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Salicylic Acid Activates a 48-kD MAP Kinase in Tobacco

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The involvement of phosphorylation/dephosphorylation in the salicylic acid (SA) signal transduction pathway leading to pathogenesis-related gene induction has previously been demonstrated using kinase and phosphatase inhibitors. Here, we show that in tobacco suspension cells, SA induced a rapid and transient activation of a 48-kD kinase that uses myelin basic protein as a substrate. This kinase is called the p48 SIP kinase (for SA-induced protein kinase). Biologically active analogs of SA, which induce pathogenesis-related genes and enhanced resistance, also activated this kinase, whereas inactive analogs did not. Phosphorylation of a tyrosine residue(s) in the SIP kinase was associated with its activation. The SIP kinase was purified to homogeneity from SA-treated tobacco suspension culture cells. The purified SIP kinase is strongly phosphorylated on a tyrosine residue(s), and treatment with either protein tyrosine or serine/threonine phosphatases abolished its activity. Using primers corresponding to the sequences of internal tryptic peptides, we cloned the SIP kinase gene. Analysis of the SIP kinase sequence indicates that it belongs to the MAP kinase family and that it is distinct from the other plant MAP kinases previously implicated in stress responses, suggesting that different members of the MAP kinase family are activated by different stresses.

INTRODUCTION

Activation of tobacco defense responses by tobacco mosaic virus (TMV) infection includes both local resistance, manifested as necrotic lesion formation resulting from host cell death at the site of infection (hypersensitive response), and systemic resistance induced in the surrounding and distal uninfected parts of the plant (systemic acquired resistance). Numerous studies have demonstrated that salicylic acid (SA) is an endogenous signal for the activation of several plant defense responses, including the synthesis of pathogenesis-related (PR) proteins (Ryals et al., 1994, 1996; Dempsey and Klessig, 1995). However, many of the components that transduce the signal between SA and *PR* genes remain to be elucidated.

One of the major pathways by which extracellular stimuli are transduced into intracellular responses is the MAP kinase signaling cascade (Herskowitz, 1995; Seger and Krebs, 1995; Vojtek and Cooper, 1995; Kyriakis and Avruch, 1996; Hirt, 1997). The basic module of a MAP kinase cascade is a specific set of three functionally interlinked kinases. Each of the three tiers of kinases contains several members. This multiplicity contributes to the specificity of the transmitted signal (Cano and Mahadevan, 1995; Seger and Krebs, 1995). In addition, the MAP kinase cascade exhibits a very steep stimulus–response curve, indicating that small changes in the level of stimulus can turn a pathway on or off (Huang and Ferrell, 1996). Therefore, the MAP kinase cascade can function as a molecular switch.

MAP kinase is activated by the dual phosphorylation of threonine and tyrosine residues in a TXY motif located between subdomains VII and VIII of the kinase catalytic domain, where X can be Glu (E), Pro (P), or Gly (G). Based on the sequence of this phosphorylation motif, three subfamilies of MAP kinases have been defined. The first subfamily consists of the extracellular signal-regulated protein kinases (ERKs) 1 and 2, which contain a TEY phosphorylation sequence. These proteins were the first members of the mammalian MAP kinase family to be cloned, and they are activated by diverse mitogens that stimulate cell division (Boulton et al., 1990; Seger and Krebs, 1995). The second subfamily consists of the Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), which contain a TPY phosphorylation sequence. The p38 and HOG1-homologous kinases comprise the third subfamily; they contain a TGY phosphorylation sequence. Both JNK/SAPK and p38/HOG1 subfamilies are involved in the responses to different stress signals (Herskowitz, 1995; Kyriakis and Avruch, 1996).

The MAP kinase family is present in a diverse array of organisms, including mammals, Xenopus, Drosophila, yeast, Dictyostelium, and plants. By using polymerase chain reaction (PCR)–based homology cloning, a variety of MAP kinase genes has been isolated from several plant species. These include *MMK1* (also known as *MsERK1* and *Msk7*), *MMK2*, *MMK3*, and *MMK4* from alfalfa (Duerr et al., 1993; Jonak et al.,

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1993, 1995, 1996), *Ntf3*, *Ntf4*, and *Ntf6* from tobacco (Wilson et al., 1993, 1995), *AtMPK1* to *AtMPK7* from Arabidopsis (Mizoguchi et al., 1993, 1994), *D5* (also known as *PsMAPK*) from pea (Stafstrom et al., 1993; Pöpping et al., 1996), *Aspk9* (also known as *AsMAP1*) from oat (Huttly and Phillips, 1995), and *PMEK1* from petunia (Decroocq-Ferrant et al., 1995).

An increasing body of evidence suggests that MAP kinases play important signaling roles in plants (Jonak et al., 1994; Nishihama et al., 1995; Stone and Walker, 1995; Hirt, 1997). Similar to the classical mammalian MAP kinases ERK1 and ERK2, MMK1, AtMPK1, and AtMPK2 are thought to be involved in cell proliferation (Jonak et al., 1993; Mizoguchi et al., 1994). Several other MAP kinases may play roles in plant stress responses. In alfalfa, the MMK4 kinase has been directly linked with cold, drought, and mechanical stresses (Bögre et al., 1996, 1997; Jonak et al., 1996). In addition, transcripts for the Arabidopsis AtMPK3 kinase accumulate after touch, cold, dehydration, and salinity stresses (Mizoguchi et al., 1996). Similarly, mRNA levels of *WIPK*, a MAP kinase homolog, accumulate after wounding (Seo et al., 1995). *WIPK* may encode a 46-kD MAP kinase activity that is rapidly activated by wounding or cutting (Seo et al., 1995; Usami et al., 1995). However, because MAP kinases are activated post-translationally by phosphorylation, further analyses are required to determine whether these elevated transcript levels are responsible for the increases in kinase activity.

In addition, stresses or phytohormones have been shown to induce several kinase activities that are believed to be MAP kinases because they preferentially phosphorylate the myelin basic protein (MBP) and are themselves phosphorylated on a tyrosine residue(s) during activation. These include the tobacco cutting (wounding)-activated p46 kinase (Seo et al., 1995; Usami et al., 1995), the fungal elicitor-activated p47 kinase from tobacco (Suzuki and Shinshi, 1995), and the abscisic acid–activated kinase from barley (Knetsch et al., 1996). In this study, we report the identification and characterization of a 48-kD kinase that is called SIP kinase (for SA-induced protein kinase). Based on sequence analyses of both the purified protein and the encoding gene, this kinase is a new member of the MAP kinase gene family. The p48 SIP kinase was rapidly and transiently activated in tobacco suspension cells by SA and may be involved in the defense response of tobacco to TMV infection. Purification of the SIP kinase has enabled us to rigorously establish that the cloned gene encodes the activated kinase and to conduct a primary characterization of plant MAP kinases.

RESULTS

SA and Its Biologically Active Analogs Activate a 48-kD Protein Kinase

Previous studies have demonstrated that in tobacco, both protein phosphorylation and dephosphorylation are required for the activation of the hypersensitive response and *PR* gene expression in response to TMV infection or SA treatment (Dunigan and Madlener, 1995; Conrath et al., 1997). To search for the kinase(s) involved in the pathway, we used an in-gel kinase activity assay. Various kinase substrates, including casein, MBP, and histone, were imbedded in the separating gel, and kinase activities were tested under different reaction conditions. Treatment of tobacco suspension cells with 250 μM SA activated a 48-kD kinase that efficiently used MBP as an artificial substrate (Figure 1A), whereas the same kinase was not activated by the addition of water (Figure 1B). This kinase is called the p48 SIP kinase. Activation of the p48 SIP kinase was very rapid and transient, peaking within 5 min and returning to basal level in ~45 min, and then decreasing further thereafter (Figures 1A and 1B). The p48 SIP kinase activity in SA-treated cells increased from 15- to 25-fold in different experiments. When casein was used as the substrate, no increases in kinase activities were detected in extracts from SA-treated cells (Figure 1C). The minimum concentration of SA required to activate this p48 kinase was 100 μM under our experimental conditions, with maximum activity obtained at 500 μM (Figure 1D). In all subsequent experiments, 250 μM SA was used because it gave near maximum activation of the SIP kinase.

To assess the biological relevance of SA induction of the p48 SIP kinase, we tested analogs of SA. Biologically active analogs of SA, such as 4-chloroSA (4-CSA) and 5-CSA, which induce enhanced disease resistance in tobacco and *PR* gene expression in both tobacco plants and tobacco suspension cells (Conrath et al., 1995), activated the SIP kinase to extents similar to that of SA (Figure 2). However, both induced a more sustained activation of the SIP kinase than did SA (data not shown). In contrast, the biologically inactive analogs 3-hydroxybenzoic acid (3-HBA) and 4-HBA were poor activators of this kinase (Figure 2).

Activation of the p48 SIP Kinase by SA Is Associated with Phosphorylation of a Tyrosine Residue(s) on the Kinase

The use of MBP as a preferred substrate suggested that the p48 SIP kinase might be a MAP kinase. Another hallmark of MAP kinases is their activation via dual phosphorylation of tyrosine and threonine residues by MAP kinase kinases (Seger and Krebs, 1995). To determine whether the SIP kinase was similarly activated, extracts from SA-treated cells were subjected to immunoblot analysis, using the phosphotyrosine-specific monoclonal antibody 4G10 (Figure 3A). Increases in the amount of a phosphotyrosine-containing 48-kD polypeptide were associated with the SA-mediated activation of the p48 SIP kinase. Furthermore, the transient activation of the p48 kinase after SA treatment (Figure 1A) correlated with a transient increase in the level of a phosphotyrosine-containing 48-kD protein (Figure 3A). These results suggest that the p48 SIP kinase is a MAP kinase.

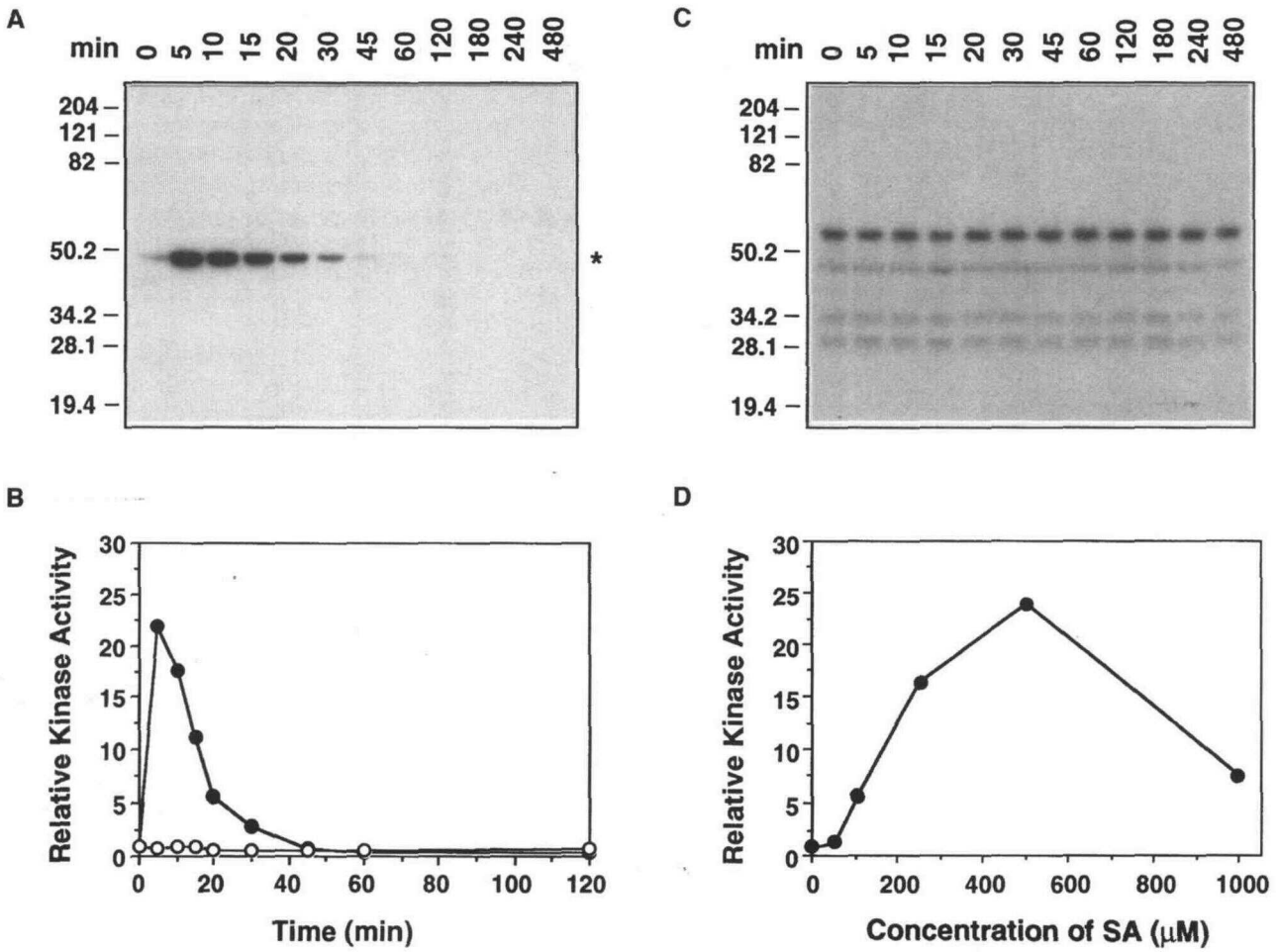

Figure 1. SA Activates a 48-kD Kinase That Preferentially Uses MBP as a Substrate.

(A) Tobacco suspension culture cells were treated with either 250 μM SA or an equal volume of water as a control. Aliquots of the culture were taken at the indicated times, and kinase activity in total cell extracts was tested with an in-gel kinase activity assay using MBP as a substrate. Only the result from SA-treated cells is shown.

(B) The p48 SIP kinase activities (●, SA treated; ○, water treated) were quantitated using a PhosphorImager, and the relative activities were plotted against time. Kinase activities were normalized to the level present at the zero time point, which was given a value of 1.

(C) The kinase activities in the same extracts from SA- or water-treated cells were tested using casein as a substrate. Only the result from SA-treated cells is shown.

(D) The dose response of p48 SIP kinase activation in tobacco suspension cells was plotted. Cells were treated with different concentrations of SA. Aliquots of culture were taken at 0 (before treatment) and 5 min, and p48 SIP kinase activities were assayed using MBP as a substrate. Data presented are fold of induction relative to the basal kinase activity at time zero.

The position of the p48 SIP kinase is indicated by an asterisk in **(A)** and in Figures 2, 3A and 3B, 5B, and 6A to 6C. In **(A)** and **(C)**, molecular mass markers at left are given in kilodaltons. Data presented in Figures 1 to 5 and 8 are from one of at least three independent experiments with similar results; the results presented in Figures 6 and 7 were replicated at least once.

To confirm these results as well as demonstrate the specificity of the 4G10 monoclonal antibody for phosphotyrosine, we coupled immunoprecipitation of phosphotyrosine-containing proteins with the in-gel kinase activity assay. Phosphotyrosine-containing polypeptides were first immunoprecipitated from extracts of SA-treated cells and then subjected to the in-gel kinase activity assay (Figure 3B). Again, the immunoprecipitated 48-kD kinase activity (Figure 3B, lanes 1 to 3) correlated with the p48 SIP kinase activity in the cell extracts (Figure 1A) as well as with the amount of a phosphotyrosine-containing 48-kD protein detected by immunoblot analysis (Figure 3A). To confirm that the antibody specifically

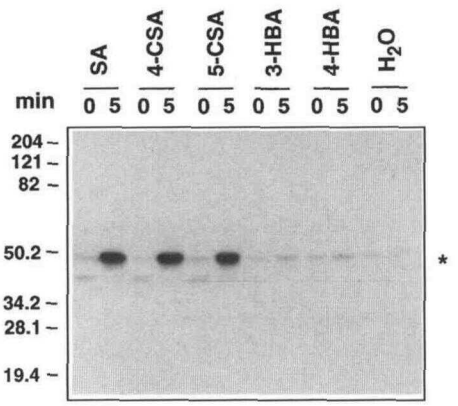

Figure 2. Only Biologically Active Analogs of SA Activate the p48 SIP Kinase.

Cells were treated with water (H_2O), 250 μM SA, or its analogs 4-CSA, 5-CSA, 3-HBA, and 4-HBA. Aliquots of culture were taken at 0 (before treatment) and 5 min. Total cell extracts were prepared, and kinase activities were assayed using MBP as a substrate. Molecular mass markers at left are given in kilodaltons.

recognized a phosphorylated tyrosine residue in the SIP kinase, we used phosphoserine, phosphothreonine, and phosphotyrosine as competitors in the immunoprecipitation experiments described above (Figure 3B, lanes 4 to 6). Only phosphotyrosine inhibited precipitation of the p48 SIP kinase, confirming the specificity of the antibody.

Purification of the Tobacco p48 SIP Kinase

The tobacco p48 SIP kinase was purified to apparent homogeneity by $(NH_4)_2SO_4$ fractionation, ultracentrifugation, and six column chromatography steps (see Methods for details). Its purification from SA-treated tobacco suspension cells is summarized in Table 1, and typical elution profiles of total protein ($A_{280\ nm}$) and kinase activity (counts per minute, with MBP as the substrate) for the six chromatography steps are shown in Figure 4. The 0 to 30% $(NH_4)_2SO_4$ fraction contained almost all of the p48 SIP kinase activity. Several kinases with similar molecular mass but much lower activity, as determined by the in-gel kinase activity assay, were present in 30 to 75% fractions from both SA-treated and control water-treated cell extracts (data not shown). The 0 to 30% $(NH_4)_2SO_4$ fraction was initially fractionated on a Q-Sepharose anion exchange column (Figure 4A). The major peak of kinase activity that eluted from this column corresponded to the p48 SIP kinase, based on its absence in the protein preparation obtained from control tobacco cells treated with water (Figure 5A). Its identity was confirmed by the in-gel kinase activity assay that revealed a major kinase of 48 kD in the Q-Sepharose fractions with high kinase activity (Figure 5B). In all of the subsequent chromatographic steps, only a single major kinase activity peak was detected by the in-solution assay, using MBP as the substrate (Figure 4).

Analysis of the pooled fractions from each purification step by SDS-PAGE revealed that the p48 SIP kinase was purified to apparent homogeneity after the final gel filtration step (Figure 6A, lane 10). More than 25,000-fold purification was required to obtain enzyme of this purity (Table 1). This result indicated that the p48 SIP kinase was ~0.004% of the total soluble protein. The final purified enzyme had a molecular mass of 48 kD (Figures 6A and 6B) and a specific activity of 340 nmol min^{-1} mg^{-1}, which is higher than purified

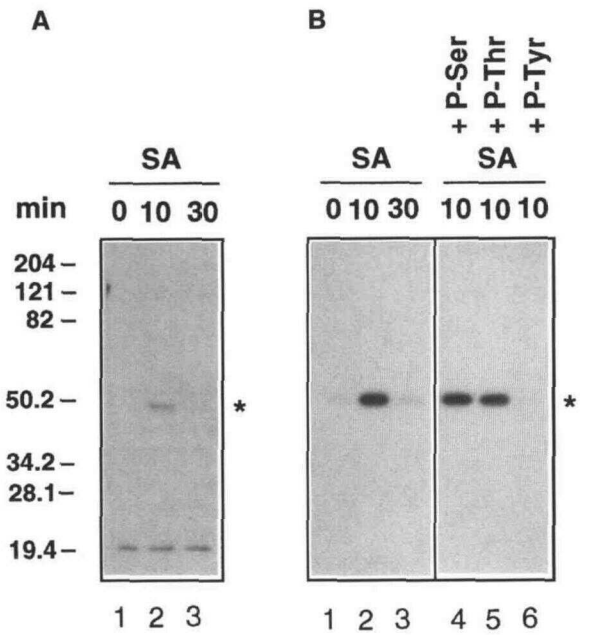

Figure 3. Activation of the p48 SIP Kinase by SA Is Associated with Phosphorylation of Tyrosine Residue(s) in the Kinase.

(A) Extracts prepared from cells sampled before treatment (0 min) or cells treated with 250 μM SA for 10 or 30 min were separated on a 10% SDS–polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes, and phosphotyrosine-containing proteins were detected by immunoblot analysis, using the phosphotyrosine-specific monoclonal antibody 4G10. Molecular mass markers indicated at left are given in kilodaltons.

(B) Phosphorylation of tyrosine residues of the p48 SIP kinase were confirmed, and the specificity of the phosphotyrosine monoclonal antibody 4G10 was tested by immunoprecipitation coupled with the in-gel kinase activity assay. Phosphotyrosine-containing proteins in total cell extracts were immunoprecipitated with 4G10 in the absence of a competitor (lanes 1 to 3) or in the presence of 1 mM phosphoserine (+P-Ser, lane 4), phosphothreonine (+P-Thr, lane 5), or phosphotyrosine (+P-Tyr, lane 6). Kinase activities in the complexes were determined by using the in-gel kinase assay. When the 4G10 antibody was omitted from the precipitation reaction, no kinase activity was precipitated from the protein extracts (data not shown).

Table 1. Purification of the p48 SIP Kinase from Tobacco Suspension Culture Cells

Fraction	Protein (mg)	Total Activity (pmol min^{-1})	Specific Activity (pmol min^{-1} mg^{-1})	Recovery (%)	Purification (fold)
Total extract[a]	1,600	20,943	13	100	1
30% $(NH_4)_2SO_4$	149	17,082	115	82	9
130,000*g*	116	16,582	143	79	11
Q-Sepharose	12	13,950	1,163	67	89
Phenyl–Sepharose HP	1.482	8,072	5,447	39	416
MonoQ HR 5/5	0.572	7,231	12,642	35	965
MBP–Sepharose	0.065[b]	6,870	105,692	33	8,068
Poly-L-lysine–agarose	0.030[b]	5,094	169,800	24	12,962
Superdex 200 HR 10/30	0.005[b]	1,698	339,600	8	25,924

[a] The starting total extract was prepared from 200 g of cells treated with SA for 5 min.
[b] The amount of protein was estimated by comparison with known standards on a Coomassie blue–stained SDS–polyacrylamide gel.

MAP kinases obtained from most other sources (Childs and Mak, 1993a, 1993b). To ensure that the 48-kD band detected on the Coomassie blue–stained SDS–polyacrylamide gels corresponded to the p48 SIP kinase, several precautions were taken. In addition to demonstrating that the p48 SIP kinase activity is present only in protein preparations from SA-treated tobacco cells, we followed the purification of both the 48-kD band (detected by Coomassie blue staining of SDS–polyacrylamide gels) and the p48 SIP kinase (monitored by the in-gel activity assay) through the last three steps of purification (data not shown). Their copurification strongly argues that they are the same protein. Moreover, because the activation of MAP kinases requires phosphorylation of a tyrosine residue in the TXY motif between subdomains VII and VIII of the kinase catalytic domain, the presence of a phosphorylated tyrosine residue in the 48-kD protein would provide further evidence that it is a MAP kinase. Immunoblot analysis of pooled fractions from each purification step, using an anti-phosphotyrosine monoclonal antibody, indicated that the 48-kD polypeptide was heavily phosphorylated on a tyrosine residue(s) (Figure 6C). Furthermore, the enrichment of tyrosine-phosphorylated 48-kD protein correlated with the purification of p48 SIP kinase activity. Thus, we conclude that the purified 48-kD protein is the p48 SIP kinase described above.

Characterization of the p48 SIP Kinase

Because of the tremendous loss of kinase activity during the concentration process and gel filtration chromatography step, the partially purified p48 SIP kinase from the poly-L-lysine-agarose chromatography step was used for characterization. This partially purified p48 SIP kinase preparation did not contain other contaminant kinases and therefore allowed the characterization of the p48 SIP kinase by the in-solution kinase assay. All further characterization of the p48 SIP kinase, described in Figures 7 and 8, used this partially purified enzyme.

The native molecular mass, as calculated from gel filtration fast protein liquid chromotography, was ~50 kD (Figure 4F). This is in good agreement with the 48 kD estimated by using SDS-PAGE. This result indicates that the p48 SIP kinase is a monomeric enzyme, as are all the other MAP kinases.

The partially purified p48 SIP kinase had a very broad optimum pH (data not shown). Even at a pH of 5.5 or 10, it retained >50% of its activity. The enzyme exhibited an absolute requirement for Mg^{2+}; Mn^{2+} could not substitute for Mg^{2+} (data not shown). Kinase activity was strongly stimulated by $MgCl_2$ up to a total concentration of 2 mM and then decreased slightly as the concentration of $MgCl_2$ increased. Stimulation by Mg^{2+} in excess of the amount necessary for the formation of a stable Mg^{2+}–ATP complex suggests an additional function for this metal ion. The purified p48 SIP kinase could not use Mg^{2+}–GTP as a phosphate (data not shown). The absolute K_m and V_{max} of the enzyme for Mg^{2+}–ATP was estimated to be 24 μM and 0.39 μmol min^{-1} mg^{-1}, respectively. Of the proteins tested for their ability to serve as in vitro substrates, only MBP proved to be a good phosphate acceptor; histone was weakly phosphorylated (Figure 7A). The extremely weak phosphorylation of casein detected in the in-solution assay, using a phosphocellulose filter (Figure 7A), may have been due to impurities in the casein preparation because no phosphorylated band could be detected when the same reaction mixtures were subjected to SDS-PAGE (data not shown). The absolute K_m for MBP was 0.19 mg/mL. Phosphoamino acid analysis using two-dimensional thin-layer electrophoresis demonstrated that only threonine residues were phosphorylated on MBP (Figure 7B). In contrast, phosphorylation of histone occurred on both serine and threonine residues (data not shown).

The SIP kinase was very sensitive to the general kinase inhibitors K252a and staurosporine, with an IC_{50} of 12 and 60

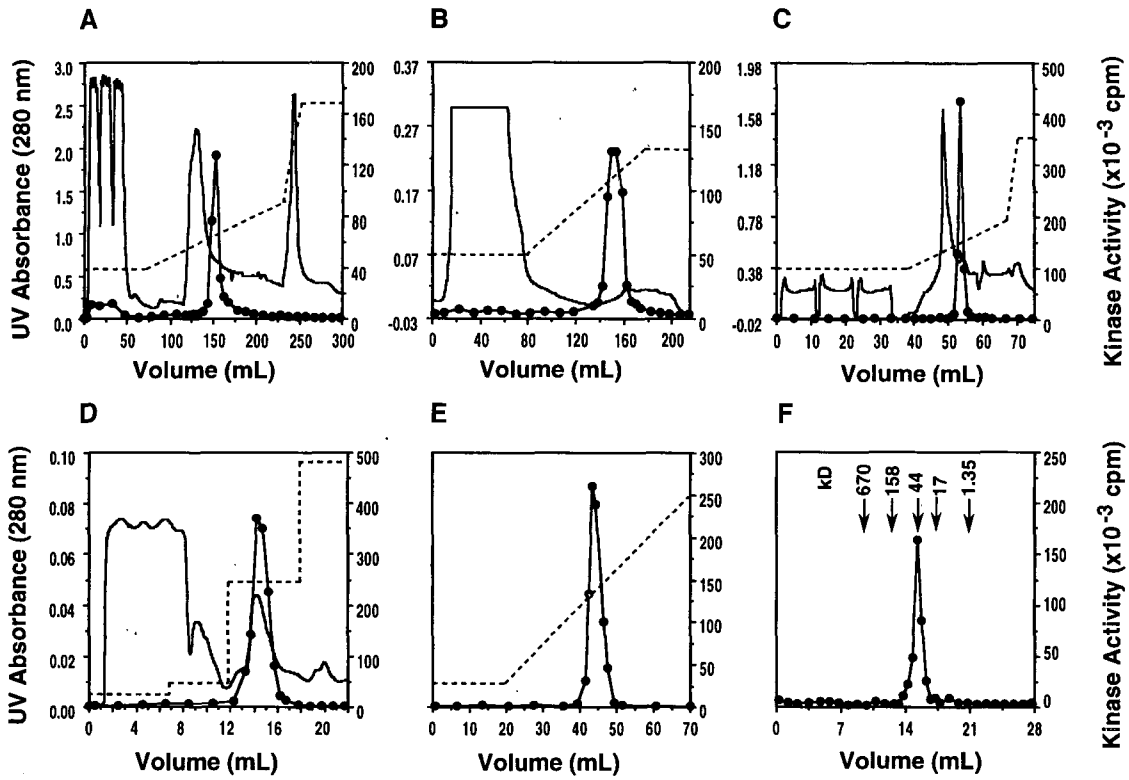

Figure 4. Elution Profiles of Protein Concentration ($A_{280\ nm}$) and Kinase Activity from Each Chromatography Step.

(A) Q-Sepharose anion exchange column.
(B) Phenyl–Sepharose HP hydrophobic interaction column.
(C) MonoQ HR 5/5 anion exchange fast protein liquid chromatography column.
(D) MBP–Sepharose affinity column.
(E) Poly-L-lysine–agarose column.
(F) Superdex 200 HR 10/30 gel filtration fast protein liquid chromotography column. The position of the molecular mass markers in kilodaltons is indicated at the top.
The dashed lines indicate the NaCl gradient profiles (as described in Methods), except in **(B)**, where it represents the ethylene glycol gradient. The kinase activity (●) was determined by the in-solution kinase assay, with MBP as substrate.

nM, respectively. In contrast, a variety of other kinase inhibitors, including the calcium-dependent protein kinase inhibitor EGTA, the tyrosine kinase inhibitor genistein, the protein kinase A peptide inhibitor TTYADFIASGRTGRRNAIHD, and the protein kinase C peptide inhibitor RFARKALRQKNVHEVKN, had no effect on its activity.

Phosphorylation of Both Tyrosine and Serine/Threonine Residues Are Required for p48 SIP Kinase Activity

To assess whether phosphorylation is required for p48 SIP kinase activation, we treated the kinase with either the tyrosine-specific protein phosphatase YOP or the serine/threonine-specific protein phosphatase PP1. Both phosphatases inactivated the SIP kinase (Figure 8). Furthermore, the inactivation of the SIP kinase by PP1 and YOP, respectively, could be prevented by the addition of okadaic acid, a PP1 inhibitor, or Na_3VO_4, a tyrosine phosphatase inhibitor. These results confirm that the inactivation of the purified SIP kinase was the result of the presence of these phosphatases.

The p48 SIP Kinase Is a Member of the MAP Kinase Family

The purified p48 SIP kinase shares several characteristics with MAP kinases, including phosphorylation of a tyrosine residue upon activation, preference for MBP as substrate, a monomeric structure, and a molecular mass within the range of 38 to 55 kD. To confirm that it is a MAP kinase, partial amino acid sequence was obtained by microsequencing of

several internal tryptic peptides (Figure 9). The sequences from two of the peptides were then used to design primers, and a fragment of the SIP kinase gene was cloned by reverse transcription–PCR. A full-length cDNA clone, which contains all of the peptide sequences obtained by microsequencing of the purified protein (Figure 9), was obtained by screening of a tobacco cDNA library. Analysis of the SIP kinase sequence shows that it contains all 11 conserved kinase subdomains found in serine/threonine kinases (Hanks and Hunter, 1995) and has the MAP kinase signature phosphorylation motif TXY preceding subdomain VIII (Figure 9). The SIP kinase shares 93% amino acid sequence identity with Ntf4 (Figure 9), a tobacco MAP kinase of unknown function previously isolated by PCR-based homology cloning (Wilson et al., 1995). However, the nucleotide sequence identity of these two genes is only 74%. Although the SIP kinase is definitely a MAP kinase, it differs from all other cloned plant MAP kinases at its N terminus. Furthermore, in conserved subdomain VIII of the kinase catalytic domain, the SIP kinase contains a proline in place of alanine, which is conserved in all of the other MAP kinases cloned in plants as well as in yeast and mammals.

The SIP kinase is clearly distinct from WIPK, another member of the tobacco MAP kinase family that has been implicated in the wounding response (Seo et al., 1995). They share only 73% amino acid sequence identity and 51% nucleotide sequence identity. These results suggest that different MAP kinases are involved in different stress responses in plants.

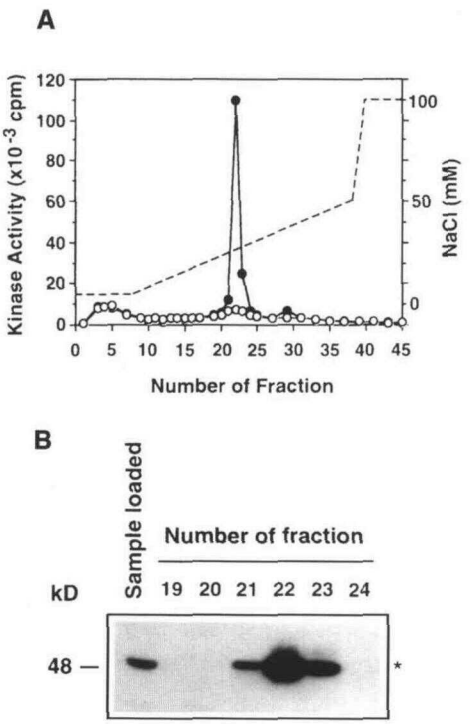

Figure 5. The Major MBP Kinase Peak Eluted from the Q-Sepharose Column Corresponds to the p48 SIP Kinase.

(A) After the ultracentrifugation step, protein samples prepared simultaneously from either SA-treated cells (●) or control water-treated cells (○) were loaded onto a HiTrapQ column connected to a fast protein liquid chromatography system. The column was then eluted with a NaCl gradient, depicted by the dashed lines. The kinase activity was determined by the in-solution kinase assay, with MBP as substrate.

(B) Selected fractions from the HiTrapQ column chromatography of the protein sample from SA-treated cells were assayed by the in-gel kinase method, with MBP as the substrate. The mass of the SIP kinase in kilodaltons is indicated at left.

DISCUSSION

Numerous studies have demonstrated that SA is an endogenous signal for the activation of several plant defense responses, including the synthesis of PR proteins (Klessig and Malamy, 1994; Ryals et al., 1994, 1996). However, many of the components in the SA signal transduction pathway remain to be elucidated. In this study, we demonstrate that a 48-kD protein kinase was rapidly activated by SA treatment of tobacco suspension cells (Figure 1). Several lines of evidence suggest that this p48 SIP kinase belongs to the MAP kinase family. Activation of the SIP kinase was associated with phosphorylation of a tyrosine residue(s) based on both immunoblotting analysis with a phosphotyrosine-specific antibody and a coupled immunoprecipitation–in-gel kinase activity assay (Figure 3). In addition, the SIP kinase preferentially phosphorylated MBP (Figure 1), was unable to use GTP as a phosphate donor, and lacked a calcium requirement for activity (data not shown). Finally, the size of this SIP kinase (48 kD) was in the range of known members of the MAP kinase family from many organisms (38 to 55 kD; Seger and Krebs, 1995).

To confirm that the p48 SIP kinase is indeed a MAP kinase, we purified it and cloned its encoding gene. The SIP kinase sequence exhibits high homology to other cloned MAP kinases, particularly the tobacco Ntf4 MAP kinase (Figure 9). Furthermore, SIP kinase contains the TEY phosphorylation sequence and conserved kinase catalytic domain associated with serine/threonine kinases. However, based on the presence of a unique N-terminal sequence and a proline in place of alanine in the conserved kinase subdomain VIII, SIP kinase appears to be a new member of the MAP kinase family. All other MAP kinases cloned from plants as well as from yeast and mammals have an alanine at this position.

The activation of p48 SIP kinase by SA was rapid and transient, with activity returning to basal levels within 45 min after stimulation. A second addition of SA at up to 4 hr after the initial treatment did not reactivate the SIP kinase, suggesting that the activation pathway was desensitized (data

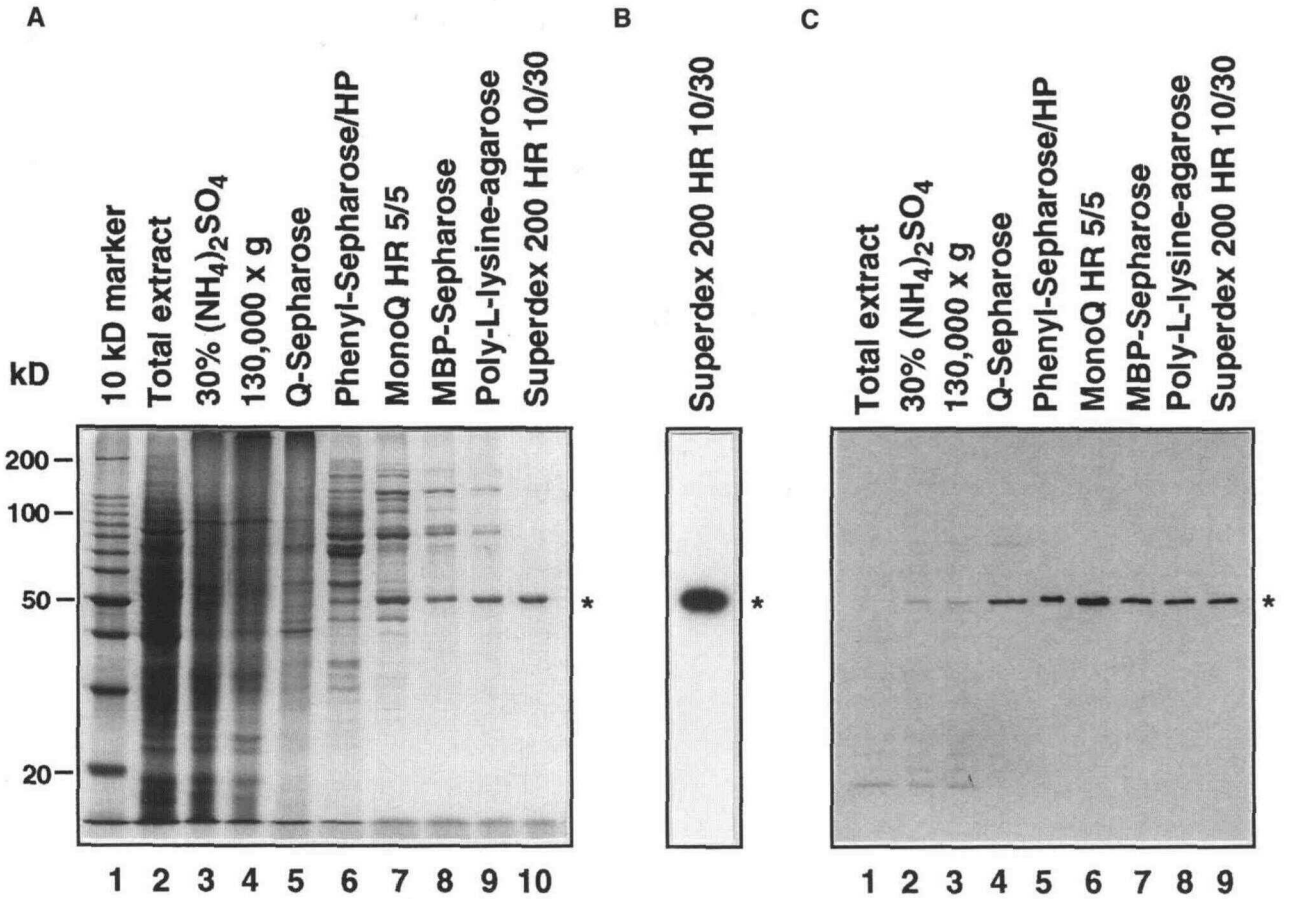

Figure 6. Analyses of Protein Composition, the Size of Purified SIP Kinase, and Phosphotyrosine-Containing Polypeptides after Different Purification Steps.

(A) For protein composition analysis, an aliquot of protein sample from each step of the purification was separated on a 10% SDS–polyacrylamide gel and stained with Coomassie blue. Lane 1 contains the 10-kD size maker (Gibco BRL); lane 2, crude extract (20 μg); lane 3, 0 to 30% $(NH_4)_2SO_4$ fraction (10 μg); lane 4, supernatant after 130,000*g* ultracentrifugation (10 μg); lane 5, pooled fractions from the Q-Sepharose column (5 μg); lane 6, pooled fractions from the phenyl–Sepharose HP column (5 μg); lane 7, pooled fractions from the MonoQ HR 5/5 column (2.5 μg); lane 8, pooled fractions from the MBP–Sepharose affinity column (1 μg); lane 9, pooled fraction from poly-L-lysine–agarose (1 μg); and lane 10, pooled fractions from the Superdex 200 HR 10/30 column (0.5 μg). The position of molecular mass markers in kilodaltons is indicated at left.

(B) To determine the size of the purified enzyme, 1 unit was loaded onto a 10% SDS–polyacrylamide gel embedded with MBP, and the kinase activity was detected by using an in-gel assay.

(C) For analysis of phosphotyrosine-containing proteins, samples from each purification step were subjected to SDS-PAGE, blotted, and probed with anti-phosphotyrosine monoclonal antibody 4G10. Lane 1 contains crude extract (5 μg); lane 2, 0 to 30% $(NH_4)_2SO_4$ fraction (5 μg); lane 3, supernatant after 130,000*g* ultracentrifugation (5 μg); lane 4, pooled fractions from the Q-Sepharose column (5 μg); lane 5, pooled fractions from the phenyl–Sepharose HP column (1 μg); lane 6, pooled fractions from the MonoQ HR 5/5 column (0.5 μg); lane 7, pooled fractions from the MBP–Sepharose affinity column (5 units, ~47 ng); lane 8, pooled fractions from poly-L-lysine–agarose (5 units, ~29 ng); and lane 9, pooled fractions from the Superdex 200 HR 10/30 column (5 units, ~15 ng).

not shown). A negative regulatory loop is likely responsible for the transient nature of the activation and the subsequent refractory period. In mammalian systems, inactivation of MAP kinases is performed by dual specificity protein phosphatases (MAP kinase phosphatases), which simultaneously dephosphorylate both the threonine and tyrosine residues of MAP kinases with high efficiency (Keyse, 1995; Groom et al., 1996). Transcription of these MAP kinase phosphatases is quickly activated by the MAP kinase signaling pathway (Bokemeyer et al., 1996).

It has previously been demonstrated that protein phosphorylation/dephosphorylation events are correlated with

the activation of defense responses in a number of plants (Dietrich et al., 1990; Felix et al., 1991; Viard et al., 1994). For example, activation of the potato *PR-10a* gene requires phosphorylation of the nuclear factor PBF-1 (Després et al., 1995), and protein kinase inhibitors blocked both the fungal elicitor-induced oxidative burst and the H_2O_2-mediated activation of defense genes in soybean suspension cells (Levine et al., 1994). In addition, the formation of TMV-induced lesions in tobacco was prevented by the phosphatase inhibitor okadaic acid (Dunigan and Madlener, 1995). Similarly, phosphatase inhibitors abolished the SA-mediated induction of *PR-1* gene expression in tobacco, whereas kinase inhibitors induced *PR-1* gene expression (Conrath et al., 1997).

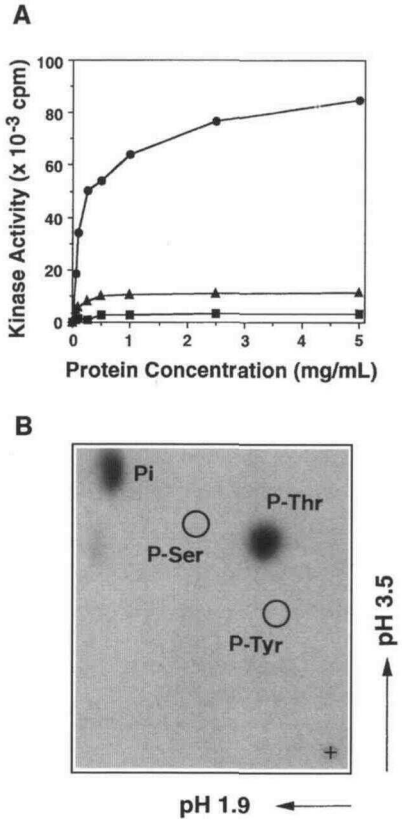

Figure 7. Analyses of the in Vitro Protein Substrate Preference and the Amino Acid Phosphorylated in MBP by the p48 SIP Kinase.

(A) Substrate preference was determined using ~0.5 units of purified enzyme for each reaction. The assay conditions were as described in Methods, except that various concentrations of MBP (●), histone (▲; type III-SS), and casein (■) were used.

(B) To identify the phosphorylated amino acids, MBP was phosphorylated in the presence of γ-^{32}P-ATP with 2 units of purified SIP kinase under standard assay conditions. After acid hydrolysis, the dried pellet was dissolved in 10 μL of a phosphoamino acid standard (1 mg/mL each of L-phosphoserine [P-Ser], L-phosphothreonine [P-Thr], and L-phosphotyrosine [P-Tyr]). The phosphoamino acids were separated by two-dimensional high-voltage thin-layer electrophoresis, as described by Sefton (1996). The circles indicate the positions of phosphoserine and phosphotyrosine as visualized by ninhydrin staining. The labeled amino acids were detected by autoradiography that matched phosphothreonine visualized by ninhydrin staining. The (+) indicates the origin.

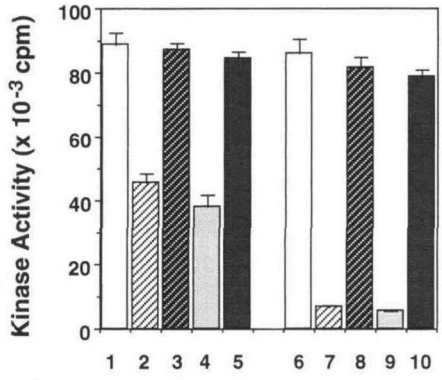

Figure 8. Inactivation of Purified p48 SIP Kinase by Phosphatase Treatment.

Approximately 20 units of the SIP kinase was treated with either the serine/threonine–specific phosphatase PP1 (0.1 units) or the tyrosine-specific protein phosphatase YOP (0.5 units) for 10 min (bars 2 to 5) or 30 min (bars 7 to 10) at 30°C in the presence or absence of a phosphatase inhibitor. Bars 1 and 6 represent the control without phosphatase or the phosphatase inhibitor; bars 2 and 7, PP1; bars 3 and 8, PP1 plus okadaic acid (1 μM); bars 4 and 9, YOP; bars 5 and 10, YOP plus Na_3VO_4 (1 mM). Before assaying for SIP kinase activity, the phosphatase inhibitors were added to all of the reactions to the same final concentrations. Each bar represents the mean of three replicates ± SE.

Because the SIP kinase is activated by SA, which plays an important role in signaling defense responses, it is tempting to speculate that the SIP kinase is also involved in the activation of these defense responses. Interestingly, a 48-kD kinase that preferentially used MBP as an artificial substrate was activated by both SA treatment and TMV infection of tobacco leaves (data not shown). We suspect that this SA/TMV-activated kinase and the SIP kinase from suspension cells are the same. However, further analyses are required to determine the identity of these proteins as well as their association with the SA signal transduction pathway. Our hypothesis that SIP kinase plays a role in SA-mediated *PR-1* gene induction seems to conflict with the finding that phosphatase inhibitors block SA induction of *PR-1* genes (Conrath et al., 1997). However, there is ample precedence for activation of the same pathway by both phosphorylation and dephosphorylation events (Hunter, 1995). A particularly relevant example is the tyrosine kinase c-Src, which activates a MAP kinase cascade (Zheng and Pallen, 1994). c-Src itself is activated by a tyrosine phosphatase (Sun and Tonks, 1994).

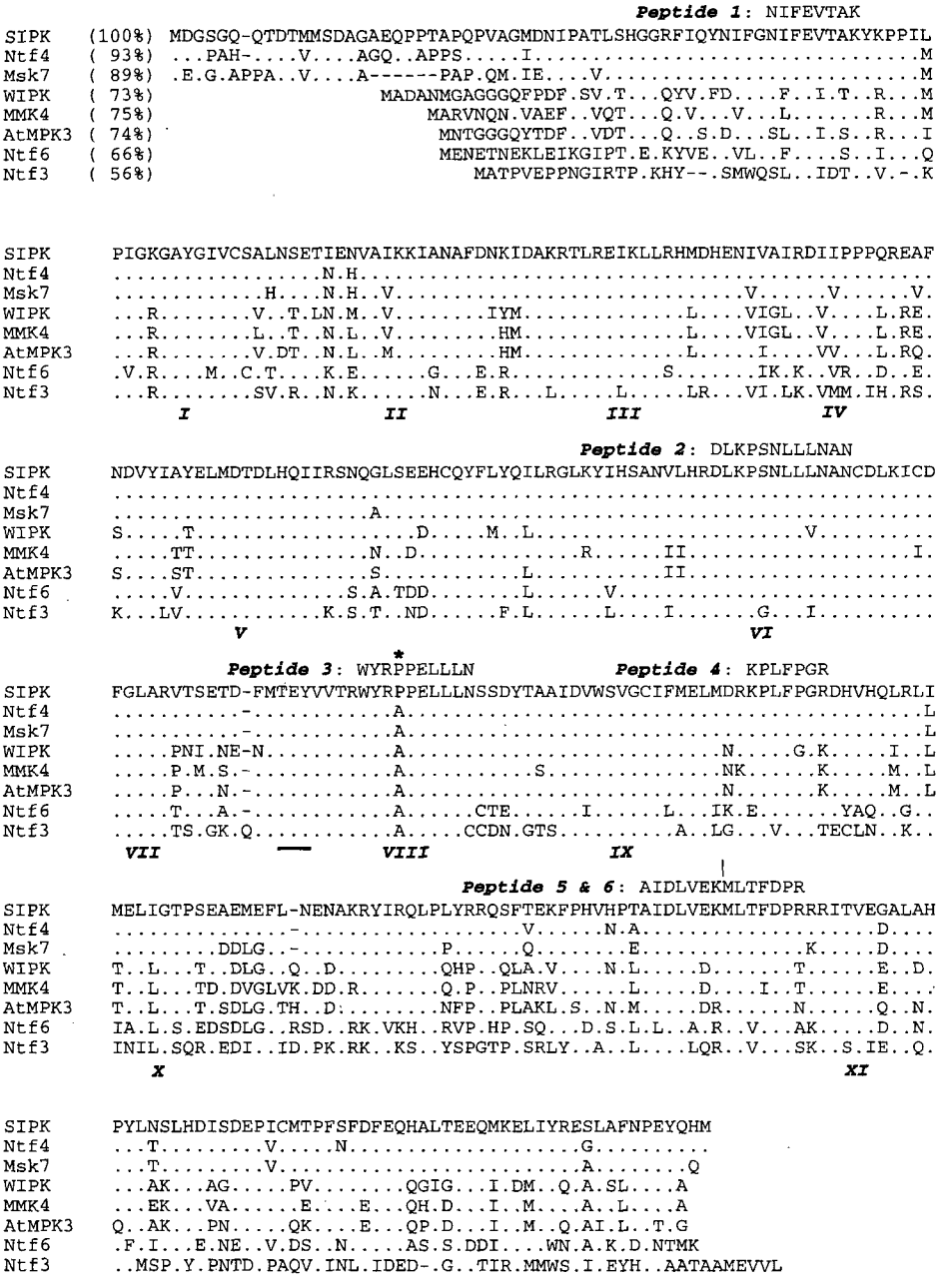

Figure 9. Alignment of the Deduced Amino Acid Sequence of the SIP Kinase (SIPK) with Other Members of the Tobacco MAP Kinase Family as Well as Stress-Related MAP Kinases from Other Plants.

The amino acid sequences of Ntf3, Ntf4, Ntf6 (Wilson et al., 1993, 1995) and WIPK (Seo et al., 1995) from tobacco, MsK7 (Duerr et al., 1993; Jonak et al., 1993) and MMK4 (Jonak et al., 1996) from alfalfa, and AtMPK3 (Mizoguchi et al., 1993) from Arabidopsis were deduced from cDNA sequences. Numbers within parentheses indicate the percentage of identity to the SIP kinase. Dots represent amino acid residues that match the SIP kinase, and dashes indicate gaps introduced to maximize alignment. The conserved TEY phosphorylation motif for MAP kinase is underlined. The unique proline residue in the conserved kinase subdomain VIII of SIP kinase is marked with an asterisk. Roman numerals indicate the 11 major conserved subdomains of serine/threonine protein kinases. The six peptide sequences obtained by microsequencing are shown on top of the SIP kinase sequence. The boundary between peptides 5 and 6 is indicated by a vertical line. The GenBank accession number for the SIP kinase cDNA is U94192.

Whereas protein tyrosine kinases and phosphatases have not been found in plants, several receptor-like serine/threonine kinases have been identified, including those encoded by the disease resistance genes *Pto* (Martin et al., 1993; Scofield et al., 1996; Tang et al., 1996) and *Xa21* (Song et al., 1995). In addition, one of the receptor-like serine/threonine kinases, RLK5 of *Arabidopsis*, has an associated phosphatase, KAPP (Stone et al., 1994). It has been suggested that the receptor-like serine/threonine kinase–phosphatase pairs mimic the receptor tyrosine kinase–phosphatase pairs in animals (Braun and Walker, 1996).

The availability of the purified SIP kinase has allowed the primary characterization of a plant MAP kinase both in its active form and in the absence of other contaminating activities. The purified protein was strongly phosphorylated on a tyrosine residue(s). Moreover, both tyrosine-specific protein phosphatase and serine/threonine–specific protein phosphatase treatments inactivated the kinase activity, suggesting that phosphorylation of these residues was required for SIP kinase activation. The purified p48 SIP kinase had a specific activity of 340 nmol min^{-1} mg^{-1}, with MBP as its phosphate acceptor. This is at least 40- and 200-fold higher than the specific activities of purified bovine brain p42 and p44 MAP kinases, respectively (Childs and Mak, 1993a). These large differences may be due in part to the efficient activation of the SIP kinase by SA and/or the ability of MBP to serve as a better substrate for the SIP kinase. In addition, the high specific activity of the SIP kinase may reflect the purification strategy, which allowed purification of the SIP kinase in 3 days. Rapid purification is critical because the phosphatase inhibitors included in the buffer were unable to completely inhibit all of the phosphatases that can inactivate the SIP kinase.

The purified SIP kinase exhibited an absolute requirement for Mg^{2+} and could barely use Mn^{2+}–ATP as a phosphate donor. This is in contrast to the cloned tobacco MAP kinases Ntf3, Ntf4, and Ntf6 (Wilson et al., 1995). For these three recombinant proteins, Mn^{2+} was a better cofactor than Mg^{2+} in both MBP phosphorylation and autophosphorylation reactions. It is possible that the SIP kinase and the other tobacco MAP kinases, Ntf3, Ntf4, and Ntf6, inherently prefer different metal ions. Alternatively, the basal and activated forms of these kinases may utilize different metal cofactors. Which of these explanations is correct cannot be determined at present because the fusion proteins expressed in *Escherichia coli* were largely in their inactive state because of the lack of upstream activating components, whereas the SIP kinase was purified in an active state. The purified p48 SIP kinase strongly phosphorylated MBP, a preferred substrate of MAP kinases. Histone was weakly phosphorylated, and casein is not a substrate for the SIP kinase. Similar substrate preferences have been reported for the tobacco Ntf3, Ntf4, and Ntf6 MAP kinases expressed in *E. coli* (Wilson et al. 1995) and the murine and sea star MAP kinases (Rossomando et al., 1991).

Two other stress-activated kinases that exhibit characteristics associated with MAP kinases have been described in tobacco. These include the p47 fungal elicitor-activated kinase (Suzuki and Shinshi, 1995) and the p46 cutting-activated kinase (Usami et al., 1995). Because purification of either of these proteins has yet to be reported, they cannot be directly compared with the purified p48 SIP kinase. Thus, it is unclear whether they differ from the SIP kinase in enzymatic properties, such as metal cofactor requirement, K_m and V_{max} values for different substrates, or inhibitor sensitivity. These properties could be used to determine whether they are the same or different MAP kinases. A gene whose transcript is induced by wounding (WIPK) has been isolated from tobacco and proposed to encode the p46 wounding-activated kinase (Seo et al., 1995). If this indeed is the case, then the wounding-activated kinase (which may be the same as the cutting-activated kinase) is different from the SIP kinase, based on a comparison of their amino acid sequences (Figure 9).

Analysis of the phylogenetic tree constructed for all cloned plant MAP kinases suggests that these kinases can be divided into two major groups (Figure 10). One group is represented by the 2,4-D–responsive AtMPK1 and AtMPK2 of Arabidopsis. These kinases may be functionally similar to the mammalian ERK1 and ERK2, which are involved in the cell proliferation in response to mitogens (Mizoguchi et al.,

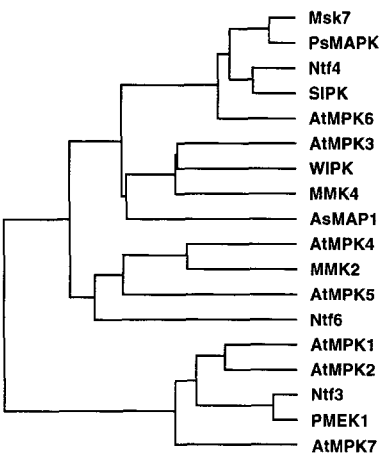

Figure 10. Relationship between All Cloned Plant MAP Kinase Members.

The phylogenetic tree shown as a dendrogram was created by the Clustal method (MegaAlign program; DNAStar, Madison, WI). The deduced amino acid sequences are from cDNA clones of *Msk7* (also termed *MsERK1* or *MMK1*; Duerr et al., 1993; Jonak et al., 1993), *AtMPK1* to *AtMPK7* (Mizoguchi et al., 1993), *PsMAPK* (also called D5; Stafstrom et al., 1993; Pöpping et al., 1996), *Ntf3* (Wilson et al., 1993), *AsMAP1* (Huttly and Phillips, 1995), *PMEK1* (Decroocq-Ferrant et al., 1995), *MMK2* (Jonak et al., 1995), *WIPK* (Seo et al., 1995), *Ntf4* and *Ntf6* (Wilson et al., 1995), and *MMK4* (Jonak et al., 1996) and for the SIP kinase (SIPK; this study).

1994). The other group can be further divided into three subgroups. The subgroup represented by AtMPK3 of Arabidopsis, WIPK of tobacco, and MMK4 of alfalfa appears to be activated by a variety of stresses, such as wounding, touch, cold, drought, and salinity (Seo et al., 1995; Bögre et al., 1996, 1997; Jonak et al., 1996; Mizoguchi et al., 1996). Interestingly, this group is also characterized by increases in their mRNA levels in response to the same stresses. The second subgroup includes Ntf4 and the SIP kinase from tobacco. Its members have N-terminal extensions of ~20 amino acids and, based on results described in this report, may also respond to stresses such as those caused by infection or SA. The function of the third subgroup is currently unknown. However, MMK2 of alfalfa can complement the yeast MPK1 (Jonak et al., 1995), which is required for yeast cell wall integrity at high temperature (Herskowitz, 1995).

In yeast and mammals, multiple MAP kinase families have been identified that participate in different signaling pathways (Cano and Mahadevan, 1995; Herskowitz, 1995). In yeast, for example, at least six independent MAP kinase pathways have been identified (Herskowitz, 1995; Ruis and Schüller, 1995). In human cells, the JNK subfamily of MAP kinases alone consists of at least 10 isoforms that are produced by alternative splicing of transcripts encoded by the *JNK1*, *JNK2*, and *JNK3* genes. The differing abilities of these isoforms to interact with the transcription factors ATF2, Jun, and Elk-1 may provide a mechanism by which various stresses cause the specific activation of different target genes (Gupta et al., 1996). Consistent with the picture emerging from studies with yeast and mammals, our study provides further evidence that different members of the plant MAP kinase family are activated by different stimuli.

METHODS

Treatment of Tobacco Cell Suspension Culture

The cell suspension culture was derived from callus tissue from leaves of *Nicotiana tabacum* cv Xanthi and grown in Murashige and Skoog medium (Gibco BRL, Gaithersburg, MD) supplemented with 1 mg/L α-naphthaleneacetic acid, 0.1 mg/L 2,4-D, 0.1 mg/L benzyladenine, and 3% sucrose. Log phase cells were used ~3 days after a 1:10 dilution. Treatment with salicylic acid (SA) and its analogs was done in the original flasks in the dark to avoid any stresses associated with transfer. At various times, 10 mL of cells (~0.2 to 0.3 g fresh weight of cells) was harvested by filtration. The cells were quickly frozen in liquid nitrogen and stored at −80°C until analysis.

Preparation of Protein Extracts

To prepare extracts from treated cells, cells (~0.2 g) with 2 volumes (w/v) of extraction buffer (100 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL antipain, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 10% glycerol, 7.5% polyvinylpolypyrrolidone) were sonicated twice for 15 sec each, with a W-375 Sonicator (Heat System-Ultrasonics, Inc., Farmingdale, NY) fitted with a microprobe at setting 4 and 80% duty cycle in a 1.5-mL microcentrifuge tube. After centrifugation at 13,000 rpm for 20 min, supernatants were transferred into clean tubes, quickly frozen in liquid nitrogen, and stored at −80°C.

For purification of the p48 SIP kinase (for SA-induced protein kinase), ~200 g of cells treated for 5 min with 250 μM SA was sonicated in 1.5 volumes of extraction buffer (w/v) until all cells were disrupted. After centrifugation at 23,000*g* for 30 min, the supernatant was transferred into clean tubes, quickly frozen in liquid nitrogen, and stored at −80°C.

Protein Concentration Assay

Unless otherwise stated, the concentration of protein extracts was determined using the Bio-Rad protein assay kit with BSA as a standard.

In-Gel Kinase Activity Assay

The in-gel kinase assay was performed as described previously (Zhang et al., 1993). Extracts containing 10 μg of protein were electrophoresed on 10% SDS–polyacrylamide gels embedded with 1 mg/mL casein, 1 mg/mL histone (type III-SS; Sigma), or 0.25 mg/mL of myelin basic protein (MBP) in the separating gel as a substrate for the kinase. After electrophoresis, SDS was removed by washing the gel with washing buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg/mL BSA, 0.1% Triton X-100 [v/v]) three times, each for 30 min at room temperature. The kinases were allowed to renature in 25 mM Tris, pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4, and 5 mM NaF at 4°C overnight with three changes of buffer. The gel was then incubated at room temperature in a 30-mL reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4) with 200 nM ATP plus 50 μCi γ-^{32}P-ATP (3000 Ci/mmol) for 60 min. The reaction was stopped by transferring the gel into 5% trichloroacetic acid (TCA) (w/v)/1% NaPPi (w/v). The unincorporated γ-^{32}P-ATP was removed by washing in the same solution for at least 6 hr with five changes. The gel was dried onto Whatman 3MM paper and exposed to Kodak XAR-5 film. Prestained size markers (Bio-Rad) were used to calculate the size of kinases. Quantitation of the relative kinase activities was done using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA).

Immunoblot Analysis with the Anti-Phosphotyrosine Antibody

Protein extracts or pooled fractions from each purification step were separated on 10% SDS–polyacrylamide gels, and the proteins were transferred to nitrocellulose membrane by semidry electroblotting. The membrane was blocked for 2 hr in TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 0.1 mM Na_3VO_4) with 10% BSA at room temperature and then incubated with the phosphotyrosine-specific monoclonal antibody 4G10 (Upstate Biotechnology Incorporated, Lake Placid, NY) in TBS buffer with BSA for 1 hr. After the blot was incubated with horseradish peroxidase–conjugated secondary antibody, the complexes were visualized using an enhanced chemiluminescence kit (Du Pont), following the manufacturer's instructions.

Immunoprecipitation Kinase Activity Assay

Protein extract (50 μg) with or without phosphoamino acid (1 mM final concentration) competitor was incubated with the 4G10 antibody in immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, 2 μg/mL antipain, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.5% Triton X-100, 0.5% Nonidet P-40) at 4°C for 4 hr on a rocker. About 25 μL packed volume of protein A–agarose was added, and the incubation was continued for another 2 hr. Agarose bead–protein complexes were pelleted by brief centrifugation. After washing with immunoprecipitation buffer three times, 1 × SDS sample buffer was added and boiled for 3 min. After centrifugation, the supernatant fraction was electrophoresed on 10% SDS–polyacrylamide gel, and the in-gel kinase assay was performed.

Purification of the p48 SIP Kinase

All purification procedures were performed in a cold room or a 4°C chamber. Protein extract from ~200 g of SA-treated cells was brought to 30% $(NH_4)_2SO_4$ saturation. After stirring slowly for 30 min, the precipitant was collected by centrifugation at 23,000*g* for 10 min. The pellets were then dissolved in a total of 30 mL of buffer A (25 mM Tris, pH 7.5, 1 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM DTT, 5% glycerol) plus 1 mM phenylmethylsulfonyl fluoride and 5 μg/mL each of antipain, aprotinin, and leupeptin. After centrifugation at 130,000*g* for 1 hr, the S130 supernatant was dialyzed against 1 liter of buffer A for 4 hr in a cold room and loaded onto a 10-mL Q-Sepharose anion exchange column (two tandemly connected 5-mL HiTrapQ columns; Pharmacia) equilibrated with buffer A plus 50 mM NaCl (Figure 4A). After washing with 30 mL of buffer A containing 50 mM NaCl, the column was eluted with a 150-mL linear gradient of 50 to 400 mM NaCl in buffer A. The p48 SIP kinase activity eluted at ~250 mM.

The fractions containing the highest kinase activity (peak fractions) were pooled, adjusted to a final concentration of 300 mM NaCl, and loaded onto a 15-mL phenyl–Sepharose HP hydrophobic interaction column (1.6 × 7.5 cm; Pharmacia) equilibrated with buffer A plus 300 mM NaCl (Figure 4B). The column was washed with 50 mL of buffer A plus 300 mM NaCl and eluted with a 100-mL linear gradient of 0 to 60% ethylene glycol and 300 to 0 mM NaCl in buffer A. The active fractions (eluting at ~40% ethylene glycol) were pooled and diluted with an equal volume of buffer A and then loaded onto a MonoQ HR 5/5 fast protein liquid chromatography anion exchange column (Pharmacia) equilibrated with buffer A plus 100 mM NaCl (Figure 4C). After washing with 5 mL of buffer A plus 100 mM NaCl, the column was eluted with a 30-mL gradient of 100 to 400 mM NaCl in buffer A. The peak fractions containing the p48 SIP kinase (~3 mL) were pooled, adjusted to a final concentration of 10 mM $MgCl_2$, and diluted with an equal volume of buffer B (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.02% Triton X-100).

The above-mentioned sample was then loaded onto an MBP–Sepharose affinity column (1 mL; prepared by coupling MBP to *N*-hydroxysuccinimide–activated Sepharose HP; Pharmacia) equilibrated with buffer B plus 50 mM NaCl (Figure 4D). The column was then step eluted with 5 mL each of buffer B plus 100, 500, or 1000 mM NaCl. The peak fractions containing the p48 SIP kinase were pooled (~2.5 mL), diluted with 3 volumes of buffer B, and loaded onto a 3.5-mL poly-L-lysine–agarose column (0.9 × 5.5 cm; Sigma). After washing with buffer B plus 50 mM NaCl, the column was eluted with a 50-mL gradient of 50 to 800 mM NaCl in buffer B (Figure 4E). The active fractions were pooled and concentrated with a Centricon filter (10,000 molecular weight cut-off; Amicon, Beverly, MA).

To further purify the p48 SIP kinase, the above-mentioned concentrated sample was loaded onto a Superdex 200 HR 10/30 fast protein liquid chromatography gel filtration column (Pharmacia) equilibrated with buffer B plus 250 mM NaCl, and the column was eluted with the same buffer at 0.5 mL/min (Figure 4F). For the measurement of the native molecular weight, the purified p48 SIP kinase was mixed with gel filtration size standards (Bio-Rad) and loaded onto the Superdex 200 HR 10/30 column. The retention time of the SIP kinase activity peak was used to calculate its molecular weight. The purified or partially purified SIP kinase after the poly-L-lysine–agarose step was stored at −20°C in buffer B plus 50% glycerol and was found to be stable for several months.

Assay of Kinase Activity in Solution

Unless specifically indicated, assays were performed at room temperature for 20 min in a final volume of 15 μL containing 0.5 mg/mL MBP, 50 μM γ-^{32}P-ATP (3000 to 6000 cpm/pmol), 25 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and enzyme. The reaction was terminated by the addition of an equal volume of 150 mM H_3PO_4; 20 μL of the mixture was then spotted onto an eight-well phosphocellulose filter strip (Pierce, Rockford, IL). After washing extensively with 150 mM H_3PO_4, the phosphoprotein was eluted with 0.4 mL of 1 N NaOH, and the radioactivity was determined by liquid scintillation counting. Total counts per minute in the reaction mixture was also determined to calculate the specific activity of the enzyme preparation. One unit of p48 SIP kinase was defined as the amount of enzyme that can catalyze the transfer of 1 pmol of phosphate from ATP to MBP in 1 min.

Identification of Phosphorylated Amino Acids in the Substrates

The procedure used to identify phosphorylated amino acids was identical to that described earlier (Zhang et al., 1993), except two-dimensional thin-layer electrophoresis was used. Protein substrates were labeled by phosphorylation in the presence of γ-^{32}P-ATP and precipitated by using 10% (w/v) TCA. After washing with 10% (w/v) TCA and acetone, the pellets were hydrolyzed in 6 N HCl for 2 hr at 110°C, dried in an evaporator (Speed-Vac; Savant Instruments, Inc., Farmingdale, NY), and then dissolved in 10 μL of a phosphoamino acids standard (1 mg/mL each of L-phosphoserine, L-phosphothreonine, and L-phosphotyrosine). The phosphoamino acids were separated by two-dimensional high-voltage thin-layer electrophoresis, as described by Sefton (1996). The position of the standards was visualized by ninhydrin (0.2% [w/v] in acetone), and the labeled amino acids were detected by autoradiography.

Treatment of the p48 SIP Kinase with Phosphatases

The phosphatase inhibitors present in the purified p48 SIP kinase preparation were first removed by dialysis against buffer B lacking β-glycerophosphate and Na_3VO_4 in a 0.5-mL 10K dialysis cassette (Slide-A-Lyzer; Pierce). For treatment with serine/threonine protein phosphatase PP1 (Calbiochem, San Diego, CA), $MnCl_2$, DTT, and BSA were added to aliquots of the SIP kinase preparation to final

concentrations of 200 μM, 5 mM, and 100 μg/mL, respectively. Then 0.1 unit of PP1 was added, and the reaction mixture was incubated at 30°C in the presence or absence of 1 μM of the phosphatase inhibitor okadaic acid (Calbiochem). For treatment with the tyrosine-specific protein phosphatase YOP (Calbiochem), NaCl, DTT, and BSA were added to purified SIP kinase to final concentrations of 150 mM, 5 mM, and 100 μg/mL, respectively. Then 0.5 unit of YOP was added, and the reaction was incubated at 30°C in the presence or absence of 1 mM of the tyrosine phosphatase inhibitor Na_3VO_4. Before assaying for the SIP kinase activity, the phosphatase inhibitors were brought to the same final concentration.

Microsequencing of Internal Tryptic Peptides

The protein in the active fractions eluted from the poly-L-lysine column was concentrated and precipitated with acetone. The pellets were then dissolved in SDS–Laemmli sample buffer and separated on a 10% SDS–polyacrylamide gel. After Coomassie Brilliant Blue R 250 staining, the 48-kD band was excised and sent to the W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT) for amino acid composition and sequence analyses. Briefly, for sequence analysis, the p48 SIP kinase was subjected to in-gel digestion with trypsin, and the resultant peptides were separated by reverse phase HPLC. After determination of molecular mass and purity by using matrix-assisted laser desorption ionization (MALDI) mass spectrometry, selected peptides were sequenced.

Cloning of the Tobacco SIP Kinase Gene

Two primers, 5′-AAYATHTTYGARGTNACNGC-3′ and 5′-CKNC-CNGGRAANARNGGYTT-3′ (where H is A, T, and C; K is T and G; N is A, T, C, and G; R is A and G; and Y is T and C), which correspond to peptide 1 and peptide 4 (Figure 9), respectively, were used to polymerase chain reaction (PCR) amplify the cDNA that was reverse transcribed from poly(A) RNA prepared from tobacco cell suspension culture. The reverse transcription–PCR product of ~600 bp was cloned into pGEM-T vector (Promega) and sequenced. A clone whose deduced amino acid sequence matched the internal peptide 2 and peptide 3 (Figure 9) was labeled with α-^{32}P-dCTP and used to screen a tobacco cDNA library under high stringency. Briefly, nylon membranes (Duralon-UV; Stratagene, La Jolla, CA), each with 5×10^4 plaque-forming units, were hybridized at 42°C for 20 hr in solution containing 20 mM Pipes, pH 6.5, 0.8 M NaCl, 50% formamide, 1% (w/v) SDS, 100 μg/mL denatured salmon sperm DNA, plus 10^6 cpm/mL of probe. After hybridization, the filters were washed once with 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate), 0.1%(w/v) SDS at room temperature, and three times, each for 15 min, with 0.1 × SSC, 0.1%(w/v) SDS at 55°C. More than 10 positive clones were obtained by screening ~10^6 plaque-forming units. Both strands of the clone containing the longest insert were sequenced using the Sequenase 2.0 kit (Amersham).

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