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Function of Mitogen-Activated Protein Kinase Gene in Biotic Stress

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Abstract: In this study, we describe the gene that codes for MAPK in *Oryza rufipogon* and *Oryza sativa* that reacts towards the blast disease (causative agent is *Magnaporthe grisea*) and brown planthoppers (*Nilaparvata lugens*). This gene has been isolated and characterized in *Oryza sativa* and in this study the same gene has been isolated from *Oryza rufipogon* using *OsMKK1* as a template and named *OrMKK1*. Through conducting this study, we found that *Oryza rufipogon* contains two copies of the *MAPK* gene in its genome. A comparative study was conducted between *OrMKK1* and *OsMKK1*; and the results showed that both these genes responded towards biotic stress. Though both the genes share a high level of amino acid similarities (94%), the kinetic reactions of both genes are different.

Key words: Biotic stress, MAP kinases, *Magnaporthe grisea*, brown planthoppers, stress modulation

INTRODUCTION

Mitogen-Activated Protein Kinase (MAPK) is an important component which is involved in various extracellularly induced reactions in plants, especially those that are induced in response to biotic and abiotic stress. The MAPK cascade ensures that there is a proper transmission of signals from the source of induction to the targeted sites of intracellular response. The basic structure of MAPK is conserved in all eukaryotes and are made up of three main classes of kinases, the MAPK Kinase Kinase (MAPKKK) that initiates the signal transduction; MAPK Kinase (MAPKK) which is the carrier of the signal itself and MAPK that delivers the signal to the intracellular targets (Zhang and Klessig, 2001; Asai *et al*., 2002).

The sequencing of the *Oryza sativa* sp. *japonica* genome has identified 15 *MAPK* genes and 8 *MAPKK* genes. Twenty *MAPK* and 10 *MAPKK* genes have been identified in the *Arabidopsis* genome alone (Hamel *et al*., 2006). The *AtMKK1* and *AtMKK2* genes that were obtained from the *Arabidopsis* genome responded towards abiotic stress such as cold, salt and injury (Teige *et al*., 2004; Hadiarto *et al*., 2006); *AtMKK4* and *AtMKK5* however were found to be involved in activating fungal elicitors (Lee *et al*., 2004; Takemoto *et al*., 2005) and *AtMKK6* and *AtMKK7* were involved in cytokinesis and development, respectively (Soyano *et al*., 2003; Melikant *et al*., 2004; Dai *et al*., 2006). In rice, *OsMKK6* is one of the few *MAPK* genes that have been characterized. There have been no reports of *MAPKK* in biotic stress caused by insects and fungi to date in rice.

The productivity of rice is largely influenced by diseases caused by various pathogens (fungi, virus and bacteria) and abiotic stress that is induced by various environmental factors such as drought and salinity (Brar and Khush, 1997). Blast, a major rice disease caused by *Magnaporthe grisea* has been known to cause reduction in yield by 10-30% a year (Thinlay *et al*., 2000). Therefore, it is important that control and prevention measures are taken to reduce the incidence of disease in plants, especially those of economic importance. 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).

In addition, the conventional breeding method has diluted the gene pool extensively that it has resulted in the generation of commercial lines that are susceptible to disease. To further escalate the situation, the change in the pathotypes of the pathogen has further contributed to the widespread of disease and the difficulty in controlling the incidence of disease amongst crops.

Oryza rufipogon, an ancestor to the cultivated rice that has a AA genome, is believed to carry many

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amenable traits such as disease resistance and stress tolerance. This rice variety has been used in various research activities to generate rice with disease resistance through the process of conventional breeding. In this study, we report the isolation of the first putative *MKK* gene (*OrMKK1*) in *Oryza rufipogon*. A comparative study was conducted to compare *OrMKK1* and the orthologous *O. sativa* (*OsMKK1*). The results obtained from this study show that the *OrMKK1* functions against pathogen and insect predation. Although, both the genes contain high levels of amino acid sequence identity (94%), they exhibit different reaction kinetics.

MATERIALS AND METHODS

Plant materials: The *Orzya rufipogon* (accession No. 101144) and *O. sativa* sp. *indica* were germinated and transferred to the greenhouse in Universiti Kebangsaan Malaysia. The seeds of *O. rufipogon* and *O. sativa* sp. *indica* were germinated on damp filter paper in a petri dish and the germinated seedlings were then transferred to tissue culture vessels that contained 45 mL Murashige and Skoog (MS) media (Duchefa, Haarlem, The Netherlands) where they were left to grow at 25°C air temperature with 16 h days^{-1} photoperiod.

The fungal infection of the 2 week old T1 transgenic plants was performed using the typical spray-inoculation method at a concentration of 10^6 spores mL^{-1} (Lee *et al*., 2001). Blast resistance was evaluated based on fungal growth in planta (Qi and Yang, 2002) as well as lesion number and size. The *O. sativa* sp. *indica* is susceptible to blast while *O. rufipogon* does not show any symptoms of the disease. For the insect predation studies, at least ten larvae of the brown planthoppers (BPH; *Nilaparvata lugens*) were placed on each T1 plant; these do not cause disease in *O. rufipogon* but cause severe disease in *O. sativa* sp. *japonica* and sp. *indica*. Resistance to insect predation was evaluated by disease symptoms exhibited by the T1 plants.

Northern and southern blot analysis: Total RNA was extracted using the Plant RNA Extraction (GibcoBRL, USA) kit. Twenty microgram of sample was electrophoresed in 1.2% formaldehyde agarose gel. The same amount of RNA was loaded into each well for the purpose of analysis and the gel was stained with ethidium bromide following electrophoresis to ensure equal loading. The northern blot assays were conducted using the 3'UTR region of the *OrMKK1* gene as a probe. *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV and *Xba*I were used to cleave the genomic DNA of *O. rufipogon* and *O. sativa*. The ORF of *OrMKK1* and *OsMKK1* does not contain a *Bam*HI restriction site, while *Eco*RI and *Eco*RV have a single site and *Bgl*II and *Xba*I have two restriction sites within the ORF. To determine the copy number of *OrMKK1* and *OsMKK1* in the *O. rufipogon* and *O. sativa* genomes, the full-length ORF of *OrMKK1* was used as a probe due to the high nucleotide sequence identity between *OrMKK1* and *OsMKK1*.

The probe was used to screen membranes containing genomic DNA of *O. rufipogon* and *O. sativa*. The northern and southern blots contained 20 μg digested RNA and 15 μg cleaved genomic DNA. The RNA and DNA was then electrophoresed and transferred onto Tropilon-Plus Nylon membranes (Applied Biosystems, USA) using the alkaline transfer methodology (Chomczynski, 1992). The hybridization process was conducted with biotinlabelled 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).

Cloning and characterization of *OrMKK1* gene: Full-length *OrMKK1* was obtained by nested PCR using the following primer sets 5'-end; 5'-AGGGATGTTTAATACCACTAC-3' and 5'-CCACTACAATGGATGATGTATATAACTATCTA-3' (nested primer), the gene-specific primers for the 3' end; 5'-GCAGCAAGGTAGGGCTCTGGAATGGTTT-3' and 5'-CAGGAAATCTGAGAGAGAGCCACCGTCC-3'(nested primers). Open Reading Frames (ORF) of *OrMKK1* and *OsMKK1* were cloned into the pGEM-T Easy cloning vector (Promega, USA). The clones were then sequenced and the sequence analysis showed that these clones were identical. The plant transformation vector pCAMBIA1300 was used to generate the plant transformation construct. The pCaM-*OrMKK1* and pCaM-*OsMKK1* construct was then transformed into *Agrobacterium* (strain EHA105) using the freeze-thaw method (Höfgen and Willmitzer, 1988). The *Agrobacterium*-mediated transformation was performed using calli derived from mature embryos of rice according to the method of Hiei *et al*. (1994).

The gene sequence of *OrMKK1* was subsequently analyzed using various bioinformatics tools. The sequence was analyzed for amino acid identity between the MKKs in *Oryza* and *Arabidopsis thaliana*. Selection of sequences for analysis was conducted using the BlastN and BlastX tool from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The complete sequence of the *OrMKK1* gene was then analyzed using the ORF finder programme from NCBI (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). A phylogenetic tree was developed using the sequence obtained as in comparison with the other sequences that were downloaded from the

NCBI database (http://www.ncbi.nlm.nih.gov/). The alignment of sequences was conducted using the ClustalX 1.81 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) (Thomson *et al*., 1997). The alignment results were further analyzed using the BioEdit ver 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/page2.html) (Hall, 1999). The phylogenetic tree was developed using the Mega4 software where the tree was generated using the UPGMA programme and Poisson correlation (Tamura *et al*., 2007).

RESULTS AND DISCUSSION

OrMKK1 is induced by the blast disease caused by *M. grisea*. Present results also show that the gene is responsive towards insect predation examined through infestation by *N. lugens*. In studying the transcript expression levels of this gene in response to the above stimuli, the 3'UTR putative *MAPK* kinase gene was used as a probe. This gene was obtained via., the PCR techniques. *OsMKK1* (ortholog *O. sativa*) was identified as the reference sequence in this research. From the northern analysis conducted, it was deduced that the expression levels of *OsMKK1* was lower than that observed in *OrMKK1* for both biotic stress examined (Fig. 1). The level of *OrMKK1* expression was several folds higher than that observed for *OsMKK1*. This could explain why in the earlier research conducted on *O. sativa*, MKKs have not been shown to have a role in pathogen infections. This is further strengthened by the observation that *O. sativa* is susceptible to the blast pathogen, while *O. rufipogon* exhibited non compatible interactions through the production of lesions. However, the level of *OsMKK1* in response to insect predation was evident though lower than the levels observed in *OrMKK1*. From the elevated transcript levels observed in *OrMKK1,* we believe that this gene has a function in biotic stress specifically toward insect predation and pathogen infections.

To obtain the full ORF of *OrMKK1*, nested PCR was conducted using the primer walking strategy to ensure that the entire gene sequence was obtained. The end results showed that the PCR product contained a 105 bp 5'-UTR, 234-bp 3'-UTR and a 1059 bp ORF that encodes a protein that is 352 amino acids long (Fig. 2). The nucleotide sequences of both *OsMKK1* and *OrMKK1* were analyzed for common restriction sites such as *Bam*HI, *Eco*RV, *Eco*RI, *Bgl*II and *Xba*I. The restriction sites were identified via., the Webcutter 2.0 software (http://rna.lundberg.gu.se/cutter2/). The molecular weight and pI of the *OrMKK1* protein sequence obtained was predicted as approximately 37 and 5.8 kDa, respectively. The *OsMKK1* protein however has been reported to have a molecular weight of 39 kDa and a pI of 5.5 (Lagergene ver. 6.0). The comparison of both sequences showed that the sequences of *OsMKK1*

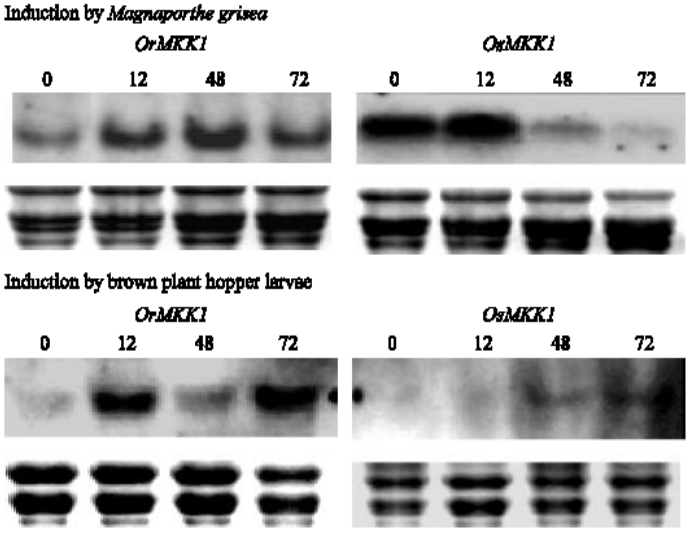

Fig. 1: Biotic stress induced by blast disease and brown planthoppers in *OrMKK1* and *OsMKK1*. The northern blot analysis were performed using the 3'-UTR of *OrMKK1* as a probe. Hours indicated above are time taken post infection or infestation. Same amount of RNA was loaded into each well as indicated by the gel picture below the northern results

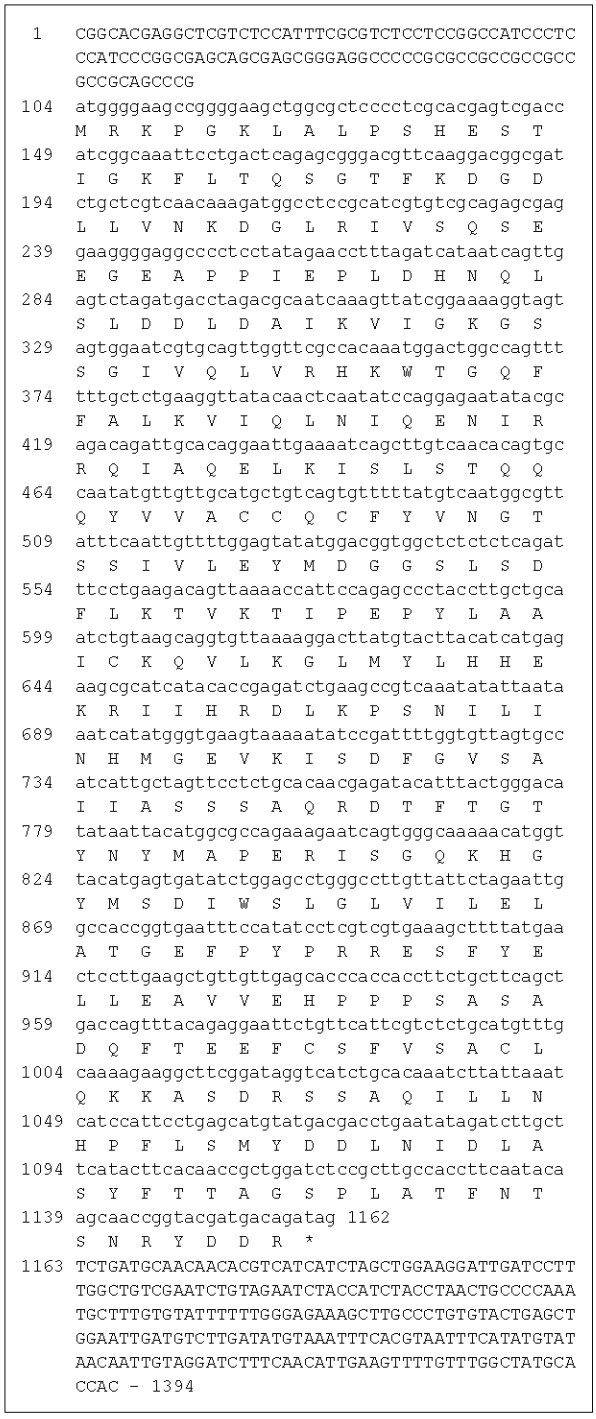

Fig. 2: The coding region *OrMKK1*. Nucleotide and predicted amino acid sequences of the coding region provided with the start and stop codon indicated in bold. The 5' and 3' UTR are in capital and italics

and *OrMKK1* have 94% sequence identity at the amino acid level. Due to the high level of identity between these genes, the *OrMKK1* gene sequence was used as a probe in the Southern analysis to determine the copy number of the gene (Fig. 3). *OsMKK1* could not be cleaved by *Bam*HI, but could be digested by *Eco*RI and *Eco*RV once and twice by *Bgl*II. A single strong hybridization band was visible when the digested genome of *O. rufipogon*

and *O. sativa* was probed with *OrMKK1* gene. This therefore indicates that both the genomes have a single copy of the *OrMKK1* and *OsMKK1* gene, respectively (Fig. 3).

We used the BlastX and BlastN programs to select and assemble complete and non redundant sets of putative *OrMKK1* orthologs. We selected *OsMKK1*, *OsMKK6* and *OsMPPK10* from rice (*O. sativa*) and *AtMEK1*, *AtMKK2*, *AtMKK3*, *AtMKK4*, *AtMKK5*, *AtMKK6* from *Arabidopsis*, that showed 94, 60, 63, 61, 57, 64, 63 and 62% amino acid identities with respect to *OrMKK1*. The phylogenetic tree generated using the amino acids of the above selected MAPKs revealed that *OrMKK1* groups together with *OsMKK1*, *OsMKK6*, *AtMEK1*, *AtMKK2* and *AtMKK6* in group A (Fig. 4). *AtMEK1* and *AtMKK2* are known to mediate cold, salt and wounding stress signalling and *AtMKK6* is implicated in cytokinesis during both meiosis and mitosis (Soyano *et al*., 2003; Teige *et al*., 2004; Hadiarto *et al*., 2006). The sequence alignment that was generated using the sequences from group A revealed a high degree of homology of the deduced *OrMKK1* protein with other MKK proteins in group A. This shows a very high level of conservation in the signalling pathway that controls disease resistance and insect predation amongst both these plants species.

Through the sequence alignment we also identified the presence of several conserved regions. In total 11 conserved catalytic subdomains that are typical in MKKs were identified. All these domains are related to functions that are either directly or indirectly related to the modulation of responses towards stress which include pathogenic and insect predation. One of the identified domains is the plant MAPKK-specific S/TXXXXXS/T motif between subdomains VII and VIII (Fig. 5). MAPKs are promiscuous serine/threonine kinases that phosphorylate a variety of substrates including

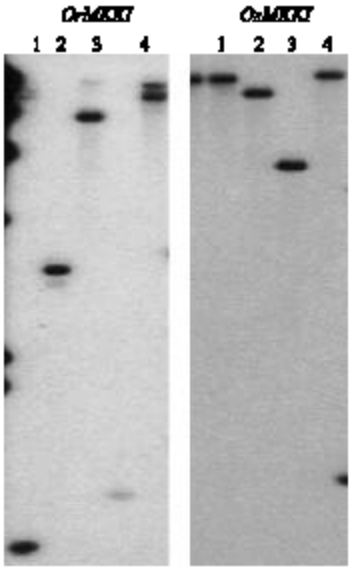

Fig. 3: *OrMKK1* and *OsMKK1* copy numbers were identified via., southern Hybridization of cleaved genomic DNA. Southern blot analysis of *OrMKK1* and *OsMKK1*. Fifteen microgram aliquots of genomic DNA were digested with *Bam*HI (1), *Eco*RI (2), *Eco*RV (3) and *Xba*I (4) for *OrMKKI*, *Bam*HI (1), *Eco*RI (2), *Eco*RV (3) and *Bgl*II (4) for *OsMKKI*

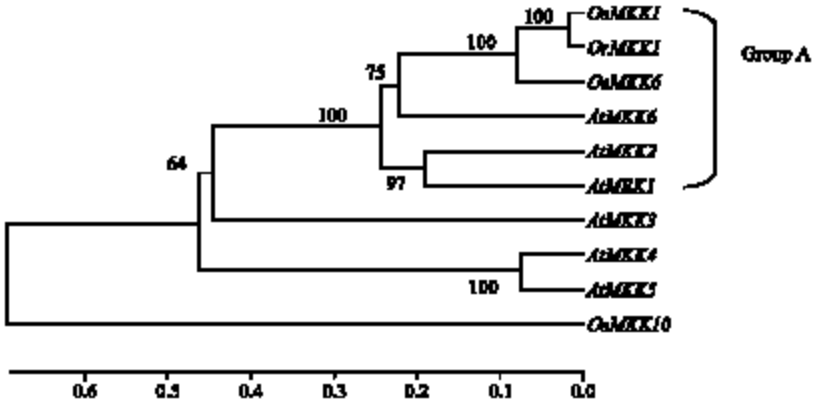

Fig. 4: Comparison of the deduced amino acid sequences of *OrMKK1* and other plant MAPK kinases of *Arabidopsis* and rice. The phylogenetic tree (shown as a dendrogram) was created using the Mega4 UPGMA programme using the Poisson correlation with previously reported plant MAPKs as follows: *OsMKK1 OsMKK6* and *OsMKK10* from rice (*O. sativa*), *AtMEK1*, *AtMKK2* and *AtMKK6* from *Arabidopsis*. Numbers on the left showing the bootstrap value generated with 1000 replications

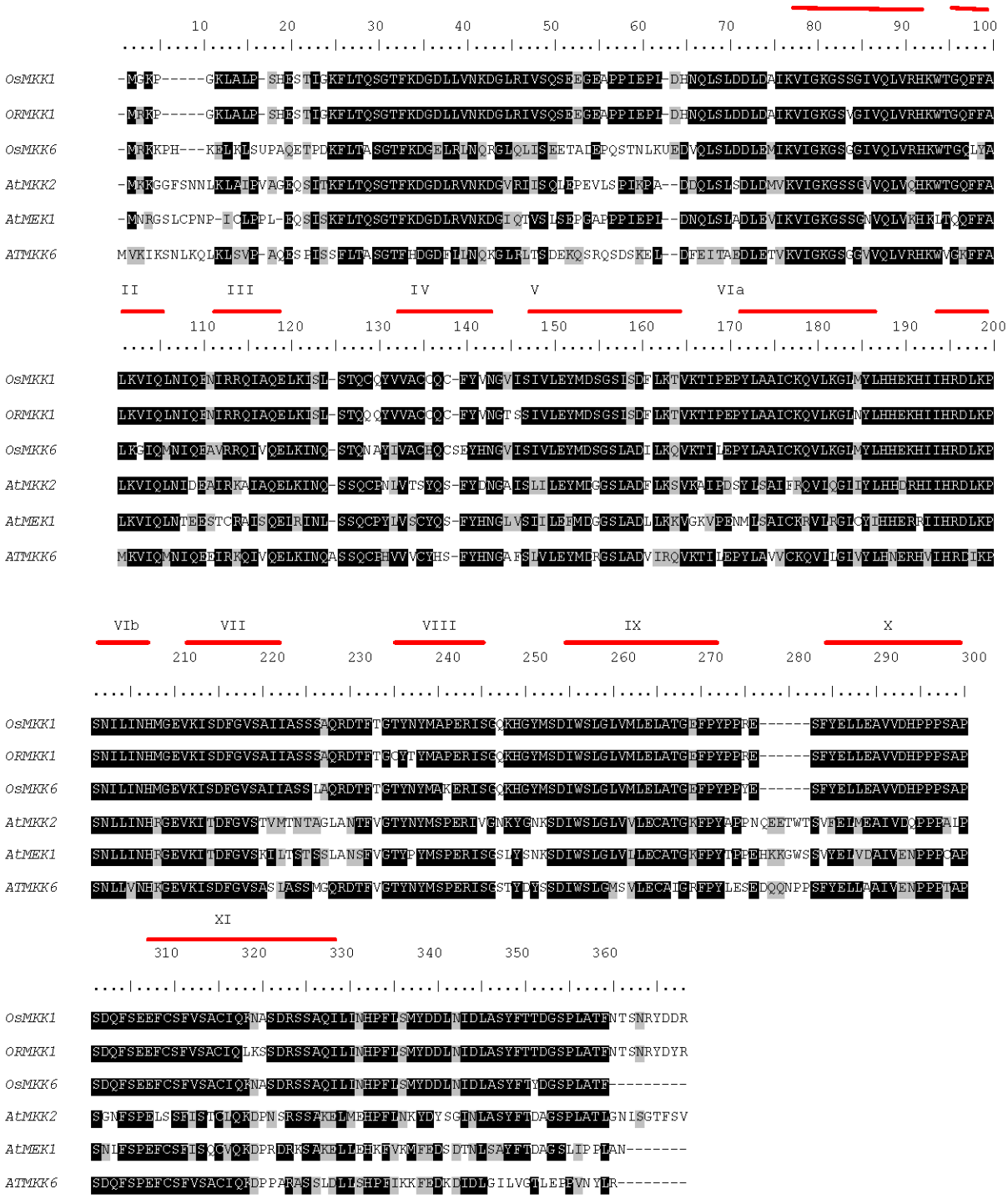

Fig. 5: Sequence alignment of the predicted *OrMKK1* protein with other MKK proteins of group A. Roman numerals designate the 11 major conserved subdomains of MKKs

transcription factors, protein kinases and cytoskeleton-associated proteins (Nakagami *et al*., 2005). The specificity of different MAPK cascades functioning within the same cell is generated through the presence of docking domains found in various components of MAPK modules and scaffold proteins. All of these carry the

features that characterize protein kinase, namely, all the 11 conserved kinase subdomains and the T-loop, which can either be TEY (exception: *OsMPK2*, which have an MEY motif instead) or a TDY motif (Rohila and Young, 2007).

As *MAPK* controls signal transduction in the defense mechanism of plants, we believe that this would involve cross talking between related defense pathways in plants. The complexity of the process involved is seen through the presence of various control domains within the gene structure. In the future research, we will be looking into the effect of signal molecules in the transduction and expression of the *MAPK* genes in *O. rufipogon* and *O. sativa*. Earlier research has shown that molecules such as jasmonate, methyl jasmonate, salicylic acid and ethylene are capable of activating the defense mechanism in plant systems. By comparing the effects induced by these molecules we hope to identify molecule(s) that are closely linked to the regulation of the MAPK cascade.

CONCLUSION

In conclusion, this is the first report of the effect of blast (*M. grisea*) and insect (*N. lugens*) predation on the induction of *OrMKK1* gene that codes for the novel MAPK kinase. Here, we have isolated and characterized the *OrMKK1* gene from the wild rice *O. rufipogon*. The level of *MKK* gene expression in *OrMKK1* was compared to the level of *OsMKK1* expressed in *O. sativa* sp. *japonica*. The higher level of gene expression of *OrMKK1* compared to *OsMKK1* over a prolonged period of time (72 h) only showed that the level of defense in *O. rufipogon* may be higher than that observed in the cultivated rice (*O. sativa* sp.). This observation would explain the exhibited disease resistance trait of the wild rice. Present results suggest that *O. rufipogon* is a suitable candidate to be used in the generation of cultivated rice species with higher level of disease and pest tolerance. The Malaysian wild rice variety of *O. rufipogon* is currently being used extensively in conventional breeding programmes worldwide as it is in Malaysia to generate cultivars with amenable traits such as disease resistance and higher yield and yield related traits.

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