A Calmodulin-Binding Mitogen-Activated Protein Kinase Phosphatase is Induced by Wounding and Regulates the Activities of Stress-Related Mitogen-Activated Protein Kinases in Rice

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The mitogen-activated protein kinase (MAPK) phosphatases (MKPs) are negative regulators of MAPKs. In dicotyledons such as Arabidopsis and tobacco, MKPs have been shown to play pivotal roles in abiotic stress responses, hormone responses and microtubule organization. However, little is known about the role of MKPs in monocotyledons such as rice. Database searches identified five putative MKPs in rice. We investigated their expression in response to wounding, and found that the expression of OsMKP1 is rapidly induced by wounding. In this study, we functionally characterized the involvement of OsMKP1 in wound responses. The deduced amino acid sequence of OsMKP1 showed strong similarity to Arabidopsis AtMKP1 and tobacco NtMKP1. Moreover, OsMKP1 bound calmodulin in a manner similar to NtMKP1. To determine the biological function of OsMKP1, we obtained osmkp1, a loss-of-function mutant, in which retrotransposon Tos17 was inserted in the second exon of OsMKP1. Unlike the Arabidopsis atmkp1 loss-of-function mutant, which shows no abnormal phenotype without stimuli, osmkp1 showed a semi-dwarf phenotype. Exogenous supply of neither gibberellin nor brassinosteroid complemented the semi-dwarf phenotype of osmkp1.

Activities of two stress-responsive MAPKs, OsMPK3 and OsMPK6, in osmkp1 were higher than those in the wild type both before and after wounding. Microarray analysis identified 13 up-regulated and eight down-regulated genes in both before and after wounding. Microarray analysis identified five genes with clear responses to wounding, indicating that OsMKP1 is involved in the negative regulation of rice wound responses.

Keywords: MAPK — MAPK phosphatase — Oryza sativa — Phosphorylation — Wound.

Introduction

The mitogen-activated protein kinases (MAPKs) are key molecules of signal transduction responses to various extracellular stimuli in eukaryotes. In plants, MAPKs are implicated in regulating growth, development and response to environmental stresses (for reviews, see Guo and Ecker 2004, Gray and Hetherington 2004, Takahashi et al. 2004, Pedley and Martin 2005). The activation of MAPKs is strictly regulated via phosphorylation of the conserved TxY motif by an upstream MAPK kinase (Widmann et al. 1999, Kyriakis and Avruch 2001). Conversely, MAPKs are inactivated by dephosphorylation of either threonine or tyrosine of the TxY motif. Numerous studies have shown that WIPK and SIPK, two tobacco MAPKs, as well as their corresponding MAPKs in other dicotyledonous species including Arabidopsis, alfalfa, tomato and parsley, are activated by a wide range of biotic and abiotic stresses such as wounding, osmotic shock, high salinity, drought, UV irradiation, ozone exposure, extreme temperature, oxidative stress and pathogen infection (Seo et al. 1999, Ichimura et al. 2000, Zhang and Klessig 2000, Tena et al. 2001, Asai et al. 2002, Jonak et al. 2002, MAPK Group 2002, Holley et al. 2003, Kroj et al. 2003, del Pozo et al. 2004). Recent studies have shown that rice OsMPK5a (also known as OsMPK2, OsMSRMK2, OsMAP1 and OsBIMK1) and OsMPK6 (also known as OsMPK2), corresponding to tobacco WIPK and SIPK, respectively, are also activated by a variety of stresses such as wounding, drought, high salinity, cold and pathogen infection (Agrawal et al. 2003, Xiong and Yang 2003, Kurusu et al. 2005, Lieberherr et al. 2005). To avoid complexity in...
describing the gene names, we refer to them as OsMPK3 and OsMPK6 in this study following the recently published nomenclature (Hamel et al. 2006). The roles of WIPK and SIPK as well as their corresponding MAPKs in other plant species have been revealed by genetic analyses. For example, silencing of WIPK or SIPK by RNA interference compromises wound-induced accumulation of jasmonic acid (Seo et al. 2007). Similarly, Xiong and Yang (2003) reported that OsMPK3-silenced rice plants have significant reductions in abiotic stress tolerances.

MAPK phosphatases (MKPs) are dual-specificity phosphatases that dephosphorylate both serine/threonine and tyrosine residues, and act as negative regulators of MAPKs (Camps et al. 2000, Keyse 2000, Theodosiou and Ashworth 2002). In contrast to many members of the MAPK family, plant MKPs probably form a small gene family. Only five MKPs are predicted in the Arabidopsis genome, including the previously reported AtMKP1, IBR5, PHS1 and DsPTP1 (Kerk et al. 2002). Mutations of the AtMKP1, IBR5 and PHS1 genes cause sensitivity to genotoxic stresses, reduced responses to the phytohormones auxin and ABA, and compromised cortical microtubule functions, respectively (Ulm et al. 2001, Monroe-Augustus et al. 2003, Naoi and Hashimoto 2004). Previously, we also showed that overexpression of NtMKP1, a putative tobacco ortholog of AtMKP1, compromised wound-induced activation of SIPK and WIPK (Yamakawa et al. 2004). This body of genetic evidence clearly revealed the important biological role of MKPs in dicotyledons. However, little is known about the role of MKPs in economically important monocotyledons such as rice. No MKP has been identified and characterized in rice. In this study, we identified five putative MKPs in rice by database searches and investigated their expression in response to wounding. Expression of OsMKP1 was rapidly induced by wounding. Studies of a retrotransposon-insertional mutant of OsMKP1 suggest that OsMKP1 negatively regulates wound responses in rice.

Results

Inactivation, but not activation of OsMPK3 and OsMPK6 depends on de novo protein synthesis

Activation of MAPKs is one of the earliest responses in wounded plants (Seo et al. 1999, Zhang and Klessig 2000, Mesiene et al. 2003). Xiong and Yang (2003) showed, using an anti-OsMPK3 antibody, that OsMPK3 is rapidly activated by wounding. However, their antibody was raised against the C-terminal 140 amino acids of OsMPK3, which are highly conserved among MAPKs, and the cross-reactivity of the antibody with other MAPK(s) was not assessed (Xiong and Yang 2003). Therefore, we first investigated whether OsMPK3 and OsMPK6, corresponding to tobacco WIPK and SIPK, respectively, are activated by wounding. To measure the specific activities of OsMPK3 and OsMPK6, antisera were raised in rabbits against peptides corresponding to the unique N-termini of OsMPK3 (OsMPK3-N) and OsMPK6 (OsMPK6-N), respectively. The specificity of the antisera was assessed by immunocomplex kinase assay and immunoblot analysis. Immunocomplex kinase assay with myelin basic protein (MBP) as a substrate showed that precipitation of OsMPK3 activity with anti-OsMPK3 antisera was specifically competed by OsMPK3-N peptide, but not by OsMPK6-N peptide, and vice versa (Fig. 1A). Neither OsMPK3 nor OsMPK6 activity was precipitated with pre-immune sera (Fig. 1A). Similarly, immunoblot analyses with recombinant OsMPK3 and OsMPK6 proteins showed that anti-OsMPK3 antisera specifically recognized recombinant OsMPK3, but not OsMPK6, and vice versa (Fig. 1B).

Having established the specificity of the antisera, the activities of OsMPK3 and OsMPK6 were measured by immunocomplex kinase assay. Activities of both OsMPK3 and OsMPK6 were transiently increased by wounding within 10 min and returned to nearly the basal level 30 min after wounding (Fig. 1C). The activity of OsMPK6 after wounding was much stronger than that of OsMPK3, although they showed similar activation patterns after wounding. Accumulation of OsMPK3 and OsMPK6 proteins was investigated by immunoblot analyses with affinity-purified antibodies. Several bands were detected with the anti-OsMPK3 antibody; however, the size of one corresponded to the predicted molecular mass of OsMPK3. Anti-OsMPK6 antibody detected a single band of the predicted size, which is absent in extracts from osmpk6 null mutant cells (Fig. 1D). The levels of OsMPK3 and OsMPK6 proteins were constant after activation by wounding (Fig. 1D).

To investigate whether activation or inactivation of OsMPK3 and OsMPK6 depends on de novo protein synthesis, plants were treated with cycloheximide (CHX) prior to wounding. As shown in Fig. 1C, activation levels of OsMPK3 and OsMPK6 by wounding in CHX-treated plants were comparable with those in control plants, but their activities remained high 2 h after wounding. Moreover, CHX treatment increased the activities of both OsMPK3 and OsMPK6 without wounding. When plants were treated with CHX for a longer time, the activities of both OsMPK3 and OsMPK6 in unwounded leaves reached levels comparable with those in leaves wounded for 10 min (data not shown). The levels of OsMPK3 and OsMPK6 proteins were constant after activation by wounding irrespective of the presence of CHX (Fig. 1D). These results suggest that the activities of both OsMPK3 and OsMPK6 are regulated post-translationally by pre-existing and de novo...
synthesized negative regulators, probably protein phosphatases.

Accumulation of OsMKP1 mRNA is rapidly induced by wounding

MKPs are dual-specificity phosphatases and act as negative regulators of MAPKs. In dicotyledons, MKPs have been shown to play pivotal roles in abiotic stress responses, hormone responses and microtubule organization (Ulm et al. 2001, Monroe-Augustus et al. 2003, Naoi and Hashimoto 2004). However, little is known about the role of MKPs in rice. We searched databases with rice genome sequences, rice full-length cDNA clones and rice expressed sequence tags (ESTs), and identified five putative MKPs that contain the catalytically essential triad. The deduced amino acid sequences of the putative dual-specificity phosphatase (DSP) catalytic domain of rice and Arabidopsis MKPs were aligned, and a phylogenetic tree was generated. As shown in Fig. 2A, rice MKPs clearly correspond to MKPs in Arabidopsis. Therefore, we designated the rice MKPs as OsMKP1, OsIBR5, OsDsPTP1, OsPHS1a and OsPHS1b, respectively (Fig. 2A).

As the inactivation of OsMPK3 and OsMPK6 depends on de novo protein synthesis, the expression pattern of rice MKPs in wounded leaves was investigated by RNA gel blot analysis. Expression of OsMKP1 was rapidly induced after wounding, whereas that of OsIBR5 was induced much later (Fig. 2B). Accumulation of OsDsPTP1 mRNA was initially decreased by wounding and then returned to the basal level (Fig. 2B). The accumulation of OsPHS1a and OsPHS1b mRNAs could not be detected by reverse transcription–PCR analysis, although we tested 5–10 combinations of primer to amplify them. As the expression of OsMKP1 was rapidly induced by wounding, we focused on OsMKP1 in the subsequent experiments.
OsMKP1 binds calmodulin (CaM) in a manner similar to tobacco NtMKP1

Previously, we reported that NtMKP1, a putative tobacco ortholog of OsMKP1, binds CaM in a Ca\(^{2+}\)-dependent manner (Yamakawa et al. 2004). As the putative CaM-binding domain is conserved in OsMKP1, we tested whether OsMKP1 binds CaM. The putative CaM-binding domain and surrounding region of OsMKP1 was expressed in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein and subjected to a CaM overlay assay. Because tobacco NtCaM1 is \(\approx99\%\) similar to rice CaM (X65016) in its primary structure, we used alkaline phosphatase-labeled NtCaM1. As shown in Fig. 3, CaM bound the GST-fused CaM-binding domain, but not GST alone, in a Ca\(^{2+}\)-dependent manner. Mutation of either Trp440 or Leu443 of NtMKP1 completely abolishes its binding to CaM (Yamakawa et al. 2004, Katou et al. 2005). The corresponding amino acids of OsMKP1 are Trp458 and Leu461, respectively. To investigate whether OsMKP1 binds CaM in a manner similar to NtMKP1, we converted Leu451, Trp458, Leu461, and Ser467 of OsMKP1 to arginine and tested their binding to CaM. As expected, mutations in Trp458 or Leu461 specifically compromised the binding of OsMKP1 to CaM (Fig. 3B). This result indicates that the interaction between MKP1 homologs and CaM is conserved among dicot and monocot plants.

Retrotransposon insertional line of OsMKP1

To reveal the biological function of OsMKP1, we obtained a retrotransposon *Tos17* insertional mutant line (NF3008) referred to as *osmkp1*. In this line, *Tos17* was found to be inserted into the second exon of OsMKP1 (Fig. 4A). Quantitative real-time PCR analysis revealed that OsMKP1 mRNA was severely reduced in *osmkp1* compared with the wild type (Fig. 4B). Moreover, we previously reported that the gelsolin-homology domain is required for the phosphatase activity of NtMKP1, a tobacco homolog of OsMKP1 (Katou et al. 2005). These results suggest that *osmkp1* is a null mutant. Unlike the *Arabidopsis* *atmkp1* loss-of-function mutant, which shows no abnormal phenotype, *osmkp1* shows a semi-dwarf phenotype with erect leaves (Fig. 4C). To confirm that the semi-dwarf phenotype of *osmkp1* is due to the loss-of-function of OsMKP1, *OsMKP1* cDNA under the control of an actin promoter was introduced into *osmkp1*. Expression of *OsMKP1* cDNA clearly restored the growth defects of *osmkp1*, although the transcript level of the transgene in complemented line 5 was about half that of the wild type. We used complemented lines 5 and 32 in further experiments as lower and higher expression lines, respectively. The semi-dwarf phenotype with erect leaves of *osmkp1* partially resembles mutants that are impaired in biosynthesis or signaling of gibberellin or brassinosteroid (BR). To investigate whether *osmkp1* has a defect in the biosynthesis of
and/or signaling of these growth hormones, \textit{osmkp1} was grown in medium supplemented with gibberellin or BR (Fig. 4D). Although exogenous application of GA3 resulted in long hypocotyls, the semi-dwarf phenotype of \textit{osmkp1} was not complemented. Similarly, the application of brassinolide (BL), a highly active BR, inhibited root growth of both the wild type and \textit{osmkp1}, as reported previously (Hong et al. 2002), but \textit{osmkp1} still showed the semi-dwarf phenotype. These results suggest that biosynthesis of these growth hormones in \textit{osmkp1} and the response of \textit{osmkp1} to these hormones are not impaired.

Increased activities of OsMPK3 and OsMPK6 in osmkp1

Rapid accumulation of \textit{OsMKP1} mRNA by wounding suggested its involvement in the inactivation of wound-activated OsMPK3 and OsMPK6 (Figs. 1, 2). Therefore, the activities of OsMPK3 and OsMPK6 in \textit{osmkp1} were compared with those in the wild type. In \textit{osmkp1}, both OsMPK3 and OsMPK6 were activated by wounding in a pattern similar to those in the wild type, but their activities were stronger than those in the wild type (Fig. 5A). Moreover, their activities in \textit{osmkp1} were stronger than those in the wild type before wounding (Fig. 5B), suggesting that OsMKP1 negatively regulates OsMPK3 and OsMPK6 both before and after wounding. To confirm that the stronger activities of OsMPK3 and OsMPK6 in \textit{osmkp1} were due to the loss of function of OsPK1, their activities in the two complemented lines were measured. As clearly shown, OsMPK3 activity in the two complemented lines was restored to levels similar to that in the wild type. In contrast, the activation of OsMPK6 in complemented line 5 was similar to that in \textit{osmkp1} although the basal activity was restored to the wild-type level. As the expression level of the \textit{OsMKP1} transgene in line 5 is about half that of endogenous \textit{OsMKP1} in the wild type (Fig. 5), complementation in this line seems to be incomplete.

It has been reported that OsMPK3-type MAPKs are regulated at transcriptional, translational and post-translational levels, whereas OsMPK6-type MAPKs are simply regulated by post-translational phosphorylation (Ichimura et al. 2000, Zhang and Klessig 2000, Jonak et al. 2002, Seo et al. 2007). To investigate whether the increased activities of OsMPK3 and OsMPK6 in \textit{osmkp1} are associated with increases in protein level, the accumulation of OsMPK3 and OsMPK6 proteins was investigated by immunoblot analysis. Accumulation of OsMPK3 protein is slightly, but reproducibly higher in \textit{osmkp1} than in the wild type, and is restored to the wild-type level in the complemented lines (Fig 5C). In contrast, the level of OsMPK6 protein was constant in all genotypes.

Constitutive expression of wound-responsive genes in osmkp1

To analyze the involvement of OsMKP1 in wound responses, we carried out a microarray analysis using the
Rice Oligo Microarray (Agilent Technologies, Inc., Palo Alto, CA, USA) containing approximately 21,500 rice genes (Kikuchi et al. 2003). Global gene expression was compared between the following samples: (i) osmkp1 vs. wild-type plants grown under unstressed conditions; and (ii) wild-type plants grown under unstressed conditions vs. wild-type plants wounded for 1, 6 or 24 h. The Cy3- and Cy5-labeled cDNA probes were prepared from mRNAs and hybridized with the microarray, and the expression profiles of the ~21,500 genes were analyzed. We used three independent samples for the microarray analysis, and the differentially expressed transcripts in osmkp1 were chosen as follows: the fold value (osmkp1/wild type) was greater than three or lower than one-third in all three samples. Thirteen and eight genes were identified as up- and down-regulated transcripts in osmkp1, respectively (Tables 1, 2). Among the 13 genes with elevated expression in osmkp1, the expression of eight genes was altered at least 3-fold after wounding; five were induced and three were repressed. Among the eight genes with reduced expression in osmkp1,
the expression of three genes was altered at least 3-fold after wounding; two were induced and one was repressed.

To confirm that the genes identified by microarray analysis are really OsMKP1-regulated genes, their transcript accumulations in the wild-type, *osmkp1* and complemented lines 5 and 32 were compared by quantitative real-time PCR analysis. As shown in Table 3, real-time PCR confirmed that the transcript levels of all OsMKP1-regulated genes except for AK070684 were altered in *osmkp1*. Moreover, transcript accumulation of all the genes except for AK064151 returned to the wild-type level in complemented line 32. In contrast, the expression levels of some genes such as clones 6, 9, 14, 18, 19 and 20 in complemented line 5 were similar to those in *osmkp1*, suggesting again that complementation in line 5 is imperfect. For OsMKP1-regulated genes that show altered expression after wounding, transcript levels in the wild type, *osmkp1*, and complemented lines 5 and 32 were compared over the time course after wounding. The expression patterns of OsMKP1-regulated genes after wounding were similar overall between *osmkp1* and the wild type (Fig. 6). The transcript levels of some wound-inducible OsMKP1-regulated genes such as AK106493, AK060800 and AK073364 were lower in the complemented lines. The expression patterns of AK099574 and AK107926 did not coincide with the results of the microarray analysis. As these genes have a number of homologs, microarray and real-time PCR might detect distinct sets of homologs.

**Discussion**

MKPs are negative regulator of MAPKs and play important roles in various physiological processes. In this study, we have shown that the rice MKP OsMKP1 regulates wound signaling probably through the regulation of two MAPKs, OsMPK3 and OsMPK6. In response to wounding, rapid and transient activation of OsMPK3 and OsMPK6 was induced post-translationally (Fig. 1C, D). In contrast, Xiong and Yang (2003) reported that wound-induced activation of OsMPK3 and OsMPK6 was induced post-translationally (Fig. 1C, D). In contrast, Xiong and Yang (2003) reported that wound-induced activation of OsMPK3 is associated with the increase in its protein level. This discrepancy may be explained by the specificity of the antibodies. They raised an anti-OsMPK3 antibody against the C-terminal 140 amino acids of OsMPK3, which is conserved among MAPKs, whereas our antibody is raised against the unique N-terminal 14 amino acids and its specificity was rigorously confirmed (Fig. 1A, B). Their antibody might detect homolog MAPK(s) in addition to OsMPK3. Activation of OsMPK3 and OsMPK6 by wounding is independent of de novo protein synthesis, whereas their inactivation requires de novo protein synthesis (Fig. 1). Similar results have been reported for tobacco wound-activated MAPK, probably SIPK (Usami et al. 1995), and alfalfa SIMK and SAMK (Meskiene et al. 2003). These results suggest that the regulatory mechanisms of stress-responsive MAPKs are highly conserved between monocots and dicots. Rapid induction of the OsMKP1 transcript by wounding (Fig. 2B) raised the possibility that OsMKP1 is involved in the inactivation of OsMPK3 and OsMPK6 activated by wounding. As expected, OsMPK3 and OsMPK6 activities in *osmkp1* loss-of-function mutant were stronger than those in the wild type both before and after wounding (Fig. 5B). However, the inactivation patterns of OsMPK3 and OsMPK6 in *osmkp1* are similar to those in the wild-type (Fig. 5B). Similarly, Ulm et al. (2002) reported that although the activity of AtMPK6 is stronger in the atm1k loss-of-function mutant than that in wild type both before and after genotoxic stresses, it still returns to the basal level. Although the transcript level of OsMKP1 in
Rice calmodulin-binding MAPK phosphatase

Table 1  List of transcripts up-regulated in osmkp1

<table>
<thead>
<tr>
<th>No.</th>
<th>Accession No.</th>
<th>Homologous to</th>
<th>osmkp1/WT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Wounding/healthy&lt;sup&gt;a,b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 h</td>
<td>6 h</td>
</tr>
<tr>
<td>1</td>
<td>AK069119</td>
<td>Unknown expressed protein</td>
<td>54.76 ± 43.92</td>
<td>0.60 ± 0.48</td>
</tr>
<tr>
<td>2</td>
<td>AK067876</td>
<td>Arabidopsis family II lipase EXL1</td>
<td>7.38 ± 0.43</td>
<td>0.70 ± 0.14</td>
</tr>
<tr>
<td>3</td>
<td>AK106493</td>
<td>Unknown expressed protein</td>
<td>7.38 ± 0.32</td>
<td>4.25 ± 5.18</td>
</tr>
<tr>
<td>4</td>
<td>AK060800</td>
<td>Rice Cab26</td>
<td>7.04 ± 5.35</td>
<td>8.10 ± 10.71</td>
</tr>
<tr>
<td>5</td>
<td>AK102404</td>
<td>Arabidopsis isp4-like</td>
<td>6.49 ± 2.51</td>
<td>0.67 ± 0.76</td>
</tr>
<tr>
<td>6</td>
<td>AK064111</td>
<td>Rice spotted leaf7b</td>
<td>6.49 ± 0.76</td>
<td>1.14 ± 0.13</td>
</tr>
<tr>
<td>7</td>
<td>AK068443</td>
<td>Unknown expressed protein</td>
<td>6.36 ± 4.46</td>
<td>3.26 ± 0.36</td>
</tr>
<tr>
<td>8</td>
<td>AK099574</td>
<td>Rice Rubisco small subunit</td>
<td>6.21 ± 1.47</td>
<td>0.57 ± 0.15</td>
</tr>
<tr>
<td>9</td>
<td>AK061531</td>
<td><em>Triticum aestivum</em> FAD7</td>
<td>5.46 ± 3.37</td>
<td>0.57 ± 0.17</td>
</tr>
<tr>
<td>10</td>
<td>AK100959</td>
<td>Rice beta-expansin (EXPB3)</td>
<td>4.29 ± 0.79</td>
<td>0.53 ± 0.15</td>
</tr>
<tr>
<td>11</td>
<td>AK065358</td>
<td><em>Gossypium hirsutum</em> RD22-like</td>
<td>4.27 ± 0.67</td>
<td>0.81 ± 0.15</td>
</tr>
<tr>
<td>12</td>
<td>AK073364</td>
<td>Unknown expressed protein</td>
<td>4.23 ± 0.70</td>
<td>1.56 ± 0.90</td>
</tr>
<tr>
<td>13</td>
<td>AK105666</td>
<td><em>Nicotiana tabacum</em> CDN41</td>
<td>3.73 ± 0.36</td>
<td>1.58 ± 0.10</td>
</tr>
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</table>

<sup>a</sup>The data represent the means ± SD of three independent samples.
<sup>b</sup>The averages above three or below one-thirds are indicated in italics or bold, respectively.

Table 2  List of transcripts down-regulated in osmkp1

<table>
<thead>
<tr>
<th>No.</th>
<th>Accession No.</th>
<th>Homologous to</th>
<th>osmkp1/WT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Wounding/healthy&lt;sup&gt;a,b&lt;/sup&gt;</th>
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<td></td>
<td></td>
<td></td>
<td>1 h</td>
<td>6 h</td>
</tr>
<tr>
<td>14</td>
<td>AK107926</td>
<td><em>Triticum aestivum</em> PR-1</td>
<td>0.12 ± 0.09</td>
<td>0.97 ± 0.30</td>
</tr>
<tr>
<td>15</td>
<td>AK069313</td>
<td>Unknown expressed protein</td>
<td>0.14 ± 0.03</td>
<td>1.27 ± 0.76</td>
</tr>
<tr>
<td>16</td>
<td>AK104862</td>
<td>Rice beta 1,3-glucanase</td>
<td>0.16 ± 0.08</td>
<td>0.82 ± 0.21</td>
</tr>
<tr>
<td>17</td>
<td>AK070656</td>
<td>Rice nicotianamine synthase 3</td>
<td>0.17 ± 0.11</td>
<td>0.37 ± 0.15</td>
</tr>
<tr>
<td>18</td>
<td>AK070684</td>
<td>Arabidopsis purple acid phosphatase</td>
<td>0.20 ± 0.04</td>
<td>0.58 ± 0.39</td>
</tr>
<tr>
<td>19</td>
<td>AK107862</td>
<td>Unknown expressed protein</td>
<td>0.21 ± 0.07</td>
<td>0.61 ± 0.34</td>
</tr>
<tr>
<td>20</td>
<td>AK064485</td>
<td>Unknown expressed protein</td>
<td>0.23 ± 0.06</td>
<td>0.50 ± 0.66</td>
</tr>
<tr>
<td>21</td>
<td>AK063251</td>
<td>Unknown expressed protein</td>
<td>0.23 ± 0.04</td>
<td>1.97 ± 0.20</td>
</tr>
</tbody>
</table>

<sup>a</sup>The data represent the means ± SD of three independent samples.
<sup>b</sup>The averages above three or below one-thirds are indicated in italics or bold, respectively.

Complemented line 32 is higher than that in the wild type, wounding induces activation of OsMPK3 and OsMPK6 in line 32 to the level comparable with the wild type. Therefore, it is likely that OsMKP1 is involved in keeping OsMPK3 and OsMPK6 activities at the basal level rather than the inactivation process of OsMPK3 and OsMPK6 activated by wounding. As MAPKs are inactivated by dephosphorylation of either threonine or tyrosine in the conserved TxY motif and none of the rice MKPs except for OsMKP1 is rapidly induced at the transcriptional level by wounding (Fig. 2B), other types of protein phosphatases, such as type 2C protein phosphatase, may be involved in the rapid inactivation process of OsMPK3 and OsMPK6 (Meskiene et al. 2003).

OsMKP1 bound CaM in a manner similar to NtMKP1 although the role of CaM binding is unclear (Fig. 3). As a putative CaM-binding domain is conserved in AtMKP1 as well as homologs in other plant species, CaM binding seems to be a unique and conserved feature of plant MKPs. However, osmkp1 shows a semi-dwarf phenotype (Fig. 4) in contrast to the Arabidopsis atmkp1 loss-of-function mutant, which shows no abnormal phenotype without stimuli (Ulm et al. 2001). The osmkp1 mutant responded to exogenously supplied gibberellin and BL; however, the semi-dwarf phenotype of osmkp1 was complemented by neither gibberellin nor BL. As biosynthesis and signaling pathways of gibberellin and BR seem to be conserved between Arabidopsis and rice (Yamamuro et al. 2000,
Hong et al. 2002, Itoh et al. 2004, Sun and Gubler 2004, Hong et al. 2005), it is unlikely that an MKP regulates biosynthesis and/or signaling of gibberellin or BR only in rice. However, it has been reported that the dwarf phenotype of a rice BR biosynthesis-deficient mutant was only partially restored by the exogenous supply of BL (Hong et al. 2002). Further analysis will be required to address this issue.

A more likely explanation for the semi-dwarf phenotype of osmkp1 is a constitutive activation of stress responses. So far a number of dwarf mutants in which stress responses are constitutively activated have been identified (Bowling et al. 1994, Petersen et al. 2000, Li et al. 2001, Song et al. 2004). In osmkp1, the activities of OsMPK3 and OsMPK6, and the protein level of OsMPK3 are increased without wounding (Fig. 5). Activation of MAPKs has been reported to induce stress responses including cell death in tobacco (Zhang and Liu 2001). Transcriptome analyses support the constitutive activation of stress responses in osmkp1. Out of 13 genes up-regulated in osmkp1, the transcript levels of five genes are clearly induced by wounding (Table 1), suggesting that wound responses are constitutively activated in osmkp1. A recent report showed that the disruption of AtMEKK1, a gene encoding Arabidopsis MAPK kinase kinase, greatly impairs growth and constitutively activates defense responses (Ichimura et al. 2006, Nakagami et al. 2006, Suarez-Rodriguez et al. 2007). Like osmkp1, the activities of AtMPK3 and AtMPK6, the putative Arabidopsis orthologs of OsMPK3 and OsMPK6, respectively, and the protein level of AtMPK3 were increased in the AtMEKK1 mutants (Ichimura et al. 2006). These results suggest that the semi-dwarf phenotype of osmkp1 seems to be caused by the activation of stress responses.

In this study, we have shown that two MAPKs, OsMPK3 and OsMPK6, are the targets of OsMKP1, suggesting that the phenotype of osmkp1 was caused by the abnormal activation of OsMPK3 and OsMPK6. However, it is possible that OsMKP1 regulates activities of more MAPKs. We identified five putative MKPs in rice

<table>
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<th>No.</th>
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<th>Line 32&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup>The data which do not coincide with microarray analysis are indicated in bold.
<sup>b</sup>The data which are not reversed to the wild-type level are indicated in italics.
(Fig. 2A), which is fewer than the number of MAPKs (17 members in rice). Similarly, *Arabidopsis* has five MKPs and 20 MAPKs (MAPK Group 2002). These disproportionate ratios of MAPK to MKP suggest that one MKP regulates multiple MAPKs in plants. Therefore, it is possible that the activity of an unidentified MAPK is increased in *osmkp1*, which causes the abnormal phenotype of *osmkp1*. Moreover, OsMKP1 and its putative orthologs in other plant species have long C-terminal extensions that seem not to be involved in the inactivation of MAPK.
(Katou et al. 2005). Therefore, it is also possible that part of the phenotype of osmkp1 is caused in a MAPK-independent manner. Further analyses such as the systematic analysis of each MAPK activity in osmkp1 and the identification of OsMKP1-interacting proteins will help us understand the exact role of OsMKP1.

Materials and Methods

Plant materials and CHX treatment

Surface-sterilized seeds of rice (Oryza sativa L. cv. Nipponbare) were germinated on agar medium. After transfer to a pot, plants were grown in an isolated greenhouse maintained at 25°C under sunlight. Five- to seven-week old plants were used for the experiments. The Tos17 insertion mutant line of OsMKP1 (NF3008; osmkp1) was isolated from a large population of rice Tos17 insertion mutants (Miyao et al. 2003). The Tos17 insertion site was confirmed by sequencing.

For CHX treatment, 2-week-old seedlings were cut at the base of the stem and immersed in water or 300μM CHX for 3 h. Leaves were cut into pieces and floated on water or CHX at 26°C under continuous light.

Identification and isolation of rice putative MKPs

Putative rice MKPs were identified by BLAST searches on the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/), and Arabidopsis Genome Research Program (RGP; http://rgp.dna.affrc.go.jp/) and the TIGR EST database (http://www.tigr.org/) based on sequence similarity to Arabidopsis MKPs. The catalytic domain of MKPs invariably contains the highly conserved sequences DX26(V/L)X(V/I)HCXAG(I/V)SRSXT(I/V)XXAY(L/I)M, in the single-letter amino acid code where X is any amino acid. The three amino acids indicated in bold have been shown to be essential for catalysis (Denu and Dixon 1995, Martell et al. 1998). Only clones having all three catalytically essential residues were chosen as rice MKPs and further characterized. As the cDNA sequence of OsMKP1 was partial, OsMKP1 cDNA was isolated from a rice seed cDNA library, prepared by using pADGAL4-21 vector and a HybridZAP-2.1 cDNA cloning kit (Stratagene, La Jolla, CA, USA). The deduced amino acid sequences of the putative DSP catalytic domain of rice and Arabidopsis MKPs were aligned using the Clustal W program (Thompson et al. 1994), and a phylogenetic tree was generated using the TreeView program (Page 1996).

Complementation of osmkp1 by OsMKP1 cDNA

The open reading frame of OsMKP1 was amplified by PCR with XbaI sites attached to the 5’ and 3’ ends, and cloned between the actin promoter fragment and a terminator at the XbaI site of the pActnos-Hm2 vector, courtesy of Dr. Makoto Matsuoka (Nagoya University). The resulting constructs were used for Agrobacterium-mediated transformation. Transformed calli were selected with 50mg l⁻¹ hygromycin.

RNA extraction and RNA gel blot analysis

Total RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s instructions. A 20μg aliquot of RNA per lane was separated on a 1.2% formaldehyde-agarose gel and transferred to a Hybond N⁺ nylon membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The blots were hybridized with 32P-labeled DNA probes specific to OsMKP1, OsIBR5, OsDsPTP1 or POX R2576 (Sasaki et al. 2004).

Microarray and real-time PCR analysis

Microarray experiments using the Rice Oligo Microarray (Agilent Technologies, Inc., Palo Alto, CA, USA) and data analysis were carried out as described previously (Hughes et al. 2001). Total RNAs were used for the preparation of Cy5- and Cy3-labeled probes.

Real-time PCR was performed with iQ SYBR Green Supermix and iCycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s recommendations. The sequences of the primers used for real-time PCR analysis are described in Supplementary Table 1.

CaM-binding assay

The putative CaM-binding domain of OsMKP1 was amplified by PCR with EcoRI and SalI sites attached to the 5’ and 3’ ends, respectively, and cloned into the corresponding sites of the pGEX4T-1 vector (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), allowing the production of GST–OsMKP1. The sequences of the primers were as follows (restriction sites are underlined): forward (5'-GGAAATTCCTCGTATAATTTCAGACGACGCAATACT-3') and reverse (5'-AGGGTGCTCACTTTAAAGCCAGGTGTGACTC-3'). Various mutations were generated using the Mutan-Super Express Km Kit (Takara Bio, Inc., Shiga, Japan) and confirmed by sequencing. The resulting constructs were used to transform Escherichia coli strain Rosetta (DE3) (Novagen, Madison, WI, USA). Expression of recombinant protein was induced with 0.5mM isopropyl β-d-thiogalactopyranoside (IPTG) at 26°C for 3 h. The CaM overlay assay was performed as described previously (Yamakawa et al. 2004), but 0.2μg ml⁻¹ of alkaline phosphatase-labeled NiCaM1 was used.

Production of recombinant proteins of MAPKs in E. coli

The open reading frames of OsMPK3 and OsMPK6 were amplified by PCR with BamHI and NotI sites attached to the 5’ and 3’ ends, respectively, and cloned into the corresponding sites of the pGEX4T-1 vector (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), allowing the production of GST–OsMPK3 and GST–OsMPK6, respectively. Expression of recombinant proteins was induced as follows: OsMPK3, 0.1mM IPTG at 26°C for 3 h; OsMPK6, 1mM IPTG at 37°C for 3 h. Recombinant proteins were purified with glutathione-Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) according to the manufacturer’s recommendations.

Production and purification of antibody

The peptides OsMPK3-N (MDGAPVAVEFRPTMTC) and OsMPK6-N (CGAPPDTEMAEEGGG), corresponding to the N-termini of OsMPK3 and OsMPK6, respectively, were synthesized and conjugated to keyhole limpet hemacyanin carrier. Polyclonal antisera were raised in rabbits. Purification of antibodies was performed as follows. Purified GST–OsMPK3 and GST–OsMPK6 (1–2mg) were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA, USA). After blocking with 5% non-fat dry milk, membranes were incubated in 4ml of antiserum at ambient temperature overnight. After washing, bound antibodies were eluted with 0.1M glycine-HCl, pH 2.5 and immediately neutralized.
Protein extraction, immunoblot analysis and MAPK activity assay

The proteins were extracted from rice leaves as described previously (Seo et al. 1999) and stored at -80°C. The concentration of the protein extracts was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

For immunoblot analyses, proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA, USA). After blocking with 5% non-fat dry milk, membranes were probed with 0.5 μg ml⁻¹ anti-OsMPK3 antibody or 1 μg ml⁻¹ anti-OsMPK6 antibody at 4°C overnight. After washing, membranes were incubated with horseradish peroxidase-labeled secondary antibody for OsMPK3 or alkaline phosphatase-labeled secondary antibody for OsMPK6. The antigen-antibody complexes were visualized using a ECL chemiluminescent kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) or by the hydrolysis of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate.

The activity of OsMPK3 and OsMPK6 was determined by immunocomplex kinase assay as described previously (Seo et al. 1999). For competition with antigen peptides, the immunocomplex kinase assay was performed in the presence of 5 μg ml⁻¹ of OsMPK3-N or OsMPK6-N peptide.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

Acknowledgments

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