ORIGINAL ARTICLE

Novel rice *OsSIPK* is a multiple stress responsive MAPK family member showing rhythmic expression at mRNA level

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Abstract We report isolation and transcriptional profiling of rice (*Oryza sativa* L.) mitogen-activated protein kinase (MAPK), OsSIPK (salicylic acid-induced protein kinase). *OsSIPK* gene is located on chromosome 6 most probably existing as a single copy in the rice genome, and encodes 398 amino acid polypeptide having the MAPK family signature and phosphorylation activation motif TEY. Steady state mRNA analyses of *OsSIPK* showed weak constitutive expression in leaves of 2-week-old rice seedlings. A time course (30–120 min) experiment using a variety of elicitors and stresses revealed that the *OsSIPK* mRNA is strongly induced by jasmonic acid (JA), salicylic acid (SA), ethephon, abscisic acid, cycloheximide (CHX), JA/SA + CHX, cantharidin, okadaic acid, hydrogen peroxide, chitosan, sodium chloride, and cold stress (12°C), but not with wounding by cut, gaseous pollutants ozone, and sulfur dioxide, high temperature, ultraviolet C irradiation, sucrose, and drought. Its transcription was also found to be tissue-specifically regulated, and followed a rhythmic dark induction in leaves. Finally, we showed that the OsSIPK protein is localized to the nucleus. From these results, OsSIPK can be implicated in diverse stimuli-responsive signaling cascades and transcription of certain genes.

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Abbreviations

ABA	Abscisic acid
AtMPK6	*Arabidopsis thaliana* MAPK6
AtMPK6	*Arabidopsis thaliana* MAPK6 gene
AtPTP1	*Arabidopsis thaliana* tyrosine phosphatase 1
CHX	Cycloheximide
CRTintP	Calreticulin-interacting protein
CT	Chitosan
EN	Endothall
ET	Ethephon
FgL	Flag leaf
FL	First leaf
HR	Hypersensitive-response
JA	Jasmonic acid

L	Leaf
LS	Leaf sheath
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MsSIMK	*Medicago sativa* salt-induced MAPK
MQ	Milli Q
NtSIPK	*Nicotiana tabacum* salicylic acid-induced protein kinase
OA	Okadaic acid
OsBIMK1	*Oryza sativa* benzothiadiazole-induced MAPK1 gene
OsBWMK1	*Oryza sativa* blast and wound-induced MAPK1 gene
OsMAPK2	*Oryza sativa* MAPK2 gene
OsMAPK4	*Oryza sativa* MAPK4 gene
OsMAPK6	*Oryza sativa* MAPK6
OsMAPK6	*Oryza sativa* MAPK6 gene
OsMSRMK2	*Oryza sativa* mutiple stress responsive MAPK2 gene
OsMSRMK3	*Oryza sativa* mutiple stress responsive MAPK3 gene
OsSIPK	*Oryza sativa* salicylic acid-induced protein kinase
OsSIPK	*Oryza sativa* salicylic acid-induced protein kinase gene
PAH	Panicles after heading
PAL	Phenylalanine ammonia-lyase
PBH	Panicles before heading
ROS	Reactive oxygen species
SA	Salicylic acid
TMV	Tobacco mosaic virus
UV-C	Ultraviolet C
WIPK	Wound-inducible protein kinase

Introduction

Diverse extracellular stimuli are transduced into intracellular responses by an evolutionary conserved mitogen-activated protein kinase (MAPK) cascade(s) in various organisms, ranging from yeast to mammals, and plants (Widmann et al. 1999; Ligterink 2000; Jonak et al. 2002). MAPK (serine/threonine PK) is the last component of the kinase cascade, which is activated by dual phosphorylation of a tripeptide motif (Thr-Xaa-Tyr), located in the activation loop (T-loop). This phosphorylation is mediated by a MAPK kinase (MAPKK) that is in turn activated by phosphorylation by a MAPKK kinase (MAPKKK). Several plant MAPKs have been isolated and characterized, and most of our understanding on the MAPK cascade (MAP-KKK-MAPKK-MAPK), which is yet to be established based on in vivo evidence, comes from intensive studies in

dicot species (Seo et al. 1995; Mizoguchi et al. 1996; Bögre et al. 1997; Ligterink 2000; Ichimura et al. 2002; Jonak et al. 2002). It is only recently that MAPKs of rice, a monocot cereal crop research model (Goff et al. 2002; Yu et al. 2002), are being cloned and characterized (Agrawal et al. 2002, 2003b, c).

The first rice MAPK, *BWMK1* (Accession No. AF177392, hereafter called *OsBWMK1* based on the adopted nomenclature for MAPKs), isolated from an indica-type cultivar IR36, was shown to be induced by blast pathogen (*Magnaporthe grisea*) infection and mechanical wounding (He et al. 1999). Recently, our group reported a MAPK, *OsMSRMK2* (multiple stress responsive; EMBL Accession No. AJ486975), induced by jasmonic acid (JA) and diverse environmental cues (Agrawal et al. 2002). Subsequently, two independent groups published three reports on the cloning and characterization of MAPKs, *OsMAPK2* (Huang et al. 2002), *OsMAPK4* (Fu et al. 2002), and *OsBIMK1* (Song and Goodman 2002). Although considerable progress has been made toward isolating rice MAPKs, taking into account the number of MAPKs in the dicot model *Arabidopsis* (20 MAPKs in its genome) (Ligterink 2000; Ichimura et al. 2002; Jonak et al. 2002), it is tempting to speculate that there are novel MAPKs still waiting to be discovered from rice.

In this study, we present identification of novel MAPK (*OsSIPK*, EMBL Accession No. AJ535841) showing differential response to wounding by cut, and is responsive not only to salicylic acid (SA) treatment but also to diverse stresses, in leaves of 2-week-old rice (cv. Nipponbare) seedlings. Furthermore, a detailed mRNA expression profile analyses strongly suggest their involvement in rice defense/stress response and development.

Materials and methods

Plant material and treatments

Rice (*Oryza sativa* L. japonica-type cv. Nipponbare; seeds obtained from the National Institute of Agrobiological Sciences, Tsukuba, Japan) were grown under white fluorescent light (wavelength 390–500 nm, 150 µmol m^{-2} s^{-1}, 12 h photoperiod) at 25°C and 70% relative humidity, and the middle portions (2 cm long) of fully expanded leaves from 2-week-old seedlings were used for all treatments as described previously, under continuous light or darkness (Hanks et al. 1988; Agrawal et al. 2002; Goff et al. 2002). For circadian/rhythmic study, the seedlings at the 2-week-old stage were left in the growth chamber for a further 3 more days. Leaf segments floated on Milli Q (MQ) water in covered Petri dishes served as a wounding by cut treatment (labeled as CUT in the Figures). Leaf segments placed in

open Petri dish without MQ water were used for drought treatment. Ultraviolet light (UV-C, 254 nm) was irradiated from a distance of 15 cm using a Hitachi (Japan) germicidal lamp (Agrawal et al. 2002). In order to study the effect of temperature pots containing 2-week-old rice seedlings were either placed under continuous light (150 µmol m^{-2} s^{-1}) in growth cabinets at 37°C (high temperature), 12°C (low temperature), and 25°C (control) (Agrawal et al. 2002). Whole seedlings were fumigated with ozone (O$_3$; 0.2 ppm) and sulfur dioxide (SO$_2$; 0.5 ppm) exactly as described previously (Millward et al. 1999; Rakwal et al. 2001). Leaves or leaf segments were sampled at the times indicated in the figures, and immediately frozen at −80°C.

Chemicals

Cantharidin (CN), okadaic acid (OA), ±JA, SA, and cycloheximide (CHX) were purchased from Sigma (St. Louis, MO, USA). Abscisic acid (ABA), ethephon (ET, an ethylene generator), hydrogen peroxide (H$_2$O$_2$), the heavy metals, copper (CuSO$_4$), cadmium (CdCl$_2$), and mercury (HgClO$_3$), and fungal elicitor chitosan (CT, water soluble, MW 3,000–30,000) were obtained from Wako Pure Chemicals (Tokyo, Japan). Endothall (EN) was obtained from BIOMOL Research Laboratories Inc. (PA, USA). Stock solutions were prepared as reported previously (Hanks et al. 1988; Agrawal et al. 2002; Goff et al. 2002).

Identification of MAPK-like genes from differentially expressed cDNA library

In our previous study on identification of *OsMSRMK2* (Agrawal et al. 2002), few clones having weak signal intensity had been selected on the presumption that some of these clones may be either closely related or novel MAPKs, and that forms the basis of this study. The phagemids (pBluescript SK$^-$) of these positive clones were rescued from phages following the in vivo excision protocol recommended by the manufacturer (Stratagene, La Jolla, CA, USA).

DNA sequencing and sequence analysis

Both strands of the recombinant phagemids were sequenced using a dye-terminator cycle-sequencing kit, and an automated capillary DNA sequencer (Genetic Analyzer ABI 310, PE Applied Biosystems, Foster City, CA, USA). All sequencing data were analyzed using Genetyx software (SDC Software Development, Tokyo, Japan). Alignment and homology of amino acid sequence was done using the MultAlin 5.4.1 (INRA) and CLUSTAL W (1.81) programs against sequences in the GenBank, and EMBL DNA database. The phylogenetic tree was constructed by the NJ (Neighbor–joining) method using the Genetyx program.

Southern analyses

Rice genomic DNA (1 µg) from rice leaves was digested with *Hin*dIII, *Eco*RI, *Xba*I, *Bgl*II, *Bam*HI, or *Pst*I, separated by electrophoresis on a 0.8% agarose gel and blotted onto a nylon membrane (Hybond-N$^+$, Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was hybridized with [α-^{32}P]dCTP-labeled (Megaprime DNA labeling system, Amersham) *OsSIPK* cDNA probe. For this, the *OsSIPK*-specific probe including the 3′-untranslated region was amplified by performing PCR with the forward primer: 5′-TGGTGTTCTATTTCAGCCTTG-3′ and the reverse primer: 5′-GTTCCAGTCTTACGATCAAC-3′). Hybridized membranes were washed with 2 × SSC and 0.1% (w/v) SDS at 65°C for 1 h, followed by an additional washing with 0.2 × SSC and 0.1% (w/v) SDS at 65°C for 1 h, and exposed to an X-ray film (Kodak, Tokyo, Japan) using two intensifying screens for 2 days at −80°C.

Northern analyses

Total RNA was isolated from rice seedling leaves using the ISOGEN RNA extraction Kit (Nippon Gene, Toyama, Japan), and blotted onto a nylon membrane (Hybond-N$^+$, Amersham, NJ, USA). Northern analyses were carried out as described previously (Agrawal et al. 2002) using the probes mentioned in Southern analyses. The hybridized membranes were washed with 2 × SSC and 0.1% SDS at 65°C for 1 h, and exposed to an X-ray film (Kodak) using two intensifying screens for 2 days at −80°C. Two independent experiments were carried out, and the extracted total RNAs were pooled for the experiment.

Localization and visualization analysis

To make the EGFP :: OsSIPK fusion protein, pK7WGF2 vector was used to clone the *OsSIPK* gene in frame with the sequence encoding the EGFP protein controlled by a CaMV35S promoter. And for the transient expression assay, the EGFP :: *OsSIPK* constructs was purified as plasmids using the Qiagen plasmid miniprep kit (Qiagen, Valencia, CA, USA). Plasmid DNAs was coated with tungsten particles and then transformed into the onion epidermal cells by particle bombardment (Bilang and Bogorad 1996). Onion epidermal cells were placed in Petri dishes containing MS media and incubated under dark condition at 28°C for 12 h. DAPI staining was performed to identify the cell nucleus. The epidermal cells expressing the EGFP :: OsSIPK fusion protein was visualized using an Olympus microscope with GFP-optimized ND filter sets (Olympus, Japan). Digital images were collected with an Olympus IX70 fluorescence microscope, an I.CAMSCOPE digital camera (Sometech, Korea), and MicroFiresoftware

(USA). The images were further processed with Adobe Photoshop 7.0 software.

Results

Identification of the *OsSIPK* gene

Knowing the importance of MAPKs in signal cascades for plant defense mechanisms against various biotic and abiotic stresses, we cloned few genes related to MAPKs in our previous studies (Agrawal et al. 2002). Of these clones obtained, we identified a novel MAPK gene *OsSIPK* (EMBL nucleotide sequence database with the Accession No. AJ535841) from JA-treated leaves of japonica-type rice (cv. Nipponbare) seedlings using differential cloning. The *OsSIPK* cDNA was 1,701-bp-long, contains an open reading frame (ORF) of 1,197 nucleotides, and encoded a protein of 398 amino acid residues with a putative molecular mass of 44858.06 and pI of 5.45 (compute pI/MW tool). The OsSIPK consisted of an ATP-binding region, a MAPK family signature, which indicates that they belong to the MAPK family, a catalytic loop, a phosphorylation motif (TEY motif) in activation loop, and CD domain. The rice genomic database search predicted that rice contains 18 MAPK isoforms. The alignment analysis based on homologies of their amino acid sequences divided them into two groups as shown in Fig. 1. Moreover, the alignment analysis of MAPKs among various species showed that CD domain of OsSIPK, which functions as a binding site of MAPKKs, is evolutionary-conserved as a DxxDE(P)xC motif (inset, Fig. 1). Among MAPKs of rice and other species, OsSIPK (OsMPK1) was grouped into MAPKs containing a TEY motif in activation loop and a CD-domain in C-terminal region, and had high homologies with NtSIPK (87% positive homology; Zhang and Klessig 1997), MsSIMK (85%; Duerr et al. 1993; Jonak et al. 1993) and AtMPK6 (84%; Mizoguchi et al. 1993; Desikan et al. 2001), which are involved in defense mechanism against biotic and abiotic stresses.

### Genomic organization and tissue specificity of the *OsSIPK* gene in rice

To view the genetic structural features of *OsSIPK* gene, we confirmed that the *OsSIPK* gene is located on chromosome 6 and consist of six exons by using the rice genomic database at Knowledge-based Oryza Molecular Biological Encyclopedia (KOME: http://cdna01.dna.affrc.go.jp/cDNA/) (Fig. 2a). The comparative analysis of the exon-intron organization of *OsSIPK* and its ortholog, *AtMPK6*, on their genomes showed that the number of exons and the size of their parallel exons between both species are the same and similar,
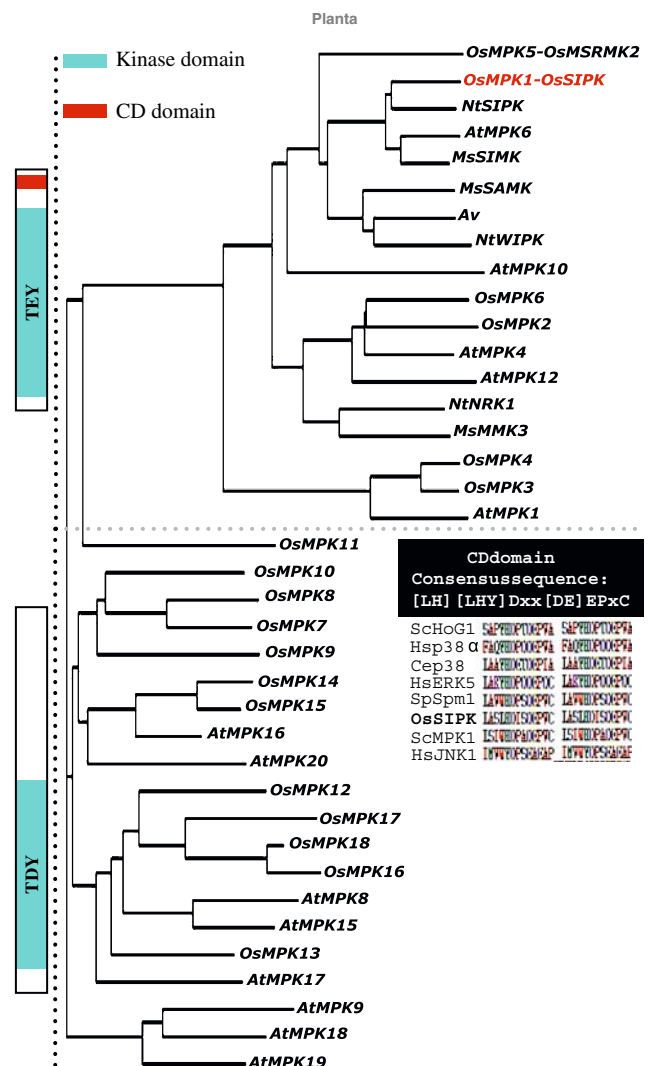
Fig. 1 Dendrogram of OsSIPK with other MAPK family members. The phylogenetic tree was constructed by NJ (Neighbor–joining) method using the Genetyx program. Predicted functional domain of MAPKs is indicated above the dendrogram. Ser/Thr Kinase domain is indicated in *blue box* containing sequence of T-loop motif and CD domain needed for MAPK docking interaction is indicated in *red*. OsMPK1-OsSIPK is marked in *red* in the dendrogram classified in TEY group. Inset: Conserved domains are analyzed by Rice Genome Automated Annotation System (RiceGAAS; http://ricegaas.dna.affrc.go.jp/) and are highlighted by *distinct color*; Consensus sequence of the MAPK CD domain and comparison of CD domain of OsSIPK with other *MAPKs* is shown

respectively (Fig. 2a). Further, for Southern analysis, rice genomic DNA (1.0 μg) digested with *Hin*dIII, *Eco*RI, *Xba*I, *Bgl*II, *Bam*HI or *Pst*I were prepared, blotted to nylon membrane, and hybridized with a probe derived from 3′-untranslated region of *OsSIPK* cDNA as shown in Fig. 2b. The result showed that although under low stringency condition (LOW), one additional band with weak intensities is shown in each line with *Hin*dIII, *Xba*I, *Bgl*II, or *Pst*I, a single dominant band was detected in each lane under high-stringency
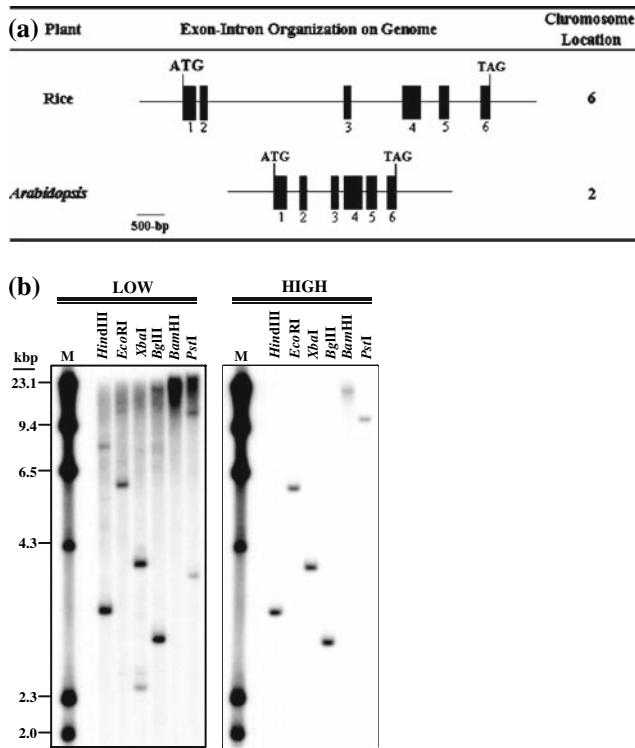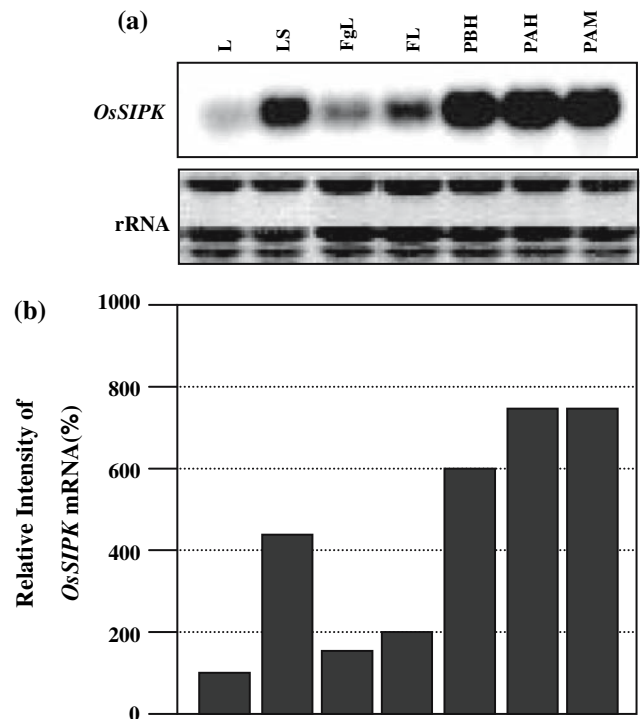
Fig. 2 Exon-intron organization of *OsSIPK* and its *Arabidopsis* homolog *AtMPK6*. The size of genomic DNA was reduced in *ratio* indicated (**a**). Genomic DNA is presented by *line* and *boxes* describe exons. Start (ATG) and stop (TAG) *codons* are marked. Southern analysis of *OsSIPK*. Genomic DNA (1.0 µg) from leaves was digested with *Hind*III, *Eco*RI, *Xba*I, *Bgl*II, *Bam*HI, or *Pst*I, separated by electrophoresis on a 0.8% (w/v) agarose gel, and blotted onto nylon membranes (Hybond-N⁺, Amersham). The membrane was hybridized to the [α-³²P]dCTP-labeled *OsSIPK* cDNA probe under low and high stringency conditions, as described in Materials and methods. *M* represents the molecular weight marker (*λ* DNA digested with *Hind*III) whose fragment size is indicated at the *left* (**b**)

Fig. 3 *OsSIPK* expression in rice tissues. **a** Samples of leaf (*L*) and leaf sheath (*LS*) of young (2-week-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) of mature plants were taken and frozen at −80°C. Blots were hybridized to [α-³²P]dCTP-labeled *OsSIPK* cDNA probe and single hybridizing bands are shown. Equal loading (20 µg) was confirmed by staining of membranes with methylene *blue*, and as a representative a part of ribosomal RNA (*rRNA*) from one membrane is shown. Northern analyses were carried out as described in Materials and methods. **b** The relative mRNA level was calculated taking the *OsSIPK* transcript in the young leaf (*L*) as 100%

condition (HIGH). These data suggest that *OsSIPK* seems to exist as a single copy in the rice genome.

Moreover, to observe the tissue-specificity of OsSIPK, we confirmed its transcript level in different tissues, young leaf (L) and shoot (leaf sheath, LS) of 14-day-old seedling, flag (FgL), first leaf (FL), and panicles at different stages of maturity of mature plants (Fig. 3). The results showed that the *OsSIPK* mRNA is expressed more in leaf sheath than in leaf at the vegetative stage. At maturity, the highest intensity appeared in panicle regardless of its maturation and the lowest signal was detected in flag leaf. These indicate that although *OsSIPK* is constitutively expressed in all of the tested tissues, its intensity is different depending on the tissue specificity.

Differential regulation of *OsSIPK* in response to diverse environmental cues

The phylogenic tree of OsSIPK showed that OsSIPK is evolutionarily very close to NtSIPK (Zhang and Klessig

1997), MsSIMK (Duerr et al. 1993; Jonak et al. 1993), and AtMPK6 (Mizoguchi et al. 1993; Desikan et al. 2001), which are implicated in responses to various biotic and abiotic stimuli (Nakagami et al. 2005 and references therein). These results made us examine the responses of *OsSIPK* to global-signaling molecules such as JA (Reymond and Farmer 1998; Agrawal et al. 2001; Rakwal et al. 2002; Weber 2002), SA (Silverman et al. 1995; Reymond and Farmer 1998; Agrawal et al. 2001), ethylene generator (ET; ethephon) (Ecker 1995; Agrawal et al. 2001), ABA (Grill and Himmelbach 1998; Agrawal et al. 2001) and H_2O_2 (Grant and Loake 2000; Agrawal et al. 2001), potent protein phosphatase inhibitors (CN and EN) (Millward et al. 1999; Agrawal et al. 2001; Rakwal et al. 2001), fungal elicitor (CT) (Hadwiger 1999; Rakwal et al. 2002), drought, high salt (NaCl), sucrose, heavy metals (copper, cadmium and mercury) and ultraviolet C (UV-C) irradiation in vitro system, and high (37°C) and low (12°C) temperature stresses in vivo system as described previously (Agrawal

et al. 2002, 2003b, c). For this, we first observed the changes of *OsSIPK* transcript level within 15 min because of the intrinsic property of MAPKs, which rapidly and transiently respond to external and internal stimuli (Agrawal et al. 2002). The result showed that the transcript levels of *OsSIPK* seem not to alter in stressed leaf fragments (data not shown). The unclear early responses of *OsSIPK* to diverse stressors implored us to further examine its behavior within a delayed time frame, from 30 to 120 min. As shown in Fig. 4, the expression of *OsSIPK* was not induced by wounding by cut within 120 min. However, it was dramatically induced by JA and SA at 60 min, then maximized at 90 min and maintained till 120 min. Ethylene generator (ET), ABA and H_2O_2 enhanced its transcript level at 30 min that was early than that seen with JA and SA, and maintained till 120 min. Moreover, a protein synthesis inhibitor, CHX, alone or together with JA or SA, induced its expression within 30 min and increased till 120 min. The treatment of fungal elicitor (CT) and NaCl, phosphatase inhibitors such as CN, EN, and OA, showed that the expression of *OsSIPK* is increased and reached the maximum at 60 or 90 min. However, drought, UV-C irradiation and sucrose did not give us clear information on *OsSIPK* expression till 120 min. Furthermore, in vivo experiments showed that the expression of *OsSIPK* was not enhanced by high (37°C) temperature, O_3- and SO_2-exposure, whereas low (12°C) temperature increased its transcript level at 30 min that was maintained till 120 min. The results suggest a differential regulation of *OsSIPK* depending on stressors.

### Rhythmically expressed OsSIPK is localized in the nucleus

The previously cloned *OsMSRMK2* is weakly and constitutively expressed regardless of light/dark circulation (Agrawal et al. 2002); whereas Fig. 5a showed that the transcription of *OsSIPK* is repetitively induced at dark period in light (12 h)/dark (12 h) circulation. To know further how light regulates the transcription of *OsSIPK*, we observed its transcript level under continuous dark condition (Fig. 5b). The result showed that the transcript level of *OsSIPK* reaches maximum at 12 h and returns to basal level after 24 h.

For the intracellular localization of OsSIPK, a reporter gene encoding *GFP* was fused to *OsSIPK* (*CaMV 35S-EGFP :: OsSIPK*), and then injected into onion (*Allium cepa*) epidermis cells by using biolistic bombardment as described in Materials and methods. The localization of OsSIPK conjugated with GFP was confirmed in transgenic individual cells by using GFP fluorescence, DAPI staining for nucleus detection, and interference contrast images for whole-cell structures. The results showed that GFP fluorescence signals appear in nucleus in transgenic onion cells containing *CaMV 35S-EGFP :: OsSIPK* gene, and in nucleus and cytoplasm in control cells containing *CaMV 35S-EGFP* gene, respectively (Fig. 6). Moreover, the comparative analysis between GFP fluorescence signals in transgenic cells over-expressing EGFP :: OsSIPK and in transgenic onion cells over-expressing EGFP :: CRTintP (calreticulin -interacting protein), which is a marker protein for nucleus localization reconfirmed that OsSIPK is located
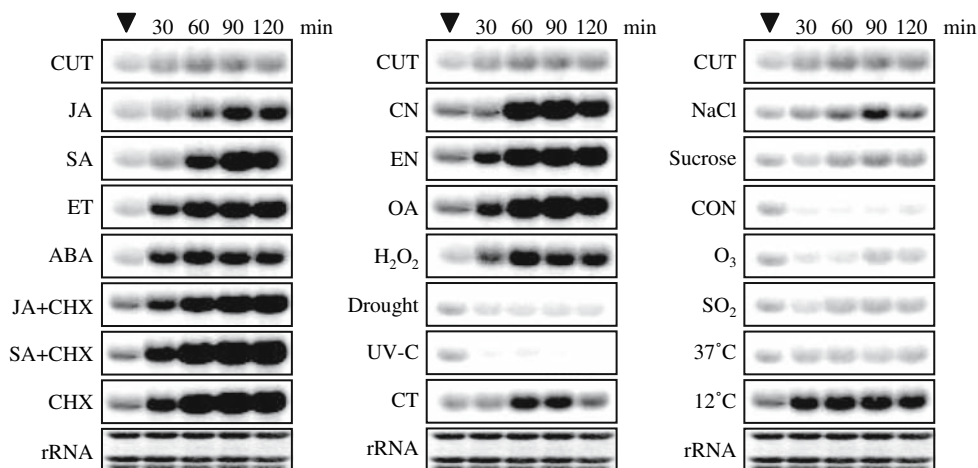
Fig. 4 Time course analysis of *OsSIPK* behavior against diverse environmental factors. Leaf segments were treated with CUT, 100 µM each of JA, SA, ABA, CHX, JA/SA + CHX, protein phosphatases inhibitors [CN, EN, and OA], 1 mM ET, 10 mM H_2O_2, drought, UV-C, 0.1% CT, 150 mM of NaCl and sucrose (in vitro). For in vivo experiments, the pots containing the 2-week-old seedlings were placed at 37 and 12°C in a controlled growth chamber, or exposed to gaseous pollutants ozone (O_3, 0.2 ppm) and sulfur dioxide (SO_2, 0.5 ppm) in a controlled fumigation chamber. Treatments were done under continuous light (150 µmol m^{-2} s^{-1}; or 350 µmol m^{-2} s^{-1} for O_3 and SO_2, CON refers to the clean air control). Equal loading and hybridization was carried out as in Fig. 3
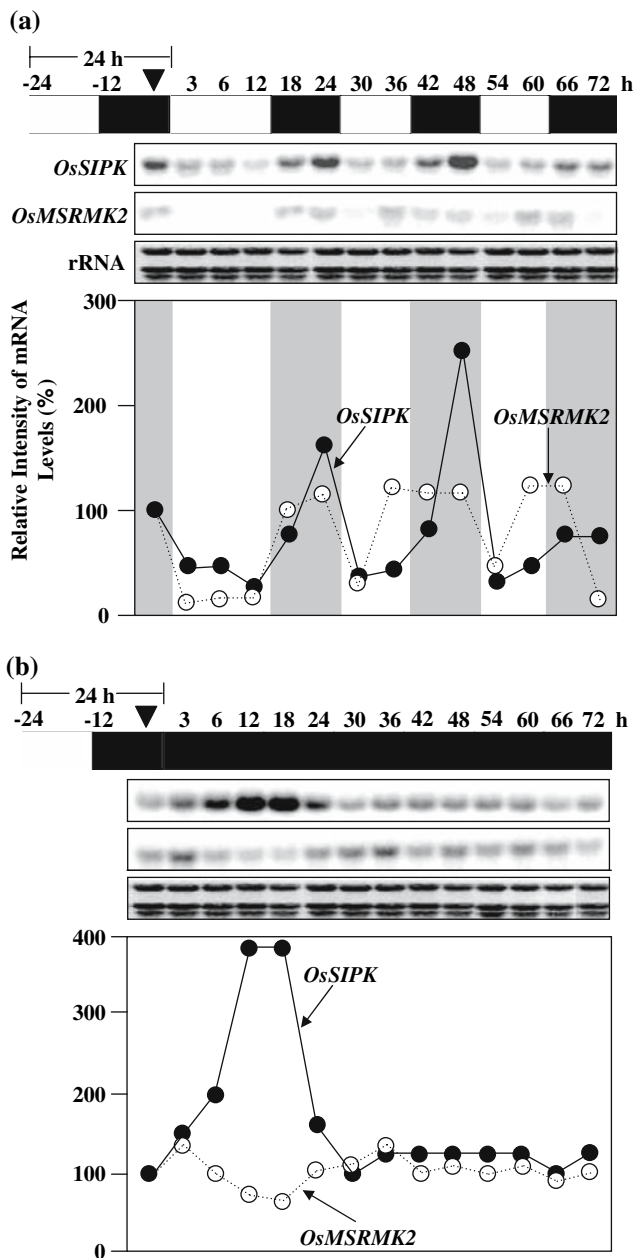
Fig. 5 Rhythmic expression of *OsSIPK*. Rice seedlings were grown and sampling of leaves was done as described in Materials and methods. Total RNA was isolated at the respective time periods under normal *light/dark cycle* (**a**) and under a shift to continuous *dark* (**b**) as given above each lane and subjected to Northern analysis as described in Fig. 3. *Arrowhead* indicates sampling at start of the experiment. Relative abundance of *OsSIPK* and *OsMSRMK2* (a comparative control) mRNAs (in %) according to the signal intensities in panel A/B is given

to nucleus (data not shown). In addition, a prediction program of protein subcellular localization (LOCtree, http://cubic.bioc.columbia.edu/cgi/var/nair/loctree) showed also that OsSIPK might be located in nucleus (Supplementary Fig. 1).

Discussion

Characteristics of the nuclear localized OsSIPK

The MAPK cascade is a crucial component of signal transduction involved in growth, development, and responses to endogenous and environmental cues in all eukaryotes. In plants, several MAPKs activated by a variety of signal molecules (JA, SA, ET, and ABA) and stressors such as pathogen attack, wounding, temperature shift, drought, salt, metal, ozone, UV-C irradiation, and reactive oxygen species (ROS) have been identified (Treisman 1996). The computational analysis of complete rice genome database revealed the existence of 18 rice MAPKs (unpublished observations; see also Liu and Xue 2007). Of rice MAPKs, we cloned several rice MAPKs and reported their transcriptional kinetics by diverse environmental stressors and signal molecules (Agrawal et al. 2002, 2003b, c). Subsequently, we cloned *OsSIPK* with high amino acid homologies to NtSIPK, AtMPK6, and MsSIMK, which are involved in the response to multiple stressors (Nakagami et al. 2005 and references therein), and revealed its location on chromosome 6 and its existence with one copy number. Particularly, considering that among rice MAPK genes, the number of exon in each gene is very various, from 2 to 12 (Liu and Xue 2007), the comparative analysis of genomic structures of *OsSIPK* and *AtMPK6* indicates that the evolutional distance of their genes may be very close. Moreover, LOCtree program, a prediction program of protein subcellular localization, predicted that OsSIPK and AtMPK6 might be located in nucleus (Supplementary Fig. 1). Actually, Schweighofer et al. (2007) showed that AtMPK6 exists in the nucleus and in the cytoplasm in *Arabidopsis* protoplast. The results suggest that an ortholog of NtSIPK, AtMPK6, and MsSIMK in rice may be OsSIPK, which is implicated in a multistressor-responsive MAPK cascade functionally.

Tissue specificity and rhythmic expression of *OsSIPK*

Lieberherr et al. (2005) reported that the transcription and the expression of OsMAPK6, which is identical to OsSIPK, were not induced, but its activity was strongly enhanced within 30 min in sphingolipid elicitor-treated rice cell culture system, and that the expression of pathogen-induced phenylalanine ammonia-lyase (PAL) is regulated by OsMAPK6 pathway. The results could not be matched with the transcript profile of *OsSIPK* in fungal elicitor-treated rice leaf fragment. However, in Fig. 3 we showed that the transcription efficiency of *OsSIPK* in green tissues, including leaf (L), flag leaf (FgL), and first leaf (FL), was lower than in leaf sheath (LS) and panicles. Particularly, the transcript levels of *OsSIPK* during light/dark rhythm was

Fig. 6 Cellular localization of EGFP :: OsSIPK in onion epidermal cells. Cells were bombarded with constructs carrying EGFP or EGFP :: OsSIPK as described in Materials and methods. To confirm the nuclear localization of the GFP fluorescence by OsSIPK, the positions of nuclei (in *blue*) were visualized by DAPI staining. Merge image confirms the GFP and DAPI staining results. Results demonstrate that OsSIPK is localized to the nucleus in the bombarded cells
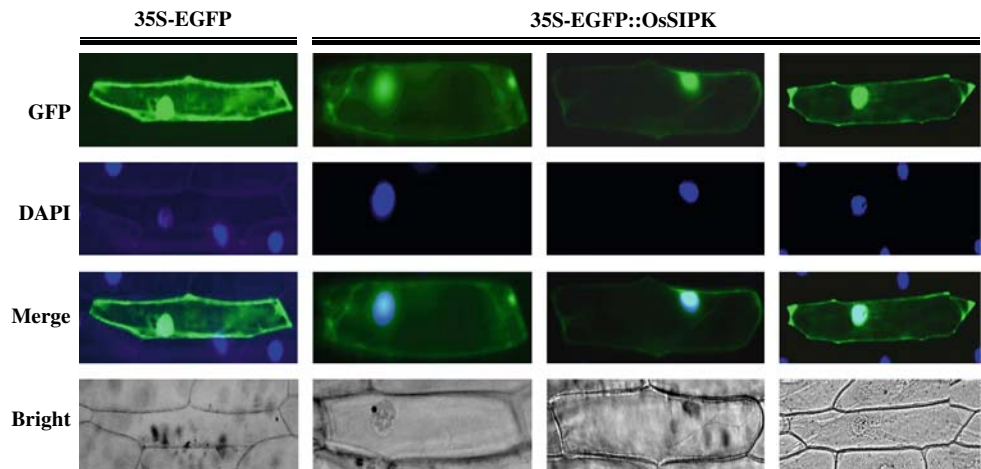
enhanced in the dark and returned to basal level in light, repetitively. Under continuous darkness, the repetitive up- and down-regulation of its transcription disappeared and its expression was transiently enhanced at between 12 and 18 h. The results indicate that the transcription of *OsSIPK* is differentially regulated depending on tissue specificity and light condition. Thus, the conditions such as tissue and light may induce the different transcription pattern of *OsMAPK6* (in cell culture) and *OsSIPK*.

Transcriptional kinetics of *OsSIPK* by various stimuli

MAPKs, the last participants of MAPK cascade, in plants, participate in signal perception and transfer and then induce rapidly and correctly necessary information for adaptation from extracellular intimidation during their lifetime (Zhang and Klessig 2001; Nakagami et al. 2005). Thus, most of MAPKs has been reported to be involved in the early response to various stimuli (Zhang and Klessig 2001; Agrawal et al. 2003a; Nakagami et al. 2005). We also reported that diverse stressors induce the dramatic increase of the transcript levels of *OsMSRMK2* and *OsBWMK1* in leaves within 15 min (Agrawal et al. 2002, 2003b). However, we found that 15 min after treatments by various stressors no significant induction of *OsSIPK* was visible (data not shown). Further experiments clearly indicated that the transcription of *OsSIPK* is maximally activated at 60 and 90 min by most of the tested stressors. These results demonstrate that *OsSIPK* may participate in relatively late response than those of *OsMSRMK2* and *OsBWMK1*.

Moreover, considering the effect of each stressor on the transcription of *OsSIPK*, signal molecules such as JA, SA, ET and ABA, and CHX, which inhibit translation elongation and assembly of polysome, enhance the accumulation of its mRNA. These effects are similar to those on other *OsMAPKs* (Agrawal et al. 2002, 2003b, c). Particularly, the CHX effect demonstrates that the transcription of the

MAPKs is inhibited by the de novo synthesized negative regulator(s). Further investigation by using three phosphatase inhibitors such as CN, EN, and OA revealed that the dephosphorylation of certain molecule(s) activate the expression of *OsSIPK*. As previously reported (Agrawal et al. 2002, 2003b, c), the result suggests that the phosphorylation/dephosphorylation of certain signal molecule by kinase/phosphatase regulates the transcription of *OsSIPK*. In addition, H_2O_2, one of major ROS, enhances the expression of *OsSIPK* as shown in Fig. 4. Recently, it has been reported that H_2O_2 inhibits the activity of *Arabidopsis* tyrosine phosphatase (AtPTP1) by the oxidation of the cysteine in its catalytic domain and thus regulates the activity of MAPK (Xu et al. 1998; Gupta and Luan 2003). High salt (NaCl), fungal elicitor (CT) and cold stresses also up-regulate the transcription of *OsSIPK*, similar to those of *AtMPK6*, *OsMSRMK2*, and *OsMSRMK3* (Nakagami et al. 2005 and references therein). However, although *OsSIPK* is assigned as a multiple stress-responsive MAPK as mentioned above, it is not induced by drought (one of the most extreme changes in the abiotic environment), sucrose, air pollution, high temperature, and UV-C irradiation. The results suggest considerable and fundamental differences among the MAPKs in sensing the stressors.

The activation of OsMAPK6 was reported to be regulated by OsRac1, which plays crucial roles in rice defense mechanisms, including production of ROS, cell death, phytoalexin production, and induction of transcription of defense-related genes (Lieberherr et al. 2005; Kawasaki et al. 1999; Ono et al. 2001; Wong et al. 2004). In addition, long-lasting activation of SIPK and delayed activation of WIPK are implicated in hypersensitive-response (HR) cell death in tobacco mosaic virus (TMV)-infected or elicitor-treated tobacco plants (Zhang and Klessig 2001). In Fig. 6 it was shown that the OsSIPK protein is localized into the nucleus. The results prove that *OsSIPK*, which responds to various stressors (Fig. 4), may interact with the active form

of OsRac1, and then might be involved in the transcription of HR-related genes.

In conclusion, cloning of *OsSIPK*, a detailed expression analysis at the transcript level and its subcellular localization analysis revealed that *OsSIPK* gene is involved in "late" response to diverse stimuli, suppressed by light signal, and localized into nucleus, which will help provide crucial cues for understanding and defining the OsSIPK pathway in rice.

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