The Plant Journal (2003) **34**, 149–160

Interaction between two mitogen-activated protein kinases during tobacco defense signaling

Yidong Liu[1], Hailing Jin[2], Kwang-Yeol Yang[1,†], Cha Young Kim[1], Barbara Baker[2] and Shuqun Zhang[1,*]
[1]*Department of Biochemistry, University of Missouri-Columbia, 117 Schweitzer Hall, Columbia, MO 65211, USA, and*
[2]*Department of Plant and Microbial Biology, University of California, Berkeley & Plant Gene Expression Center, USDA-ARS, 800 Buchanan Street, Albany, CA 94710, USA*

Received 8 October 2002; revised 3 January 2003; accepted 8 January 2003.
*For correspondence (fax +1 573 884 4812; e-mail zhangsh@missouri.edu).
†Current address: Department of Biology, Utah State University, Logan, UT 84322, USA.

Summary

Plant mitogen-activated protein kinases (MAPKs) represented by tobacco wounding-induced protein kinase (WIPK) have unique regulation at the level of transcription in response to stresses. By using transcriptional and translational inhibitors, it has been shown previously that *WIPK* gene expression and *de novo* protein synthesis are required for the high-level activity of WIPK in cells treated with elicitins from *Phytophthora* spp. However, regulation of *WIPK* expression and the role(s) of WIPK in plant disease resistance are unknown. In this report, we demonstrate that *WIPK* gene transcription is regulated by phosphorylation and de-phosphorylation events. Interestingly, salicylic acid-induced protein kinase (SIPK) was identified as the kinase involved in regulating *WIPK* gene expression based on both gain-of-function and loss-of-function analyses. This finding revealed an additional level of interaction between SIPK and WIPK, which share an upstream MAPKK, NtMEK2. Depending on whether WIPK shares its downstream targets with SIPK, it could either function as a positive feed-forward regulator of SIPK or initiate a new pathway. Consistent with the first scenario, co-expression of WIPK with the active mutant of NtMEK2 leads to accelerated hypersensitive response (HR)-like cell death in which SIPK also plays a role. Mutagenesis analysis revealed that the conserved common docking domain in WIPK is required for its function. Together with prior reports that (i) WIPK is activated in *NN* tobacco infected with tobacco mosaic virus, and (ii) PVX virus-induced gene silencing of *WIPK* attenuated *N* gene-mediated resistance, we concluded that WIPK plays a positive role in plant disease resistance, possibly through accelerating the pathogen-induced HR cell death.

Keywords: mitogen-activated protein kinase, salicylic acid-induced protein kinase, wounding-induced protein kinase, hypersensitive response.

Introduction

The active defense of plants against invading pathogens often includes rapid hypersensitive response (HR) cell death, the activation of a complex array of defense genes, and the production of antimicrobial phytoalexins (Baker *et al.*, 1997; Martin, 1999; Scheel, 1998; Somssich and Hahlbrock, 1998; Yang *et al.*, 1997). In addition to these local responses, the uninfected portions of the plant usually develop systemic acquired resistance (Ryals *et al.*, 1996; Yang *et al.*, 1997). The activation of these defense responses is initiated by the plant's recognition of pathogens, which is mediated either by a gene-for-gene interaction between a plant resistance (*R*) gene and a pathogen avirulence (*Avr*) gene, or by the perception of a non-race-specific elicitor, such as flagellin by its receptor, FLS2. Signals generated from such interactions are transduced into cellular responses via several interlinked pathways (Baker *et al.*, 1997; Dangl and Jones, 2001; Gómez-Gómez and Boller, 2000; Hammond-Kosack and Jones, 1996; Martin, 1999; Scheel, 1998; Staskawicz *et al.*, 1995).

Mitogen-activated protein kinase (MAPK) cascades are major pathways downstream of sensors/receptors that transduce extracellular stimuli into intracellular responses in yeast and animal cells (Chang and Karin, 2001; Davis, 2000; Herskowitz, 1995; Widmann *et al.*, 1999). While a number of important families of mammalian kinases are absent in plants, they contain an expanded family of MAPKs with more than 20 different members in *Arabidopsis* genome (MAPK Group, 2002; Tena *et al.*, 2001; The

© 2003 Blackwell Publishing Ltd

Arabidopsis Genome Initiative, 2000; Zhang and Klessig, 2001). These MAPKs are likely to play various roles in plant growth, development, and responses to external and endogenous stimuli (Hirt, 1997; Mizoguchi *et al.*, 1997; Tena *et al.*, 2001; Zhang and Klessig, 2001).

Several distinct MAPK cascades have been implicated in the regulation of plant disease resistance, either positively or negatively (Asai *et al.*, 2002; Frye *et al.*, 2001; Innes, 2001; Petersen *et al.*, 2000; Tena *et al.*, 2001; Zhang and Klessig, 2001). Infection of resistant tobacco plants that carry the *N* resistance gene with tobacco mosaic virus (TMV) leads to the activation of two tobacco MAPKs, salicylic acid-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK) (Zhang and Klessig, 1998b). In addition, both MAPKs are activated by Avr9, a fungal Avr factor from *Cladosporium fulvum* in a Cf-9-dependent manner (Romeis *et al.*, 1999). Other non-host-specific elicitors, such as elicitins from *Phytophthora* spp., fungal cell wall-derived elicitors, xylanase from *Trichoderma viride*, and bacterial elicitor harpin could also activate one or both MAPKs (Lee *et al.*, 2001; Suzuki *et al.*, 1999; Zhang *et al.*, 1998, 2000), suggesting that these two MAPKs are converging points after the perception of different pathogens and pathogen-derived elicitors. The orthologs of SIPK and WIPK in other plant species, including AtMPK6 and AtMPK3 in *Arabidopsis*, and SIMK and SAMK in alfalfa, have also been shown to be activated by various stress stimuli (Cardinale *et al.*, 2000; Desikan *et al.*, 2001; Kovtun *et al.*, 2000; Ligterink *et al.*, 1997; Mizoguchi *et al.*, 1996; Munnik *et al.*, 1999; Nühse *et al.*, 2000; Stratmann and Ryan, 1997).

The gene-for-gene specific activation of SIPK and WIPK by TMV and avr9 suggests their role(s) in disease resistance (Romeis *et al.*, 1999; Zhang and Klessig, 1998b). The inhibition of SIPK and WIPK activation by staurosporine and K-252a suppresses the HR-like cell death in tobacco suspension cells treated with fungal elicitin. Furthermore, by adding inhibitors at different times after the elicitin treatment, it was shown that the prolonged activation of SIPK and/or delayed activation of WIPK are required for tobacco cells to commit to a cell death program (Zhang *et al.*, 2000). Prolonged activation of MAPK was also associated with HR-like cell death of tobacco cells treated with xylanase, a fungal elicitor from *T. viride* and *Arabidopsis* cells treated with harpin, a bacterial elicitor from *Pseudomonas syringae* pv. *syringae* (Desikan *et al.*, 2001; Suzuki *et al.*, 1999). More direct evidence for the role of SIPK and WIPK in HR came from a conditional gain-of-function study of NtMEK2^{DD}, the constitutively active mutant of their upstream kinase NtMEK2 (Yang *et al.*, 2001). Using a similar approach, activation of SIPK alone was shown to be sufficient to induce HR-like cell death (Zhang and Liu, 2001). However, the HR-like phenotype is delayed in the absence of WIPK activity, suggesting that WIPK activation might play a role in accelerating the cell death process.

Our previous attempts to alter the activity of WIPK by overexpressing it were unsuccessful, which is in contrast to SIPK, suggesting that WIPK has differential regulation at the post-translational level, even though they share an upstream kinase, NtMEK2 (Zhang and Liu, 2001). The other difference is that *WIPK* is activated at the transcriptional level by various stress stimuli, while *SIPK* gene expression remains constant during plant response to stresses (Romeis *et al.*, 1999; Zhang and Klessig, 1998a,b; Zhang *et al.*, 2000). We found that in unstressed plants or suspension cells, WIPK protein is present at very low basal levels. In response to pathogen infection or treatment of pathogen-derived elicitors, the cellular level of WIPK protein increases, which is preceded by the increase of *WIPK* transcript (Zhang and Klessig, 1998b; Zhang *et al.*, 2000). By the use of actinomycin D, an inhibitor of gene transcription, and cycloheximide, an inhibitor of protein translation, it was shown that the high-level activation of WIPK protein in cells requires both gene transcription and *de novo* protein biosynthesis (Zhang *et al.*, 2000). However, how *WIPK* transcription is regulated and the role of WIPK in plant disease resistance are unknown.

In this report, we show that *WIPK* gene transcription is regulated by phosphorylation and de-phosphorylation events. Interestingly, SIPK was identified as the kinase involved in regulating *WIPK* gene expression based on both gain-of-function and loss-of-function analyses. When WIPK was co-expressed with NtMEK2^{DD}, high levels of WIPK activity were detected, which were associated with accelerated HR-like cell death. Together with our previous reports that (i) WIPK is activated in *NN* tobacco infected with tobacco mosaic virus specifically during resistance response, and (ii) PVX virus-induced gene silencing of *WIPK* attenuated *N* gene-mediated resistance (Jin *et al.*, 2003), we concluded that WIPK plays a positive role in tobacco disease resistance, possibly via accelerating the HR cell death.

Results

A phosphorylation event is required for the activation of WIPK *gene transcription in tobacco cells treated with elicitins from* Phytophthora *spp.*

One of the early pieces of evidence for the involvement of plant MAPK in stress signaling was the transcriptional activation of *WIPK* by wounding (Seo *et al.*, 1995). Later, *WIPK* transcript was shown to be induced by various elicitors and pathogens (Romeis *et al.*, 1999; Zhang and Klessig, 1998b; Zhang *et al.*, 2000). Its orthologs in other plant species, such as *MMK4* from alfalfa, *AtMPK3* from *Arabidopsis*, and *ERMK* from parsley (*Petroselinum crispum*) are similarly induced at the mRNA level (Jonak *et al.*, 1996; Ligterink *et al.*, 1997; Mizoguchi *et al.*, 1996). Their counterpart in wheat, *TaWCK1*, is activated transcriptionally by a fungal

elicitor as well (Takezawa, 1999). These results suggest that stress-induced transcription of *WIPK* or its orthologs evolved early in plants before the divergence of dicots and monocots and might play an important role in plant defense responses.

Our previous report showed that staurosporine and K-252a, two kinase inhibitors, could block the increase in WIPK activity and cell death in tobacco cells treated with elicitins, a group of small proteinaceous non-race-specific elicitors from *Phytophthora* spp. We assumed that these inhibitors suppressed the activity of upstream MAPKK of WIPK and therefore prevented the activation of WIPK (Zhang *et al.*, 2000). Quite unexpectedly, we later found that the induction of WIPK protein by elicitin was also suppressed by these kinase inhibitors (Figure 1a, lower panel), which should be the direct cause for the suppression of WIPK activity (Figure 1a, upper panel; this panel is identical to Figure 4 in Zhang *et al.* (2000). It is reproduced here for easy comparison as the result shown in the lower panel was for the same protein extracts.). In cells pre-treated with DMSO, the solvent used for inhibitor stocks, cryptogein or parasiticein led to a significant increase in WIPK protein after 3 h as determined by immunoblot analysis using WIPK-specific antibody, which is consistent with previous reports (Zhang *et al.*, 2000). However, no or very little WIPK protein accumulated at 3 or 8 h in cells pre-treated with either kinase inhibitor (Figure 1a, lower panel). RNA blot analysis revealed that the *WIPK* mRNA accumulation was blocked as well (Figure 1b). The increase in *WIPK* mRNA and, to a lesser extent, WIPK protein at 8 h in cells pre-treated with staurosporine or K-252a (Figure 1) is related to the degradation of these kinase inhibitors over time because the addition of a second dose of kinase inhibitor could further delay the increase (data not shown). Based on the fact that the increase in *WIPK* mRNA in tobacco cells after elicitin treatment can be blocked by actinomycin D (Zhang *et al.*, 2000), we conclude that it is the *WIPK* gene activation, rather than the change in mRNA stability, that is dependent on a kinase activity. The decrease of *WIPK* mRNA in cells pre-treated with DMSO at 8 h is caused by the death of cells in the suspension cultures (Figure 1b; Zhang *et al.*, 2000).

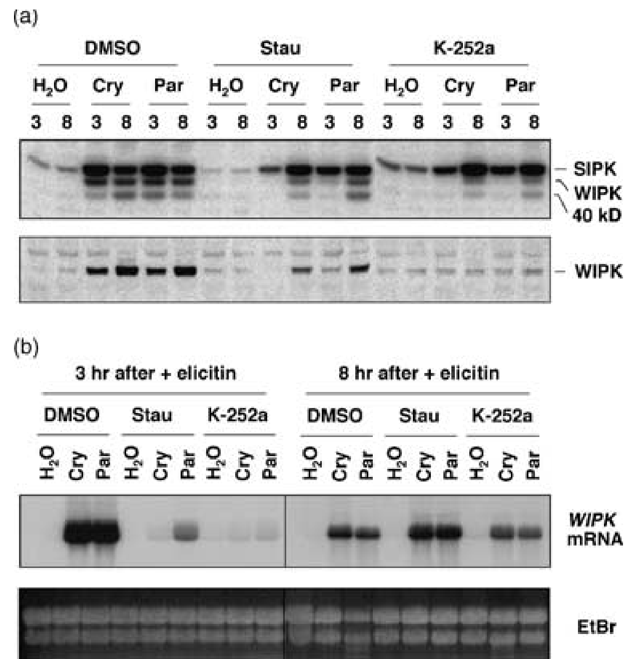

Figure 1. Kinase inhibitors block elicitin-induced *WIPK* gene transcription and protein accumulation.
(a) Inhibition of elicitin-induced WIPK activation by kinase inhibitors is associated with the blockage of WIPK protein accumulation. Tobacco cells were pre-treated with kinase inhibitor, either K-252a (1 μM) or staurosporine (1 μM), for 5 min. In the control, an equal volume of DMSO, the solvent used to prepare the inhibitor stocks, was added. Cryptogein (Cry) or parasiticein (Par) was added at zero time to the final concentration of 25 nM, and two sets of samples were collected at 3 and 8 h. Protein extracts were prepared from one set of samples, and the kinase activity was determined by an in-gel kinase assay with MBP as a substrate (upper panel). The levels of WIPK protein were determined by immunoblot analysis using WIPK-specific antibody, Ab-p44N (lower panel).
(b) Inhibition of elicitin-induced *WIPK* gene expression by kinase inhibitors. Total RNA was prepared from the second set of samples. Equal amounts of RNA (10 μg) were electrophoresed on a 1.2% formaldehyde-agarose gel and transferred to a Zeta-Probe membrane (Bio-Rad). The steady-state levels of *WIPK* were determined by RNA blot analysis. An ethidium bromide (EtBr)-stained gel was used to show equal loading of samples. The degradation of rRNA seen at 8 h was caused by cell death. Note: The loading sequence for RNA blot analysis was different from that for protein analysis.

SIPK activation precedes and correlates with the WIPK *gene expression*

The activation of *WIPK* gene expression by stresses in tobacco is preceded by the increase of SIPK activity (Romeis *et al.*, 1999; Zhang and Klessig, 1998a,b; Zhang *et al.*, 2000). In addition, suppression of SIPK activity by kinase inhibitors correlated with the inhibition of elicitin-induced *WIPK* gene activation (Figure 1). These results indicate that SIPK might be the kinase involved in the regulation of *WIPK* transcription. To strengthen this correlation, experiment with delayed addition of the kinase inhibitor was performed. In this experiment, staurosporine was added either at the same time (0 h), or 0.5, 1, 2, and 3 h after the addition of cryptogein. Samples were taken at 0, 0.5, 1, 2, 4, and 8 h after the addition of kinase inhibitor, except for the control where no kinase inhibitor was added. In-gel kinase assay (Figure 2, left panels) and RNA blot analysis of *WIPK* (Figure 2, right panels) showed a close correlation between SIPK activity and *WIPK* transcript levels. Two hours after the addition of cryptogein, the addition of kinase inhibitor could no longer suppress either the activation of SIPK or the steady-state level of *WIPK* mRNA (Figure 2), which suggests that the signaling pathway controlling *WIPK* gene expression was fully activated.

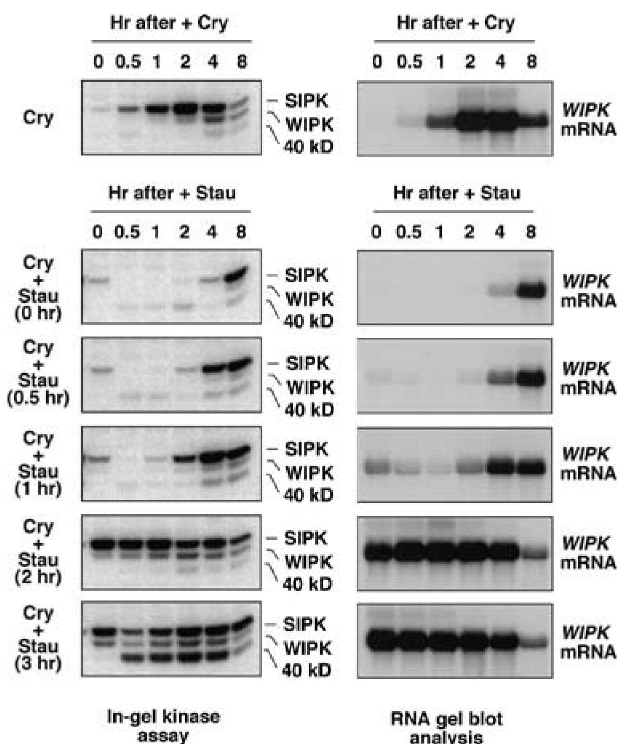

Figure 2. Inhibition of *WIPK* gene activation by kinase inhibitors correlated with the inhibition of SIPK activity.
To correlate SIPK activation and *WIPK* gene expression after elicitin treatment, experiments with delayed kinase inhibitor treatment were performed. In this experiment, cryptogein (Cry) was added at zero time and a kinase inhibitor, staurosporine (Stau, 1 μM) was added either at the same time (0 min), or 0.5, 1, 2, and 3 h after the addition of the cryptogein. Two sets of samples were taken at the indicated times after the addition of staurosporine. Protein extracts were prepared from one set of samples, and the kinase activity was determined by an in-gel kinase assay with MBP as a substrate (left panel). Total RNA was prepared from the second set of samples. *WIPK* transcript levels were determined by RNA blot analysis (right panel).

In this experiment, staurosporine can inhibit both the activation of SIPK and the activity of SIPK. Two hours after elicitin treatment, SIPK was fully activated in cells as demonstrated by in-gel kinase assay (Figure 2, left, fifth and sixth panels). However, the kinase inhibitor added at this time could still suppress the activity of SIPK *in vivo* (Zhang and Klessig, 1997). The failure of staurosporine to reduce the level of *WIPK* mRNA after 2 h of elicitin treatment suggests one of the following two scenarios. First, the SIPK substrates were fully phosphorylated 2 h after the addition of elicitin. The phosphorylated substrates stayed active even after SIPK was inhibited by staurosporine, which could maintain the high rate of *WIPK* transcription in cells. Alternatively, *WIPK* mRNA is very stable. As a result, although the *de novo* transcription of *WIPK* gene was blocked after staurosporine was added at this late time, the steady-state level of *WIPK* mRNA remained constant.

Phosphatase inhibitors activate SIPK and induce WIPK expression

MAPK activity is regulated by reversible phosphorylation and de-phosphorylation. Several reports demonstrated that the treatment of cells with phosphatase inhibitors induces the activity of one or two MAPKs (Cazalé *et al.*, 1999; Suzuki *et al.*, 1999; Taylor *et al.*, 2001). In tobacco cells treated with either calyculin A or okadaic acid, transient activation of an MAPK was observed (Figure 3, top panel). This MAPK was confirmed to be SIPK by immune-complex kinase assay (data not shown). The activation of SIPK was followed by the increase of *WIPK* mRNA and protein (Figure 3, middle and bottom panels). Here, a relatively transient activation of SIPK was associated with long-lasting increase of *WIPK* transcripts. One possible explanation is that phosphatase inhibitors also blocked the de-phosphorylation of SIPK substrate(s) and therefore the turning-off of *WIPK* transcription. In cells treated with calyculin A or okadaic acid, we did not observe the increase in *WIPK* activity, although the WIPK protein was induced (Figure 3, top and bottom panels). This is consistent with our previous observation that the increase in WIPK activity requires elevated levels of its upstream kinase (Zhang and Liu, 2001; Zhang *et al.*, 2000). In cells treated with the phosphatase inhibitor, the increase in SIPK activity is likely because of the inhibition of its inactivation and there is no net increase in the upstream kinase activity. Alternatively, there is only a very transient activation of the upstream MAPKK, possibly NtMEK2, after phosphatase inhibitor treatment. However, the temporal difference between the transient activation of NtMEK2 and the accumulation of WIPK protein prevents the activation of the newly synthesized WIPK. In contrast to our observation that a single MAPK, i.e. SIPK, is

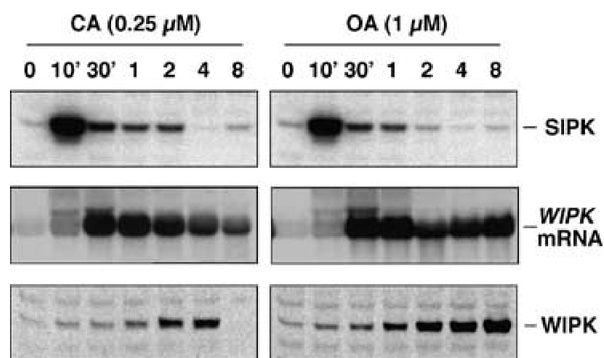

Figure 3. Phosphatase inhibitor-induced *WIPK* gene transcription is preceded by the activation of SIPK.
Tobacco cells were treated with phosphatase inhibitor, calyculin A (CA, 0.25 μM) or okadaic acid (OA, 1 μM). Samples were harvested at the indicated times for protein and total RNA preparation. SIPK activity was determined by an in-gel kinase assay with MBP as a substrate (top panel). *WIPK* transcript levels were determined by RNA blot analysis (middle panel). The levels of WIPK protein were determined by immunoblot analysis using WIPK-specific antibody, Ab-p44N (bottom panel).

activated by phosphatase inhibitors, Cazalé *et al.* (1999) and Taylor *et al.* (2001) reported the activation of two MAPKs, possibly SIPK and WIPK, by calyculin A. The difference in the observation is likely because of the experimental procedures. In these two reports, cells were filtered/washed before the addition of the inhibitor. Our data showed that the filtration/washing of tobacco cells activates SIPK and induces WIPK protein (Zhang *et al.*, 2000). The presence of WIPK protein before the phosphatase inhibitor treatment may lead to the simultaneous activation of SIPK and WIPK by calyculin A treatment.

Activation of SIPK by NtMEK2^{DD} leads to an increase in WIPK *mRNA and protein, which is then activated by the same MAPKK*

Despite good correlative evidence from pharmacologic studies, more direct evidence for the involvement of SIPK in *WIPK* gene expression was needed. In tobacco leaves transiently transformed with *NtMEK2^{DD}*, SIPK and WIPK are activated simultaneously after the induction of *NtMEK2^{DD}* expression (Yang *et al.*, 2001). This is because WIPK protein has already been induced after *Agrobacterium* infiltration (data not shown). We were expecting that in permanent *NtMEK2^{DD}* transgenic plants where *Agrobacterium* was absent, the SIPK function could be examined alone. However, to our surprise, we still saw the activation of WIPK along with SIPK upon the induction of *NtMEK2^{DD}* expression (Figure 4, second panel; the first and second panels of this figure are the same as those of Figure 1a of Jin *et al.* (2003). They are reproduced here for easier comparison because the third and fourth panels are from the same set of samples.). A careful examination of the data however revealed that the activation of WIPK was delayed, which is similar to that in *NN* tobacco plants infected with TMV or in cells treated with elicitin (Zhang and Klessig, 1998b; Zhang *et al.*, 2000). This result suggests that the activation of SIPK, which pre-exists in the cells, by NtMEK2^{DD} may trigger the activation of *WIPK* expression. To prove this, we analyzed the levels of *WIPK* mRNA and protein in the same samples. As shown in Figure 4 (third panel), *WIPK* gene transcription was turned on after the SIPK activation by NtMEK2^{DD}. The 2 h delay for the SIPK activation was the time required for NtMEK2^{DD} protein biosynthesis after DEX treatment. The accumulation of WIPK protein followed (Figure 4, fourth panel), which was concurrent with the appearance of WIPK activity (Figures 4, second panel). In the control NtMEK2^{KR} plants, none of these events were observed, although NtMEK2^{KR} protein was induced to similar levels after DEX treatment (Figure 4). The decrease in NtMEK2^{DD} protein, SIPK and WIPK activity, *WIPK* transcript, and SIPK and WIPK protein in *NtMEK2^{DD}* plants 24 h after DEX treatment was because of the cell death and dehydration of the leaves.

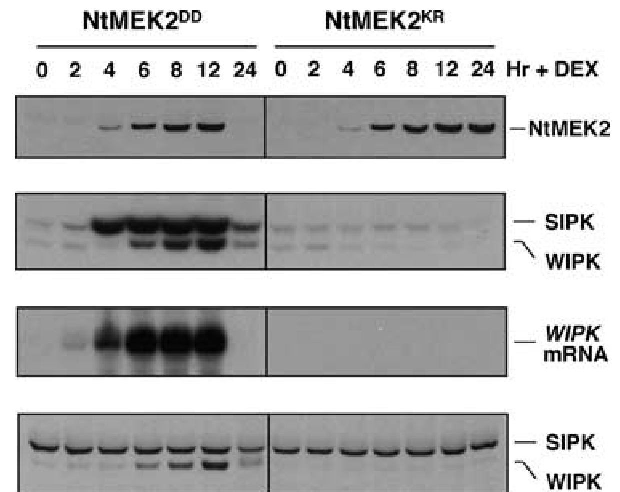

Figure 4. Activation of endogenous SIPK by NtMEK2^{DD} in permanent transgenic plants is followed by *WIPK* gene activation, WIPK protein accumulation, and increase of WIPK activity.
Permanent *NtMEK2^{DD}* transgenic plants were treated with DEX (30 μM), and two sets of leaf discs were harvested at the indicated times for protein and total RNA preparations. As a control, permanent NtMEK2^{KR} transgenic plants were treated side-by-side. The induction of transgene was monitored by immunoblot analysis using anti-Flag antibody (first panel). The activation of endogenous SIPK and WIPK was determined by an in-gel kinase assay with MBP as a substrate (second panel). The activation of *WIPK* gene expression was determined by RNA-gel blotting analysis (third panel). The levels of SIPK and WIPK proteins were assayed by immunoblot analysis using an equal mixture of SIPK- and WIPK-specific antibodies (fourth panel).

SIPK is required for TMV-induced WIPK protein accumulation

In addition to gain-of-function evidence, loss-of-function analysis demonstrated that SIPK is required for TMV-induced WIPK protein accumulation. Previously, we showed that the suppression of *SIPK* or *WIPK* by PVX-induced gene silencing compromised *N*-gene-mediated resistance of TMV in *N. benthamiana*::*NN* plants (Jin *et al.*, 2003). Similar to TMV infection of *N. tabacum* cv. Xanthi (*NN*) (Zhang and Klessig, 1998b), *N. benthamiana*::*NN* plants challenged with TMV also accumulated high levels of WIPK protein 2 days post-TMV inoculation (Figure 5a, top panel). However, when SIPK in *N. benthamiana*::*NN* plants was silenced by virus-induced gene silencing (VIGS), the induction of WIPK protein by TMV infection was suppressed (Figure 5a, middle panel). This result demonstrated that SIPK is required for pathogen-induced WIPK accumulation. The day 2 samples were harvested from *Agrobacterium*-infiltrated leaves. As in leaves used for transient transformation experiments, WIPK protein accumulated because of the effect of *Agrobacterium*. In contrast, in leaves inoculated with *Agrobacterium* carrying pGrWIPK construct, no WIPK protein accumulation was detected (Figure 5a, bottom panel). This might be a result of rapid local gene silencing of *WIPK*. However, the local silencing of SIPK

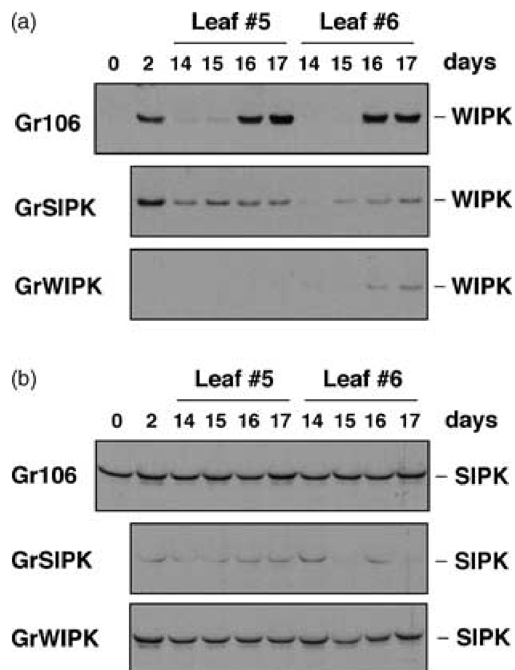

Figure 5. Suppression of SIPK by PVX virus-induced gene silencing inhibited the TMV-induced WIPK protein accumulation.
N. benthamiana::NN plants were infiltrated with *Agrobacterium* carrying empty binary PVX vector (pGr106) or a construct with fragment of *SIPK* (pGrSIPK) or *WIPK* (pGrWIPK). Fourteen days later, the upper leaves that show the silencing of target gene were inoculated with GFP-tagged TMV. Samples were collected just before (0 day), or 2, 14, 15, 16, and 17 days after *Agrobacterium* inoculation. The samples collected 14, 15, 16, and 17 days after *Agrobacterium* inoculation corresponded to 0, 1, 2, and 3 days after TMV inoculation. Day 2 samples were from local *Agrobacterium*-inoculated leaves. Day 14, 15, 16, and 17 samples were from leaf #5 and #6, which were assayed independently and gave similar results. After the protein extracts were prepared, the levels of (a) WIPK and (b) SIPK proteins were determined by immunoblot analysis using WIPK- and SIPK-specific antibodies.

observed on day 2 did not suppress the *Agrobacterium*-induced WIPK protein accumulation (Figure 5a,b, middle panels). One possible interpretation is that the induction of WIPK protein by *Agrobacterium*, which is detectable within a few hours after *Agrobacterium* infiltration, preceded the reduction of SIPK protein after gene silencing.

In plants where *WIPK* gene was silenced (Figure 5a, bottom panel), the level of SIPK was not reduced (Figure 5b, bottom panel), demonstrating the specificity of gene silencing. This conclusion is also supported by evidence at the mRNA level (Jin *et al.*, 2003). It is interesting to see that in SIPK-suppressed leaves, the basal levels of WIPK protein before TMV inoculation were a little higher than those in the controls (Figure 5a, upper and middle panels). This result also indicates that the inhibition of WIPK protein accumulation in SIPK-suppressed leaves after TMV infection was not because of the non-specific cross-suppression. Based on the data presented here, which include (i) pharmacological studies using kinase and phosphatase inhibitors, (ii) the conditional gain-of-function study using *NtMEK2^{DD}* transgenic plants, and (iii) the loss-of-function study using VIGS, we conclude that SIPK is involved in regulating *WIPK* gene expression and protein accumulation in response to stress stimuli.

Activation of ectopically expressed WIPK accelerates the HR-like cell death

Prolonged activation of SIPK and delayed activation of WIPK are associated with HR-cell death induced by avirulent pathogen or pathogen-derived elicitors (Suzuki *et al.*, 1999; Zhang and Klessig, 1998b; Zhang *et al.*, 2000). In the absence of WIPK activity, cell death induced by SIPK activation is delayed, suggesting that WIPK might function in accelerating the cell death (Zhang and Liu, 2001). Our previous attempts to alter WIPK activity by overexpressing it under either CaMV 35S promoter or steroid-inducible promoter have failed. In both cases, we obtained transgenic lines that overexpress WIPK either constitutively when 35S promoter was used (Zhang, S. and Klessig, D.F., unpublished data) or under induced condition when pTA7002 vector was used (Zhang and Liu, 2001). One interpretation for these results is that WIPK activation requires the activation of its upstream kinase NtMEK2.

As a result, we attempted to co-express WIPK and NtMEK2^{DD} by transforming *WIPK* into *NtMEK2^{DD}* transgenic plants using *Agrobacterium*-mediated transient transformation. In this experiment, we chose to use *NtMEK2^{DD}*-22 line, which expresses NtMEK2^{DD} at a relatively low level after the application of DEX, and cell death phenotype requires about 24–36 h to develop instead of the 12–16 h for lines with higher levels of inducibility. *WIPK* transgene was constructed in pTA7002 vector and tagged with a Flag epitope at the N-terminus as previously described (Zhang and Liu, 2001). As negative controls, two WIPK mutants, WIPK^{KR}, in which the catalytically essential Lys (K) in the kinase subdomain II was replaced with Arg (R), and WIPK^{AF}, in which the activation motif TEY was replaced with AEF, were also constructed. Both mutants were tagged with Flag epitope at the N-termini as well. As shown in Figure 6(a, top panel), co-expression of Flag-tagged WIPK (F-WIPK) with NtMEK2^{DD} resulted in the activation of F-WIPK, which migrates in between the endogenous SIPK and WIPK. Immune-complex kinase assay using anti-Flag antibody confirmed its identity (data not shown). As expected, F-WIPK^{KR} and F-WIPK^{AF} remained inactive when they were co-expressed with NtMEK2^{DD}. Immunoblot analysis using anti-Flag antibody demonstrated that F-WIPK, F-WIPK^{KR}, and F-WIPK^{AF} were expressed at similar levels (Figure 6a, middle panel). This result also provided experimental evidence that the TEY motif is required for the activation of WIPK by its upstream MAPKK. The

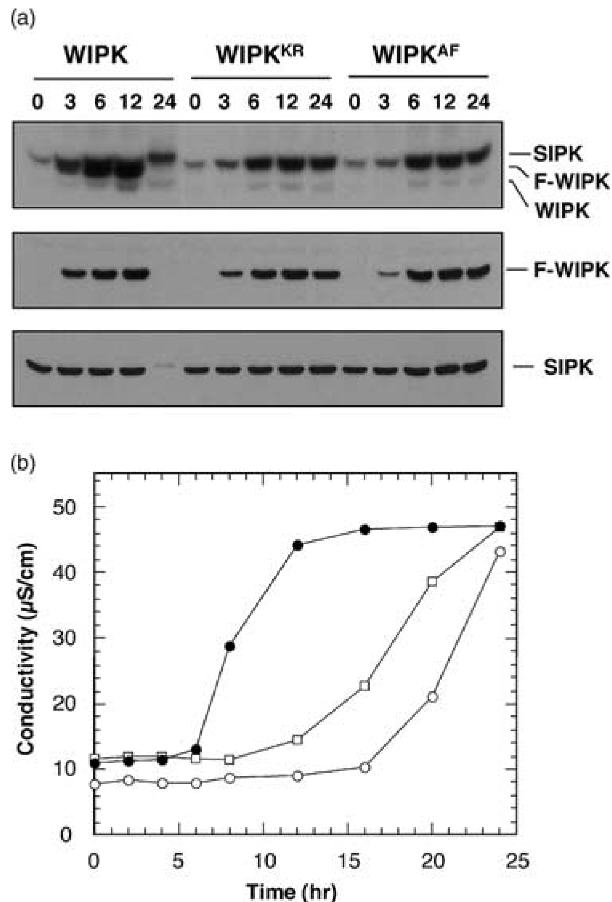

Figure 6. Co-expression of WIPK with $NtMEK2^{DD}$ accelerated HR-like cell death in tobacco.
(a) Activation of ectopically expressed WIPK by $NtMEK2^{DD}$. T_2 plants from $NtMEK2^{DD}$-22 transgenic line were transiently transformed with Flag-tagged WIPK, or its inactive mutant $WIPK^{KR}$ or $WIPK^{AF}$, by *Agrobacterium*-mediated transformation. DEX (30 μM) was applied 48 h later, and samples were taken at the times indicated. The MAPK activities in the leaf tissue were determined by in-gel kinase assay with MBP as a substrate (top panel). The expression of Flag-WIPK was monitored by immunoblot analysis using anti-Flag antibody (middle panel). The level of SIPK protein was determined by immunoblot analysis using SIPK-specific antibody, Ab-p48N (bottom panel).
(b) Co-expression of *WIPK* accelerated HR-like cell death in *$NtMEK2^{DD}$* plants after DEX treatment. Cell death was quantified by measuring electrolyte leakage. Filled circle (●), co-transformed with WIPK; open square (□), co-transformed with $WIPK^{KR}$; open circle (○), control without *Agrobacterium* infiltration.

disappearance of F-WIPK at 24 h (Figure 6a, middle panel, lane 5) was because of the cell death and dehydration of the tissue. The expression of the permanently transformed Flag-tagged $NtMEK2^{DD}$ was much lower than that of F-WIPK and therefore did not show up in autoradiogram with suitable exposure for WIPK. As controls, the levels of SIPK were also determined, which remained constant except in leaf tissues co-transformed with F-WIPK for 24 h when tissues were dehydrated and little protein could be extracted (Figure 6a, bottom panel). The up-shift of SIPK (Figure 6a, lane 5 of top and bottom panels) was because of the loss of the abundant Rubisco, the large subunit of which migrates just above SIPK and pushes the SIPK band down a little in SDS–PAGE.

Cell death in leaf tissues co-expressing F-WIPK and $NtMEK2^{DD}$ was much faster than that in the controls. We used the electrolyte leakage to monitor the progress of cell death quantitatively. The amount of electrolytes leaked into bathing water to the total electrolytes released after complete tissue collapse should represent the percentage of cell death. As shown in Figure 6(b), the time required for the leakage of half of the electrolyte was reduced from approximately 22 h to approximately 8 h when WIPK was co-expressed with $NtMEK2^{DD}$. Leaves co-transformed with $WIPK^{KR}$ also showed acceleration of cell death, in which the time for half electrolyte leakage was shortened to approximately 16 h. This is likely because of the presence of *Agrobacterium* as $WIPK^{KR}$ is inactive.

Common docking (CD) domain is essential for the WIPK activation by NtMEK2

SIPK always shows basal-level activity in cells as determined by the in-gel kinase assay. Overexpression of SIPK resulted in the elevation of SIPK activity and cell death, which is likely a result of the basal-level activity of NtMEK2 in the cells (Zhang and Liu, 2001). However, overexpression of WIPK under the same condition did not lead to the increase in its activity, indicating that the activation of WIPK requires high activity of its upstream kinase. Domain swapping experiments demonstrated that the C-terminal sequence of WIPK contains the sequence determinant for this differential regulation (Zhang and Liu, 2001). To determine if the CD domain in the C-terminus of WIPK is required for its activation by $NtMEK2^{DD}$, mutants with the conserved Asp (D) residues substituted with Asn (N), $WIPK^{2N}$, the WIPK(D337N) mutant, and $WIPK^{NN}$, the WIPK(D334N/D337N) double mutants, were co-expressed with $NtMEK2^{DD}$ in tobacco leaves. As shown in Figure 7(a, top panel), the activation of $WIPK^{NN}$ by $NtMEK2^{DD}$ was greatly diminished, while that of $WIPK^{2N}$ stayed at a level similar to that of WIPK. This result suggested that either the first Asp residue or both are required for the WIPK activation by its upstream kinase. The expression of F-WIPK, F-$WIPK^{2N}$, and F-$WIPK^{NN}$ from the transient transformation was at similar levels as determined by immunoblot analysis with anti-Flag antibody (Figure 7a, middle panel). The level of SIPK protein remained constant except in tissues that were dehydrated, and little protein could be extracted (Figure 7a, bottom panel, lane 5). In leaves co-transformed with F-$WIPK^{2N}$, SIPK protein was still detected 24 h after DEX treatment. In this case, the tissue had collapsed, but not dehydrated yet. The up-shift of SIPK was because of the degradation of Rubisco, the large subunit of which

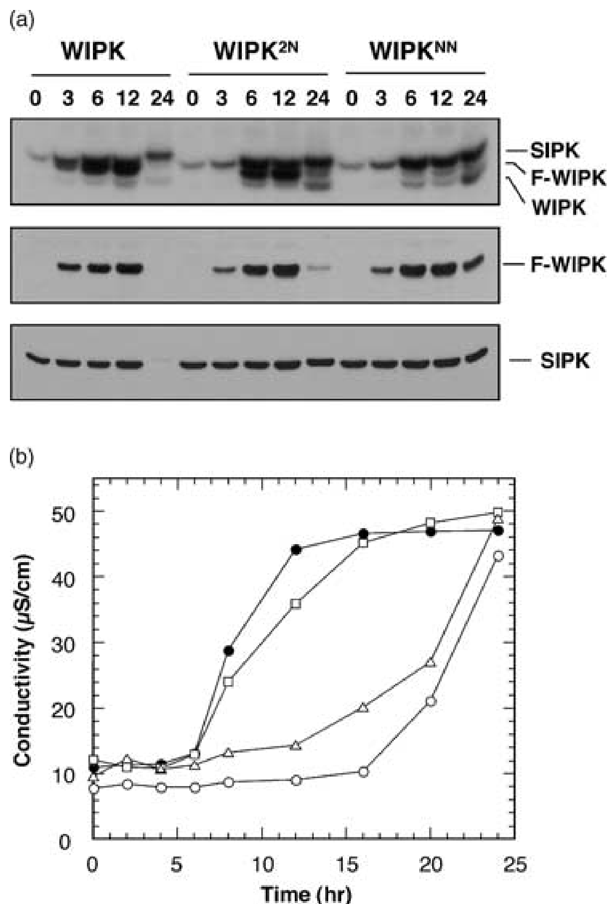

Figure 7. The common docking (CD) domain of WIPK is required for its function *in vivo*.
(a) The conserved Asp residue in the CD domain of WIPK is essential for its activation by NtMEK2^{DD}. To study the importance of Asp residues in the CD domain of WIPK, we replaced the conserved Asp (D) residues in this domain with Asn (N). WIPK^{2N}, the WIPK(D337N) mutant, and WIPK^{NN}, the WIPK(D334N/D337N) double mutant, were transiently transformed into T_2 plants from NtMEK2^{DD}-22 transgenic line. Transgene expression was induced by the application of DEX (30 μM) 48 h later, and samples were taken at the times indicated. The MAPK activities in the leaf tissue were determined by in-gel kinase assay with MBP as a substrate (top panel). The expression of transiently transformed WIPK was monitored by immunoblot analysis using anti-Flag antibody (middle panel). The level of SIPK protein was determined by immunoblot analysis using SIPK-specific antibody, Ab-p48N (bottom panel).
(b) Mutation of the Asp residue in the CD domain of WIPK compromised its ability in accelerating the HR-like cell death. Cell death was quantified using electrolyte leakage by measuring the conductivity of the water. Filled circle (●), co-transformed with WIPK; open square (□), co-transformed with WIPK^{2N}; open triangle (△), co-transformation with WIPK^{NN}, and open circle (○), control without *Agrobacterium* infiltration.

migrates just above SIPK and pushes the SIPK band down a little in SDS–PAGE (Figure 7a, bottom panel, lane 10).

The cell death as indicated by electrolyte leakage was first detected in leaves co-transformed with WIPK. The progress of cell death in leaves co-transformed with WIPK^{2N} was similar to that in WIPK. In contrast, cell death in leaves co-transformed with WIPK^{NN} showed significant delay, which is similar to that with WIPK^{KR} mutant (Figures 6b and 7b), suggesting that the conserved Asp residues in the CD domain are required for WIPK function *in vivo*.

Discussion

Stress-induced *WIPK* transcription is preceded by the increase in SIPK activity. In addition, the kinetics of *WIPK* gene activation and the induction of SIPK activity match as well, i.e. prolonged activation of SIPK is associated with long-lasting activation of *WIPK* transcription such as in cells treated with elicitins, whereas transient activation of SIPK is followed by a transient activation of *WIPK* transcription such as in wounded leaves (Zhang and Klessig, 1998a; Zhang *et al.*, 2000). In this study, we showed that *WIPK* gene activation in tobacco suspension cells treated with elicitin is dependent on a kinase activity whose kinetics of activation correlates with that of SIPK (Figures 1 and 2). In addition, treatment of cells with protein phosphatase inhibitors activated SIPK, which was followed by *WIPK* transcription and WIPK protein accumulation (Figure 3). These results lead to our hypothesis that SIPK regulates the transcription of *WIPK*. Consistent with this, the activation of pre-existing SIPK by NtMEK2^{DD} in transgenic plants resulted in the expression of *WIPK* gene, which was followed by an increase in WIPK protein and WIPK activity. Furthermore, when SIPK was silenced by VIGS, TMV-induced WIPK accumulation was abolished, demonstrating that SIPK is required for pathogen-induced *WIPK* transcription. This finding revealed an additional level of interaction between SIPK and WIPK, which share an upstream MAPKK, NtMEK2 (Yang *et al.*, 2001).

Figure 8 depicts our current model illustrating the interrelationship and roles of SIPK and WIPK in plant defense responses. SIPK, NtMEK2, and the unidentified upstream MAPKKK(s) pre-exist in cells. Upon the perception of invading pathogens, NtMEK2 is activated by its upstream MAPKKK(s), which in turn activates the pre-existing SIPK. Activation of SIPK turns on the transcription of *WIPK* gene, which leads to the accumulation of WIPK protein. The newly synthesized WIPK protein is then activated by the active NtMEK2. There are cases where the accumulation of WIPK protein is not followed by the increase of its activity, such as in cells treated with fungal cell wall elicitor or phosphatase inhibitors (Figure 3) (Zhang *et al.*, 2000). This is likely because of the decrease of NtMEK2 activity by the time the WIPK protein accumulates to a significant level. Consistent with this speculation, the activation of SIPK is also transient in these cells (Zhang *et al.*, 1998, 2000). Through an unknown mechanism, the prolonged activation of SIPK and delayed activation of WIPK could lead to the generation of reactive oxygen species, which is likely to be the direct cause of cell death (Ren *et al.*, 2002). Besides

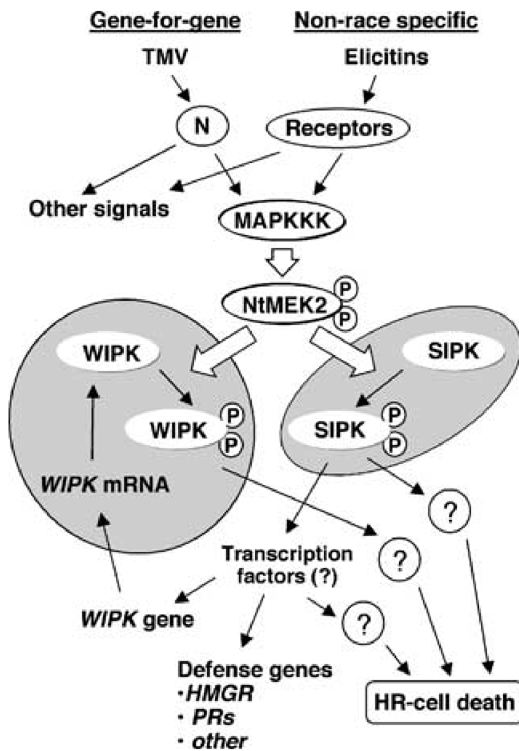

Figure 8. Model depicts the functions and interrelationship between SIPK and WIPK in tobacco defense signaling.
Plant cells detect the invading pathogens through either gene-for-gene interaction or the binding of non-race-specific elicitor to its receptor. Signals generated from both types of interactions lead to the activation of NtMEK2 by unidentified MAPKKK(s). Active NtMEK2 phosphorylates and activates the pre-existing SIPK. SIPK activation leads to the transcription of *WIPK* gene, possibly via the phosphorylation activation of a transcription factor. The increase of *WIPK* transcripts is followed by the accumulation of WIPK protein, which is activated by NtMEK2, the same MAPKK that activates SIPK. Based on gain-of-function analysis, WIPK functions as a positive feed-forward regulator of SIPK in plant defense responses. Such a role for WIPK might rely on a simple dose–response relationship if WIPK shares the same substrate(s) as SIPK. Alternatively, WIPK could act on a new set of substrates, which accelerates the HR-like cell death. Besides *WIPK*, SIPK is likely to be involved in the regulation of other defense genes, such as *HMGR*, either directly or indirectly.

regulating the expression of *WIPK*, SIPK is likely to be involved in the activation of several other defense genes either directly or indirectly (Yang *et al*., 2001).

Based on gain-of-function analysis, prolonged activation of SIPK alone is sufficient to induce HR-like cell death. However, the cell death phenotype is delayed in the absence of WIPK activity (Zhang and Liu, 2001). Here, we demonstrated that the co-expression of WIPK with NtMEK2^{DD} resulted in high-level activation of WIPK, which leads to accelerated cell death. As WIPK and SIPK shared a common upstream kinase and the activation of SIPK induced *WIPK* gene expression and *de novo* protein synthesis, we conclude that WIPK may function as a positive feed-forward regulator of SIPK in plant defense signaling. There are two possible scenarios for the action of WIPK in this pathway. One is that WIPK shares the same substrate(s) with SIPK and the effect of WIPK is mainly through an additive dose-dependent response. Alternatively, WIPK has unique substrate(s), and the activation of WIPK turns on a new pathway or pathways that accelerate the cell death. The determination of the exact mechanism awaits the identification of the substrate(s) of these two interlinked MAPKs.

In this report, we focus on the regulation of the induced level of *WIPK* expression. However, we did notice lower level of WIPK protein in untreated cells (Figures 1a and 3, zero time point). It is difficult to determine whether this low-level accumulation of WIPK protein is because of the prior stresses imposed on the cells, such as the constant mechanical shaking of cell suspension cultures, or because of the basal-level expression unrelated to stresses. It is also interesting for us to notice that the level of WIPK protein was a little higher in SIPK VIGS plants before TMV infection (Figure 5a). One possible interpretation is that, in the absence or presence of only a reduced level of SIPK, basal-level expression of WIPK in the cells was elevated to compensate the loss of SIPK. In a recent report, it was concluded that the suppression of SIPK by RNA interference does not alter the level of WIPK protein (Samuel and Ellis, 2002), which apparently contradicts our results. This discrepancy could arise from the differences in treatments and experimental procedures. The level of WIPK protein is high before ozone treatment, and ozone does not induce WIPK at the protein level (Samuel and Ellis, 2002). The high level of WIPK protein before ozone treatment could be related to prior exposure of plants to stress(s) and the induction of WIPK protein. Under this condition, additional stress treatment may not be able to elevate WIPK protein to a higher level. It is equally possible that the disagreement is caused by the difference between the specificity and sensitivity of the two WIPK antibodies used in the two studies. Our WIPK antibody can recognize recombinant WIPK protein at a subnanogram range, and the specificity of the antibody has been rigorously demonstrated by competition experiments (Zhang and Klessig, 1998a,b). In contrast, the antibody used in the study performed by Samuel and Ellis (2002) stained submicrogram level (as estimated from the Coomassie Blue-stained gel shown in Figure 1 of Seo *et al*., 1999) of recombinant WIPK only weakly. However, a band of about equal intensity was detected in 50 μg of total protein (Seo *et al*., 1999), which indicates that WIPK is highly abundant in tobacco leaves (approximately 1 μg in 50 μg total protein). This is not consistent with our estimation of WIPK protein in induced tobacco leaves (approximately 0.5 ng in 10 μg total protein), which raises the concern whether the band detected using this antibody is indeed WIPK. It is possible that this antibody is not sensitive enough to detect the real WIPK in the total protein extracts.

There are situations where only SIPK becomes activated, such as in tobacco cells subjected to salt or osmotic stress or treated with fungal cell wall elicitors (Hoyos and Zhang, 2000; Mikolajczyk *et al.*, 2000; Zhang *et al.*, 1998). In these cases, the activation of SIPK is transient, which might be a result of transient activation of NtMEK2 in the cells. When plants are challenged by avirulent pathogens, such as TMV infection of *NN* tobacco plants, or cells treated with elicitins from *Phytophthora* spp. that induce HR cell death, sustained activation of SIPK is associated with a delayed activation of WIPK. However, there was no instance that we observed the activation of WIPK without the SIPK activation. This is because SIPK and WIPK share the same upstream kinase and SIPK pre-exists in cells, whereas WIPK is inducible. In the absence of WIPK protein, activation of NtMEK2 will only lead to an increase in SIPK activity. However, if cells have been pre-exposed to stresses and WIPK protein has already accumulated in cells, WIPK could be activated by NtMEK2 with the same kinetics as that of SIPK by a second stimulus. This phenomenon is also seen in plants transiently transformed with $NtMEK2^{DD}$ (Yang *et al.*, 2001). In this experiment, *Agrobacterium*, which was used to deliver the transgene, could induce the expression of endogenous *WIPK*. Once $NtMEK2^{DD}$ was induced by the application of DEX, simultaneous activation of SIPK and WIPK was observed (Yang *et al.*, 2001).

Previously, we demonstrated that although wounding transiently activates *WIPK* gene expression, little or no WIPK activity could be detected by in-gel kinase assay (Zhang and Klessig, 1998a). Instead, SIPK was found to be the major MAPK activated by wounding. The size difference between SIPK and WIPK allows the easy separation of these two MAPKs in both the in-gel kinase assay and immunoblot analysis (Figure 4, second and fourth panels). Based on this study, the transient activation of *WIPK* expression after wounding is also likely a result of SIPK activation. Consistent with this, wounding-induced SIPK activation precedes the induction of *WIPK* expression (Zhang and Klessig, 1998a). In contrast to wounding-induced transient *WIPK* gene expression, pathogen or pathogen-derived elicitors that induce HR cell death lead to long-lasting activation of *WIPK* gene expression, which is followed by *de novo* synthesis of WIPK protein and delayed activation of WIPK activity (Zhang and Klessig, 1998b; Zhang *et al.*, 2000). As a result, we believe that WIPK is likely to be involved in plant disease resistance rather than wounding responses because the activity is the one that ultimately determines if an MAPK is involved in a particular response. On the other hand, although wounding-induced *WIPK* gene expression is unlikely to be involved in wounding responses directly, *WIPK* gene activation by wounding might affect the responses of plants to a second stimuli, i.e. it might be involved in the cross-talks between different stress-signaling pathways in plants.

The regulation of *WIPK* and its orthologs in other plant species at the transcriptional level is very unique among MAPKs. The significance of such dual regulation could be twofold. First, it allows one MAPKK, NtMEK2, to control two different MAPKs separately under certain conditions. When plants are first exposed to abiotic stresses, such as wounding, high salinity, and osmotic shock, only SIPK becomes activated by NtMEK2 because WIPK protein is absent in the cells. Secondly, the multiple steps required for the production of active WIPK could prevent its accidental activation, which might be detrimental to the cell survival. During plant disease resistance, sustained upstream signal leads to the long-lasting activation of SIPK and delayed activation of WIPK, which are involved in committing cells to the HR cell death program based on our correlative and gain-of-function analysis (Yang *et al.*, 2001; Zhang *et al.*, 2000). To further our understanding of the regulation of SIPK and WIPK in response to a stress stimulus, it is important for us to identify both the positive regulator(s), i.e. upstream MAPKKK(s), and the negative regulator(s), i.e. MAPKK phosphatase(s), of NtMEK2, which plays a critical role in determining the activity of SIPK and WIPK.

Experimental procedures

Treatment of tobacco cell suspension culture

Tobacco cell suspension culture was maintained and treated as previously described (Zhang *et al.*, 2000). Log-phase cells were used 3–4 days after a 1 : 10 dilution. Treatments with the elicitins (25 nM) and/or inhibitors were done in the original flasks in the dark to avoid any stresses associated with transfer. For inhibitor studies, the cells were treated at the times indicated by the addition of stock solutions in DMSO. At various times, 10 ml cells (approximately 0.3 g fresh weight) were harvested by filtration. The cells were quickly frozen in liquid nitrogen and stored at −80°C until analysis.

Agrobacterium-mediated transformation

Permanent $NtMEK2^{DD}$ transgenic plants in *N. tabacum* cv. Xanthi nc [NN] background (Jin *et al.*, 2003) were grown at 22°C in a walk-in growth chamber programed for a 14 h light cycle. Six- to eight-week-old T_2 plants were used for co-transformation experiments. Various mutants of wounding-induced protein kinase (*WIPK*) were generated by QuickChange site-directed mutagenesis (Stratagene) as previously described (Yang *et al.*, 2001; Zhang and Liu, 2001). *Agrobacterium tumefaciens* LBA4404 carrying different constructs in pTA7002 steroid-inducible promoter (Aoyama and Chua, 1997) was grown overnight in YM medium (yeast extract, 0.04%; mannitol, 1%; NaCl, 1.7 mM; $MgSO_4$, 0.8 mM; K_2HPO_4, 2.2 mM; pH 7.0) containing 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} kanamycin, and 100 μM acetosyringone. Cells were collected by centrifugation (4000 ***g***), re-suspended to OD_{600} of 0.8 in MS medium (pH 5.9) with 100 μM acetosyringone, and infiltrated into the fully expanded leaves. The expression of transgene was induced by infiltration of dexamethasone (DEX, 30 μM) 40–48 h later. Samples for protein and RNA preparation were collected at indicated times and quickly frozen in liquid N_2 and stored at −80°C until use.

PVX virus-induced gene silencing

Transgenic *Nicotiana benthamiana* plants carrying *N* gene (*N. benthamiana::NN*) were grown at 24°C in a growth cabinet under 16 h light/8 h dark cycle. The PVX-mediated VIGS experiments were performed as previously described (Jin *et al.*, 2003). Fourteen days after the inoculation of *Agrobacterium* carrying pGr106 constructs, the fifth and sixth leaves were inoculated with TMV-GFP. Leaf tissues were collected 1, 2, and 3 days post-TMV inoculation for analysis.

Protein extraction, immunoblot analysis, and kinase assays

Protein was extracted from leaf tissue and stored at −80°C (Zhang and Klessig, 1998a). The concentration of protein extracts was determined using the Bio-Rad protein assay kit with BSA as the standard. Immunoblot analysis, in-gel kinase activity assay, and immune complex kinase activity assay were performed as described previously (Yang *et al.*, 2001).

Determination of electrolyte leakage

Eight leaf discs were taken at indicated times and floated in 5 ml Millipore water in a 15 ml conical tube after being rinsed briefly in de-ionized water. After 1 h incubation with mild shaking, the conductivity of the water solution was measured using a conductivity meter.

RNA blot analysis

RNA was extracted using Trizol reagent according to the manufacturer's instructions. Ten micrograms of total RNA per lane was separated on 1.2% formaldehyde-agarose gels and transferred to a Zeta-Probe membrane (Bio-Rad). The level of *WIPK* transcript was detected by hybridization with α-[^{32}P]-dCTP random primer-labeled cDNA probe as described previously (Zhang and Klessig, 1998b).

Acknowledgements

We thank N.-H. Chua for providing the pTA7002 vector and D. Baulcombe for providing the pGr106 vector. This research was supported by National Science Foundation Grant MCB-9974796, IBN-0133220, and the Missouri Agricultural Experiment Station project MO-BCSL0572 to S. Zhang and USDA CRIS 5335-22000-005–00D to B. Baker. C.Y. Kim is supported by Molecular Biology Post-doctoral Fellowship from the University of Missouri.

References

Aoyama, T. and Chua, N.-H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J.* **11**, 605–612.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J. (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature*, **415**, 977–983.

Baker, B., Zambryski, P., Staskawicz, B. and Dinesh-Kumar, S.P. (1997) Signaling in plant–microbe interactions. *Science*, **276**, 726–733.

Cardinale, F., Jonak, C., Ligterink, W., Niehaus, K., Boller, T. and Hirt, H. (2000) Differential activation of four specific MAPK pathways by distinct elicitors. *J. Biol. Chem.* **275**, 36734–36740.

Cazalé, A.-C., Droîllard, M.J., Wilson, C., Heberle-Bors, E., Barbier-Brygoo, H. and Laurière, C. (1999) MAP kinase activation by hypoosmotic stress of tobacco cell suspensions: towards the oxidative burst response? *Plant J.* **19**, 297–307.

Chang, L. and Karin, M. (2001) Mammalian MAP kinase signaling cascades. *Nature*, **410**, 37–40.

Dangl, J.L. and Jones, J.D.G. (2001) Plant pathogens and integrated defense responses to infection. *Nature*, **411**, 826–833.

Davis, R. (2000) Signal transduction by the JNK group of MAP kinases. *Cell*, **103**, 239–252.

Desikan, R., Hancock, J.T., Ichimura, K., Shinozaki, K. and Neill, S.J. (2001) Harpin induced activation of the *Arabidopsis* mitogen-activated protein kinases AtMPK4 and AtMPK6. *Plant Physiol.* **126**, 1579–1587.

Frye, C.A., Tang, D. and Innes, R.W. (2001) Negative regulation of defense responses in plants by a conserved MAPKK kinase. *Proc. Natl. Acad. Sci. USA*, **98**, 373–378.

Gómez-Gómez, L. and Boller, T. (2000) FLS2: a LRR receptor-like kinase involved in recognition of the flagellin elicitor in *Arabidopsis*. *Mol. Cell*, **5**, 1–20.

Hammond-Kosack, K.E. and Jones, J.D.G. (1996) Resistance gene-dependent plant defense responses. *Plant Cell*, **8**, 1773–1791.

Herskowitz, I. (1995) MAP kinase pathways in yeast: for mating and more. *Cell*, **80**, 187–197.

Hirt, H. (1997) Multiple roles of MAP kinases in plant signal transduction. *Trends Plant Sci.* **2**, 11–15.

Hoyos, M.E. and Zhang, S. (2000) Calcium-independent activation of salicylic acid-induced protein kinase and a 40-kilodalton protein kinase by hyperosmotic stress. *Plant Physiol.* **122**, 1355–1363.

Innes, R.W. (2001) Mapping out the roles of MAP kinases in plant defense. *Trends Plant Sci.* **6**, 392–394.

Jin, H., Kim, C.Y., Liu, Y., Yang, K.-Y., Baker, B. and Zhang, S. (2003) Activation of a mitogen-activated protein kinase pathway is required for *N*-gene mediated resistance in tobacco. *Plant J.* (in press).

Jonak, C., Kiegeri, S., Ligterink, W., Barker, P.J., Huskisson, N.S. and Hirt, H. (1996) Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl. Acad. Sci. USA*, **93**, 11274–11279.

Kovtun, Y., Chiu, W.-L., Tena, G. and Sheen, J. (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. USA*, **97**, 2940–2945.

Lee, J., Klessig, D.F. and Nürnberger, T. (2001) A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene *HIN1* independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. *Plant Cell*, **13**, 1079–1093.

Ligterink, W., Kroj, T., zur Nieden, U., Hirt, H. and Scheel, D. (1997) Receptor-mediated activation of a MAP kinase in pathogen defense of plants. *Science*, **276**, 2054–2057.

MAPK Group (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci.* **7**, 301–308.

Martin, G.B. (1999) Functional analysis of plant disease resistance genes and their downstream effectors. *Curr. Opin. Plant Biol.* **2**, 273–279.

Mikolajczyk, M., Awotunde, O.S., Muszynska, G., Klessig, D.F. and Dobrowolska, G. (2000) Osmotic stress induces rapid activation of a salicylic acid-induced protein kinase and a homolog of protein kinase ASK1 in tobacco cells. *Plant Cell*, **12**, 165–178.

Mizoguchi, T., Ichimura, K. and Shinozaki, K. (1997) Environmental stress response in plants: the role of mitogen-activated protein kinases. *Trends Biotechnol.* **15**, 15–19.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumota, K. and Shinozaki, K. (1996) A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, **93**, 765–769.

Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A. and Hirt, H. (1999) Distinct osmo-sensing protein kinase pathways are involved in signalling moderate and severe hyper-osmotic stress. *Plant J.* **20**, 381–388.

Nühse, T., Peck, S.C., Hirt, H. and Boller, T. (2000) Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK6. *J. Biol. Chem.* **275**, 7521–7526.

Petersen, M., Brodersen, P., Naested, H. ***et al.*** (2000) *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. *Cell*, **103**, 1111–1120.

Ren, D., Yang, H. and Zhang, S. (2002) Cell death mediated by mitogen-activated protein kinase pathway is associated with the generation of hydrogen peroxide in *Arabidopsis*. *J. Biol. Chem.* **277**, 559–565.

Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H. and Jones, J. (1999) Rapid Avr9- and Cf-9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound and salicylate responses. *Plant Cell*, **11**, 273–287.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.-Y. and Hunt, M.D. (1996) Systemic acquired resistance. *Plant Cell*, **8**, 1809–1819.

Samuel, M.A. and Ellis, B.E. (2002) Double jeopardy: both overexpression and suppression of a redox-activated plant mitogen-activated protein kinase render tobacco plants ozone sensitive. *Plant Cell*, **14**, 2059–2069.

Scheel, D. (1998) Resistance response physiology and signal transduction. *Curr. Opin. Plant Biol.* **1**, 305–310.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H. and Ohashi, Y. (1995) Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. *Science*, **270**, 1988–1992.

Seo, S., Sano, H. and Ohashi, Y. (1999) Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. *Plant Cell*, **11**, 289–298.

Somssich, I.E. and Hahlbrock, K. (1998) Pathogen defence in plants – a paradigm of biological complexity. *Trends Plant Sci.* **3**, 86–90.

Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G. and Jones, J.D.G. (1995) Molecular genetics of plant disease resistance. *Science*, **268**, 661–667.

Stratmann, J.W. and Ryan, C.A. (1997) Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors. *Proc. Natl. Acad. Sci. USA*, **94**, 11085–11089.

Suzuki, K., Yano, A. and Shinshi, H. (1999) Slow and prolonged activation of the p47 protein kinase during hypersensitive cell death in a culture of tobacco cells. *Plant Physiol.* **119**, 1465–1472.

Takezawa, D. (1999) Elicitor- and A23187-induced expression of WCK-1, a gene encoding mitogen-activated protein kinase in wheat. *Plant Mol. Biol.* **40**, 921–933.

Taylor, A.T.S., Kim, J. and Low, P.S. (2001) Involvement of mitogen-activated protein kinase activation in the signal-transduction pathways of the soya bean oxidative burst. *Biochem. J.* **355**, 795–803.

Tena, G., Asai, T., Chiu, W.-L. and Sheen, J. (2001) Plant mitogen-activated protein kinase signaling cascades. *Curr. Opin. Plant Biol.* **4**, 392–400.

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **108**, 796–815.

Widmann, C., Gibson, S., Jarpe, M.B. and Johnson, G.L. (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* **79**, 143–180.

Yang, K.-Y., Liu, Y. and Zhang, S. (2001) Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. *Proc. Natl. Acad. Sci. USA*, **98**, 741–746.

Yang, Y., Shah, J. and Klessig, D.F. (1997) Signal perception and transduction in plant defense responses. *Genes Dev.* **11**, 1621–1639.

Zhang, S. and Klessig, D.F. (1997) Salicylic acid activates a 48 kD MAP kinase in tobacco. *Plant Cell*, **9**, 809–824.

Zhang, S. and Klessig, D.F. (1998a) The tobacco wounding-activated MAP kinase is encoded by *SIPK*. *Proc. Natl. Acad. Sci. USA*, **95**, 7225–7230.

Zhang, S. and Klessig, D.F. (1998b) *N* resistance gene-mediated *de novo* synthesis and activation of a tobacco MAP kinase by TMV infection. *Proc. Natl. Acad. Sci. USA*, **95**, 7433–7438.

Zhang, S. and Klessig, D.F. (2001) MAPK cascades in plant defense signaling. *Trends Plant Sci.* **6**, 520–527.

Zhang, S. and Liu, Y. (2001) Activation of salicylic acid-induced protein kinase, a mitogen-activated protein kinase, induces multiple defense responses in tobacco. *Plant Cell*, **13**, 1877–1889.

Zhang, S., Du, H. and Klessig, D.F. (1998) Activation of tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora* spp. *Plant Cell*, **10**, 435–449.

Zhang, S., Liu, Y. and Klessig, D.F. (2000) Multiple levels of tobacco WIPK activation during the induction of cell death by fungal elicitins. *Plant J.* **23**, 339–347.