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ORIGINAL PAPER

OsPFA-DSP1, a rice protein tyrosine phosphatase, negatively regulates drought stress responses in transgenic tobacco and rice plants

Bing Liu · Jieqiong Fan · Yang Zhang · Peiqiang Mu · Peng Wang · Jianbin Su · Huihuang Lai · Shaowu Li · Dongru Feng · Jinfa Wang · Hongbin Wang

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Abstract Dephosphorylation plays a pivotal role in regulating plant growth, development and abiotic/biotic stress responses. Here, we characterized a plant and fungi atypical dual-specificity phosphatase (PFA-DSP) subfamily member, OsPFA-DSP1, from rice. OsPFA-DSP1 was determined to be a functional protein tyrosine phosphatase (PTP) in vitro using phosphatase activity assays. Quantitative real-time PCR and GENEVESTIGATOR analysis showed that *OsPFA-DSP1* mRNA was induced by drought stress. Transfection of rice protoplasts showed that OsPFA-DSP1 accumulated in both the cytoplasm and nucleus. Ectopic overexpression of *OsPFA-DSP1* in tobacco increased sensitivity to drought stress and insensitivity to ABA-induced stomatal closure and inhibition of stomatal opening. Furthermore, overexpression of *OsPFA-DSP1* in rice also increased sensitivity to drought stress. These results indicated that OsPFA-DSP1 is a functional PTP and may act as a negative regulator in drought stress responses.

Keywords Protein tyrosine phosphatase · PFA-DSPs · Drought stress · OsPFA-DSP1

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B. Liu and J. Fan contributed equally to this work.

B. Liu · J. Fan · Y. Zhang · P. Mu · P. Wang · J. Su · H. Lai · S. Li · D. Feng · J. Wang · H. Wang (✉)
State Key Laboratory of Biocontrol, Key Laboratory of Gene Engineering of Ministry of Education and Guangdong Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, 510275 Guangzhou, People's Republic of China
e-mail: wanghb@mail.sysu.edu.cn

Introduction

Plants constantly encounter diverse abiotic stresses, such as drought, salt and low temperature in the process of growth and development. To survive these conditions, plants employ a complex set of distinct signaling pathways that trigger stress specific tolerance or avoidance in the organism as a whole (Singh et al. 2010). Reversible protein phosphorylation is the most common mechanism in these signaling transduction pathways, which is catalyzed by protein kinases and protein phosphatases (Kaasik et al. 2001; Luan 2002; Stoker 2005).

Much progress has been made towards understanding the roles of protein kinases, particularly mitogen-activated protein kinases (MAPKs) in many cellular processes, including cytoskeleton rearrangement, phytohormone signaling and stress response (Bartels et al. 2010). However, little is known about protein phosphatases, especially the protein tyrosine phosphatase (PTP) family in plants (Monroe-Augustus et al. 2003; Sokolov et al. 2006). For example, the MAPK phosphatases (MPKs) participate in plant responses to abiotic stresses. The *mkp1* mutant is tolerant to salinity compared with wild type *Arabidopsis*, suggesting the involvement of AtMKP1 in regulation of plant responses to salinity challenges (Ulm et al. 2002). Silencing of *AtMKP2* has been shown to result in ozone sensitivity, indicating its role in oxidative stress response (Lee and Ellis 2007). In rice, *OsMKP1* is rapidly induced by wounding and negatively regulates wound responses (Katou et al. 2007). In recent years, some PTPs have been identified, and these studies suggested that PTPs play important roles in regulating signal transduction pathways and in controlling growth and development of plants (Stoker 2005; Gupta et al. 2002; Sugiyama et al. 2008). The first found plant tyrosine-specific PTP, AtPTP1 in

Arabidopsis thaliana, is encoded by a stress-responsive gene, which is upregulated by high salt and downregulated by cold (Xu et al. 1998). The recombinant AtPTP1 protein can be reversibly inactivated by hydrogen peroxide (H_2O_2), suggesting that AtPTP1 may be a major target for oxidative stress in plants (Gupta and Luan 2003). In *Phalaenopsis amabilis*, the classical PTP gene, *PaPTP1*, was isolated and implicated to play a role in response to wounding (Fu et al. 2011). In wheat, TMKP1 is induced in the sensitive durum wheat variety Mahmoudi and repressed in the tolerant variety Om Rabia3 under salt and osmotic stresses, suggesting that it may play an active role in modulating plant cell responses to these conditions (Zaidi et al. 2010).

Protein tyrosine phosphatases comprise a large and diversified superfamily, including many subfamilies, which have different substrate specificities, such as phosphoproteins, lipids, deoxyribonucleic acids and carbohydrates (Alonso et al. 2004). There are three types of lipid phosphatases in land plants: tumor suppressor phosphatase and tensin homologue deleted in chromosome 10 (PTEN), myotubular myopathy related protein (MTMR) and plant and fungi atypical dual-specificity phosphatase (PFA-DSP) (Roma-Mateo et al. 2007).

The PFA-DSP lipid phosphatases have special substrate specificities for dephosphorylating phosphatidylinositol and comprise a subfamily of PTP that only exists in plants and fungi (Roma-Mateo et al. 2007). AtPFA-DSP1 has displayed in vitro activity towards pyronitrophenyl phosphate (pNPP) and phosphoinositides. In addition, AtPFA-DSP1 has a strong structural and sequence similarity to cysteine phosphatases, but its activity is highly resistant to inhibition by SH-group inhibitors, suggesting that it may act via a different mechanism (Aceti et al. 2008). SIW14, a *Saccharomyces cerevisiae* PFA-DSP, is involved in actin cytoskeleton organization and endocytosis in response to nutrient deprivation and stress conditions (Care et al. 2004). However, no physiological function has been reported for plant PFA-DSPs to date. In this study, we characterized a *PFA-DSP* in rice (*Oryza sativa*), *OsPFA-DSP1*, and overexpressed or silenced it in rice to evaluate its function during abiotic stress. The phosphatase activity and subcellular location of OsPFA-DSP1 were determined, and genetic techniques were used to study its biological functions in transgenic rice and tobacco plants. Our results suggested that OsPFA-DSP1 may function as a negative regulator in response to drought stress.

Materials and methods

Plant materials and growth conditions

Seeds of rice (*O. sativa* cv. Nipponbare) were germinated on 1/2 MS solid medium at 26°C under long-day conditions (16 h light/8 h dark cycle) for stress and hormone treatments. The 10 day-old seedlings were incubated in solutions containing 20% PEG 6000, 250 mM NaCl or 100 μM abscisic acid (ABA) and sampled at designated times. The 20% PEG 6000 solution was used to induce drought stress in plants. The soil grown wild type tobacco and rice, and transgenic tobacco and rice were in a greenhouse with a 16 h light/8 h dark cycle at 28°C.

Semi-quantitative PCR and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from rice seedlings with/without various stress-treated using Trizol (Invitrogen, USA). First-strand cDNAs were synthesized from total RNA using PrimeScript RTase (Invitrogen, USA) according to the manufacturer's protocol. qRT-PCR was performed with a BIO-RAD iQ5 system (BIO-RAD, USA) using SYBR Premix Ex Taq (TaKaRa, Japan) to monitor double-stranded DNA products. The transcript levels of *OsPFA-DSP1* were normalized to the transcript levels of *β*-actin. Each data point had three replicates, and the experiments were repeated twice. The results from the repeated experiments were consistent, and those from one set of experiments are shown. The gene-specific primers were as follows: OsPFA-DSP1, 5′-GCTCCGTCCTGTGCCTCT-3′ and 5′-GCCTC ACGGATTCTATCTTCA-3′; *β*-actin (internal control), 5′-CCTGACGGAGCGTGGTTAC-3′ and 5′-CCAGGGCG ATGTAGGAAAGC-3′.

The following gene-specific primers for were used for semi-quantitative reverse transcription PCR analysis: OsPFA-DSP1, 5′-TATATGCGGCAGGAGGCCAC-3′ and 5′-GCCGTCAACATGAGAATGATGC-3′; *β*-actin, 5′-GG TATTGTTAGCAACTGGGATG-3′ and 5′-GATGAAAG AGGGCTGGAAGA-3′.

Generation of transgenic tobacco and rice

For overexpression analysis, the *OsPFA-DSP1* cDNA was cloned with primers 5′-GAGAAGCTTATGCGGCAGGA GGCC-3′ and 5′-GCATCTAGATCAACATGAGAATG ATG-3′, introducing *Hin*d III and *Xba* I sites (underlined), and inserted into the pCAMBIA 1301 binary vector along with a cauliflower mosaic virus (CaMV) 35S promoter. The resulting 35S::*OsPFA-DSP1* vector was introduced into *Agrobacterium* EHA105, and transformed into tobacco and rice as described (Jones et al. 1995; Toki et al. 2006). To obtain *OsPFA-DSP1* RNAi rice plants, one fragment of sense and antisense *OsPFA-DSP1* cDNA were cloned into the pRiAct1 vector (Zhong et al. 2007), and then the promoter-RNAi fragment digested with *Kpn*I and *Stu*I was transferred into the pCAMBIA1301 binary vector cut with *Kpn*I and *Pml*I. The primers are as follows: sense fragment,

5′-TCTAAGCTTGCGCTTTGCTGCTGCC-3′ and 5′-ATACTGCAGCCCGATGCATCCCTCT-3′, with *Hin*dIII and *Pst*I sites (underlined), respectively; anti-sense fragment, 5′-TCTACGCGTGCGCTTTGCTGCTGCC-3′ and 5′-ATAGTCGACCCCGATGCATCCCTCT-3′, with *Mlu*I and *Sal*I sites (underlined), respectively. These resulting constructs were introduced into *Agrobacterium* EHA105 and then transformed into rice as previously described (Toki et al. 2006). Transgenic tobacco and rice seedlings were screened on 1/2 strength MS solid medium containing 50 μg/L hygromycin.

Expression and purification of the recombinant OsPFA-DSP1 proteins

The coding region of *OsPFA-DSP1* cDNA was amplified with primers 5′-GAGGAATTCATGCGGCAGGAGGCC-3′ and 5′-GCAGTCGACTCAACATGAGAATGATGC-3′, introducing *Eco*RI and *Sal*I sites (underlined), respectively, and then inserted into the pGEX4T-1 (GE Healthcare, USA) vector, which contains a glutathione S-transferase (GST) tag sequence. The recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3), and the expressed proteins were purified according to the handbook of the GST gene fusion system (GE healthcare).

Phosphatase assays

Protein phosphatase activity was determined by using GST-OsPFA-DSP1 fusion protein and pNPP as substrate (Aceti et al. 2008). This assay was performed in 96-well microplates in 160 μl reaction mixtures containing 50 mM Tris–HCl (pH 7.0) buffer at 37°C for 2 h. The reaction was monitored by measuring the increase of absorbance at 405 nm due to the production of *p*-nitrophenol. For determination of kinetic constants, 5 μg of recombinant GST-OsPFA-DSP1 fusion proteins were used in the phosphatase assay. Kinetic parameters were determined by non-linear curve fitting using GraphPad Prism (version 4.02 for Windows, www.graphpad.com). For PTP specific determination, phosphatase activity was measured in the absence or in the presence of PTP inhibitor sodium vanadate (1 mM) and protein phosphatase inhibitor okadaic acid (300 nM). For assaying oxidation sensitivity, the reaction was performed with purified GST-OsPFA-DSP1 protein and pNPP as substrate after incubation for 2 h at 37°C in the presence of the indicated concentrations of H_2O_2 and dithiothreitol (DTT).

Promoter-GUS analysis

The promoter of *OsPFA-DSP1* gene was obtained from Osiris (http://www.bioinformatics2.wsu.edu/cgi-bin/Osiris/cgi/home.pl) and cloned with primers, 5′-ACGGTAGGGGTGGTAATGGGC-3′ and 5′-GTGCAGTGCATATTCGATGAATC-3′. The promoter was then inserted at the *Eco*RI and *Pst*I sites in the expression vector pCAMBIA 1381 to drive the expression of the *GUS* reporter gene. The resulting construct was introduced into rice by *Agrobacterium*-mediated transformation (Toki et al. 2006). The GUS assay was performed at different developmental stages as described (Jefferson et al. 1987).

Subcellular localization analysis

The coding sequence of *OsPFA-DSP1* was cloned into pXDG (Chen et al. 2009) with primers 5′-TATATGCGGCAGGAGGCCAC-3′ and 5′-GCCGTCAACATGAGAATGATGC-3′. The resulting 35S::*GFP-OsPFA-DSP1* vectors were transfected into rice protoplasts, which were isolated from 10 day-old rice green seedlings as described (Zhang et al. 2011). Approximately 10 h after transfection, the nuclei of protoplasts were stained with 100 μg/mL 4′, 6-diamidino-2-phenylindole (DAPI) for 15–30 min. Protoplasts were then observed using an Olympus fluorescent microscope and visualized by Olympus DP2-BSW software. GFP and DAPI were excited by blue light and UV light, respectively.

Drought stress and ABA treatment in transgenic tobacco plants

Four-week-old wild type and transgenic tobacco plants were used for drought stress and ABA treatment experiments. Water-loss rates of detached leaves were measured by monitoring the fresh weight loss at indicated time points. For stomata aperture measurement, leaf disks of 1 cm diameter prepared from healthy and fully-expanded leaves of wild type and *35S::OsPFA-DSP1* plants were floated on open buffer (10 mM MES-KOH, pH 6.15) in the dark for 2 h to promote stomata closure and then transferred to another open buffer (10 mM MES-KOH, 50 mM KCl, pH 6.15) with 20 or 50 μM ABA under light for 2 h. The leaf disks were dried quickly on filter paper. Sections of abaxial epidermis were then harvested by adherent tape transferred to glass slides and observed immediately using a Nikon YS-100 optical microscope.

Drought stress treatment in transgenic rice plants

Three-leaf stage seedlings grown in rice nutrient solution (Shaheen and Hood-Nowotny 2005) containing 20% PEG 6000 were used to simulate drought stress for 10 days. Plants grown on soil at the heading stage were subjected to drought stress for 7 days, and relative conductivity was determined as previously described (Morsy et al. 2005).

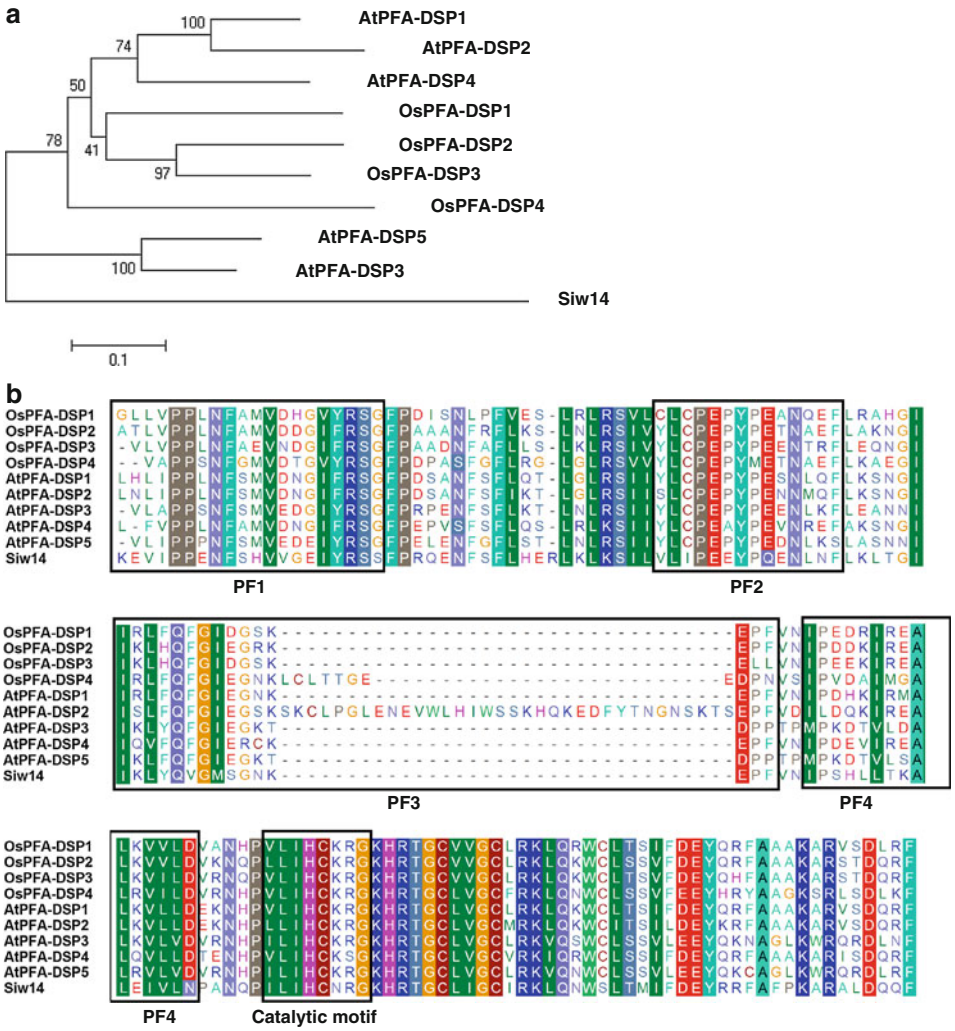

Fig. 1 Phylogenetic analysis and conserved motifs of the PFA-DSP family proteins in *O. sativa* and *A. thaliana*. **a** A phylogenetic tree of PFA-DSP proteins was generated by using the MEGA 4.0 platform with the neighbor-joining method. Siw14 was used as an outgroup. Bootstrap probabilities were obtained from 500 replicates. A *scale bar* is shown below the tree. **b** Members of the *O. sativa* and *A. thaliana* PFA-DSP family share four conserved motifs (PF1–PF4) found in the PRINTS database (identifier: PFDSPHPHTASE). Accession numbers: OsPFA-DSP1, Os09g0135700; OsPFA-DSP2, Os02g0771400; OsPFA-DSP3, Os06g0208700; OsPFA-DSP4, Os12g0420300; AtPFA-DSP1, At1g05000; AtPFA-DSP2, At2g32960; AtPFA-DSP3, At3g02800; AtPFA-DSP4, At4g03960; AtPFA-DSP5, At5g16480; Siw14, YNL032 W

Additionally, three-leaf stage seedlings were air-dried for 2 h at 28°C under continuous light of 150 μmol m^{-2} s^{-1}. Room temperature chlorophyll fluorescence was measured using an IMAGING-PAM chlorophyll fluorometer (MAXI Version; Walz, Efeltrich, Germany). Before measurements, the leaves were adapted to the dark for 10 min. For each measurement, a small light beam was applied, and the minimal fluorescence level (F_o) was measured. F_m was then measured by applying a saturation light pulse. Functional damage to the plants was assayed by calculating the ratio of variable to maximum fluorescence (F_v/F_m) as a measure of the maximal photochemical efficiency of PSII.

Results

OsPFA-DSP1 is a member of the *PFA-DSP* family

Four homologues in *O. sativa*, OsPFA-DSP1, OsPFA-DSP2, OsPFA-DSP3 and OsPFA-DSP4, and five homologues in *A. thaliana*, AtPFA-DSP1, AtPFA-DSP2, AtPFA-DSP3, AtPFA-DSP4 and AtPFA-DSP5, were found by BLASTP search using the OsPFA-DSP1 amino acid sequence and phylogenetic analysis (Fig. 1a). The Siw14 of *S. cerevisiae* was selected as the out group and served as a reference for determination of the evolutionary relationships among these PFA-DSP proteins. OsPFA-DSP1 is a 222-amino-acid protein that has been annotated as a putative DSP of unknown function on the basis of possessing the catalytic signature motif for DSPs (Fig. 1b). The amino acid sequence alignment showed that OsPFA-DSP1 contains four conserved and characteristic motifs of members of the PFA-DSP family, PF1–PF4 (Fig. 1b). These results indicated that OsPFA-DSP1 is a member of PFA-DSP family.

OsPFA-DSP1 with tyrosine-specific protein phosphatase activity in vitro

The full-length *OsPFA-DSP1* cDNA was isolated from *O. sativa* by reverse transcription PCR and cloned into pGEX4T-1. After isopropyl β-D-thiogalactopyranoside

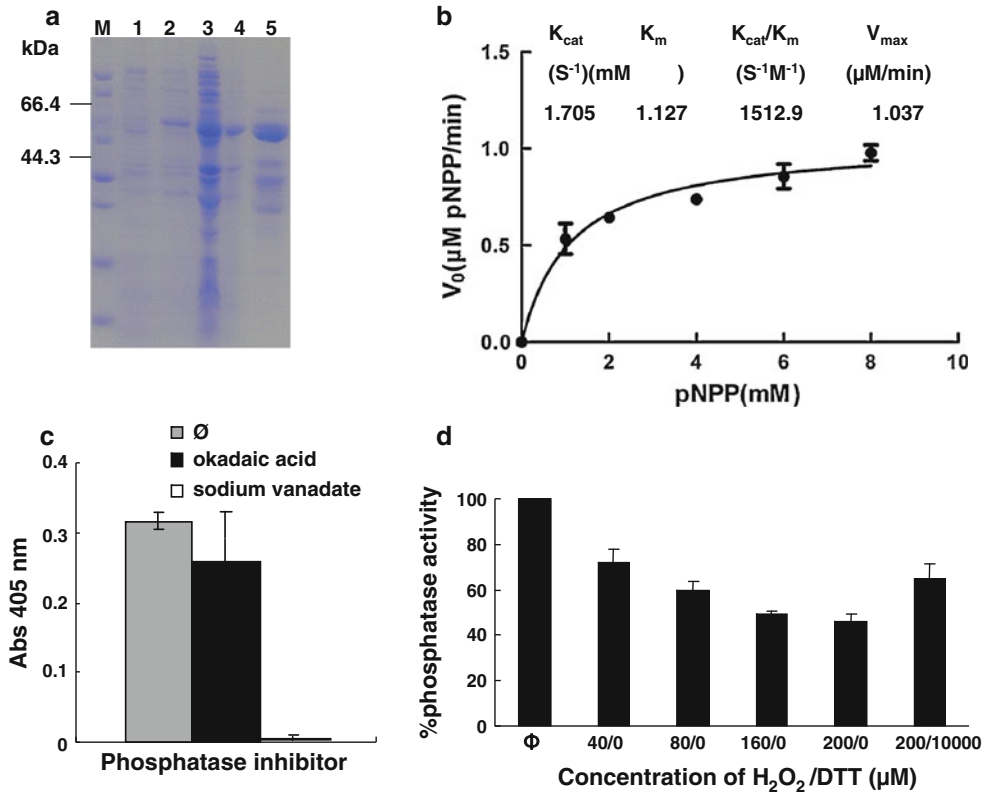

Fig. 2 Purification and phosphatase activity of recombinant GST-OsPFA-DSP1 protein. **a** Expression and purification of GST-OsPFA-DSP1 in *E. coli*. The non-induced (*lane 1*) or induced (*lane 2*) proteins of *E. coli* BL21 cells transformed with GST-OsPFA-DSP1 expression vector. The soluble (*lane 3*), insoluble (*lane 4*) and purified GST-PFA-DSP1 (*lane 5*) proteins were analyzed by 12% SDS-PAGE. Protein molecular masses are shown on the left. **b** Phosphatase assay of GST-OsPFA-DSP1. Reactions were carried out in 50 mM Tris–HCl (pH 7.0) with pNPP substrate. Initial velocities were measured at different pNPP concentrations ranging from 1 to 8 mM, and the data points were fitted to the Michaelis–Menten equations by non-linear regression. **c** Inhibition of GST-OsPFA-DSP1 phosphatase activity assay. The phosphatase activity of GST-OsPFA-DSP1 was measured in the absence (Ø) or presence of phosphatase inhibitors as indicated. **d** Sensitivity of GST-OsPFA-DSP1 to oxidation. GST-OsPFA-DSP1 was incubated with pNPP for 2 h at 37°C in the presence of the indicated concentrations of H_2O_2 or DTT. Activity was measured and the activity inhibition ratio calculated. Ø indicates a mock treatment without H_2O_2 or DTT. In all cases, bars represent the mean of at least three experiments, and error bars represent standard deviations

(IPTG) induction, the recombinant GST-OsPFA-DSP1 fusion protein was purified by using glutathione Sepharose beads (Fig. 2a). The protein phosphtase activity of GST-OsPFA-DSP1 was performed by using pNPP as a substrate and GST-OsPFA-DSP1 was active with a *Km* of 1.127 mM (Fig. 2b).

To determine whether OsPFA-DSP1 is a tyrosine-specific protein phosphatase, phosphatase activity assays were conducted using different phosphatase inhibitors. The catalytic activity of GST-OsPFA-DSP1 was reduced to 81% in the presence of the Ser/Thr phosphatase inhibitor okadaic acid, while it dramatically decreased to 1% in the presence of the PTP inhibitor sodium vanadate (Fig. 2c). The results showed that OsPFA-DSP1 is a tyrosine-specific protein phosphatase.

In oxidation sensitivity assays, the catalytic activity of GST-OsPFA-DSP1 was reduced to 46% following the addition of 200 mM H_2O_2, and the phosphatase activity recovered to 65% with addition of the reducing agent DTT (Fig. 2d). These results indicated that OsPFA-DSP1 is inactivated by oxidation, which is considered a general mechanism of PTP inactivation (Peters et al. 2003).

To determine the effect of metal ions on the activity of GST-OsPFA-DSP1 protein, various ions and their chelator EDTA were added to the reaction system. As shown in Table 1, Na^+, Mg^{2+}, Al^{3+} or EDTA had no significant effect on the phosphatase activity of GST-OsPFA-DSP1, which suggested that OsPFA-DSP1 is a metal independent PTP (Table 1).

Expression profiles and subcellular localization assays

To determine the expression pattern of *OsPFA-DSP1*, qRT-PCR was carried out and *Promoter*$_{OsPFA\text{-}DSP1}$*::GUS*

Table 1 Effect of metal ions on phosphatase activity of GST-OsPFA-DSP1 proteins

Chemicals	Concentration (mM)	Control GST-OsPFA-DSP1 (%)
None	0	100.00
NaF	20	77.42
$MgCl_2$	5	93.55
$AlCl_3$	0.1	90.32
EDTA	10	109.38

Using pNPP as a substrate, the phosphatase activity of GST-OsPFA-DSP1 was measured as A_{405} in the presence of various chemicals relative to the control (set as 100%)

transgenic plants were generated. *OsPFA-DSP1* transcripts were apparently induced by 20% PEG 6000 (to simulate drought), ABA and NaCl, with expression peaking around 36 h after treatment with 20% PEG 6000, 4 h after treatment with ABA and 6 h after treatment with NaCl (Fig. 3a). We also used GENEVESTIGATOR V3 to analyze the expression profiles of *OsPFA-DSP1* (Hruz et al. 2008). The probe Os.38393.1.S1_at of *OsPFA-DSP1* was used to analyze an *O. sativa* microarray database (OS_51 K: Rice Genome 51 K array) with abiotic stress and hormone treatments. *OsPFA-DSP1* was found to be upregulated by drought, salt and trans-zeatin, downregulated by heat, with not obvious response to cold, indole-acetic acid (IAA), kinetin (KT), naphthylacetic acid (NAA) and gibberellin (GA) (Fig. 3b).

In histochemical staining of the $Promoter_{OsPFA\text{-}DSP1}$*::GUS* transgenic plants, GUS activity was observed in whole young seedlings, including roots, stems and leaves, while it was little detected in mature seedlings (between heading stage and flowering stage) (Fig. 3c). Similar results were observed in GENEVESTIGATOR analysis, in which nine development stages were shown. The *OsPFA-DSP1* was mainly expressed in seedlings, booting stage and dough stage, while expressed little in other stages (Fig. 3d).

To investigate the subcellular localization of OsPFA-DSP1, GFP-OsPFA-DSP1 was transiently expressed in rice green protoplasts. GFP alone has been reported in both the cytoplasm and nucleus (Launholt et al. 2006; Seibel et al. 2007), which was confirmed in rice green protoplasts in our study (Fig. 4). GFP-OsPFA-DSP1 showed a similar localization pattern as GFP (Fig. 4), filling both the cytoplasm and nucleus, which could be seen by blue DAPI staining. In addition, the previously reported protein dual-specificity phosphatases, such as LePP5, IBR5 and PP2C, also have cytoplasmic and nucleus localization analyzed by fusing with fluorescent proteins (De la Fuente van Bentem et al. 2003; Lee et al. 2009; Brock et al. 2010). These data indicated that OsPFA-DSP1 may target to both the cytoplasm and nucleus.

Ectopic overexpression of *OsPFA-DSP1* in tobacco increases sensitivity to drought stress

To understand the biological function of *OsPFA-DSP1*, transgenic tobacco plants constitutively expressing *OsPFA-DSP1* (*35S::OsPFA-DSP1*) were generated through *Agrobacterium*-mediated transformation. *OsPFA-DSP1* mRNA accumulation in transgenic plants (OX 3, OX 13 and OX 25) were determined by semi-quantitative reverse transcription PCR. OX 25 showed the highest expression level of *OsPFA-DSP1* (Fig. 5a) and thus was selected for further analysis. There were no obvious phenotype differences between wild type plants and *35S::OsPFA-DSP1* plants under normal growth conditions (Fig. 5b). Four-week-old transgenic plants and wild type plants were subjected to drought stress by withholding water for 7 days. As shown in Fig. 5b, after 7 days of drought stress treatment, *35S::OsPFA-DSP1* plants showed less vigorous growth and were more wilted than wild type plants, indicating that ectopic overexpression of *OsPFA-DSP1* in tobacco plants resulted in increased sensitivity to drought stress.

Subsequently, we measured the water loss rates of detached leaves from the wild type plants and *35S::OsPFA-DSP1* plants. The detached leaves of *35S::OsPFA-DSP1* plants lost water more quickly than those of the wild type (Fig. 5c), suggesting that ectopic overexpression of *OsPFA-DSP1* in tobacco plants may increase sensitivity to drought stress through enhancing water loss.

During drought, ABA levels increase dramatically in plants and are involved in stomata behavior (Zeevaart 1980). Low ABA concentrations promote stomatal closure, and high ABA concentrations suppress light-induced stomatal opening (Garcia-Mata and Lamattina 2007). Thus, the stomata apertures of transgenic and WT tobacco were examined. Stomata apertures between the two types of plants were not significantly different without ABA treatment. When treated with 20 μM ABA, the stomatal opening of wild type plants was inhibited to a great extent, while the stomatal opening in *35S::OsPFA-DSP1* plants were statistically significantly larger than that of wild type plants ($P < 0.05$) (Fig. 5d). Similar results were observed with 50 μM ABA treatment. These results indicated that OsPFA-DSP1 in transgenic plants may be involved in the ABA signaling pathway in response to drought.

Overexpression of *OsPFA-DSP1* in rice also results in sensitivity to drought stress

To investigate the physiological function of OsPFA-DSP1, we generated and selected three independent lines of *OsPFA-DSP1*-overexpressing and *OsPFA-DSP1*-RNAi rice plants for further study, respectively. A 406-bp corresponding to 97 bp of the 3′-terminal of *OsPFA-DSP1*

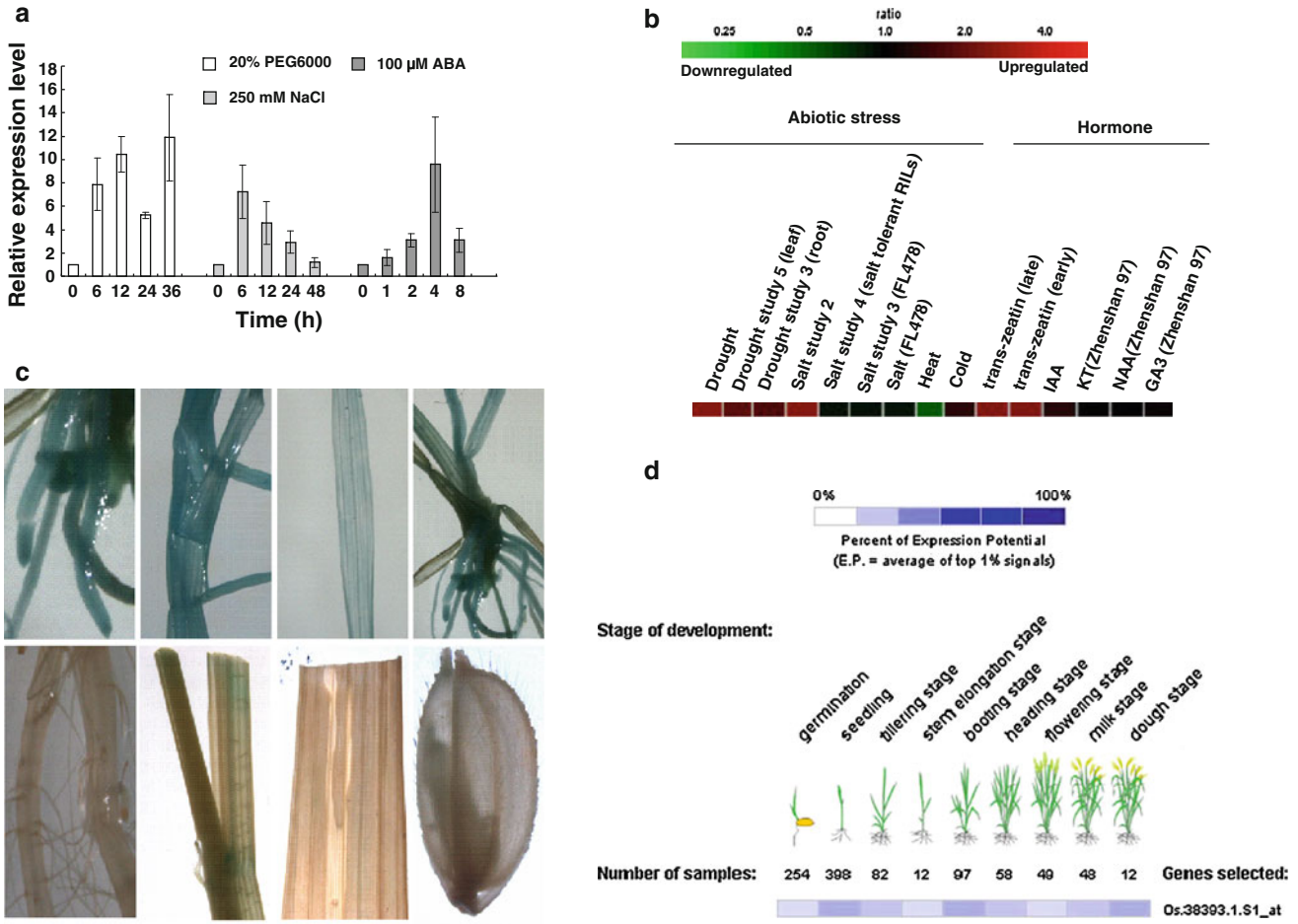

Fig. 3 Expression patterns of *OsPFA-DSP1* in rice. **a** Expression of *OsPFA-DSP1* in response to various abiotic stresses using qRT-PCR. Ten-day-old seedlings were subjected to drought (20% PEG 6000), NaCl (250 mM) or ABA (100 μM). **b** Inducible expression patterns of *OsPFA-DSP1*. The expression profiles were obtained from an OS_51 K: rice genome 51 K array as reported by GENEVESTIGATOR V3, which illustrates different expression levels of *OsPFA-DSP1* under different abiotic stresses and hormones treatments. Results are presented as a heat map, with *red* indicating higher levels and *green* indicating lower levels of transcript accumulation. **c** Tissue expression pattern of *OsPFA-DSP1* by GUS staining in Promoter$_{OsPFA\text{-}DSP1}$*::GUS* transgenic rice plants. Images in the upper panel represent young root, stem, leaf and seedling (*left* to *right*). Lower panels represent mature root, stem, leaf and spike (*left* to *right*). **d** Developmental expression patterns of *OsPFA-DSP1*. The expression profiles obtained from an OS_51 K: Rice Genome 51 K array as reported by GENEVESTIGATOR V3, which illustrates different expression levels of *OsPFA-DSP1* in different developmental stages. Results are given as a heat map with *blue*/*white* coding that reflects absolute signal values, where darker shades of *blue* represent stronger expression (color figure online)

coding sequence and 309 bp of the 3′-UTR sequence was used to specifically silence *OsPFA-DSP1* by BLAST search. By qRT-PCR analysis, relative *OsPFA-DSP1* expression levels of independent transgenic plants lines OX-4, OX-10 and OX-11 showed 23, 18 and fourfold increases, respectively, compared to that of wild type (Fig. 6a). Relative *OsPFA-DSP1* expression levels of the three RNAi plant lines were 10–20% compared to that of WT plants (Fig. 6b). Three-leaf stage rice seedlings grown in nutrient solution were transferred to the nutrient solution containing 20% PEG 6000 to simulate drought stress. After 10 days of treatment, *OsPFA-DSP1*-overexpressing plants exhibited more wilting than wild type and *OsPFA-DSP1*-RNAi plants (Fig. 6c). Additionally, the relative conductivity of *OsPFA-DSP1*-overexpressing plants lines OX-10 and OX-11 increased to 17 and 8%, respectively, with the value of OX-10 being significantly higher than that of wild type plants ($P < 0.05$). Meanwhile, the *OsPFA-DSP1*-RNAi plants had similar values of relative conductivity to that of wild type plants (Fig. 6d).

Plants at the heading stage were also subjected to drought stress. *OsPFA-DSP1*-overexpressing plants began to wilt after 4 days of drought, while the wild type and *OsPFA-DSP1*-RNAi plants did not show wilting until 7 days of drought (Fig. 6e). The relative conductivity increased from 3 to 58% in *OsPFA-DSP1*-overexpressing

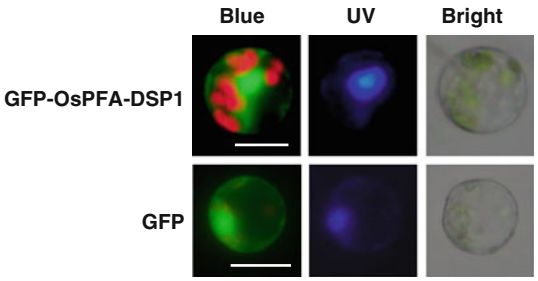

Fig. 4 Subcellular localization of OsPFA-DSP1. GFP or GFP-OsPFA-DSP1 fusion proteins were transiently expressed in rice protoplasts and observed using an Olympus fluorescent microscope. *Green* signal indicates GFP, *red* signal indicates chlorophyll fluorescence, which is also excited by *blue* light. The DAPI signal (nucleus) indicated in *blue* is excited by UV light. Bright field images are also shown. *Scale bars* 10 μm (color figure online)

plants, while it only increased from 3 to 25% in wild type plants. The *OsPFA-DSP1* RNAi-2 plants had lower relative conductivity after drought stress, increasing from 4 to 15% (Fig. 6f). The increased drought sensitivity of *OsPFA-DSP1*-overexpressing plants was further verified by measuring changes of chlorophyll fluorescence. Lower F_v/F_m was also observed in *OsPFA-DSP1*-overexpressing plants compared to wild type and *OsPFA-DSP1* RNAi plants under drought stress (Fig. 6g). These results suggested that OsPFA-DSP1 participates in and may be a negative regulator in the plant response to drought stress.

Discussion

Like protein kinases, phosphatases also perform pivotal functions in signal transduction networks at different plant developmental stages and under various stress conditions (Singh et al. 2010). Most phosphatases have been described in *Arabidopsis*, but few have been characterized in rice. Here, we identified a rice PFA-DSP, OsPFA-DSP1, which

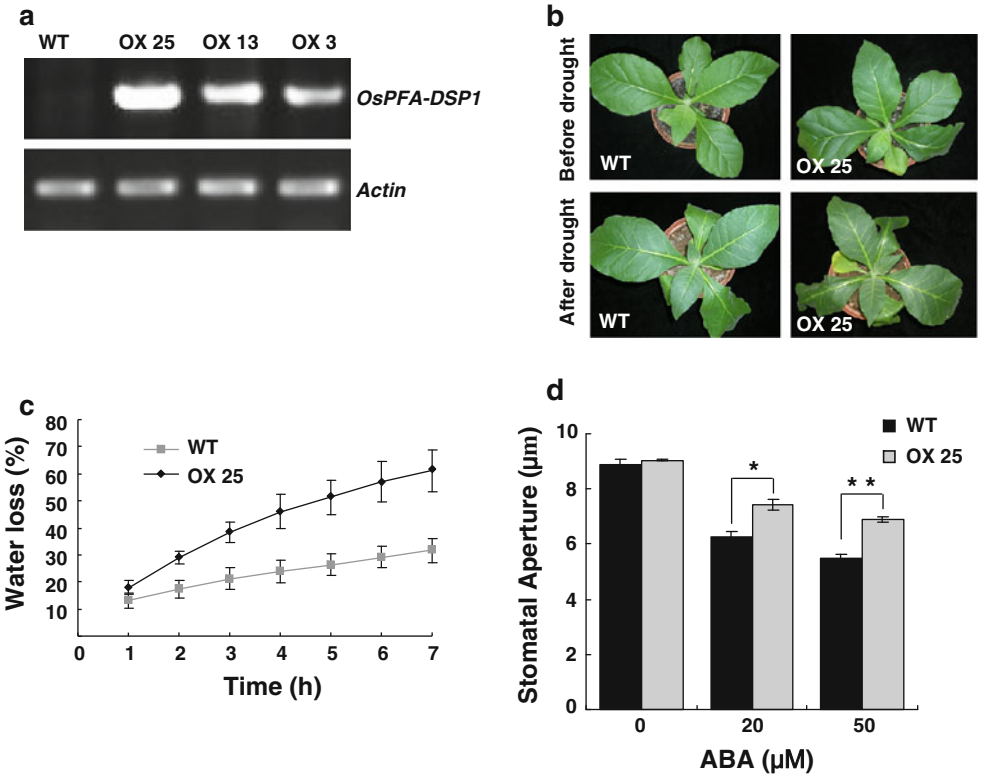

Fig. 5 Ectopic expression of *OsPFA-DSP1* increased sensitivity to drought stress in transgenic tobacco plants. **a** Semi-quantitative reverse transcription PCR detection of *OsPFA-DSP1* transcripts in transgenic tobacco lines OX 25, OX 13 and OX 3. **b** Phenotypes of wild type and *35S::OsPFA-DSP1* transgenic tobacco under drought stress. Four-week-old wild type and *35S::OsPFA-DSP1* plants were supplied with the same volume of water and then drought treated for 7 days in natural conditions, followed by watering for recovery. Images of representative plants after the seventh day of drought stress are shown. **c** Water loss rate of detached leaves from wild type plants and *35S::OsPFA-DSP1* plants at indicated time points under normal conditions. **d** Stomatal aperture of wild type and *35S::OsPFA-DSP1* transgenic OX 25 line under ABA treatment. Leaf disks of 1 cm diameter were prepared from healthy and fully-expanded leaves of wild type and transgenic line OX 25 plants, and floated in open buffer (10 mM MES-KOH, pH 6.15) in the dark for 2 h to promote stomata closure, then transferred to another open buffer (10 mM MES-KOH, 50 mM KCl, pH 6.15) with 20 μM or 50 μM ABA under light for 2 h ($n = 3$ independent experiments with 80 stomata at each data point). $^*P < 0.05$ and $^{**}P < 0.01$, compared with control plants given the same treatment

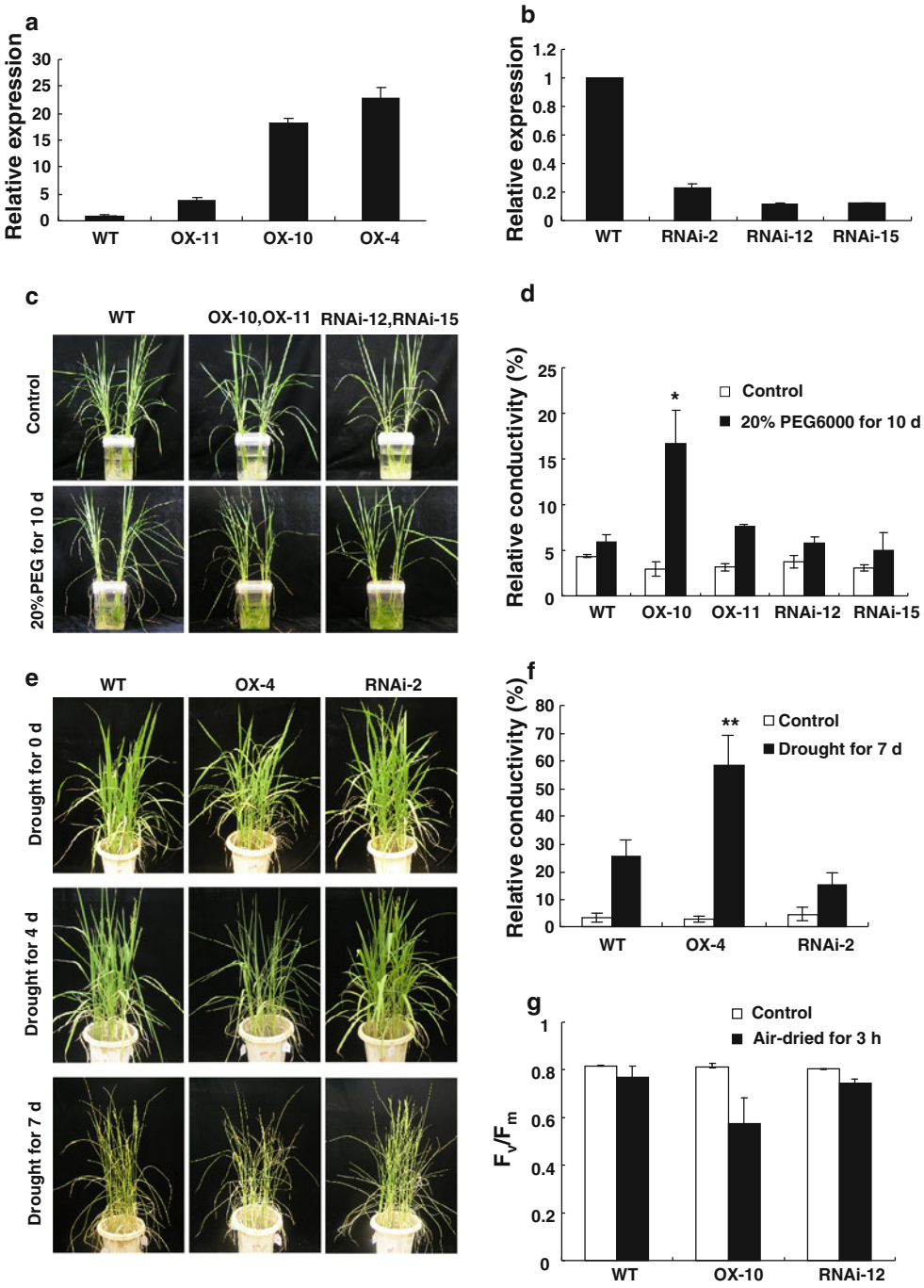

Fig. 6 Overexpression of *OsPFA-DSP1* in rice conferred sensitivity to drought stress. *OsPFA-DSP1* transcripts in **a** *OsPFA-DSP1*-overexpressing and **b** *OsPFA-DSP1*-RNAi plants were detected by qRT-PCR. **c** Phenotype of three-leaf stage wild type, *OsPFA-DSP1*-overexpressing and *OsPFA-DSP1*-RNAi plants treated with 20% PEG 6000 to simulate drought for 10 days. **d** Relative conductivity of rice leaves after 20% PEG 6000 treatment for 10 days. **e** Wild type, *OsPFA-DSP1*-overexpressing and *OsPFA-DSP1*-RNAi plants were subjected to natural drought stress at the heading stage. **f** Relative conductivity of rice leaves after drought stress treatment for 7 days. **g** Changes in chlorophyll fluorescence of wild type, *OsPFA-DSP1*-overexpressing and *OsPFA-DSP1*-RNAi plants during drought stress. Three-leaf stage seedlings were air-dried for 3 h at 28°C under continuous light of 150 μmol m^{-2} s^{-1}. Each data point represents the mean ± SE of triplicate experiments ($n = 4$). *$P < 0.05$ and **$P < 0.01$, compared with control plants given the same treatment

has PTP activity and may function as a negative regulator in response to drought stress.

OsPFA-DSP1 belongs to the PFA-DSP subfamily, displaying high homology with *Arabidopsis* AtPFA-DSP1 (Fig. 1a), of which the phosphatase catalytic properties has been characterized in vitro (Aceti et al. 2008; Roma-Mateo et al. 2007). Here, we demonstrated that OsPFA-DSP1 also has phosphatase activity in vitro (Fig. 2b). The catalytic activity of OsPFA-DSP1 was inhibited by the PTP specific inhibitor sodium vanadate, but not significantly affected by the serine/threonine phosphatase inhibitor okadaic acid (Fig. 2c), suggesting that OsPFA-DSP1 is a PTP. Oxidation is an important regulatory mechanism for the PTP family of enzymes (den Hertog et al. 2005), and OsPFA-DSP1 activity was also shown to be sensitive to oxidation (Fig. 2d).

Examination of the expression patterns under various stress conditions and in different plant organs may provide insights into the underlying physiological, biochemical and molecular mechanism of stress tolerance and regulation of

development (Singh et al. 2010). *OsPFA-DSP1* transcripts were mainly expressed in seedlings, booting stage and dough stage, and strongly upregulated by drought, high salt, ABA and trans-zeatin treatments (Fig. 3). These results indicated that *OsPFA-DSP1* may be involved in the response to multiple abiotic stresses, especially drought stress, which was also consistent with the results of a previous microarray analysis (Singh et al. 2010).

Tobacco plants ectopically overexpressing *OsPFA-DSP1* exhibited lower drought tolerance than did the wild type (Fig. 5b), while there were no obvious differences between these plants in other conditions (NaCl and H_2O_2 treatments, data not shown). This phenotype may have been conferred by the ectopic overexpression of *OsPFA-DSP1* in tobacco, but this idea was dismissed as *OsPFA-DSP1*-overexpressing rice plants were also sensitive to drought stress (Fig. 6c and e).

Drought is a major environmental stress that affects growth and development of plants. Under drought stress, stomatal pores located on the plant epidermis regulate the loss of water by transpiration, and reversible phosphorylation participates in the regulation of stomatal aperture. During drought, plants accumulate ABA, which induces the rapid closing of stomata (Cominelli et al. 2005), and a high concentration of ABA inhibits light-induced stomatal opening (Garcia-Mata and Lamattina 2007). In contrast to wild type tobacco plants, *OsPFA-DSP1*-overexpressing tobacco showed high insensitivity in ABA-induced stomatal closure and inhibition of stomatal opening (Fig. 5d) that may result in increased water loss by transpiration (Fig. 5c). *OsPFA-DSP1*-overexpressing tobacco exhibited stomata behavior similar to that in the *AtMPK3* knockdown plant, which displayed insensitivity to ABA-induced inhibition of stomatal opening (Gudesblat et al. 2007). These results indicated that OsPFA-DSP1 may be involved in ABA-mediated stomata behavior under drought stress.

In *OsPFA-DSP1*-overexpressing rice plants, the relative conductivities of three independent lines OX-4, OX-10 and OX-11 were both improved compared to that of wild type (Fig. 6d, f). The improvement in relative conductivity of OX-11 was not significant compared to that of OX-4 and OX-10, as the relative expression level of *OsPFA-DSP1* in OX-11 was also lower than that of OX-4 and OX-10 (Fig. 6a). Notably, the relative conductivity and phenotype of the *OsPFA-DSP1*-RNAi plants were not significantly different from wild type (Fig. 6c–f), perhaps due to the functional redundancy of other homologues in rice (Fig. 1a). In addition, *OsPFA-DSP1* overexpression resulted in lower F_v/F_m levels (Fig. 6g), consistent with the high water loss rate in the transgenic tobacco plants (Fig. 5c). Nevertheless, before our study, there has been no evidence for the specific role of PTP in drought stress, although several kinases, such as OsMPK5, AtMPK4 and AtMPK6, have been reported to be involved in the drought stress response (Xiong and Yang 2003; Ichimura et al. 2000). Our results indicated that *OsPFA-DSP1* is involved in drought stress responses and may function as a negative regulator.

Drought stress is a complex process involving a large number of pathways that subtly make changes and send signals around the plant over a period of time. Therefore, it is difficult to mimic true drought stress conditions. The 20% PEG 6000 treatment also simulates osmotic stress, and even allowing the tissue to dessicate in dry air does not faithfully replicate natural drought stress. However, combining the results obtained from 20% PEG 6000 treatment and natural drought stress should be informative.

In conclusion, we have identified a rice PFA-DSP, OsPFA-DSP1, which was demonstrated to be a functional PTP in vitro, and *OsPFA-DSP1* mRNA was shown to be induced by drought, high salt and ABA. Overexpression of *OsPFA-DSP1* in rice and tobacco plants increased plant sensitivity to drought stress, indicating that OsPFA-DSP1 functions as a negative regulator in response to drought stress. However, to further understand the role of OsPFA-DSP1 in the plant response to drought stress, it will be crucial to identify the upstream and downstream effectors in OsPFA-DSP1-related signaling pathways and to demonstrate whether OsPFA-DSP1 functions in the drought response through its PTP activity. As one of the PFA-DSPs, OsPFA-DSP1 should be further studied to identify its physiological targets, such as proteins and phosphoinositides.

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