A Raf-Like MAPKKK Gene DSM1 Mediates Drought Resistance through Reactive Oxygen Species Scavenging in Rice1[C][W][OA]

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Mitogen-activated protein kinase (MAPK) cascades have been identified in various signaling pathways involved in plant development and stress responses. We identified a drought-hypersensitive mutant (drought-hypersensitive mutant1 [dsm1]) of a putative MAPK kinase kinase (MAPKKK) gene in rice (Oryza sativa). Two allelic dsm1 mutants were more sensitive than wild-type plants to drought stress at both seedling and panicle development stages. The dsm1 mutants lost water more rapidly than wild-type plants under drought stress, which was in agreement with the increased drought-sensitivity phenotype of the mutant plants. DSM1-RNA interference lines were also hypersensitive to drought stress. The predicted DSM1 protein belongs to a B3 subgroup of plant Raf-like MAPKKKs and was localized in the nucleus. By real-time PCR analysis, the DSM1 gene was induced by salt, drought, and abscisic acid, but not by cold. Microarray analysis revealed that two peroxidase (POX) genes, POX22.3 and POX8.1, were sharply down-regulated compared to wild type, suggesting that DSM1 may be involved in reactive oxygen species (ROS) signaling. Peroxidase activity, electrolyte leakage, chlorophyll content, and 3,3′-diaminobenzidine staining revealed that the dsm1 mutant was more sensitive to oxidative stress due to an increase in ROS damage caused by the reduced POX activity. Overexpression of DSM1 in rice increased the tolerance to dehydration stress at the seedling stage. Together, these results suggest that DSM1 might be a novel MAPKKK functioning as an early signaling component in regulating responses to drought stress by regulating scavenging of ROS in rice.

Plants are exposed to diverse abiotic stresses including drought, salt, and adverse temperatures throughout their life cycles. For stress response and tolerance, plants have evolved a variety of biochemical and physiological mechanisms. Among the stresses, drought is a major environmental factor limiting productivity and distribution of plants (Boyer, 1982). Many stress-responsive genes have been identified and altered gene expression plays an important role in plant drought resistance (Zhu, 2002; Li et al., 2008; Lee et al., 2009); however, the biological functions of most of the stress-responsive genes are largely unknown.

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kinase domain (KD) and a long N-terminal regulatory domain (RD). In contrast, MEKK family members have less conserved protein structure (Jouannic et al., 1999). In recent years, the roles of individual plant MAPKKK proteins in diverse plant signal transduction pathways have been explored. The NPK1 gene from tobacco (Nicotiana tabacum) has previously been shown to play an important role in regulating cell division (Banno et al., 1993; Nishihama et al., 2001, 2002; Suyano et al., 2003). More recently, Shou et al. (2004) reported that overexpression of the KD of NPK1 enhances abiotic stress tolerance in transgenic maize (Zea mays). The Arabidopsis ANP1 gene is homologous to the tobacco NPK1 gene. ANP1 is responsive to oxidative stress and is involved in negative regulation of the auxin signal transduction pathway (Kovtun et al., 1998, 2000). OMTK1, a MAPKKK activated by hydrogen peroxide (H2O2) in alfalfa (Medicago sativa), can specifically activate the MAPK MMK3 in the protoplast, resulting in cell death (Nakagami et al., 2004). To date, only two Raf family MAPKKKs, namely CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) and ENHANCED DISEASE RESISTANCE1 (EDR1), have been published. The CTR1 gene negatively regulates ethylene responses. The Arabidopsis mutant ctrl1 exhibits characteristic constitutive ethylene responses in the absence of ethylene (Kieber et al., 1993). Furthermore, CTR1 has been found to interact with the His-KD of ETR1 and the ethylene response sensor (ERS1) in vitro (Clark et al., 1998). The EDR1 was identified and the edr1 mutant was shown to be resistant to powdery mildew (Frye and Innes, 1998). In addition, EDR1 negatively regulated salicylic acid-inducible defense responses in Arabidopsis (Frye et al., 2001), but its substrates are still unknown.

Rice (Oryza sativa) is one of the most important crops in the world, especially in Asia. One of the main environmental factors that affects rice yield is water deficit or drought stress. Drought resistance is a complex quantitative trait. Although genetic mapping of drought resistance-related traits of rice has been reported (Yue et al., 2005, 2006) and many drought stress-related genes in rice have been identified, the molecular basis of drought response and adaptation in rice is largely unclear. Recent studies have suggested that many protein kinases are involved in drought resistance in rice. Among them, members of the calcium-dependent protein kinase (CDPK), calcineurin B-like protein-interacting protein kinase (CIPK), and MAPK families have been identified as conferring stress resistance in rice. For example, overexpression of OsCDPK7 in rice results in enhanced stress tolerance at the seedling stage (Saijo et al., 2000); overexpression of three CIPK genes (OsCIPK03, OsCIPK12, and OsCIPK15) enhances tolerance to cold, drought, and salt stress, respectively, in transgenic rice (Xiang et al., 2007); overexpression of a MAPK family gene OsMAPK5α in rice leads to increased OsMAPK5a kinase activity and enhanced tolerance to drought, salt, and cold stresses (Xiong and Yang, 2003). OsMAPK44, another rice MAPK gene, is highly inducible by salt and drought treatment and OsMAPK44 overexpression in rice plants generates increased tolerance to salt stress (Jeong et al., 2006b). However, evidence for MAPKKK genes being involved in stress resistance of rice has not been reported.

In this study, we identified a rice drought-sensitive mutant (drought-hypersensitive mutant1 [dsm1]). The dsm1 mutant showed enhanced drought sensitivity in both the seedling stage and the panicle development stage. DSM1 encodes a putative MAPKKK belonging to the Raf family of protein kinases. Our results demonstrate that DSM1 may act as an early signal regulating the responses to drought stress in rice. Our data also indicate that drought hypersensitivity of dsm1 is partially due to a decrease in reactive oxygen species (ROS) scavenging.

RESULTS

Identification of a Drought-Sensitive Mutant in Rice

To reveal the potential functions of MAPK cascades in drought-stress signaling in rice, we collected a total of 15 T-DNA insertion mutants (japonica rice Zhonghua11 [ZH11] background) corresponding to six putative MAPKKK genes (Supplemental Table S1) available in the T-DNA mutant library in the Rice Mutant Database (Wu et al., 2003). These mutants were prescreened for drought resistance under field conditions. Under normal growth conditions, most of the mutant lines showed no obvious phenotype change (or segregation) compared to the wild-type ZH11. Under drought stress (water withheld from 4-week-old plants for 10 d), one of the mutant lines for a predicted MAPKKK gene (LOC_Os02g50970) showed increased sensitivity to drought stress, and this mutant, dsm1-1 (dsm1, allele 1), was selected for further analysis. We collected a second T-DNA mutant allele of this gene (dsm1-2; Hwayoung [HY] background) from the Pohang University of Science and Technology Rice T-DNA Insertion Sequence Database (POSTECH RISD; An et al., 2003; Jeong et al., 2006a). Sequence analysis indicated that the insertion site of the dsm1-1 mutant is located in exon 12, 7,174 bp downstream of ATG, and the T-DNA of the dsm1-2 mutant is inserted in intron 10, 6,734 bp downstream of ATG (Fig. 1A). Transcription of DSM1 in dsm1 mutants was checked by semiquantitative reverse transcription (RT)-PCR using RNA templates isolated from homozygous mutants. The mRNA was not detectable in either mutant, suggesting that the expression of DSM1 was abolished in the mutants (Fig. 1B).

When grown under normal growth conditions, neither of the dsm1 mutants displayed any obvious phenotype change (Fig. 2A, top). To verify the drought-sensitivity phenotype, homozygous mutant (dsm1/dsm1) and wild-type (DSM1/DSM1) lines derived from DSM1/dsm1 genotype plants for both...
mutant alleles were grown in sandy-mixed soil along with wild-type rice ZH11 or HY. The 4-week-old plants were subjected to drought treatment by stopping irrigation for 10 to 15 d. Under the moderate stress condition, the \textit{dsm1} mutant lines showed more severe wilting and a lesser degree of recovery after rewattering than the wild-type genotypes (Fig. 2A, bottom), and the phenotype cosegregated with the genotype (data not shown). After severe drought-stress treatment, we found that only about 17\% of the mutant plants survived, while wild-type plants had a significantly higher survival rate (approximately 63\%; Fig. 2B). Subsequently, we measured water loss rates of detached leaves from the wild type and the \textit{dsm1} mutant lines. Results showed that the detached leaves of the \textit{dsm1} mutants lost water slightly faster than wild type (Fig. 2C). Drought sensitivity of \textit{dsm1} mutants was also tested at the panicle development stage. The \textit{dsm1}-1 and wild type were planted in a paddy field with a removable rain-off shelter and in PVC tubes filled with sandy soil as described previously (Hu et al., 2006). During drought stress, \textit{dsm1}-1 plants showed earlier leaf rolling and wilting than wild-type plants (Fig. 2D). Statistical analysis of the yield and spikelet fertility also suggested that \textit{dsm1}-1 exhibited hypersensitivity to drought stress at the panicle development stage (Fig. 2E and F). In the field, the total grain yield of \textit{dsm1}-1 was reduced by about 22\% when compared to the wild type; the spikelet fertility of \textit{dsm1}-1 (45\%) was also significantly ($P < 0.01$) lower than that of wild-type plants (60\%; Fig. 2E). In the PVC tubes (more severe drought stress applied), the mutant showed 60\% less yield than the wild type (\textit{DSM1/ DSM1}), and the spikelet fertility of the mutant was only about half that in the wild type (Fig. 2F). Nevertheless, no significant difference in yield and fertility was observed between the mutant and wild-type plants under normal growth conditions. We also checked the sensitivity of the \textit{dsm1}-1 mutant to salt stress, and results showed that the mutant was more sensitive to salt stress than the wild type (Supplemental Fig. S1), but no significant difference was observed for cold stress (data not shown).

Suppression of DSM1 Resulted in Enhanced Sensitivity to Drought Stress

To further examine the functions of DSM1 in rice, DSM1-RNA interference (RNAi) rice plants were produced with a 400 bp open reading frame region of DSM1 as the targeted interference region. Among 24 \textit{T0} RNAi plants checked, five showed significantly decreased expression of DSM1 (Fig. 3A). Three independent RNAi lines (RNAi-10, RNAi-11, and RNAi-20) were selected for drought testing at the seedling stage. Results showed that the DSM1-RNAi lines were also hypersensitive to drought stress (Fig. 3B), which was in agreement with the phenotype of \textit{dsm1} mutant plants. The survival rate of DSM1-RNAi lines was only 10\% to 15\%, whereas 60\% to 70\% of wild-type plants recovered (Fig. 3C). Consistent with this result, detached leaves of DSM1-RNAi lines lost water more quickly than the wild-type leaves (Fig. 3D). Taken together, these results suggest that DSM1 plays essential roles in drought resistance in rice.

\textbf{DSM1 Expression Profile and Subcellular Localization of DSM1}

As \textit{dsm1} showed hypersensitivity to drought stress, we examined the expression level of DSM1 under drought as well as other treatments including cold, salt, and abscisic acid (ABA) at the seedling stage. As shown in Figure 4A, the DSM1 transcript level increased after drought and salt treatment, albeit not very strongly (about 2- to 3-fold). DSM1 was only slightly induced by ABA. Contrary to the induction of DSM1 expression by salt and drought stresses, DSM1 was not induced by cold stress but rather slightly suppressed. These results seem to be consistent with the \textit{dsm1} phenotypes in stress responses and indicate that DSM1 may function mainly in drought- and salt-stress responses.

To investigate the tissue-specific expression pattern of DSM1 in rice, we generated transgenic rice plants with a construct (P\textsubscript{DSM1}::GUS) in which the GUS reporter gene was under the control of a DSM1 promoter. Histochemical staining for GUS activity was performed in five independent transgenic plants. Results showed that strong GUS expression was observed in the stamen, pistil, mature leaves, and roots (Fig. 4, C–F), and weak GUS expression was detected in the hull (Fig. 4B), suggesting a constitutive expression pattern of DSM1 in rice. This result was supported by microarray data of the gene in the genomic expression.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Identification of \textit{dsm1} T-DNA insertion mutants. A, Schematic diagram of DSM1 gene and two alleles of T-DNA insertion mutants, \textit{dsm1}-1 and \textit{dsm1}-2. LB, Left border; RB, right border. Exons and introns are indicated in blue and white, respectively. B, RT-PCR analysis of DSM1 in ZH11, HY, \textit{dsm1}-1, and \textit{dsm1}-2. Actin1 was used as internal control. [See online article for color version of this figure.]}\end{figure}
Figure 2. Increased drought sensitivity of dsm1 at both seedling and heading stages. A, Five-leaf stage wild-type (ZH11 or HY) and dsm1 mutant plants were growing in barrels filled with a mixture of soil and sand (1:1). The left and right half of each barrel was planted with wild-type and mutant plants, respectively. Plants were not watered for 12 d, followed by rewatering for 7 d. The photograph was taken before drought stress and after recovery. B, Comparison of survival rate of wild type (ZH11 or HY) and dsm1 mutants (dsm1-1 [ZH11 background] or dsm1-2 [HY background]) after drought stress. Values are means ± se (n = 3). **P < 0.01 (t test). C, Water loss from detached leaves of wild type (ZH11 or HY) and dsm1 mutants (dsm1-1 [ZH11 background] or dsm1-2 [HY background]) at indicated time points. D, ZH11 and dsm1 plants were subjected to drought stress at heading stage. The photograph was taken on day 20 after rewatering. E, Spikelet fertility and yield of ZH11 and dsm1-1 under drought stress in paddy field with a removable rain-off shelter. Values are means ± sd (n = 32). **P < 0.01 (t test). F, Spikelet fertility and yield of wild type (WT; DSM1/DSM1 [ZH11 background]), heterozygous, and homozygous dsm1-1 under drought stress in PVC tubes filled with sandy soil. Values are means ± sd (n = 12). **P < 0.01 (t test).
To determine the subcellular localization of DSM1, the DSM1 cDNA was fused in frame to the GFP marker gene under the control of cauliflower mosaic virus 35S promoter. In the transgenic rice expressing DSM1-EGFP, the fluorescence was observed only in the nuclei (Fig. 5A). To confirm this result, rice protoplasts prepared from an etiolated shoot were cotransformed with 35S:sGFP-DSM1 and 35S:CFP-GHD7 by polyethylene glycol treatment. GHD7 was used as a marker since it has been reported as a transcription factor localized in the nucleolus (Xue et al., 2008). As shown in Figure 5B, green fluorescence produced by sGFP-DSM1 overlapped with blue fluorescence produced by CFP-GHD7, which further confirmed that DSM1 is a nuclear protein.

DSM1 Features a Raf-Like MAPKKK

DSM1 encodes a putative MAPKKK of 1,111 amino acids with a molecular mass of 123 kD. Based on domain scanning against the Pfam database (http://pfam.sanger.ac.uk/) and comparison with other plant MAPKKKs, DSM1 consists of a C-terminal KD (amino acids 838–1,090) and an N-terminal putative RD (Fig. 6A). DSM1 was compared with reported MAPKKKs from other plants (Supplemental Fig. S2). The results showed that DSM1 contains all 11 subdomains common to all known protein kinases (Hanks and Quinn, 1991). Alignment of the full-length protein sequence of DSM1 with the well-characterized plant MAPKKKs suggests that DSM1 has high sequence similarity in both the KD and the RD to EDR1 (73% identity in KD and 36% identity in RD) and CTR1 (65% identity in KD and 42% identity in RD). Both EDR1 and CTR1 belong to the B3 subgroup of plant Raf-like MAPKKKs (Ichimura, 2002). In contrast, DSM1 showed relatively low sequence identity with other plant MAPKKK proteins. For example, DSM1 shared only 31% and 30% identity, respectively, with AtMEKK1 and NPK1 even in the conserved KD. A phylogenetic tree based on the conserved KD and the position of the KD also placed DSM1 into the Raf-like MAPKKK family (Fig. 6B).

Raf-like MAPKKKs in plants have been reported to possess kinase activities. CTR1 and its KD display autophosphorylation activity, as well as the ability to phosphorylate myelin basic protein (MBP) in vitro. The EDR1 KD also can autophosphorylate (Covic et al., 1999; Tang and Innes, 2002; Huang et al., 2003). To determine if DSM1 has kinase activity, the KD was expressed in Escherichia coli strain BL21, but the expressed protein was highly insoluble (data not shown). To overcome this problem, transgenic rice

Figure 3. Suppression of DSM1 showed increased sensitivity to drought stress. A, Transcript level of DSM1 was measured by RT-PCR in DSM1-RNAi plants (ZH11 background). Numbers 8, 10, 11, 17, and 20 indicated independent RNAi transgenic plants. Actin1 was used as internal control. B, Five-leaf stage ZH11 and DSM1-RNAi plants were subjected to no watering for 12 d, followed by rewatering for 7 d. Surviving seedlings were photographed. C, Comparison of survival rate of ZH11 and DSM1-RNAi plants after drought stress. Values are means ± SE (n = 3). **P < 0.01 (t test). D, Water loss from detached leaves of ZH11 and DSM1-RNAi plants at indicated time points.
were generated expressing the DSM1 KD with a FLAG tag (provided by Sun and Zhou, 2008) fused at the carboxyl terminus. The KD-FLAG protein was immunoprecipitated from a total protein extract of the KD-FLAG transgenic plants with anti-FLAG M2 affinity gel. Immunoprecipitated proteins were separated by SDS-PAGE with immunoprecipitated protein extract from wild type as a negative control. Silver staining of the SDS-PAGE gel showed a clearly visible band (slightly larger than the predicted 43-kD fusion protein) only in the transgenic plants (Fig. 7A). The band was further confirmed to be KD of DSM1 by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) following trypptic digestion (Supplemental Fig. S3). The purified protein was subjected to the kinase assay as described previously (Huang et al., 2003). MBP is a universal substrate that has been widely used to examine kinase activity (Eichberg and Iyer, 1996). Our results demonstrated that MBP was strongly phosphorylated by the DSM1 KD and that the DSM1 KD had autophosphorylation activity regardless of the presence or absence of MBP (Fig. 7B). These results together suggest that DSM1 functions as a typical Raf-like MAPKKK in rice.

Microarray Analysis of dsm1 Plants

It is known that MAPK cascade-mediated signaling often leads to regulation of gene expression through phosphorylation of transcriptional regulators. To identify genes with altered expression levels in the dsm1 mutant, microarray (Affymetrix Rice GeneChip) analysis was performed to compare the expression profiles between the dsm1 mutant and wild type (the DSM1/DSM1 genotype in the progenies of DSM1/dsm1 plants) under both normal and drought-stress conditions. Results suggest that only a small portion of genes in the rice genome show significant expression level changes in the dsm1 mutant compared to the wild type under normal conditions. Only 12 genes (0.02% of all rice genes) were down-regulated by more than 2.5-fold in the mutant (Supplemental Table S2). However, the transcription levels of many well-studied abiotic stress-related marker genes (such as RD22, RAB21, and RAB16c) were not changed in the dsm1 mutant compared to the wild type. Among the down-regulated genes in the dsm1 mutant, two peroxidase (POX) genes POX22.3 and POX8.1, which showed predominant expression during resistant interactions (Chittoor

Figure 4. Expression pattern of DSM1. A, Expression of DSM1 under abiotic stress. Two-week-old seedlings were subjected to cold (4°C), salt (200 mM NaCl in the hydroponic culture medium), ABA (100 μM), and drought stress (removing the water supply from the plants). Relative expression levels of DSM1 were examined by quantitative real-time PCR. B, DSM1 promoter:::GUS expression patterns in transgenic rice plants (ZH11 background). GUS staining was shown in hull (1), pistil (2), stamen (3), root (4), and mature leaves (5).
et al., 1997), were dramatically down-regulated (15- and 5.3-fold, respectively). Several genes annotated as encoding expression proteins and retrotransposon proteins were also down-regulated in the dsm1 mutants, but their relevance to stress resistance is not clear. Even though we noticed that 47 genes (0.08% of all rice genes) were up-regulated (more than 2-fold) in the mutant under normal conditions (Supplemental Table S3), no obvious link or relevance to abiotic stress was found for these genes based on their predicted function and expression features. By contrast, the dsm1 mutant showed significantly altered gene expression compared with wild-type plants under drought stress. A total of 678 genes (1.18% of all rice genes) and 126 genes (0.22% of all rice genes) showed up- and down-regulated expression, respectively, in dsm1 mutants compared to wild-type plants under stress conditions (Supplemental Tables S4 and S5). Among these, a number of stress-responsive genes were altered in the dsm1 mutants, and these genes encode proteins with diverse functions such as transcription factors, protein kinases, calcium-binding proteins, stress-inducible proteins, antioxidant enzymes, and cell wall biosynthetic proteins. To confirm the microarray results and to have a closer view of the two POX genes, the expression of POX22.3 and POX8.1 were assessed by real-time PCR in the dsm1 and wild-type seedling plants treated with or without drought stress for 72 h. Consistent with the microarray results, the transcript levels of POX22.3 and POX8.1 were significantly lower in the mutants than in wild type under both normal and drought-stress conditions (Fig. 8). A number of other genes, including two genes encoding protein Brittle-1 (LOC_Os09g32200) and ATP-binding protein (LOC_Os01g54420), were also confirmed by real-time PCR for their expression level changes in the mutant (Fig. 8).

The dsm1 Mutant Is Sensitive to Oxidative Stress

 Peroxidases are involved in defense against abiotic stresses through scavenging of ROS (Hiraga et al.,

Figure 6. Protein sequence analysis of DSM1. A, Schematic diagram of functional domains. B, Phylogenetic tree of MAPKKK members in plants. The phylogenetic tree was created in MEGA4 software with the neighbor-joining method. Numbers indicate percentage values after 1,000 replications. Scale indicates amino acid substitutions per position. Prefixes on protein names indicate species of origin: At, Arabidopsis; Bn, Brassica napus; Nt, N. tabacum; Os, O. sativa; Le, S. lycopersicum var. lycopersicum; Cm, Cucumis melo; Fs, Fagus sylvatica; Ah, Arachis hypogaea.

Figure 7. DSM1 KD displayed kinase activity. A, Silver-stained SDS-PAGE analysis of immunopurified DSM1 KD protein (with FLAG) from ZH11 (lane 2) and transgenic rice expressing DSM1 KD (lane 3). Molecular weight marker (lane 1) is indicated on the left. B, Immunopurified DSM1 KD protein was mixed with MBP (lane 1) or buffer alone (lane 2) in kinase reaction. Phosphorylated protein was analyzed by SDS-PAGE, then followed by autoradiography. The arrows indicate the position of the proteins.
In the *dsml* mutants, the expression levels of *POX22.3* and *POX8.1* and a few other genes encoding putative oxidoreductases were significantly reduced. This suggests that the hypersensitivity of the *dsml* mutant to drought stress may result from reduced capacity for ROS scavenging. To test this, POX activities in the soluble protein extracts from the drought-stressed and nonstressed leaves of *dsml* and wild-type plants were measured. Peroxidase activity was increased after drought stress in both *dsml* and wild-type plants; however, significantly lower POX activity was detected in the *dsml* plants than in the wild-type plants (Fig. 9A).

It has been determined previously that the POX activity in plants is correlated with increased tolerance to oxidative stresses (Kim et al., 2008). Therefore, the response of *dsml* plants to oxidative stress was analyzed. Methyl viologen (MV) is a well-known oxidative stress inducer that inhibits electron transport during photosynthesis and generates H$_2$O$_2$ in chloroplasts under light (Chen et al., 2006). The leaves from plants grown in soil were floated on 4 $\mu$m MV and electrolyte leakage, an indicator of membrane damage, was measured every 4 h. The results showed that the *dsml* leaves exhibited significantly higher electrolyte leakage than the wild-type leaves (Fig. 9B). After 24 h of MV treatment, more than 49% of ions leaked from cells in the *dsml*-1 mutant, whereas ion leakage of the wild type was less than 36%. The sensitivity to MV was also checked in intact plants. Two-week-old seedlings of *dsml* and wild-type were exposed to 10 $\mu$m MV. At 1 d after MV treatment, necrosis was observed on the leaves of *dsml*-1, while all wild-type leaves were still green (Fig. 9C). At 3 d, the *dsml*-1 mutants showed much more severe chlorosis and damage than wild-type plants (Fig. 9C). After severe oxidative stress (2-week-old seedlings treated with 30 $\mu$m MV for 3 d and followed by recovery for 2 d), most of the mutant plants died while the wild-type plants recovered very quickly (Fig. 9D). A severe decrease of chlorophyll content (35% chlorophyll retained compared to untreated plants) was observed in the *dsml*-1 plants exposed to 30 $\mu$m MV for 3 d, whereas no obvious decrease of chlorophyll content was noted in wild-type plants after the same treatment (Fig. 9E).

ROS are known to cause damage to cellular membranes. It is possible that the membrane damage in the *dsml*-1 plants caused by oxidative stress may be associated with accumulation of ROS. Therefore, we determined the accumulation of H$_2$O$_2$ by 3,3′-diaminobenzidine (DAB) staining and superoxide by nitroblue tetrazolium staining. Under normal growth conditions, no obvious H$_2$O$_2$ was detected in either wild-type or *dsml*-1 plants. After MV stress (30 $\mu$m MV treatment for 72 h), H$_2$O$_2$ was detected in both the wild type and the mutant, but accumulation of brown precipitate (DAB staining) in the mutant was much larger than in the wild type (Fig. 9F). However, no significant difference in nitroblue tetrazolium staining was observed between the mutant and wild type (data not shown). Together, these observations demonstrate the enhanced sensitivity of the *dsml* mutant to oxidative stress resulted from reduced ability of ROS (at least H$_2$O$_2$) scavenging.

**Overexpression of DSM1 Improves Tolerance to Dehydration Stress**

Genetic and physiological analysis of the *dsml* mutant implies that DSM1 may play a positive role in the regulation of stress resistance in rice. We predicted that overexpression of DSM1 might result in an increased stress tolerance. To test this hypothesis, transgenic plants were generated by overexpressing the full-length DSM1 fused with the FLAG tag under the control of the maize ubiquitin promoter. A total of 43 independent transgenic plants were obtained and the expression level of DSM1 was verified by western blot with antibody against the FLAG tag. Three independent transgenic lines (denoted with numerals 9, 38,
Figure 9. The dsml mutants showed hypersensitivity to oxidative stress. A, The POX activity in ZH11 and dsml-1 during drought stress. Values are means ± se (n = 3). **P < 0.01 (t test). FW, Fresh weight. B, Analysis of MV stress-related electrolyte leakage in ZH11 and dsml-1 at indicated time. Values are means ± se (n = 4). **P < 0.01 and *P < 0.05 (t test). C, Leaf phenotype of ZH11 and dsml-1 plants (three-leaf stage) after MV stress (10 μM MV for 4 d). D, Phenotype of ZH11 and dsml-1 (three-leaf stage) plants after MV stress (30 μM MV for 4 d and recovery for 2 d). E, Total chlorophyll contents at 96 h after 30 μM MV stress. Values are means ± se (n = 3). **P < 0.01 (t test). WT, Wild type. F, DAB staining for H2O2 in unstressed and MV-stressed leaves of ZH11 and dsml-1 plants.
and 42) with higher expression (Fig. 10A) were selected for further analyses.

We first tested the tolerance of DSM1-overexpression plants under mannitol (120 mM) treatment, which mimics drought stress in terms of dehydration and oxidative outcomes. We observed that the shoots of the overexpression plants were slightly shorter than those of wild-type plants in normal Murashige and Skoog (MS) medium, but the wild-type plants exhibited more serious growth inhibition than the overexpression plants under the stress conditions (Fig. 10B). Statistical analysis suggested that the overexpression lines had significantly less suppression of shoot growth (58% to 65% of normal growth) than wild type (only about 45% of the normal growth) under the mannitol treatment (Fig. 10C). These results suggest that overexpression of DSM1 has a positive effect on stress tolerance.

**DISCUSSION**

Plants have developed sophisticated signaling pathways to deal with environmental stress. Protein kinases such as CDPKs, CIPKs, and MAPK cascades have been shown to have important roles in abiotic stress signaling as well as in many other signaling processes. MAPK cascades are evolutionarily conserved and involved in signaling pathways related to biotic and abiotic stress responses. Compared with those in yeast (*Saccharomyces cerevisiae*) and mammalian, however, only a limited number of genes in MAPK cascades have been identified in plants (Ichimura, 2002).

Here, we report the identification and characterization of a drought-sensitive mutant (*dsm1*) in rice. A database search and sequence analysis suggests that DSM1 contains a conserved protein KD and features a B3 subgroup of plant Raf-like MAPKKK. In Arabidopsis, six genes encoding MAPKKK proteins have been identified (Ichimura, 2002). But there are only two members of the B3 subgroup of plant Raf-like MAPKKKs reported with known function: (1) CTR1 and its tomato (*Solanum lycopersicum* var. *lycopersicum*) ortholog negatively regulate ethylene responses and play a role in defense response (Kieber et al., 1993; Leclercq et al., 2002; Lin et al., 2008), and (2) EDR1, which is involved in salicylic acid-inducible defense response. The *edr1* mutant showed resistance to powdery mildew and senesced early in response to exogenous ethylene (Frye and Innes, 1998; Frye et al., 2001). Protein kinase activity has been found for many reported MAPKKKs (Covic et al., 1999; Tang and Innes, 2002; Huang et al., 2003). Here we also demonstrated that the DSM1 KD can phosphorylate itself and MBP. To the best of our knowledge, DSM1 is the first member of the MAPKK family to be identified as having drought-stress signaling function in plants.

To study the function of DSM1, we identified two T-DNA insertion mutant alleles for the DSM1 gene. Meanwhile, DSM1-RNAi plants were also generated to genetically confirm the mutant phenotype. The results revealed that both of the *dsm1* mutants and the DSM1-RNAi plants displayed hypersensitivity to drought stress in seedling and panicle developmental stages. Even though the mutants showed slightly increased sensitivity to salt stress at the seedling stage,
no significant difference in seed germination or seedling growth was observed between wild-type and mutant plants when they were treated with ABA, salt, cold, and heat stress (data not shown). These findings suggest that DSM1 may mainly function in drought-stress signaling through an ABA-independent pathway. In many previous studies, knock out of MAPKKK genes often resulted in aberrant morphologies when the mutants were grown under normal growth conditions. For example, a mutant of the Arabidopsis CTR1 gene exhibited constitutive triple responses in the absence of ethylene (Kieber et al., 1993). Double mutant (anp2 anp3) for two MAPKKK genes displayed an overall reduction in total plant size and disrupted cell division, but the mutant of a single gene was not significantly different from the wild type (Krysan et al., 2002). Most recently, a mekk1 mutant was reported with abnormal phenotypes including extremely dwarfed, curled leaves and early senescence (Nakagami et al., 2006). We also examined the phenotypes of the two dsm1 mutants under normal growth conditions. However, neither of the dsm1 mutants displayed any obvious phenotypic change throughout the life cycle of rice. Although it is possible that functional redundancy may exist for DSM1 and other rice MAPKKK genes with additional function, our results suggested that DSM1 plays essential roles in drought-stress signaling in rice since a single mutation of this gene caused hypersensitivity to drought stress.

Phylogenetic analysis suggests that DSM1 belongs to a subfamily of MAPKKK in plants in which only two proteins, CTR1 and EDR1, have been functionally characterized. Tang and Innes failed to obtain overexpression plants on transformation of the full-length cDNA of EDR1 in either the wild type or an edr1 mutant of Arabidopsis (Tang and Innes, 2002). It was proposed that overexpression of EDR1 was either lethal to Arabidopsis or blocked some step in the transformation process. A similar lethal phenotype was observed in the overexpression of MEKK1 in Arabidopsis under the 35S Cauliflower mosaic virus or a dexamethasone-inducible promoter (Nakagami et al., 2006). Unlike the overexpression of EDR1 and MEKK1, overexpression of DSM1 in rice yielded no obvious phenotypic changes under normal growth conditions, suggesting that DSM1 has evolved with different function in rice compared to its closely related homologs in Arabidopsis. Indeed, overexpression of DSM1 enhanced tolerance to dehydration stress caused by mannitol. Western-blot analysis with anti-FLAG antibody showed that the increased DSM1 protein level was linked to increased mannitol tolerance. Together with the mutant phenotypes, these results suggest that DSM1 participates in positive control of drought resistance in rice. Even though the survival rates of the DSM1 overexpression lines were similar to those of the wild type after drought stress at the seedling stage under soil conditions (data not shown), the effect of DSM1 overexpression on drought stress under different conditions remains to be determined since drought resistance is a complex trait that is affected by developmental stages. In addition, constitutively increasing transcript or protein level of a protein kinase may not necessarily lead to significant phenotypic gains since most protein kinases need to be activated to exert functions.

It was previously shown that overexpression of NPK1, a MAPKKK family gene, increased tolerance to freezing, heat shock, and salt stress (Kovtun et al., 2000). DSM1 is significantly different from NPK1, not only in functional domain composition but also in the spectrum of functions in stress resistance. Our results suggest that DSM1 functions specifically in drought and oxidative stresses. To elucidate the mechanisms of dsm1 in drought resistance, we measured Pro content, stomata number, and stomatal conductance of dsm1 mutants. However, no significant difference was found for these measurements between the mutants and wild-type plants in response to drought stress (data not shown). Therefore, we further checked the global expression profiles of dsm1 and wild-type plants. Even though the expression of a number of genes was significantly increased or decreased in the mutant compared to wild type, most of these genes appear to be irrelevant to stress resistance based on annotations or expression patterns of these genes. However, we noticed that the expression of two POX-encoding genes, POX22.3 and POX8.1, were markedly reduced in the dsm1 mutant. This clue led us to test oxidative stress response and ROS scavenging of the mutant.

ROS homeostasis is well known to be associated with oxidative stresses. When ROS is low in cells, they act as signal molecules. Under serious biotic and abiotic stress, ROS can be overproduced and cause damage of cell growth by oxidizing proteins, lipids, and DNA (Pollé, 2001; Mittler et al., 2004). Therefore, plants have developed sophisticated systems to protect themselves from oxidative stress by adjusting ROS homeostasis. These systems include numerous scavenging enzymes such as superoxide dismutase, POX, ascorbate peroxidase, glutathione peroxidase, catalase, and low Mn, antioxidants (Noctor and Foyer, 1998; Apel and Hirt, 2004; Mittler et al., 2004). Peroxidases scavenge ROS by catalyzing oxidoreduction between H2O2 and reductants. Plant POXs exist as a large family of isozymes and are involved in a broad range of physiological processes (Lagrimini et al., 1990; Young et al., 1995; Quiroga et al., 2000; Passardi et al., 2005). Peroxidases have been implicated in defense against abiotic stresses through increased scavenging of ROS (Hiraga et al., 2001). For example, overexpression of sweet potato (Ipomoea batatas) POX gene swpa4 led to 50-fold higher POX activity compared with the wild-type plant. The transgenic plants showed a significantly enhanced tolerance to a variety of abiotic and biotic stresses (Kim et al., 2008). Most recently, a metallothionein protein in rice was reported to confer enhanced tolerance to drought and oxidative stress by increasing activities of antioxidants catalase, POX, and
ascorbate peroxidase (Yang et al., 2009). POX22.3 and POX8.1 are POX genes that are predominantly expressed during resistant interactions (Chittoor et al., 1997). Here, we found that the expression levels of these two genes were dramatically down-regulated in the dsm1 mutant compared to the wild type, and the POX activity in dsm1 was also significantly decreased. In accordance with the reduced POX activity, dsm1 showed markedly increased sensitivity to oxidative stress as indicated by the significant increase of chlorophyll degradation, chlorosis, and ion leakage. Overall, our results indicated that dsm1 mutant plants are more sensitive to drought and oxidative stresses due to an increase in ROS damage caused by the reduced POX activity. Several studies have demonstrated that MAPK cascades are involved in ROS signaling or homeostasis regulation. OMTK1, a MAPKKK activated by H$_2$O$_2$ in alfalfa, channels oxidative stress signaling by direct MAPK interaction (Nakagami et al., 2004). Studies of MEKK1 revealed that MEKK1 functions in integrating ROS homeostasis with plant development and hormone signaling (Ichimura et al., 2006; Nakagami et al., 2006). A recent study suggested that overexpression of a maize MAPKK gene, ZmMPK7, in transgenic tobacco can enhance protection provided by the POX defense systems against ROS-mediated injury during osmotic stress (Zong et al., 2009). Here we show that DSM1 plays a critical role in mediating oxidative stress signaling and drought resistance in rice. We have attempted to identify DSM1-interacting proteins by yeast two-hybrid screening and pair-wise interaction testing with rice MAPKKs, but neither experiment resulted in positive DSM1-interaction proteins. Of special note, DSM1 is localized in the nucleus, which contrasts to previously reported MAPKKs thought to be localized in cytoplasm. Although the direct phosphorylation targets or interacting proteins of DSM1 remain to be revealed, our results suggest that DSM1 may be a novel nuclear protein kinase mediating oxidative stress signaling by a different pathway from traditional MAPK cascades.

In conclusion, this study establishes that DSM1 is a novel nuclear protein kinase with sequence similarity to Raf-like MAPKKs that plays critical roles in drought and oxidative stress resistance in rice by directly or indirectly regulating POX22.3 and POX8.1 expression and ROS scavenging. Efforts are ongoing to identify the target proteins of DSM1 to reveal the complete pathway of DSM1 in drought and oxidative stress signaling.

MATERIALS AND METHODS

Plant Materials

The japonica rice (Oryza sativa) cultivars HY and ZH11 were used in this study. The dsm1-1 (ZH11 background) and dsm1-2 (HY background) mutant seeds were obtained from the Rice Mutant Database (http://rmd.ncpgr.cn) and POSTECH RISD (http://www.postech.ac.kr/life/pg/risd/), respectively. Homozygous mutants were identified and used in further analyses.

Stress Treatments

To measure the transcript level of the DSM1 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 drought (removal of the water supply from the plants), salt (addition of 200 ms NaCl in the hydroponic culture medium), cold (exposure of the plants to 4°C), and 100 μM ABA followed by sampling at the designated time.

DSM1 overexpression and RNAi transgenic plants were selected by germinating seeds on MS medium containing 50 mg L$^{-1}$ hygromycin. Wild-type and homozgyous mutants were grown on MS medium or grown in soil.

For drought stress at the seedling stage, mutant/transgenic plants and wild-type plants were growing in the same barrels filled with a mixture of soil and sand (1:1). At the five-leaf stage, the water supply was depleted to allow drought stress to develop. After recovery, surviving seedlings were photographed and analyzed. Water-loss rates of detached leaves from the wild type and the mutant were measured by monitoring the fresh weight loss at indicated time points. The drought stress at the panicle development stage was conducted by growing plants in PVC tubes (1 m in height and 20 cm in diameter) and drought-stress method was the same as described previously (Hu et al., 2006; Yue et al., 2006).

To evaluate mannitol stress tolerance, germinated seeds were transplanted in MS medium supplemented with 125 mM mannitol. After 10 d of growth, shoot length was measured.

For the oxidative treatment, rice seedlings at the two-leaf stage were submersed into a 10 or 30 μM MV solution. After 3 d, the seedlings were transferred to distilled water for recovery and photographed.

RNA Isolation and RT-PCR

Total RNA was isolated from rice leaves using TRIzol reagent (Invitrogen). cDNA templates were synthesized using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Real-time quantitative RT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems) using SYBR Premix Ex Taq (Takara) according to the manufacturer’s protocol. Rice Actin1 gene was used as the endogenous control. The relative quantization method (ΔΔCT) was used to evaluate quantitative variation of replicates examined. For semiquantitative RT-PCR, PCR amplifications were performed in 50 μL volumes with the following protocol: one cycle of 94°C for 4 min, 25 to 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min. The gene-specific primers are listed in Supplemental Table S6.

Plasmid Construction and Rice Transformation

A full-length cDNA of DSM1 was obtained from the Knowledge-based Orzya Molecular biological Encyclopedia (http://cDNA01.dna.affrc.go.jp/cDNA/). To generate the DSM1 full-length and KD overexpression constructs, a 1.8-kb fragment containing the entire coding region or only the KD was amplified from the full-length cDNA clone by PCR. The fragments were inserted into the p3301U-FLAG vector as described previously (Sun and Zhou, 2008). To investigate DSM1 gene expression, a genomic DNA sequence corresponding to 2,100 bp upstream of the predicted ATG codon of the open reading frame was cloned into pCAMBIA-3912 binary vector. For subcellular localization, the coding region of DSM1 cDNA was prepared by PCR, and cloned into the GATEWAY destination vector pHWG2.0 binary vector by recombination reaction (Invitrogen; Karimi et al., 2005). DSM1-RNAi construct was made by introducing a fragment targeting 400 bp open reading frame region of DSM1 into the pDS1301 vector (Chu et al., 2006). The constructs were introduced into japonica rice ZH11 by Agrobacterium-mediated transformation (Hei et al., 1994). The GUS assay was performed using the histochemical staining method as described previously (Sun and Zhou, 2008). To investigate DSM1 gene expression, a genomic DNA sequence corresponding to 2,100 bp upstream of the predicted ATG codon of the open reading frame was cloned into pCAMBIA-3912 binary vector. For subcellular localization, the coding region of DSM1 cDNA was prepared by PCR, and cloned into the GATEWAY destination vector pHWG2.0 binary vector by recombination reaction (Invitrogen; Karimi et al., 2005). DSM1-RNAi construct was made by introducing a fragment targeting 400 bp open reading frame region of DSM1 into the pDS1301 vector (Chu et al., 2006). The constructs were introduced into japonica rice ZH11 by Agrobacterium-mediated transformation (Hei et al., 1994). The GUS assay was performed using the histochemical staining method as described previously (Sun and Zhou, 2008).

Plant organ, tissue, or young shoots were incubated in staining buffer (50 mM sodium phosphate at pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mg mL$^{-1}$ of X-Rhodamine, 100 μg mL$^{-1}$ of chloramphenicol, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, and 20% methanol) at 37°C overnight and then washed with 70% ethanol to remove chlorophyll. GUS images were taken with a fluorescence stereomicroscope (Leica MF FLIII). To determine the subcellular localization of DSM1, more than 20 DSM1-GFP transgenic plants were obtained. Young roots of 2-week-old transgenic plants were examined for GFP fluorescence under a Leica TCSSP2 fluorescence. Rice protoplast transformation was performed according to the method of Zhou et al. (2009) with minor modifications (Zhou et al., 2009).
Immunoprecipitation and Kinase Assay

Rice leaves (2 g) were ground into powder in liquid nitrogen and mixed with 4 mL of protein extraction buffer (100 mM HEPES, 5 mM EDTA, 5 mM EGTA, 10 mM dithiothreitol [DTT], 10 mM Na$_2$VO$_4$, 10 mM NaF, 50 mM P-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 mM $\gamma$-ATP, 0.1% Triton X-100, 5 pg mL$^{-1}$ leupeptin, 10% [v/v] glycerol). The extract was centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant was incubated with 40 mL anti-FLAG M2 affinity gel overnight at 4°C. The resin was washed three times with Tris-buffered saline (pH 7.5, Tris-HCl, 150 mM NaCl, pH 7.4). Proteins were eluted with 150 mg mL$^{-1}$ 3X Flag peptide according the manufacturer’s instructions (Sigma). Eluted proteins were subjected to SDS-PAGE and the interesting bands were analyzed by MALDI-TOF-MS.

For the kinase assay, 2 g of purified DSM1 protein was added into 20 mL of kinase buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 10 mM MnCl$_2$, 1 mM DTT, 2 pg of MBP, 0.2 mM ATP, 1 $\mu$M $\gamma$-$\beta$-$\alpha$ATP). The reaction was incubated at room temperature for 30 min and terminated by the addition of 5 $\mu$L of sample buffer and heating at 100°C for 5 min. After separation on a 15% SDS-PAGE gel, the gel was dried at 60°C for 2 h. The $\gamma$-$\beta$-ATP-labeled bands were detected using Kodak x-ray film.

Microarray

Two independent biological replicates of 6-week-old dom1-1 and wild-type plants under both normal and drought-stress conditions were used for microarray experiments. For microarray analysis, the process for experiment followed the standard protocol of Affymetrix GeneChip service (CapitalBio). To find differentially expressed genes, the signal ratio of each gene between the wild type and the mutant was calculated. Genes exhibiting more than 2-fold enhanced or 2.5-fold reduced transcription level in two biological replicates are listed in Supplemental Tables S2 to S5.

Western-Blot Analysis

Total leaf proteins were extracted using extraction buffer (100 mM HEPES, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na$_2$VO$_4$, 10 mM NaF, 50 mM P-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 mg mL$^{-1}$ aprotinin, 5 pg mL$^{-1}$ leupeptin, 10% [v/v] glycerol). The total protein was separated on 12% SDS-PAGE gel and then transferred to polyvinylidene difluoride membrane. The membrane was incubated with anti-FLAG antibody and western blot was conducted according to Hou et al. (2009).

Measurement of POX Activity, Electrolyte Leakage and Chlorophyll Content, and DAB Staining

To measure POX activity, enzymes were extracted using 0.05 M potassium phosphate buffer (pH 7.0). The homogenate extract was centrifuged at 12,000g for 15 min at 4°C. The supernatant was used as the enzyme extract. Peroxidase activity was performed according to the method of Kwak et al. (1995) with minor modifications (Kwak et al., 1995). The reaction mixture (in a total volume of 5 mL) contained the enzyme extract (0.1 mL), 0.05 mM potassium phosphate buffer (pH 5.5, 2.9 mL), 2% (v/v) H$_2$O$_2$ (1 mL), and 0.05 mM guaiacol (1 mL) as substrates. The oxidation of guaiacol was monitored and absorbance measured at 470 nm every 30 s. For electrolyte leakage, six leaves from six plants were placed in 10 mL tubes containing 6 mL of 4 mM MV. The conductivity of the MV solution was determined with an ion leakage meter (Leici) at the designated time. Total electrolyte leakage was measured after boiling the sample for 15 min. The percentage of electrolyte leakage caused by MV treatment was determined by the ratio of ion leakage at a given time and total ion leakage. Chlorophyll content was carried out according to the method described by Lichtenthaler (1987). For DAB staining, leaves were vacuum infiltrated with 0.1 mg mL$^{-1}$ 3,3’-diaminobenzidine in 50 mM Tris-acetate buffer, pH 5.0. Samples were incubated for 24 h at room temperature in the light. To remove chlorophylls, the stained samples were transferred to 100% ethanol and incubated at 100°C for 15 min. Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AK100519.

Supplemental Data

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

Supplemental Figure S1. The dom1-1 mutants showed increased sensitivity to salt stress (three-leaf stage plants were treated with 100 mM NaCl).

Supplemental Figure S2. Alignment of DSM1 with plants known function MAPKKs.

Supplemental Figure S3. Identification of DSM1 KD using MALDI-TOF-MS.

Supplemental Table S1. General information about MAPK cascades T-DNA mutants.

Supplemental Table S2. Genes down-regulated in dom1-1 mutant lines compared to wild type (DSM1/DSM1) in normal condition.

Supplemental Table S3. Genes up-regulated in dom1-1 mutant lines compared to wild type (DSM1/DSM1) in normal condition.

Supplemental Table S4. Genes down-regulated in dom1-1 mutant lines compared to wild type (DSM1/DSM1) in drought stress.

Supplemental Table S5. Genes up-regulated in dom1-1 mutant lines compared to wild type (DSM1/DSM1) in drought stress.

Supplemental Table S6. Primers used in this study.

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LITERATURE CITED


