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Double Jeopardy: Both Overexpression and Suppression of a Redox-Activated Plant Mitogen-Activated Protein Kinase Render Tobacco Plants Ozone Sensitive

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In plants, the role of mitogen-activated protein kinase (MAPK) in reactive oxygen species (ROS)–based signal transduction processes is elusive. Despite the fact that ROS can induce MAPK activation, no direct genetic evidence has linked ROS-induced MAPK activation with the hypersensitive response, a form of programmed cell death. In tobacco, the major ROS-induced MAPK is salicylate-induced protein kinase (SIPK). We found through gain-of-function and loss-of-function approaches that both overexpression and RNA interference–based suppression of SIPK render the plant sensitive to ROS stress. Transgenic lines overexpressing a nonphosphorylatable version of SIPK were not ROS sensitive. Analysis of the MAPK activation profiles in ROS-stressed transgenic and wild-type plants revealed a striking interplay between SIPK and another MAPK (wound-induced protein kinase [WIPK]) in the different kinotypes. During continuous ozone exposure, abnormally prolonged activation of SIPK was seen in the SIPK-overexpression genotype, without WIPK activation, whereas strong and stable activation of WIPK was observed in the SIPK-suppressed lines. Thus, one role of activated SIPK in tobacco cells upon ROS stimulation appears to be control of the inactivation of WIPK.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) modules form a key part of the eukaryotic signal transduction network that links environmental inputs to a wide range of modifications of cellular functions, ranging from cell division to cell death. In plants, MAPK signaling has been implicated in defense against pathogens and herbivores, in cellular responses to auxin, abscisic acid, and other phytohormones, in cell cycle control, in the induction of programmed cell death, and in responses to abiotic stresses such as UV light and ozone (Zhang and Klessig, 1997; Kovtun et al., 1998; Romeis et al., 1999; Heimovaara-Dijkstra et al., 2000; Samuel et al., 2000; Nishihama et al., 2001; Yang et al., 2001; Miles et al., 2002).

A variety of stress responses have been found to involve the rapid activation of a specific subset of plant MAPKs, notably Arabidopsis MPK6 (Ichimura et al., 2000; Kovtun et al., 2000; Nühse et al., 2000; Yuasa et al., 2001) and its orthologs in other species, such as salicylic acid–induced protein kinase (SIPK) in tobacco (Zhang and Klessig, 1998a, 1998b; Romeis et al., 1999; Mikolajczyk et al., 2000; Samuel et al., 2000; Zhang et al., 2000) and salt stress-induced MAPK (SIMK) in alfalfa (Cardinale et al., 2000). Because many biotic and abiotic stressors (virus infection, treatment with microbial elicitors, wounding, and osmotic stress) elicit a very rapid oxidative burst in plant cells, the apparent convergence of disparate stress signals on this particular MAPK node may be related to the sensitive response of MPK6/SIPK to redox perturbation.

Exposure to ozone immediately creates an oxidizing environment in plant tissues and triggers an array of cellular responses, including the accumulation of antioxidants, elicitation of pathogenesis-related proteins, deposition of phenols, induction of ethylene synthesis, suppression of primary metabolic activities such as photosynthesis, and eventually cell death (Darrall, 1989; Schraudner et al., 1992; Conklin and Last, 1995; Sharma and Davis, 1997; Tuomainen et al., 1997). Ozone enters the plant mesophyll through the stomata and diffuses through inner air spaces. In the cell wall and plasmalemma, it is converted spontaneously to reactive oxygen species (ROS) by contact with either water or membrane components (Sharma and Davis, 1997). The ozone-induced cell death process is influenced by the interaction of multiple signaling molecules, including salicylic acid, jasmonic acid, and ethylene (Orvar and Ellis, 1997; Overmyer et al., 2000; Rao et al., 2000).

One of the earliest responses elicited by ozone and other ROS generators in plants is the activation of specific MAPKs (Samuel et al., 2000; Desikan et al., 2001). The primary ROS-activated tobacco MAPK has been identified as the 46-kD

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SIPK; a second MAPK, the 44-kD wound-induced protein kinase (WIPK), usually responds more weakly (Kumar and Klessig, 2000; Samuel et al., 2000).

The rapid activation of these MAPKs suggests that their action on downstream targets could be important for the modulation of the cellular response to increased oxidative damage, but direct evidence for that role is lacking in plants. No intracellular substrates have been identified for either SIPK or WIPK, nor have loss-of-function genotypes been assessed for their ability to control redox stress. Stable overexpression or suppression of SIPK or WIPK in transgenic tobacco apparently did not result in the alteration of its activity (Yang et al., 2001). By contrast, transient overexpression of SIPK or its upstream activator, NtMEK2, in an active form has been shown to lead to the activation of either SIPK or both SIPK and WIPK, with associated induction of defense genes and hypersensitive response (HR)–like cell death (Yang et al., 2001; Zhang and Liu, 2001). This finding suggests that SIPK may play a role as a positive regulator in the cell death pathway.

The previously reported inability to produce SIPK-suppressed lines, and the lack of phenotype or alteration of SIPK activity reported for overexpression lines (Yang et al., 2001), have suggested that the normal functioning of this kinase may be essential for cell survival. However, we report here, using RNA interference (RNAi) technology, the recovery and analysis of transgenic tobacco plants in which SIPK is either overexpressed ectopically or largely eliminated. These plants display distinctive ozone response phenotypes that confirm the importance of SIPK activation for the effective control of ROS damage and also reveal an unexpected interplay between the activities of SIPK and WIPK.

RESULTS

Infiltration of fully grown tobacco leaves with a suspension of *Agrobacterium tumefaciens* cells carrying a SIPK-FLAG overexpression construct resulted in the accumulation of the epitope-tagged SIPK protein in the infiltrated tissue within 48 h. In unstressed cells, endogenous SIPK was not phosphorylated at the TXY motif found in the activation loop of the kinase, as indicated by the absence of any signal in the control lane of a protein gel blot (Figure 1C) prepared using an anti-pMAPK antibody that specifically recognized the doubly phosphorylated protein. In the infiltrated tissue, however, at least a portion of the pool of SIPK became activated by 48 h after infiltration, with even greater activation observed by 72 h. In the same period, the infiltrated zones showed signs of tissue collapse, and by 96 h, these zones became completely necrotic (Figure 1A).

When leaves were coinfiltrated with Agrobacterium carrying the SIPK-FLAG overexpression construct plus an RNAi construct that targeted SIPK, both expression and activation of SIPK-FLAG were suppressed completely (Figures 1B and 1C). The cell death induced by the overexpression of SIPK-FLAG in the infiltrated zones also was eliminated (Figure 1A).

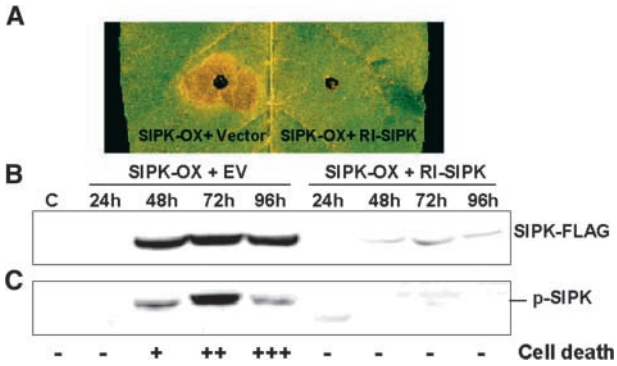

Figure 1. Post-Transcriptional Gene Silencing-Induced Suppression of Cell Death Triggered by Transient Overexpression of SIPK.

Coinfiltration with Agrobacterium containing the SIPK-FLAG construct along with the SIPK-RI construct inhibited the cell death process induced by transient overexpression of SIPK-FLAG alone **(A)**. Protein samples extracted at different times after infiltration of the constructs were immunoblotted with either anti-FLAG antibody **(B)** or phospho-MAPK-specific antibody (anti-pERK) **(C)**. −, +, ++, and +++ indicate the extent of visible lesions appearing in the infiltrated zones. EV, agrobacterium carrying empty vector.

The cell death associated with the spontaneous activation of SIPK in overexpression (OX) transgenic cells suggested that it might be difficult to recover stably transformed lines using this construct, but cocultivation of tobacco leaf discs with the appropriate Agrobacterium culture and selection on kanamycin yielded a number of transgenic lines that were found to ectopically express a range of levels of SIPK-FLAG (Figure 2A). No spontaneous activation of SIPK was detected in these lines, all of which displayed normal growth and development phenotypes.

Transformation of tobacco leaf discs with the SIPK-RI construct also yielded stable transgenic lines, although with a sharply reduced frequency. In the recovered RI lines, silencing of endogenous SIPK expression was observed to varying degrees, ranging from partial reduction in both SIPK mRNA and protein to elimination of both products (Figures 3B and 3C). The specificity of this silencing was shown by the continued expression in most of the recovered RI lines of the closely related *NTF4* MAPK gene, whose cDNA sequence is 89% identical to that of *SIPK* (Figure 3D). The RI lines again showed largely normal growth and development phenotypes, although the most severely suppressed lines showed some modest tendency to dwarfing (data not shown).

Plants of both the OX and RI lines showed no signs of spontaneous cell death under normal growth conditions. However, exposure of mature OX or RI leaves to levels of ozone that caused no visible injury to wild-type plants (500 parts per billion [ppb]) resulted in the rapid appearance of small necrotic lesions on leaves of both the transgenic

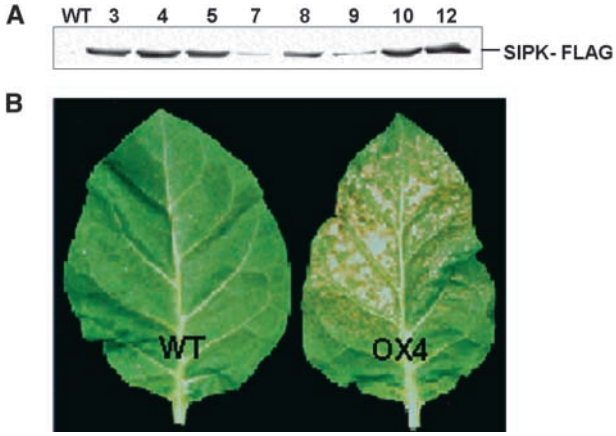

Figure 2. Transgenic Tobacco Plants Overexpressing SIPK-FLAG Show Increased Ozone Sensitivity.

(A) Proteins (40 μg) extracted from leaves of the different OX lines ectopically expressing SIPK-FLAG were immunoblotted using anti-FLAG antibody.

(B) Transgenic tobacco line OX4 and wild-type tobacco (Xanthi-nc) plants ($n = 25$) were exposed to ozone (500 ppb) for 8 h. The treated leaves were photographed 24 h after exposure.

WT, wild type.

genotypes (Figures 2B and 3E). The kinetics of this oxidative stress damage were quite different. Lesions consistently appeared on the leaves of OX plants as early as 4 to 6 h, but visually similar lesions only appeared on RI leaves ~24 h later. In plants challenged with lower ozone concentrations (250 ppb), an analogous pattern was observed except that the necrotic responses were delayed until 48 h (OX) and 72 h (RI) (data not shown). When leaf discs prepared from the wild-type, OX, and RI genotypes were assayed for the loss of membrane integrity and associated ion leakage resulting from ozone exposure (500 ppb), differential timing of the damage response also was observed (Figure 4).

To assess in situ the relative levels of hydrogen peroxide accumulation induced by ozone exposure, control and ozone-treated leaf halves were infiltrated with 3,3′-diaminobenzidine solution. The staining patterns revealed no detectable levels of hydrogen peroxide in untreated leaves of any of the genotypes or in leaves of wild-type plants after 8 h of ozone exposure. However, strong 3,3′-diaminobenzidine staining was observed in both the OX and RI lines after ozone treatment (Figure 4B).

The observation that overexpression of SIPK-FLAG in infiltrated leaves was accompanied by the spontaneous activation of MAPK and by cell death raised the question of whether activation of the ectopically expressed protein was necessary for the induction of cell death. Therefore, site-directed mutagenesis was used to create a version of SIPK-FLAG in which the TEY motif found in the activation loop of SIPK had been converted to an AEF sequence. This modification yielded a kinase that retained a low level of basal activity when the recombinant protein was assayed in vitro against myelin basic protein (Figure 5A), but it could not be activated further through dual phosphorylation of the activation loop by upstream MAPK kinases. Unlike the SIPK-FLAG construct, when transiently expressed in tobacco leaves, the SIPK(AEF)-FLAG construct failed to cause cell death in the infiltrated zone (data not shown).

Stably transformed tobacco plants expressing high levels of SIPK(AEF)-FLAG also were recovered readily after Agrobacterium cocultivation, and these plants displayed no visibly altered phenotype. Despite accumulating similar levels of the epitope-tagged kinase (Figure 5B), the ozone sensitivity of these SIPK(AEF) transgenic lines did not differ from that of wild-type plants (data not shown). This finding indicates that the heightened ozone sensitivity observed in SIPK-OX transgenic lines requires not only that the ectopically expressed kinase be expressed at high levels within the plant cell but that it have the capacity to become activated.

The activation status of both SIPK and WIPK in tobacco tissue extracts can be assessed either on protein gel blots using a phosphospecific antibody or by immunoprecipitation with antibodies that discriminate between SIPK and WIPK, followed by in gel or in vitro kinase activity assays. When the various transgenic and wild-type tobacco lines were monitored during a 30-min period of ozone exposure, striking differences in the pattern of kinase activation were observed among these genotypes (Figure 6).

As reported previously (Samuel et al., 2000), ozone treatment led to the rapid activation of SIPK in leaves of wild-type plants. This was accompanied by a much weaker activation of the smaller kinase, WIPK (Figure 6A). In the OX4 genotype, ozone exposure also led to SIPK activation, but the level of activation appeared to be depressed relative to the wild-type response, despite the presence of far greater amounts of ectopically expressed SIPK in the OX cells (Figures 6B and 6C). No activation of WIPK was detected in the OX tissue samples.

The SIPK(AEF) genotype presented a kinase activation profile that was very similar to that of the wild type. This indicates that flooding the cell with a nonactivatable version of SIPK (a potential dominant-negative form) does not interfere with the ability of the upstream MAPK cascade elements to transmit oxidant-induced signals to their cognate MAPKs.

Exposure of the RI genotype to ozone, on the other hand, yielded a very different MAPK activation profile. Very weak or no SIPK activation was detected, as would be predicted for a genotype in which SIPK expression has been suppressed by post-transcriptional gene silencing (Figures 6A and 6C). Instead, ozone exposure produced strong and specific activation of WIPK. The identity of these highly activated kinases in ozone-treated leaves of each genotype was confirmed through immunoprecipitation of the 30-min ozone-treated protein extracts with either SIPK- or WIPK-specific antibodies, followed by in gel kinase assays (Figures 6D and 6E).

Aside from the unexpected massive activation of WIPK, the stability of that activation also was strikingly different in

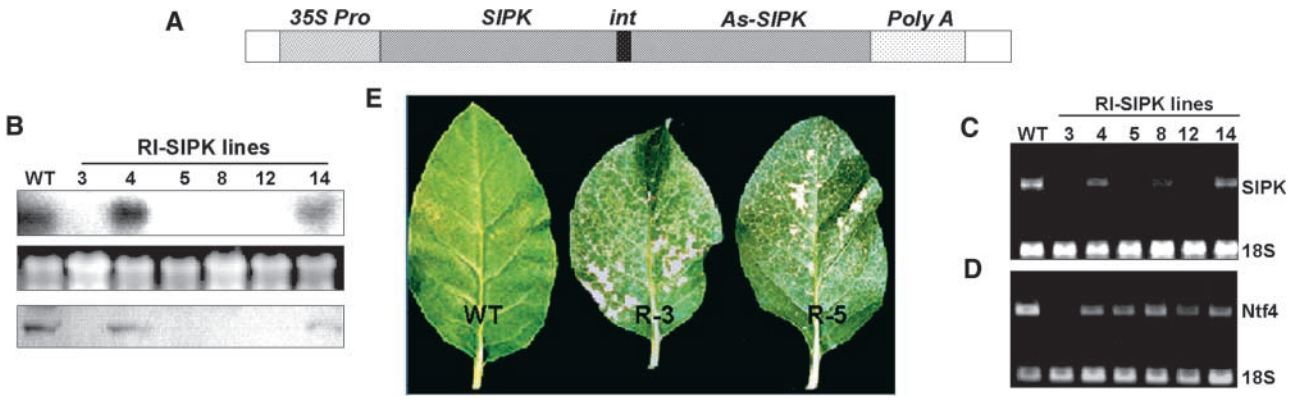

Figure 3. SIPK-Suppressed Lines Also Are Sensitive to Ozone.

(A) RNAi construct under the control of the 35S promoter of *Cauliflower mosaic virus*.

(B) SIPK is suppressed in four of the six PCR-positive lines. RNA gel blot analysis was performed using total RNA (15 μg) extracted from wild-type and SIPK-RI lines and probed with the radiolabeled C-terminal fragment of the *SIPK* ORF. Autoradiography revealed essentially no SIPK mRNA in four of the six PCR-positive lines (top). Ethidium bromide staining of the gel showed equal loading of RNA (middle). Immunoblot analysis of protein samples from the same lines indicated the absence of detectable amounts of SIPK protein in all four SIPK-suppressed lines (bottom).

(C) Similar results were observed when reverse transcriptase–mediated PCR was conducted using *SIPK*-specific primers.

(D) *NTF4* gene expression in the SIPK-suppressed lines was analyzed by reverse transcriptase–mediated PCR using gene-specific primers.

(E) SIPK-suppressed lines R3 and R5 display ozone-sensitive phenotypes. Plants ($n = 15$) of SIPK-suppressed tobacco transgenic lines R3 and R5, together with wild-type plants, were exposed to ozone (500 ppb) for 8 h per day for 2 days. The treated leaves were photographed 24 h after the end of 2 days of exposure.

WT, wild type.

this genetic background. Normally, when oxidants trigger a rapid activation of SIPK, it is a transient response. The activation is effectively lost within 1 h, even under conditions of continuous oxidant stimulus, as seen in Figure 7A (wild-type lane). However, in the RI genotype, WIPK was not only activated rapidly but the pool of this MAPK remained continuously active for up to 8 h after the initiation of the response (Figure 7A, RI lane). Although normally there is far less WIPK than SIPK present in tobacco leaves (Zhang and Klessig, 1998b), the high activation signal observed in the RI tissue extracts did not appear to reflect increased levels of WIPK protein in this genotype compared with wild-type plants, as assessed by protein gel blot analysis (Figure 7C).

Interestingly, kinase activation by ozone in the OX genotype also was prolonged abnormally, relative to that seen in ozone-treated wild-type plants, but in this case, the active kinase was SIPK rather than WIPK (Figure 7B). In addition, unlike the hyperactivated WIPK pool, the extended activation of SIPK in the OX line was more transient and disappeared within 4 h. This is approximately the time at which visible lesions began appearing on ozone-treated OX leaves.

Examination of the temporal response of the two genes (*GST* [glutathione *S*-transferase] and *cAPX* [cytosolic ascorbate peroxidase]) whose expression was induced strongly by ozone treatment revealed that the loss of SIPK signaling in the RI genotype resulted in a delayed response in the expression of both genes. In the OX line, the prolonged activation of SIPK signaling resulted in the suppression of *GST* induction, whereas *APX* gene expression was unaffected (Figures 8A and 8B).

DISCUSSION

Plant cells must deal constantly with ROS from a range of sources, including photooxidation, mitochondrial electron transport, flavin oxidase by-products, and environmental insults such as UV light, ozone, and ionizing radiation. Against this background, ROS pulses ("oxidative bursts") also can occur within cells, usually as very early responses to localized challenges to cellular integrity such as wounding and pathogen assault. These pulses may serve in multiple functions, including activation of redox protection mechanisms, modulation of intracellular signal transduction pathways, and transmission of systemic signals to neighboring cells.

A severe oxidative challenge that overwhelms local protective measures ultimately will lead to cell death. The archetype for this outcome is the HR response induced during incompatible host–pathogen interactions. Similar lesions are induced by exposure to increased levels of ozone or UV light. The exact process by which cellular integrity fails is unclear, but the notion that HR represents a form of genetically programmed cell death is supported by the identification of nu-

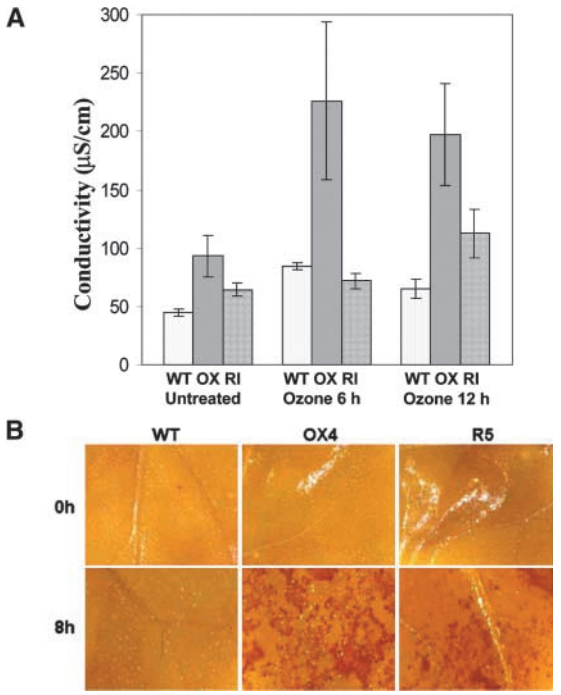

Figure 4. Quantitation of Ozone-Induced Cell Death and Hydrogen Peroxide Accumulation in SIPK Kinotypes.

(A) Ion leakage from leaf discs (five each) of the third and fourth leaves of wild-type, OX4, and RI5 lines was assessed as an indicator of the loss of membrane integrity 6 and 12 h after the initiation of ozone exposure (500 ppb). The data presented are means and standard deviations from three independent experiments.
(B) 3,3′-Diaminobenzidine staining to detect hydrogen peroxide accumulation in ozone-treated leaves of SIPK kinotypes.
WT, wild type.

merous mutants affected in the process of lesion formation (Richberg et al., 1998).

The correlation of ROS pulses with the cell death process has been described extensively. Treatments such as chilling, wounding, pathogen infection, UV irradiation, and ozone exposure rapidly induce ROS accumulation in plant cells, followed later by lesion development. However, despite these correlative observations, a functional link between ROS accumulation and local lesion formation has yet to be defined.

It is striking that so many stresses that elicit ROS accumulation in plant cells consistently appear to activate MAPK modules as one of their earliest effects (Seo et al., 1995; Zhang and Klessig, 1998b; Allan et al., 2001; Desikan et al., 2001; Orozco-Cardenas et al., 2001). The MAPK observed most consistently to be activated by both applied stresses and ROS is SIPK in tobacco (Samuel et al., 2000; Miles et al., 2002) or its apparent orthologs in other species, such as MPK6 in Arabidopsis (Kovtun et al., 2000; Yuasa et al., 2001) and SIMK in alfalfa (Cardinale et al., 2000). This pattern suggests that SIPK activation might play an important role in determining the response and ultimate fate of the stressed cells.

Links between ROS-associated cell death and MAPK signaling have been reported for a number of nonplant systems. Hydrogen peroxide–induced cell death in cultured mammalian oligodendrocyte cells is inhibited by PD98059, a specific inhibitor of MEK, the upstream kinase of the ERK1/2 MAPK (Bhat and Zhang, 1999), whereas delayed and prolonged activation of p44 and p42 MAPKs is critical for genistein-induced programmed cell death in rat primary cortical neurons (Linford et al., 2001). Similarly, delayed and persistent activation of ERK1/2 is associated with glutamate-induced oxidative cytotoxicity in neuronal cell lines (Stanciu et al., 2000).

There also is evidence that ROS-activated MAPKs may play analogous roles in plant cells. Cell death induced in Arabidopsis cell suspension cultures by treatment with a bacterial elicitor (harpin) is inhibited when the cells are treated with the MEK inhibitor PD98059 (Desikan et al., 1999), whereas pretreatment of tobacco cells with staurosporine, a general protein kinase inhibitor, suppresses the cell death normally induced by exposure to fungal elicitors (Suzuki et al., 1999).

Genetic manipulation experiments also have implicated MAPK activation in the cell death process. In Arabidopsis plants overexpressing constitutively active forms of the MAPK kinases AtMEK4 and AtMEK5 under the control of an inducible promoter, HR-like lesions appeared after induction with dexamethasone, and lesion formation was preceded by the activation of endogenous MAPKs and the accumulation of hydrogen peroxide (Ren et al., 2002). Transient overexpression of a constitutively active form of a MAPK kinase (NtMEK2) in tobacco also led to the sustained activation of MAPKs, identified

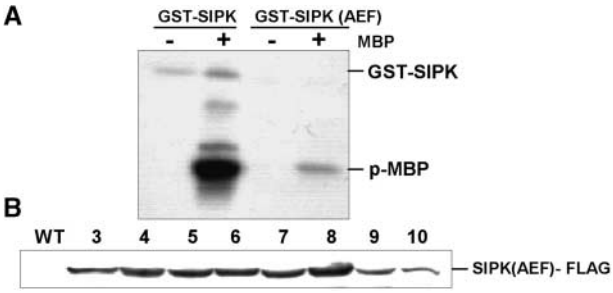

Figure 5. Activity and Expression of Mutagenized SIPK.

(A) The recombinant SIPK activation loop mutant is less active than wild-type SIPK. Myelin basic protein (MBP)-phosphorylating activities of SIPK and SIPK(AEF) were measured by incubating recombinant proteins (5 μg) with 5 μg of MBP, as described in Methods. The phospho-MBP product was visualized through autoradiography after SDS-PAGE fractionation.
(B) SIPK(AEF) transgenic lines show high expression of the transgene product. Proteins (40 μg) extracted from the different lines overexpressing SIPK(AEF)-FLAG were fractionated by SDS-PAGE and immunoblotted using anti-FLAG antibody.
WT, wild type.

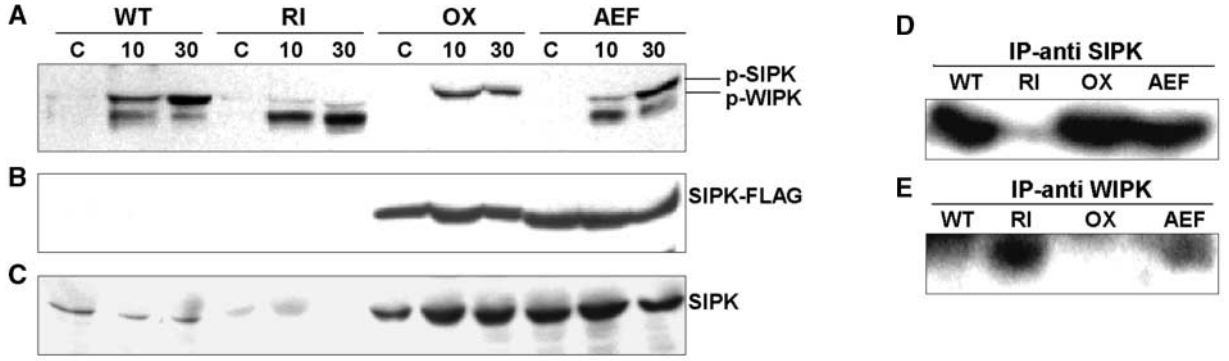

Figure 6. Differential Ozone-Induced Activation of SIPK and WIPK in SIPK Kinotypes.

(A) Crude protein extracts prepared from ozone-exposed tissues from T1 lines of the different SIPK kinotypes (RI5, OX4, and AEF8) and the wild type were resolved on a 10% polyacrylamide gel, blotted, and probed with an anti-phospho-ERK antibody to recognize phospho-MAPK forms.
(B) The same blot was probed subsequently using an anti-FLAG antibody to detect ectopic expression of the transgene product in the different kinotypes.
(C) A replicate protein gel blot was analyzed using a SIPK-specific antibody, revealing high expression of SIPK forms in the overexpressor lines and its absence in the SIPK-suppressed lines.
(D) and **(E)** Protein samples prepared from ozone-exposed (30 min) tissues from the different kinotypes were immunoprecipitated with either SIPK-specific **(D)** or WIPK-specific **(E)** antibodies. The immunoprecipitates were subjected to an in gel kinase assay, as described in Methods.
WT, wild type.

as SIPK and WIPK, and to the death of the infiltrated tissue (Yang et al., 2001). Transient overexpression of SIPK itself was shown subsequently to result in the formation of HR-like lesions, but only in young leaves (Zhang and Liu, 2001).

We have confirmed that ectopic SIPK overexpression leads to the appearance of high levels of the activated kinase in Agrobacterium-infiltrated tobacco tissue and to rapid cell death (Figure 1). On the other hand, when stably transformed tobacco plants were produced that overexpressed epitope-tagged SIPK (Figure 2), they displayed no visible phenotype. When exposed to ozone, however, the transgenic SIPK-OX plants proved to be much more sensitive than the nontransgenic parental line, indicating that ROS-induced cell death was controlled less effectively in the overexpression genotype.

Although this pattern is consistent with the results of NtMEK2 or SIPK-OX transient expression, its physiological relevance remains uncertain, because we know little about the effects of the accumulation of nonphysiological levels of active signal components on cellular function. To unambiguously identify a functional relationship between ROS activation of SIPK and ROS-induced cell death, we turned to the creation of defined loss-of-function mutants.

The modification of SIPK function in transgenic tobacco plants using either conventional gene-silencing methods (cosuppression and antisense-mediated suppression) or overexpression of dominant-negative forms proved ineffective (Yang et al., 2001) (data not shown). However, expression of an intron-containing "hairpin RNA" (Smith et al., 2000) designed to target a unique tract within the SIPK coding sequence yielded a number of transgenic plants in which SIPK expression was suppressed severely and specifically through post-transcriptional gene silencing. Loss of SIPK had no obvious phenotypic consequences for plants grown under normal greenhouse conditions.

Given the sensitivity of SIPK-OX lines to ozone, it might have been predicted that the absence of this kinase would have no effects, or perhaps even positive effects, on the ozone sensitivity of the SIPK-RI lines. Instead, after ozone treatments that induced no visible damage on wild-type plants, the SIPK-RI lines developed numerous lesions on their middle leaves within 24 h. Thus, the inability of the suppressed genotype to generate and activate SIPK compromises the cell's ability to manage ROS stress and to control cell death, although apparently on a different time scale from that observed in SIPK-OX plants.

Which facet of ROS-stress management has been compromised in SIPK-OX and SIPK-RI plants is not clear. No constitutive hydrogen peroxide accumulation was detected in any of the genotypes, suggesting that their heightened ozone sensitivity is not the consequence of a preexisting accumulation of ROS. Instead, it appears that alteration of the normal ozone-induced MAPK activation process, through either unregulated overexpression or suppression, creates an inability to cope with increased redox stress. Examination of the transcriptional activity of two genes whose mRNAs accumulate rapidly after ozone exposure showed that the response of both genes was affected differently (Figure 8).

Expression of *cAPX*, which encodes a major ROS-scavenging enzyme, was induced less effectively by ozone in RI plants, whereas it was unaffected in the OX line. Antisense suppression of *cAPX* was shown previously to create hypersensitivity to both ozone (Orvar and Ellis, 1997) and pathogens (Mittler

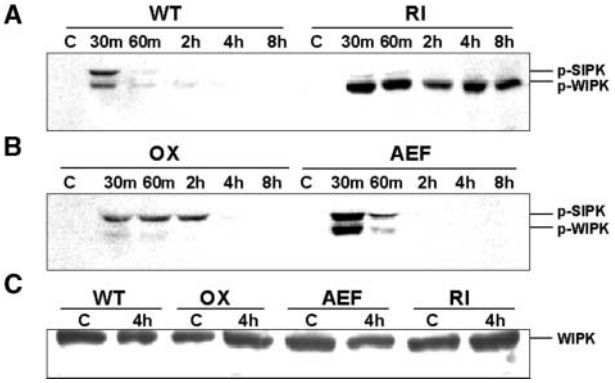

Figure 7. Loss of SIPK Has Differential Effects on the Expression of Ozone-Induced Genes and Leads to the Hyperactivation of WIPK.

(A) and **(B)** Extended ozone exposure reveals strong and prolonged activation of WIPK in the RI line. A temporal profile of the phosphorylation status of SIPK and WIPK was generated through anti-pERK immunoblotting of crude proteins extracted from tissues of either wild-type and RI lines **(A)** or OX and AEF lines **(B)** exposed to ozone for different times (0 to 8 h).

(C) Alteration of SIPK does not lead to changes in the amount of WIPK. Protein extracts from untreated and 4-h ozone-treated tissues from different kinotypes were immunoblotted with anti-WIPK antibody. WT, wild type.

et al., 1999) in transgenic tobacco plants. On the other hand, ozone-induced expression of *GST*, a general cellular protectant, was suppressed strongly in the *OX* line, but its expression was delayed markedly in the RI line. In Arabidopsis, both hydrogen peroxide and ozone induce GST expression (Clayton et al., 1999; Grant et al., 2000), and this expression has been demonstrated to require the activity of an unidentified 48-kD MAPK and calcium ion influx. Calcium channel activity also is essential for the ROS activation of SIPK in tobacco (Samuel et al., 2000).

The delayed response of the antioxidant genes in the RI line could result in increased early accumulation of ROS (Figure 4B), which could lead to a necrotic cell death process. In the OX line, although the *cAPX* gene response to ozone appeared to be normal, the antioxidant response clearly was unable to contain the increasing ROS levels associated with extended SIPK activation (Figure 4B). MAPK activation has been linked previously to increased ROS accumulation in Arabidopsis (Ren et al., 2002). A broader comparison of transcript profiles should generate useful insights into other connections between the transmission of redox signals by SIPK and the ability of the cell to avoid oxidative cell death.

Another aspect of the link between SIPK activation and cell death is revealed in the pattern of MAPK activation in ROS-stressed plants. The activation of SIPK by ozone occurred within 10 min in SIPK-OX plants but was not reversed for 4 h, by which time cell death already was becoming visible. This outcome is similar to the association of the prolonged activation of mammalian ERK with the induction of programmed cell death in neurons (Stanciu et al., 2000). Considered together with the results of the transient expression experiments, this finding demonstrates that the unregulated continuous activity of SIPK within plant cells profoundly affects normal homeostatic mechanisms.

The absence of SIPK in the SIPK-RI genotype also led to premature cell death under redox stress conditions, but in this case, the hyperactivated species observed was WIPK rather than SIPK. There have been other indications that WIPK plays a central role in plant stress signaling. This gene was identified originally on the basis of its rapid and transient induction upon wounding of tobacco leaves (Seo et al., 1995), and the gene product was shown later to be activated transiently by wounding (Seo et al., 1999) and by various other stresses (Romeis et al., 1999; Zhang et al., 2000). WIPK activation usually is accompanied by the activation of SIPK, but SIPK and WIPK do not always respond in unison; some oxidative stresses appear to activate SIPK preferentially and leave WIPK unaffected (Kumar and Klessig, 2000; Samuel et al., 2000).

WIPK activity, either alone or together with SIPK, has been suggested to be involved in the induction of cell death in cultured tobacco cells by specific fungal elicitor treatments (Zhang et al., 2000). Pretreatment of the elicited cells with staurosporine and K252A (protein kinase inhibitors) completely suppressed both WIPK activation and cell death. However, transient overexpression of WIPK did not result in its activation and failed to induce cell death in infiltrated tobacco leaves, unlike overexpression of SIPK (Zhang and Liu, 2001). In another study, the stable overexpression of WIPK in transgenic tobacco was accompanied by the constitutive expression of protease inhibitor II and the accumulation of methyl jasmonate

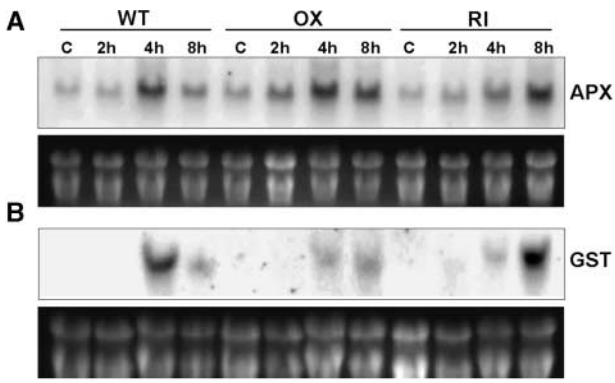

Figure 8. Alteration of SIPK Signaling Affects the Expression of GST and cAPX.

RNA gel blot analysis of the accumulation of cAPX **(A)** and GST mRNA **(B)** in wild-type, SIPK-overexpressing, and SIPK-suppressed transgenic tobacco. Plants were exposed to ambient air (C) or 500 ppb of ozone for 2, 4, and 8 h, and total RNA was harvested from the third and fourth leaves.

WT, wild type.

(Seo et al., 1999), but the oxidative stress sensitivity of the WIPK-OX lines was not reported.

How SIPK elimination leads to the prolonged hyperactivation of WIPK is unknown, but various possibilities suggest themselves. If NtMEK2 is the sole upstream MAPK kinase responsible for the activation of both SIPK and WIPK, these two MAPKs may normally compete for binding to NtMEK2. However, basal levels of SIPK in unstimulated tobacco cells are much higher (10-fold) than those of WIPK (Zhang and Klessig, 1998b). In the absence of competition from SIPK, activation of WIPK by NtMEK2 activation in SIPK-RI cells may be much more efficient than usual. This scenario also might explain why WIPK remains largely inactivated in ozone-treated SIPK-OX tissues in which an excess of SIPK is present. However, although this model accounts for WIPK hyperactivation, it does not necessarily explain why that activation is prolonged abnormally.

Alternatively, one of the normal roles of activated SIPK may be the direct or indirect regulation of WIPK activity. Both dual-specificity phosphoprotein phosphatases (MKP) and Ser/Thr phosphatases have been implicated in inactivating MAPK pathways in mammalian and plant models (Brondello et al., 1997; Meskiene et al., 1998; Ulm et al., 2001; Westermarck et al., 2001). If SIPK activity is required for the induction or activation of a protein phosphatase that normally acts upon phospho-WIPK, the absence of SIPK from oxidant-stressed SIPK-RI cells would create a situation in which WIPK could be activated by its cognate MAPK kinase but could not be inactivated subsequently.

In this regard, it is interesting that Arabidopsis plants in which a dual-specificity phosphatase (AtMKP-1) has been mutated by T-DNA insertional mutagenesis display increased activation of an unidentified ~49-kD MAPK and are more susceptible to ROS-generating stresses (e.g., UV light) (Ulm et al., 2001). On the other hand, MP2C, an alfalfa Ser/Thr phosphatase belonging to the PP2C class, has been shown to be a negative regulator of the MAPK pathway involving stress-activated MAPK, an apparent ortholog of WIPK (Meskiene et al., 1998). Both classes of protein phosphatase could be involved in cross-regulation mechanisms. Resolution of this question, and of the relative importance of the loss of SIPK activity versus the enhancement of WIPK activity in controlling oxidant-induced cell death, will require the development and analysis of other relevant single and multiple loss-of-function genotypes. These studies are now under way.

METHODS

Plant Material and Treatment

Tobacco (*Nicotiana tabacum*) plants of all genotypes were grown for 6 weeks in soil under controlled environmental conditions (25/20°C, 16-h-light/8-h-dark cycle) and then exposed to ozone (500 parts per billion) and harvested as described previously (Orvar and Ellis, 1997).

Recombinant Protein Production

The open reading frame (ORF) of salicylate-induced protein kinase (*SIPK*) was amplified by reverse transcriptase–mediated (RT) PCR using gene-specific primers and RNA isolated from untreated leaves of tobacco cv Xanthi-nc. The amplicon was cloned in frame into the expression vector pGEX 4T-3. Mutations in the activation loop of SIPK were introduced using a PCR-mediated approach, taking advantage of the unique NheI restriction site close to the activation loop. The mutational primers were designed so that the mutant form would code for AEF instead of TEY at amino acid positions Thr-218 and Tyr-220.

The *SIPK*(AEF) gene construct then was cloned into pGEX 4T-3. The recombinant glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21 cells by induction with 0.1 mM isopropylthio-β-galactoside for 4 h at 25°C, followed by purification according to the manufacturer's protocol (Amersham Pharmacia). The different constructs were sequenced to confirm the changes and the absence of mismatches.

Intron-Spliced Hairpin Loop RNA–SIPK Construct

The double-stranded RNA interference construct was tailored through a PCR-mediated approach using the N-terminal sequence of the *SIPK* ORF. A minimal intron based on the splice junctions and flanking regions of the fourth intron of *AtMPK6* (the Arabidopsis ortholog of SIPK) was incorporated into the sense-strand primer. The sense strand then was amplified using a primer combination that generated an EcoRI cleavage site and intron-XbaI sequence on the opposite ends of the product, whereas the antisense strand was amplified using a primer combination that added BamHI and XbaI sites on the opposite ends of the product. These two products were directionally cloned into EcoRI-BamHI–processed Bin19/pRT101 through a triple ligation, which placed the RNA interference construct under the control of the 35S promoter of *Cauliflower mosaic virus* (Figure 3A).

Binary Vector Construction and Plant Transformation

The different SIPK overexpression constructs were tagged with a C-terminal FLAG epitope through a PCR-mediated approach, followed by ligation into the plant expression vector Bin19/pRT101, which contains an *nptII*-selectable marker. All of the constructs were sequenced to confirm the presence of appropriate changes. The recombinant binary vector was used to transform competent *Agrobacterium tumefaciens* (EHA105) cells by a freeze-thaw transformation procedure.

Agrobacterium-mediated transformation of tobacco (cv Xanthi-nc) was performed using a leaf disc cocultivation procedure. Transformants were selected on half-strength Murashige and Skoog (1962) culture medium containing 50 mg/L kanamycin. Surviving plantlets were screened by PCR using 35S forward and gene-specific reverse primer combinations. Positive transformants then were screened by protein gel blot analysis (see below) using an anti-FLAG antibody for the SIPK overexpression lines and anti-SIPK antibodies to assess the RI suppression lines.

The confirmed transgenic lines were transferred to soil and grown to maturity, and seeds were collected. The T1 seeds were germinated on half-strength Murashige and Skoog (1962) medium with 50 mg/L kanamycin, and antibiotic-resistant plants were transferred to soil and grown under controlled conditions.

Transient Transformation Using Agrobacterium Infiltration

Four- to 6-week-old wild-type tobacco plants (cv Xanthi-nc) were used for infiltration experiments as described previously (Yang et al., 2001). This involved leaf infiltration with a mixed culture (OD of 0.4 at 600 nm) of Agrobacterium EHA105 containing the SIPK-FLAG overexpression construct plus an equal population of Agrobacterium containing either the empty vector or the SIPK-RI construct. At the indicated times (Figure 1), the infiltrated area was cut from the leaf, frozen in liquid nitrogen, and stored at −80°C until further analysis.

RNA Gel Blot and RT-PCR Analysis

Total RNA (15 μg) was resolved on 1% agarose-formaldehyde gels, blotted, and probed as described previously (Orvar and Ellis, 1997). The 600-bp C-terminal fragment of the *SIPK* ORF and PCR-amplified fragments of the cytosolic ascorbate peroxidase (c*APX*) and *GST* were used as probes. Gene-specific primers were used to amplify the ORFs of *cAPX* (forward, 5′-AGAACAATTGCTATGGGTAAGTG-3′; reverse, 5′-GCAAGCTTAAGCTTCAGCAAAT-3′) and *GST* (forward, 5′-ATGGCGATCAAAGTCCATGGTA-3′; reverse, 5′-TTTTTGCAG-CTTCTCCAATCCC-3′) using cDNA as the template.

The cDNA was synthesized from total RNA extracted from control and ozone-exposed tissues of the different genotypes/treatments using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). RT-PCR was performed using gene-specific primers designed to target either *SIPK* (25 cycles) or *NTF4* (30 cycles). The number of cycles was adjusted so that the amplification was within the linear range. As an internal control, 18S ribosomal cDNA was amplified using a 1:4 ratio of 18S-specific primers to competitor's DNA fragments provided by Ambion (Austin, TX).

Protein Extraction and Protein Gel Blot Analysis

Total protein extracts were prepared (40 to 80 μg) and used for protein gel blot analysis as described previously (Samuel et al., 2000). A primary antibody dilution of 1:1000 was used for anti-pERK (New England Biolabs, Beverly, MA), and a dilution of 1:5000 was used for anti-SIPK, anti–wound-induced protein kinase (WIPK) (Seo et al., 1999; Y. Ohashi, personal communication), and anti-FLAG (Sigma) antibodies.

Immune Complex Kinase Assay

Immunoprecipitations were performed as described previously (Samuel et al., 2000) using 250 μg of extracted protein together with 5 μg of either anti-SIPK or anti-WIPK antibodies. The immunoprecipitates were analyzed in an in gel kinase assay as described previously using myelin basic protein as the substrate (Zhang and Klessig, 1997).

In Vitro Kinase Assays

GST fusion proteins (5 μg) of the wild-type and mutant SIPK(AEF) were incubated with 5 μg of myelin basic protein and 10 μCi of γ-^{32}P–labeled ATP (>5000 Ci/mmol) (Amersham Pharmacia) in a 20-μL reaction mixture (20 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 2 mM Na_3VO_4, and 20 mM β-glycerophosphate) at 30°C for 30 min. The reaction was stopped with 6 × SDS loading buffer, and the samples were resolved on a 15% polyacrylamide gel, blotted onto a nylon membrane, and visualized by autoradiography.

Ion-Leakage Assay

Five leaf discs (9 mm) were cut from each of the third and fourth leaves of ozone-exposed and untreated plants of the wild type, OX4, and RI5 lines. The 10 leaf discs were incubated in 5 mL of deionized water at 25°C on a gyratory shaker at 110 rpm for 4 h, and the conductivity of the solution was measured as described previously (Mittler et al., 1999).

In Situ Staining for Hydrogen Peroxide

Hydrogen peroxide was visualized in situ by 3,3′-diaminobenzidine staining performed essentially according to Torres et al. (2002). Leaf halves were collected after 8 h of ozone exposure (500 parts per billion) and vacuum infiltrated with the 3,3′-diaminobenzidine (1 mg/mL) solution. Infiltrated leaves were placed under high humidity until brown precipitation was observed (5 to 6 h) and then fixed with a solution of ethanol:lactic acid:glycerol (3:1:1, v/v) for 2 days, followed by further clearing in methanol. Unless indicated otherwise, all experiments were repeated with consistent results.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

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