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Activation of the Tobacco SIP Kinase by Both a Cell Wall–Derived Carbohydrate Elicitor and Purified Proteinaceous Elicitins from *Phytophthora* spp

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Two purified proteinaceous fungal elicitors, parasiticein (an α elicitin) and cryptogein (a β elicitin), as well as a fungal cell wall–derived carbohydrate elicitor all rapidly activated a 48-kD kinase in tobacco suspension cells. The maximum activation of this kinase paralleled or preceded medium alkalization and activation of the defense gene phenylalanine ammonia–lyase (*PAL*). In addition, the two elicitins, which also induced hypersensitive cell death, activated a 44- and a 40-kD kinase with delayed kinetics. By contrast, the cell wall–derived elicitor only weakly activated the 44-kD kinase and failed to activate the 40-kD kinase. The size and substrate preference of the 48-kD kinase are reminiscent of the recently purified and cloned salicylic acid–induced protein (SIP) kinase, which is a member of the mitogen-activated protein kinase family. Antibodies raised against a peptide corresponding to the unique N terminus of SIP kinase immunoreacted with the 48-kD kinase activated by all three elicitors from *Phytophthora* spp. In addition, the cell wall elicitor and the salicylic acid–activated 48-kD kinase copurified through several chromatography steps and comigrated on two-dimensional gels. Based on these results, all three fungal elicitors appear to activate the SIP kinase. In addition, inhibition of SIP kinase activation by kinase inhibitors correlated with the suppression of cell wall elicitor–induced medium alkalization and *PAL* gene activation, suggesting a regulatory function for the SIP kinase in these defense responses.

INTRODUCTION

Plant cells exposed to elicitors, whether crude fungal cell wall fragments or defined molecules such as purified proteins and avirulence gene products, respond with a battery of cellular changes (Dixon and Lamb, 1990; Yu, 1995; Hammond-Kosack and Jones, 1997; Yang et al., 1997). Some of these responses, such as changes in ion fluxes and the generation of reactive oxygen species, occur very rapidly and may involve events that occur primarily at the post-translational level (Levine et al., 1994; Mehdy, 1994; Viard et al., 1994; Jabs et al., 1997). Other responses, such as the accumulation of phytoalexins and synthesis of chitinases, glucanases, and other pathogenesis-related proteins, involve induction of gene expression (Dixon and Lamb, 1990; Yang et al., 1997).

Several lines of evidence have implicated the involvement of protein phosphorylation in regulating the elicitor-stimulated defense responses, including those induced by elicitins. Elicitins are a family of small extracellular proteins produced by the pathogenic fungal genus *Phytophthora* (Yu, 1995). In tobacco, elicitins induce a hypersensitive-like response and confer protection against subsequent infection by microbial pathogens. Treatment of plant cells with either purified elicitins or cell wall–derived carbohydrate elicitors has been shown to cause rapid changes in phosphoprotein profiles (Dietrich et al., 1990; Felix et al., 1991, 1994; Viard et al., 1994). The inhibition of protein phosphorylation by K-252a and staurosporine correlates with the blockage of (1) medium alkalization, (2) reactive oxygen species generation, and (3) defense gene activation, including that of phenylalanine ammonia–lyase (*PAL*) (Grosskopf et al., 1990; Felix et al., 1991; Viard et al., 1994; Suzuki et al., 1995). Conversely, protein phosphatase inhibitors can mimic the effects of fungal elicitors, presumably by promoting protein phosphorylation (Felix et al., 1994).

Potential substrates for protein kinases responding to fungal elicitors include DNA binding proteins, such as KAP-1 and KAP-2. Both proteins recognize the H-box element contained within the elicitor-responsive chalcone synthase promoter. Dephosphorylation of KAP-1 and KAP-2 alters the mobility of their protein–DNA complexes (Yu et al., 1993). In addition, phosphorylation of G/HBF-1, a soybean basic leucine zipper transcription factor, by a bacterial pathogen–induced serine kinase enhances its ability to bind the chalcone synthase *chs15* promoter (Dröge-Laser et al.,

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1997). Similarly, elicitor-induced phosphorylation of the nuclear factor PBF-1 is required for effective binding and activation of the potato *PR-10a* gene (Després et al., 1995). A kinase with characteristics of mammalian protein kinase C is implicated in this process (Subramaniam et al., 1997). Unfortunately, in all of these cases, the identity of the kinase(s) is not yet known.

The mitogen-activated protein (MAP) kinase cascade is one of the major pathways by which extracellular stimuli are transduced into intracellular responses in yeast and mammalian cells (Herskowitz, 1995; Seger and Krebs, 1995; Kyriakis and Avruch, 1996). MAP kinases (MAPKs) are activated by dual phosphorylation of threonine and tyrosine residues in a TXY motif located between subdomains VII and VIII of the kinase catalytic domain by MAP kinase kinase (MAPKK). MAPKK, in turn, is activated by MAP kinase kinase kinase (MAPKKK). In mammals, two of the three subgroups of the MAPK family, the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) and the p38 kinase are activated in response to various stress signals, including UV and ionizing radiation, hyperosmolarity, oxidative stress, and cytokines. The outcome of SAPK/JNK and/or p38 activation depends on the magnitude and duration of their activation. Transient activation of these MAPKs induces various defense responses and allows the cells to adapt to an adverse environment. By contrast, persistent activation leads to apoptosis (Xia et al., 1995; Chen et al., 1996; Kyriakis and Avruch, 1996; Rosette and Karin, 1996; Nishina et al., 1997).

An increasing body of evidence suggests that MAPKs also play important signaling roles in plants, particularly during the activation of various stress-associated responses (Jonak et al., 1994; Nishihama et al., 1995; Stone and Walker, 1995; Hirt, 1997; Mizoguchi et al., 1997; Zhang and Klessig, 1997). In alfalfa, the MMK4 kinase has been 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* accumulate after wounding (Seo et al., 1995). *WIPK* may encode a 46-kD MAPK activity that is rapidly activated by wounding or cutting (Seo et al., 1995; Usami et al., 1995). However, because MAPKs are activated post-translationally by phosphorylation, further analyses are required to determine whether elevated transcript levels are responsible for the increases in kinase activity.

MAPKs have also been implicated in defense responses to microbial pathogens. The Pep25 elicitor derived from the 42-kD glycoprotein secreted by *P. sojae* activates a 45-kD MAPK in parsley cells that is believed to be encoded by the *ERMK* gene (Ligterink et al., 1997). Treatment of tobacco cells with a cell wall elicitor from *P. infestans* activates the expression of defense genes, which can be blocked by staurosporine (Suzuki et al., 1995). A 47-kD protein kinase with characteristics of a MAPK is activated in these fungal cell wall elicitor–treated cells (Suzuki and Shinshi, 1995), but the identity of this kinase and its encoding gene remain unknown. We have identified a 48-kD MAPK in tobacco, termed SIP kinase (for salicylic acid [SA]–induced protein kinase), which is activated by SA, an endogenous signaling molecule involved in defense responses (Zhang and Klessig, 1997). SIP kinase was purified to homogeneity, and its corresponding gene was cloned based on the partial amino acid sequence of the purified protein. This has allowed us to rigorously establish that the cloned gene encodes the activated kinase.

In this study, we report that a cell wall–derived carbohydrate elicitor and two purified proteinaceous elicitins, parasiticein and cryptogein, from *Phytophthora* spp activated a 48-kD kinase in tobacco cells. Moreover, this 48-kD kinase is identical to the SA-activated SIP kinase. By using the kinase inhibitors K-252a and staurosporine, we demonstrate that inhibition of SIP kinase activation by these inhibitors correlates with depression of medium alkalization and *PAL* gene activation induced by the fungal cell wall elicitor. This result argues that the SIP kinase may participate in the activation of these elicitor-induced defense responses in tobacco.

RESULTS

Several Fungal Elicitors Activate a 48-kD Protein Kinase and Induce Defense Responses in Tobacco Cells

Activation of plant defense responses by a variety of fungal elicitors appears to involve protein phosphorylation. The identity of the responsible kinases is unknown, and an increasing body of evidence from studies in yeast and mammalian systems implicates the participation of MAPKs in response to stresses; therefore, we set out to determine whether several different elicitors from phytopathogenic *Phytophthora* spp might also act through MAPKs. Because MAPKs preferentially use the myelin basic protein (MBP) as an artificial substrate, we used an in-gel kinase activity assay with MBP (Zhang and Klessig, 1997). Three elicitors were tested. They were a crude cell wall–derived carbohydrate elicitor from *P. parasitica*; a purified 10-kD proteinaceous α elicitin, parasiticein, from *P. parasitica*; and a purified β elicitin, cryptogein, from *P. cryptogea.*

Treatment of tobacco suspension cells with the fungal cell wall elicitor rapidly activated a 48-kD kinase, with activity peaking within $\sim$10 min (Figures 1A and 1B). Thereafter, activity declined to approximately half maximum and remained at that level for $\sim$2 hr before returning to the basal level between 4 and 8 hr after treatment. Parasiticein (Figure 1C) and cryptogein (Figure 1D) also induced the rapid activation of a 48-kD kinase. Its activation was more prolonged, remaining at high levels for $\sim$4 hr before declining in conjunction with the appearance of hypersensitive cell death (Figure 2C). A 44- and a 40-kD kinase were also activated by these

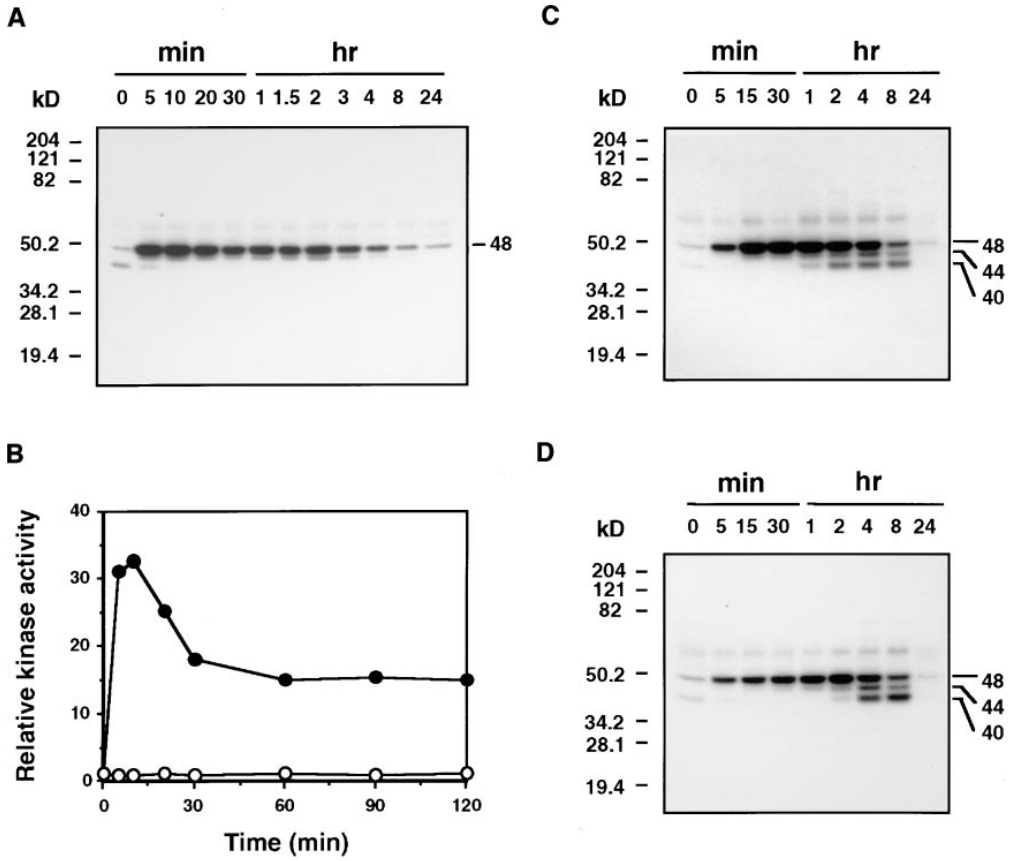

Figure 1. Treatment with a Fungal Cell Wall Elicitor or Either Elicitin Activates a 48-kD Protein Kinase in Tobacco Cells.

(A) Tobacco cells were treated with the fungal cell wall–derived elicitor, and aliquots were taken at the indicated times. Kinase activity was determined with an in-gel kinase activity assay using MBP as a substrate. Only results from the elicitor-treated cells are shown for the in-gel kinase activity assay. The sizes of the molecular mass markers (at left) and the activated kinase (at right) are given in kilodaltons.

(B) The 48-kD kinase activities in elicitor-treated (filled circle) and H_2O-treated (open circle) cells 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) Tobacco cells were treated with 50 nM parasiticein, and aliquots were taken at the indicated times. Kinase activity was determined as given in **(A)**. The sizes of the molecular mass markers (at left) and the activated kinase (at right) are given in kilodaltons.

(D) Tobacco cells were treated with 50 nM cryptogein, and aliquots were taken at the indicated times. Kinase activity was determined as given in **(A)**. The sizes of the molecular mass markers (at left) and the activated kinase (at right) are given in kilodaltons.

Data presented in Figures 1 to 10 are from one of at least two independent experiments with similar results.

two elicitins. Their activation was delayed, with peak activity detected at ~4 hr, just before the onset of cell death. The fungal cell wall elicitor also activated a 44-kD kinase, but only transiently and to a much lesser extent than that seen with the elicitins. There was no evidence for activation of a 40-kD kinase by the cell wall elicitor, even after a very long exposure of the autoradiogram (data not shown).

To determine whether the activation of one or more of these kinases might be involved in the induction of defense responses by the different elicitors, we monitored the kinetics of medium alkalization (Figure 2A) and induction of *PAL* gene expression (Figure 2B). All three elicitors induced significant medium alkalization, which was evident within 15 min and plateaued ~2 hr after treatment. Increased levels of *PAL* mRNA were detected by 30 min, and they peaked at 4 hr. Because elicitins are well-characterized inducers of tobacco cell death, this defense-associated response was also analyzed (Figure 2C). Parasiticein and cryptogein induced significant cell death by 8 hr; by 24 hr after treatment, 90 to 95% of the cells had died. In contrast, the fungal cell wall elicitor had little effect on cell viability up to 24 hr after treatment.

The rapid activation of the 48-kD kinase paralleled or slightly preceded the rapid induction of medium alkalization

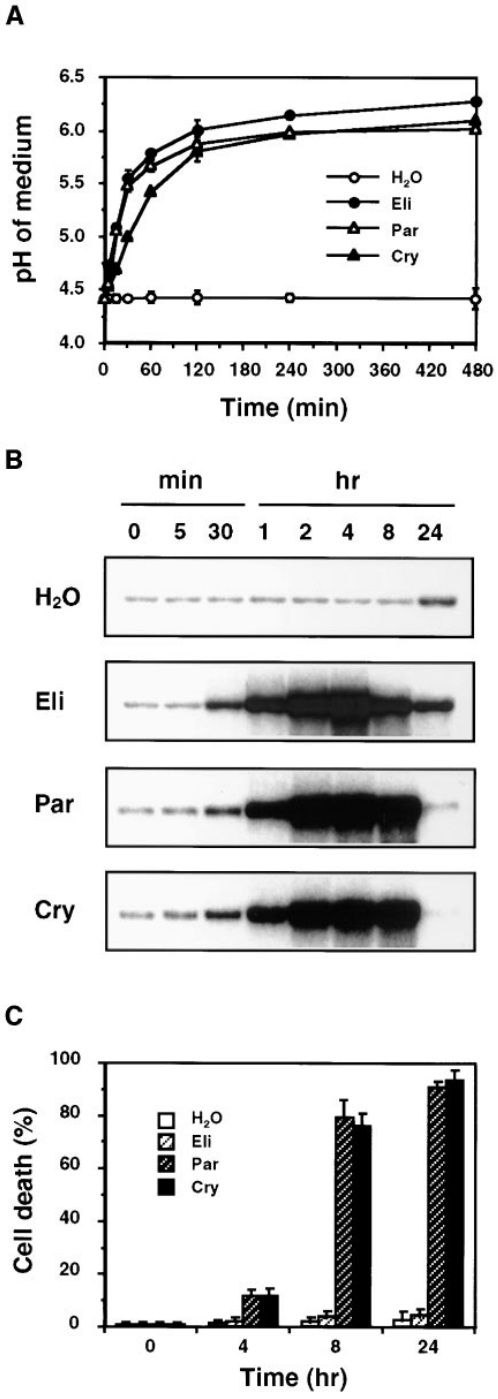

Figure 2. All Three Fungal Elicitors Induce Defense Responses in Tobacco Suspension Cells.

(A) Alkalization of the extracellular medium after treatment with the fungal cell wall–derived elicitor or elicitins. Tobacco suspension culture cells were treated with the fungal cell wall elicitor (Eli; 50 μg of glucose equivalent per mL), parasiticein (Par; 50 nM), cryptogein (Cry; 50 nM), or an equal volume of H_2O as a control. Aliquots were taken at the indicated times, and the pH of the medium was determined after the cells were removed by filtration. Data represent the mean of three replicates ±SE.

(B) *PAL* gene expression after fungal elicitor treatment. Total RNA was prepared from cells harvested by filtration and used for gel blot analysis. Twenty micrograms of RNA was separated on 1.2% formaldehyde–agarose gels and transferred to a Zeta probe membrane. The blots were hybridized with random primer–labeled *PAL* cDNA.

(C) Elicitin-induced hypersensitive cell death in tobacco suspension cells. Cell viability was examined by the fluorescein diacetate method at the indicated times. Data represent the mean of three replicates ±SE.

and *PAL* gene expression. Furthermore, of the three kinases, only the 48-kD kinase was strongly activated by all three inducers of these two defense responses. Thus, the 48-kD kinase appeared to be the most likely candidate for participation in the induction of these early defense responses, whereas the 44- and 40-kD kinases may participate in late responses such as hypersensitive cell death induced by elicitins.

The Fungal Cell Wall Elicitor–Activated 48-kD Kinase Copurifies and Comigrates with SIP Kinase

The size of the 48-kD elicitor-activated kinase is identical to that of the SIP kinase, which was recently purified and its corresponding gene cloned (Zhang and Klessig, 1997). The possibility that the cell wall elicitor– and the SA-activated kinases were the same was tested by determining whether they would copurify through several chromatographic steps. Extracts prepared from cells treated for 5 min with either the cell wall elicitor or SA were subjected to ultracentrifugation at 130,000*g* (S130) and desalting before fractionation by MonoQ anion exchange fast-protein liquid chromatography (FPLC). A single peak of protein kinase activity was detected with each extract, which eluted at the same position in the salt gradient (data not shown). To confirm that the kinase activities indeed copurified off the MonoQ column, we mixed together equal amounts of each extract before applying to the column. Both activities eluted together as a single sharp peak at ~250 mM NaCl (Figure 3). MonoQ column chromatography previously has been used successfully to separate closely related MAPKs, such as extracellular signal-regulated protein kinases (ERKs), JNKs, and a p38 kinase under similar conditions (Ahn et al., 1990; Bogoyevitch et al., 1995; Heidenreich and Kummer, 1996). These two kinase activities also copurified upon subsequent phenyl–Sepharose column chromatography (data not shown).

To further establish whether the cell wall elicitor– and SA-activated kinases were the same, a portion of the peak fraction from the MonoQ column for each extract was subjected to two-dimensional gel electrophoresis, either separately

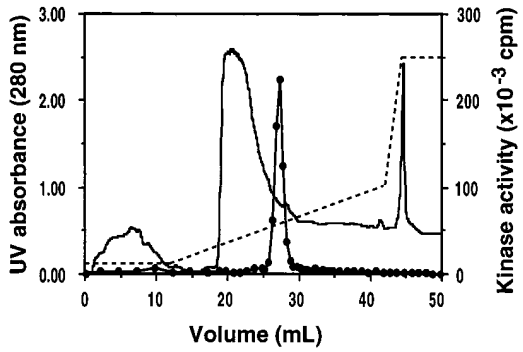

Figure 3. The Fungal Cell Wall Elicitor– and SA-Activated Kinase Activities Copurify on a MonoQ FPLC Column.

Equal amounts of protein extracts (25 mg each) from elicitor- or SA-treated cells were mixed and loaded onto a MonoQ HR5/5 FPLC column. After washing, the column was eluted with an NaCl gradient in buffer A. The solid line without data points represents the protein concentration ($A_{280\ nm}$), and the dashed line denotes the NaCl gradient profile. Kinase activity (filled circles) was determined by an in-solution kinase assay, using MBP as the substrate.

(Figures 4A and 4B) or after mixing (Figure 4C). Because the kinase activity in the S130 fraction precipitated at the top of the isoelectric focusing tube gel for the first dimension, the partially purified MonoQ fractions were used. A single kinase activity, which migrated as a spot with slight tailing, was detected with all three samples, including the mixture. These results indicate that both kinases have identical molecular weights and isoelectric points.

SIP Kinase–Specific Antibodies Recognize the 48-kD Kinase Activated by All Three *Phytophthora* Elicitors

The copurification and the comigration on two-dimensional gels of the cell wall elicitor– and SA-activated kinases suggest that they are identical. To provide more direct evidence for this assumption, we used antibodies directed against peptides corresponding to the unique N terminus or more conserved C terminus of SIP kinase. Antibodies targeted to the N-terminal peptide (p48N, MDGSGQQTDTMMSDAGAE-QPPTAP) and C-terminal peptide (p48C, KELIYRESLAFN-PEYQHM) of SIP kinase (Figure 5A) were raised in rabbits and affinity purified. The specificity of the anti-p48N (Ab-p48N) and anti-p48C (Ab-p48C) antibodies was assessed by immunoblot analyses against a panel of different tobacco MAPKs. For this purpose, the cloned *SIPK*, *Ntf4*, *WIPK*, and *NtMPK6* (a new MAPK isolated from tobacco; H. Du, S. Zhang, and D.F. Klessig, unpublished data) genes were expressed as histidine (His)-tagged fusion proteins in *Escherichia coli* and affinity purified (Figure 5B). The His tag plus the linker sequence add ~3.6 kD to the N terminus of each protein. The deduced molecular masses of the recombinant proteins, including the His tag, are 48.8 (SIPK), 48.6 (Ntf4), and 46.4 kD (WIPK). The *Ntf4*- and *SIPK*-encoded proteins migrated as slightly larger proteins (~3 kD) on an SDS–polyacrylamide gel (Figure 5B), as previously reported for SIP kinase (Zhang and Klessig, 1997). Note that the SIPK and Ntf4

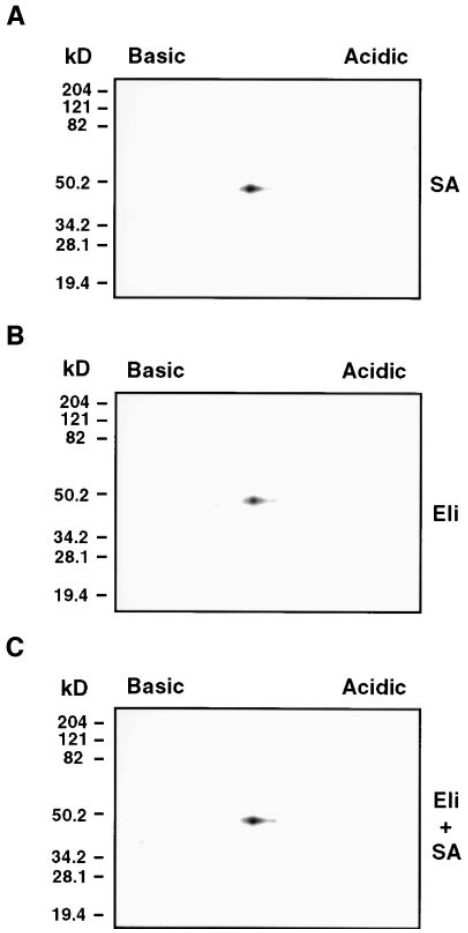

Figure 4. The Fungal Cell Wall Elicitor–Activated Kinase Comigrates with the 48-kD SIP Kinase on Two-Dimensional Electrophoresis Gels.

(A) Two-dimensional in-gel kinase activity assay of the SA-activated kinase. One unit of enzyme from the pooled MonoQ fractions with the highest levels of kinase activity was mixed with an equal volume of isoelectric focusing sample buffer and loaded onto an isoelectric focusing tube gel. After electrofocusing, the tube gel was mounted onto a 10% SDS–polyacrylamide gel with MBP embedded in the separating gel. After electrophoresis, the kinase activity was detected by an in-gel kinase activity assay.

(B) Two-dimensional in-gel kinase activity assay of the elicitor (Eli)-activated kinase.

(C) Two-dimensional in-gel kinase activity assay of a mixture of equal amounts of the elicitor- and SA-activated kinases.

The sizes of the molecular mass markers at left are given in kilodaltons.

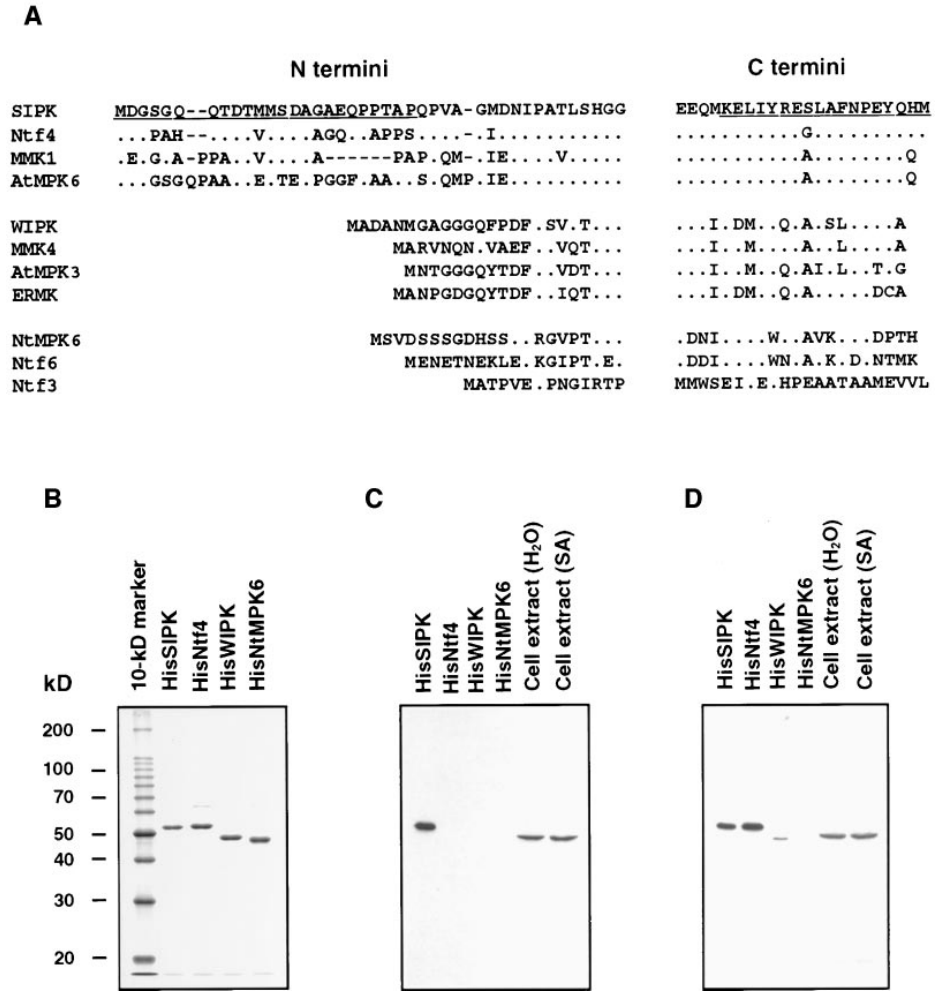

Figure 5. Specificity of Antibodies to the N Terminus (Ab-p48N) and C Terminus (Ab-p48C) of SIP Kinase.

(A) Alignment of the N- and C-terminal sequences of SIP kinase with other members of the tobacco MAPK family as well as stress-related MAPKs from other plants. The amino acid sequences from the N and C termini of SIP kinase (SIPK; Zhang and Klessig, 1997), WIPK (Seo et al., 1995), NtMPK6 (H. Du, S. Zhang, and D.F. Klessig, unpublished data), Ntf3, Ntf4, and Ntf6 (Wilson et al., 1993, 1995) from tobacco, MMK1 and MMK4 from alfalfa (Duerr et al., 1993; Jonak et al., 1993, 1996), AtMPK6 and AtMPK3 from Arabidopsis (Mizoguchi et al., 1993), and ERMK from parsley (Ligterink et al., 1997) were deduced from cDNA sequences. Dots represent amino acid residues that match SIP kinase, and dashes indicate gaps introduced to maximize alignment. Sequences corresponding to the peptides used to raise antibodies are underlined.

(B) SDS-PAGE analysis of His-tagged recombinant MAPKs of tobacco. Equal amounts (0.5 μg) of recombinant SIPK, Ntf4, WIPK, and NtMPK6 proteins were separated on a 10% SDS–polyacrylamide gel and stained with Coomassie Brilliant Blue R 250. The position of the 10-kD molecular mass marker ladder in kilodaltons is indicated at the left.

(C) Ab-p48N specifically recognizes SIP kinase. Five nanograms each of HisSIPK, HisNtf4, HisWIPK, and HisNtMPK6 or 20 μg of protein extracts from tobacco cells treated with H_2O or SA was analyzed by immunoblotting with Ab-p48N. After incubation with a horseradish peroxidase–conjugated secondary antibody, the complex was visualized using enhanced chemiluminescence.

(D) Ab-p48C recognizes SIPK and Ntf4 equally well and weakly cross-reacts with WIPK. A duplicate blot to that shown in **(C)** was reacted with Ab-p48C.

proteins are ~5 kD larger than the *WIPK*- and *NtMPK6*-encoded proteins based on migration rates (Figure 5B); this is consistent with their aberrant migration rates (corresponding to ~3 kD) and the presence of an 18–amino acid extension at their N termini (Figure 5A).

The immunoblot analyses with this panel of recombinant MAPKs demonstrated that the Ab-p48N is highly specific. Ab-p48N recognized only the His-tagged SIPK (Figure 5C), which is consistent with the fact that this antibody was raised against SIP kinase's unique N-terminal sequence

(Figure 5A). In contrast, Ab-p48C reacted equally well with His-tagged SIPK and Ntf4 (Figure 5D), which is consistent with the very high conservation of C-terminal sequences between these two proteins. Ab-p48C also reacted weakly with HisWIPK but failed to recognize HisNtMPK6, paralleling their decrease in sequence identity to the C terminus of SIPK (Figure 5A). Because the SIP kinase is post-translationally activated by phosphorylation (Zhang and Klessig, 1997), it was not surprising that both antibodies reacted with a 48-kD protein present in approximately equal amounts in H_2O- or SA-treated tobacco cells (Figures 5C and 5D; see below for more detailed analyses).

Having established the specificity of these antibodies, we then used them to determine whether the fungal cell wall elicitor–activated kinase was identical to SIP kinase. These experiments used an immunocomplex kinase assay in which extracts from H_2O-, cell wall elicitor–, or SA-treated cells were reacted with the antibodies. The resultant antigen–antibody complexes were then precipitated with protein A–agarose beads and washed extensively before addition to a kinase assay mixture with γ-^{32}P-ATP and MBP as substrates. The reaction mixture, including the phosphorylated MBP, was then fractionated by SDS-PAGE (Figure 6). Ab-p48C and, most importantly, Ab-p48N immunoprecipitated not only the SA-activated kinase but also the fungal cell wall elicitor–activated kinase. To demonstrate further the specificity of the antibodies and their reaction with the cell wall elicitor– or SA-activated kinases, we used p48N and p48C as competitor peptides in the immunoprecipitation reaction. Peptide p48N blocked immunoprecipitation of the cell wall elicitor–activated as well as SA-activated kinases by Ab-p48N but not by Ab-p48C, and peptide p48C blocked immunoprecipitation by Ab-p48C but not by Ab-p48N.

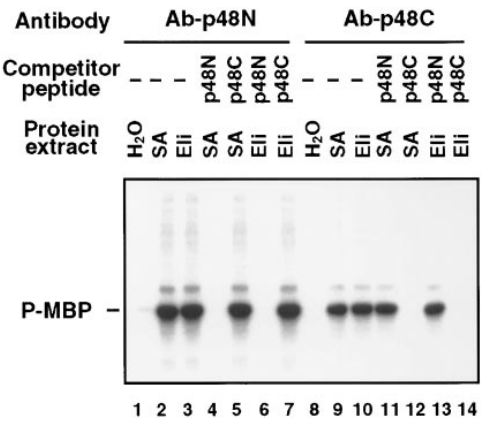

Figure 6. SIP Kinase–Specific Ab-p48N as Well as Ab-p48C Immunoprecipitate the Fungal Cell Wall Elicitor– and SA-Activated Kinases.

Protein extracts (50 μg) from H_2O-, SA-, and elicitor (Eli)-treated cells were immunoprecipitated with either Ab-p48N (lanes 1 to 7) or Ab-p48C (lanes 8 to 14) in the absence ([−]; lanes 1 to 3 and 8 to 10) or presence of peptides p48N (lanes 4, 6, 11, and 13) or p48C (lanes 5, 7, 12, and 14). Kinase activity of the immunocomplex was subsequently assayed as described in Methods, and the phosphorylated MBP (P-MBP) was visualized by autoradiography.

Both antibodies were also used with the immunocomplex kinase assay to follow the kinetics of activation of the 48-kD kinase after treatment with SA (Figure 7A) or the cell wall elicitor (Figure 7B). This assay was also used to demonstrate that the SIP kinase–specific Ab-p48N as well as Ab-p48C immunoreacted with both the parasiticein- and cryptogein-activated kinases (Figures 7C and 7D), confirming their identity to the SIP kinase. The activation kinetics and magnitude of kinase activity observed in these assays correlated well with those obtained by the in-gel kinase assays for all three *Phytophthora* elicitors (Figure 1) and SA-treated cells (Zhang and Klessig, 1997).

SIP Kinase Is Activated Post-Translationally by Both the Fungal Cell Wall Elicitor and SA

In addition to post-translational activation of MAPKs in yeast, plant, and mammalian cells, several MAPKs have been reported to be activated at the mRNA level in plants (Seo et al., 1995; Jonak et al., 1996; Mizoguchi et al., 1996; Bögre et al., 1997). Therefore, we examined both mRNA and protein levels of SIP kinase during activation by SA and the cell wall elicitor (Figures 8A and 8B). The steady state levels of the *SIPK* transcript remained unchanged after activation by either stimuli. Similarly, immunoblot analysis using the SIP kinase–specific Ab-p48N revealed that protein levels also remained constant, arguing that post-translational modification was responsible for SIP kinase activation. Because yeast and mammalian MAPKs are activated by dual phosphorylation of the TXY motif between subdomains VII and VIII of the catalytic kinase domain, we tested whether tyrosine phosphorylation was associated with activation by either stimulus. To do so, extracts from H_2O-, SA-, or fungal cell wall elicitor–treated cells were immunoprecipitated with Ab-p48N, and the immunoprecipitates were then subjected to immunoblot analysis using an anti-phosphotyrosine monoclonal antibody, 4G10 (Figure 8C). The specificity of this immunoprecipitation reaction was monitored by adding the competitor peptides p48N and p48C. As anticipated, tyrosine phosphorylation was associated with SIP kinase activation by the cell wall elicitor as well as by SA. This is consistent with our previous data showing that the purified SIP kinase from SA-treated cells was phosphorylated on a tyrosine residue(s) (Zhang and Klessig, 1997).

Inhibition of SIP Kinase Activation by Kinase Inhibitors Correlates with Suppression of Medium Alkalization and *PAL* Gene Activation

Because SIP kinase activation requires its phosphorylation by a MAPKK, which in turn is activated by a MAPKKK,

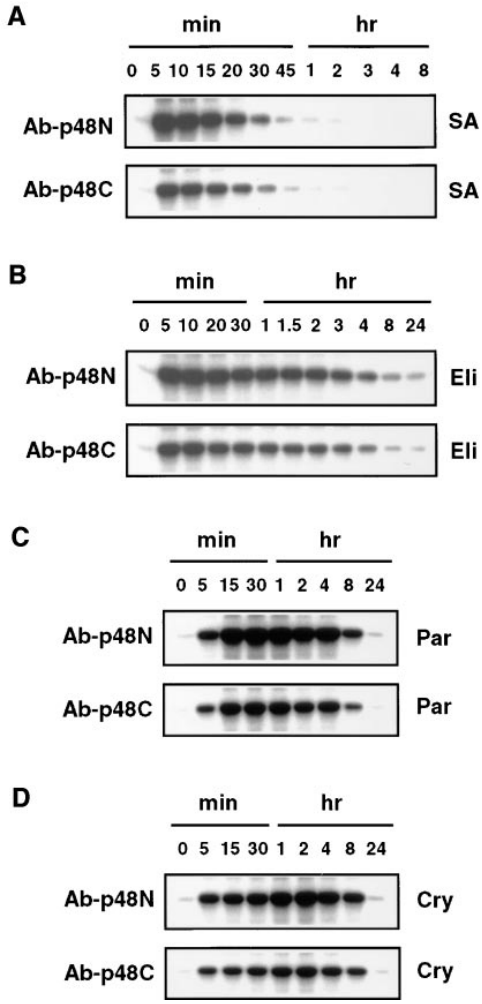

Figure 7. Activation of SIP Kinase by the Fungal Cell Wall Elicitor, Parasiticein, and Cryptogein.

(A) SA-mediated activation of a kinase immunoprecipitated by antibodies raised against SIP kinase. Protein extracts (50 μg) from cells treated with SA for various times were immunoprecipitated with 2.5 μg of either Ab-p48N or Ab-p48C. Kinase activity of the immunocomplex was assayed as described in Methods.

(B) Prolonged activation by the fungal cell wall elicitor (Eli) of a kinase immunoprecipitated by antibodies raised against SIP kinase. Protein extracts (50 μg) from cells treated with the elicitor for various times were immunoprecipitated with 2.5 μg of either Ab-p48N or Ab-p48C, and kinase activity of the immunocomplex was assayed as described in **(A)**.

(C) Sustained activation by parasiticein (Par) of a kinase immunoprecipitated by antibodies raised against SIP kinase. Protein extracts (50 μg) from cells treated with parasiticein for various times were immunoprecipitated with 2.5 μg of either Ab-p48N or Ab-p48C. Kinase activity of the immunocomplex was assayed as described in **(A)**.

(D) Sustained activation by cryptogein (Cry) of a kinase immunoprecipitated by antibodies raised against SIP kinase. Protein extracts (50 μg) from cells treated with cryptogein for various times were immunoprecipitated with 2.5 μg of either Ab-p48N or Ab-p48C. Kinase activity of the immunocomplex was assayed as described in **(A)**.

kinase inhibitors were used to test whether SIP kinase participates in defense response induction by the fungal cell wall elicitor. Tobacco cells were pretreated with K-252a, staurosporine, or 0.05% DMSO (in which the inhibitor stock solutions were made) for 5 min followed by addition of the fungal cell wall elicitor or water (as a control). At various times, cells were harvested for preparation of protein and RNA, and the pH of the medium was determined. Pretreatment with either kinase inhibitor blocked the rapid activation of SIP kinase by the elicitor (Figure 9). Similarly, both inhibitors suppressed induction by the elicitor of medium alkalization (Figure 10A) and *PAL* gene expression (Figure 10B), suggesting that SIP kinase is involved in induction of these two defense responses.

The blockage of SIP kinase activation suggests that the SIP kinase kinase and/or SIP kinase kinase kinase are sensitive to these kinase inhibitors. In addition, we have previously demonstrated that SIP kinase itself is very sensitive to K-252a and staurosporine, with an IC_{50} (concentration required for 50% inhibition) of 12 and 60 nM, respectively (Zhang and Klessig, 1997). Therefore, suppression of the defense responses may have resulted from the combined effects of inhibition of SIP kinase activation and SIP kinase activity. Prolonged treatment (⩾4 hr) with the kinase inhibitors alone resulted in slight activation of *PAL* (Figure 10B and data not shown), perhaps reflecting potential side effects of these kinase inhibitors. Either inhibitor alone or in combination with the cell wall elicitor did not affect cell viability (data not shown).

DISCUSSION

In this study, we demonstrate that a 48-kD kinase is activated in tobacco suspension cells after treatment with either a cell wall elicitor or two elicitins derived from the fungal genus *Phytophthora.* Both elicitins and, to a lesser extent, the fungal cell wall elicitor also induced a 44-kD kinase; a 40-kD kinase was activated solely by the elicitins (Figure 1). The size and substrate preference of the 48-kD kinase were similar to that of SIP kinase, a recently identified MAPK that is rapidly and transiently activated by SA treatment of tobacco suspension cells (Zhang and Klessig, 1997). Further analyses demonstrated that these kinase activities copurified on MonoQ (Figure 3) and phenyl-Sepharose columns (data not shown) and comigrated on two-dimensional electrophoresis gels (Figure 4). Moreover, a SIP kinase–specific antibody, Ab-p48N, which was made against the unique N terminus of SIP kinase, immunoprecipitated the 48-kD kinase activated by SA, cell wall elicitor, and elicitin treatments (Figures 6 and 7). In addition, activation of the 48-kD kinase by these inducers was not associated with increases in mRNA or protein levels but rather with the phosphorylation of a tyrosine residue(s) on the 48-kD kinase protein (Figure 8; data not shown). This is consistent with the previous demonstration

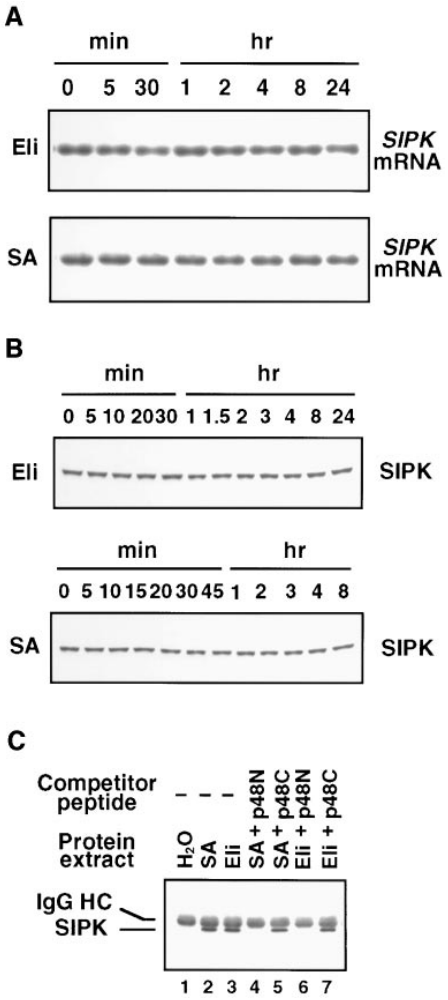

Figure 8. Post-Translational Activation of SIP Kinase by the Elicitor and SA.

(A) The transcript level of the *SIPK* gene remains constant during the course of SIP kinase activation. Total RNA was extracted at the indicated times from cells treated with either the fungal cell wall elicitor (Eli) or SA. Twenty micrograms of total RNA was separated on a denaturing formaldehyde–agarose gel, transferred to a Zeta probe membrane, and sequentially hybridized with the 5′ untranslated region and then the full-length *SIPK* cDNA. Both gave the same result; only the autoradiogram produced with the full-length cDNA probe is shown.
(B) SIPK protein levels remain unchanged during the activation of SIP kinase by the cell wall elicitor and SA. Twenty micrograms of protein extracts from cells treated with either the elicitor or SA was separated by SDS-PAGE. After blotting to nitrocellulose, the 48-kD SIP kinase was detected with Ab-p48N.
(C) Activation of the 48-kD SIP kinase by the cell wall elicitor and SA is associated with its tyrosine phosphorylation. Protein extracts (50 μg) from H_2O-, SA-, and cell wall elicitor (Eli)–treated cells (5 min) were immunoprecipitated with Ab-p48N in the absence ([−]; lanes 1 to 3) or in the presence (+) of peptides p48N (lanes 4 and 6) or p48C (lanes 5 and 7). The phosphotyrosine in the immunocomplex was detected by reaction with monoclonal antibody 4G10. The upper band corresponds to the heavy chain of rabbit IgG (IgG HC).

that SIP kinase is activated at the post-translational level by a phosphorylation event (Zhang and Klessig, 1997). Based on these results, the 48-kD kinase activated by the fungal cell wall elicitor and the two elicitins is the same as the SA-activated SIP kinase. Because tobacco is amphidiploid, it is possible that the 48-kD MAPK activated by SA, the cell wall elicitor, and elicitins is encoded by both *SIPK* and its corresponding gene from the other parent of tobacco. The copurification, comigration on two-dimensional gel, and immunoprecipitation with the SIP kinase–specific antibody argue that if there are two genes for the 48-kD MAPK, then their product is very similar, if not identical.

Treatment of tobacco suspension cells with the fungal cell wall elicitor and elicitins also induced media alkalization and *PAL* gene expression, two responses associated with plant disease resistance (Figures 2A and 2B). At much later times, the elicitins also induced cell death (Figure 2C). Interestingly, activation of the 48-kD SIP kinase paralleled or slightly preceded media alkalization and *PAL* gene expression, suggesting that it is involved in their induction. In contrast, activation of the 44- and 40-kD kinases by elicitins occurred

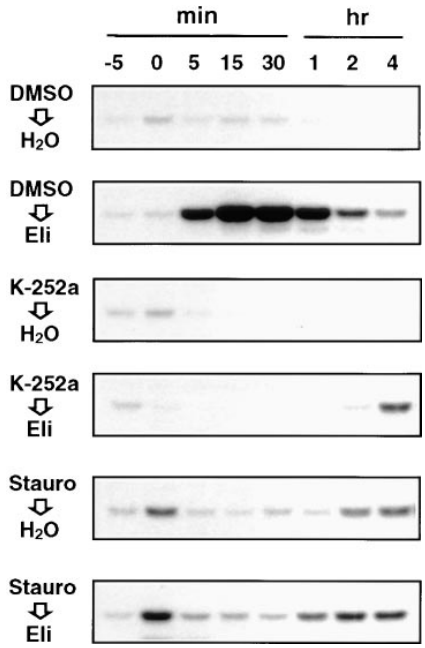

Figure 9. Kinase Inhibitors Block the Activation of SIP Kinase by the Fungal Cell Wall Elicitor.

Tobacco cells were pretreated for 5 min with K-252a (0.5 μM in 0.05% DMSO), staurosporine (Stauro; 0.5 μM in 0.05% DMSO), or DMSO (0.05%) as the control, followed by the addition of either the fungal cell wall elicitor (Eli; 25 μg of glucose equivalent per mL) or H_2O as the control. At the times indicated, aliquots were taken, cells were harvested by filtration, and protein extracts were prepared. Kinase activity was determined with an in-gel kinase activity assay using MBP as a substrate.

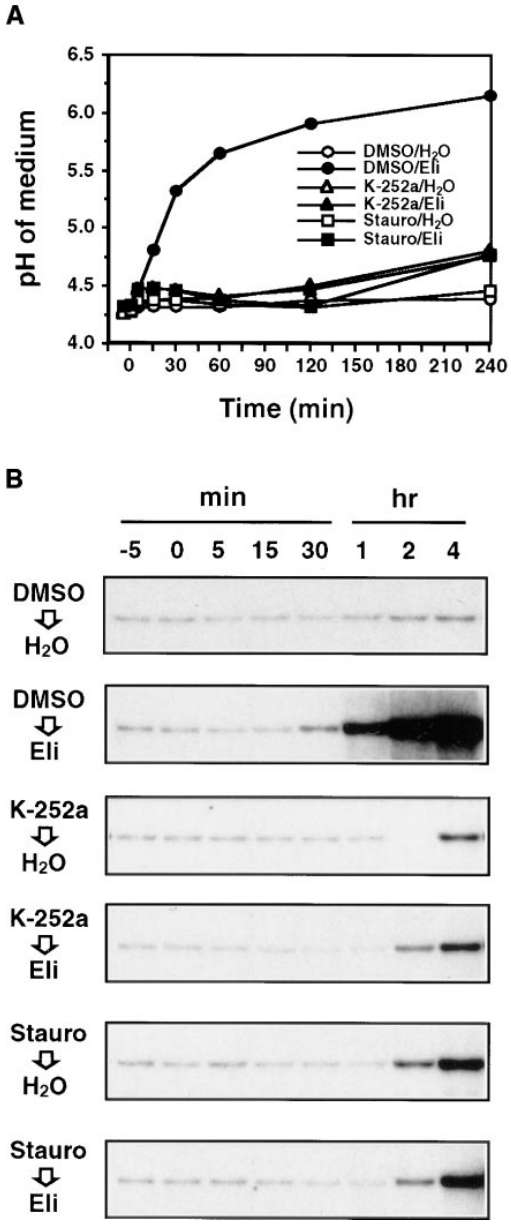

Figure 10. Pretreatment with Either K-252a or Staurosporine Inhibits the Fungal Cell Wall Elicitor–Induced Defense Responses.

(A) Inhibition of the fungal cell wall elicitor–induced medium alkalization by kinase inhibitors. Tobacco suspension culture cells were treated as given in Figure 9. The pH of the medium was determined after the cells were removed for protein and RNA preparation by filtration (open circle, DMSO/H_2O; filled circle, DMSO/elicitor [DMSO/Eli]; open triangle, K-252a/H_2O; filled triangle, K-252a/Eli; open square, staurosporine/H_2O [Stauro/H_2O]; filled square, Stauro/Eli).
(B) Inhibition of *PAL* gene activation by kinase inhibitors. Total RNA was prepared from cells harvested by filtration and used for gel blot analysis. Twenty micrograms of RNA was separated on 1.2% formaldehyde–agarose gels and transferred to a Zeta probe membrane. The blots were hybridized with random primer–labeled *PAL* cDNA.

well after these two defense responses were induced but concurrent with the hypersensitive cell death. Consistent with the participation of 48-kD kinase in induction of these defense responses, the protein kinase inhibitors K-252a and staurosporine were found to block fungal cell wall elicitor–induced SIP kinase activation (Figure 9) and suppress medium alkalization and *PAL* gene expression induced by this elicitor (Figure 10).

Activation of SIP kinase is one of the earliest responses of tobacco cells after exposure to the fungal cell wall elicitor, elicitins, or SA, with peak activity being reached within 5 to 10 min. This very rapid activation by the cell wall elicitor and elicitins together with the very similar initial kinetics of activation by SA, the cell wall elicitor, and elicitins argue against a model in which the elicitor and elicitins activate SIP kinase through increases in SA levels. The fungal cell wall elicitor and elicitins induced medium alkalization and *PAL* gene expression, whereas only the elicitins induced cell death (Figure 2). By contrast, SA failed to induce any of these responses (data not shown). How is activation of SIP kinase by different inducers associated with such distinct intracellular responses? One possibility is that in the case of SA, additional components or signals are necessary for activation of these responses. This hypothesis is consistent with the finding that SA can potentiate several defense responses, such as *PAL* gene expression and hypersensitive cell death activated by pathogen infection, whereas SA is incapable of inducing these defense responses by itself (Kauss et al., 1992; Mur et al., 1996; Naton et al., 1996; Shirasu et al., 1997).

An alternative explanation is that the differences in the kinetics (duration) of SIP kinase activation may specify the responses. Although the magnitude of SIP kinase activation was similar for all three fungal elicitors and SA, the duration was markedly different (Figure 1). Stimulation of SIP kinase by SA was transient, with activity peaking by 5 min and returning to basal levels within 45 min of treatment (Zhang and Klessig, 1997). By contrast, SIP kinase activity after fungal cell wall elicitor treatment was more prolonged, with ~50% of the peak activity remaining 2 hr after treatment and a gradual return to basal level occurring by 8 hr after treatment (Figures 1A and 1B). In comparison, both elicitins induced a high-level, persistent activation of SIP kinase until hypersensitive cell death (Figures 1C and 1D).

In mammals, there is considerable precedence for induction of different cellular responses to a wide variety of stimuli through a limited number of MAPKs. For example, UV light, ionizing radiation, osmotic stress, heat stress, oxidative stress, and cytokines, including tumor necrosis factor (TNF) and interleukin-1 (IL-1), activate one or sometimes both of the SAPK/JNK and p38 kinases, which belong to two subfamilies of the mammalian MAPK family (Kyriakis and Avruch, 1996). Similarly, many different growth factors elicit their intracellular responses through kinases belonging to a third MAPK subfamily, ERKs (Marshall, 1995). Currently, there are only a few known members of this subfamily (e.g., ERK1 and ERK2), and they are all associated with cell proliferation.

The ability of such a limited number of mammalian MAPKs to transduce various stimuli into different responses appears to be determined to a large extent by the duration and/or magnitude of MAPK activation (Marshall, 1995; Chen et al., 1996). For instance, treatment of PC12 cells with nerve growth factor (NGF) leads to an outgrowth of neurites and the eventual cessation of cell division (i.e., cell differentiation), whereas treatment with epidermal growth factor (EGF) leads to cell proliferation. The different cellular responses to NGF and EGF are determined by the duration of ERK activation. ERK activation is sustained for several hours after NGF stimulation, but it is short-lived after EGF treatment (Marshall, 1995). Similarly, activation of SAPK/JNK kinase by a variety of stress stimuli is rapid and transient and leads to responses that allow the cell to adapt to its environment (Kyriakis and Avruch, 1996). In contrast, prolonged activation of this kinase initiates apoptosis (Chen et al., 1996).

There are potentially many different ways for regulation of the duration of MAPK activation. In mammalian systems, inactivation of MAPKs is performed by dual-specificity protein phosphatases (MAPK phosphatases, such as CL-100 and its homologs), which simultaneously dephosphorylate both the threonine and tyrosine residues of MAPKs with high efficiency (Keyse, 1995; Groom et al., 1996). Transcription of these MAPK phosphatases is quickly activated by the MAPK signaling pathway (Bokemeyer et al., 1996; Grumont et al., 1996). Dual-specificity phosphatases have not yet been identified in higher plants; however, a homolog has been cloned from the green alga Chlamydomonas (Haring et al., 1995). Thus, the prolonged activation of SIP kinase by the elicitins, and to a lesser extent by the fungal cell wall elicitor, could be brought about if these inducers activated SIP kinase but not a corresponding MAPK phosphatase. In contrast, SA might induce both of these activities, thereby ensuring that SIP kinase is activated transiently. Consistent with this negative regulation model is the finding that SIP kinase activity after SA activation rapidly drops below the original basal level observed before stimulation (Zhang and Klessig, 1997).

The activation kinetics for mammalian MAPK have also been shown to be affected by receptor number, downregulation of the receptor, or differential usage of the signaling pathways downstream of the receptor (Marshall, 1995; Vojtek and Cooper, 1995; Kyriakis and Avruch, 1996). Binding proteins or potential receptors for both a β-glucan elicitor (an active compound of cell wall elicitors) and an elicitin have been identified in plants (Wendehenne et al., 1995; Umemoto et al., 1997). Perhaps these different elicitors are perceived by different receptors whose downstream pathways converge into one or more MAPK cascades early in the stress response signaling pathway, just as in mammals and yeast. Exactly how the fungal cell wall elicitor, both elicitins, and SA activate the same kinase is unclear and must await the identification of signal transduction components upstream of SIP kinase, including the elicitor and SA receptors and/or effectors.

In addition to the duration of SIP kinase activation, differential activation of the 44- and 40-kD kinases by the three groups of inducers may also play a critical role in influencing which cellular responses are induced. Although the elicitins induced both the 44- and the 40-kD kinases, the fungal cell wall elicitor induced only the 44-kD kinase. Moreover, activation of this 44-kD kinase by the fungal cell wall elicitor was transient, and it occurred to a substantially lesser extent than that observed with the elicitins (Figure 1). In contrast, SA treatment activated neither the 44- nor the 40-kD kinases (Zhang and Klessig, 1997).

What is the relationship between SIP kinase and other stress-related MAPKs? A 47-kD kinase activity with characteristics of a MAPK has been shown to exhibit prolonged activation kinetics when tobacco cells are treated with a cell wall–derived elicitor from *P. infestans.* Approximately 40% of the peak activity for this kinase was detected 1 hr after treatment, the last time point reported (Suzuki and Shinshi, 1995). We suspect that this 47-kD kinase is the same as that activated by the cell wall elicitor from *P. parasitica*, namely, SIP kinase.

A 46-kD wounding-activated MAPK from tobacco has been identified, and a gene that may encode this kinase, called *WIPK*, has been cloned (Seo et al., 1995). Based on differences between *WIPK* and *SIPK* sequences, it has been suggested that these genes belong to two distinct subgroups of plant MAPKs (Zhang and Klessig, 1997). *SIPK* and its subgroup members encode proteins with an ~20–amino acid extension at their N termini that are not present in those encoded by *WIPK* subgroup members. In addition, the *SIPK* and *WIPK* subgroups appear to be activated differently. Members of the *SIPK* subgroup are not induced at the mRNA level by stimuli, whereas *WIPK* and its orthologs are (Seo et al., 1995; Jonak et al., 1996; Mizoguchi et al., 1996; Bögre et al., 1997; Ligterink et al., 1997; Figure 8A). *SIPK* orthologs include the alfalfa *MMK1* and Arabidopsis *AtMPK6* genes, whereas *WIPK* subgroup members include the alfalfa *MMK4*, Arabidopsis *AtMPK3*, and parsley *ERMK* genes (Figure 5A; Ligterink et al., 1997; Zhang and Klessig, 1997).

Very recently, Ligterink et al. (1997) reported the identification of a 45-kD MAPK from parsley cells that is rapidly activated by a 25–amino acid peptide (Pep25) derived from the 42-kD glycoprotein elicitor secreted by *P. sojae.* A gene termed *ERMK*, which may encode this kinase, has been isolated using the alfalfa *MMK4* cDNA as a heterologous probe. However, the correlation between *ERMK* and the Pep25-activated kinase has yet to be rigorously established. A severalfold increase in *ERMK* transcripts was observed within 30 min of Pep25 treatment, but this seems too slow to account for the increase in kinase activity within 3 min (Ligterink et al., 1997). Furthermore, although the antibody raised against the C-terminal 10 amino acids of MMK4 (termed M7) immunoreacted with the *E. coli*–expressed recombinant ERMK protein, which shares seven out of 10 amino acids with MMK4 at its C terminus, this antibody is also

likely to recognize MMK1, another member of the MAPK family from the same species. MMK1, which belongs to the SIP kinase subgroup, shares eight of its 10 C-terminal amino acids with MMK4 (Figure 5A). We found that Ab-p48C, which is directed against the C-terminal 18 amino acids of SIPK, recognized WIPK because of the sequence conservation at the C terminus between these two MAPKs (Figure 5D).

Currently, the details as to how SIP kinase transmits the signal to facilitate differential responses to the elicitins, fungal cell wall elicitor, and SA remain to be defined, in addition to the upstream components in this MAPK cascade. Like other MAPKs, the SIP kinase may utilize multiple substrates, whose presence may determine which intracellular responses are activated. Identification of these substrates as well as the upstream components should greatly facilitate our understanding of how plants perceive and transduce foreign and endogenous signals associated with defense responses to microbial pathogens.

METHODS

Treatment of Tobacco Cell Suspension Culture

The cell suspension culture was maintained and treated as previously described (Zhang and Klessig, 1997). Log phase cells were used at 3 days after a 1:10 dilution in fresh medium. Treatment with the elicitors or salicylic acid (SA) 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) were harvested by filtration. The cells were quickly frozen in liquid nitrogen and stored at −80°C until analysis. For kinase inhibitor studies, the cells were pretreated with K-252a or staurosporine for 5 min by addition of 1 mM stock solutions in DMSO to a final concentration of 0.5 μM.

The fungal cell wall elicitor was prepared from a heat-released cell wall fraction (a gift from J. Chappell, University of Kentucky, Lexington) of the fungal pathogen *Phytophthora parasitica* and quantitated as previously described (Zhang et al., 1993). The elicitor was used at a final concentration of 50 μg of glucose equivalents per mL of cell suspension culture or as stated in the legend to Figure 9. Parasiticein and cryptogein were generous gifts from B. Tyler and E. Doyle (University of California at Davis). Both elicitins were used at a final concentration of 50 nM.

Preparation of Protein Extracts

To prepare extracts from treated cells, we mixed cells with two 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-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg/mL antipain, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 10% glycerol, and 7.5% polyvinylpolypyrrolidone) and sonicated with a sonicator (model W-375; Heat System-Ultrasonics, Inc., Farmingdale, NY) until all of the cells were disrupted. After centrifugation at 20,000*g* for 20 min, supernatants were transferred into clean tubes, quickly frozen in liquid nitrogen, and stored at −80°C.

Protein Kinase Activity Assay

Kinase activity assays were performed as described previously (Zhang and Klessig, 1997). Briefly, for the in-gel kinase activity assay, extracts containing 10 μg of protein were electrophoresed on 10% SDS–polyacrylamide gels embedded with 0.25 mg/mL of myelin basic protein (MBP) in the separating gel as 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, and 0.1% Triton X-100 [v/v]) three times for 30 min each 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 30 mL of reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, and 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 (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 the kinases. Quantitation of the relative kinase activities was done using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA).

In-solution kinase activity assays were performed at room temperature for 20 min in a final volume of 15 μL containing 0.5 mg/mL of 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 Chemical Co., 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 the 48-kD 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.

Measurement of Medium Alkalization and Cell Viability Assay

The pH of the medium was measured after the cells were removed by filtration through two layers of MiraCloth (Calbiochem) by using a pH meter (digital ionalyzer model 501; Orion Research Inc., Cambridge, MA). Data presented are averages of three replicates ±SE.

Cell viability was assayed as described by Widholm (1972). Briefly, a drop of cells was taken at the indicated times and mixed with a drop of fluorescein diacetate solution (0.01% [w/v] in culture medium) on a microscope slide. The live cells, which fluoresce bright green, were viewed under an epifluorescence microscope (Zeiss Axioskop 20; Carl Zeiss, Inc., Thornwood, NJ). Three fields, each containing >100 cells without big clumps, were counted. The percentage of dead cells was calculated as the average ±SE.

RNA Gel Blot Analysis

RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD), following the manufacturer's instructions. Twenty micrograms of total RNA per lane was separated on 1.2% formaldehyde–agarose

gels and transferred to Zeta probe membranes (Bio-Rad). Blots were hybridized with random primer–labeled inserts consisting of either a full-length cDNA (for *PAL* and *SIPK*) or a 5′ untranslated region (for *SIPK*), as previously described (Zhang et al., 1993).

MonoQ Fast Protein Liquid Chromatography

Protein extracts from the elicitor- or SA-treated cells were centrifuged, either separately or as a mixture containing equal amounts of both, at 130,000*g* for 1 hr. The S130 supernatant was passed through a desalting column (Bio-Rad) equilibrated with buffer A (25 mM Tris, pH 7.5, 1 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM DTT, and 5% glycerol) plus 1 mM PMSF and 5 μg/mL each of antipain, aprotinin, and leupeptin. Approximately 50 mg of S130 fraction was loaded onto a MonoQ HR 5/5 fast-protein liquid chromatography (FPLC) anion exchange column (Phamacia) equilibrated with buffer A plus 50 mM NaCl. After washing with 5 mL of buffer A plus 50 mM NaCl, the column was eluted with a 30-mL gradient of 50 to 400 mM NaCl, followed by a 3-mL gradient of 400 to 1000 mM NaCl, and then 5 mL of 1000 mM NaCl in buffer A.

Two-Dimensional In-Gel Kinase Activity Assay

A Bio-Rad mini two-dimensional gel apparatus was used according to the manufacturer's instructions. Approximately 0.5 units of enzyme were mixed with an equal volume of sample buffer (9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte [Bio-Rad], and 0.4% Bio-Lyte 3/10 ampholyte) and loaded onto an isoelectric focusing tube gel (9.2 M urea, 4% total acrylamide [94.6% acrylamide and 5.4% bisacrylamide], 2% Triton X-100, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonia persulfate, and 0.1% TEMED [Sigma]). After electrophoresis at 500 V for 20 min and then 750 V for 3.5 hr, the tube gel was equilibrated in SDS-PAGE sample loading buffer for 10 min at room temperature and mounted onto a 10% SDS–polyacrylamide gel with 0.25 mg/mL MBP in the separating gel. The kinase activity was then detected by the in-gel kinase activity assay described above.

Expression and Purification of Recombinant Tobacco MAPKs

Fragments of *Ntf4*, *WIPK*, and *NtMPK6*, a new tobacco MAPK, were isolated by using primers corresponding to the conserved MAPK sequences by reverse transcription–polymerase chain reaction (RT-PCR). Full-length clones were obtained by screening a tobacco cDNA library. A BamHI restriction site was introduced in front of the ATG start codons of the *SIPK*, *Ntf4*, *WIPK,* and *NtMPK6* genes by PCR, and the products were cloned into the pGEM-T vector (Promega). To ensure that no errors were introduced by PCR, each construct was checked by sequencing. For each clone, the fragment generated by a BamHI (5′ end) and XhoI (3′ end) digestion was ligated in-frame into the pET-28a(+) vector (Novagen, Madison, WI). The junction between the histidine (His) tag and MAPK start codon was confirmed by sequencing. To produce His tag fusion proteins, BL21 cells transformed with pET-28a(+)/MAPK were induced with 10 mM isopropyl β-D-thiogalatopyranoside for 3 hr. HisMAPK proteins were purified by using a nickel affinity column (Novagen), concentrated, and dialyzed against buffer A overnight at 4°C.

Antibody Production and Immunoblot Analysis

The peptides p48N (MDGSGQQTDTMMSDAGAEQPPTAP) and p48C (KELIYRESLAFNPEYQHM), corresponding to the N terminus and C terminus of SIP kinase, respectively, were synthesized and conjugated to keyhole limpet hemacyanin carrier. Polyclonal antisera were raised in rabbits and purified by affinity column chromatography (Zymed Laboratory Inc., South San Francisco, CA).

For immunoblot analysis, 20 μg of total protein per lane was separated on 10% SDS–polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by semi-dry electroblotting. After blocking for 1 hr in TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) with 5% nonfat dried milk (Carnation, Glendale, CA) at room temperature, the membranes were incubated with either Ab-p48N or Ab-p48C antibody (0.2 μg/mL final concentration in TBS buffer) for 1 hr. After washing in TBS buffer four times, the blots were incubated with a horseradish peroxidase–conjugated secondary antibody (Sigma; 1:10,000 dilution), and the complexes were visualized using an enhanced chemiluminescence kit (Du Pont) and following the manufacturer's instructions.

To detect tyrosine phosphorylation of the Ab-p48N immunoprecipitated SIP kinase, we separated samples equivalent to starting material of ~30 μg of total extract by 10% SDS-PAGE. After electroblotting to a nitrocellulose membrane, phosphotyrosine-containing proteins were detected as reported previously (Zhang and Klessig, 1997).

Immunocomplex Kinase Activity Assay

Protein extract (50 μg) with or without peptide competitor (20 μg/mL final concentration) was incubated with Ab-p48N or Ab-p48C (2.5 μg) 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, and 0.5% Nonidet P-40) at 4°C for 2 hr on a rocker. Approximately 20 μL of packed volume of protein A–agarose was added, and incubation was continued for another 2 hr. Agarose bead–protein complexes were pelleted by brief centrifugation and washed three times with immunoprecipitation buffer and then three times with reaction buffer. Kinase activity in the complex was assayed at room temperature for 20 min in a final volume of 25 μL containing 0.1 mg/mL of MBP and 10 mM of ATP with 1 μCi of γ-^{32}P-ATP. The reaction was stopped by the addition of SDS-PAGE sample loading buffer. After electrophoresis on a 15% SDS–polyacrylamide gel, the phosphorylated MBP was visualized by autoradiography.

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