A STRESS-RESPONSIVE NAC1-Regulated Protein Phosphatase Gene Rice Protein Phosphatase18 Modulates Drought and Oxidative Stress Tolerance through Abscisic Acid-Independent Reactive Oxygen Species Scavenging in Rice

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Plants respond to abiotic stresses through a complexity of signaling pathways, and the dephosphorylation mediated by protein phosphatase (PP) is an important event in this process. We identified a rice (Oryza sativa) PP2C gene, OsPP18, as a STRESS-RESPONSIVE NAC1 (SNAC1)-regulated downstream gene. The ospp18 mutant was more sensitive than wild-type plants to drought stress at both the seedling and panicle development stages. Rice plants with OsPP18 suppressed through artificial microRNA were also hypersensitive to drought stress. Microarray analysis of the mutant revealed that genes encoding reactive oxygen species (ROS) scavenging enzymes were down-regulated in the ospp18 mutant, and the mutant exhibited reduced activities of ROS scavenging enzymes and increased sensitivity to oxidative stresses. Overexpression of OsPP18 in rice led to enhanced osmotic and oxidative stress tolerance. The expression of OsPP18 was induced by drought stress but not induced by abscisic acid (ABA). Although OsPP18 is a typical PP2C with enzymatic activity, it did not interact with SNF1-RELATED PROTEIN KINASE2 protein kinases, which function in ABA signaling. Meanwhile, the expression of ABA-responsive genes was not affected in the ospp18 mutant, and the ABA sensitivities of the ospp18 mutant and OsPP18-overexpressing plants were also not altered. Together, these findings suggest that OsPP18 is a unique PP2C gene that is regulated by SNAC1 and confers drought and oxidative stress tolerance by regulating ROS homeostasis through ABA-independent pathways.

Abiotic stresses, such as drought or salinity, cause intensive losses to agricultural production worldwide. Plants respond to these stresses at both the cellular and molecular levels by perception and transduction of stress signals through a complexity of signaling pathways. Activation of a large number of stress-responsive regulatory genes and synthesis of various functional proteins by these signaling transduction mechanisms lead to diverse physiological and metabolic responses so as to confer tolerance to the environmental stresses (Xiong et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Reversible protein phosphorylation is an important event in signal transduction, and it regulates virtually all cellular activities in eukaryotic systems (Luan, 2003). For protein phosphorylation, a protein kinase transfers the terminal phosphate group of an ATP molecule to the hydroxyl group on a Ser, Thr, or Tyr side chain of the protein. The reverse reaction or dephosphorylation is instead catalyzed by a protein phosphatase (PP; Alberts et al., 2002). The attachment to or removal of a phosphate group from a protein often has profound effects on the structure and thereby, the functional properties of the protein (Luan, 2003). Moreover, as a result of the combined activities of protein kinases and PPs, the phosphate groups on proteins are continually turning over, being added, and then rapidly removed (Alberts et al., 2002). Such phosphorylation cycles are important in allowing the phosphorylated proteins to switch rapidly from one state to another, which allows plants to respond to stress stimuli rapidly and accurately.

According to the substrate specificity, PPs can be categorized into Ser/Thr and Tyr phosphatases. The earliest biochemical studies in animal systems defined two major types (PP1 and PP2) of Ser/Thr phosphatases based on their substrate specificity and pharmacological properties (Cohen, 1989). The PP2-type phosphatases can be further classified into PP2A, PP2B, and PP2C simply by their dependence on divalent cations: PP2B and PP2C are activated by Ca$^{2+}$ and Mg$^{2+}$, respectively, whereas PP2A, like PP1, does not require divalent cations for activation (Luan, 2003). A unique feature of plant Ser/Thr phosphatases is the abundance and diversity of PP2C-type
enzymes (Luan, 2003). In the Arabidopsis (Arabidopsis thaliana) genome, there are 76 PP2Cs that are clustered into 10 groups (A–J; Schweighofer et al., 2004). In the rice (Oryza sativa) genome, 90 PP2Cs were identified and classified into 11 groups (A–K; Singh et al., 2010). Plant PP2C proteins have been shown to have critical roles in signaling pathways involved in hormone and stress responses, organ formation, and flower development (Luan, 2003; Schweighofer et al., 2004). In Arabidopsis, group A PP2Cs, including ABSCISIC ACID INSENSITIVE1 (ABI1), are well characterized and described as negative regulators of abscisic acid (ABA) signaling pathways involved in diverse processes, including seed germination, stomatal movement, and stress responses (Leung et al., 1997; Saez et al., 2004; Kuhn et al., 2006; Zhang et al., 2012). ABI1 and PP2CA are also essential for plant tolerance to several abiotic stresses, including salt, drought, and freezing (Strizhov et al., 1997; Gosti et al., 1999; Tähtiharju and Palva, 2001). The group B member ARABIDOPSIS SER/THR PHOSPHATASE OF TYPE 2C 1 (AP2C1) inactivates the mitogen-activated protein kinases (MAPKs) MPK4 and MPK6 involved in regulating stress hormone levels and defense responses (Schweighofer et al., 2007). 

Medicago sativa PP2C (MP2C), a wound-induced alfalfa (Medicago sativa) PP2C with homology to AP2C1, also acts as a negative regulator of MAPK pathways (Meskiene et al., 2003). Group C includes the Arabidopsis PP2C proteins POLTERGEIST and POLTERGEIST-like, which are integral components of the CLAVATA/WUSCHEL-RELATED HOMEobox signaling pathway that is essential for maintenance of both the root and shoot stem cells (Yu et al., 2003; Song et al., 2008). The kinase-associated PP is a membrane-anchored PP2C that interacts with several receptor-like kinases and functions as a negative regulator of receptor-like kinase signaling pathways (Stone et al., 1994; Shah et al., 2002). Kinase-associated PP is also a component of a unique Na⁺ adaptation pathway (Manabe et al., 2008). Among 90 predicted PP2Cs in rice, very few members have been reported. XA21 BINDING PROTEIN15 encodes a PP2C that negatively regulates the XA21-mediated innate immune response in rice (Park et al., 2008). DCWI1 (for DOWN-REGULATED GENE11 IN CW-TYPE CYTOPLASMIC MALE STERILE) encodes a PP2C and has a role as a mitochondrial signal transduction mediator in pollen germination (Fujii and Toriyama, 2008). 

BENZOTHIAZIAZOLE-INDUCED PROTEIN PHOSPHATASE 2C genes (OsBIPP2C1 and OsBIPP2C2) are significantly induced by benzothiazole, one of the defense-related signal molecules in plants, and over-expression (OE) of OsBIPP2C1 or OsBIPP2C2 in transgenic tobacco (Nicotiana tabacum) plants resulted in increased disease resistance (Hu et al., 2006b, 2009). To date, however, no PP2C involved in drought stress has been reported in rice.

Our previous study reported that overexpression of STRESS-RESPONSIVE NAC1 (SNAC1), a stress-responsive NAC (for NAM, ATAC, and CUC2) transcription factor gene, resulted in increased stomatal closure and improved drought and salt tolerance (Hu et al., 2006a). In this study, a rice group F2 PP2C gene, OsPP18, was characterized as a target gene of the transcription factor SNAC1. We show that SNAC1 can bind to the promoter of the OsPP18 gene and activate its expression. The ospp18 mutant showed increased drought and oxidative sensitivity through the reduced activity of reactive oxygen species (ROS) scavenging enzymes, whereas OE of OsPP18 enhanced osmotic and oxidative tolerance in transgenic rice. A significant finding is that OsPP18 mediates drought and oxidative stress responses by regulating ROS homeostasis through ABA-independent pathways.

RESULTS

OsPP18 Is Regulated by SNAC1

OsPP18 (LOC_Os02g05630) is a predicted PP2C gene that is up-regulated in SNAC1-overexpressing plants based on microarray analysis (Hu et al., 2006a). Up-regulation of OsPP18 in the SNAC1-overexpressing plants was confirmed by real-time quantitative reverse transcription (qRT)-PCR analysis (Fig. 1A). However, the expression of OsPP18 was significantly down-regulated in the SNAC1-artificial microRNA (amiRNA) transgenic plants with the expression of SNAC1 suppressed (Fig. 1B). These results indicate that the expression of OsPP18 is positively regulated by SNAC1.

The NAC recognition sequence and core DNA-binding sequence (CDBS) for the NAC protein have been identified in Arabidopsis (Tran et al., 2004). Structure analysis of the SNAC1 NAC domain revealed structural similarities with the reported structure of the Arabidopsis ANAC NAC domain (Chen et al., 2011). To test whether SNAC1 can bind to the promoter of OsPP18, a genomic fragment of the OsPP18 promoter containing the CDBS located upstream of the start codon (Fig. 1C) was fused upstream of the HIS3 minimal promoter to serve as a reporter construct, designated as pHIS2-P<sub>OsPP18</sub> and the pGAD-SNAC1 plasmid (containing the putative DNA-binding domain of SNAC1 fused to the GAL4 activation domain upstream of the HIS3 minimal promoter to serve as an effector construct). The two constructs were cotransformed into the yeast (Saccharomyces cerevisiae) strain Y187. The cotransformants were able to grow on synthetic dropout/Leu⁻/Trp⁻/His⁻ medium with 30 mM 3-amino-1, 2, 4-Triazole, whereas the negative control could not (Fig. 1D), indicating that SNAC1 could bind to the OsPP18 promoter in yeast. Furthermore, chromatin immunoprecipitation (ChIP) PCR assays indicated that the SNAC1 protein could bind to the promoter fragment of OsPP18 (Fig. 1E). These results suggest that OsPP18 is a downstream gene of SNAC1.

OsPP18 Encodes a PP2C

The genomic sequence of OsPP18 consists of eight exons, and the open reading frame is predicted to encode a protein with 348 amino acids with high similarity to PP2C. The PP2C catalytic domain of OsPP18 is located between amino acids 82 and 321, which are
predicted to have phosphatase catalytic activity. PP2C is an Mn$^{2+}$- or Mg$^{2+}$-dependent protein Ser/Thr phosphatase, and metal-associating residues were revealed by analysis of the crystal structure of human PP2Cα (Das et al., 1996). Although the sequence identity between the catalytic domains of OsPP18 and the corresponding catalytic region of human PP2Cα (amino acids 23–284) was only 33%, all of the metal-associating residues (Glu-37, Asp-38, Asp-60, Gly-61, Asp-239, and Asp-282) of human PP2Cα were conserved in OsPP18 (Supplemental Fig. S1A).

OsPP18 belongs to the group F2 of the PP2C gene family in rice according to Singh et al. (2010). There are five PP2Cs from Arabidopsis and seven PP2Cs from rice in the group F2. Sequence alignment of the PP2C domain of OsPP18 with other evolutionarily related phosphatases also suggests that OsPP18 belongs to group F2 rather than group A, which contains ABI1, ABI2, HYPERSENSITIVE TO ABA1 (HAB1), and HAB2 that are known as negative regulators of ABA signaling (Hubbard et al., 2010; Supplemental Fig. S1B). OsPP18 and Arabidopsis HOPW1-1-INTERACTING2 (WIN2) are in the same cluster (with 83% identity to WIN2). WIN2 interacts with HopW1-1, which is a modular Pseudomonas syringae Avr effector for eliciting a resistance response in Arabidopsis and partially required for HopW1-1-induced disease resistance (Lee et al., 2008). To date, the functions of the rice group F2 PP2Cs, including OsPP18, have not been reported.

First, we examined whether OsPP18 is a functional PP2C phosphatase. The recombinant glutathione-S-transferase (GST)-OsPP18 and GST proteins were purified for the phosphatase activity assay by measuring the release of phosphate from a phosphorylated synthetic peptide (Donella Deana et al., 1990). The results showed that, in the presence of the phosphorylated peptide substrate, GST-OsPP18 but not GST alone catalyzed the release of phosphate (Fig. 2B). This reaction was inhibited by the Ser/Thr phosphatase inhibitor sodium fluoride but not by vanadate, a Tyr phosphatase inhibitor (Fig. 2B). We further incubated the recombinant GST-OsPP18 and GST proteins with the substrate in various reaction buffers, which were optimized for the activity assays of different types of PP classes. The results indicated that OsPP18 enzyme activity was detected only in PP2C buffer and not in PP2A and PP2B buffers (Fig. 2C). Moreover, the presence of Mg$^{2+}$ in the PP2C buffer was required for the full activity of OsPP18. These results show that OsPP18 encodes an enzymatically active PP2C.

The Expression Profile and Subcellular Localization of OsPP18

Because OsPP18 is a direct target gene of a stress-inducible transcription factor SNAC1, the expression of OsPP18 under various abiotic stresses was investigated in rice ‘Zhonghua 11’ (ZH11) by quantitative (q)PCR. The results showed that the OsPP18 transcript level strongly increased after drought stress but was not significantly changed under other stress treatments (Fig. 3A).
submergence treatment. Under various phytohormone treatments, OsPP18 was transiently induced only by jasmonic acid (JA) and salicylic acid (SA), and of special note, it was not induced by ABA (Fig. 3B).

The expression profile of OsPP18 during the entire lifecycle of rice was also investigated by extracting the relative expression values of OsPP18 from the microarray dataset in the Collection of Rice Expression Profiles database (Wang et al., 2010). In total, 30 representative organs/tissues of rice 'Minghui 63' were selected for expression-level analysis of OsPP18, and a dynamic spatio-temporal expression pattern was observed (Supplemental Fig. S2). The expression of OsPP18 was very high (signal value > 15,000) in the stamen at 1 d before flowering. Relatively high expression (signal value > 3,500) of OsPP18 was detected in leaves and sheaths at the secondary-branch primordium differentiation stage, leaves from plants with 4- to 5-cm young panicles, and hull at 1 d before flowering.

According to Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/; Chou and Shen, 2010), OsPP18 was predicted to be located in chloroplast, cytoplasm, and nucleus. To examine the location of OsPP18 in vivo, yellow fluorescent protein (YFP)-tagged OsPP18 and cyan fluorescent protein (CFP)-tagged GHD7 (a well-defined nuclear protein in rice; Xue et al., 2008) or YFP-tagged OsPP18 and CFP were cotransformed into rice protoplasts. As shown in Supplemental Figure S3, yellow fluorescence produced by OsPP18-YFP overlapped with cyan fluorescence produced by CFP and partly overlapped with cyan fluorescence produced by CFP-GHD7 but did not overlap with the autofluorescence of chlorophyll. These results indicated that OsPP18 is targeted to the cytoplasm and nucleus.

The ospp18 Mutant Is Hypersensitive to Drought Stress

To reveal the function of OsPP18 during the stress response, a transfer DNA (T-DNA) insertion mutant, ospp18 (‘Dongjin’ [DJ] background), was collected from the Rice T-DNA Insertion Sequence Database (POSTECH RISD; Jeon et al., 2000). Flanking sequence analysis indicated that the T-DNA was inserted in the first intron 281 bp downstream of the start codon (Fig. 4A). The transcription of OsPP18 was significantly impaired in the mutant (Fig. 4B).

Three homozygous mutant (ospp18/ospp18) lines and three wild-type sibling (OsPP18/OsPP18) lines derived from the heterozygous mutant (OsPP18/ospp18) were chosen for drought stress treatment at different developmental stages. Young seedlings of the homozygous mutant lines were planted along with wild-type sibling seedlings with the same age and vigor. The four leaf-stage plants were subjected to drought treatment by stopping irrigation for 10 d. Under drought stress conditions, all of the mutant lines showed earlier and more severe wilting symptoms compared with the wild-type siblings (Fig. 4C). After recovery by rewatering, 50% to

Figure 2. OsPP18 encodes a PP2C. A, Phylogenetic tree of group F2 PP2Cs in Arabidopsis and rice. The phylogenetic tree was created in MEGA5 software with the neighbor-joining method based on the conserved PP2C domain of PP2C. Group A PP2Cs, ABI1, and ABI2 from Arabidopsis and OsPP48 and OsPP76 from rice were defined as outgroups. Numbers indicate percentage values after 1,000 replications. Sequences used in this analysis were as follows: OsPP18, OsPP91 (LOC_Os06g48300), OsPP70 (LOC_Os04g56450), OsPP11 (LOC_Os01g43100), OsPP80 (LOC_Os05g50970), OsPP86 (LOC_Os06g13530), OsPP87 (LOC_Os06g33549), OsPP48 (LOC_Os03g16170), and OsPP76 (LOC_Os05g38290) from rice and WIN2 (At4g31750), AtPP2C71 (At5g24940), AtPP2C69 (At5g10740), AtPP2C76 (At5g3140), AtPP2C11 (At1g43900), ABI1 (At4g26080), and ABI2 (At5g57050) from Arabidopsis. B, OsPP18 protein is a Ser/Thr phosphatase; 5 μg of GST-OsPP18 or GST was incubated with no substrate, 100 μM substrate, 100 μM substrate plus 1 mM sodium vanadate, or 100 μM substrate plus 50 mM sodium fluoride (NaF). C, The OsPP18 protein is a PP2C; 5 μg of GST-OsPP18 or GST was incubated with 100 μM substrate in various buffers specific for PP2A, PP2B, and PP2C activity. A phosphorylated synthetic peptide was used as a substrate in phosphatase activity assay. Error bars indicate SE based on three replicates.

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70% of the wild-type plants were recovered, whereas only 20% of the \textit{ospp18} mutant plants were recovered (Fig. 4D), which was significantly (Student’s \textit{t} test, \textit{P} < 0.01) less than the wild type. The drought sensitivity of the \textit{ospp18} mutant was also tested at the panicle differentiation stage with the mutant and the wild type grown in a paddy field under a removable rain-off shelter. During the drought stress treatment, the growth of the \textit{ospp18} plants was slower than that of the wild-type plants (Fig. 4E). After drought stress treatment, the mutant plants showed significantly (Student’s \textit{t} test, \textit{P} < 0.01) reduced plant height, tiller number, and aboveground biomass compared to the wild-type plants, whereas no significant difference was observed under normal growth conditions (Fig. 4, F–H). The relative seed-setting rate, a ratio of the seed-setting value under stress conditions to that under normal conditions, was also significantly (Student’s \textit{t} test, \textit{P} < 0.01) lower in the \textit{ospp18} mutant than in the wild type after drought stress treatment (Fig. 4I). When the drought stress treatment was applied at the flowering stage, similar results were obtained (Supplemental Fig. S4).

We also examined for a possible physiological basis related to the increased drought sensitivity of the \textit{ospp18} mutant. The rate of water loss was not significantly different in the wild type and the \textit{ospp18} mutant (data not shown). Accumulation of ROS is a frequent consequence of drought stress, and excess ROS can result in oxidative damage to cellular membranes and other cellular components, which ultimately leads to cell death. To determine whether ROS accumulation is related to the phenotype, malondialdehyde (MDA), a product of lipid peroxidation, was measured. The results showed that the MDA level was significantly (Student’s \textit{t} test, \textit{P} < 0.01) higher in the \textit{ospp18} mutant than in the wild type under drought stress conditions (Fig. 4J), suggesting that ROS overaccumulation triggered by the drought stress contributed to the increased drought sensitivity.

To further verify the function of OsPP18, an amiRNA strategy (Warthmann et al., 2008) was used to knock down the expression of OsPP18 in rice. The expression of OsPP18 in 12 T$_0$ OsPP18 amiRNA (amiR) plants was checked by qPCR (Supplemental Fig. S5A), and three independent transgenic lines (amiR-OsPP18-5, amiR-OsPP18-9, and amiR-OsPP18-11) were selected for drought resistance testing. The results showed that the amiR-OsPP18 lines were also hypersensitive to drought stress at the seedling stage (Fig. 5A). The average survival rate of the amiR-OsPP18 families was only about 20%, significantly lower than the wild type, which had a survival rate of about 60% (Fig. 5B).

Figure 3. Expression of OsPP18 under various abiotic stress (A) and phytohormone (B) treatments. Four leaf-stage rice seedlings were subjected to drought stress (removing the water supply from the plants), salt (200 mM NaCl), cold (4°C), heat (42°C), H$_2$O$_2$ (1% [v/v]), UV, wound, submergence, ABA (100 \textmu M), JA (100 \textmu M), and SA (100 \textmu M) treatments. The expression levels are normalized to an Actin endogenous control. Error bars indicate SE based on three replicates.
To check if the drought hypersensitivity phenotype is related to osmotic stress (a physiological stress that commonly occurs during drought stress), the ospp18 mutant and wild-type seedlings were grown in medium with 150 mM mannitol. The mutant plants showed more severe suppression in shoot growth under the mannitol-triggered osmotic stress, whereas no significant difference in growth performance was observed between the ospp18 mutant and the wild type under normal growth conditions (Supplemental Fig. S6, A and B). Similar results were obtained for the OsPP18 amiRNA plants (Supplemental Fig. S6, C and D). These results suggest that OsPP18 may also play a positive role in osmotic stress tolerance, which contributes to the drought stress resistance in rice.

**Expression Profile Changes in the ospp18 Mutant**

It is known that PPs participate in regulating many signaling pathways, which may finally lead to alterations...
in gene expression. To identify genes with altered expression levels in the ospp18 mutant, microarray analysis was performed to compare the expression profiles of two independent ospp18 mutant lines and the wild type (OsPP18/OsPP18 genotype derived from OsPP18/ospp18 plants). At a threshold of a 2-fold change of expression level, 54 and 163 genes were up-regulated and down-regulated, respectively, in both of the independent homozygous mutant lines compared with the wild type under both conditions (Supplemental Fig. S7A; Supplemental Tables S1 and S2). Among 217 genes with expression-level changes, 87, 55, and 38 genes are responsive to drought, salt, and cold stresses, respectively, based on the data in the public microarray database (http://www.ncbi.nlm.nih.gov/geo; GSE6901). Gene ontology (GO) analysis revealed that the genes belonging to three categories of biological processes were significantly overrepresented (hypergeometric test; P < 0.01; Supplemental Table S3): response to stimulus (abiotic, endogenous, and biotic stimulus), metabolism (lipid metabolism, amino acid and derivative metabolism, and carbohydrate metabolism), and signal transduction. Especially, the drought-responsive expression patterns for 55% of the genes in the response to stimulus category were altered in the mutant (Supplemental Fig. S7B). We selected 10 genes from several functional categories to validate the microarray data by qPCR (Supplemental Fig. S7C), and the qPCR results were in agreement with the microarray data.

Among the genes with altered expression levels in the mutant, eight transcription factor genes, including members from the AP2, TIFY, BASIC LEUCINE ZIPPER (bZIP), BASIC HELIX-LOOP-HELIX, and zinc finger families, were down-regulated. A TIFY family gene OsTIFY11a, a positive regulator of salt and dehydration stresses tolerance (Ye et al., 2009), was down-regulated by 2.27-fold in the mutant. A few GDSL-like lipase genes predicted to be involved in lipid metabolism but unknown for their roles in abiotic stress tolerance were down-regulated in the mutant. Many stress-related genes encoding diverse proteins, such as thaumatin, actin-depolymerization factor, heavy metal-associated protein, and protease inhibitor, were also down-regulated in the mutant, albeit that the exact functions of these proteins in stress tolerance remain to be revealed. We also noticed that the expression levels of some genes encoding ROS scavenging enzymes were changed in the mutant. For example, three GST genes and two peroxidase genes were down-regulated.

The ospp18 Mutant Is Sensitive to Oxidative Stress

GSTs are the ubiquitous enzymes that play a key role in cellular detoxification. The GST genes detected in the microarray were further investigated for their expression changes in the ospp18 mutant under normal and drought stress conditions by qPCR. The results showed that the expression levels of the GST genes were significantly reduced in the ospp18 mutant under both normal and drought stress conditions (Fig. 6A). The reduced expression of the GST genes and severe oxidative damage in the ospp18 mutant caused by drought stress (Fig. 4) indicated that the hypersensitivity of the ospp18 mutants to drought stress may have mainly resulted from a reduced capacity for ROS scavenging. To check this hypothesis, GST activities in the drought-stressed and unstressed leaves of ospp18 and wild-type plants were measured. The results indicated that GST activity was significantly lower in the ospp18 mutant than in the wild type under both conditions (Fig. 6B). We also measured the activity of other ROS scavenging enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and peroxidase, in the ospp18 mutant and wild-type plants. Among these ROS scavenging enzymes, the activity of GR was significantly lower in the ospp18 mutant than in the wild type under both the drought stress and nonstress conditions (Fig. 6B).

The reduced activity of GST and GR might contribute to the decreased tolerance to oxidative stresses. To confirm, the response of ospp18 to oxidative stress was investigated by using MV, a well-known oxidative stress inducer in plants. The results showed that the mutant leaves exhibited significantly higher electrolyte leakage than the wild-type leaves (Fig. 6C). After 72 h of...
MV treatment, more than 80% of the ions leaked from cells of the ospp18 mutant, whereas for the wild type, it was less than 55%. The response of the ospp18 mutant to oxidative stress was also examined in intact plants. Germinated wild-type and ospp18 seeds were sowed on Murashige and Skoog (MS) medium containing 2 μM MV. After 7 d, ospp18 plants exhibited an etiolating phenotype and a severe decrease of chlorophyll (only 19% of chlorophyll was retained compared with untreated plants), whereas the wild-type seedlings only showed a slight decrease of chlorophyll (42% of chlorophyll was retained compared with untreated plants; Fig. 6, D and E). Nevertheless, the chlorophyll content was not significantly different in the wild type and the ospp18 mutant under normal growth conditions. In another oxidative stress testing, three leaf-stage seedlings of the wild type and the ospp18 mutant were treated with 100 mM hydrogen peroxide (H2O2). After 4 d, the leaves of the ospp18 mutant showed severe chlorosis and rolling, whereas no obvious necrosis or damage was observed for the wild type (Fig. 6F). Accumulation of H2O2 was then determined by 3,3′-diaminobenzidine (DAB) staining. After 100 mM H2O2 treatment for 4 d,
significantly more H$_2$O$_2$ had accumulated in the leaves of the ospp18 mutant (Fig. 6G). The higher accumulation of H$_2$O$_2$ was in accordance with the phenotype of the ospp18 mutant under oxidative stress. These results suggest that the phenotype of severe damage in the ospp18 mutant under oxidative stress may be caused by an increase in ROS.

**Overexpression of OsPP18 Improves Tolerance to Osmotic and Oxidative Stresses**

Up-regulation of OsPP18 by SNAC1 and the drought-sensitivity phenotype of the ospp18 mutant prompted us to test the effect of OsPP18-OE on stress tolerance. Transgenic plants were generated by OE of the full-length complementary DNA (cDNA) of OsPP18 under the control of the maize (Zea mays) ubiquitin promoter. In total, 11 independent transgenic plants were obtained, and the expression level of OsPP18 was verified by qPCR. Two independent lines (OsPP18-OE-4 and OsPP18-OE-7; Supplemental Fig. S5B) were selected for additional analyses.

For testing osmotic stress tolerance, OsPP18-OE and wild-type plants were grown on MS medium containing 150 mM mannitol. The OsPP18-OE plants showed significantly less suppression of shoot growth (50%–60% of normal growth) than the wild type (only 36% of the normal growth) under the mannitol treatment (Fig. 7, A and B). The tolerance of the OsPP18-OE plants to the MV-induced oxidative stress was also tested. When OsPP18-OE and wild-type plants were grown on MS medium containing 2 μM MV for 7 d, the wild type exhibited an etiolation phenotype and a severe decrease in chlorophyll content (only 10% of chlorophyll was retained compared with untreated plants), whereas 33% to 39% of chlorophyll was retained in the OsPP18-OE plants (Fig. 7, C and D). Under oxidative stress, the detached leaves of OsPP18-OE plants exhibited significantly less electrolyte leakage and accumulated less H$_2$O$_2$ compared with wild-type leaves (Fig. 7, E and F). Moreover, two GST genes, which were repressed in the ospp18 mutant, were up-regulated in the OsPP18-OE plants. Additionally, two genes encoding SOD were also up-regulated in the OsPP18-OE plants (Fig. 7G). These results suggest that OE of OsPP18 may improve tolerance to osmotic and oxidative stresses, contrary to the phenotypes of the ospp18 mutant.

**OsPP18 Functions Independent of ABA Signaling**

Recently, a core ABA signaling pathway composed of rice PYRABACTIN-RESISTANT MUTANT LIKE/REGULATORY COMPONENTS OF ABA RECEPTOR5 (RCAR5), OsPP2C30, OSMOTIC STRESS/ABA-ACTIVATED PROTEIN KINASE2 (SAPK2), and RICE ABRE BINDING FACTOR for ABA-dependent gene regulation was identified in rice through interaction and transient gene expression assays (Kim et al., 2012). To determine whether OsPP18 is involved in ABA signaling, like OsPP2C30, pairwise interaction tests of OsPP18 with members of the rice SAPK family were conducted by using a yeast two-hybrid system (Fig. 8A). Six rice SAPKs, including SAPK2, were analyzed, but none of the SAPKs showed an interaction with OsPP18. These results indicate that OsPP18 may not function like OsPP2C30 or ABI1 in the ABA signaling. Importantly, the sensitivity of ospp18 and OsPP18-OE plants to ABA was not different compared with the wild-type plants (Fig. 8B). To further exclude the possibility of OsPP18 on ABA signaling, the expression of a few well-known ABA-responsive genes in rice, including RESPONSIVE TO ABA PROTEIN21 (RAB21), LOW TEMPERATURE INDUCED PROTEIN9 (LIP9), and OsbZIP23 (Mundy and Chua, 1988; Rabbani et al., 2003; Xiang et al., 2008), was checked in the ospp18 mutant. The qPCR results indicated that the ABA-induced expression of these genes was not affected in the ospp18 mutant (Fig. 8C), further confirming that OsPP18 may not be involved in ABA signal transduction. These results together suggest that the function of OsPP18 in response to drought and oxidative stresses is ABA independent.

**DISCUSSION**

**OsPP18 May Be a Downstream Gene of SNAC1**

Plants have evolved complicated regulatory networks to respond to various abiotic stresses. Transcription factors play important roles in the abiotic stress response by activating various genes related to stress tolerance. SNAC1 is an important transcription factor involved in drought response, and SNAC1-overexpressing rice exhibits significantly enhanced drought resistance under field conditions (Hu et al., 2006a). Here, we found that a PP2C gene OsPP18 may be a downstream gene of SNAC1, which is supported by several complementary experimental results. The expression of OsPP18 was up-regulated in the SNAC1-overexpressing plants and repressed in the SNAC1 amiRNA plants, and the SNAC1 protein was able to bind to the OsPP18 promoter in both yeast and rice (Fig. 1). Both SNAC1 and OsPP18 were induced by drought, high salinity, and low temperature (Hu et al., 2006a; Fig. 3). The ospp18 mutant and amiR-OsPP18 plants were sensitive to drought stress, which is just contrary to the drought-resistant phenotype of SNAC1-overexpressing plants. Furthermore, both the SNAC1- and OsPP18-overexpressing plants showed enhanced tolerance to oxidative stress (Supplemental Fig. S8). In addition, the expression level of OsPP18 in the SNAC1-overexpressing plant was also higher than that in the wild type under drought stress condition (Supplemental Fig. S9). All of these results indicate that OsPP18 is a target gene of SNAC1.

Recently, a rice homolog of SIMILAR TO RCD ONE (SRO; similar to RADICAL-INDUCED CELL DEATH1), OsSRO1c, was characterized as another target gene of SNAC1 (You et al., 2013). OsSRO1c was induced in guard cells by drought stress and reduced water loss by promoting stomatal closure through H$_2$O$_2$ accumulation (You et al., 2013). In this study, we found that the water loss rate was not significantly different between the wild type and
the ospp18 mutant (data not shown), indicating that OsPP18 may not be involved in the regulation of stomatal movement. However, the ospp18 mutant showed increased sensitivity to oxidative stress, and OE of OsPP18 enhanced tolerance to oxidative stress. Thus, in addition to reduced water loss, enhanced tolerance to drought-induced ROS may also contribute to the improved drought tolerance mediated by SNAC1.
OsPP18 Is a Positive Regulator of Stress Tolerance Independent of ABA Signaling

Sequence analysis suggests that OsPP18 is a typical PP with the PP2C catalytic domain located between amino acids 82 and 321. We showed that OsPP18 has dephosphorylation activity (Fig. 2, B and C). Phylogenetic analysis suggests that OsPP18 belongs to group F2 of plant PP2C, in which only WIN2 in Arabidopsis has been functionally characterized. In this work, the expression profiles of OsPP18 under multiple abiotic stresses and phytohormone treatments were examined by qPCR; the results showed that OsPP18 was strongly induced by drought (Fig. 3A), and it was transiently induced by JA and SA treatments. However, it was not induced by ABA treatment (Fig. 3B). These results suggest that OsPP18 is a unique drought-responsive PP2C gene in rice.

PPs are key components of a number of signaling pathways, including responses to environmental stimuli (Schweighofer et al., 2004). Some plant PP2Cs have been characterized with regulatory roles in stress tolerance. ABI1 and PP2CA are two important PP2Cs for plant tolerance to several abiotic stresses, including salt, drought, and freezing, by regulation of ABA signaling (Strizhov et al., 1997; Gosti et al., 1999; Tähtiharju and Palva, 2001). OE of an ABI1 ortholog in the liverwort Marchantia polymorpha resulted in reduced freezing and osmotic stress tolerance (Tougane et al., 2010). OE of a maize PP2C gene ZmPP2C2 in tobacco enhanced tolerance to cold stress (Hu et al., 2010), whereas OE of another maize PP2C gene ZmPP2C in Arabidopsis decreased tolerance to salt and drought stresses (Liu et al., 2009). OE of Fagus sylvatica FsPP2C1 in Arabidopsis increased ABA insensitivity (González-García et al., 2003), whereas constitutive expression of FsPP2C2 in Arabidopsis led to hypersensitivity to ABA (Reyes et al., 2006). To date, all of the reported PP2Cs, except ZmPP2C2 and FsPP2C1, were negative regulators of stress tolerance. The positive effect of ZmPP2C2
and FsPP2C1 on stress tolerance was investigated only at the seed germination stage, whereas the actual mechanisms of these PP2Cs in stress tolerance remains unclear. In this study, the ospp18 mutant was sensitive to drought stress at not only the seedling stage but also, the young panicle differentiation and flowering stages (Fig. 4, C–I; Supplemental Fig. S4). The ospp18 mutant was also sensitive to osmotic stress (Supplemental Fig. S6, A and B). The phenotype of OsPP18 amiRNA plants under drought and osmotic stresses was in agreement with that of the ospp18 mutant (Fig. 5; Supplemental Fig. S6, C and D). We also tested the tolerance of ospp18 under high salinity and cold stress, but no significant difference was observed between the ospp18 and the wild-type plants (data not shown). In contrast to the drought sensitivity of the ospp18 mutant, OsPP18-overexpressing plants showed improved tolerance to osmotic stress (Fig. 7, A and B). All of these results suggest that OsPP18 is a positive regulator of drought tolerance in rice.

The core ABA signaling module consisting of the PYRABACTIN-RESISTANT MUTANT/RCAR receptors, PP2Cs, and SAPKs is responsible for the early ABA signaling, which is supported by abundant genetic, physiological, biochemical, and structural evidence (Hubbard et al., 2010). Similar ABA signaling components were also identified in rice (Kim et al., 2012). Most of the reported abiotic stress-related PP2Cs, such as ABI1, PP2CA, ZmPP2C, FsPP2C1, and FsPP2C2, mediate the stress responses through the regulation of ABA signaling pathways (Gosti et al., 1999; Tähtiharju and Palva, 2001; González-García et al., 2003; Reyes et al., 2006; Liu et al., 2009). Here, we found that OsPP18, a typical PP2C, could not interact with SAPK2 (Fig. 8A; a component of ABA signal transduction in rice identified by Kim et al. [2012]) or other SAPKs in rice. Meanwhile, the expression of OsPP18 was not induced by ABA (Fig. 3B), and the ABA sensitivity of the ospp18 mutant and OsPP18-overexpressing plants was not affected (Fig. 8B). In addition, the ABA-induced expression of well-known ABA-responsive genes, such as RAB21, LIP9, and OsbZIP23, was not affected in the ospp18 mutant (Fig. 8C). These results together indicate that OsPP18 may not be a component of ABA signal transduction. To the best of our knowledge, OsPP18 is the first member of the PP2C family to be identified as a positive regulator of drought stress tolerance function in an ABA-independent pathway in plants.

OsPP18 Mediates Drought Stress Tolerance by Regulation of ROS Homeostasis

Production of ROS is a frequent event in plants suffering from diverse abiotic stresses (Mittler, 2002). ROS are important signaling molecules to control various processes, such as stomatal movement, but also, harmful molecules that cause oxidative damage to cells (Apel and Hirt, 2004). Numerous studies have revealed the involvement of ROS scavenging capacity in plant tolerance to abiotic stresses. For example, OE of the rice CALCULUM-DEPENDENT PROTEIN KINASE12 increased salt tolerance by regulating the expression of genes for ROS scavenging and production enzymes to reduce the accumulation of ROS (Asano et al., 2012). Loss of function of an MAPK kinase gene, DROUGHT-HYPERSENSITIVE MUTANT1, caused increased sensitivity to drought stress, mainly because of the increase in ROS damage (Ning et al., 2010). The drought-sensitive phenotype of the ospp18 mutant was not related to stomatal aperture, and the leaf water loss rate was not significantly different between the wild type and the ospp18 mutant (data not shown). Increased MDA levels in the ospp18 mutant under drought stress conditions (Fig. 4J) implied that more severe oxidative damage occurred in the cells of the ospp18 mutant during the drought stress treatment. Relevant to this finding, detached leaves of the ospp18 mutant exhibited higher electrolyte leakage than the wild type after MV treatment (Fig. 6C), and the ospp18 seedlings exhibited a larger decrease in the levels of chlorophyll and accumulated more H2O2 under oxidative stress (Fig. 6, D–G). To the contrary, OsPP18-overexpressing plants showed enhanced tolerance to oxidative stress (Fig. 7, C–F). These results showed that the function of OsPP18 in drought tolerance may be associated with the regulation of antioxidation ability.

To maintain cellular redox homeostasis and detoxification of excess ROS, plants have evolved antioxidation systems involving arrays of ROS scavenging enzymes, such as SOD, CAT, GST, GR, and glutathione peroxidase (Mittler, 2002; Apel and Hirt, 2004). GST plays an important role in the reduction of organic hydroperoxides formed during oxidative stress using the tripeptide glutathione (GSH) as a cosubstrate or coenzyme (Dixon et al., 2002). OE of a GST gene from Escherichia coli in transgenic tobacco enhanced salt and cold tolerance (Le Martret et al., 2011). In another study, transgenic Arabidopsis plants overexpressing a mustard (Brassica juncea) GST gene (BgGSTT2) exhibited tolerance to HgCl2 and paraquat (Gong et al., 2005). In the ospp18 mutant, the expression of GST genes and the activity of GST were significantly reduced (Fig. 6, A and B). The activity of GR, an enzyme that reduces glutathione disulfide to the sulfhydryl form GSH, was also reduced in the mutant (Fig. 6B). These results suggest that the reduced activity of ROS scavenging enzymes may significantly contribute to excessive H2O2 accumulation and greater oxidative damage in the ospp18 mutant, which is associated with the increased sensitivity of the mutant to drought stress.

In conclusion, OsPP18 is regulated by SNAC1 and positively affects drought and oxidative stress tolerance by regulating ROS homeostasis through ABA-independent pathway(s) in rice. Compared with the well-known ABA-dependent negative regulator PP2Cs (such as ABI1) in stress responses, OsPP18 provides a unique and core component to investigate the ABA-independent regulatory networks in stress responses. Additional identification of the upstream and downstream components of OsPP18 may completely elucidate the role of ABA-independent PP2C-mediated signaling pathways underlying the responses of plants to diverse stresses.
MATERIALS AND METHODS

Plant Materials and Stress Treatments

The japonica rice (Oryza sativa) DJ, ZH11, and ‘IRAT109’ were used in this study. Mutant ospp18 (DJ background) seeds were obtained from the POSTECH RSD (http://www.postech.ac.kr/life/plg/risd/). The homozygous mutant and the wild-type genotype segregated from the heterozygous mutant were identified by PCR analysis using a pair of genomic primers flanking the insertion site and a primer on the T-DNA.

To measure the transcript level of OsPP18 under various stresses, ZH11 plants were grown in the greenhouse with a 14-h-light/10-h-dark cycle. Plants at the four-leaf stage were treated with various stresses and phytohormones. For drought stress, the seedlings were grown for 7 d without watering and sampled at 0, 3, 5, and 7 d. For salt stress, the seedlings were irrigated with 200 mM NaCl solution and sampled at 0, 3, 6, and 12 h. For cold stress, seedlings were transferred to a growth chamber at 4°C and sampled at 0, 3, 6, and 12 h. For light stress, seedlings were transferred to a growth chamber at 42°C and sampled at 0, 10, and 30 min and 1 h after treatment. For UV stress, seedlings were transferred to a growth chamber at 4°C and sampled at 0, 3, 6, and 12 h. For cold stress, seedlings were transplanted in a phytohormone treatment, leaves were sprayed with 100 μM MVA, JA, or SA and sampled at 0, 1, 3, and 6 h. For submergence stress, seedlings were completely submerged in a plastic tank filled with water and sampled at 0, 12, 24, and 72 h after stress initiation. For phytohormone treatment, leaves were sprayed with 100 μM ABA, JA, or SA and sampled at 0, 1, 3, 6, and 12 h.

For stress testing, OsPP18-OE and amiRNA transgenic plants were selected by germinating on MS medium containing 50 mg L⁻¹ hygromycin. The wild type and homozygous mutants were grown on MS medium or in soil. For drought stress testing at the seedling stage, mutant/ transgenic and wild-type plants were grown in the same barrel filled with a mixture of soil and sand (1:1). The water supply was stopped to allow drought stress to develop at the four-leaf stage. After all of the leaves wilted, plants were recovered (by rewatering) and photographed, and the survival rate was recorded. Drought stress testing at the young panicle differentiation and the survival rate was recorded. Drought stress testing at the young panicle differentiation and the survival rate was recorded. Drought stress testing at the young panicle differentiation and the survival rate was recorded.

Plasmid Construction and Rice Transformation

A full-length cDNA of OsPP18 was amplified from rice cultivar IRAT109 by RT-PCR, and the sequence-confirmed PCR fragment was inserted into the pU1301 vector under the control of a maize ubiquitin gene promoter for OE. The primers are listed in Supplemental Table S4. The amiR-OsPP18 was constructed by using the previously described method (Wartmann et al., 2008). A 21-mer sequence targeting the 3′-untranslated region of OsPP18 was used to replace the endogenous miRNA and miRNA* of Osa-MIR528 designed by the Web MicroRNA Designer platform (WMD; Ossowski et al., 2008). The resulting amiRNA was cloned into pU1301. Both of the OE and amiRNA constructs were transformed into ZH11, a japonica rice line can be easily transformed by the Agrobacterium spp.-mediated transformation method (Hei et al., 1994). The generation of SNAC1-repressed transgenic plants by an amiRNA strategy has been previously described in detail (You et al., 2013).

In Vivo Binding Assay of SNAC1

For ChiP assays, ZH11 plants were used for chromatin extraction and immunoprecipitation as described previously (You et al., 2013). Briefly, the aerial parts of three leaf-stage rice seedling were treated with formaldehyde, and the nuclei were isolated and sonicated using an Ultrasonic Crashner Noise Isolating Chamber (SCTNZT). The soluble chromatin fragments were isolated and preabsorbed with sheared salmon sperm DNA/protein A-agarose (Sigma-Aldrich) to remove nonspecific binding. Immunoprecipitations with anti-SNAC1 rabbit polyclonal antibody (New-East Biosciences) and anti-IgG antibody were prepared as described. The precipitated DNA was analyzed by PCR using specific primer sets (Supplemental Table S4). Typically, 26 to 28 cycles were performed for the PCR.

Physiological Measurements

The water loss rates of detached leaves were measured by monitoring the fresh weight lost at the indicated time points. Oxidative damage was estimated by measuring the content of MDA as described previously (Du et al., 2010). MDA content was detected by DAB staining as described previously (Ning et al., 2010). For electrolyte leakage, leaves were placed in 10-mL tubes containing 6 mL of 5 mM MV. The conductivity of the MV solution was determined with an ion leakage meter (Leici) at the designated time as described previously (Ning et al., 2010). The percentage of electrolyte leakage caused by MV treatment was determined by the ratio of ion leakage at a given time to the total ion leakage. Total electrolyte leakage was measured after boiling the sample for 15 min. Chlorophyll content was measured according to the method described by Knudson et al. (1977). Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium according to the previously described method (Giannopolitis and Ries, 1977). The activity of ROS scavenging enzymes, such as peroxidase, glutathione peroxidase, CAT, GST, and GR, and the content of GSH were measured using a kit from the Nanjing Jiancheng Bioengineering Institute. The GST and GR activities were recorded as units per milligram protein. ¹ One unit of GST activity was defined as the amount of enzyme depleting 1 μmol GSH in 1 min. One unit of GR activity was defined as the amount of enzyme depleting 1 μmol NADPH within 1 min.

Quantification of Gene Expression

The TRIZol reagent (Invitrogen) was used according to the manufacturer's instructions to extract total RNAs. Before RT, total RNA was treated with amplification-grade DNase I (Invitrogen) for 15 min to degrade any possible contaminating residual genomic DNA. cDNA templates were synthesized using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Real-time RT-qPCR was performed on a 7500 Real-Time PCR System (Applied Biosystems) using SYBR Premix Ex Taq (TaKaRa) according to the manufacturer’s protocol. The rice Actin1 gene (LOC_Os03g00865) was used as the endogenous control. The relative expression levels were determined as described previously (Livak and Schmittgen, 2001). The gene-specific primers are listed in Supplemental Table S4.

Microarray Analysis

Two independent biological replicates of the ospp18 mutant and wild-type plants were used for microarray experiments. The process of microarray analysis was conducted according to the standard protocol of the Affymetrix GeneChip service (CapitalBio). The analysis of differential gene expression and GO category overrepresentation was performed using the MAS 3.0 molecule annotation system (http://bioinfo.capitalbio.com/mas3/). The genes exhibiting more than a 2-fold expression change in two biological replicates are listed in Supplemental Tables S1 and S2. Representative differentially expressed genes were confirmed by real-time RT-qPCR.

Biochemical Assays in Saccharomyces cerevisiae

Yeast (Saccharomyces cerevisiae) one-hybrid assays were performed using the Matchmaker One-Hybrid System (Clontech). The OsPP18 promoter fragment was fused upstream to the HIS3 minimal promoter and served as a reporter construct. SNAC1 was fused to the GAL4 activation domain in the pGADT7-Rec2 vector and cotransformed with the reporter vector (pPH2-P-GAL4) into V817 yeast cells for determination of the DNA-protein interactions. The primers used for the yeast constructs are listed in Supplemental Table S4. The yeast two-hybrid assays were performed using the ProQuest Two-Hybrid System (Invitrogen). The coding region of OsPP18 was amplified and cloned into pDEST32 using Gateway technology (Invitrogen) to generate a bait vector with OsPP18 fused to the GAL4 DNA binding domain. The plasmids that contained SAPKs fused to the GAL4 activation domain (Tang et al., 2012) were cotransformed with the bait plasmid into the yeast strain MV203 according to the method described previously (Gietz et al., 1997). The protein-protein interaction was determined by colony-lift filter assays (β-gal assay) and the growth of transformants on synthetic complete selection medium containing 20 μg 3-amino-1,2,4-triazole and lacking Leu, Trp, and His.
PP2C Activity Assay

To obtain the recombinant protein from bacteria, GST-fused OsPP18 were constructed into the pGEX-4T-1 vector (GE Healthcare) and expressed in Escherichia coli (BL21). GST fusion protein production was induced by 0.1 mM isopropyl β-D-thiogalactoside at 30°C. Bacterial lysates were applied to glutathione Sepharose (GE Healthcare), and GST fusion proteins were eluted with 10 mM glutathione following the manufacturer’s instructions.

Phosphatase activity was measured according to the instructions provided by the manufacturer using a nonradioactive Ser/Thr phosphatase assay system (Promega). The purified recombinant protein was incubated for 15 min at 30°C with or without Ser/Thr Phosphopeptide in various buffers as designed (Fig. 2). The color was allowed to develop for 15 min, and the absorbance was measured at 600 nm with a Tecan M200 Plate Reader (Tecan). The buffers used were or without the Ser/Thr Phosphopeptide in various buffers as designed (Fig. 2). The color was allowed to develop for 15 min, and the absorbance was measured at 600 nm with a Tecan M200 Plate Reader (Tecan). The buffers used were

Subcellular Localization of OsPP18

To investigate the subcellular localization of the OsPP18 protein, we modified the pM999-35 vector by inserting a short DNA fragment that contains restriction enzyme recognition sites for KpnI and EcoRI. The full open reading frame of OsPP18 was cloned into the modified pM999-35 vector and fused with the YFP reporter gene. Plasmids were purified using the QIAGEN Plasmid Midi Kit according to the manufacturer’s protocol. The rice protoplast isolation and transformation protocol was described previously (You et al., 2013). The expression of the fusion construct was monitored after 16 h of incubation in the dark, and images were captured with a confocal microscope (TCS SP2, Leica).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Multiple sequence alignment and sequence comparison.

Supplemental Figure S2. Subcellular localization of OsPP18.

Supplemental Figure S3. Increased drought sensitivity of opsp18 mutant at the flowering stage.

Supplemental Figure S4. Transcript level of OsPP18 in amiR-OsPP18- and OsPP18-overexpressing plants.

Supplemental Figure S5. Suppression of OsPP18 showed increased sensitivity to osmotic stress.

Supplemental Figure S6. Expression analysis of opsp18 mutant.

Supplemental Figure S7. SNAC1-overexpressing plants showed enhanced tolerance to oxidative stress.

Supplemental Figure S8. Expression of OsPP18 in SNAC1-overexpressing and amiR-SNAC1 plants under drought stress condition.

Supplemental Table S1. Genes up-regulated in opsp18 mutant compared with the wild type.

Supplemental Table S2. Genes down-regulated in opsp18 mutant compared with the wild type.

Supplemental Table S3. GO analysis of the differentially expressed genes.

Supplemental Table S4. Primer sequences used in this study.

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