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Identification of Putative MAPK Kinases in *Oryza minuta* and *O. sativa* Responsive to Biotic Stresses

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The mitogen-activated protein kinase (MAPK) signaling cascade is critical for regulating plant defense systems against various kinds of pathogen and environmental stresses. One component of this cascade, the MAP kinase kinases (MAPKK), has not yet been shown to be induced in plants following biotic attacks, such as those by insects and fungi. We describe here a gene coding for a blast (*Magnaporthe grisea*)- and insect (*Nilaparvata lugens*)-responsive putative MAPK kinase, *OmMKK1* (*Oryza minuta* MAPKK 1), which was identified in a library of *O. minuta* expressed sequence tags (ESTs). Two copies of *OmMKK1* are present in the *O. minuta* genome. They encode a predicted protein with molecular mass 39 kDa and p*I* of 6.2. Transcript patterns following imbibition of plant hormones such as methyl jasmonic acid (MeJA), ethephone, salicylic acid (SA) and abscisic acid (ABA), as well as exposure to methyl viologen (MV), revealed that the expression of *OmMKK1* is related to defense response signaling pathways. A comparative analysis of *OmMKK1* and its *O. sativa* ortholog *OsMKK1* showed that both were induced by stress-related hormones and biotic stresses, but that the kinetics of their responses differed despite their high amino acid sequence identity (96%).

Keywords: Blast; Brown Planthopper; MAPK Signaling Cascade; Regulation of Gene Expression; Wild Rice.

Introduction

The mitogen-activated protein kinase (MAPK) signaling network is a crucial component of plant responses to various extracellular stimuli, and one of the main pathways for responding to biotic and abiotic stresses. MAPK cascades operate as efficient signal transmission networks linking extracellular stimuli with intracellular targets. The basic structure of the MAPK pathway is conserved in all eukaryotes and consists of three classes of kinases: a MAPK kinase kinase (MAPKKK), that initiates signaling; a MAPK kinase (MAPKK), the signaling carrier, and a MAPK that conveys the signal to intracellular targets (Asai *et al.*, 2002; Zhang and Klessig, 2001). The completion of sequencing projects revealed 15 MAPK genes and 8 MAPKK genes in the *Oryza sativa* genome, and 20 MAPKs and ten MAPKKs in the Arabidopsis genome (Hamel *et al.*, 2006; Ichimura, 2002). The genes coding for MAPKs have been the focus of intensive study in Arabidopsis and *O. sativa* (Agrawal *et al.*, 2003a ; Asai *et al.*, 2002). In Arabidopsis, six of the known MAPKKs have been found to be involved in diverse functions: *AtMKK1* and *AtMKK2* mediate abiotic stresses such as cold, salt and wounding (Hadiarto *et al.*, 2006; Teige *et al.*, 2004); *AtMKK4* and *AtMKK5* are activated by fungal elicitors (Lee *et al.*, 2004; Takemoto *et al.*, 2005), and *AtMKK6* and *AtMKK7* are involved in cytokinesis and development, respectively (Dai *et al.*, 2006; Melikant *et al.*, 2004; Soyano *et al.*, 2003). In rice, however, only one (*OsMKK6*) of the eight known MAPKKs has been characterized (Wen *et al.*, 2002), and to date there is no report of any plant MAPKK being induced by biotic stresses such as insects and fungal pathogens.

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Abbreviations: MAPK, mitogen-activated protein kinase; MAPKK, MAP kinase kinase.

Like all food crops, the productivity of rice (*Oryza sativa*) is adversely affected by a number of pathogens (fungi, insects, bacteria and viruses) and abiotic stresses (wounding, salt, temperature) (Brar and Khush, 1997). The most important of the former is blast disease (*Magnaporthe grisea*), which can cause productivity losses of 10–30% annually (Thinlay, 2000). The brown planthopper (BPH, *Nilaparvata lugens*) is also a notorious insect pest of rice, and severe infestation of rice plants by this insect causes hopperburn, the main cause of serious losses of rice crops throughout Asia (Backus *et al.*, 2005). Consequently it is of great economical and social importance that strategies are developed for overcoming pathogen attacks and that these strategies are incorporated into breeding programs. However, the genetic diversity of cultivated rice species has been reduced due to intensive crop interbreeding among elite lines, with the result that cultivated rice has only a limited ability to resist plant pathogens and pests. This inability to respond to biotic stresses is only aggravated by the constant alterations of disease pathotypes.

The wild relatives of cultivated crop species have received considerable attention as potential sources of genetic diversity because they are known to contain a large proportion of the existing genetic variation of these species, including resistance to various stresses. *Oryza minuta*, a wild relative of cultivated rice (AA genome), has the BBCC genome and is known to carry resistance to various kinds of pathogens such as blast and bacterial blight. Hence it has been used to transform cultivated rice lines to resistance to blast and bacterial blight (Vaughan, 1994).

In an earlier publication we reported an analysis of 5,211 leaf-expressed sequence tags (ESTs) of *O. minuta* (Cho *et al.*, 2004). We now describe the identification in these ESTs of the first putative MKK gene (*OmMKK1*, ABI93775) shown to be induced by both blast and brown planthopper. Comparative analysis of *OmMKK1* and its *O. sativa* ortholog (*OsMKK1*, AK111598) revealed that *OmMKK1* is induced by stress-related hormones and biotic-stresses, but that the kinetics of expression of *OsMKK1* differed from those of *OmMKK1* despite their 96% amino acid sequence identity. Our observations confirm the value of *O. minuta* as a genetic resource.

Materials and Methods

Plant materials Seedlings of *Orzya minuta* (accession no. 101144) and *O. sativa* cv. Hwasung were grown in a glasshouse at the National Crop Experiment Station (NCES) in Korea. For the fungal infection experiments, a conidial suspension (5×10^4 conidia ml^{-1} in 0.1% Tween-20; a mixture of four races of rice blast, *Magnaporthe grisea* KJ101, KJ102, KJ197 and KI409) was sprayed in abundance onto the leaves of 4-week-old seedlings. The rice blast is compatible with *O. sativa* cv. Hwasung but incompatible with *O. minuta*. At least ten larvae of the brown planthopper (BPH; *Nilaparvata lugens*) Korean biotype1 were placed on each plant; these do not cause disease in *O. minuta* but cause severe disease in *O. sativa*. The blast and BPH treatments were performed at the National Crop Experiment Station in Suwon, Korea.

Sterilized seeds of *O. minuta* and *O. sativa* cv. Hwasung were germinated on moistened filter paper in a Petri dish at 25°C under dark conditions for 1 week and then transferred onto four pieces of gauze (5 × 5 cm) in two Incu tissue culture frames (model 310071; SPL Lifesciences, Seoul, Korea) containing 45 ml of liquid Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands) and cultivated at 25°C under a 16/8-h (light/dark) photoperiod. The seedling roots were exposed to four different plant hormones, methyl jasmonic acid (MeJA), ethephone, salicylic acid (SA) and abscisic acid (ABA), as well as methyl viologen (MV) in MS liquid medium, by adding concentrated stock solution (10×) to the liquid MS culture medium to final concentrations of 100 μM (MeJA), 5 mM (ethephone), 200 μM (SA), 100 μM (MV) and 100 μM (ABA), and by spraying the same chemicals in 0.1% Tween-20 on seedling leaves; mock controls were incubated in MS medium, and sprayed with 0.1% Tween-20 solution as above, without addition of hormones.

Cloning and identification of the *OmMKK1* gene Based on the EST data and Northern blot analysis we selected a blast- and BPH-induced gene (*OmMKK1*) for further study. Full-length *OmMKK1* was obtained by 5′-rapid amplification of cDNA ends (RACE)-PCR according to Frohman *et al.* (1988), with minor modifications. An amplified phagemid *O. minuta* leaf cDNA library (Cho *et al.*, 2004) was used as RACE pool, and RACE-PCR was carried out with the following primers: phagemid vector (Stratagene, USA) primer for the 5′-end; 5′-AGG GAT GTT TAA TAC CAC TAC-3′ and 5′-CCA CTA CAA TGG ATG ATG TAT ATA ACT ATC TA-3′ (nested primer), the gene-specific primers for the 3′ end; 5′-GCA GCA AGG TAG GGC TCT GGA ATG GTT T-3′ and 5′-CAG GAA ATC TGA GA GA G AG CCA CCG TCC-3′ (nested primers). The PCR products were cloned into the pGEM-T Eazy cloning vector (Promega, USA). Three independent RACE experiments were performed for each analysis, and five clones per RACE-PCR were selected and sequenced. Sequence analysis showed that the five independent clones were identical and contained the expected overlap with the original EST sequence. Full-length cDNA was obtained by end-to-end PCR with the primers nearest to the 5′- or 3′-end, using newly synthesized first-strand cDNAs from the pooled RNAs from *O. minuta* leaves infested with blast and BPH as template. Open reading frames (ORF) of *OmMKK1* and *OsMKK1* were cloned using the cDNA of *O. minuta* or *O. sativa*, respectively, and the following primers (containing *Bam*HI sites, underlined): 5′-end of *OmMKK1,* 5′-CGC GGATCC ATG GGG AAG CCG GGG AAG CTG-3′; 3′-end of *OmMKK1,* 5′-GC GGATCC CTA TCT GTC ATC GTA CCG GTT-3′; 5′-end of *OsMKK1,* 5′-CGC GGATCC ATG GGG AAG CCG GGG AAG CT-3′; 3′-end of *OsMKK1,* 5′-GC GGA-

TCC CTA TCT GTC ATC ATA TCG GTT G-3′.

Northern and Southern analysis Total RNAs were extracted using TRIZOL reagent (GibcoBRL, USA). 20 μg samples were separated by electrophoresis on a 1.2%-for-maldehyde-agarose gel. To confirm equal loading of the RNA, the gel was stained with ethidium bromide following electrophoresis. The 3′-untranslated region (UTR) of *OmMKK1* was used as a probe for the Northern hybridization analysis. Restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV and *Xba*I were used to digest the genomic DNAs of *O. minuta* and *O. sativa.* Based on the ORF sequences of *OmMKK1* and *OsMKK1*, *Bam*HI has no cleavage site in the ORF; both *Eco*RI and *Eco*RV have one cleavage site and *Bgl*II and *Xba*I each have two. To determine the copy number of *OmMKK1* and *OsMKK1* in the *O. minuta* and *O. sativa* genomes, the full-length ORF of *OmMKK1* was used as probe in each case because of the high nucleotide sequence identity (95%) between *OmMKK1* and *OsMKK1*.

For Northern and Southern analyses, the extracted RNAs (20 μg) and each of the genomic DNA digests (15 μg) were subjected to electrophoresis and blotted onto a Tropilon-Plus Nylon membrane (Applied Biosystems, USA) by the alkaline transfer method (Chomczynski, 1992). Hybridization was carried out using biotin-labeled probes at 68°C for 16 h, and the signals were detected on X-ray film (Fujifilm, Japan) using the Southern-Light and Southern-*Star* Systems (Applied Biosystems, USA).

Reverse transcription-PCR A 2-μg aliquot of total RNA was used for the first-strand cDNA synthesis following incubation at 65°C for 10 min. cDNA was synthesized in 20-μl reaction volumes containing 0.8 μl SuperScript III (Invitrogen, Carlsbad, Calif.), 10 mM dithiothreitol, 0.8 μl poly (dT) primer, 0.5 mM dNTPs and reaction buffer (5×) at 50°C for 80 min. The PCR analyses were performed under different thermocycling conditions for each gene; one cycle of 95°C for 5 min, followed by 21–30 cycles (gene-dependent step) of 95°C for 15 s, 58°C for 15 s and 72°C for 30 s (*EF1α*, 25 cycles; *OmMKK1* and *OsMKK1*, 27 cycles). *EF1α* (Elongation factor 1-α) was employed as a loading control for the RT-PCR amplification (Nicot *et al.*, 2005). The primers for the RT-PCR of *OmMKK1* and *OsMKK1* were designed from the genomic sequence of *OsMKK1* so that *OmMKK1* and *OsMKK1* could be amplified from the cDNA templates but not the genomic DNA templates: rice *EF1α*: 5′-GAT GAA CAA GAG GTC CTT CA-3′ and 5′-GAG GAC TCC AGT CTC AAC AC-3′; rice *MKK1*: 5′-ACC AAT TCC AAT ACA CAA GAC TTG ACA GGC C-3′and 5′-GCT AAA ATC AAT CGT GAG TGA CAA ACA TGG GC-3′; *OsOPR1*: 5′-GGC AGG GTC TCC ACC ACA GA-3′ and 5′-CGA ACG GCG ATA ATC TGA TTC C-3′.

Results and Discussion

***OmMKK1* is induced by blast (*M. grisea*) and BPH (*N. lugens*) infections** Based on the EST data of Cho *et al.* (2005), we identified 20 genes involved in the response of *O. minuta* to biotic stresses by Northern analysis using total RNAs extracted from BPH- and blast-treated *O. minuta* leaves (data not shown). Probes for Northern hybridization were produced from cDNA clones containing the 3′-UTR based on the EST sequence data. A putative MAPK kinase gene in the *O. minuta* leaf ESTs, which was induced by both blast and BPH treatments, was selected for study (Fig. 1A) and eventually renamed *O. minuta* MAP kinase kinase *1* (*OmMKK1*). *OsMKK1* (an *O. sativa* ortholog) was also identified for comparison. Northern analysis showed that *OsMKK1* was induced to a lower level than *OmMKK1* by both BPH and blast, which is compatible with *O. sativa* cv. Hwasung but incompatible with *O. minuta*.

The insert size of the EST clone was 1.1 kbp. To obtain the full-length ORF we carried out 5′-RACE-PCR and obtained an extended 294-bp DNA fragment. The full-length cDNA was obtained by end-to-end PCR with primers nearest to the 5′- or 3′-end; this resulted in the identification of a 103-bp 5′-UTR, 232-bp 3′-UTR and 1059-bp ORF encoding 352 amino acids (Fig. 1B). The deduced protein was predicted to have an estimated molecular mass of 39 kDa and a p*I* of 6.2 (Lagergene ver. 6.0; DNASTAR, USA). The nucleotide sequence of the cDNA has been deposited in GenBank under accession number ABI93775. The deduced protein of *OsMKK1* was predicted to have an estimated molecular mass of 39 kDa and a p*I* of 5.5 (Lagergene ver. 6.0) and found to have 96.6% amino acid sequence identity with *OmMKK1*.

Because of the high nucleotide sequence identity between *OmMKK1* and *OsMKK1*, we used the full-length ORF of *OmMKK1* as a probe for Southern analysis to investigate the copy numbers of these genes in each genome (Fig. 2B). AK111598 (*OsMKK1*) is not cut by *Bam*HI, but is cut once by *Eco*RI and *Eco*RV and twice by *Bgl*II. Two bands were generated by *Bam*HI, three bands by *Eco*RI and *Eco*RV, and four bands by *Bgl*II, indicating that there are two copies of *OsMKK1* in the *O. sativa* genome. For *OmMKK1*, we chose *Xba*I instead of *Bgl*II, based on the sequence comparison (Fig. 2A). Two bands were generated by *Bam*HI, three bands by *Eco*RI and *Eco*RV, and four bands by *Xba*I, suggesting that *OmMKK1* exist as two copies in the *O. minuta* genome (Fig. 2B).

Sequence comparison and phylogenetic analysis of the putative *OmMKK1* protein Using the BlastX and BlastN programs we succeeded in assembling a complete and non-redundant set of putative *OmMKK1* orthologs. *OmMKK1* shared 96%, 61%, 65%, 64%, 59%, 66%, 65% and 65% amino acid identities with *OsMKK1* (AK111598) and *OsMKK6* (AK059461) from rice (*O. sativa*), *AtMKK1* (At4g26070), *AtMKK2* (At4g29810) and *AtMKK6* (At5g 56580) from Arabidopsis, AAF67262 from *Nicotiana tabacum*, CAA04261 from *Lycopersicon esculentum* and

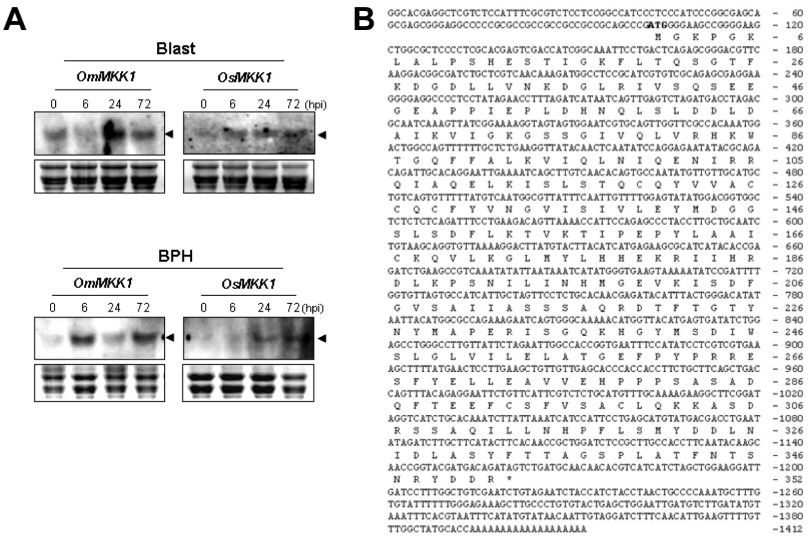

Fig. 1. *OmMKK1* and *OsMKK1* are induced by biotic stresses in *Oryza minuta* and *O. sativa*. **A.** Expression patterns of *OmMKK1* and *OsMKK1* in response to brown planthopper (BPH) and blast treatments; Northern blot analyses were performed using the 3′-untranslated region (UTR) of *OmMKK1* cDNA as a probe. *Arrows* indicate target transcripts. Equal loading of RNA was confirmed by visualizing rRNA on a gel stained with ethidium bromide: *blast*, *Magnaporthe grisea*; *BPH*, brown planthopper (*Nilaparvata lugens*); *hpi*, hours post-infestation or infection. **B.** Nucleotide and predicted amino acid sequences of *OmMKK1* cDNA: *bold italics*, start codon of *OmMKK1*; *asterisk*, stop codon of *OmMKK1*.

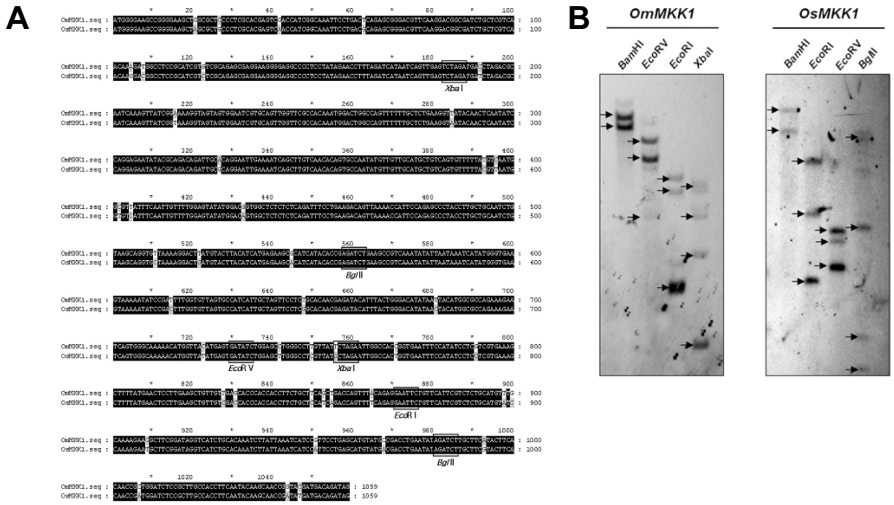

Fig. 2. Copy numbers of *OmMKK1* and *OsMKK1*. **A.** Comparison of the full open reading frame (ORF) nucleotide sequences of *OmMKK1* with *OsMKK1*. Alignment was performed with the Lasergene Megalign Program (DNASTAR). Restriction enzyme sites are indicated by *boxes* and *nomenclatures*. **B.** Southern blot analysis of *OmMKK1* and *OsMKK1*. 15-μg aliquots of genomic DNA were digested with *Bam*HI, *Eco*RI, *Eco*RV, *Bgl*II or *Xba*I. The digested and blotted DNA was hybridized with full-length ORF-specific probes of *OmMKK1* and *OsMKK1*, respectively.

AJ293275 from *Medicago sativa*, respectively. A phylogenetic analysis of the amino acid sequences of the reported plant MKK homologs revealed that *OmMKK1* groups with *OsMKK1*, *OsMKK6*, *AtMKK1*, *AtMKK2* and *AtMKK6* in Group A (Fig. 3A). AtMKK1 and AtMKK2 are known to mediate cold, salt and wounding stress signaling, and AtMKK6 is implicated in cytokinesis during both meiosis and mitosis (Hadiarto *et al.*, 2006; Soyano *et al.*, 2003; Teige *et al.*, 2004). Sequence alignment of the deduced *OmMKK1* protein with the other MKK proteins of group A revealed a high degree of homology of the deduced *OmMKK1* protein with other MKK proteins in Group A. OmMKK1 contains the 11 conserved catalytic subdomains typical of MKKs as well as the plant MAPKK-specific

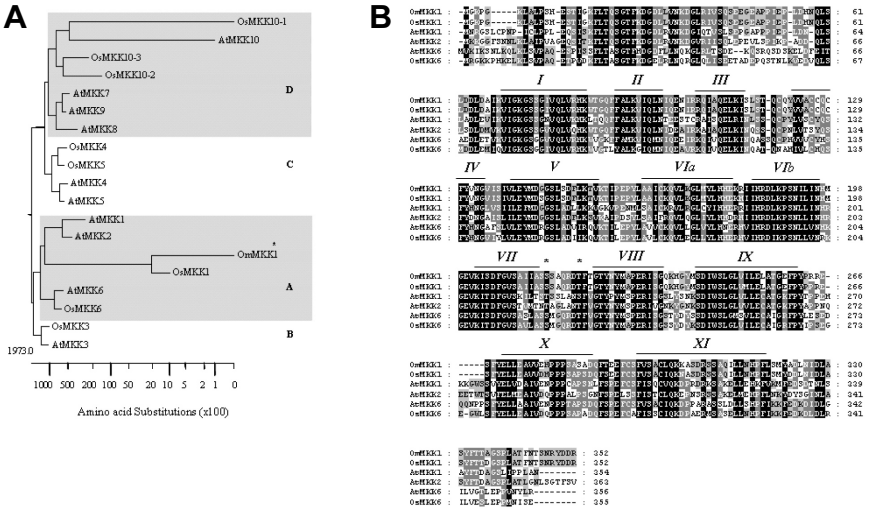

Fig. 3. Comparison of the deduced amino acid sequences of *OmMKK1* and other plant MAPK kinases of Arabidopsis and rice. **A.** The phylogenetic tree (shown as a dendrogram) was created using the Jotun Hein algorithm in the Lagergene Megalign program (DNASTAR) with previously reported plant MAP kinase kinases as follows; *AtMAKK1* (At4g26070), *AtMAKK2* (At4g29810), *AtMAKK3* (At5g40440), *AtMAKK4* (At1g51660), *AtMAKK5* (At3g21220), *AtMAKK6* (At5g56580), *AtMAKK7* (At1g18350), *AtMAKK8* (At3g06230), *AtMAKK9* (At1g73500), *AtMAKK10* (At1g32320), *OsMAKK1* (Os06g05520), *OsMAKK3* (Os06g27890), *OsMAKK4* (Os02g54600), *OsMAKK5* (Os06g09180), *OsMAKK6* (Os01g32660), *OsMAKK10-1* (Os02g46760), *OsMAKK10-2* (Os03g12390) and *OsMAKK10-3* (Os03g50550). Evolutionary distance is expressed as the number of substitutions per amino acid. **B.** Sequence alignment of the predicted *OmMKK1* protein with other MKK proteins of group A. *Roman* numerals designate the 11 major conserved subdomains of MKKs, *asterisks* indicate the plant MAPK kinase-specific S/TXXXXXS/T motif between subdomains *VII* and *VIII*. Alignment was performed with the Lagergene Megalign Program (DNASTAR).

S/TXXXXXS/T motif between subdomains *VII* and *VIII* (Fig. 3B).

Hamel *et al.* (2006) reported that that there are eight members of the MKK gene family in the rice genome. OsMKK6 (AK059461) has been reported to function as a modulator of a low-temperature signaling pathway with *OsMPK3* (Wen *et al.*, 2002). The *OmMKK1* and *OsMKK*1 genes are therefore valuable assets as novel MKK members induced by fungal (blast) and insect (BPH) pathogens.

Characterization of the biotic stress-related hormone responses of *OmMKK1* and *OsMKK1* Plants defend themselves against herbivorous insects and fungal pathogens by a broad spectrum of defense mechanisms that include both preexisting physical and chemical barriers and inducible defense responses. The plant hormones JA, ethylene (ET) and SA are important global signaling molecules involved in inducible defense responses (Glazebrook, 2005; Kessler and Baldwin, 2002; Thomma *et al.*, 2001). MV (methyl viologen, paraquoat) leads to the production of reactive oxygen intermediates (ROIs) such as H_2O_2, O_2^- and OH^- (Suntres, 2002). These ROIs are crucial to plant defense responses such as HR-related cell death against pathogen attack(s) (Lamb and Dixon, 1997; Levine *et al.*, 1994). The defense responses of Arabidopsis against attacks of insect and fungal pathogens involve complex interactions of positively and negatively regulated JA-,

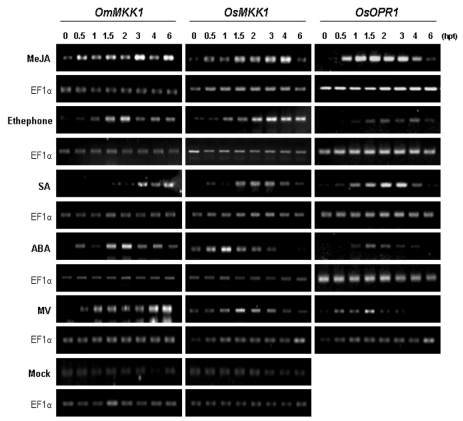

Fig. 4. Differential expression patterns of *OmMKK1* and *OsMKK1*. Total RNA was extracted from 4-week seedlings after treatment with methyl jasmonic acid (MeJA, 100 μM), ethephone (5 mM), salicylic acid (SA, 200 μM), abscisic acid (ABA, 100 μM) and methyl viologen (MV, 100 μM) in MS liquid medium, or mock treatments with MS liquid medium. *hpt* Hours post-treatment. Rice *EF1α* was used as an internal control. Primers used for the RT-PCR were designed from the consensus regions between *O. minuta* and *O. sativa*: rice *EF1α*, 5′-GAT GAA CAA GAG GTC CTT CA-3′ and 5′-GAG GAC TCC AGT CTC AAC AC-3′; rice *MKK1*, 5′-ACC AAT TCC AAT ACA CAA GA C TTG ACA GGC C-3′ and 5′-GCT AAA ATC AAT CGT GAG TGA CAA ACA TGG GC-3′; *OsOPR1*: 5′-GGC AGG GTC TCC ACC ACA GA-3′ and 5′-CGA ACG GCG ATA ATC TGA TTC C-3′.

ET- and SA-dependent pathways (De Vos *et al.*, 2005; Thomma *et al.*, 2001). Apart from a very few reports, little is known about blast fungus- or herbivorous insect-induced defense mechanisms (Kim and Sohn, 2005; Moreno *et al.*, 2005; Ning *et al.*, 2006).

To determine if the BPH- and blast-induced *OmMKK1* and *OsMKK1* genes are also induced by methyl jasmonate (MeJA), SA and MV, we cultured 4-week-old seedlings of *O. minuta* and 2-week-old seedlings of *O. sativa* in MS medium supplemented with these plant hormones (Fig. 4A). Total RNAs were prepared from whole plants treated for varying times, and the changes in the steady-state level of mRNAs were monitored by RT-PCR using gene-specific primers. *OmMKK1* was expressed at a basal level in non-treated leaves, and its expression increased progressively in response to increasing lengths of exposure of MeJA, SA, ethephone and MV. However, expression was similar in root, leaf blade or leaf sheath (data not shown).

Comparative analyses by RT-PCR revealed that *OmMKK1* and *OsMKK1* were both induced by the five treatments (MeJA, ethephone, SA, ABA and MV) but that their expression patterns differed. Both genes were induced rapidly and strongly within 30 min of the start of JA treatment but *OmMKK1* was induced earlier than *OsMKK1* by ethephone and later by SA, ABA and MV. These results suggest that the induction of *OmMKK1* and *OsMKK1* by BPH and the blast fungus is somehow linked to the JA-, ET-, SA-, ABA- and MV-related signaling pathways.

OsOPR1 (AJ557138), which is important in the octadecanoid pathway related to biosynthesis of JA and is known to be responsive to all five of the above treatments (Agrawal *et al.*, 2003b), was used as a comparative control. The RT-PCR results with *OsOPR1* confirmed that all chemicals and hormones were applied successfully (Fig. 4).

In conclusion, this is the first report of two blast (*M. grisea*)- and insect (*N. lugens*)-responsive genes, *OmMKK1* and *OsMKK1*, that code for novel MAPK kinases in *O. minuta* and *O. sativa*, respectively. Based on our observations that both *OmMKK1* and *OsMKK1* were induced by MeJA, ethephone, MV, SA, and ABA as were in *OsOPR1* (AJ557138), and were induced by biotic stresses. We propose that these two putative MAPK kinases could be used as foundation stones for investigating defense-related MAP kinase signaling cascades in rice.

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