Journal of Agricultural Science (2017), **155**, 966–977. © Cambridge University Press 2017
doi:10.1017/S0021859617000041

The APETALA2/ethylene-responsive factor transcription factor OsDERF2 negatively modulates drought stress in rice by repressing abscisic acid responsive genes

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(*Received 19 February 2016; revised 8 December 2016; accepted 18 January 2017; first published online 13 February 2017*)

SUMMARY

APETALA2/ethylene-responsive factor (AP2/ERF) family transcription factors play a vital role in plant growth and in response to hormones and abiotic stresses. In the current research, it is reported that OsDERF2, one of the drought-responsive ERF, is a member of the DREB sub-family. OsDERF2 is a nuclear-localized protein and has transcriptional activity in yeast. Expression of *OsDERF2* was induced by drought and inhibited by abscisic acid (ABA). However, *OsDERF2* RNA interference (RNAi) knock-down transgenic lines enhanced tolerance to drought stress at seedling stage and were much more sensitive to ABA treatment, which may result from the increased ABA level *in vivo*. The basic leucine zipper (bZIP) transcription factor family plays an important role in the ABA signalling pathway of abiotic stress. Quantitative real-time polymerase chain reaction analysis revealed that the bZIP family gene *OsbZIP20* and ABA-response gene *OsABA45* were up-regulated 25 times and 120 times, respectively, in *OsDERF2* RNAi knock-down lines under drought stress, which were up-regulated five and seven times in wild type under drought stress. The current data reveal that OsDERF2 negatively modulates drought stress response in an ABA-mediated pathway through regulating gene expression of other ABA-response transcription factors.

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population. Although total global rice production demonstrates annual increases, there are many biotic and abiotic factors affecting yield. The most influential abiotic stresses are drought, salinity, cold and heat stresses. In particular, drought stress resulting from the absence of rainfall for long periods or deficits in usable water resources can affect crop yield significantly (Hadiarto & Tran 2011; Joo *et al*. 2013). In plants, drought stress causes a decrease of water potential in tissue, which induces a series of physiological responses, such as growth and development inhibition, decrease in chlorophyll content, inhibition of photosynthesis and stomatal closure (Zhang *et al*. 2013; Lim *et al*. 2014). Meanwhile, the expression of unique genes or groups of genes and their expression patterns show different responses under drought stress (Do *et al*. 2014; Oono *et al*. 2014).

In plants, APETALA2/ethylene-responsive factor (AP2/ERF) is a large family of transcription factors that include AP2, ERF, DREB and RAV sub-family members. The AP2 sub-family members possess two repeats of the AP2/ERF domain, ERF and DREB sub-family proteins contain a single AP2/ERF domain and RAV sub-family proteins have an additional B3 DNA-binding domain. The difference between ERF and DREB members is the binding sequence: the ERF proteins bind to AGCCGCC, while the DREB proteins recognize A/GCCGAC (Dey & Corina Vlot 2015). Based on the conserved AP2/ERF DNA-binding domain, 170 AP2/ERF family genes have been identified by phylogenetic analysis of the rice genome (Rashid *et al*. 2012). The AP2/ERF family proteins play a vital role in plant growth and enable plants to tolerate ambient changes (Licausi *et al*. 2013), and use different pathways in response to hormone

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changes and biotic and abiotic stresses (Mizoi *et al*. 2012). It has previously been reported that an AP2/ERF factor, AtERF7, plays an important role in abscisic acid (ABA) responses and acts as a repressor of gene transcription (Song *et al*. 2005). Two jasmonate-responsive AP2 factors, AaERF1 and AaERF2, positively regulate artemisinin biosynthesis in *Artemisia annua* L. (Yu *et al*. 2012). OsDERF1 modulates ethylene biosynthesis and drought tolerance through directly regulating two ERF repressors, OsERF3 and OsAP2-39, in rice and AtERF11 is a negative regulator of ABA-mediated ethylene synthesis via interaction with ACS2/5 promoters in *Arabidopsis* (Li *et al*. 2011*b*; Wan *et al*. 2011). In addition, DREB1A and DREB2A have functions in low-temperature and drought stress responses in *Arabidopsis*, and SiDREB2 contributes to drought tolerance in foxtail millet (Liu *et al*. 1998; Lata *et al*. 2011). DREB2A and DREB2B are induced by high-salt stress, while AtERF98 enhances salt tolerance through modulation of ascorbic acid synthesis (Nakashima *et al*. 2000; Zhang *et al*. 2012). RAV factors play roles in the disease defence pathway in tomato (Li *et al*. 2011*a*). Submergence tolerance regulator Sub1A is another ERF transcription factor, which also improves drought tolerance (Xu *et al*. 2006; Fukao *et al*. 2011). The findings detailed above provide substantial evidence that each AP2/ERF family protein has a distinctive functional role in the regulation of diverse physiology processes, thus further dissection of the function of AP2/ERF proteins will deepen the understanding of plant responses to abiotic stresses.

Previously, 12 drought-responsive AP2/ERF genes (DERF) were identified using expression data for stress treatment in rice seedlings. Among these genes, OsDERF1 negatively modulates ethylene biosynthesis and drought tolerance through transcriptional regulation of *OsERF3* and *OsAP2-39* (Wan *et al*. 2011). In the present study, the functional analysis of *OsDERF2* (*LOC_Os04g46440*) using RNA interference (RNAi) knock-down transgenic plants is reported. The results provide evidence that OsDERF2 confers negative regulation in drought stress through transcriptional repression of ABA response-related genes in rice.

MATERIALS AND METHODS

Plant material and drought stress treatment

Rice (*Oryza sativa*) seeds of wild-type Nipponbare (WT) and transgenic lines were used in the drought stress treatment, as previously described (Wan *et al*. 2011). After germination at 30 °C for 2 days, germinated seeds were transplanted on sandy soil in a greenhouse at 26 °C and 16 h light/8 h dark. The 2-week-old seedlings were exposed to successive drought by withholding water supply. For the control, all seedlings were maintained under normal growth conditions with water. When the seedlings began to wilt at the 6th day, drought phenotypes among different lines were observed. After all the seedlings showed varying degrees of stress symptoms up to the 9th day, plants were re-watered for 7 days to allow recovery, and then the growth status and rate of survival was recorded as images and analysed.

Phylogenetic analysis

Sequences of DREB members were searched using the basic local alignment search tool (BLAST) in the GenBank protein database (http://www.ncbi.nlm.nih.gov/BLAST/) and the results inspected manually. These sequences were aligned with ClustalW using default parameters. A phylogenetic tree was constructed using MEGA 5·0 with the neighbour-joining method. Bootstrap analysis was performed with 100 replicates, and bootstrap values on the tree are shown as percentages.

Sub-cellular localization analysis

The coding sequence of *OsDERF2* was cloned into the pGDG vector to construct green fluorescent protein (GFP) fusion with OsDERF2. The fusion construct (35S::GFP-OsDERF2) and control (35S::GFP) were transformed into onion epidermal cells with an *Agrobacterium*-mediated system, incubated on 1/2 strength Murashige and Skoog (MS) medium for 24 h at 26 °C in darkness, and the fluorescence of GFP was observed using a Leica TCS-SP4 laser scanning confocal microscope.

Generation of transgenic plants

RNA interference transgenic plants were generated as described in Ding *et al*. (2007). The less conserved region at the C-terminus (located at amino acids 178-217 of OsDERF2) was used to interfere with gene expression. The resulting plasmid was transformed into *Agrobacterium* and WT Nipponbare calli were used as the recipients for *Agrobacterium*-mediated transformation. Transformed plants with

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reduced expression levels were detected using quantitative real-time polymerase chain reaction (qRT-PCR) as described by Wan *et al*. (2011). T3 transgenic lines were used in the present study and denoted as RIs, and the different lines are indicated by numbers.

Transcriptional activity detection in yeast

Different truncations (including the activating domain) were fused in-frame to the DNA-binding domain vector pGBKT7. The fusion plasmids were transformed into yeast AH109 as described by the manufacturer (Clontech, USA). The transformants were grown on selective medium plates at 30 °C for 3 days.

Abscisic acid and drought treatment for gene expression analysis

Wild-type seedlings cultured in water for 7 days were used for *OsDERF2* gene expression analysis. For ABA treatment, plants were transferred into water with 50 μM ABA, while an equal volume of absolute ethanol was added to water for the controls. For the drought treatment, seedlings were taken out of the water and kept on filter paper. Samples were collected at 0, 0·5, 1, 2 and 3 h after drought treatment and 0, 0·5, 1, 2, 4 and 8 h after ABA treatment. Gene expression analysis was performed by qRT-PCR. The gene-specific primers are shown in Table 1.

Abscisic acid sensitivity test

For the ABA sensitivity test of transgenic rice seedlings, geminated plants of transgenic rice and the wild-type control at the same growth stage were transferred to MS medium containing different concentrations of ABA (0, 2 and 10 μM). The seedlings were grown for 3 days in a growth chamber and root lengths were measured.

Measurement of malondialdehyde and proline contents

The malondialdehyde (MDA) and proline contents of plants were detected following polyethylene glycol (PEG) treatment for 5 days as described in Madhava Rao & Sresty (2000).

Detection of endogenous abscisic acid levels in plant

Leaves of 2-week-old seedlings (0·2 g) were frozen in liquid nitrogen and ground finely, followed by extraction with 1 ml extraction mixture (2-propanol: H_2O:concentrated hydrochloric acid (HCl) = 2 : 1 : 0·002, vol/vol/vol). The extraction samples were shaken at 100 rpm for 30 min at 4 °C, followed with addition of 1 ml dichloromethane and shaken for 30 min at 4 °C. After centrifugation at 13 000 ***g*** for 5 min, 900 μl of the solvent was transferred from the lower phase and the solvent mixture concentrated. The samples were dissolved in 0·1 ml methanol and ABA was measured as described in Pan *et al*. (2010).

RESULTS

OsDERF2 belonging to the DREB family is inducible by drought stress

As described in the previous study, 12 drought-responsive ERF genes (DERF) were identified using expression data for stress treatment in rice seedlings (http://www.ricearray.org) (Wan *et al*. 2011). Among these genes, *OsDERF1* modulates drought response through negatively affecting ethylene production (Wan *et al*. 2011). *OsDERF2* (*LOC_Os04g46440*) encodes a 217 amino acid protein. Amino acids 46–109 contain a typical AP2 DNA-binding domain, and residues 34–40 contain a nuclear localization signal (Fig. 1(*a*)). NCBI (National Center for Biotechnology Information) BLASTp results and classification using MEGA 5·0 showed that OsDERF2 was similar to DREB members of the AP2/ERF family, such as LOC_Os02g43970, LOC_Os10g41130, OsDREB1A, OsDREB1B, OsDREB1C, AtTINY, AtABI4, AtDREB2A, AtDREB2B and ZmDBF2, which contain an AP2 domain (Fig. 1(*b*)).

Sequence analysis of *OsDERF2* showed that residues 34–40 PKKRPRN is a nuclear localization signal. To determine the sub-cellular localization of OsDERF2, the coding sequence of *OsDERF2* was fused to *GFP* in the pGDG vector. The onion cells transformed with the control *p35S::GFP* displayed fluorescence throughout the cells, but fluorescence in the onion cells transformed with *p35S::GFP-OsDERF2* was restricted exclusively to the nucleus (Fig. 2(*a*)), demonstrating that OsDERF2 is a nuclear-localized protein as predicted.

The full-length, different deletions, including N-terminal of 190 amino acids, N-terminal of 149 amino acids and N-terminal of 110 amino acids of OsDERF2 were fused to the GAL4 DNA-binding domain resulting in the plasmids of pGBKT7-OsDERF2, pGBKT7-N190, pGBKT7-N149 and pGBKT7-N110, respectively. These plasmids were then transformed into yeast strain

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Table 1. *Oligo nucleotides used in the present study*

Oligo name	Oligo nucleotide (5′–3′)
The specific primers of vector construction	
OsDERF2RNAi-F	GCTACTAGTCTCCTGGACCTGAGATACGA
OsDERF2RNAi-R	GAGAGATCTGGGATAGTCGAAGATAAGATGC
OsDERF2-F (BglII)	TGGAAGATCTATGGACGACTCCCACGACCTG
OsDERF2-R (EcoRI)	TCCGGAATTCTCAGTAATCCCACAGCATGGGCTC
OsDERF2-F (EcoRI)	TCCGGAATTCATGGACGACTCCCACGACCTG
OsDERF2-R (BglII)	TGGAAGATCTTCAGTAATCCCACAGCATGGGCTC
OsDERF2-R190 (BglII)	TGGAAGATCTCGAGAGGCTCGAGGACGTCT
OsDERF2-R149 (BglII)	TGGAAGATCTGGAGCAGGTGTCGGGGCT
OsDERF2-R110 (BglII)	TGGAAGATCTCGGGAGCAGGTGCGCGA
OsDERF2-R (HindIII)	ATCCCAAGCTTCAGTAATCCCACAGCATGGGCTC
qRT-PCR primers	
OsACTIN-F	TCCAAGCAGCATGAAGATCA
OsACTIN-R	CACATAAGAGAGTGACGTACA
OsDERF2-F	AGCACGCTGTTCGACCTCCC
OsDERF2-R	TCAGTAATCCCACAGCATGGGCT
OsbZIP15-F (RT)	CCCACATCAGCTACCAATCT
OsbZIP15-R (RT)	GCATGGACCACCCTCTATATC
OsbZIP20-F (RT)	CAGTGAGTCGCTGTTGGATATAG
OsbZIP20-R (RT)	CGGTTGGAAACCATCCTTCT
OsbZIP33-F (RT)	CCTTGAGAGCAAAGGTGAAGA
OsbZIP33-R (RT)	GCTGAGGGATGACATATCAGAAG
OsbZIP52-F (RT)	TCCACAGAAACAAAGCGAATAAG
OsbZIP52-R (RT)	GACCTGTGATTCGAGTTCAGATA
OsbZIP58-F (RT)	CGACTCTCGTCCTCCTATCTT
OsbZIP58-R (RT)	GGCACACTTCTTCGTCTTCT
OsbZIP71-F (RT)	TGTGTGCCCTAACTGACATCCTGA
OsbZIP71-R (RT)	AAGTCTATGGGTGGCTGGTTCCAT
OsbZIP88-F (RT)	AGGAACAGTCAGATGATGATGG
OsbZIP88-R (RT)	CCTGGCTGATTCCCGATTT
OsABI5-F	GGAGAACGCTCGTCTCAAAG
OsABI5-R	CTAGTGCCACACCAGAAGCA
OsABA45-F	CAAGTGATGATCGGATCGAA
OsABA45-R	CACAATAGCGACCTCGACAA
AAO1-F	TTCGCCATTTGTTCGTAA
AAO1-R	CAGAGGAGGTTGCTCAAG
AAO2-F	CCCTTGACGCCAACACTG
AAO2-R	CCGCTTTCGCCACTTATT
AAO3-F	CGCCTGGTAAAGTGTCTA
AAO3-R	AATTGCTCCTTGAGTGGT
SDR1-F	TGACAGCCAGGGACGAGA
SDR1-R	TCAGCCAACCGAGAAACG
SDR2-F	CGCCCAAGGAGTAGATAACA
SDR2-R	GACAGCAGCAGAGGCAGTAA
SDR3-F	TAGCCATCTTCGCCACC
SDR3-R	GCAAAGGGACTCAACAGC
ZEP1-F	ATAGATGATGGCAACAAGGTAA
ZEP1-R	TCAATGTCAGGAGGCACAA
NCED1-F	CTCACCATGAAGTCCATGAGGCTT
NCED1-R	GTTCTCGTAGTCTTGGTCTTGGCT
NCED3-F	CCCCTCCCAAACCATCCAAACCGA

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Table 1. (*Cont.*)

Oligo name	Oligo nucleotide (5′–3′)
NCED3-R	TGTGAGCATATCCTGGCGTCGTGA
NCED5-F	ACATCCGAGCTCCTCGTCGTGAA
NCED5-R	TTGGAAGGTGTTTTGGAATGAACCA

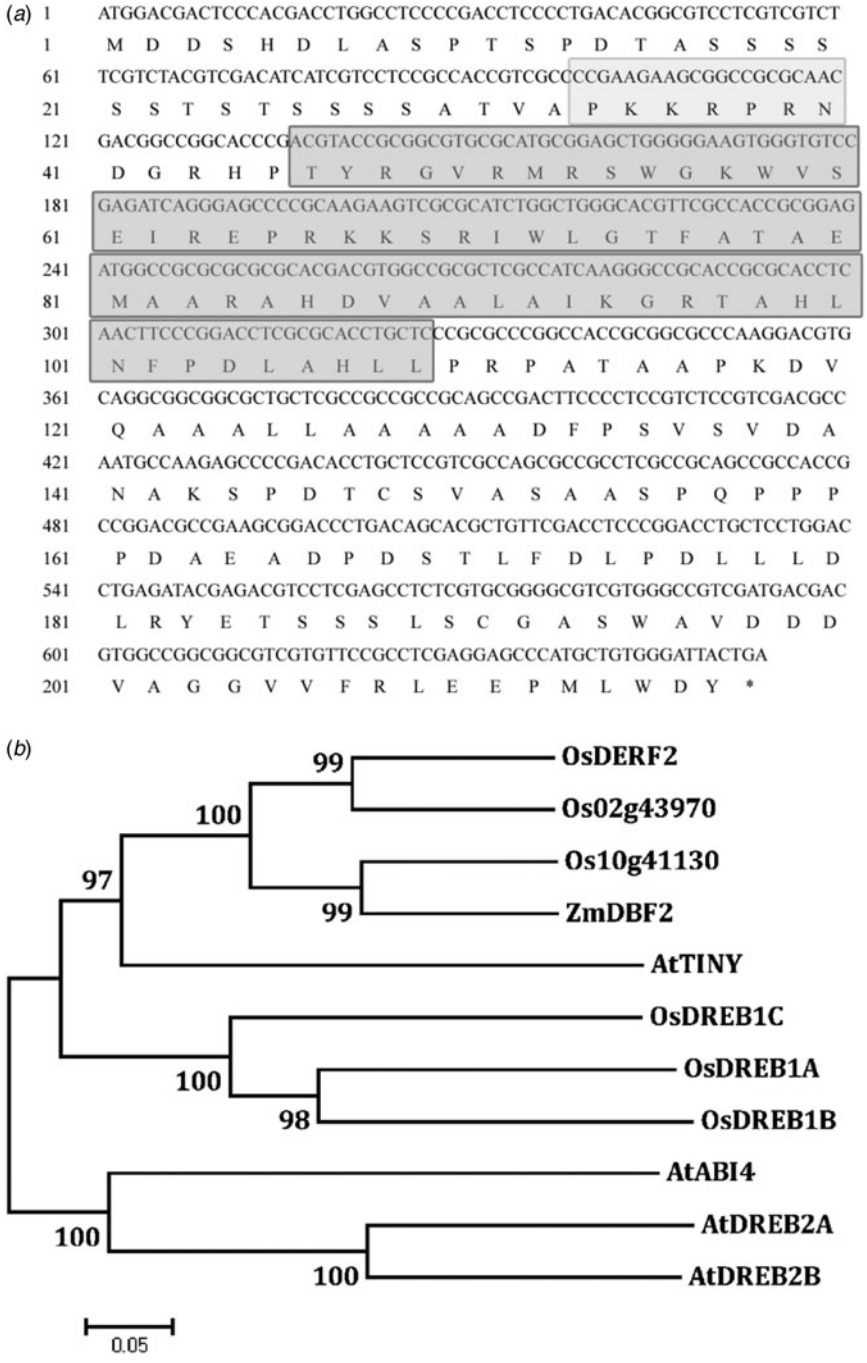

Fig. 1. Sequence analysis of OsDERF2 protein. (*a*) Nucleotide and protein sequences of OsDERF2. Light grey box indicates nuclear localization signal, and dark grey box indicates APETALA2 domain; (*b*) phylogenetic relationships among plant DREB members from rice, maize and *Arabidopsis*. Bootstrap values from 100 replicated are shown at each node.

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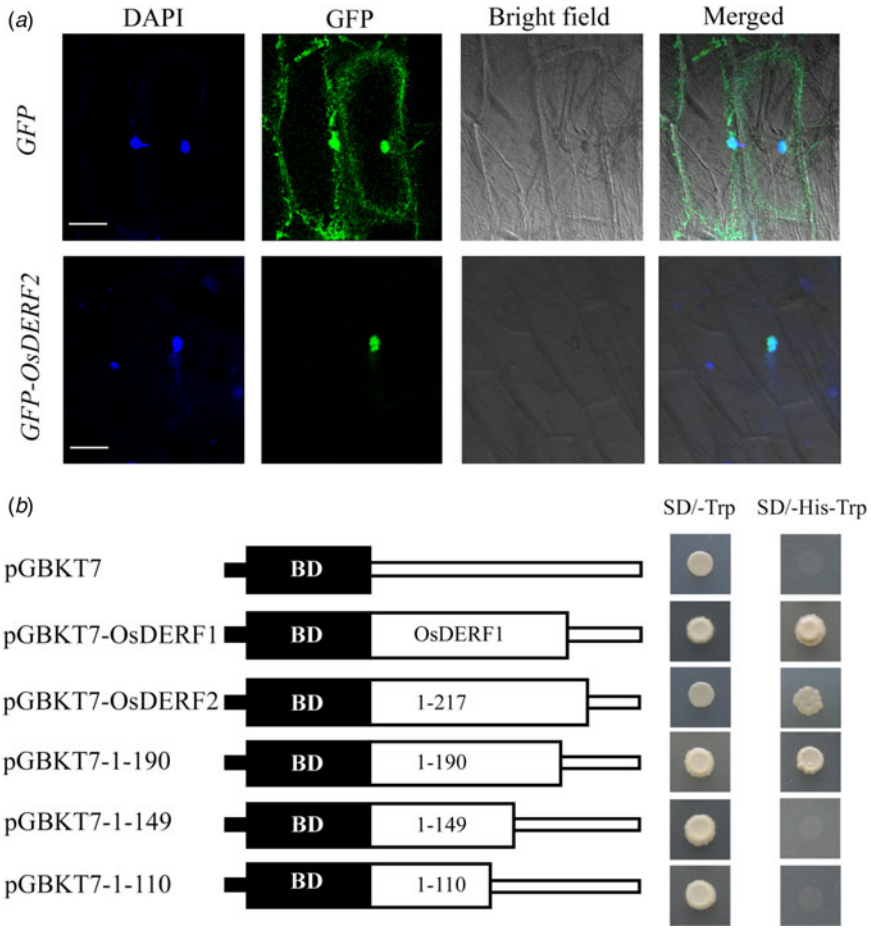

Fig. 2. Subcellular localization and transcriptional activity of OsDERF2 protein. (*a*) Nuclear localization of the OsDERF2 protein as revealed by GFP fusion protein. The constructs *GFP* and *GFP-OsDERF2* were expressed in onion epidermal cells using an *Agrobacterium*-mediated system. GFP fluorescence was detected using confocal microscopy. Scale bars, 1 μm; (*b*) transcriptional activity of OsDERF2 in yeast. Left panel shows the schematic diagrams of various constructs used for transactivation. OsDERF1 was used as positive control. BD indicates GAL4 DNA-binding domain. Right panel shows the yeast growth on the SD/-Trp and SD/-His-Trp medium. Colour online.

AH109, with plasmid pGBKT7 and the previously reported pGBKT7-OsDERF1 (Wan *et al*. 2011) as negative and positive controls, respectively. The transformants of pGBKT7-OsDERF1, pGBKT7-OsDERF2 and pGBKT7-N190 could grow on the SD/-Trp and SD/-His-Trp medium, while the transformants of pGBKT7-N149, pGBKT7-N110 and the negative control pGBKT7 could not grow on the SD/-His-Trp medium (Fig. 2(*b*)). These results indicated that *OsDERF2* contains an activation domain in amino acids 149–190, possibly functioning as a transcriptional activator.

The expression of *OsDERF2* in different tissues of rice was determined with qRT-PCR and the results showed that transcripts of *OsDERF2* were highly expressed in seedling leaves and sheaths, 25 and 10 times as much, respectively, as that in roots (Fig. 3(*a*)). Therefore, the tissues including leaves and sheaths were used to analyse the transcript level of *OsDERF2* under different treatments. To investigate the function of *OsDERF2,* the promoter sequence (2000 base pairs upstream of the initiation codon) was analysed using the PLACE database (for motifs found in plant cis-acting regulatory DNA elements, now supplanted by TENOR – http://tenor.dna.affrc.go.jp/). The results showed that the promoter of *OsDERF2* contains multiple putative stress-responsive cis-acting elements, including MYC, MYB and ABRE recognition sites. Further qRT-PCR analysis revealed that the gene expression of *OsDERF2* was inhibited by ABA, and the solvent of ABA was used as a control. Although the expression of *OsDERF2* obviously decreased at 0·5 h and then increased until 4 h under ABA treatment, it decreased seriously at 8 h (Fig. 3(*b*)). In drought-treated seedlings, *OsDERF2* expression was

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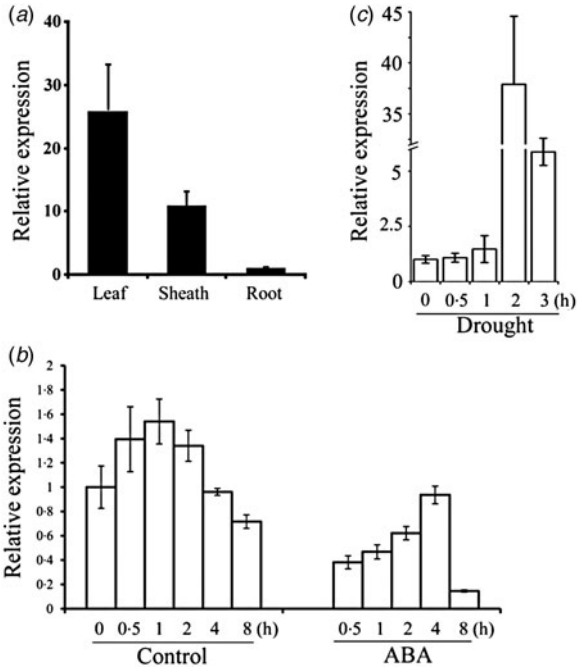

Fig. 3. Detection of *OsDERF2* transcripts in different tissues of rice seedlings and in response to ABA and drought stress. (*a*) Expression of *OsDERF2* in different tissues; (*b*) expression of *OsDERF2* in response to 50 μM ABA for 8 h, ABA solvent is used as control; (*c*) expression of *OsDERF2* in response to drought for 3 h. The bars represent (±) S.E.

strongly induced and peaked at 3 h (Fig. 3(*c*)). These data indicate that *OsDERF2* might be involved in drought response through the ABA signalling pathway.

OsDERF2 negatively modulates rice drought response

To determine the regulatory function of *OsDERF2* in abiotic stress, *OsDERF2* RNA interference transgenic rice (RI) were generated. The RI lines with expression levels of *OsDERF2* decreased to 30–60% of WT were selected for the current research (Fig. 4). RI-2, RI-4, RI-5 and RI-6 showed 50, 60, 55 and 30% reductions in expression of *OsDERF2*, respectively. RI-2, RI-4 and RI-5 were used for the ABA sensitivity test. The lines, including RI-4, RI-5 and RI-6 were used in drought stress treatments and ABA-level detection. There are no obvious differences between these RI lines and WT in terms of plant development at the seedling and heading stages. However, in the drought stress tolerance treatment, when the WT seedling leaves withered, most of the RI lines still grew well. After the drought-treated seedlings were re-watered, the difference in growth status between WT and RI lines was increased (Fig. 5(*a*)). The survival rates of all RI lines

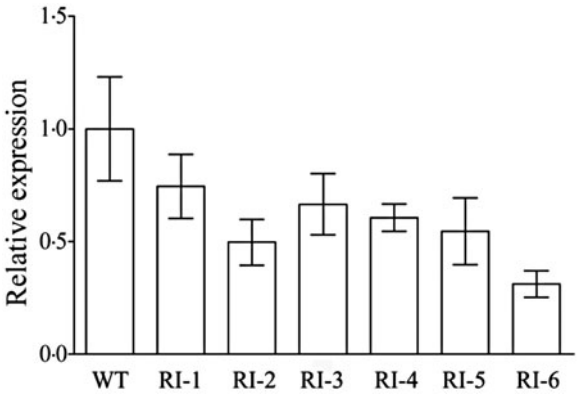

Fig. 4. Relative expression levels of *OsDERF2* in different RNAi transgenic lines. The gene expression in the WT was assigned a value of 1. The bars represent (±) S.E.

(RI-4, RI-5 and RI-6) were more than 85%, whereas that of WT was only 55% (Fig. 5(*b*)). These results indicated that OsDERF2 regulated rice tolerance to drought negatively. Free radicals accumulating in plants under drought stress would lead to the damage of DNA, proteins and lipids, and MDA is an end product of membrane lipid peroxidation (Wan *et al*. 2011). The MDA content of seedlings grown under normal and PEG treatment were investigated and the results showed that there were no obvious changes among WT and RI lines under normal growth conditions. After PEG treatment, although both WT and RI lines showed increased MDA content, the increase was higher in WT (up to two times) than in RI lines (1·5 times) (Fig. 5(*c*)), indicating that *OsDERF2* enhances the production of oxidative stress. Meanwhile, proline is crucial for osmotic adjustment (Wei *et al*. 2014). To determine whether *OsDERF2* negatively modulates tolerance to drought through affecting osmolyte accumulation, the proline content was measured and found to increase up to 1·4 times in WT and 1·8 times in RI lines (Fig. 5(*d*)), suggesting that *OsDERF2* reduces the accumulation of osmolytes to modulate drought tolerance.

Enhanced response of *OsDERF2* RNA interference lines to abscisic acid in root growth

Abscisic acid is a key regulator of plant adaptation to stress and different aspects of plant growth and development. Under stress conditions, plants synthesize ABA in various organs and initiate defence mechanisms, such as the regulation of stomatal aperture and expression of defence-related genes involved in resistance to

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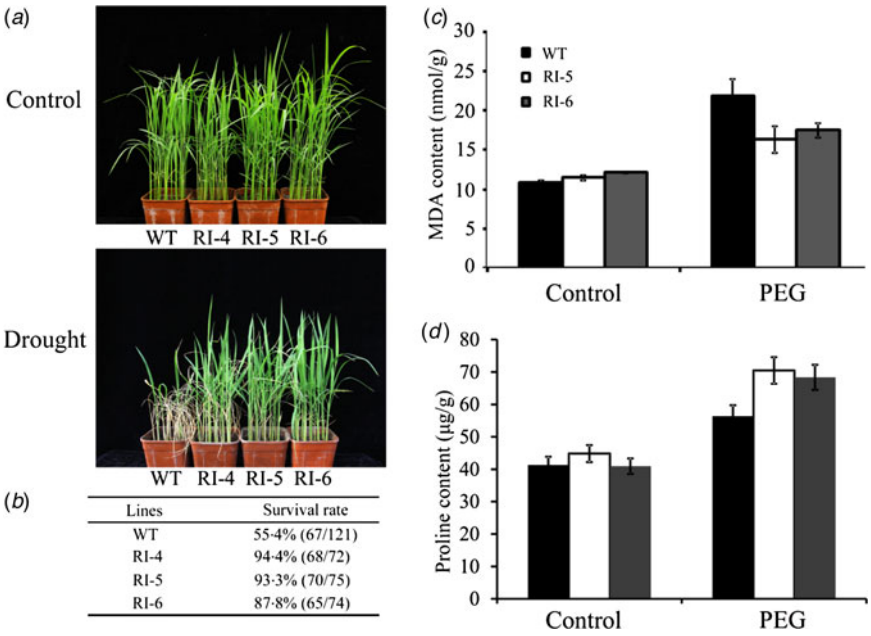

Fig. 5. Regulation of *OsDERF2* in drought stress response. (*a*) WT and RI lines were subjected to drought stress for 9 days, followed by recovery for 7 days. Control: rice plants were grown under normal conditions. Drought: plants had the daily water supply withheld; (*b*) survival rate of all plants used in (*a*) after re-watering. The numbers in parentheses shows the survival seedlings/total tested plant; (*c*) MDA content of WT and RI plants under normal conditions or treatment with 15% PEG 6000 for 5 days; (*d*) proline content of WT and RI plants under normal condition or treatment with 15% PEG 6000 for 5 days. Data are the average of three replicates, and there were 10 plants per replicate. The bars represent (±) S.E. Colour online.

environmental stresses. Since expression of *OsDERF2* was inhibited by ABA, and RI lines of *OsDERF2* were more tolerant to drought stress, further tests were conducted to investigate whether *OsDERF2* is involved in ABA sensitivity, which is an important aspect of the ABA-mediated regulation pathway. Three RI lines (RI-2, RI-4 and RI-5) and WT were used to test the effect of ABA on seedling development. The germinated seedlings were treated with different concentrations of ABA (0, 2 and 10 μM), and the growth of RI lines was more inhibited than that of WT (Fig. 6(*a*)). The root length of RI lines was significantly shorter than WT plants under ABA treatment, and no apparent difference was observed under normal growth conditions. The root length decreased by 10 and 60% in WT at 2 and 10 μM ABA, and by 30 and 90% in RI lines (Fig. 6(*b*)). These results suggested that the RI lines of *OsDERF2* were more sensitive to ABA than WT.

Increased abscisic acid levels in *OsDERF2* RNA interference lines

Increase of ABA levels in plants could enhance drought stress tolerance, due to the closure of stomata and accumulation of numerous proteins, such as late embryogenesis abundant (LEA), for osmotic adjustment (Verslues & Bray 2006). Based on the enhanced ABA sensitivity of *OsDERF2* RI lines, the ABA contents of two RI lines were measured, in order to confirm whether the drought tolerance of *OsDERF2* RI lines is ABA mediated. The data showed that the endogenous ABA levels increased significantly in RI-5 and RI-6, compared with that in WT seedlings (Fig. 7(*a*)). Although the genes involved in ABA biosynthesis, including *NCED3, NCED5, AAO2* and *SDR1* in RI-6 had been slightly down-regulated, the expression of *AAO3* was up-regulated 2·5-fold in RI-6 (Fig. 7(*b*)). These results proved that the transcriptional expression of the genes involved in ABA biosynthesis failed to contribute to ABA accumulation in *OsDERF2* RNAi lines.

Under abiotic stress, ABA concentration goes up and ABA receptors bind ABA, followed by release of SnRK2, which activates basic leucine zipper (bZIP) transcription factors (Kim *et al*. 2015). A total of 75 bZIPs have been identified and classified into 10 groups in *Arabidopsis thaliana*. Most of the ABRE binding bZIPs belongs to group A (Lu *et al*. 2009); however, bZIPs in other groups also have functions in ABA response in rice (Liu *et al*. 2014). To further confirm the difference of ABA levels between WT

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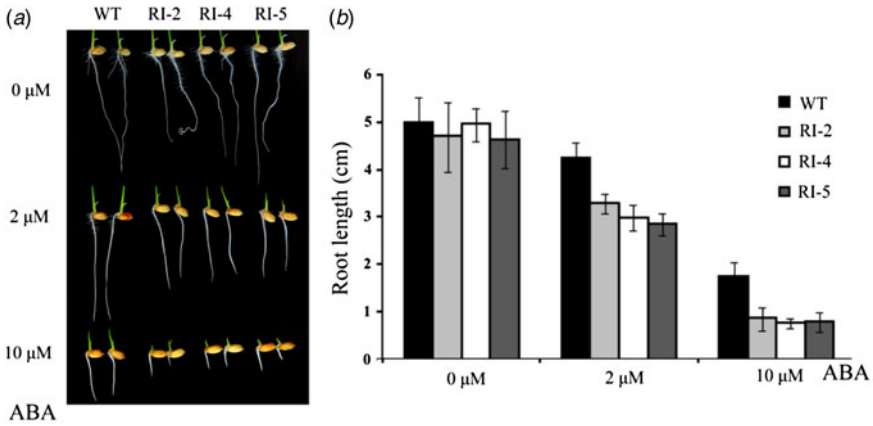

Fig. 6. ABA sensitivity of *OsDERF2*-RNAi transgenic rice. (*a*) Root growth of 3-day-old seedlings on MS medium with or without ABA (0, 2 and 10 μM); (*b*) root lengths of seedlings in (*a*). All experiments were repeated with three biological replicates. WT, wild type. RI-2, RI-4 and RI-5 are independent RNAi lines of *OsDERF2*. The bars represent (±) S.E. Colour online.

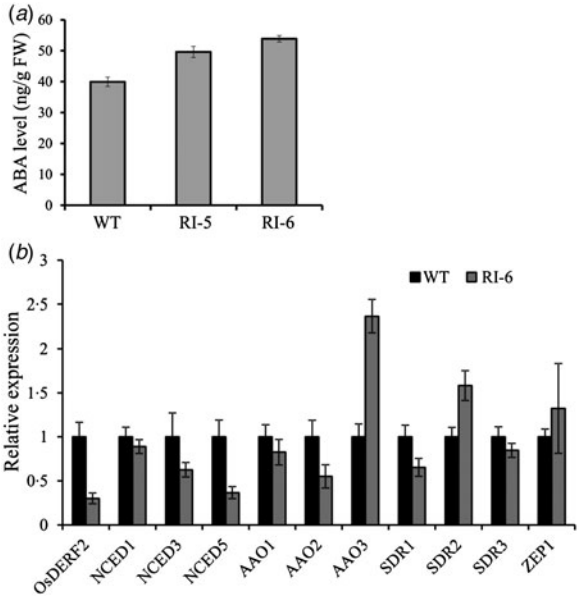

Fig. 7. ABA level of *OsDERF2*-RNAi transgenic rice. (*a*) ABA level of WT and *OsDERF2*-RNAi transgenic lines. FW indicates fresh weight. (*b*) Transcriptional expression levels of genes involved in ABA biosynthesis in rice detected by qRT-PCR. The expression of each gene in the WT was assigned a value of 1. ABA and RNA were extracted from the roots and leaves of 2-week-old seedlings. The bars represent (±) S.E.

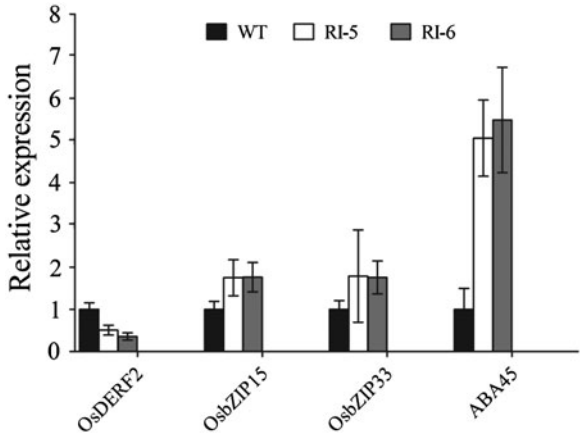

Fig. 8. The up-regulated ABA-response genes in the *OsDERF2*-RNAi lines. The expression of each gene in the WT was assigned a value of 1. The bars represent (±) S.E.

and RI lines, the expression levels of ABA-response genes, including bZIP transcription factor family genes were detected through qRT-PCR. It was found that the expression levels of *OsbZIP15, OsbZIP33* and *OsABA45* were up-regulated in the *OsDERF2* RI lines (Fig. 8). *OsbZIP15* and *OsbZIP33* belong to group C. Gene expression levels under normal and drought treatment were also measured. The results showed that most *OsbZIP* genes and their downstream genes were up-regulated after drought treatment in both WT and RI-6 seedlings; however, the transcripts of *OsbZIP20* and *OsABA45* were up-regulated about five- and eightfold, respectively, in the WT seedlings, and 25- and 130-fold, respectively, in the RI-6 seedlings (Fig. 9). These results suggested that *OsDERF2*-modulated gene expressions involved in ABA response through regulated ABA accumulation, which may contribute to drought tolerance in rice.

DISCUSSION

In the current study, a rice AP2/ERF protein OsDERF2 was identified, which is located in the nucleus and has

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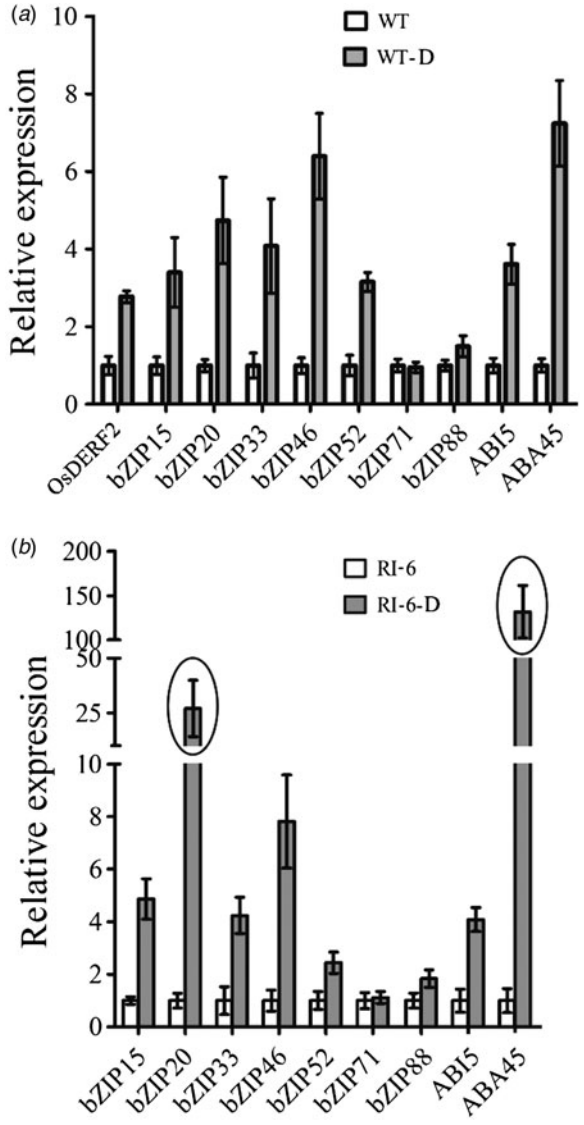

Fig. 9. Transcriptional expression levels of ABA-response genes in WT and *OsDERF2*-RNAi line under normal and drought stress (indicated as D) by qRT-PCR. The expression of each gene under normal condition was assigned a value of 1. The bars represent (±) S.E.

a transcriptional activity domain between amino acids 149–190. DREB sub-family members are involved in two separate signal transduction pathways under low temperature and drought. It has also been found that the expression of *DREB* genes is induced by abiotic stress at different time periods (Agarwal *et al*. 2006). *OsDERF2* is a novel transcription factor in the DREB sub-family. The expression of *OsDERF2* is induced by drought, while its RNAi lines show more tolerance to drought stress, indicating that *OsDERF2* acts as a negative regulator in drought stress. Wan *et al*. (2011) reported that *OsDERF1* over-expression lines show more sensitivity to drought and RNAi lines show more tolerance to drought than the wild type. However, the expression of *OsDERF1* is induced by drought stress (Wan *et al*. 2011). Another example is *OsbZIP71*, which is repressed under saline conditions, while constitutive over-expression of *OsbZIP71* improved plant tolerance to salt (Liu *et al*. 2014). DREBs are generally positive regulators in abiotic stresses, but *OsDERF2* negatively modulates drought tolerance in rice. Therefore, it is proposed that *OsDERF2* may activate some repressors involved in drought response.

Many rice genes have been identified as drought responsive, which include the genes encoding for aquaporins, AP2/ERF-, bZIP-, NAC- and MYB-type transcription factors, LEA proteins, osmoprotectant-synthesizing enzymes, protein kinases, metallothionein and metallothionein-like proteins, and cytochrome P450 family proteins (Hadiarto & Tran 2011). The phytohormone ABA plays a crucial role in the adaptive response to abiotic stresses such as drought, cold and high salinity. It is also involved in various processes of plant growth, including seed maturation, dormancy, inhibition of cell division and germination (Zou *et al*. 2008). RNAi lines of *OsDERF2* showed more sensitivity to ABA and accumulated more ABA than in the WT; meanwhile, these lines improved plant tolerance to drought stress. However, the expression of ABA biosynthesis genes is not related to the increased ABA level in *OsDERF2* RNAi lines. It is known that ABA accumulation depends on production, degradation and transportation in roots (Shi *et al*. 2015); therefore, *OsDERF2* may be involved in ABA catabolism or transportation. This should be the subject of further research. It implies that *OsDERF2* has functions in ABA accumulation and ABA response, which is one reason for the higher tolerance of *OsDERF2* RI lines under drought stress.

The biosynthesis of ABA is induced by drought and the resultant activation of two regulatory ABA-dependent gene expressions. One is the bZIP/ABRE system and the other is MYC/MYB. The bZIP family plays an important role in the ABA signalling pathway of abiotic stress. For example, *OsbZIP23* (Xiang *et al*. 2008), *OsbZIP46* (Tang *et al*. 2012), *OsbZIP72* (Lu *et al*. 2009), *OsbZIP12/OsABF1* (Amir Hossain *et al*. 2010), *OsABI5* (Zou *et al*. 2008) and *OsbZIP71* (Liu *et al*. 2014) play important roles in ABA signal transduction and osmotic stress responses. In the current study, it was shown that the levels of *OsbZIP15* and *OsbZIP33* expression were up-regulated in *OsDERF2*-RNAi lines, which can form heterodimers with *OsbZIP71* (Liu *et al*. 2014). *OsbZIP71* has no transcriptional activity and needs

https://doi.org/10.1017/S0021859617000041 Published online by Cambridge University Press

other bZIPs to activate downstream genes. Therefore, *OsDERF2* may affect the activity of *OsbZIP71* by modulating gene expressions of *OsbZIP15* and *OsbZIP33*. Under drought stress, expression levels of *OsbZIP20* and *OsABA45* increased much more in the *OsDERF2*-RNAi line than that in WT plants. *OsbZIP20* (RITA-1) displays broad binding specificity for palindromic ACGT elements, and plays a role in the regulation of rice genes expressed in developing rice seeds (Izawa *et al*. 1994). The promoter sequence of *OsbZIP20* (2000 base pairs upstream of the transcription start site) was analysed using the PLACE database. The results showed that the promoter of *OsbZIP20* contains ABA-responsive cis-acting elements ABRE and AP2/ERF binding sites, including DRE, GCC and RAV1. Therefore, it is speculated that *OsbZIP20* is not only involved in seed development but also in stress responses. *OsABA45* is a GRAM domain containing an ABA-responsive protein, in which the promoter of this encoding gene contains two copies of the CGCG box (Wang *et al*. 2011). The CGCG box is regulated by calmodulin and involved in the transcription regulation of multiple abiotic stress responsiveness (Yang & Poovaiah 2002). In particular, many GC-rich motifs with a core motif of CGCG have been found to be over-represented in the promoter of the ABA- and stress-induced gene (Cuming *et al*. 2007). These data further indicate that *OsDERF2* has negative regulation in the response of the rice to drought environment through ABA-mediated pathway.

ACCESSION NUMBERS

The GenBank accession numbers are as follows: OsDERF2, LOC_Os04g46440; OsbZIP15, LOC_Os02g07840; OsbZIP20, LOC_Os02g16680; OsbZIP33, LOC_Os03g58250; OsbZIP52, LOC_Os06g45140; OsbZIP58, LOC_Os07g08420; OsbZIP71, LOC_ Os09g13570; OsbZIP88, LOC_Os12g40920; OsABI5, LOC_Os01g64000; OsABA45, LOC_Os12g29400; OsDREB1A, LOC_Os09g35030; OsDREB1B, LOC_ Os09g35010; OsDREB1C, LOC_Os06g03670; AtDREB2A, At5g05410; AtDREB2B, At3g11020; AtABI4, At2g40220; AtTINY, At2g44940.

The current work was supported by Grant Special Foundation of Transgenic Plants in China (grant numbers 2014ZX08009-15B and 2014ZX08001-003) and the National Science Foundation of China (grant number 31171465).

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