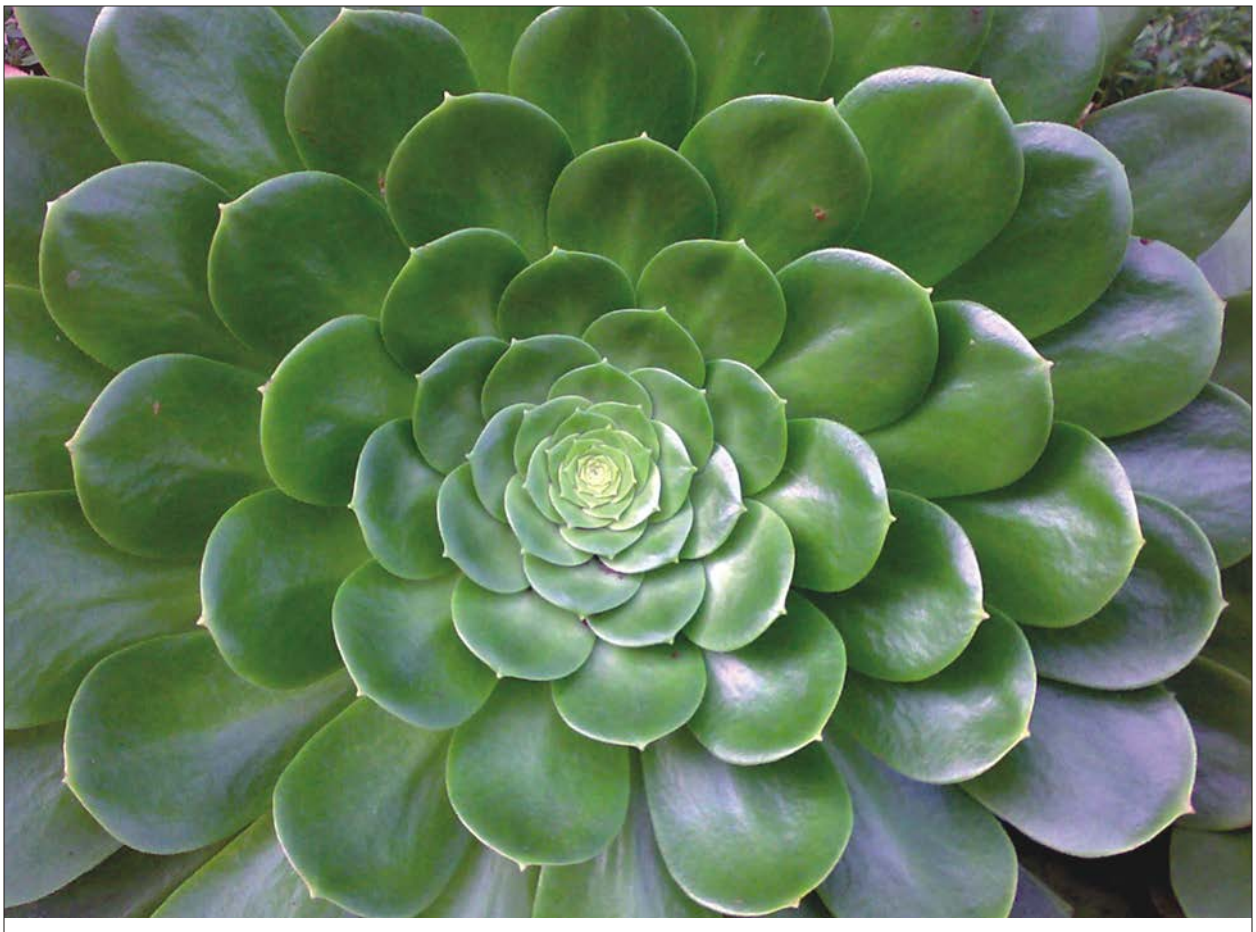

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Effect of Signal Molecules and Hormones on the Expression of Protein Kinase Gene *OrMKK1* in Rice

K. Nadarajah and N.M. Kassim
School of Biosciences and Biotechnology, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Darul Ehsan, Malaysia

Abstract: A putative protein kinase gene was isolated from *Oryza rufipogon* using the *OsMKK1* as template for primer synthesis. This gene was used to analyse the involvement of signal molecules and hormones in signal transduction of mitogen-activated protein kinase. The 352 amino acids long MAPK has a molecular weight of 37 kDa and a pI value of 6.1. The gene sequence contained a dual-phosphorylation activation motif TDY (Thr-Asp-Tyr) and four activity domains (catalytic loop, activation loop, ATP binding site and substrate binding site). The 5'UTR of the gene was also analysed and was shown to contain the MYBCORE, ERE, GT-1 and GATA box, all of which have a role to play in MAPK function. Here we have treated the *OrMKK1* lines with Jasmonic Acid (JA), salicylic acid (SA), ethylene (ET), benzothiadiazole (BTH) and abscisic acid (ABA) to determine the involvement of these molecules and hormones in MAPK signal transduction. *OrMKK1* gene was induced by JA, SA, BTH and ET but was delayed and weak in ABA. The highest level of expression is seen in JA treated plants. The transcript level of this gene was also studied in various tissues and organs of rice and the results show that the gene is developmentally regulated as clearly seen from the Northern analysis conducted on rice tissues. The results from this study suggest that *OrMKK1* may be activated by signal molecules and hormones and this gene may play a role in the plant defense mechanism.

Key words: *Oryza sativa*, MAPK, defense response, motifs, domains

INTRODUCTION

Mitogen-Activated Protein Kinases (MAPK) have been reported to play an important role in the plant signal transduction pathways. The various extracellular and intracellular signals perceived by the plant are then transduced and enlarged through the process of phosphorylation that relays the signal through the MAPK cascades that eventually results in the activation of specific transcription factors that turn on or regulate the expression of downstream genes (Tena *et al*., 2001). The MAPK-mediated signaling pathways were reported to be involved in cell division, cellular differentiation, metabolism and both biotic and abiotic stress responses (Peter, 2001; Rohila and Yang, 2007). The MAPK cascade is conserved through evolution from unicellular to complex eukaryotic organisms and usually constitutes protein kinases from three different subfamilies including MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase) and MAPK. Although, detailed steps of MAPK cascades have yet to be elucidated in a given plant species, specific upstream MAPKKs for a few well-characterized MAPKs have been determined (Agrawal *et al*., 2002).

In the past decade, genes encoding MAP kinases and other components of MAPK cascades in the signal transduction pathways in plants have been identified. Their functions in a variety of cellular and physiological activities as well as stress responses have been elucidated (Hirt, 1997; Nadarajah *et al*., 2009a, b). Seo *et al*. (1999) identified a wound-inducible MAP kinase (WIPK

Corresponding Author: Dr. Kalaivani Nadarajah, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi Selangor Malaysia

wound-induced protein kinase) in tobacco that is regulated by the synthesis of Jasmonic Acid (JA) (Zhang *et al*., 2000). A stress induced seed storage gene, *AtVSP* was also jamonate regulated in *Arabidopsis thaliana* (Nadarajah and Turner, 2003; Nadarajah, 2001). Another signaling molecule, Salicylic Acid (SA), has been shown to play a role in plant defense responses against pathogens. SA is involved in the activation of Systemic Acquired Resistance (SAR) in plants and is involved in the activation of SA-dependent defense genes. A SA-Induced Protein Kinase (SIPK) belonging to MAPK family was identified in suspension cells of tobacco and the activity of this gene was activated by SA using Myelin Basic Protein (MBP) as the substrate (Zhang and Klessig, 2001).

Many MAPK genes are also reported with roles in the defense responses to fungal, bacterial and viral pathogens in various plant species (Lee *et al*., 2001; Nadarajah *et al*., 2009a; Nürnberger *et al*., 1994; Zhang and Klessig, 2001). The activation of these MAPKs genes occur through the process of phosphorylation at threonine (T) and tyrosine (Y) residues that are located between subdomains VII and VIII in all MAPKKs (Payne, 1991). The phosphorylation domain in plant MAPKs has the signature TXY motif which contains either glutamic acid (E) or aspartic acid (D) at the X location. On the basis of sequence variation in the kinase domain that has been studied so far, the feature of phosphorylation motif can be used to classify the MAPKs to five subgroups (Nadarajah *et al*., 2009b; Thompson *et al*., 1994). MAPKs of subgroup V have a TDY (Thr-Asp-Tyr) phosphorylation motif and a C-terminus domain potentially involved in protein- protein interactions.

In this study, a full length gene of a novel MAPK gene, designated *OrMKK1* was isolated from *Oryza rufipogon*. *OrMKK1* and *OsMKK1* (from *Oryza sativa indica*) have a high level of sequence identity (94%) (Nadarajah *et al*., 2009a). The analysed gene was transformed into rice and the T3 generation of this line was used to determine the effect of signal molecules and hormones on the expression profile of *OrMKK1*. Here, we also studied the differential expression profile of the gene in various tissues and organs to determine if the gene is developmentally regulated.

MATERIALS AND METHODS

This project was conducted from May 2008 to 31 December 2008 at the Plant Molecular Biology Laboratory, Level 4, Biology Building at Universiti Kebangsaan Malaysia. The plants used in this study were grown in controlled conditions in the Plant Biotechnology Laboratory Facilities of Institute of Biological Systems (INBIOSIS), Universiti Kebangsaan Malaysia.

Isolation of *OrMKK1*

The *OrMKK1* gene was isolated via PCR using the following primer set 5'-AGGGATGTTTAATACCACTAC-3' and 5'-CAGGAAATCTGAGAGAGAGCCACCGTCC-3'. The amplified fragment of the gene was cloned into the pGEM-T Easy vector (Promega, USA). The DNA sequencing was performed on both ends of the fragments with T7 and SP6 primers in the ABI PRISM 3700 automatic sequencer (Applied Biosystems, USA). The sequence data was analysed against the rice genomic sequence to determine the full-length sequence of the *OrMKK1* gene.

Analysis of *OrMKK1*

The gene sequence of *OrMKK1* was subsequently analysed using various bioinformatics tools. The sequence was analysed for amino acid identity between the MKKs in *Oryza* sp. and certain other plant species. Sequences 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 analysed using the ORF finder programme from NCBI (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The motif content in the promoter region was determined via the UTR Blast (http://bighost.area.ba.cnr.it/BIG/Blast/BlastUTR.html) and UTR Scan Programme (http://bighost.area.ba.cnr.it/BIG/UTRScan/). Both these programmes were available at http://www.hsls.pitt.edu/guides/genetics/obrc/dna/motifs_regulatory_sites/sequence_analysis_gene

_prediction (Health Science Library System University of Pittsburg). The 3D-structure of the protein was determined via the CDD v2.16 -27036 PSSMs search database at http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml . The structure was then viewed via the Cn3D software at http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml.

Transformation of *OrMKK1* into Rice

The binary vector, pCAMBIA1300 was used in generating the rice transformation construct. The pCaM-OrMKK1 construct was 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 *Oryza sativa indica* according to the method of Hiei *et al*. (1994)

Materials and Treatment

The OrMKK1 T3 transgenic lines were grown at 28°C and 16 hd^{-1} photoperiod. Seedlings at four-leaf stage were sprayed with jasmonic acid (0.1 mM), salicylic acid (1 mM), benzothiadiazole (BTH, 0.3 mM), ethylene (ET, 0.1 mM) and abscisic acid (ABA, 0.1 mM) respectively. The leaf tissues were sampled at time points of 0, 0.5, 1, 2, 4 and 12 h after spraying and placed immediately in liquid nitrogen before RNA extraction.

Extraction of Total RNA and Northern Blot Assay

RNA was extracted using the Plant RNA Extraction Kit (GibcoBRL, USA). Total RNA (about 20 μg) that was obtained from JA, SA, BTH, ET and ABA treated plantlets was separated on 1.2% agarose gel containing formaldehyde and then transferred onto nylon membranes. 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. Northern blot analysis was also conducted on different rice tissues and organs to analyse the differential expression of *OrMKK1* gene in development.

The northern blotting was conducted by transferring onto Tropilon-Plus Nylon membranes (Applied Biosystems, USA) the electrophoresed product using the alkaline transfer methodology (Chomczynski, 1992). The hybridization process was conducted with 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).

RESULTS

Isolation and Sequence Analysis of *OrMKK1*

A fragment of ~1.5 kb in length was amplified and the sequence data indicated that this fragment was ~ 1 414 bp in length and the longest ORF was 1 059 nucleotides long. The database search revealed that *OrMKK1* had 94% sequence identity with *OsMKK1* (*Oryza sativa japonica MKK gene*). The predicted protein of *OrMKK1* is 352 amino acids (aa) in length with an estimated molecular mass of 54 kDa and a calculated pI of 6.1 as shown in Fig. 1. In order to define the promoter region controlling the expression and the transacting factors that interact with them, a motif analysis was conducted using a number of online softwares. This analysis identified several elements in its 5'UTR. The TATA box is located between -45 to -38 in the promoter region of *OrMKK1*. There were other motifs that were identified via the UTR Scan and UTR Blast System. These were the GATA box, GT-1, MYBCORE and ERE motifs (Fig. 1).

Comparison of OrMKK1 protein with known MAP kinases showed that it contained all the 11 conserved subdomains of MAPK (Fig 1). Unlike most other plant MAPKs however, the dual-phosphorylation sites between subdomains VII and VIII of *OrMKK1* is TDY (Thr-Asp-Tyr) instead

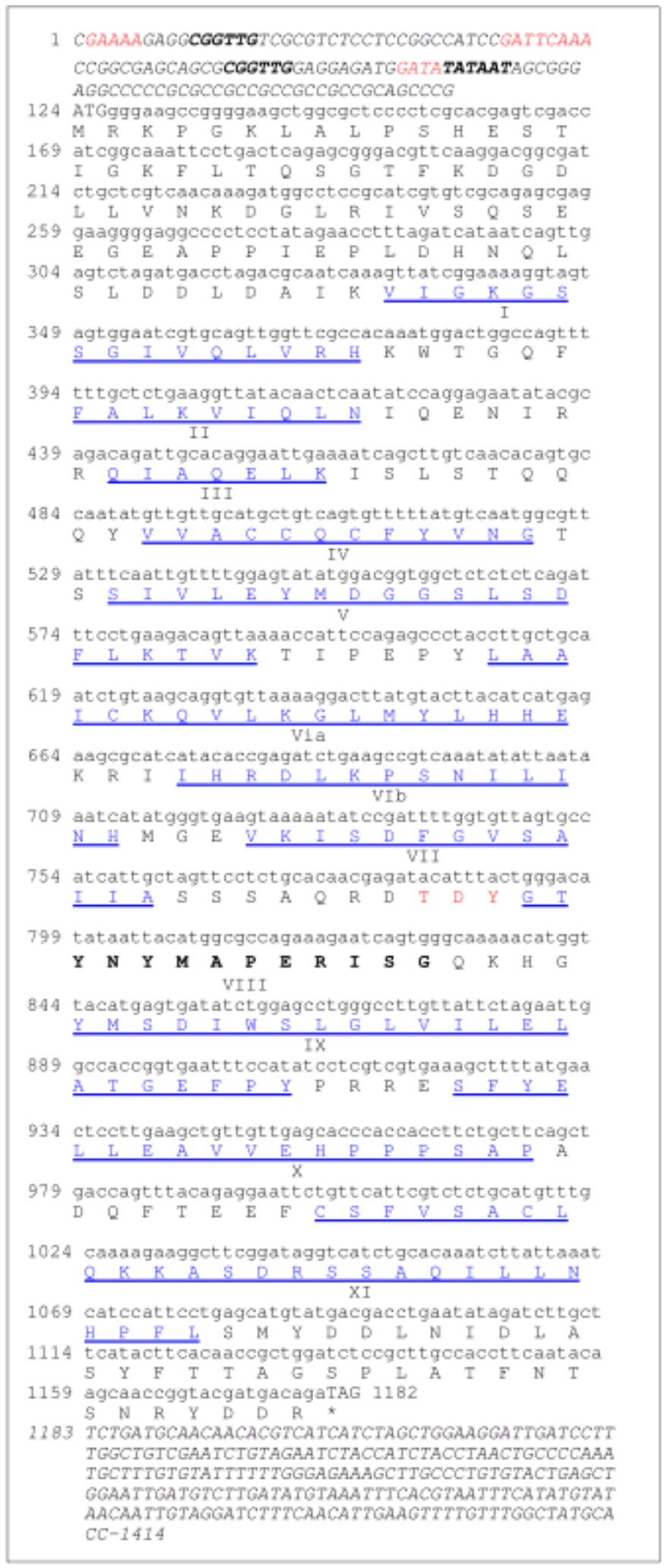

Fig. 1: The coding region *OrMKK1*. Nucleotide and predicted amino acid sequences of the coding region provided with the start and stop codons indicated in bold and capital. Location of the 11 MAPK domains are in blue underlined, each numbered in Roman numerals. The TDY site is indicated in red. The 5' and 3' UTR are in italics. The 5'UTR of *OrMKK1* contains motifs: *GAAAA* – GT-1 box; *CGGTTG* – MYBCORE; *GATTCAAA* – ERE; *GATA* – GATA box; *TATAAT* – TATA box

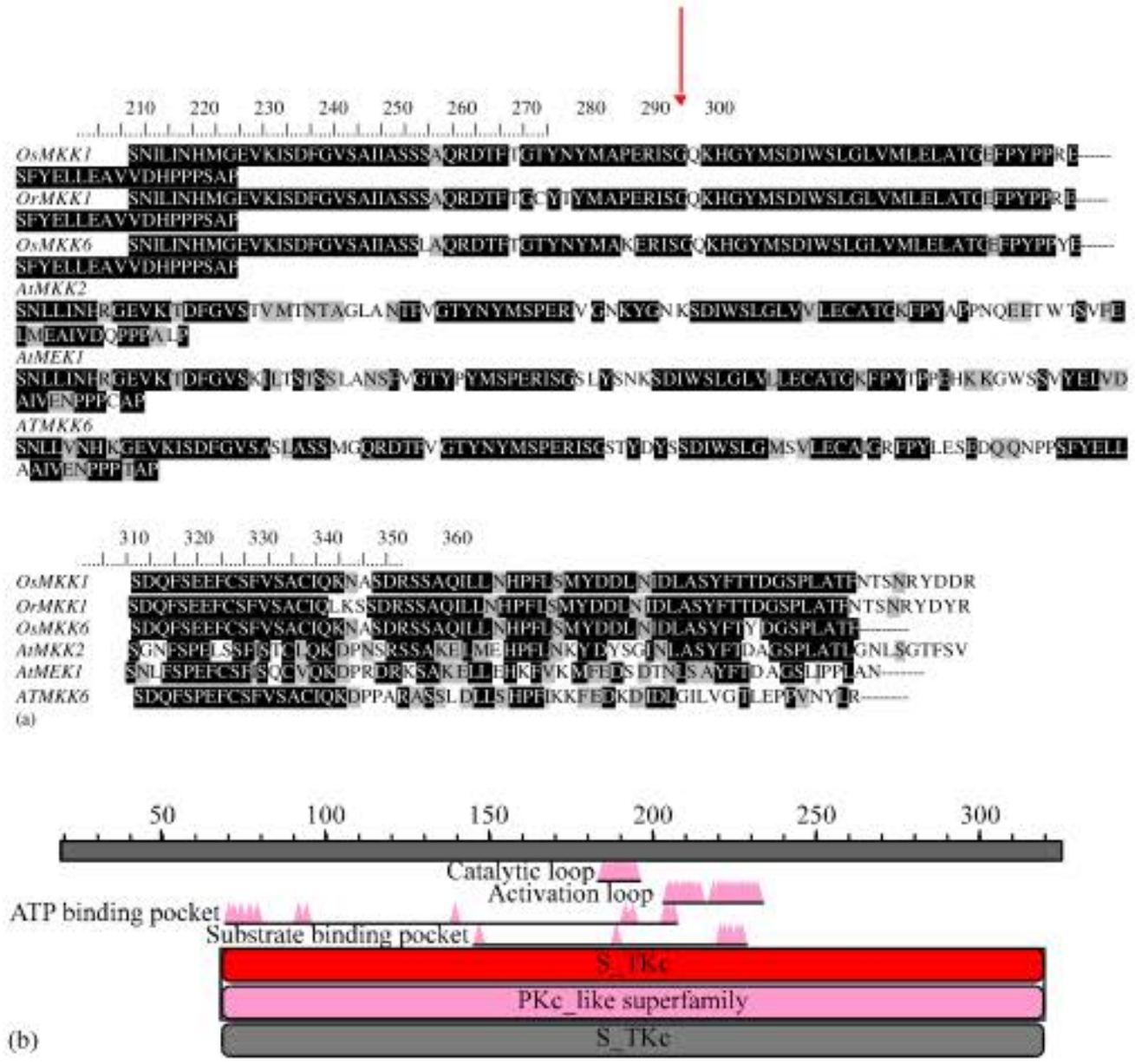

Fig. 2: (a) The sequence alignment between *OrMKK1* and other MAPKs in *Orysa sativa indica* (*OsMKK1*), *Oryza sativa japonica* (*OsMKK6*) and *Arabidopsis thaliana* (*AtMEK1, AtMKK2*, *AtMKK6*). Location of starting point for C terminus indicated with arrow. (b) A diagrammatic representation of the location of the four important domains in the ORF of *OrMKK1*. The diagram indicates the location of catalytic loop, activation loop, ATP binding pocket and substrate binding pocket

of the TEY (Thr-Glu-Tyr), TPY (Thr-Pro-Tyr), or TGY (Thr-Gly-Tyr) sites that are found in most of the reported MAPKs. In addition, *OrMKK1* has a long C-terminus region (about 116 aa in length) that is linked to the protein kinase domain (Fig. 2a). These two features suggest that *OrMKK1* is a new member of the subgroup V MAPK in rice.

The protein is structurally divided into 4 domains which are the catalytic loop, activation loop, ATP binding site and substrate binding site. All these domains are located between the amino acid 70 to 240 of the 352 aa long ORF of *OrMKK1* (Fig. 2b). The 3 dimensional-structure of this protein exhibiting the location of these sites is given in Fig. 3a-d. The catalytic loop contains the ATP, Mg^{2+}, catalytic Asp and serine substrate (2PHK) site, while the activation loop consists of the activation segment and P+1 loop and one or more of the critical phosphorylation sites (e.g. the CK2 kinase activation segment). The ATP binding pocket and the substrate binding pocket both contain phosphorylase kinases with the Mg^{2+} -ATP analog complex (2PHK).

Expression Level of *OrMKK1* in Response to Stress Treatments

The T3 generation of ORMKK1 (rice carrying the pCaM-OrMKK1 construct) was sprayed with JA, SA, BTH, ET and ABA. Twenty microgram of RNA was loaded into each lane and the

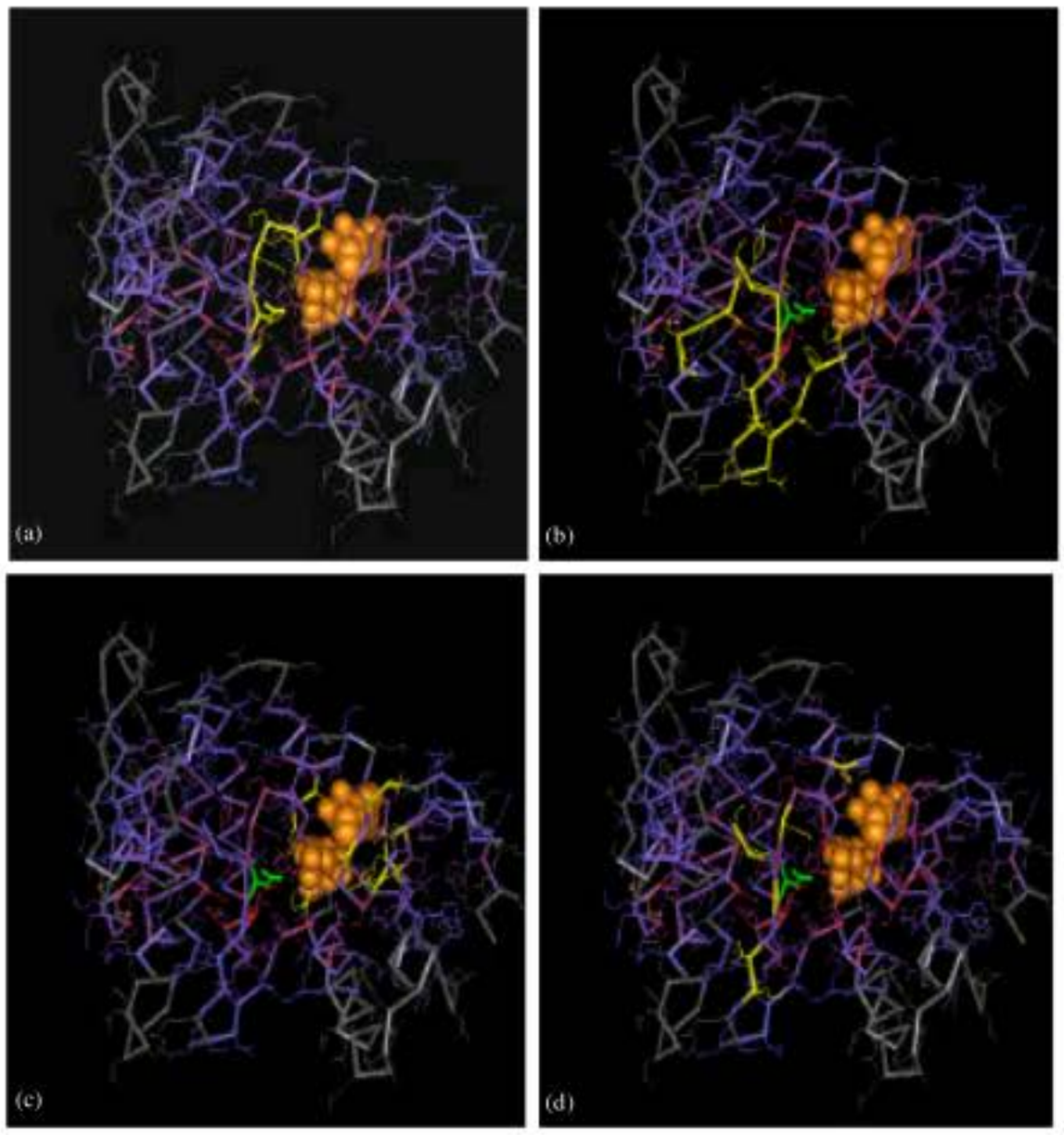

Fig. 3: Three dimensional (3D) Protein Structure of OrMKK1. The locations of the four domains are given in the pictures above. The structures were obtained using the Cn3D 4.1 Program in NCBI. The domains are highlighted in yellow and are located around the Mg^{2+} core in the centre (circles in orange) (a) Catalytic loop, (b) Activation loop, (c) ATP binding pocket and (d) Substrate binding pocket

electrophoresed product was blotted onto nylon membranes. The northern blot analysis suggests that *OrMKK1* was induced by JA, SA BTH and ET as shown in Fig.4a-d. In the JA treatment, transcript of *OrMKK1* increased significantly at 30 min after treatment and was maintained at high level expression till 4hrs and later dropped to low expression level at 12 h post treatment (Fig. 4a). In the SA treatment, the transcript level of this gene increased at 30 min after treatment and peaked at 1 h before gradually declining to low level expression from 4 hrs onwards (Fig. 4b). The expression of *OrMKK1* was also analysed with BTH, a chemical that reacts in a similar manner to SA. The results obtained in BTH treated plants peaked at 0.5 hrs and peaked again at 4hrs and dropped at 12 hrs. The level of expression, however, was slightly lower in BTH treated plants compared to SA (1-2-folds lower) (Fig. 4c). When treated with ET, the transcript levels peaked at 30 min and then gradually dropped to low level expression at 12 h (Fig. 4d). The ABA hormone, however, did not seem to affect the transcript level of this gene until the 12th h, where low level expression was noticed (Fig. 4e). Therefore, the expression to ABA is delayed and weak.

Developmental Regulation of *OrMKK1*

In order to determine if the *OrMKK1* gene is developmentally regulated, the expression of this gene was examined in different plant organs and tissues such as young leaf (L) and shoot (leaf sheath, LS) of 14-day-old seedlings, flag leaf (FgL), first leaf (FL) and panicles at different stages in maturity of the plants (Fig. 5). The northern analysis showed that *OrMKK1* is expressed in the vegetative stage

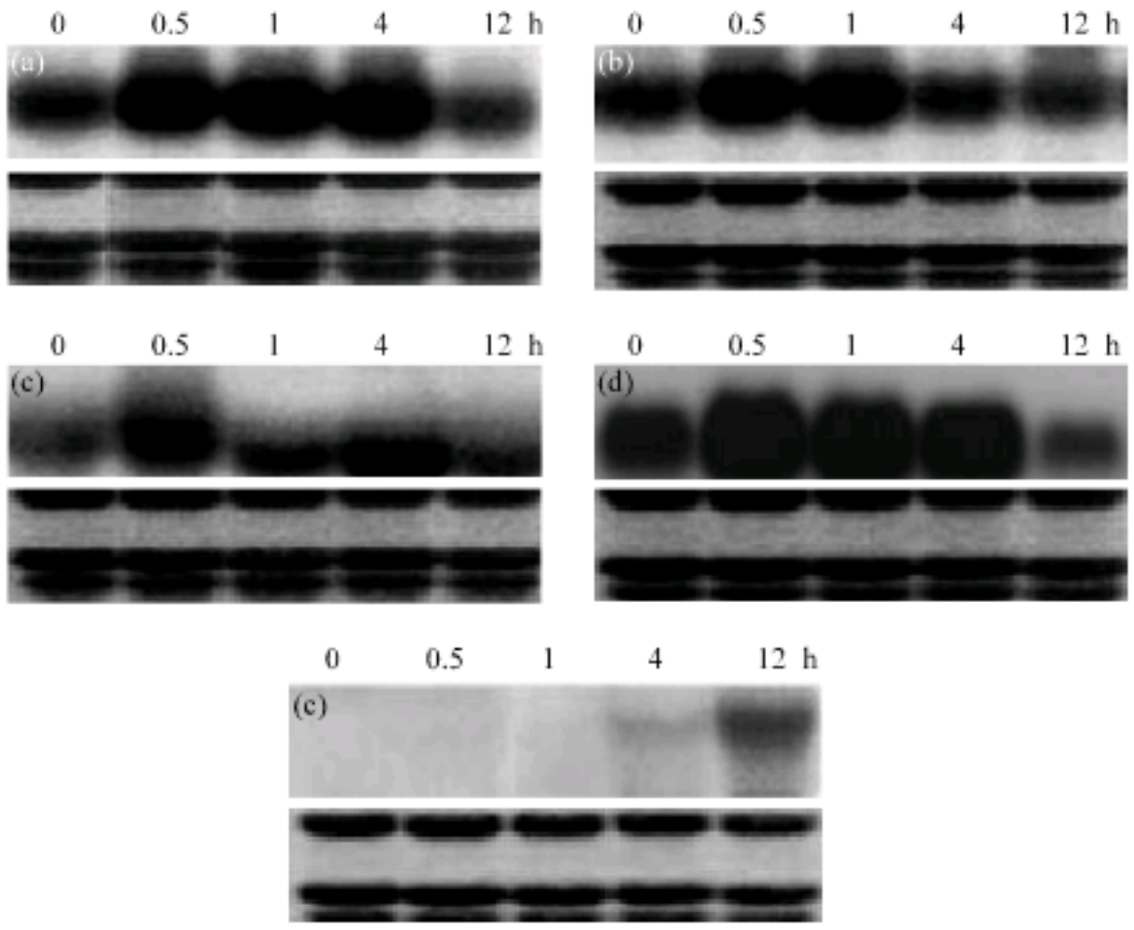

Fig. 4: Time course analysis of *OrMKK1* in response to treatment with (a) JA, (b) SA, (c) BTH, (d) ET and (d) ABA. Induced Leaf segments were treated with JA, SA, ET, BTH and ABA. Concentrations used in this experiment are as mentioned in materials and methods. Seedlings were grown at 16 hd^{-1} photoperiod at 28°C. rRNA was used as control to ensure that all wells were loaded equally

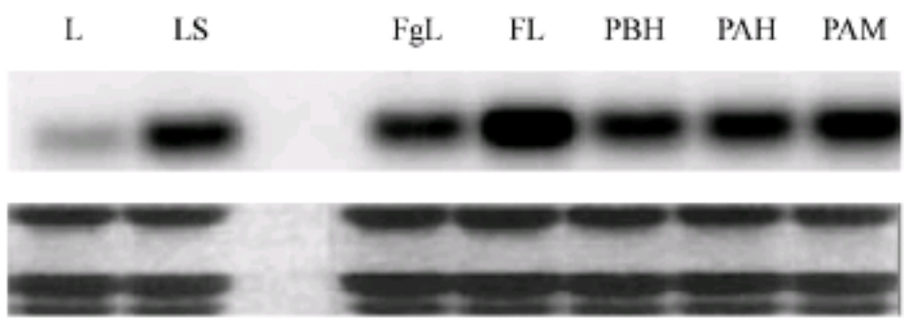

Fig. 5: Developmentally regulated *OrMKK1* expression in rice tissues. (A) Samples of leaf (L) and leaf sheath (LS) of young (two-weak-old) seedlings and the flag leaf (FgL) and first leaf (FL) and panicles before heading (PBH), after heading (PAH) and at maturity (pollination stage, PAM). rRNA was used to ensure that the same amount of RNA was loaded into each well

in leaf and leaf sheath. The transcript level in the leaf sheath was much higher than that in the leaf (>4-folds higher). *OrMKK1* was expressed in the first leaf of rice over the flag leaf at maturity. The amount of transcript detected was between 2-3 folds higher then that observed in flag leaf of rice. The panicle however showed increased expression with maturation. The expression level was higher in panicle at maturity (PAM) followed by Panicle at Heading (PAH) and Panicle Before Heading (PBH), respectively. These results suggest that *OrMKK1* is developmentally regulated in rice (Fig. 5).

DISCUSSION

In this study, a full-length gene encoding a putative MAPK (*OrMKK1*) was isolated, analysed and transformed into rice. Comparison of the amino acid sequence of *OrMKK1* with other reported

plant MAPKs indicates that *OrMKK1* carries a phosphorylation motif of TDY instead of TEY (commonly found in most MAPKs) and an extended C-terminus region linked to the kinase domain, which are the two common features of subgroup V MAPKs in plants.

A motif analysis conducted on the 5'UTR of *OrMKK1* identified several motifs in this region. The light responsive GT-1 and GATA box was found in the promoter region of *OrMKK1*. The GATA box has been identified in highly expressing genes in several plant species (Benfey and Chua, 1990). This motif is required for high level, light regulated and tissue specific expressing genes and has been found in promoters of photoregulated genes. The promoter also contains ERE (ethylene) and MYBCORE motif (drought or water stress). This finding indicates that the *OrMKK1* may play a pivotal role in hormonal signaling pathways and can be activated by abiotic stresses and other forms of physical stresses. The *OrMKK1* is also induced by wounding (Nadarajah *et al*., 2009a). In the wound signaling pathway, ABA and ethylene levels are elevated. These hormones are involved in the healing process in damaged tissues (Leon *et al*., 2001). This may explain the presence of the ERE motif in this gene.

As serine/threonine protein kinases, the enzymatic activities of these proteins are controlled by phosphorylation of specific residues in the activation segment of the catalytic domain (Nuhse *et al*., 2000). This sometimes happens in combination with reversible conformational changes in the C-terminal auto-regulatory tail (Ichimura, 2002). The ATP binding motif and the substrate binding sites are important in the function of the MAP kinase. Energy is required to fuel the process of phosphorylation in the MAPKs. The locations of these domains have been determined for *OrMKK1* where the 3-D structure and the amino acid locations for these domains have been provided in the results segment above.

The MAP kinase signaling cascade is recognized for its role in many stress-related signal transductions. Several lines of evidence have suggested that MAPK genes can be induced by more than one signaling molecule (such as SA and JA), hormone (such as ABA, ethylene and GA), or abiotic stresses. Typical examples are *SIPK* (Zhang and Klessig, 2001; Kumar and Klessig, 2000) (induced by SA and NO) and *WIPK* (Zhang *et al*., 2000) (induced by ethylene and fungal elicitor) from tobacco, OsEDR1 (Kim *et al*., 2003; Song and Goodman, 2002) (induced by JA, SA, ABA, H_2O_2 and ethylene), *OsBIMK1* (Song and Goodman, 2002) (induced by BTH and blast fungus) and *OsMAPK5* (He *et al*., 1999; Lee *et al*., 2001; Xiong and Yang, 2003) (induced by blast fungus, ABA, JA and SA).

The data from this study suggests that *OrMKK1* is transiently but strongly induced by JA, SA, BTH and ET. The response was however weak towards ABA. In our previous study this gene is induced by biotic stress such as fungal infections, insect predation and wounding (Nadarajah *et al*., 2009a; Hadiarto *et al*., 2006; Lee *et al*., 2008). Unlike other reported rice MAP kinases, such as *OsMAPK5* (Xiong and Yang, 2003), *OrMKK1* is not induced by abiotic stresses (Nadarajah *et al*., 2009b) but by JA, SA, BTH and ET. Since both JA and SA are endogenous- signaling molecules involved in plant defense responses (Feys and Parker, 2000; Kunkel and Brooks, 2002), the results in this study suggest that *OrMKK1* may be a component of a novel MAPK-signaling cascade that is associated with JA, SA and ET defense signaling in rice. Further functional analysis of *OrMKK1 is* however required to claim the discrete role of this gene in JA-, SA- and ET-mediated signal transduction pathways and their involvement in the defense signaling and defense responses in rice (Agrawal *et al*., 2002; Ryu *et al*., 2006).

Rice was the first plant species where a JA-inducible MAPK was identified (Agrawal *et al*., 2002). The fact that JA induces *OrMKK1* substantiates the presence of MAPK cascade for JA signaling in rice and strengthens earlier conclusions by other research groups that JA is a critical endogenous signal in rice defense/stress pathway (Agrawal *et al*., 2002; Lee *et al*., 2008; Nakagami *et al*., 2005). Around 20 MAPKs have been revealed after completion of the *Arabidopsis* (dicot model) genome (Asai *et al*., 2002), but none of them has been shown to be JA-inducible.

Regulation of *OrMKK1* by SA implies a SA-dependent MAPK cascade in rice and a role for SA in rice plant defense/stress responses (Agrawal *et al*., 2002; Silverman *et al*., 1995). Considering the fact that both drought and ABA down-regulate *OrMKK1*, it is hypothesised that drought did not increase endogenous ABA in these plants but may have done so in the OrMKK2 lines (Nadarajah *et al*., 2009b).

Ethylene signal transduction involves ETR1, a two-component histidine protein kinase receptor that functions upstream of the negative regulator *CTR1* (Huang *et al*., 2003). The similarity of ETR1 to members of the Raf family of mitogen-activated protein kinase kinase kinases (MAPKKKs) suggests that ethylene signaling in plants involves a MAPK pathway, but no direct evidence for this has been provided. In this study we have shown that *OrMKK1* is activated by ethylene in rice (Chang *et al*., 1993; Chang, 1996). In *Medicago*, the ACC-activated MAPKs were SIMK and MMK3, while in *Arabidopsis* MPK6 and another MAPK were identified. *Medicago* SIMKK specifically mediated ACC-induced activation of SIMK and MMK3 (Kiegerl *et al*., 2000). Transgenic *Arabidopsis* plants over expressing SIMKK have constitutive MPK6 activation and ethylene-induced target gene expression. SIMKK over-expressing lines resemble *ctr1* mutants in showing a triple response phenotype in the absence of ACC (Huang *et al*., 2003; Kiegerl *et al*., 2000). The MPK6 was not activated by ACC in *etr1* mutants and the *ein2* and *ein3* mutants showed normal activation profiles. In contrast, *ctr1* mutants showed constitutive activation of MPK6 (Clark *et al*., 1998). This data indicates that a MAPK cascade is part of the ethylene signal transduction pathway in plants. In addition, through the differential screening in rice the *OrMKK1* seems to be expressed differently at different stages of plant development in different tissues and organs.

In conclusion, the activation of *OrMKK1*, a MAPK gene from *Oryza rufipogon* is shown to be regulated by signal molecules (JA, SA, BTH) and hormones (ethylene). Through the differential analysis conducted on different rice tissues and organs, it is clear that *OrMKK1* is developmentally regulated. This enables us to conclude that the *OrMKK1* is a gene that may be involved in the plant defense signaling mechanism and is collectively regulated by various pathways that are controlled by different signals and hormones (JA, SA, ET-induced pathways) and is differentially regulated in organs and tissues.

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