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SIMKK, a Mitogen-Activated Protein Kinase (MAPK) Kinase, Is a Specific Activator of the Salt Stress–Induced MAPK, SIMK

Stefan Kiegerl,^a Francesca Cardinale,^a Christine Siligan,^a Andrea Gross,^b Emmanuel Baudouin,^{a,1} Aneta Liwosz,^a Staffan Eklöf,^{a,2} Sandra Till,^a Laszlo Bögre,^{a,3} Heribert Hirt,^{a,4} and Irute Meskiene^a

^a Institute of Microbiology and Genetics, Vienna Biocenter, University of Vienna, 1030 Vienna, Austria

^b Institute of Genetics, Faculty of Biology, D-33501 Bielefeld, Germany

In eukaryotes, mitogen-activated protein kinases (MAPKs) play key roles in the transmission of external signals, such as mitogens, hormones, and different stresses. MAPKs are activated by MAPK kinases through phosphorylation of MAPKs at both the threonine and tyrosine residues of the conserved TXY activation motif. In plants, several MAPKs are involved in signaling of hormones, stresses, cell cycle, and developmental cues. Recently, we showed that salt stress–induced MAPK (SIMK) is activated when alfalfa cells are exposed to hyperosmotic conditions. Here, we report the isolation and characterization of the alfalfa MAPK kinase SIMKK (SIMK kinase). SIMKK encodes an active protein kinase that interacts specifically with SIMK, but not with three other MAPKs, in the yeast two-hybrid system. Recombinant SIMKK specifically activates SIMK by phosphorylating both the threonine and tyrosine residues in the activation loop of SIMK. SIMKK contains a putative MAPK docking site at the N terminus that is conserved in mammalian MAPK kinases, transcription factors, and phosphatases. Removal of the MAPK docking site of SIMKK partially compromises but does not completely abolish interaction with SIMK, suggesting that other domains of SIMKK also are involved in MAPK binding. In transient expression assays, SIMKK specifically activates SIMK but not two other MAPKs. Moreover, SIMKK enhances the salt-induced activation of SIMK. These data suggest that the salt-induced activation of SIMK is mediated by the dual-specificity protein kinase SIMKK.

INTRODUCTION

Protein phosphorylation is one of the major mechanisms for controlling cellular functions in response to external signals. In eukaryotes, a specific class of serine/threonine protein kinases, the mitogen-activated protein kinases (MAPKs), is involved in many of these processes. A general feature of MAPK cascades is their composition of three functionally linked protein kinases. A MAPK is phosphorylated and thereby activated by a MAPK kinase (MAPKK), which itself becomes activated by another serine/threonine protein kinase, a MAPK kinase kinase. MAPKKs are dual-specificity kinases that phosphorylate and thereby activate MAPKs on both the threonine and tyrosine residues of the TXY phosphorylation motif. MAPKs have a bilobed structure in which the ATP is bound in the cleft between the two lobes and the C-terminal lobe binds the substrate. The crystal structure of

ERK2, a mammalian MAPK, suggested the first explanations for why the unique dual-phosphorylation event is necessary for the activation of this group of protein kinases (Zhang et al., 1994). Thr183 and Tyr185 of ERK2 are contained in a loop that connects kinase subdomains VII and VIII. Whereas Thr183 is exposed on the surface of the loop and therefore is accessible to the MAPKK MEK1, Tyr185 is buried in a hydrophobic pocket. Because both residues have to be phosphorylated for ERK2 to be activated, MEK1 has first to phosphorylate Thr183; the resulting conformational change of ERK2 makes Tyr185 accessible for subsequent phosphorylation (Canagarajah et al., 1997).

Signaling through MAPK cascades can lead to various different effects, including differentiation and cell division (Robinson and Cobb, 1997), but MAPK pathways are also involved in responding to various stresses. Nuclear targets of MAPKs can be various transcription factors, such as Ste12, c-Jun, Elk-1, and c-Myc (Karin and Hunter, 1995), but targets may also be other protein kinases, such as MAPK-activated protein kinase-2 (Stokoe et al., 1992), proteins including the epidermal growth factor receptor and the Ras exchange factor Sos, and upstream components of the MAPK cascade, such as Raf1 and MEK1 (Whitmarsh and Davis, 1996). Although many MAPK cascades have been

¹ Current address: Laboratoire de Biologie Végétale et Microbiologie, Université de Nice–Sophia Antipolis, Parc Valrose, 06108 Nice Cedex 2, France.

² Current address: Lönholmmsg 3, SE-55450 Jönköping, Sweden.

³ Current address: School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK.

⁴ To whom correspondence should be addressed. E-mail hehi@gem.univie.ac.at; fax 43-1-4277-9546.

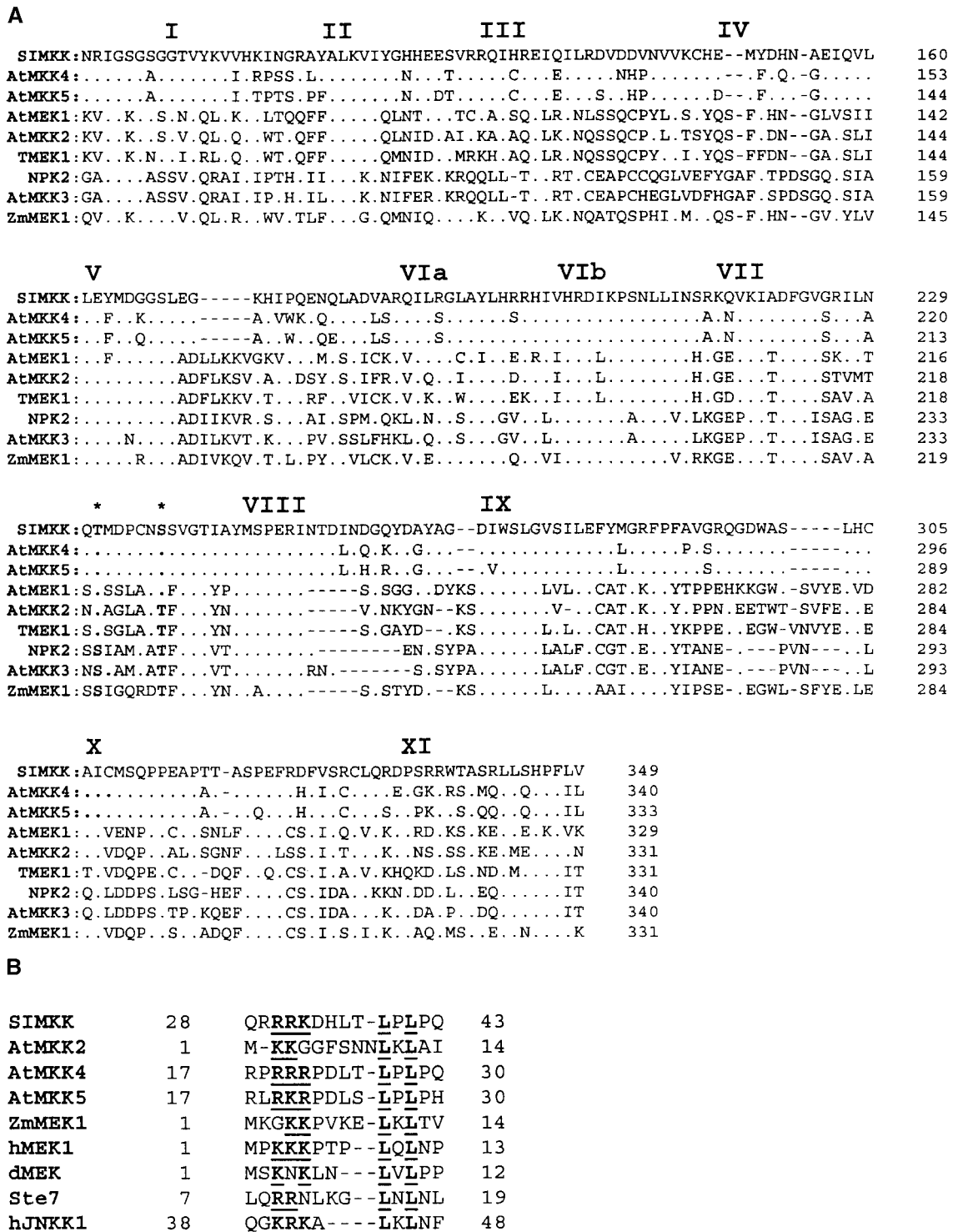


Figure 1. Alfalfa SIMKK Belongs to a Subfamily of Plant MAPKs and Has a Conserved MAPK Docking Motif.

(A) Multiple alignment of the deduced catalytic core protein sequences of alfalfa SIMKK with AtMEK1 (Morris et al., 1997), AtMKK2, AtMKK3, AtMKK4, and AtMKK5 (Ichimura et al., 1998a) from Arabidopsis; TMEK1 (Hackett et al., 1998) from tomato; NPK2 (Shibata et al., 1995) from tobacco; and ZmMEK1 (Hardin and Wolniak, 1998) from maize. Identical amino acids are indicated by dots, gaps are indicated by dashes, and

defined in yeast and animals, their composition in plants remains unclear. Members of the "three-kinase module" can be found in plants (reviewed in Ligterink and Hirt, 2000). Considerable progress has been made in understanding plant MAPKs, and various studies have demonstrated that MAPKs play a role in several aspects of development (Wilson et al., 1997), cell division (Banno et al., 1993; Calderini et al., 1998; Bögre et al., 1999), and the action of hormones, including auxin (Mizoguchi et al., 1994), abscisic acid (Knetsch et al., 1996), gibberellic acid (Huttly and Phillips, 1995), ethylene (Kieber et al., 1993; Chang, 1996), salicylic acid (Zhang and Klessig, 1997), and jasmonic acid (Seo et al., 1995, 1999; Stratmann and Ryan, 1997). MAPKs are also activated by biotic and abiotic stresses, such as cold and drought (Jonak et al., 1996) and wounding (Seo et al., 1995, 1999; Usami et al., 1995; Bögre et al., 1997; Zhang and Klessig, 1998), and during plant-pathogen interaction (Ligterink et al., 1997; Zhang et al., 1998; Romeis et al., 1999).

To date, several MAPKKs have been isolated from different plants, including AtMEK1 (Morris et al., 1997), AtMCK2, AtMCK3, AtMCK4, and AtMCK5 from Arabidopsis (Ichimura et al., 1998a, 1998b), TMEK1 from tomato (Hackett et al., 1998), NPK2 from tobacco (Shibata et al., 1995), and ZmMEK1 from maize (Hardin and Wolniak, 1998). Despite the cloning of these genes, the identity of the pathways on which the kinases function is still unclear, as is which MAPKs are activated by them. A possible MAPK cascade has been defined by the yeast two-hybrid analysis and functional complementation tests of yeast mutants (Mizoguchi et al., 1998). Although AtMEK1, but not another Arabidopsis MAPKK, could phosphorylate and thereby activate AtMPK4 (Huang et al., 2000), it still has to be proven whether these kinases constitute a cascade in plants. Recently, a novel MAPKK was isolated by a two-hybrid screen with a tobacco MAPK, but the recombinant MAPKK could not phosphorylate or activate the respective MAPK (Liu et al., 2000).

We reported previously the identification and characterization of the salt stress-induced MAPK (SIMK) from alfalfa (Munnik et al., 1999). To determine the upstream activator of SIMK, we set up a two-hybrid screen using SIMK as bait. Here, we report the isolation and characterization of the alfalfa MAPKK termed SIMKK (SIMK kinase). SIMKK encodes a functional protein kinase that specifically activates SIMK in vitro and in vivo. Furthermore, SIMKK specifically phosphorylates SIMK on the

BD fusion	AD fusion	β -gal filter assay	Growth on Ade ⁻
SIMK	SIMKK	blue	••
MMK2	SIMKK	white	
MMK3	SIMKK	white	
SAMK	SIMKK	white	
SIMKK	SIMKK	white	
SIMKK	pGAD424	white	
SIMK	pGAD424	white	
pGBT9	SIMKK	white	
pGBT9	SIMK	white	

Figure 2. Two-Hybrid Interaction Assay between Alfalfa MAPKs and SIMKK.

GAL4 activation domain fusions were cotransformed with GAL4 binding domain fusions into PJ69-4A and tested for interaction either in a β -galactosidase (β -gal) filter lift assay or by growth on Ade⁻. The first column shows the GAL4 binding domain (BD) fusions that were analyzed for interaction with the GAL4 activation domain (AD) fusions of the second column. blue indicates interaction in the β -galactosidase filter lift assay. Growth on Ade⁻ indicates interaction.

threonine and tyrosine residues of the activation loop, establishing that SIMKK is a specific dual-specificity protein kinase of SIMK.

RESULTS

SIMKK Interacts with SIMK in the Yeast Two-Hybrid System

In an attempt to isolate the activating kinase of SIMK, the open reading frame of SIMK was fused to the GAL4 binding domain of pGBT9, a bait plasmid. The yeast strain PJ69-4A was transformed with this plasmid and an alfalfa pGAD424 cDNA library expressing the plant proteins as a fusion with the GAL4 activation domain. Approximately 150,000 transformants were screened by plating them on selective medium lacking adenine, leucine, and tryptophan. Potentially positive clones were tested for false-positive clones by plasmid rescue (Robzyk and Kassir, 1992) and retransformation

Figure 1. (continued).

the putative phosphorylation sites are indicated by asterisks. The 11 catalytic subdomains are represented by roman numerals above the respective regions.

(B) Multiple alignment of putative MAPK docking sites in various MAPKs. The MAPK docking site consensus sequence is K/R-K/R-K/R-X(1-5)-L/I-X-L/I (Jacobs et al., 1999). Numbers indicate the first and last residues in each protein and domain. The putative MAPK docking site of SIMKK was compared with the motifs of Arabidopsis AtMCK2, AtMCK4, and AtMCK5 (Ichimura et al., 1998a); maize ZmMEK1 (Hardin and Wolniak, 1998); *Drosophila melanogaster* MEK (Tsuda et al., 1993); human MEK1 (Zheng and Guan, 1993) and JNKK1 (Lin et al., 1995); and *Saccharomyces cerevisiae* Ste7 (Teague et al., 1986).

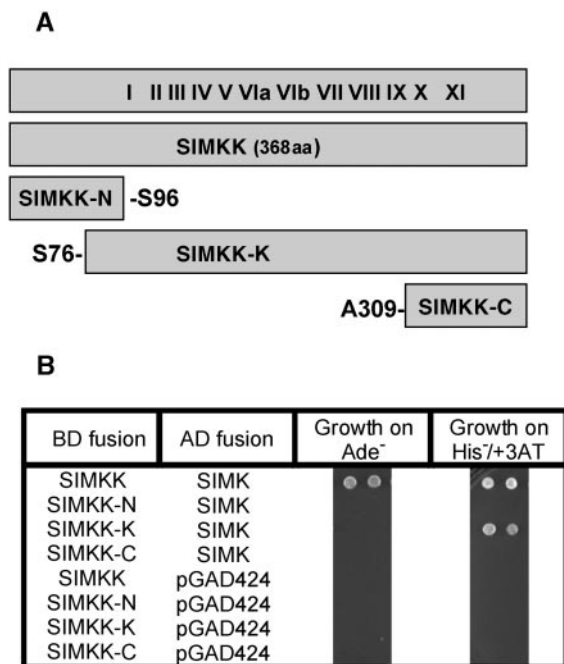


Figure 3. The N-Terminal MAPK Docking Site of SIMKK Is Not Essential for Interaction with SIMK.

(A) SIMKK and three truncated versions of SIMKK were used for yeast two-hybrid interaction analysis with SIMK. The three SIMKK constructs contained either the noncatalytic N-terminal (SIMKK-N) or catalytic C-terminal (SIMKK-C) domain or a truncated version of SIMKK that lacked the N-terminal domain (SIMKK-K). SIMKK-N, SIMKK-K, and SIMKK-C correspond to the coding regions of SIMKK from 1 to 96, 76 to 368, and 309 to 368, respectively.

(B) Two-hybrid interaction assay between SIMK, SIMKK, and truncated versions of SIMKK. The SIMKK constructs were fused to the GAL4 binding domain of pGBT9, transformed into PJ69-4A together with SIMK-pGAD424, and tested for interaction with SIMK by growing transformants on SD/Ade⁻ and SD/His⁻ + 3-AT. The first column shows the GAL4 binding domain (BD) fusions that were analyzed for interaction with the GAL4 activation domain (AD) fusions in the second column. Growth on selective medium indicates interaction.

into PJ69-4A. Transformants were plated on SD/His⁻ + 3-AT (medium lacking histidine but containing 10 mM 3-aminotriazole) and SD/Ade⁻ (medium lacking adenine). Three clones were obtained that could grow on medium lacking either adenine or histidine. Sequencing of the inserts of the isolated plasmids revealed that one of the cDNAs potentially encoded a protein of the MAPKK family and therefore was termed SIMKK. As shown in Figure 1A, the 1.5-kb cDNA contains the entire ORF of SIMKK, coding for a protein of ~42 kD. The protein contains the typical features of MAPKKs: 11 catalytic subdomains and two putative phosphorylation sites (indicated by asterisks in Figure 1A) that are targeted by the upstream activating kinase.

An alignment of the catalytic core of SIMKK with other MAPKKs from plants shows the great homology within this gene family. The greatest sequence homology was observed with AtMKK4 and AtMKK5 from Arabidopsis (Ichimura et al., 1998a).

As shown in Figure 1B, the N terminus of SIMKK also contains a DEJL motif—K/R-K/R-K/R-X(1-5)-L/I-X-L/I—known to function as a MAPK docking site in mammals (Jacobs et al., 1999) and conserved in animal and yeast MAPKKs. The MAPK docking site appears to be conserved in several different plant MAPKKs, because not only SIMKK but also Arabidopsis AtMKK2, AtMKK4, and AtMKK5 and maize ZmMEK1 contain a putative MAPK docking motif.

SIMKK Interacts Specifically with SIMK

To determine whether the interaction between SIMKK and SIMK is specific, we tested whether SIMKK can interact with three other alfalfa MAPKs, MMK2 (Jonak et al., 1995), MMK3 (Bögge et al., 1999), and SAMK (Jonak et al., 1996). For this purpose, MMK2, MMK3, and SAMK were fused to the GAL4 binding domain of pGBT9 and transformed into

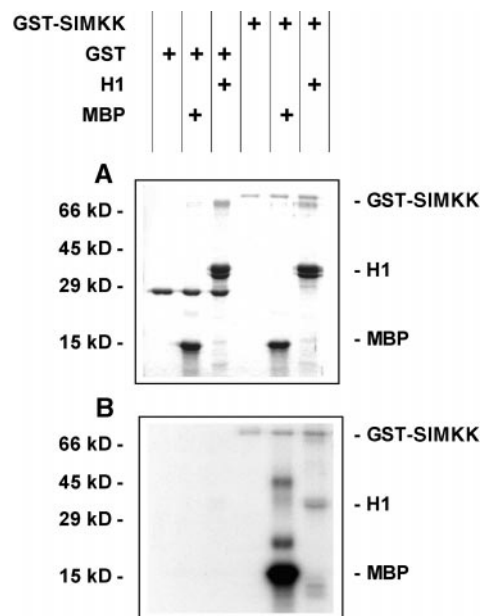


Figure 4. SIMKK Encodes an Active Kinase.

SIMKK was cloned into pGEX-3x and expressed as a GST fusion protein. GST was expressed as a control. Affinity-purified GST or GST-SIMKK was incubated in the presence of γ -³²P-ATP with MBP or histone H1 (H1). After SDS-PAGE, the reaction products were analyzed as indicated.

(A) Coomassie blue staining.

(B) Autoradiography.

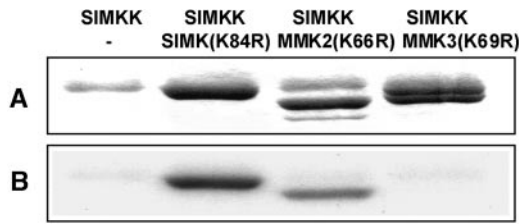


Figure 5. SIMKK Phosphorylates SIMK.

In vitro kinase assays of GST-SIMKK alone and kinase-negative MAPKs as substrates. GST-SIMKK was incubated in the presence of γ - 32 P-ATP alone or with SIMK(K84R), MMK2(K66R), or MMK3(K69R). After SDS-PAGE, the reaction products were analyzed as indicated.

(A) Coomassie blue staining.

(B) Autoradiography.

the yeast strain PJ69-4A, which already contained SIMKK-pGAD424. Colonies were tested for growth on Ade⁻ and for interaction in a β -galactosidase filter lift assay. SIMK-pGBT9 was used as a positive control, and the empty vector pGAD424 was used as a negative control. As shown in Figure 2, only cells cotransformed with SIMK-pGBT9 and SIMKK-pGAD424 were able to grow on Ade⁻ and interacted in the β -galactosidase filter lift assay.

N-Terminal MAPK Docking Site of SIMKK Helps but Is Not Essential for Interaction with SIMK

The noncatalytic N terminus of *Xenopus* MAPKK XMEK is important for interaction with the respective frog MAPK (Fukuda et al., 1997). Docking sites for ERK/MEK and JNK/JNKK interaction have been defined (Xia et al., 1998; Jacobs et al., 1999). One of these interaction motifs is the so-called DEJL motif, which is also found in the N terminus of SIMKK (Figure 1B). To investigate whether the DEJL motif of SIMKK is important for the interaction with SIMK, three truncated forms of SIMKK were produced by polymerase chain reaction (Figure 3A). The three different SIMKK constructs contained either the noncatalytic N-terminal (SIMKK-N) or the C-terminal (SIMKK-C) domain or a truncated version of SIMKK that lacked the N-terminal noncatalytic domain (SIMKK-K). The SIMKK constructs were fused to the GAL4 binding domain of pGBT9 and subsequently transformed into PJ69-4A along with SIMK-pGAD424. The SIMKK constructs were tested for interaction with SIMK by growing transformants on Ade⁻ and His⁻ + 3-AT plates. Full-length SIMKK was used as a positive control, and the empty vector pGAD424 was used as a negative control. In addition, SIMKK-N, SIMKK-K, and SIMKK-C were also fused to the GAL4 binding domain and tested in combination with the empty vector pGAD424 for autoactivation. PJ69-4A cells that were cotransformed with SIMKK-pGBT9 and SIMK-

pGAD424 could grow on Ade⁻ or His⁻ + 3-AT. In contrast, PJ69-4A cells transformed with SIMK-pGAD424 and either SIMKK-N-pGBT9 or SIMKK-C-pGBT9 could not grow under these conditions. On the other hand, PJ69-4A cells transformed with SIMK-pGAD424 and SIMKK-K could still grow on His⁻ + 3-AT, but they lost their ability to grow on Ade⁻ (Figure 3B). These results indicate that the N-terminal extension of SIMKK alone is not sufficient for interaction with SIMK. However, because deletion of the N terminus of SIMKK decreases the interaction with SIMK, the N terminus still contributes to the interaction of SIMKK with SIMK.

SIMKK Encodes a Functional Protein Kinase

To demonstrate that SIMKK encodes a functional protein kinase, we cloned SIMKK into pGEX-3x and produced a bacterially expressed glutathione S-transferase (GST)-SIMKK fusion protein. The affinity-purified GST-SIMKK protein (Figure 4B) was used for in vitro kinase assays with myelin basic protein (MBP) and histone H1 as substrates (Figure 4A). SIMKK could autophosphorylate (Figure 4B) and phosphorylate MBP and histone H1, although it showed a preference for MBP (Figure 4B). To exclude the possibility that contaminating protein kinases were present in the affinity-purified GST-SIMKK fraction, affinity-purified GST was also prepared; however, it showed neither autophosphorylation nor protein kinase activity toward MBP or histone H1 (Figure 4B). These data show that SIMKK encodes an active protein kinase that can phosphorylate MBP.

SIMKK Phosphorylates SIMK in Vitro

To determine whether SIMKK recognizes SIMK as a substrate in vitro, we expressed SIMK into pGEX-3x; it was expressed as a GST fusion protein in *Escherichia coli*. When affinity-purified GST-SIMK protein was incubated with GST-SIMKK in the presence of γ - 32 P-ATP, an increase of the 32 P-labeled SIMK was observed (data not shown), indicating that SIMKK can phosphorylate SIMK in vitro. To discriminate between SIMK autophosphorylation and labeling by SIMKK, we produced a kinase-negative form of SIMK by in vitro mutagenesis, replacing the lysine residue K84 with arginine. MMK2 and MMK3, two other MAPKs from alfalfa, were also mutated, at positions K66 and K69, respectively. SIMK(K84R), MMK2(K66R), and MMK3(K69R) were cloned into pGEX-3x and expressed as GST fusion proteins (Figure 5A). The affinity-purified GST-SIMK(K84R), GST-MMK2(K66R), and GST-MMK3(K69R) were first tested for their kinase activities with MBP as substrate. In contrast to the wild-type GST fusion proteins of SIMK, MMK2, and MMK3 (Jonak et al., 1995), the mutant versions of these kinases showed no autophosphorylation and no phosphorylation of MBP (data not shown). When used as a substrate for

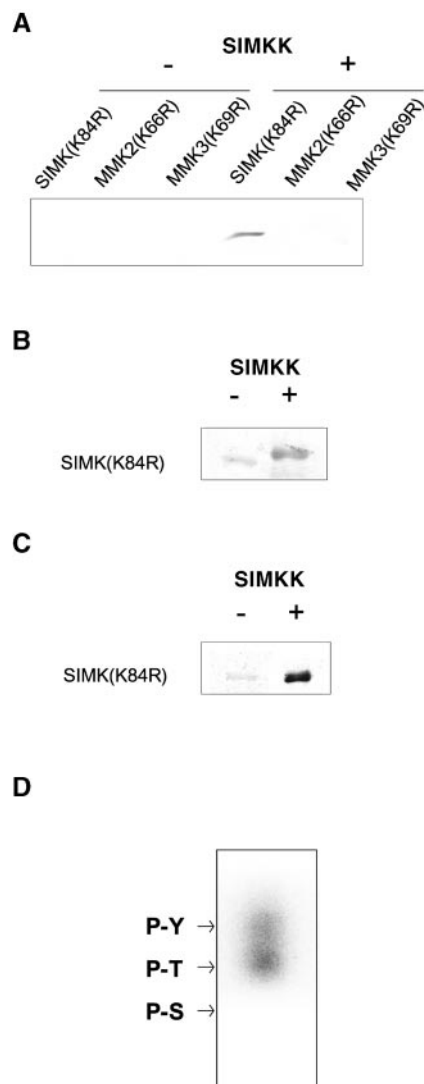


Figure 6. SIMKK Phosphorylates SIMK at the Threonine and Tyrosine Residues of the Activation Loop.

For phosphorylation analysis, the kinase-inactive MAPKs SIMK(K84R), MMK2(K66R), and MMK3(K69R) were incubated in the presence of ATP without (–) or with (+) SIMKK.

(A) Phosphorylation of the TEY motif of the MAPKs was subsequently analyzed by immunoblotting with a pTEpY phosphospecific antibody.

(B) to (D) Phosphorylation of the TEY motif of SIMK(K84R) was analyzed by immunoblotting with a phosphotyrosine-specific antibody **(B)**, immunoblotting with a phosphothreonine-specific antibody **(C)**, and thin-layer chromatography of the phosphorylated amino acids **(D)**. P-Y, P-T, and P-S indicate the mobility of phospho-tyrosine, phospho-threonine, and phospho-serine, respectively.

in vitro kinase assays with SIMKK, SIMKK strongly phosphorylated GST-SIMK(K84R) and to a lesser extent phosphorylated GST-MMK2(K66R) (Figure 5B).

SIMKK Phosphorylates SIMK on Both the Threonine and Tyrosine Residues of the Activation Loop

MAPKs are dual-specificity protein kinases that activate MAPKs by phosphorylating both the threonine and tyrosine residues of the TXY motif in the activation loop between subdomains VII and VIII. To determine whether SIMKK acts as a dual-specificity kinase and whether this phosphorylation is specific for SIMK, we performed a series of immunoblotting experiments with different antibodies.

For phosphorylation analysis of the MAPKs, the kinase-negative GST fusion proteins of SIMK, MMK2, and MMK3 were incubated with or without GST-SIMKK. The phosphorylation of the TEY motif subsequently was analyzed by immunoblotting the MAPKs with a set of three antibodies. The monoclonal pTEpY-specific antibody selectively recognizes only those MAPKs that carry phosphorylated threonine and tyrosine residues at the TEY motif of the activation loop. After incubating SIMK(K84R), MMK2(K66R), and MMK3(K69R) in the absence of SIMKK, the pTEpY antibody detected no signal (Figure 6A), demonstrating the inability of the mutated kinases to autophosphorylate at the TEY motif. After incubation of the MAPKs with SIMKK, the pTEpY antibody exclusively decorated SIMK(K84R) (Figure 6A). These data show that SIMKK is a dual-specificity kinase that specifically phosphorylates the TEY motif of SIMK.

To demonstrate that the phosphorylation of SIMK by SIMKK occurs on both the tyrosine and threonine residues, we analyzed phosphorylated GST-SIMK(K84R) by immunoblot analysis with either a phosphotyrosine or a phosphothreonine antibody. Increases in both tyrosine (Figure 6B) and threonine (Figure 6C) phosphorylation of GST-SIMK(K84R) were observed after incubation with SIMKK. One-dimensional phosphopeptide analysis also indicated that SIMKK phosphorylates SIMK(K84R) on both threonine and tyrosine (Figure 6D), confirming that SIMKK acts as a dual-specificity protein kinase of SIMK.

In Vivo Activation of SIMK, but Not MMK2 or MMK3, by SIMKK

To determine whether SIMKK can activate SIMK in vivo, we coexpressed SIMK and SIMKK in parsley protoplasts. Protein extracts from transformed protoplasts were produced, and SIMK-hemagglutinin (HA) was immunoprecipitated with an anti-HA antibody. SIMK activity was determined by in vitro kinase assays with MBP as a substrate. In cells that were transformed with the vector alone, the HA antibody was unable to precipitate any MBP kinase activity (Figure 7A, lane 1). Ectopically expressed SIMK alone showed rela-

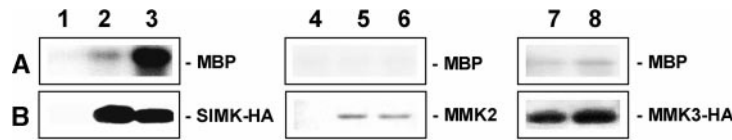


Figure 7. SIMKK Specifically Activates SIMK in Vivo.

Parsley protoplasts were transiently transformed with the empty vector pSH9 (lanes 1 and 4), pSH9-SIMK-HA (lane 2), pSH9-SIMK-HA and pRT101-SIMKK (lane 3), pSH9-MMK2 (lane 5), pSH9-MMK2 and pRT101-SIMKK (lane 6), pSH9-MMK3-HA (lane 7), and pSH9-MMK3-HA and pRT101-SIMKK (lane 8). SIMK-HA, MMK2, MMK3-HA, and SIMKK were expressed under the control of the 35S promoter.

(A) SIMK activity was determined from protein extracts by immunoprecipitation with an HA antibody followed by an *in vitro* kinase assay with MBP as a substrate.

(B) The same extracts as in **(A)** were immunoblotted with either HA or MMK2 antibody.

tively low kinase activity (Figure 7A, lane 2). In contrast, severalfold greater SIMK activity was obtained when cells were cotransformed with both SIMK and SIMKK (Figure 7A, lane 3). To determine whether SIMKK is a specific activator of SIMK, the experiments were repeated with MMK2 (Figure 7A, lanes 5 and 6) and MMK3 (Figure 7A, lanes 7 and 8). In contrast to the SIMK results, no increases in MMK2 or MMK3 activity were observed after cotransformation with SIMKK (Figure 7A, lanes 6 and 8, respectively). The differences in SIMK, MMK2, and MMK3 kinase activities were not caused by different protein amounts, as shown by immunoblotting the protein extracts with either the HA or the MMK2 antibody (Figure 7B); this indicates that SIMKK is a specific activator of SIMK.

SIMKK Enhances the Salt-Induced Activation of SIMK

SIMK is a salt-inducible MAPK that is activated by moderate hyperosmotic stress conditions. Treatment of cells with NaCl at 250 mM activates SIMK, which reaches maximum activity in ~ 10 min (Munnik et al., 1999). To determine whether SIMKK may be involved in mediating the activation of SIMK by salt, parsley cells were transiently transfected with empty vector (Figure 8, lane 1), the SIMK-HA expression vector alone (Figure 8, lanes 2 and 3), or the expression vectors of SIMK-HA and SIMKK (Figure 8, lanes 4 and 5). After immunoprecipitation of SIMK with HA antibody, SIMK kinase activity was determined by *in vitro* kinase assays with MBP as a substrate. When expressed alone, SIMK showed very little kinase activity (Figure 8A, lane 2) and was only slightly induced by salt stress treatment (Figure 8A, lane 3). Although SIMK could be activated by coexpression with SIMKK (Figure 8A, lane 4), salt stress enhanced this activation considerably (Figure 8A, lane 5). Immunoblotting the protein extracts with HA antibody (Figure 8B) showed that similar concentrations of SIMK protein were present in the cell extracts and cannot explain the differences in SIMK activities observed in the immunokinase assays. Together, these data suggest that SIMKK is involved in mediating the salt-induced activation of SIMK.

DISCUSSION

Unlike animals, plants are sessile organisms. To survive changes in their environment, plants have evolved complex and sophisticated sensing and adaptation systems that allow them to respond and adapt to many stress situations. Such stresses include abiotic factors such as cold, drought, wounding, UV radiation, and osmotic stress and biotic factors such as pathogens. Stress responses are characterized by a complex spatial and temporal pattern of events encompassing immediate early responses, which occur within seconds and minutes, and include the opening of ion channels and the formation of reactive oxygen intermediates. These events are followed by the onset of the transcriptional activation of certain genes and the production of certain plant hormones, such as ethylene and jasmonic acid. Late responses occur within hours and days and include the expression of genes that regulate various metabolic pathways or are involved directly in stress responses (Somssich and Hahlbrock, 1998; Maleck and Dietrich, 1999).

MAPKs play important roles in mediating stress responses in animals and yeast. MAPKs are also activated by different stresses in plants (Jonak et al., 1999). MAPK activation is among the first detectable signaling events. Recently, the SIMK MAPK pathway was identified as being activated by increased salt concentrations (Munnik et al., 1999). In this article, we provide evidence that SIMKK is a specific activator of SIMK. SIMKK was isolated by screening an alfalfa two-hybrid cDNA library with SIMK as bait. We demonstrated that the interaction between SIMKK and SIMK is specific, because no interaction was observed with three other MAPKs. Furthermore, SIMKK is a functional dual-specificity protein kinase that phosphorylates SIMK on both the threonine and tyrosine residues of the activation loop.

In animals, MAPK docking sites can be found in MAPKKs, phosphatases, and MAPK substrates, including MAPK-activated protein kinase-2 and transcription factors. The MAPK docking motif is characterized by a cluster of positively charged amino acids outside of the catalytic domain (Jacobs

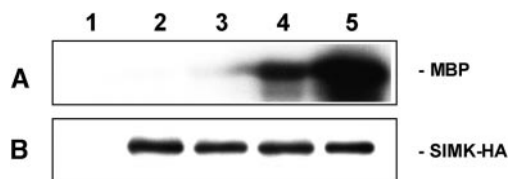


Figure 8. SIMKK Enhances Salt-Induced Activation of SIMK in Vivo.

Parsley protoplasts were transiently transformed with the empty vector pSH9 (lane 1), pSH9-SIMK-HA (lanes 2 and 3), and pSH9-SIMK-HA and pRT101-SIMKK (lanes 4 and 5). SIMK-HA and SIMKK were expressed under the control of the 35S promoter. Cells were treated for 10 min with 250 mM NaCl (lanes 3 and 5).

(A) SIMK activity was determined from protein extracts by immunoprecipitation with an HA antibody followed by an *in vitro* kinase assay with MBP as a substrate.

(B) The same extracts as in **(A)** were immunoblotted with HA antibody.

et al., 1999). Recently, substrate docking sites constituting two negatively charged amino acids were identified within mammalian MAPKs (Tanoue et al., 2000). When substrates, activators, and regulators of ERK2 were mutated in their MAPK docking sites, these proteins lost their ability to coimmunoprecipitate with ERK2. The N-terminal noncatalytic part of SIMKK also contains a putative MAPK docking site. However, interaction between SIMK and SIMKK was still possible in the absence of the docking site, although to a lesser degree. These results suggest that additional, as yet unidentified sites must be involved in the SIMKK-SIMK interaction. Identification of these additional MAPK docking sites should increase our understanding of the mechanism of MAPK functioning.

Recently, SIPKK, a tobacco MAPKK, was isolated and found to interact with SIPK, a tobacco homolog of SIMK (Liu et al., 2000). SIPK and SIPKK interacted in the yeast two-hybrid system, and SIPK could be coimmunoprecipitated with SIPKK from bacterial extracts. A GST fusion protein of SIPKK could phosphorylate MBP *in vitro*, thereby demonstrating that SIPKK is a functional protein kinase. However, SIPKK was unable to phosphorylate or activate SIPK, suggesting that SIPKK is not the activator of SIPK.

MAPKs in all eukaryotes are activated by a post-translational process involving the phosphorylation of a threonine and a tyrosine residue of the so-called TXY motif between subdomains VII and VIII. Phosphorylation of the TXY motif is performed by MAPKKs, which are dual-specificity kinases. Using bacterially expressed GST fusion proteins of SIMKK and SIMK, we demonstrated that SIMKK phosphorylates SIMK on both the threonine and tyrosine residues of the TXY motif. Furthermore, because two other MAPKs from alfalfa were not phosphorylated by SIMKK, SIMKK was shown to be a dual-specificity protein kinase of SIMK.

In vivo activation of MAPKs by a MAPKK has not been shown in plants. One study combined yeast two-hybrid analysis with results obtained from functional complementa-

tion tests of yeast mutants (Mizoguchi et al., 1998). Although these results suggest that the components investigated may be part of a given signaling cascade, complementation occurs under strong selection pressure and therefore might not reflect the situation *in vivo*. Several *in vitro* experiments have suggested that SIMKK is an activator of SIMK, and strong evidence for such a function was obtained in transient coexpression assays. These experiments demonstrated that SIMKK can activate SIMK *in vivo*. Because two other alfalfa MAPKs, MMK2 and MMK3, were not activated by SIMKK under these conditions, SIMKK appears to be a specific activator of SIMK.

SIMK is a salt-inducible MAPK that responds to moderate hyperosmotic conditions (Munnik et al., 1999). To determine whether SIMKK may be involved in mediating the salt-induced activation of SIMK, we performed coexpression experiments with SIMKK and SIMK in the presence and absence of salt stress. Although SIMK was only slightly activated by salt stress, coexpression of SIMKK and SIMK resulted in considerably greater SIMK activation than that achieved by SIMKK alone. These results are consistent with the notion that SIMKK is a specific activator of the salt-inducible MAPK pathway. Because salinity is becoming one of the major limiting factors in agricultural productivity, identification of the molecular mechanisms of salt stress signaling not only serves as an important contribution to basic science but also provides new ways to improve the salt tolerance of vulnerable crop plants.

METHODS

Isolation and Sequence Analysis of Salt Stress-Induced Mitogen-Activated Protein Kinase Kinase (SIMKK)

Full-length SIMK (salt stress-induced mitogen-activated protein kinase [MAPK]) was used as bait to screen a yeast two-hybrid cDNA expression library prepared from suspension-cultured cells from alfalfa (*Medicago sativa*) (Hybri-ZAP; Stratagene). Yeast strain PJ69-4A (James et al., 1996) was transformed with an efficiency of 15,000 colony-forming units per microgram of library DNA, and ~150,000 transformants were screened directly for growth on medium lacking adenine, leucine, and tryptophan (SD/Ade⁻/Leu⁻/Trp⁻). Plasmids of putatively positive clones were rescued and retransformed into yeast. Transformants were plated on medium lacking histidine but containing 10 mM 3-aminotriazole (SD/His⁻ + 3-AT) and on SD/Ade⁻. Positive clones were fully sequenced (T7 sequencing kit; Pharmacia).

Cloning of SIMKK, SIMK, MMK2, MMK3, SAMK, and Truncated SIMKK Forms into pGAD424 and pGBT9

By polymerase chain reaction (PCR), the open reading frame (ORF) of SIMKK was cloned as an SmaI/XhoI fragment into pGAD424 (Clontech, Palo Alto, CA) and pGBT9 (Clontech) by using the following primers: 5' primer (MEK4x4), TATACCCGGGAATGAGGCCGATTCAGCTTC; and 3' primer (MEK4x3), TTTCCCGGGCTCGAGGAC-

GAAGTAAGAAGAAAGTGATCTTG. SIMK and MMK2 were cloned as a BamHI fragment, as described previously (Jonak et al., 1995). MMK3 was cloned as a BamHI fragment by using the following primers: 5' primer (M14x1), AAAAGGATCCGTAACAGAATAATCATG, and 3' primer (M14x2), ATATGGATCCGACCATTGTGCCAAGTC; SAMK was cloned with 5' primer (MMK4x1), TTTTGGATCCCAATGGCC-AGAGTTAACC, and 3' primer (M17x2), TATGGATCCCTAAGCATA-CTCAGGATTG. A truncated SIMKK with a noncatalytic N-terminal domain (SIMKK-N) was cloned with the 5' primer MEK4x4; the 3' primer (MEK4n3) was TTTTGGATCCCTCCGCTTCCGATCCGGTT. Truncated SIMKK lacking the noncatalytic N-terminal domain (SIMKK-K) was cloned with the 5' primer (MEK4k5), TTTTCCCGG-GGAGTCAACAGCTAGTGATT, and the 3' primer MEK4x3. Truncated SIMKK with the C-terminal domain (SIMKK-C) was cloned with the 5' primer (MEK4c5), ATATCCCGGGGACTGCTTCTCCGGAGT-TTAGG, and the 3' primer MEK4x3. After PCR, the ORFs of the protein kinase constructs were fully sequenced.

Cloning of SIMKK, SIMK, MMK2, and MMK3 into pGEX-3x and Expression as Glutathione S-Transferase Fusion Proteins

SIMKK was cloned as an SmaI/SmaI PCR fragment into pGEX-3x (Pharmacia) by using the following primers: 5' primer (MEK4x1), TATACCCGGGGAGATGAGGCCGATTCAGCTTC; and 3' primer (MEK4x2), TTTTCCCGGGTACATTGACGAAGTAAGAAG. SIMK, MMK2, and MMK3 were cloned as a BamHI/BamHI fragment into pGEX-3x by using the ORFs described above. Expression of glutathione S-transferase (GST) fusion proteins in *Escherichia coli* and affinity purification were performed as described (Ausubel et al., 1999). Protein concentrations were determined with a Bio-Rad detection system.

Yeast Strains, Growth Conditions, Transformation, and β -Galactosidase Filter Assay

SD medium was prepared as described (Sherman et al., 1979). Yeast strain PJ69-4A (James et al., 1996) was used for selection on Ade⁻ or His⁻ + 3-AT. Strain HF7C (Feilolter et al., 1994) was taken for the β -galactosidase filter assay. Transformation of yeast cells was performed as described by Schiestl and Gietz (1989). The filter assays were performed as described by Breeden and Nasmyth (1985).

In Vitro Mutagenesis

To produce kinase-negative versions of MAPKs, the conserved lysine residues K84, K66, and K69 of SIMK, MMK2, and MMK3, respectively, were changed to arginine by in vitro mutagenesis using the Altered Sites kit (Promega), as described by the manufacturer. The following oligonucleotides were synthesized for the in vitro mutagenesis reaction: SIMKLOF, 5'-GCATTGCAATCTTCATAACCGCG-ACATGTTC-3'; MMK2LOF, 5'-GCCAATCTTCCTAATGGCGAC-3'; and MMK3LOF, 5'-CGCCAATCTTCCTATCGCAACG-3'.

In Vitro Phosphorylation Assays

GST fusion proteins were incubated in different combinations for 30 min at room temperature in 20 mM Hepes, pH 7.4, 15 mM MgCl₂, 5

mM EGTA, 1 mM DTT, 10 μ M ATP, and 2 μ Ci of γ -³²P-ATP. The molar ratio of enzyme to substrate was 1:5. The reaction volume was 20 μ L. The reaction was stopped by adding 5 \times SDS sample buffer and heating for 5 min at 95°C. Samples were either frozen at -20°C or analyzed directly by SDS-PAGE on 10% gels.

Protein Blotting and Immunodetection of the Phosphorylated GST-MAPKs

After separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by tank blotting (overnight at 30 mA; Bio-Rad). The transfer buffer contained 50 mM boric acid and 50 mM Tris base. For identification of phosphorylated MAPKs, the membranes were washed for 5 min in TBS-Tween (0.01 M Tris, pH 8.0, 0.15 M NaCl, and 0.05% Tween 20) and blocked for 20 min in TBS-Tween containing 0.3% fat-free milk powder. After washing three times for 20 min each, blots were incubated for 1.5 hr with biotin-conjugated goat anti-mouse or goat anti-rabbit antibody (1:1000; Sigma) diluted in TBS-Tween. Washing was done three times as described above. Then, blots were incubated in streptavidin-horseradish peroxidase conjugate (1:500) and washed again. The staining reaction was performed in a freshly prepared solution of 3,3'-diaminobenzidine (Sigma) diluted in TBS plus 0.03% hydrogen peroxide. The antibodies used were a monoclonal phospho-MAPK antibody (1:1000; New England Biolabs, Beverly, MA), a monoclonal phosphotyrosine antibody (1:2000; Sigma), and a polyclonal phosphothreonine antibody (1:1000; Zymed, San Francisco, CA).

Phosphoamino Acid Analysis

After in vitro phosphorylation of affinity-purified GST-SIMK protein by GST-SIMK(K84R) in the presence of ³²P- γ -ATP, the reaction was stopped by protein precipitation with 20% trichloroacetic acid in the presence of 50 μ g of BSA. After exhaustive washing with 10% trichloroacetic acid, the proteins were hydrolyzed under argon with 6 N HCl at 110°C for 1 hr. The HCl was eliminated by evaporation under vacuum and successive washes with water. Amino acids were resuspended in chromatography solvent (isobutyric acid/0.5 M NH₄OH, 5:3 [v/v]), and phosphoamino acid composition was resolved by autoradiography after one-dimensional chromatography on cellulose plates.

Transient Expression Assays

The ORFs of SIMK and MMK3 were fused to the C-terminal triple hemagglutinin (HA) epitope and cloned as a NcoI/BamHI fragment into pSH9 (Holtorf et al., 1995). The ORFs of SIMKK and MMK2 were cloned as XhoI/XbaI and EcoRI/KpnI fragments into pRT101 (Töpfer et al., 1987).

Transient expression and cotransformation experiments were performed with protoplasts from suspension-cultured parsley cells. Protoplasts were prepared as described (Dangl et al., 1987). Fifteen to 30 μ g of plasmid DNA was used for cotransformations; a 35S β -glucuronidase reporter construct (Holtorf et al., 1995) was used to evaluate the transformation efficiency. Experiments were performed at least three times with samples from different protoplast preparations.

Immunokinase Assays

Extracts were prepared from transformed protoplasts 12 to 16 hr after transformation. Protein extracts were prepared as described (Jonak et al., 1996). Protoplast extracts, containing 150 μg of total protein, were immunoprecipitated with either 5 μL of HA antibody (BABC0, Richmond, CA) or 2 μL of MMK2 antibody (5 μg of protein A-purified antibody) (Jonak et al., 1996) and 20 μL of protein G- or protein A-Sepharose beads (suspended in 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% Tween 20, 5 mM NaF, 10 $\mu\text{g}/\text{mL}$ leupeptin, and 10 $\mu\text{g}/\text{mL}$ aprotinin) for 2 hr at 4°C. The beads were washed three times with buffer I (20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, and 1% Triton X-100), once with the same buffer but containing 1 M NaCl, and once with kinase buffer (20 mM Hepes, pH 7.5, 15 mM MgCl_2 , 5 mM EGTA, and 1 mM DTT). Kinase reactions of the immunoprecipitated proteins were performed in 10 μL of kinase buffer containing 1 mg/mL myelin basic protein (MBP), 0.1 mM ATP, and 2 μCi of γ - ^{32}P -ATP. The protein kinase reactions were performed at room temperature for 30 min. The reactions were stopped by the addition of 5 \times SDS sample buffer. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

Protoplast extracts containing SIMK-HA, MMK2, and MMK3-HA were immunoblotted either with HA antibody, as recommended by the manufacturer (BABC0), or with MMK2 antibody, as described (Jonak et al. 1996).

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SIMKK, a Mitogen-Activated Protein Kinase (MAPK) Kinase, Is a Specific Activator of the Salt Stress –Induced MAPK, SIMK

Stefan Kiegerl, Francesca Cardinale, Christine Siligan, Andrea Gross, Emmanuel Baudouin, Aneta Liwosz, Staffan Eklöf, Sandra Till, Laszlo Bögre, Heribert Hirt and Irute Meskiene

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