Tobacco Transcription Factor WRKY1 Is Phosphorylated by the MAP Kinase SIPK and Mediates HR-Like Cell Death in Tobacco

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The salicylic acid-induced protein kinase (SIPK) of tobacco, which is a mitogen-activated protein kinase (MAPK), is activated by various biotic and abiotic treatments. Overexpression of SIPK has been shown to trigger cell death. In this study, a targeted yeast two-hybrid approach identified the tobacco transcription factor WRKY1 as a potential substrate. SIPK phosphorylated WRKY1, which resulted in enhanced DNA-binding activity of WRKY1 to its cognate binding site, a W box sequence from the tobacco chitinase gene *CHN50*. SIPK-mediated enhancement of WRKY1 DNA-binding activity was inhibited by staurosporine, a general kinase inhibitor. Co-expression of SIPK and WRKY1 in *Nicotiana benthamiana* led to more rapid cell death than expression of SIPK alone, suggesting that WRKY1 is involved in the formation of hypersensitive response-like cell death and may be a component of the signaling cascade downstream of SIPK.

Plants contain an arsenal of defenses that can be used to prevent colonization by an invading pathogen. The ability of a plant to recognize an invading avirulent pathogen and induce defense responses is initiated by the direct or indirect interaction between a plant-encoded resistance (R) protein and a pathogen-encoded avirulence (Avr) protein (Flor 1942; Keen 1990; Nimchuk et al. 2003). One of the most visible responses triggered by this interaction is the hypersensitive response (HR), a form of programmed cell death that leads to necrotic lesion formation at the site or sites of pathogen entry (Dangl et al. 1996; Richberg et al. 1998). In addition to the HR, many resistance-associated phenomena occur in the inoculated leaf, including ion fluxes, increases in reactive oxygen species, accumulation of salicylic acid (SA), and activation of many defense genes, including the pathogenesis-related (*PR*) genes (Dempsey et al. 1999; Hammond-Kosack and Jones 1996).

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Subsequent to these events, the uninoculated leaves of the plant develop a long-lasting, broad-range resistance known as systemic acquired resistance (SAR).

The interaction between tobacco and *Tobacco mosaic virus* (TMV) serves as a model system to elucidate the disease resistance-signaling pathway in plants. Tobacco cultivars resistant to TMV contain the *N* resistance gene, which encodes a protein containing a nucleotide binding site (NBS) and C-terminal leucine-rich repeats (LRRs) (Whitham et al. 1994). Recently, virus-induced gene silencing (VIGS) was used to identify several downstream components in this signaling pathway. These include EDS1, RAR1, SGT1, Hsp90, a component of the Skp1/Cullin/F-box protein (SCF)-type E3 ubiquitin ligase, and two components of the COP9 signalosome (Liu et al. 2002a,b, 2004a; Peart et al. 2002a,b). The defense signaling molecules SA, jasmonic acid (JA), and ethylene also are implicated in the *N*-mediated resistance pathway (Liu et al. 2002a, 2004b).

Components of two different mitogen-activated protein kinase (MAPK) cascades also are required for *N* gene-mediated resistance to TMV (Jin et al. 2003; Kim and Zhang 2004; Liu et al. 2004b; Yang et al. 2001; Zhang and Liu 2001). In yeast and mammalian cells, MAPK cascades form major pathways through which external signals are transduced into intracellular responses (Chang and Karin 2001; Davis 2000; Tena et al. 2001; Widmann et al. 1999; Zhang and Klessig 2001). The basic MAPK cascade unit in all eukaryotes consists of three kinases, a MAPK, its phosphorylating kinase MAPK kinase (MAPKK), and its phosphorylating kinase MAPKK kinase (MAPKKK). Silencing of the tobacco NTF6 (a MAPK), MEK1 (a MAPKK), or NPK1 (a MAPKKK), which constitute a cascade module, compromised *N*-mediated resistance to TMV (Jin et al. 2002; Liu et al. 2004b). A second cascade consisting of two MAPKs, SA-inducible protein kinase (SIPK), and wound-inducible protein kinase (WIPK), as well as their activating kinase NtMEK2, also has been implicated in *N*-mediated resistance. Both SIPK and WIPK are activated in tobacco resisting TMV infection (Zhang and Klessig 1998) and, through the use of kinase inhibitors, have been linked to HR cell death after elicitor treatment (Zhang et al. 2000). Furthermore, overexpression of SIPK or a constitutively active mutant of NtMEK2 (NtMEK2^{DD}) induced constitutive HR cell death and defense gene expression (Jin et al. 2003; Kim and Zhang 2004; Yang et al. 2001; Zhang and Liu 2001). By contrast, silencing the expression of *SIPK*, *WIPK*, or *NtMEK2* attenuated TMV resistance (Jin et al. 2003).

Recently, selected isoforms of 1-aminocyclopropane-1-carboxylic acid synthase (ACS), the rate-limiting enzyme of eth-

ylene biosynthesis, have been shown to be substrates for *Arabidopsis* MPK6, a SIPK ortholog (Liu and Zhang 2004). Phosphorylation of ACS2 and ACS6 by MPK6 leads to the accumulation of ACS proteins and elevated ethylene production (Liu and Zhang 2004). In addition, nuclear extracts of tobacco expressing a constitutively active NtMEK2 displayed increased binding activity to the W box, a binding site for WRKY proteins (Kim and Zhang 2004). WRKY proteins are a plant-specific family of transcription factors that bind the W box sequence ((T)TGAC(C/T)); W boxes have been identified in the promoters of various pathogen- and wounding-responsive genes, including members of the *PR* gene family and *NPR1* (Eulgem et al. 2000; Rushton and Somssich 1998; Yu et al. 2001). Further linking WRKY proteins with N-mediated resistance is the recent demonstration that VIGS-induced silencing of *NbWRKY1*, *NbWRKY2*, or *NbWRKY3* suppressed resistance to TMV; the size of HR lesions also was reduced in *NbWRKY1*-silenced plants (Liu et al. 2004b). However, it currently is unclear whether MAPKs activate WRKY proteins directly or indirectly. Some ACS isoforms are substrates for

MAPKs (Liu and Zhang 2004); therefore, MAPKs could activate WRKY proteins indirectly via elevated ethylene levels. Consistent with the possibility that induced W box-binding activity does not require direct phosphorylation of WRKY proteins, phosphatase treatment did not affect the increased W box-binding activity detected in constitutively active *NtMEK2^{DD}*-expressing tobacco (Kim and Zhang 2004). Instead, because these transgenics, like TMV- or SA-treated tobacco, accumulate more transcripts for several *WRKY* genes, increased W box-binding activity was attributed to increased *WRKY* gene expression (Chen and Chen 2000; Kim and Zhang 2004; Yang et al. 1999). In contrast, phosphatase treatment of tobacco nuclear extracts nearly abolished the TMV- or SA-induced W box-binding activity of a *WRKY* DNA-binding activity, arguing that phosphorylation is important for WRKY activation (Yang et al. 1999).

To investigate the mechanism through which MAPKs transduce the resistance signal, we have used a targeted yeast two-hybrid approach to identify potential SIPK substrates. Here we report that SIPK binds and phosphorylates WRKY1, which enhances its DNA-binding activity. Moreover, coexpression of WRKY1 in *Nicotiana benthamiana* accelerated cell death induced by overexpression of SIPK alone.

RESULTS

SIPK interacts with WRKY1.

Previous efforts to identify SIPK- (and WIPK)-interacting proteins using an untargeted yeast two-hybrid screen led to the identification of several interacting proteins (F. L. H. Menke and D. F. Klessig, *unpublished results*); however, no obvious transcription factors were identified. To facilitate identification of transcription factors that interact with SIPK (or WIPK), we employed the Cytotrap yeast two-hybrid system (Aronheim et al. 1997). The Cytotrap system is ideally suited to test protein-protein interaction between transcription factors and other proteins, because the interaction is independent of transcription activation and takes place in the cytoplasm. Four different members of the tobacco WRKY family, *WRKY1*, *WRKY2*, *WRKY3*, and *WRKY4*, and two members of the tobacco ethylene response element-binding protein (EREBP) family, *EREBP1* and *EREBP2*, were cloned into the prey vector of the Cytotrap system and introduced into yeast strain *cdc25*, with *SIPK* or *WIPK* cloned in the bait vector. Only yeast coexpressing *SIPK* and *WRKY1* were able to grow at both the permissive temperature of 25°C and the selective temperature of 37°C (Fig. 1A and data not shown). In contrast, yeast coexpressing *WRKY1* and either *WIPK* or the negative control *MafB* (Aronheim et al. 1997) failed to grow at 37°C (Fig. 1A). To test whether this weak interaction between SIPK and WRKY1 observed in yeast was meaningful, their interaction was analyzed in vitro using a coimmunoprecipitation assay. The ability of a SIPK-specific antibody to immunoprecipitate [³⁵S]-labeled WRKY1 in the presence of recombinant SIPK protein, but not in its absence (Fig. 1B), suggests that WRKY1 is an interacting partner of SIPK. In contrast, the WIPK-interacting protein W136 (F. L. H. Menke and D. F. Klessig, *unpublished data*) was not coimmunoprecipitated with SIPK (Fig. 1B, lane 3), thus demonstrating the specificity of the assay.

SIPK phosphorylates WRKY1 in vitro.

To determine whether the interaction between SIPK and WRKY1 leads to phosphorylation of WRKY1, in vitro phosphorylation experiments were performed. Incubation of recombinant WRKY1 protein with SIPK purified from tobacco suspension cells led to phosphorylation of WRKY1 (Fig. 2A, lane

Fig. 1. WRKY1 interacts with salicylic acid-induced protein kinase (SIPK). **A**, Cytotrap yeast two-hybrid interaction of WRKY1 with SIPK. WRKY1 was coexpressed with MAFB, wound-inducible protein kinase (WIPK), or SIPK in yeast strain *cdc25H* and grown at 25°C (permissive) or 37°C (restrictive) conditions. pM-WRKY1, *WRKY1* subcloned in the prey vector pMyr; pS-MAFB/WIPK/SIPK, *MAFB/WIPK/SIPK* subcloned in the bait vector pSos. **B**, Co-immunoprecipitation of WRKY1 with SIPK. [³⁵S]-labeled WRKY1 or [³⁵S]-labeled W136 protein was incubated with SIPK-specific antibody α -p48N in the presence (lanes 3 and 5) or absence (lanes 4 and 6) of SIPK and complexes were pulled down with protein A agarose and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% gel. Lanes 1 and 2 show the amount of input protein for WRKY1 and W136, respectively. The arrow indicates the position of the WRKY1 protein. Size markers are shown on the right of the panel in kilodaltons.

3). Incubation of WRKY1 with recombinant SIPK, however, did not result in phosphorylation, unless SIPK was preincubated with constitutively active NtMEK2^{DD} (Yang et al. 2001) and ATP (Fig. 2B and C). Because NtMEK2^{DD} alone did not phosphorylate WRKY1 in vitro (Fig. 2B, lane 3 and C, lane 1), we

Fig. 2. Salicylic acid-induced protein kinase (SIPK) phosphorylates WRKY1 in vitro. **A**, Activated SIPK purified from tobacco (tSIPK) phosphorylates WRKY1, but not TGA2, in vitro. WRKY1 or TGA2 was incubated with tSIPK in the presence of [γ -³²P]-ATP. The reaction mixture subsequently was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel. Positions of size markers are shown on the right of the panel in kilodaltons. **B**, Phosphorylation of WRKY1 by recombinant SIPK requires MEK2^{DD} activation of SIPK. WRKY1 was incubated with SIPK, MEK2^{DD}, or both in the presence of [γ -³²P]-ATP. The reaction mixture subsequently was resolved by SDS-PAGE on a 10% gel. Position of WRKY1 and SIPK are indicated on the left and size markers are shown on the right of the panel in kilodaltons. **C**, Activated recombinant SIPK phosphorylates WRKY1 but not TGA2. TGA2 and WRKY1 were incubated with MEK2^{DD} and [γ -³²P]-ATP in the presence or absence of SIPK. Reaction mixtures were subsequently resolved by SDS-PAGE on a 10% gel.

concluded that WRKY1 is phosphorylated by SIPK, which first must be activated by its upstream MAPKK, MEK2. Incubation of MEK2^{DD}, MEK2^{DD}-activated SIPK, or highly purified tobacco SIPK with TGA2, an *Arabidopsis* bZIP transcription factor, did not result in detectable phosphorylation (Fig. 2A and C), arguing that SIPK selectively phosphorylates WRKY1 in vitro. Note that radiolabeling of MEK2^{DD}-activated SIPK with [γ -³²P]-phosphate (Fig. 2B, lane 1 and C, lane 2) was observed only in cases where SIPK was able to phosphorylate the substrate, suggesting that interaction with a substrate is required for this additional phosphorylation of SIPK. MEK2^{DD}-activated SIPK is phosphorylated on threonine and tyrosine in the TxY motif of the activation loop with unlabeled ATP by pre-incubation with MEK2^{DD}. Subsequent incubation with [γ -³²P]-ATP and the substrate resulted in the phosphorylation of both the substrate and SIPK with [γ -³²P]-phosphate. Although we do not know why phosphorylation of SIPK by MEK2^{DD} is enhanced in the presence of WRKY1, this detectable phosphorylation of SIPK could be due to i) phosphorylation of partially phosphorylated SIPK, ii) exchange of unlabeled phosphate for [γ -³²P]-phosphate in the TxY motif of the activation loop, or iii) phosphorylation on additional amino acid residues upon complex formation with the substrate.

Phosphorylation of WRKY1 by SIPK enhances its binding to W box sequence.

Phosphorylation affects the DNA-binding activity of many transcription factors (Brivanlou and Darnell 2002); therefore, we tested whether SIPK-mediated phosphorylation of WRKY1 altered its ability to bind a W box sequence (*Pchn*) from the tobacco class I basic chitinase gene *CHN50*. WRKY1 previously was shown to bind this *Pchn* probe in a sequence-specific manner (Yang et al. 1999). Electrophoretic mobility shift assay (EMSA) revealed that recombinant WRKY1 alone binds the *Pchn* probe (Fig. 3, lane 6). Note that a minimum amount of WRKY1 was used to observe the effect of SIPK on the

Fig. 3. Salicylic acid-induced protein kinase (SIPK)-dependent phosphorylation enhances WRKY1 DNA-binding activity. Recombinant WRKY1 and TGA2 proteins were incubated with probe corresponding to a W box sequence (*Pchn*) from the tobacco class I basic chitinase gene *CHN50*. Electrophoretic mobility shift assay was performed on all of the recombinant proteins following either no pretreatment (lanes 2 and 6), pretreatment with recombinant SIPK only (lanes 3 and 7), pretreatment with MEK2^{DD}-activated recombinant SIPK (lanes 4 and 8), or pretreatment with SIPK purified from tobacco in the absence (lanes 5 and 9) or presence (lane 10) of 1 μ M staurosporine. As a negative control (C), no recombinant protein was present in lane 1. Reaction mixtures were fractionated by polyacrylamide gel electrophoresis on a 10% gel. Positions of the bound and free probe are indicated on the right of the panel.

binding activity of WRKY1 to avoid the binding activity being saturated in the absence of SIPK. In the presence of SIPK highly purified from tobacco, the DNA-binding activity of WRKY1 was enhanced substantially (Fig. 3, lane 9). The DNA-

binding activity of WRKY1 also was enhanced by recombinant SIPK activated by NtMEK2^{DD}, although to a lesser extent (Fig. 3, lane 8). The addition of staurosporine, a general kinase inhibitor, substantially suppressed this SIPK-mediated increase in the DNA-binding activity (Fig. 3, lane 10), suggesting that SIPK-mediated phosphorylation of WRKY1 promotes its DNA-binding activity. In contrast, pre-incubation with neither highly purified nor recombinant SIPK led to binding of *Pchn* by TGA2 (Fig 3, lanes 3, 4, and 5). Taken together, these results suggest that SIPK-mediated phosphorylation of WRKY1 promotes its DNA-binding activity.

In vivo localization and activity of WRKY1 and SIPK.

Several plant MAPKs have been shown to be localized in the nucleus, suggesting that their substrates also are present in this location (Lee et al. 2004). To assess whether SIPK and WRKY1 are localized in the nucleus, their coding sequences were fused at the C-terminus with that of green fluorescent protein (GFP) and expressed under the cauliflower mosaic virus 35S promoter. Analysis of transgenic tobacco BY2 suspension cells revealed that SIPK:GFP was present in both the cytosol and nucleus, whereas WRKY1:GFP was localized predominantly in the nucleus (Fig. 4A). Transient expression of WRKY1:GFP in *N. benthamiana* in the absence or presence of SIPK coexpression also resulted in predominant nuclear localization of WRKY1-GFP (data not shown).

Zhang and Liu (2001) previously showed that transient overexpression of SIPK caused HR-like cell death and activation of defense gene expression. In light of this observation and the phosphorylation (Fig. 2) and activation (Fig. 3) of WRKY1 by SIPK, we tested whether transient expression of WRKY1 would facilitate induction of host cell death by SIPK overexpression (Fig. 4B). Expression of GFP (Fig. 4B, infiltrated area 1) or WRKY1:GFP (area 4) alone or together (areas 2 and 3) did not induce cell death by 4 days post infiltration (dpi) with *Agrobacterium* spp. (Fig. 4B, left leaf), nor did coexpression of GFP and SIPK (area 5). Co-expression of SIPK with the lowest level of WRKY1:GFP also did not lead to cell death by 4 dpi (area 6). In contrast, when more WRKY1:GFP was coexpressed in the presence of SIPK, cell death was induced in a dose-dependent manner (areas 7 and 8). The protein levels for WRKY1:GFP and SIPK were monitored by immunoblot analysis with α GFP and α SIPK antibodies, respectively (Fig. 4C). Overexpression of WRKY1:GFP or SIPK alone, but not GFP, eventually induced cell death after a week or more (data not shown). These results suggest that SIPK and WRKY1 act in concert to trigger HR-like cell death, possibly through direct phosphorylation of WRKY1 by SIPK.

DISCUSSION

Based on phylogenetic analyses, plant MAPKs recently were divided into four groups (Ichimura et al. 2002). MAPKs composing group A, including tobacco SIPK and its *Arabidopsis* ortholog MPK6, are associated primarily with environmental and hormonal stress responses. For example, SIPK and MPK6 are activated by wounding, elevated levels of ozone, bacterial and fungal elicitors, and viral infection (Asai et al. 2002; Ellis et al. 2002; Romeis et al. 1999; Zhang and Klessig 1998), and they have been implicated in signaling resistance to infection by bacterial, fungal, oomycete, and viral pathogens (Asai et al. 2002; Jin et al. 2002; Menke et al. 2004). Recent reverse genetic studies also have implicated group A, as well as B, MAPKs in *R* gene-mediated resistance in *N. benthamiana* and tomato (Ekengren et al. 2003; Liu et al. 2004b; Pedley and Martin 2004). To date, a number of transcription factors, including TGA, WRKY, Myb, and EREBP, have been

Fig. 4. Transient coexpression of salicylic acid-induced protein kinase (SIPK) and WRKY1 accelerates development of hypersensitive response-like cell death. **A**, SIPK and WRKY1 colocalize in the nucleus. Tobacco BY2 cells were transformed with SIPK:green fluorescent protein (GFP) or WRKY1:GFP fusion constructs under the cauliflower mosaic virus 35S promoter. Transformed cells stained with 4,6-diamidino-2-phenylindole (DAPI) were analyzed under a fluorescent microscope. Images were made under white light and UV light with filters to visualize GFP or DAPI fluorescence. Bars indicate 50 μ m. **B**, Transient coexpression of WRKY1:GFP or GFP in the absence (left panel) or the presence (right panel) of SIPK in *Nicotiana benthamiana*. Areas 1 and 5 contain GFP, areas 4 and 8 contain WRKY1:GFP, areas 2 and 6 contain a 3:1 ratio of GFP to WRKY1:GFP, and areas 3 and 7 contain a 1:3 ratio of GFP to WRKY1:GFP. The SIPK expression vector was introduced into all eight areas; however, its expression was induced only in areas 5 to 8 due to coinfiltration of 30 μ M Dex. Pictures were taken at 4 days after infiltration. **C**, Western blot analysis showing WRKY1:GFP, GFP, and SIPK protein levels in *N. benthamiana*. Protein extracts from *N. benthamiana* leaf discs were prepared from a similar set of samples (shown in B) at 2 days after infiltration and were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 12% gel. The lane numbers correlate with the numbers shown in B. Arrows indicate the expected position of WRKY1:GFP (top arrow) and GFP (bottom arrow). Size markers are shown on the left of the panel in kilodaltons.

shown to act downstream of various MAPKs (Asai et al. 2002; Cheong et al. 2003; Ekengren et al. 2003; Kim and Zhang 2004; Liu et al. 2004b). However, the only identified substrates for plant MAPKs are the *Arabidopsis* ethylene biosynthetic enzyme ACS6, which is the limiting enzyme in ethylene biosynthesis (Liu and Zhang 2004), and rice OsEREBP1 (Cheong et al. 2003). Here, we report the interaction of SIPK with the tobacco WRKY1 transcription factor, which belongs to group I of the WRKY superfamily (Eulgem et al. 2000), and show that this interaction leads to phosphorylation of WRKY1, which increases WRKY1's binding activity to its cognate binding site. Moreover, coexpression of SIPK and WRKY1 in *N. benthamiana* resulted in accelerated cell death, suggesting that SIPK and WRKY1 participate in the HR in planta.

It is interesting to note that, although we did not detect any interaction between SIPK and either NtEREBP1 or NtEREBP2, Cheong and associates (2003) reported interaction between OsEREBP1 and BWMK1, a group D MAPK from rice. The discrepancy between these results may be due to the unusual nature of rice OsEREBP1 and BWMK1. OsEREBP1 shares only approximately 20% homology with other EREBPs, and BWMK1, which is only distantly related to SIPK, is unusual because it has a TDY activation motif rather than the common TEY motif, and also an extended C-terminal domain (Cheong et al. 2003). Interestingly, phosphorylation of OsEREBP1 did not require prior activation of BWMK1, whereas WRKY1 phosphorylation was dependent on the activation of SIPK by MEK2. Thus, although SIPK and BWMK1 are activated by similar treatments, including fungal elicitor, hydrogen peroxide, and SA (Cheong et al. 2003; Samuel et al. 2000; Zhang and Klessig 1997; Zhang et al. 1998), they appear to be components of distinct signaling pathways.

WRKY transcription factors have been implicated in the regulation of plant defense gene expression (Rushton and Sommsich 1998; Rushton et al. 1996; Singh et al. 2002). Their cognate binding site, the W box, can be found in the promoters of genes associated with resistance (Dong et al. 2003; Kalde et al. 2003; Kim and Zhang 2004; Maleck et al. 2000; Petersen et al. 2000). Moreover, using an *Arabidopsis* spp. protoplast transient expression system, Asai and coworkers (2002) identified a complete MAPK cascade and two WRKY proteins (WRKY22 and WRKY29) that act downstream of the bacterium flagellin receptor FLS2 to confer resistance to pathogens. The activity of WRKY proteins appears to be regulated at several levels and, for some WRKY proteins, it may involve phosphorylation. For example, the pep25 elicitor from *Phytophthora* spp. induces the accumulation of at least two distinguishable PcWRKY1 protein isoforms in parsley (Turck et al. 2004). Immunoprecipitation coupled with two-dimensional gel electrophoresis showed that these coexisting forms each can be separated into several distinct species that may correspond to different phosphorylation states of PcWRKY1. Moreover, induction of W box-binding activity in tobacco by SA or TMV treatment is almost completely abolished by phosphatase treatment (Yang et al. 1999). On the other hand, the induction of several defense genes and the elevated W box-binding activity observed in nuclear extracts from MEK2^{DD}-overexpressing tobacco does not appear to require phosphorylation-mediated activation of WRKY proteins, because phosphatase treatment did not suppress W box-binding activity (Kim and Zhang 2004). Instead, the elevated binding activity corresponded with a rapid increase in transcripts for several WRKY genes, including *NtWRKY3*, *NtWIZZ*, *NtDBP3*, and *NtSubD48*. A likely reason for these conflicting results is that monitoring W box-binding activity 6 h after induction of MEK2^{DD} (Kim and Zhang 2004) is too late to observe any effect of activated SIPK on WRKY1. Almost 25% of the 72 *Arabidopsis* WRKY proteins are induced transcriptionally within 2 h by

pathogen infection or SA treatment (Dong et al. 2003; Kalde et al. 2003); thus, the enhanced W box-binding activity seen at 6 h after induction likely contains a significant contribution from de novo synthesized WRKY proteins, which may overshadow the contribution of WRKY proteins activated by SIPK-mediated phosphorylation. Moreover, Cormack and associates (2002) reported that elicitor-induced transcriptional activation of five parsley WRKY proteins was differentially sensitive to cycloheximide, suggesting that these proteins are subject to distinct activation mechanisms. Consistent with this view, an in vivo binding assay using chromatin immunoprecipitation (ChIP) showed that W boxes in the *PcPR1-1* gene were occupied by different WRKY proteins depending on the specific stimulus (Turck et al. 2004); they proposed that WRKY proteins act in a network of mutually competing participants for the binding site in a stimulus-dependent manner.

Although searching for substrates is an obvious approach to gain insight into the function of a kinase, the list of plant MAPK substrates is still very limited. This may be because many of the MAPK-substrate interactions are very transient and unstable in order to facilitate rapid enzyme kinetics. Indeed, we found that the SIPK-WRKY1 interaction was very weak, being just detectable in the Cytotrap system (Fig. 1A). To date, only two potential plant MAPK substrates have been reported using yeast two-hybrid systems, OsEREBP1 (Cheong et al. 2003) and WRKY1 (this report).

Like Zhang and Liu (2001), we found that prolonged, elevated levels of SIPK resulted in HR-like cell death in *N. benthamiana* (data not shown). Although the components downstream of SIPK in the cell death pathway have yet to be determined together, the results of several studies, including ours, suggested that WRKY1 may be a participant. Silencing WRKY1 in *N. benthamiana* results in reduced lesion size in TMV-inoculated leaves (Liu et al. 2004b), whereas we described that SIPK phosphorylates and activates WRKY1, and coexpression of WRKY1 and SIPK results in more rapid induction of cell death than overexpression of either alone. Together with earlier studies, this suggests that WRKY1 participates in a cell death signal transduction cascade.

MATERIALS AND METHODS

DNA constructs.

SIPK and *WIPK* were subcloned into the Cytotrap (Stratagene, La Jolla, CA, U.S.A.) bait vector pSOS by introducing a *Bam*HI site upstream of the start codon by polymerase chain reaction (PCR) using pBK-SIPK or pBK-WIPK (Zhang and Klessig 1998; Zhang and Klessig 1997) as templates and with forward primers *Bam*HI-WIPK, *Bam*HI-SIPK, and T7-rev. PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and excised with *Bam*HI and *Xho*I and ligated into pSOS digested with *Bam*HI and *Sal*I. Similarly, *WIPK* and *SIPK* were subcloned into the pMYR prey vector by introducing an *Eco*RI site upstream of the start codon using forward primers *Eco*RI-WIPK and *Eco*RI-SIPK. PCR products were cloned into pGEM-T Easy (Promega), excised with *Eco*RI and *Xho*I, and ligated into pMYR. A similar strategy was used to clone the transcription factors *WRKY1-4* and *EREBP1* and 2 into pSOS and pMyr. Forward primers used to introduce appropriate restriction sites upstream of the start codon were *Eco*RI-WRKY1, *Bam*HI-WRKY1, *Eco*RI-WRKY2, *Bam*HI-WRKY2, *Eco*RI-WRKY3, *Bam*HI-WRKY3, *Sma*I-WRKY4, *Bam*HI-WRKY4, *Nco*I-EREBP1, *Bam*HI-EREBP2, *Sma*I-EREBP1, and *Eco*RI-EREBP2.

For the GFP fusion constructs, the following primers were used to amplify the open reading frame of WRKY1 and SIPK: *Xba*I-WRKY1, WRKY1-*Bam*HI, *Xba*I-SIPK, and SIPK-

*Bam*HI. PCR products were cloned into pGEM-T-Easy (Promega), excised with *Xba*I and *Bam*HI, and ligated to pCAMBIA2300-smGFP (pCAMBIA-GFP) (Kang and Klessig 2005) to create pCAMBIA-WRKY1:GFP and pCAMBIA-SIPK:GFP. The first 2,861 nucleotides of the TEV genome containing all of *P1* and *HC-Pro* and the beginning of the *P3* gene was mobilized from pRTL-0027 (Kasschau and Carrington 2001) by digestion with *Xho*I and *Bam*HI, and ligated to a binary vector, pBA002 (Hare et al. 2003), to create pBA-HC-Pro, which was used to suppress silencing of transient expression in *N. benthamiana*.

To obtain a dexamethasone (Dex)-inducible expression construct, SIPK was excised from pBK-SIPK with *Spe*I and *Xba*I and ligated in the Dex-inducible expression vector pTA7002 (Aoyama and Chua 1997) digested with *Spe*I. Sequences for the amplified DNAs were verified using an automated sequencer (ABI3100; Perkin-Elmer, Boston).

Yeast two-hybrid screen.

Yeast strain *cdc25H* (Stratagene) harbors a temperature-sensitive mutation in the *cdc25* gene, the yeast homologue for human *Sos* (*hSos*), and does not grow at 37°C. WRKY1 was expressed as a hybrid protein with an N-terminal myristoylation site, whereas MAFB, WIPK, and SIPK were expressed as hybrid proteins fused to hSOS. After cotransformation with the pSOS or pMyr constructs according to manufacturer's instructions, *cdc25H* cells were plated onto solid minimal medium with glucose and lacking Uracil and Leucine (SD-UL) and grown for 2 days at 25°C. Subsequently, the yeast cells were replica plated onto SD-UL and SD-UL with galactose and raffinose instead of glucose (SD_{gal}-UL) and grown at 37°C for up to 7 days. Protein-protein interactions were detected by recruitment of the human *Sos* gene product (*hSos*) to the membrane of the cell, which activates the Ras pathway and allows growth at 37°C.

Coimmunoprecipitation.

For coimmunoprecipitation experiments, SIPK was subcloned in pET28a (Novagen, Madison, WI, U.S.A.) using *Eco*RI and *Xho*I, creating an N-terminal His-tagged version of the protein. This His-tagged SIPK was expressed in *Escherichia coli* strain BL21(DE3) and purified by standard Nickel affinity chromatography (Novagen) using the 6× His tag present in pET28a. *WRKY1* and *WRKY3* also were cloned into pET28a using *Eco*RI and *Xho*I, and these constructs were used as template for a TnT quick-coupled in vitro transcription or translation system using rabbit reticulocyte lysate (Promega). In vitro translation was done in the presence of [³⁵S]-methionine to obtain radiolabeled proteins. An aliquot of the [³⁵S]-labeled protein was incubated with 0.5 µg of recombinant SIPK and 2.5 µg of SIPK antibody (α-p48N) (Zhang and Klessig 1998) in immunoprecipitation (IP) buffer (50 mM Tris, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100, Leupeptin and Antipain at 10 µg/ml, 0.4 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol [DTT]) for 90 min at 4°C in a head-over-tail tumbler. Protein A agarose (Sigma) was added and the incubation continued for 60 min. Complexes briefly were spun down in a microcentrifuge and the supernatant was removed. The pellet was washed with the IP buffer and spun down as before and the washing was repeated three times. The final pellet was resuspended in 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, boiled for 5 min, and resolved by SDS-PAGE on a 10% gel. After electrophoresis, the gel was dried and exposed to X-ray film (X-Omat, Kodak, Rochester, NY, U.S.A.).

Purification of tobacco SIPK.

The activated SIPK from the tobacco suspension cells was purified as described by Zhang and Klessig (1997) using am-

monium sulfate fractionation, Q-Sepharose, phenyl-Sepharose, MonoQ, and myelin basic protein (MBP)-Sepharose chromatography. The activity and purity of SIPK in the purified fractions was monitored using in-gel and immune-complex kinase assays using various substrates, including bovine serum albumen, casein, histone, and MBP.

In vitro phosphorylation.

Purified protein extracts of His-tagged versions of MEK2^{DD}, WRKY1, and TGA2 were made as described above for SIPK. For in vitro phosphorylation experiments with activated recombinant SIPK, 1 µg of SIPK was pre-incubated with 0.5 µg of MEK2^{DD} in kinase buffer (25 mM Tris, pH 7.5, 10 mM MnCl₂, 1 mM EGTA, and 1 mM DTT) in the presence of 1 mM ATP at 30°C for 20 min. In vitro phosphorylations were done with 1 µg of activated recombinant SIPK or approximately 5 ng of SIPK purified from tobacco, 0.5 µg of MEK2^{DD}, and 1 µg of substrate in the kinase buffer in the presence of 0.2 µl of [³²P]-ATP (3000 Ci/mmol, 10 mCi/ml) and 20 µM ATP for 30 min at 30°C. For EMSA, the same procedure was carried out without [³²P]-ATP, and 1 µM staurosporine was added for the kinase inhibition. For in vitro phosphorylation assay, SDS-PAGE loading buffer was added and samples were resolved by SDS-PAGE on a 10% gel. The gel was dried and exposed to film as described above.

Growth conditions.

Tobacco BY2 suspension cells were maintained by transferring 5 ml of a 7-day-old culture into 50 ml of fresh medium (1× Murashige and Skoog salts, MES at 500 mg/liter (pH 5.7), thiamin at 1 mg/liter, myoinositol at 100 mg/liter, K₂HPO₄ at 180 mg/liter, 2,4-dichlorophenoxyacetic acid at 2.21 mg/liter, and sucrose at 30 g/liter) and growing the culture at 22°C in darkness at 120 rpm. *N. benthamiana* plants were grown at 24°C in a greenhouse with a light-and-dark cycle of 16 and 8 h, respectively.

Agrobacterium-mediated transient expression.

Agrobacterium-mediated transient expression in *N. benthamiana* was performed as described by Sessa and associates (2000). *Agrobacterium tumefaciens* strain GV2260 (final optical density at 600 nm = 0.5) was used to syringe infiltrate 5-week-old *N. benthamiana* leaves. Final *Agrobacterium* mixture for the infiltration consisted of 30% (vol/vol) pBA-HC-Pro, 30% (vol/vol) pTA-SIPK, and 40% (vol/vol) pCAMBIA-GFP or pCAMBIA-WRKY1:GFP. SIPK expression was induced by adding 30 µM Dex (Sigma) in ethanol to the final infiltration mixture. Infiltration mixtures without Dex also contained the same amount of ethanol. *N. benthamiana* leaves were collected for immunoblot assay 2 days after infiltration. A similar set of *N. benthamiana* leaves was kept an additional 2 days for cell death observation.

Tobacco BY2 cells were transformed with GFP fusion constructs by *Agrobacterium*-mediated transformation as previously described by van der Fits and associates (2000). Selection for transgenic BY2 cells was done on medium containing 0.7% plant tissue agar and antibiotics (hygromycin at 50 µg/ml, vancomycin at 100 µg/ml, and cefotaxim at 400 µg/ml), and grown for 7 days in the dark. Emerging transgenic callus was transferred into fresh medium containing hygromycin and grown to the appropriate density, then subcultured as described above.

Localization experiments.

Transgenic BY2 cells (5 to 7 days old) expressing SIPK-GFP or WRKY1-GFP were treated with 4,6-diamidino-2-phenylindole (DAPI) and analyzed with a fluorescent micro-

scope (IX70, Olympus, Tokyo) fitted with filters to visualize GFP and DAPI. Images were acquired with a digital camera (MagnaFire, Olympus) mounted on the microscope and image capturing software (MagnaFire, ver 2.1; Olympus).

EMSA.

The double-stranded oligonucleotide was generated by primed synthesis of the oligonucleotide P_{CHN0} using P₃₄ in a reaction mixture containing 10 µCi [α -P³²] dATP; 20 µM unlabeled dATP; 50 µM each dCTP, dGTP, and dTTP; 2 units of Klenow fragment (NEB); and the buffer supplied by the manufacturer. Phosphorylation of TGA2 and WRKY1 recombinant proteins are described above in the section for *in vitro* phosphorylation. Following the reaction for 30 min at 25°C, the probe was purified with a MicroSpin G-50 column (Amersham, Piscataway, NJ, U.S.A.). Binding reactions and gel analysis were done as described (Wang et al. 1998).

Immunoblot analysis.

Leaf discs of *N. benthamiana* 1 cm in diameter were ground in 50 µl of 2× SDS-PAGE loading buffer. Immunoblot was done as described (Lin et al. 1995) using an enhanced chemiluminescence method (Amersham) with the following modification. A polyvinylidene difluoride membrane (Millipore, Billerica, MA, U.S.A.) was probed with 10,000-fold diluted polyclonal α GFP antibody (Abcam, Cambridge) or α SIPK antibody (Zhang and Klessig 1998) and, subsequently, with 15,000-fold diluted α rabbit antibody conjugated to horseradish peroxidase (Amersham) as a secondary antibody. ECL-Plus (Amersham) and X-ray film (X-Omat, Kodak) were used to develop images.

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