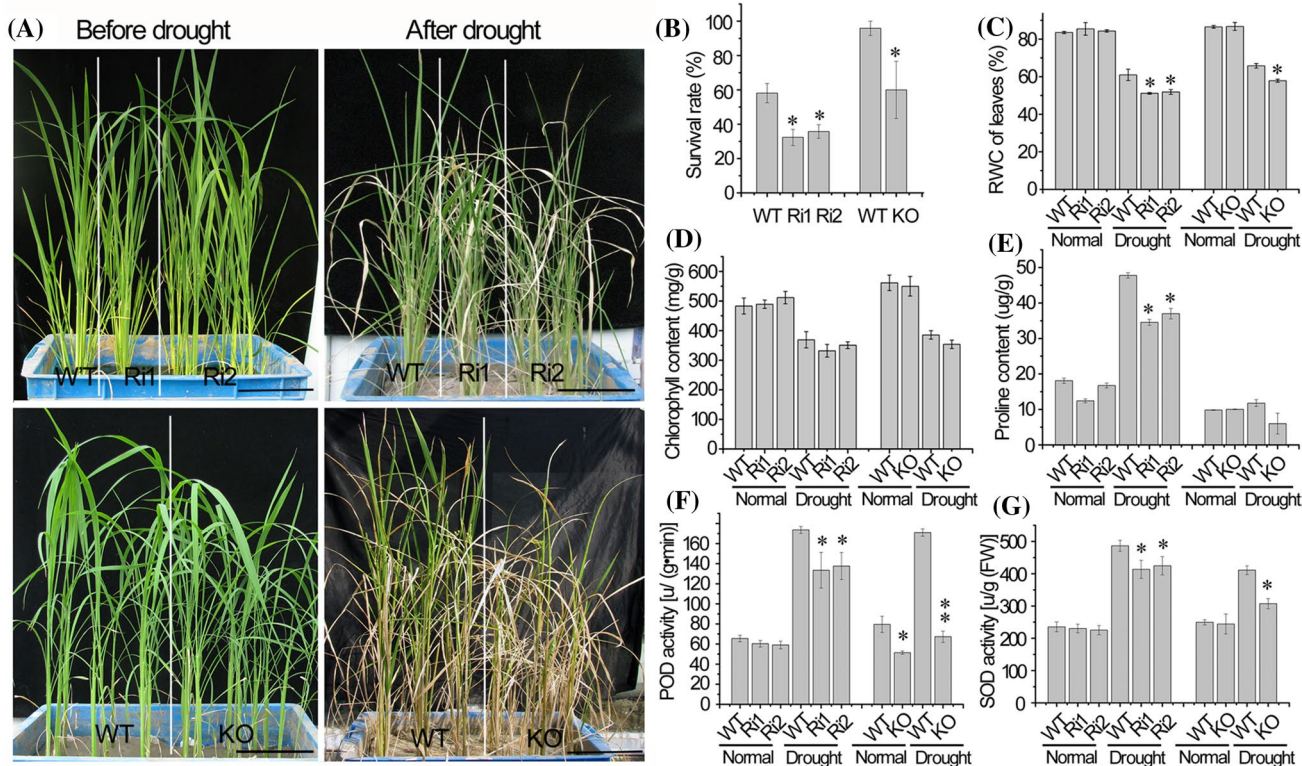


Fig. 5 Disruption of *OsERF101* reduced rice drought tolerance. **a** Phenotype of *OsERF101* RNAi (Ri) lines and the knockout (KO) mutant under normal and drought conditions. Scale bars: 10 cm. **b** The survival rate of different plants under drought stress at the reproductive stage. **c** The relative water contents (RWC) of leaves at the reproductive stage. **d** The chlorophyll content of leaves at the reproductive stage. **e** The proline content of different plants at the reproductive stage. **f** The POD activities of different plants at the reproductive stage. **g** The SOD activities of different plants at the reproductive stage. Drought stress experiments were done in triplicates. Values were shown as means \pm SD ($n \geq 40$) in **b–g**. Significance of difference between WT control and the transgenic lines or the knockout mutant under the same conditions was analyzed by Student's *t*-test, * $P \leq 0.05$, ** $P \leq 0.01$

stabilization (Verbruggen and Hermans 2008; Zhou et al. 2009). We also found that the proline content was increased in the overexpression lines but decreased in the RNAi and mutant plants compared to wild type control. In support of this, the proline biosynthetic genes *OsP5CS1* and *OsOAT* were transcriptionally up-regulated and the catabolic gene *OsP5CDH* was down-regulated in the overexpression lines, and as a contrast, *OsP5CS1* and *OsP5CDH* genes had the opposite changes in KO mutant. Taken together, *OsERF101* could protect plant cells by promoting ROS clearing and osmo-protectant accumulation, and *OsERF101* is a positive regulator of drought response.

Improving grain yield is an important goal for breeding rice elite cultivar, and one of the important processes affecting crop yield is the reproductive process. The stress tolerance properties at this stage largely determine the final output of crop growth. Therefore, seeking for genes which can improve reproductive development under stresses is important for molecular breeding. *OsERF101* is predominantly expressed in flowers under normal growth conditions, and the RNA in situ hybridization results also showed that it

is expressed in tapetum and microspores, indicating a possible role of this gene in male development. This speculation is supported by the evidence that RNA interference and knock out mutation of *OsERF101* reduced the pollen fertility under normal growth conditions. *OsERF101* is induced by drought stress in flowers (Jin et al. 2013) and in leaves, and overexpression of this gene improves the pollen fertility and seed setting rate under drought stress, suggesting a dual role of *OsERF101* in reproductive development and in drought responses. Our results demonstrated that *OsERF101* regulates male development process under drought stress.

ABA regulates the expression level of a large number of genes under abiotic stresses (Manavalan et al. 2012; Seiler et al. 2011; Wilkinson and Davies 2002; Yoshida et al. 2010). In ABA signaling pathway, ABA2 is involved in ABA biosynthesis (Cheng et al. 2002; Gonzalez-Guzman et al. 2002); whereas ABA8OX1 and ABA8OX3 are responsible for ABA degradation (Kushiro et al. 2004). We found that the expression levels of both ABA synthesis and degradation genes were increased in the flowers of *OsERF101*-overexpression lines. It is likely that drought stress increased

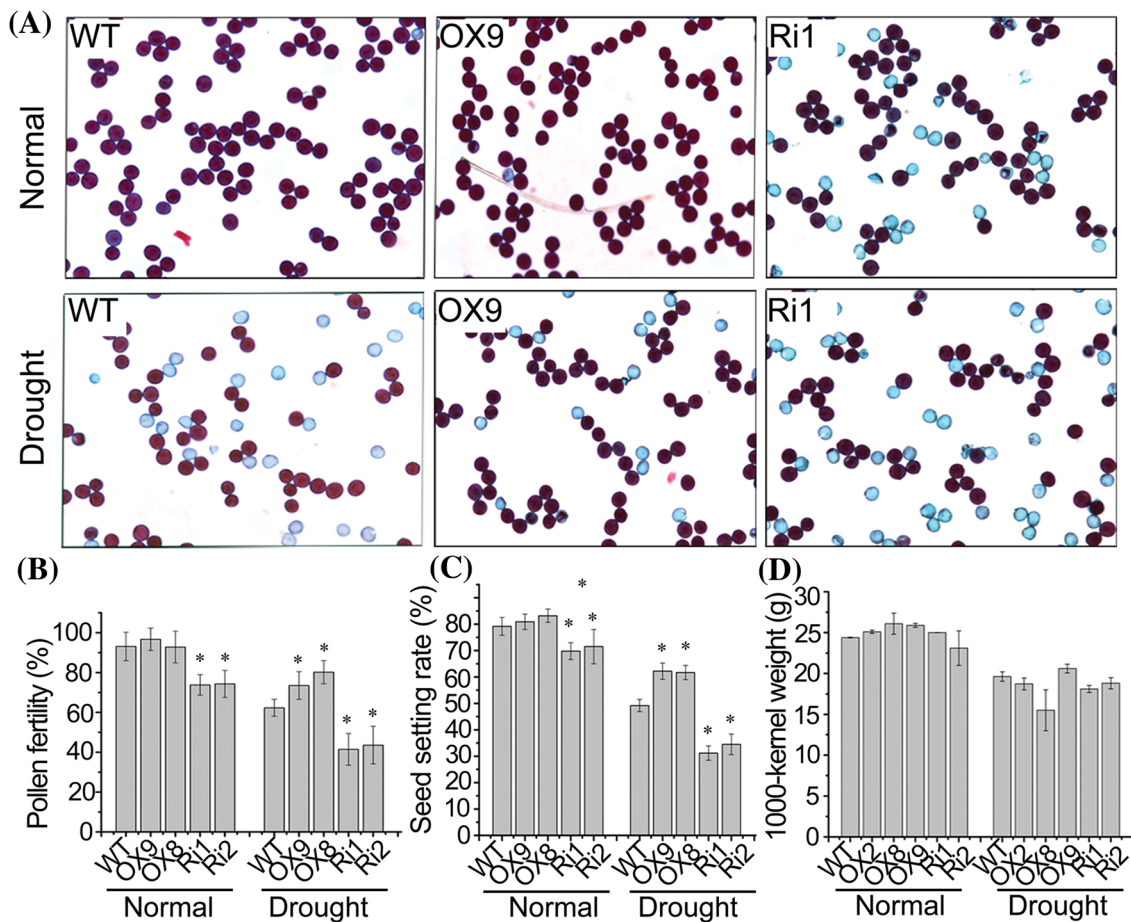


Fig. 6 *OsERF101* enhanced rice reproductive-stage drought tolerance. **a** Alexander red staining showing the fertility of pollen grains. The viable pollen grains were stained as dark purple and inviable blue. **b** Pollen fertilities of different lines under normal and drought conditions. **c** Seed setting rates of different lines under normal and drought conditions. **d** 1000-kernel weights of different lines under

normal and drought conditions. The experiments were done in triplicates and values were shown as means \pm SD ($n \geq 20$) in **b–d**. Significance of difference between WT control and the transgenic lines under the same conditions was analyzed by Student's *t*-test, * $P \leq 0.05$, ** $P \leq 0.01$

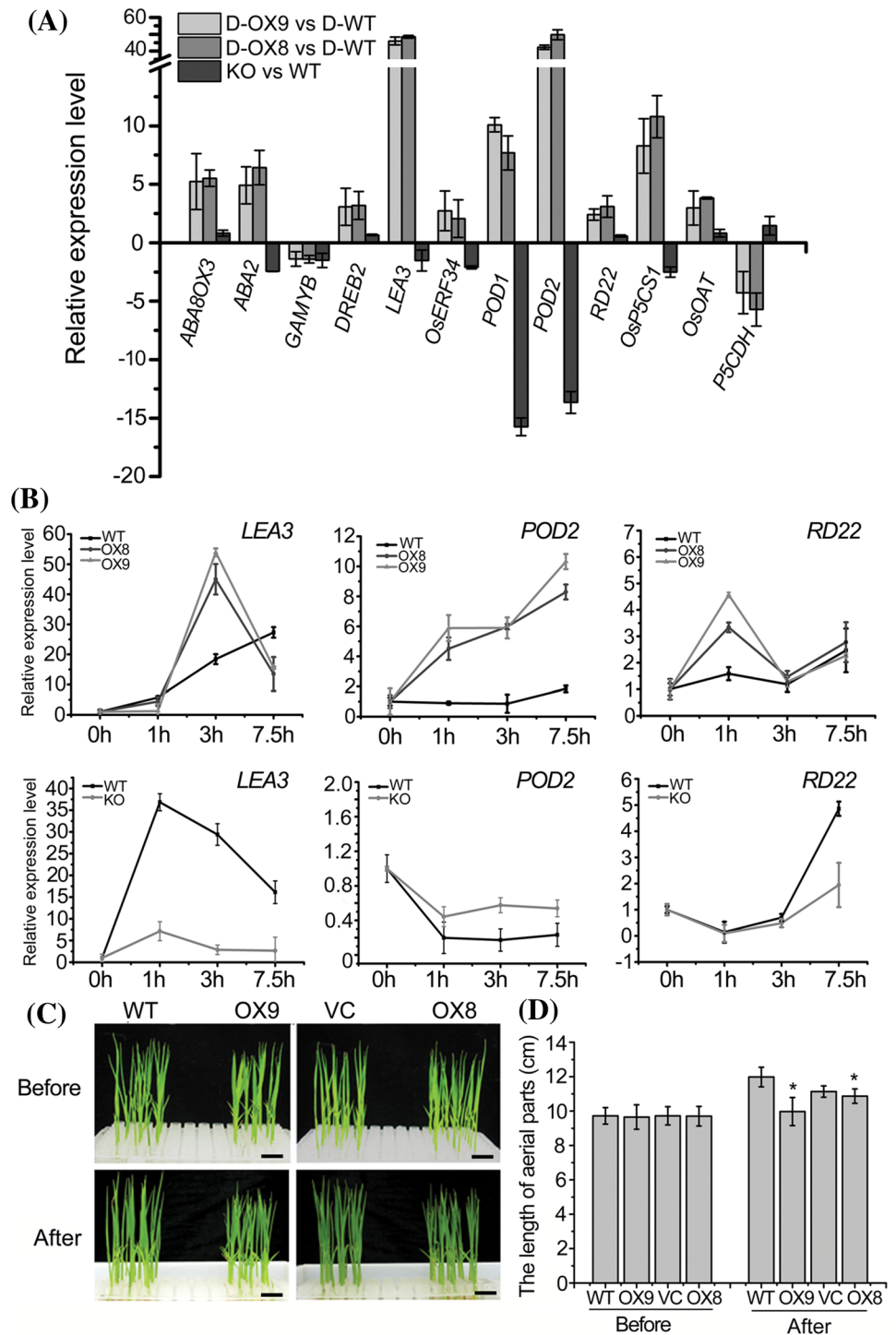
ABA level in the overexpression lines and it conversely activated the expression of ABA degradation genes such as *ABA8OX3* for protecting the anthers from the destructive impact of excess ABA on reproductive development (Ji et al. 2011). The *OsERF101*-overexpression plants also showed ABA-sensitive phenotype (Fig. 7b), suggesting that the ABA metabolism or ABA signaling in these plants were influenced. qRT-PCR results revealed that drought stress induced the expression of five ABA-responsive genes more strongly in the overexpression plants than in wild-type plants (Fig. 7), further supporting a role of *OsERF101* in ABA signaling pathway. Considering that *OsERF101* is an ABA-inducible gene, the function of *OsERF101* in ABA signaling pathway remains further investigation.

Interestingly, *OsERF101*, as a transcription factor, is localized both in cytosol and in nucleus based on our results (Supplementary Fig. S2) and another report (Wamaita et al.

2012). It remains unknown why it is in cytosol. Wamaita et al. proposed that *OsERF101* was phosphorylated by *OsMAPK3/6* in both nucleus and cytoplasm in rice innate immunity (Wamaita et al. 2012). It is worthy of further study whether this modification is related to its localization status. Other dually-localized protein with transcriptional activity have also been reported, such as *Arabidopsis* bZIP28, NAC089 and PIF7 (Leivar et al. 2008; Liu et al. 2007; Yang et al. 2014). There are various mechanisms underlying the translocation behavior of transcription factors. Studying whether *OsERF101* changes its localization under drought stress will provide new insights on its action of mode.

In summary, our results revealed that *OsERF101* positively regulates drought responses. *OsERF101* increases the expression levels of drought-responsive genes for synthesizing proline and for eliminating harmful ROS to

Fig. 7 ABA signaling pathway was affected in the transgenic plants. **a** Expression level changes of stress-responsive genes in the flowers of the overexpression lines and the knockout mutant compared with wild type control. The flowers of stage 5–8 after drought treatment for 1 week were harvested for qRT-PCR. Values indicate means \pm SD from three biological replicates. **b** Time-dependent expression curves of ABA-responsive genes after 20% PEG6000 treatment at the vegetative stage. Leaves from 2-week seedlings were analyzed. Values indicate means \pm SD from three biological replicates. **c** Phenotype of three-leaf-stage rice seedlings treated by 50 μ M ABA for 2 days. **d** Seedling growth measurements after ABA treatment. VC has similar *ERF101* expression level to wild type and was used as a vector control. Values were shown as means \pm SD ($n \geq 40$). Significance of difference between WT or VC control and the transgenic lines under the same conditions was analyzed by Student's *t*-test. * $P \leq 0.05$, ** $P \leq 0.01$



protect vegetative and reproductive tissues against water deficit stress.

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