Research Note

Molecular Cloning and Characterization of a Tobacco MAP Kinase Kinase That Interacts with SIPK

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A tobacco MAP kinase termed SIPK (salicylic acid-induced protein kinase) is activated in response to a variety of stress signals, including pathogen attack and wounding (S. Zhang and D. F. Klessig, *Proc. Natl. Acad. Sci. USA* 95:7225-7230, 1998; S. Zhang and D. F. Klessig, *Proc. Natl. Acad. Sci. USA* 95:7433-7438, 1998). Using the yeast two-hybrid system, we have identified a gene encoding a protein that interacts with SIPK but not the wounding induced protein kinase (WIPK), which is another tobacco MAP kinase. Sequence analysis indicated that this SIPK-interacting protein is a member of the MAP kinase kinase family; thus, it was named SIPK kinase (SIPKK). Co-immunoprecipitation experiments demonstrated that SIPKK and SIPK interact in vitro. Consistent with its putative function as a kinase, SIPKK phosphorylated myelin basic protein in vitro. Interestingly, SIPKK was induced at the mRNA level after *Tobacco mosaic virus* (TMV) infection or wounding, albeit with kinetics that are too slow to account for the activation of SIPK following these stimuli.

Mitogen-activated protein (MAP) kinase cascades play important roles in signal transduction pathways, such as those leading to stress responses and cell proliferation, in mammalian and yeast systems. These cascades are composed of three functionally linked kinases: MAP kinase (MAPK); MAP kinase kinase (MAPKK); and MAP kinase kinase kinase (MAPKKK). After stimulation by extracellular stimuli, MAPKKK activates MAPKK by the phosphorylation of serine and serine/threonine residues in the SXXXS/T motif. As a dual-specificity kinase, MAPKK then activates MAPK through the phosphorylation of threonine and tyrosine residues in the TXY motif located between kinase subdomains VII and VIII. Activated MAPK then modulates cellular responses by the phosphorylation of various enzymes or transcription factors. In this way, extracellular stimuli are transduced into intracellular responses. In mammalian systems, there are at least

three characterized MAPK cascades, and in yeast at least six MAPK cascades have been identified (Davis 1994; Herskowitz 1995; Hirt 1997).

Homologues of the components constituting MAPK cascades in animals have also been identified in plants. For example, CTR (Kieber et al. 1993) and NPK1 (Banno et al. 1993) are structurally related to MAPKKKs. In addition, MAPKK homologues, such as NPK2 from tobacco (Shibata et al. 1995), ZmMEK from maize (Hardin and Wolniak 1998), an MAPKK from tomato (Hackett et al. 1998), and several MAPKKs from Arabidopsis (Mizoguchi et al. 1997), have been cloned. At least 20 MAPK homologues have also been cloned in various plant species, including Arabidopsis (Mizoguchi et al. 1993, 1994), tobacco (Wilson et al. 1993; Zhang and Klessig 1997), alfalfa (Duerr et al. 1993; Jonak et al. 1993, 1995), pea (Stafstrom et al. 1993), oat, parsley, and petunia (Decroocq-Ferrant et al. 1995). Based on their amino acid sequences, these MAPKs have been classified into three groups (Hirt 1997).

A variety of studies have demonstrated that MAPKs are involved in several aspects of plant growth and development, including pollen development (Wilson et al. 1997), hormone action, and cell division (Bögre et al. 1999). They also are activated in response to a variety of stress stimuli, such as wounding and pathogen attack. For example, MMK4 of alfalfa is involved in cold, drought, and mechanical stresses (Bögre et al. 1996, 1997; Jonak et al. 1996). A salicylic acid (SA)-induced protein kinase termed SIPK, which was purified from tobacco suspension cells and the corresponding gene cloned (Zhang and Klessig 1997), was shown to be activated by various signals, including SA, fungal elicitors, *Tobacco mosaic virus* (TMV) infection, Cf-9-mediated recognition of the *Cladosporium fulvum*-encoded Avr 9 peptide, and wounding (Zhang and Klessig 1997; Zhang et al. 1998; Zhang and Klessig 1998a, 1998b; Romeis et al. 1999). In all cases examined, SIPK was activated exclusively at the posttranslational level by phosphorylation. The tobacco WIPK is also activated posttranslationally by a subset of these stimuli. Additionally, WIPK is induced at the mRNA level by wounding (Seo et al. 1995, 1999; Zhang and Klessig 1998a).

To identify the kinase responsible for SIPK activation, as well as other SIPK-interacting proteins, we used the yeast two-hybrid system. The entire SIPK open reading frame was fused in-frame to the carboxy terminus of the DNA-binding

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(DB) domain of GAL4 in the “bait” plasmid pAS2-1 and transformed into yeast strain PJ69-4A, which contains the reporter genes *GAL1-HIS3*, *GAL2-ADE2*, and *GAL7-LAC Z* (James et al. 1996). While the GAL4-SIPK fusion alone did not activate transcription of the *GAL2-ADE2* reporter gene, it induced a slight increase in expression of *GAL7-LAC Z* (Table

Table 1. Quantitative β -galactosidase assay of SIPK and SIPKK interaction

GAL4 DNA binding domain fusion (bait) ^a	GAL4 activation domain fusion (prey) ^a	β -Galactosidase activity ^b
pAS2-1-SIPK	pACT2-SIPKK	23.0 $\pm$ 0.3
pAS2-1-WIPK	pACT2-SIPKK	4.9 $\pm$ 0.5
pVA3-1	pTD-1	42.7 $\pm$ 2.2
pAS2-1	pACT2-SIPKK	2.2 $\pm$ 0.4
pAS2-1-SIPK	pACT2	3.7 $\pm$ 0.2
pAS2-1	pACT2	1.7 $\pm$ 0.2

^a The GAL4 DNA binding domain in pAS2-1 and GAL4 activation domain in pACT2 are as described in the Clontech user's manual.

^b Data shown are average $\pm$ SE calculated from three independent assays.

1) and sufficient *GAL1-HIS3* expression to allow growth in the absence of exogenous histidine (His⁺). However, histidine auxotrophy could be restored by growing the cells in 20 mM 3-aminotriazole (3-AT), a chemical inhibitor of the *HIS3* product. A tobacco cDNA library, in which the cDNA inserts were cloned into a site 3' of the GAL4 transcription activation (AD) domain in plasmid pACT2, was used for the “prey” library. Yeast colonies were sequentially selected for His⁺ and adenine (Ade⁺) prototrophs followed by a liquid β -galactosidase assay. By screening approximately three million transformants, we identified one positive clone. Preliminary sequence analysis of this SIPK-interacting clone revealed the presence of motifs characteristic of MAPK kinases; thus, this clone was designated SIPK kinase (SIPKK).

To test whether *SIPKK* encoded a protein that could autoactivate reporter gene expression, it was retransformed into yeast strain PJ69-4A. Yeast containing SIPKK and the empty bait vector (pAS2-1) failed to grow on SD (synthetic dropout) media lacking either histidine (His⁻) or adenine (Ade⁻) (Fig. 1); thus, SIPKK alone could not activate these

Fig. 1. Interaction of SIPKK with SIPK in the yeast two-hybrid system. The entire SIPK and WIPK open reading frames were fused in-frame to the C terminus of the DNA-binding (DB) domain of GAL4 in pAS2-1 by initially releasing SIPK and WIPK from the pGEM-T vector (Zhang et al. 1998) with *Bam*HI and *Xba*I, and cloning these fragments into the corresponding sites of the Bluescript (KS) vector. *Not*I/*Eco*RI fragments from the resultant plasmids were then generated and cloned into corresponding sites of pLexA vectors (Clontech, Palo Alto, CA). *Eco*RI/*Sa*I fragments were then generated from these plasmids (with a partial digestion for SIPK) and cloned into pAS2-1 vectors (Clontech). The prey for this two-hybrid assay consisted of a tobacco leaf cDNA library cloned into the pACT2 vector. The resultant bait- and prey-containing plasmids were then transformed into yeast strain PJ69-4A by the method of Gietz and Schiestl (1995) and the yeast colonies were grown for 3 days on SD (synthetic dropout) Trp⁻ Leu⁻ His⁻ medium containing 20 mM 3-AT to inhibit low levels of *HIS3* activity. Three million transformants were screened and 150 His⁺ clones were identified. Seven of these were found to be both Ade⁺ and LAC Z⁺. After plasmid rescue (Robzyk and Kassir 1992) and retransformation into PJ69-4A with pAS2-1-SIPK, one transformant, called SIPKK, was again able to grow on SD Trp⁻ Leu⁻ His⁻ with 20 mM 3-AT and SD Trp⁻ Leu⁻ Ade⁻ media. Growth in His⁻ and Ade⁻ plates indicates reporter gene activation. pAS2-1 and pACT2 are bait and prey vectors without inserts. pVA3-1 and pTD1-1 encode two fusion proteins that are known to interact (Clontech).

reporter genes. In contrast, yeast co-expressing SIPKK and SIPK were prototrophic for histidine and adenine. As expected, yeast expressing SIPK plus the empty prey vector (pACT2) did not grow in the absence of histidine or adenine. Expression of the *GAL7-LAC Z* reporter gene was then quantified with the β -galactosidase assay (Table 1). Yeast containing both SIPK and SIPKK had six- and 10-fold higher levels of β -galactosidase activity than yeast expressing either SIPK or SIPKK and the corresponding empty prey or bait vectors. Moreover, the level of β -galactosidase activity exhibited by yeast expressing both SIPK and SIPKK was nearly half of that exhibited by the positive control (pVA3-1 plus pTD1-1). Taken together, these data indicate that *SIPKK* encodes a protein that physically interacts with SIPK in the yeast two-hybrid system.

To investigate whether SIPKK specifically interacts with SIPK in the yeast two-hybrid system, *WIPK*, which encodes another tobacco MAP kinase (Seo et al. 1995), was cloned into pAS2-1, and then co-transformed with pACT2-SIPKK into yeast strain PJ69-4A. In contrast to yeast expressing both SIPK and SIPKK, the *GAL1-HIS3* and *GAL2-ADE2* reporter genes were not activated in yeast expressing both *WIPK* and SIPKK (Fig. 1). Similar results were obtained with the *GAL7-LAC Z* reporter gene (Table 1). These results suggest that the interaction between SIPK and SIPKK is specific.

To verify the observed interaction between SIPKK and SIPK in the yeast two-hybrid system, co-immunoprecipitation experiments were performed (Fig. 2). cDNAs encoding SIPK and SIPKK were cloned in-frame into the PET 28a(+) and PET 28c(+) expression vectors, respectively, and expressed in *Escherichia coli*. The resultant SIPK and SIPKK fusion proteins were then incubated together, along with an SIPKK antibody. After several washes, the co-immunoprecipitates were separated on sodium dodecyl sulfate-polyacrylamide gels and were reacted with antibodies for SIPKK or SIPK (Ab-p48N; Zhang et al. 1998). The SIPKK antibody recognized a 48-kDa protein, which corresponds to the expected molecular mass of the SIPKK fusion protein. Moreover, since this antibody recognized the 48-kDa protein only in assays containing SIPKK and did not cross-react with SIPK (Fig. 2B), it appears to be specific for SIPKK. Addition of the SIPKK antibody to assays containing SIPK and SIPKK resulted in the immunoprecipitation of both SIPKK (Fig. 2B) and SIPK, at the previously reported size of approximately 52 kDa (Fig. 2A; Zhang et al. 1998). Thus, SIPK and SIPKK interact in vitro.

The 1.6-kb *SIPKK* cDNA clone was sequenced in its entirety and shown to contain a 1,071-bp open reading frame, a 123-bp 5' untranslated region, and a 421-bp 3' untranslated region including a poly (A) tail (GenBank accession number AF165186). Translation of this open reading frame generates a protein with a molecular mass of 39.3 kDa. A BLAST search of current data bases with the deduced SIPKK amino acid sequence revealed significant homology to several MAPKKs (Fig. 3). Of these, an MAPKK from tomato exhibited the greatest similarity to SIPKK (89%). Comparisons of the SIPKK sequence with those of other MAPKKs revealed that SIPKK contains the 11 subdomains characteristic of protein kinases (Fig. 3; Hanks and Quinn 1990). Moreover, the TFVGT motif that is conserved in a number of MAPKKs (Nishihama et al. 1995) is present in subdomain VIII. Southern blot analysis with the *SIPKK* cDNA revealed that this

MAPKK most likely belongs to a multigene family in tobacco (data not shown).

To determine whether SIPKK encodes a functional kinase, an in vitro kinase assay was performed. Bacterially expressed,

Fig. 2. SIPK co-immunoprecipitates with SIPKK. A 19-amino-acid peptide (DLGSYFTSAGPPLATLTEL), corresponding to the C terminus of the SIPKK protein, was synthesized and conjugated to keyhole limpet hemacyanin carrier. Polyclonal antisera were raised in two rabbits and purified by affinity column chromatography (Zymed Laboratory, San Francisco). SIPK antibody (Ab-p48N) was produced as described previously (Zhang et al. 1998). Co-immunoprecipitation experiments were performed essentially as described by Bower et al. (1996) except that 5 μ g of SIPKK antibody was added to 100 μ g of total soluble protein produced in *Escherichia coli* expressing SIPK and/or SIPKK proteins and prepared according to the manufacturer's instructions (Novagene, Madison, WI). Immunoblot analysis was performed essentially as described by Zhang et al. (1998) except that the membranes were reacted with (A) the SIPK-specific antibody (Ab-p48N) or (B) the SIPKK antibody at a concentration of 0.5 μ g/ml. Molecular mass markers are shown in kDa.

SIPKK (100)	MKKG	SLAPNLKLSL	PPPDEVNLSK	FLTESGTFKD	.GDLLVNRDG	VRIVSQS..E	VEAPSVIQPS
Tomato MAPKK (89)	MKKG	SFAPNLKLSL	PPPDEVALSK	FLTESGTFKD	.GDLLVNRDG	VRIVSQS..E	VAAPSVIQPS
MAP2k β (66)	MKKG	GFSNNLKLA	PVAGEQSITK	FLTQSGTFKD	.GDLRVNKDG	VRIIISQL..E	PEVLSPIKPA
MAP kinase kinase 2 (66)	MKKG	GFSNNLKLA	PVAGEQSITK	FLTQSGTFKD	.GDLRVNKDG	VRIIISQL..E	PEVLSPIKPA
MEK1 (63)	MNRG	SLCPN.PICL	PPL.EQSISK	FLTQSGTFKD	.GDLRVNKDG	IQTIVSLS..E	PGAPPPIEPL
ZmMEK1 (56)	MKKG	KPVKELKLT	P.AQETPVDK	FLTAGSGTFKD	.GELRLNQSG	LRLISEENG	EDESTKLKVE
MAP kinase kinase 4 (44)	MRPIQSPPGV	SVPVKSRPR	RPDLTLPLPQ	RDVSLAVPLP	LPPTSGGSGG	SSGSAPSSGG	SASSTNTNSS
MAP kinase kinase 5 (43)	MKPIQSPSGV	ASPMKNRLRK	RPDLSLPLPH	RDVALAVPLP	LPPPS....	SSSSAPAS.S	SAISTNISA.
MAP kinase kinase α (43)	MKPIQSPSGV	ASPMKNRLRK	RPDLSLPLPH	RDVALAVPLP	LPPPS....	SSSSAPAS.S	SAISTNISA.
I							
SIPKK (100)	DNQLCLADFE	AVKVGKNG	GIVRLVQHKW	TGQFFALKAI	QMNIEESMRK	HIAQELRINQ	SSQVPYVVIS
Tomato MAPKK (89)	DNQLCLADFE	SSKVIGKNG	GIVRLVQHKW	TGQFFALKVI	QMNIDESMRK	HIAQELRINQ	SSQCPYVVIC
MAP2k β (66)	DDQLSLSDLD	MVKVIGKSS	GVVQLVQHKW	TGQFFALKVI	QLN DEAIRK	ATAQELKINQ	SSQCPNLVTS
MAP kinase kinase 2 (66)	DDQLSLSDLD	MVKVIGKSS	GVVQLVQHKW	TGQFFALKVI	QLNIDEAIRK	ATAQELKINQ	SSQCPNLVTS
MEK1 (63)	DNQLSLADLE	VIKVGKSS	GNVQLVKHKL	TQQFFALKVI	QLNTEESTCR	ATSQELRINL	SSQCPYLVSC
ZmMEK1 (56)	DVQLSMDLE	MIQVIGKSG	GVVQLVRHKW	VGTLFALKGI	QMNIESVRK	QIVQELKINQ	ATQSPHVMC
MAP kinase kinase 4 (44)	IEAKNYSIDL	RGNRIGSGAG	GTVYKVIHRP	SSRLYALKVI	YGNHEETVRR	QICREIEILR	DVNHPNVVKC
MAP kinase kinase 5 (43)	..AKSLSELE	RVNRIGSGAG	GTVYKVIHTP	TSRPFALKVI	YGNHEDTVRR	QICREIEILR	SVDHPNVVKC
MAP kinase kinase α (43)	..AKSLSELE	RVNRIGSGAG	GTVYKVIHTP	TSRPFALKVI	YGNHEDTVRR	QICREIEILR	SVDHPNVVKC
II							
SIPKK (100)	YQSFF..DNG	AISIILEYMD	GGSLADFLKK	VKTIPERYLA	AICKQVLKGL	WYLHHEKHII	HRDLKPSNLL
Tomato MAPKK (89)	YQSFF..DNG	AISLILEYMD	GGSLADFLKK	VKTIPERFLA	VICKQVLKGL	WYLHHEKHII	HRDLKPSNLL
MAP2k β (66)	YQSFY..DNG	AISLILEYMD	GGSLADFLKS	VKAIPDSYLS	AIFRQVLQGL	IYLLHHRHII	HRDLKPSNLL
MAP kinase kinase 2 (66)	YQSFY..DNG	AISLILEYMD	GGSLADFLKS	VKAIPDSYLS	AIFRQVLQGL	IYLLHHRHII	HRDLKPSNLL
MEK1 (63)	YQSFY..HNG	LVSIILEFMD	GGSLADLLKK	VGKVPENMLS	AICKRVLRGL	CYIHHERRII	HRDLKPSNLL
ZmMEK1 (56)	HQSFY..HNG	VIYLVLEYMD	RGLADIVKQ	VKTILEPYLA	VLCQVLEGL	LYLHHQRHVI	HRDIKPSNLL
MAP kinase kinase 4 (44)	HEMF..DQNG	EIQVLLLEFMD	KGSL....EG	AHVWKEQQLA	DLSRQILSGL	AYLHS.RHIV	HRDIKPSNLL
MAP kinase kinase 5 (43)	HDMF..DHNG	EIQVLLLEFMD	QGSL....EG	AHIWQEQLA	DLSRQILSGL	AYLHR.RHIV	HRDIKPSNLL
MAP kinase kinase α (43)	HDMF..DHNG	EIQVLLLEFMD	QGSL....EG	AHIWQEQLA	DLSRQILSGL	AYLHR.RHIV	HRDIKPSDLL
III							
SIPKK (100)	INHRGDVKIT	DFGVSAVLAS	TSGLANTFVG	TYNMSPERI	L....GGAY	GYRSDIWSLG	LVLLECATGV
Tomato MAPKK (89)	INHRGDVKIT	DFGVSAVLAS	TSGLANTFVG	TYNMSPERI	S....GGAY	DYKSDIWSLG	LVLLECATGH
MAP2k β (66)	INHRGEVKIT	DFGVSTVMTN	TAGLANTFVG	TYNMSPERI	V....GNKY	GNKSDIWSLG	LVLLECATGK
MAP kinase kinase 2 (66)	INHRGEVKIT	DFGVSTVMTN	TAGLANTFVG	TYNMSPERI	V....GNKY	GNKSDIWSLG	LVLLECATGK
MEK1 (63)	INHRGEVKIT	DFGVSKILTS	TSSLANSFVG	TYPYMSPERI	S....GSLY	SNKSDIWSLG	LVLLECATGK
ZmMEK1 (56)	VNRKGEVKIT	DFGVSAVLA	SSIQQRDTFVG	TYNMSPERI	S....GSTY	DYKSDIWSLG	LVLLECAIGR
MAP kinase kinase 4 (44)	INSAKNVKIA	DFGVSRLA	QTMDCPNSSVG	TIAYMSPERI	NTDLNQGKYD	GYAGDIWSLG	VSILEFYLGR
MAP kinase kinase 5 (43)	INSAKNVKIA	DFGVSRLA	QTMDCPNSSVG	TIAYMSPERI	NTDLNHGRYD	GYAGDVWSLG	VSILEFYLGR
MAP kinase kinase α (43)	INSAKNVKIA	DFGVSRLA	QTMDCPNSSVG	TIAYMSPERI	NTDLNHGRYD	GYAGDVWSLG	VSILEFYLGR
IV							
SIPKK (100)	FPYSPQADE	GWVNVYELME	TIVDQPAFSA	PPDQFSPQFC	SFISACVQKD	QKDRLSANEL	MRHPFITMYD
Tomato MAPKK (89)	FPYKPPEGDE	GWVNVYELME	TIVDQPEPCA	PPDQFSPQFC	SFISACVQKH	QKDRLSANL	MSHPFITMYD
MAP2k β (66)	FPYAPPNQE	TWTSVFELME	AIVDQPPPAL	PSGNFSPELS	SFISTCLQKE	PNSRSSAKEL	MEHPFLNKYD
MAP kinase kinase 2 (66)	FPYAPPNQE	TWTSVFELME	AIVDQPPPAL	PSGNFSPELS	SFISTCLQKE	PNSRSSAKEL	MEHPFLNKYD
MEK1 (63)	FPYTPEHKK	GWSSVYELVD	AIVENPPPCA	PSNLFSPFC	SFISQCVQKD	PRDRKSASEL	LEHFKVVMFE
ZmMEK1 (56)	FPYIPSEG.E	GWLSFYELLE	AIVDQPPPSA	PADQFSPEFC	SFISSCIQKD	PAQRMSASEL	LNHPFLKKFE
MAP kinase kinase 4 (44)	FPFPVSRQGD	.WAS...LMC	ATCMSQPPEA	PATA.SQEFR	HFVSCCLQRE	PGKRRSAMQL	LQHPFILRAS
MAP kinase kinase 5 (43)	FPFAVSRQGD	.WAS...LMC	AICMSQPPEA	PATA.SQEFR	HFVSCCLQSD	PPKRWSAQQL	LQHPFILKAT
MAP kinase kinase α (43)	FPFAVSRQGD	.WAS...LMC	AICMSQPPEA	PATA.SQEFR	HFVSCCLQSD	PPKRWSAQQL	LQHPFILKAT
V							
SIPK (100)	DLIDLGSYF	TSAGPPLATL	TEL				
Tomato MAPKK (89)	DQDIDLGSYF	TSAGPPLATL	TEL				
MAP2k β (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MAP kinase kinase 2 (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MEK1 (63)	DSDTNLSAYF	TDAGSLIPPL	AN				
ZmMEK1 (56)	DKDLNLGILV	ENLEPPMNIP	E				
MAP kinase kinase 4 (44)	PSQNRSPQNL	HQLLPPPRPL	SSSSSPTT				
MAP kinase kinase 5 (43)	GGPNL	RQMLPPPRPL	PSAS				
MAP kinase kinase α (43)	GGPNL	RQMLPPPRPL	PSAS				
VI							
SIPK (100)	DLIDLGSYF	TSAGPPLATL	TEL				
Tomato MAPKK (89)	DQDIDLGSYF	TSAGPPLATL	TEL				
MAP2k β (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MAP kinase kinase 2 (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MEK1 (63)	DSDTNLSAYF	TDAGSLIPPL	AN				
ZmMEK1 (56)	DKDLNLGILV	ENLEPPMNIP	E				
MAP kinase kinase 4 (44)	PSQNRSPQNL	HQLLPPPRPL	SSSSSPTT				
MAP kinase kinase 5 (43)	GGPNL	RQMLPPPRPL	PSAS				
MAP kinase kinase α (43)	GGPNL	RQMLPPPRPL	PSAS				
VII							
SIPK (100)	DLIDLGSYF	TSAGPPLATL	TEL				
Tomato MAPKK (89)	DQDIDLGSYF	TSAGPPLATL	TEL				
MAP2k β (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MAP kinase kinase 2 (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MEK1 (63)	DSDTNLSAYF	TDAGSLIPPL	AN				
ZmMEK1 (56)	DKDLNLGILV	ENLEPPMNIP	E				
MAP kinase kinase 4 (44)	PSQNRSPQNL	HQLLPPPRPL	SSSSSPTT				
MAP kinase kinase 5 (43)	GGPNL	RQMLPPPRPL	PSAS				
MAP kinase kinase α (43)	GGPNL	RQMLPPPRPL	PSAS				
VIII							
SIPK (100)	DLIDLGSYF	TSAGPPLATL	TEL				
Tomato MAPKK (89)	DQDIDLGSYF	TSAGPPLATL	TEL				
MAP2k β (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MAP kinase kinase 2 (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MEK1 (63)	DSDTNLSAYF	TDAGSLIPPL	AN				
ZmMEK1 (56)	DKDLNLGILV	ENLEPPMNIP	E				
MAP kinase kinase 4 (44)	PSQNRSPQNL	HQLLPPPRPL	SSSSSPTT				
MAP kinase kinase 5 (43)	GGPNL	RQMLPPPRPL	PSAS				
MAP kinase kinase α (43)	GGPNL	RQMLPPPRPL	PSAS				
IX							
SIPK (100)	DLIDLGSYF	TSAGPPLATL	TEL				
Tomato MAPKK (89)	DQDIDLGSYF	TSAGPPLATL	TEL				
MAP2k β (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MAP kinase kinase 2 (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MEK1 (63)	DSDTNLSAYF	TDAGSLIPPL	AN				
ZmMEK1 (56)	DKDLNLGILV	ENLEPPMNIP	E				
MAP kinase kinase 4 (44)	PSQNRSPQNL	HQLLPPPRPL	SSSSSPTT				
MAP kinase kinase 5 (43)	GGPNL	RQMLPPPRPL	PSAS				
MAP kinase kinase α (43)	GGPNL	RQMLPPPRPL	PSAS				
X							
SIPK (100)	DLIDLGSYF	TSAGPPLATL	TEL				
Tomato MAPKK (89)	DQDIDLGSYF	TSAGPPLATL	TEL				
MAP2k β (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MAP kinase kinase 2 (66)	YSGINLASFY	TDAGSPLATL	GNLSGTFSV				
MEK1 (63)	DSDTNLSAYF	TDAGSLIPPL	AN				
ZmMEK1 (56)	DKDLNLGILV	ENLEPPMNIP	E				
MAP kinase kinase 4 (44)	PSQNRSPQNL	HQLLPPPRPL	SSSSSPTT				
MAP kinase kinase 5 (43)	GGPNL	RQMLPPPRPL	PSAS				
MAP kinase kinase α (43)	GGPNL	RQMLPPPRPL	PSAS				
XI							

Fig. 3. Sequence comparison between SIPKK and other putative plant MAPKKs. Amino acid sequences of SIPKK, the tomato MAPKK (Hackett et al. 1998), MAP2K β , MAP kinase kinase 2 (Ichimura et al. 1998), MEK1 (Morris et al. 1997), ZmMEK1 (Hardin and Wolniak 1998), MAP kinase kinase 4 (Ichimura et al. 1998), MAP kinase kinase 5 (Ichimura et al. 1998), and MAP kinase kinase α (Jouannic et al. 1996) were deduced from cDNA sequences and aligned with the PILEUP program (Wisconsin GCG package, Madison, WI). Amino acid percent identity to SIPKK is shown in parentheses. Dots represent gaps introduced to maximize alignment. The 11 conserved subdomains characteristic of protein kinase (I–XI) are noted in Roman numerals. The conserved motif found in many MAPKKs is underlined in the SIPKK sequence.

His-tagged SIPK and SIPKK were affinity purified on a nickel column and incubated in a kinase assay with [γ - 32 P]ATP and myelin basic protein (MBP), which serves as a phosphorylation substrate for many MAPKs (Zhang et al. 1998). As shown in Figure 4, the SIPKK fusion protein was able to phosphorylate MBP (Fig. 4, lane 1), indicating that *SIPKK* encodes a kinase. SIPK did not exhibit detectable autophosphorylation activity (Fig. 4, lane 2). By contrast, the SIPK fusion protein, which serves as the positive control, phosphory-

Fig. 4. SIPKK can phosphorylate MBP. Bacterially expressed SIPK and SIPKK proteins were purified with a nickel affinity column by the method of Zhang et al. (1998). In vitro protein kinase assays were performed essentially as described by Crews et al. (1992) except that 1 μ g of affinity purified recombinant protein was incubated with 20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM dithiothreitol, 2 μ g of aprotinin per ml, 10 μ M unlabeled ATP, and 10 μ Ci [γ - 32 P-ATP] in the presence or absence of 1 μ g of MBP per μ l. Lane 1, SIPKK plus MBP; lane 2, SIPKK minus MBP; lane 3, SIPK plus MBP; lane 4, SIPK K-R plus MBP. Protein molecular markers are shown in kDa.

Fig. 5. SIPKK is induced at mRNA level by *Tobacco mosaic virus* (TMV) infection and wounding. **A**, Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) grown for 6 to 8 weeks at 22°C were infected with TMV and maintained at 32°C for 48 h, then shifted back to 22°C (hps, h post temperature shift), as previously described (Zhang and Klessig 1998b). Disks from infected leaves were collected at different times post temperature shift and used for isolation of total RNA according to the Trizol method (Gibco BRL, Gaithersburg, MD). Ten micrograms of total RNA per lane was separated on 1.2% agarose-formaldehyde gels, transferred to nylon membranes, and probed with the full-length SIPKK cDNA clone labeled by the random primer method (Feinberg and Vogelstein 1983). To allow normalization for differences in loading among lanes, filters were stripped and reprobed with a rDNA probe generated as described previously (Shah et al. 1997). **B**, Six- to 8-week-old tobacco plants grown at 22°C were wounded according to the method of Usami et al. (1995). At different times after wounding, total RNA was isolated and analyzed as described above.

lated MBP as well as itself (Fig. 4, lane 3). Unfortunately, numerous attempts to demonstrate that SIPKK could phosphorylate SIPK in vitro were unsuccessful, even when the sensitivity of the assay was increased by the use of the SIPK K-R mutant. This mutant, which was generated by mutating the lysine (K) at amino acid 89 residue of SIPK to arginine (R), lacks autophosphorylation activity and, therefore, reduces the background. Our inability to show SIPK phosphorylation by SIPKK is consistent with the findings of others, who have isolated several MAPKKs and MAPKs from Arabidopsis but have encountered difficulties demonstrating MAPKK-mediated phosphorylation of an MAPK in vitro (Mizoguchi et al. 1998; Ichimura et al. 1998). There is only one report demonstrating phosphorylation and activation of a plant MAPK by a plant MAPKK (Gupta et al. 1998).

Previous studies have demonstrated that SIPK and other MAPKs belonging to the SIPK subgroup are activated strictly at the posttranslational level following stress stimuli (Zhang and Klessig 1997; Zhang et al. 1998; Zhang and Klessig 1998a, 1998b). By contrast, WIPK and its subgroup members exhibit increases in both their enzymatic activity and mRNA levels in response to various stimuli (Jonak et al. 1996; Bögre et al. 1997; Seo et al. 1995; Ligterink et al. 1997; Zhang and Klessig 1998a, 1998b). For most of these MAPKs, the relatively slow accumulation of transcripts appears unlikely to account for the very rapid increase in kinase activity. However, increases in the mRNA levels of WIPK have been shown to precede both protein accumulation and kinase activation in tobacco resisting TMV infection (Zhang and Klessig 1998b). In addition, it was recently demonstrated that transcripts for MEK1, an MAPKK from Arabidopsis, accumulate in response to wounding (Morris et al. 1997). Thus, we wished to determine whether stress stimuli, such as TMV infection or wounding, increased the levels of SIPKK mRNA.

To facilitate SIPKK mRNA analysis in TMV-infected tobacco, the plants were maintained at 32°C for 2 days and then shifted to room temperature (22°C). Because tobacco grown under elevated temperatures are unable to resist TMV infection, such a shift causes a strong, synchronous induction of many defense responses, including the accumulation of SA and pathogenesis-related (PR) proteins, the restriction of viral movement, and the development of necrotic lesions (Kassanis 1952; Gianinazzi 1970; Malamy et al. 1992). Analysis of mRNA isolated from TMV-infected plants revealed a modest increase in SIPKK transcripts that occurred around 5 h post temperature shift (hps; Fig. 5A). SIPKK mRNA levels then remained elevated for the remainder of the sampling period. By contrast, no increase in SIPKK transcripts was observed in mock-inoculated, temperature-shifted plants (Fig. 5A). Wounding was also observed to induce the accumulation of SIPKK transcripts. An increase in SIPKK transcripts was detected by 30 min after wounding and this level was maintained for the rest of the time course (Fig. 5B).

Although TMV infection and wounding have previously been shown to induce SIPK activity, the modest increase in SIPKK mRNAs following these stresses is too slow to play a role in this phenomenon. In TMV-infected tobacco, SIPK activity increased within 2 to 3 hps (Zhang and Klessig 1998b), while rises in SIPKK transcripts were not detected until 5 hps. Similarly, although wounding induced SIPK activity within 5 min (Zhang and Klessig 1998a), SIPKK transcripts did not

accumulate until after 30 min. These results, however, do not rule out a role for SIPKK in activating SIPK. In animals, MAPKK activation occurs exclusively at the posttranslational level (Seger and Krebs 1995). Additionally, SIPK activation by a wide variety of stimuli occurs in the absence of detectable increases in mRNA levels (Zhang et al. 1998; Zhang and Klessig 1998a, 1998b). Thus, TMV infection and wounding may rapidly activate SIPKK at the posttranslational level; this activated SIPKK could then activate SIPK. Future experiments will clearly be required to help elucidate the role of SIPKK in transducing signals through the SIPK cascade.

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