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A subset of hypersensitive response marker genes, including *HSR203J*, is the downstream target of a spermine signal transduction pathway in tobacco

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Summary

A cellular signal transduction pathway induced by the polyamine, spermine (Spm), and transmitted by mitochondrial dysfunction is proposed in tobacco. In this investigation, we further resolve the pathway by identifying a subset of hypersensitive response (HR) marker genes as downstream components. In a previous report, we identified harpin-induced 1 (*HIN1*) and two closely related genes as responsive to Spm. Other HR marker genes, *HSR203J*, *HMGR*, *HSR201*, and *HSR515*, are also Spm-responsive. Induction of these HR marker genes, including *HIN1*, by Spm was suppressed by pre-treatment with antioxidants, calcium channel blockers, inhibitor of mitochondrial permeability transition pore openings, and blockers of amine oxidase/polyamine oxidase. Such quenching is also observed for Spm-induced activation of two mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase (SIPK), and wound-induced protein kinase (WIPK), and upregulation of the *WIPK* gene, suggesting that all these components are part of the same signaling pathway. Furthermore, gain-of-function and loss-of-function studies on MAPK cascade members reveal that the expression of Spm-induced HR marker genes varies with respect to involvement of SIPK/WIPK activation.

Keywords: hypersensitive response, mitochondria, signal transmitter, spermine, target gene, tobacco.

Introduction

The three major polyamines (PAs) in plants include putrescine (Put), spermidine (Spd), and spermine (Spm). These compounds are implicated in the promotion of growth and development through interactions with various biomolecules by virtue of their basic nature (Tabor and Tabor, 1984). In addition, PAs are implicated in various processes against (a)biotic stress conditions (Bouchereau *et al*., 1999; Urano *et al*., 2003; Walters, 2000, 2003a,b).

Despite the continued interest in the role of PAs in plants exposed to (a)biotic stress only limited information is currently available. Most studies on compatible–incompatible interactions between plants and pathogens describe the changes in PA content and enzymatic activities involving PA biosynthesis, as observed in the case of 'green islands' formed on cereal leaves infected with biotrophic fungal pathogens, such as rust and powdery mildew. Greenland and Lewis (1984) showed that rust (*Puccinia hordei*)-infected barley leaf tissues contained six to seven times higher Spd levels compared with healthy tissues, implying a relationship between Spd and green island formation. Walters *et al.* (1985) demonstrated that all PA content increased in barley leaves infected with the powdery mildew fungus, *Erysiphe graminis*, consistent with the increase in enzymatic activities of arginine decarboxylase (ADC), ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (SAMDC). Studies on plant–viral pathogen interactions by Negrel *et al.* (1984) disclosed that ODC activity was 20-fold enhanced in tobacco leaves exhibiting hypersensitive response (HR) to

Tobacco mosaic virus (TMV) infection. Consistent with this report, the *ODC* gene was upregulated during the HR process (Yoda *et al.*, 2002). Using the same experimental system, Torrigiani *et al.* (1997) observed maximum levels of free- and conjugated-Put and Spd in the necrotic area. The authors suggested that high levels of PA conjugates are required for necrotic lesion development, which restricts virus movement and prevents systemic infection.

In the above studies, PAs are confirmed as 'functional molecules' but not 'signaling molecules'. However, experiments by Yamakawa *et al.* (1998) indicate that Spm functions as a signal molecule to transduce defense responses. The authors identified Spm as an endogenous inducer of pathogenesis-related (PR) proteins during TMV-induced HR. Moreover, the group demonstrated that exogenously applied Spm induced PR proteins and conferred TMV resistance to host plants. In view of this finding, we examined the possibility of PA functioning as signaling molecules in more detail. The data obtained so far support our working hypothesis. Specifically, of the three PAs, Spm induces mitochondrial dysfunction, and subsequently, activation of salicylic acid (SA)-induced protein kinase (SIPK; Zhang and Klessig, 1997) and wound-induced protein kinase (WIPK; Seo *et al.*, 1995) (Takahashi *et al.*, 2003). These two mitogen-activated protein kinases (MAPKs) are involved in the regulation of both defense gene expression and HR-like cell death (Lee *et al.*, 2001; Ren *et al.*, 2002; Yang *et al.*, 2001). Using a differential hybridization approach, harpin-induced protein 1 (*HIN1*) (Gopalan *et al.*, 1996) and two closely *HIN1*-related genes were isolated as possible downstream targets in the Spm-signaling pathway. We confirmed that these three *HIN1* family genes were upregulated during TMV-induced HR (Pontier *et al.*, 1999; Takahashi *et al.*, 2004). Based on the above evidence, we hypothesize that Spm is one of the signal transmitters in defense responses against phytopathogens.

In this study, we address the possibility that other HR-specific genes are Spm-responsive, and demonstrate that in addition to *HIN1*, a subset of HR marker genes, including *HSR203J*, is responsive to Spm. Pharmacological and biochemical analyses reveal that these HR marker genes are positioned downstream of Spm-triggered mitochondrial malfunction. Moreover, data from gain-of-function and loss-of-function studies on members of the MAPK cascade indicate that the Spm-induced signaling pathway has at least two distinct branches with respect to involvement of SIPK/WIPK activation.

Results

Expression of a subset of HR marker genes is induced by Spm

We have shown that Spm specifically activates two MAPKs, SIPK and WIPK (Takahashi *et al.*, 2003), and induces expression of *HIN1* (Gopalan *et al.*, 1996), a well-known HR marker gene, and two closely related genes, *HIN9* and *HIN18* (Takahashi *et al.*, 2004). Our results strongly indicate that this compound acts as a signal transmitter in defense responses against pathogens. In this study, we investigate whether other HR marker genes respond to Spm. The probes employed were *HSR203J* (Pontier *et al.*, 1994), *HMGR* encoding 3-hydroxy-3-methylglutaryl CoA reductase, the first and rate-limiting enzyme in the mevalonic acid-derived terpenoid biosynthetic pathway (Kang *et al.*, 1998), *HSR201*, which displays high similarity to a tomato gene involved in fruit ripening (Czernic *et al.*, 1996) and encodes benzoyl-coenzyme A: benzyl alcohol benzoyl transferase (D'Auria *et al.*, 2002), and *HSR515* encoding an isoform of cytochrome P450 (Czernic *et al.*, 1996). All four genes were responsive to Spm. Transcripts started to accumulate at 6 h after Spm treatment and were maintained at high levels up to 24 h (Figure 1). *HSR203J* and *HMGR* were expressed at basal levels in mature tobacco leaves, whereas almost null expression of *HSR201* and *HSR515* was observed under physiological conditions. Induction kinetics of the latter two genes was slower than those of the former two genes as well as that of *HIN1* (Takahashi *et al.*, 2004). Two other PAs, Put and Spd, did not affect gene expression (Figure 2a). SA is a key molecule in defense responses against pathogens in tobacco plants. Accordingly, we analyzed the involvement of SA in Spm-induced expression of the four genes. In *NahG*-transgenic tobacco plants (Friedrich *et al.*, 1995) with undetectable levels of SA, all four genes were induced by Spm. However, induction was partly impaired in the cases of

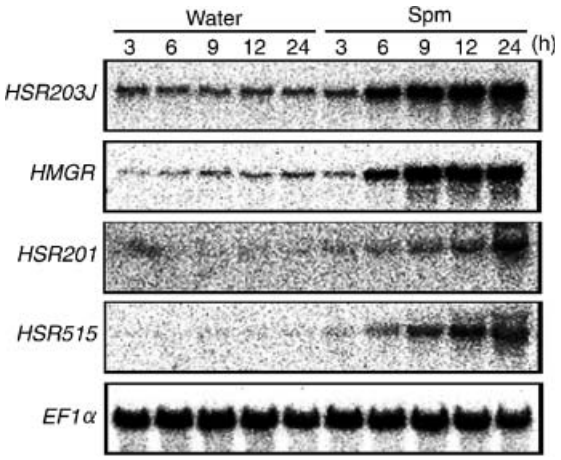

Figure 1. Northern analysis of the expression of *HSR203J, HMGR, HSR201,* and *HSR515* genes after the addition of spermine (Spm) to tobacco leaf disks. Total RNA samples were extracted at the indicated times from control and Spm (0.5 mM)-treated tobacco leaf disks. Aliquots (10 μg each) were separated on formaldehyde–1% agarose gels, and transferred onto membranes. Blots were hybridized with radiolabeled full-length *HSR203J, HMGR, HSR201* and *HSR515* cDNA. Hybridization with the tobacco *EF1α* gene served as a loading control.

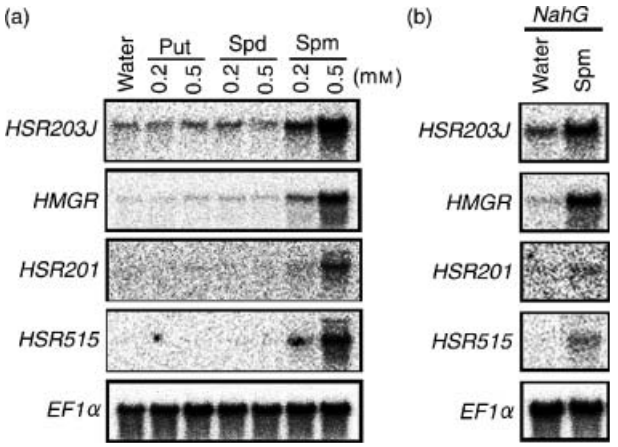

Figure 2. Specificity of polyamine (PA) compounds regarding the expression of *HSR203J*, *HMGR*, *HSR201* and *HSR515* genes and involvement of salicylic acid in spermine (Spm)-induced expression of hypersensitive response marker genes.
(a) Tobacco leaf disks were treated for 12 h with putrescine (Put), spermidine (Spd) or Spm (0.2 or 0.5 mM) and samples were analyzed as described in Figure 1. The tobacco *EF1α* gene served as a loading control.
(b) Leaf disks from *NahG* transgenic tobacco plants were treated with Spm (0.5 mM) for 12 h and samples were analyzed as in (a).

HSR201 and *HSR515*. The finding indicates that SA does not affect Spm-induced *HSR203J* and *HMGR* gene expressions, but is partially involved in the expression of *HSR201* and *HSR515* (Figure 2a,b).

Antioxidants and calcium channel blockers suppress Spm-induced expression of the five HR marker genes

Recent studies provide evidence that the production of reactive oxygen species (ROS) and/or influx of extracellular Ca^{2+} occur during pathogen-induced defense responses. Moreover, these compounds are involved in the activation of subsequent defense-associated responses in various plants (Blume *et al.*, 2000; Bolwell, 1999; Dat *et al.*, 2000; Lamb and Dixon, 1997; Mithöfer *et al.*, 1999; Xu and Heath, 1998). Using specific bioprobes, we have shown that the activation of SIPK and WIPK by Spm is completely dependent on ROS production and Ca^{2+} influx (Takahashi *et al.*, 2003). The involvement of ROS and Ca^{2+} influx in the induction of five HR marker genes, including *HIN1*, by Spm was examined using a similar approach. Pre-treatment of tobacco leaf disks with two antioxidants for 1 h, specifically, flavone (Minagawa *et al.*, 1992) and *N*-acetylcysteine (Wendehenne *et al.*, 1997), partially or totally suppressed expression of the five HR marker genes induced by Spm (Figure 3a). The calcium channel blockers, La^{3+} and Gd^{3+}, additionally attenuated Spm-specific induction of the HR marker genes (Figure 3b). The results collectively imply that both ROS generation and Ca^{2+} influx occur upstream of Spm induction for all five HR marker genes.

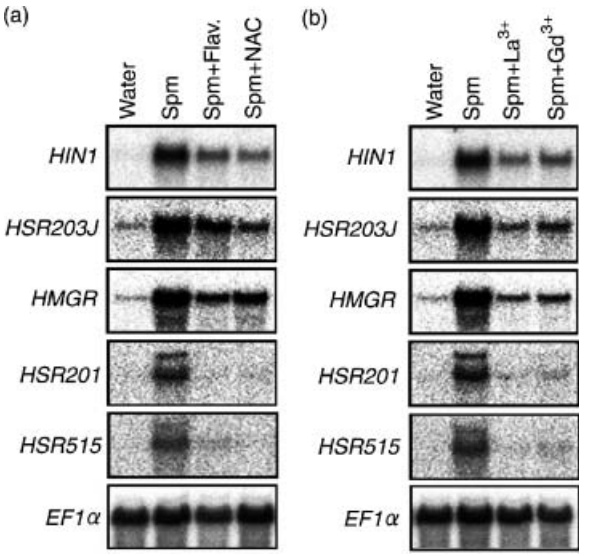

Figure 3. Effects of antioxidants and calcium channel blockers on spermine (Spm)-induced expression of *HIN1*, *HSR203J*, *HMGR*, *HSR201*, and *HSR515* genes.
(a) Effects of antioxidants on the expression of five hypersensitive response (HR) marker genes. Leaf disks were treated with flavone (1 mM) or *N*-acetylcysteine (2 mM) for 1 h prior to Spm addition (0.5 mM) and further incubated for 12 h. Total RNA (10 μg) was separated on a formaldehyde–1% agarose gel and transferred onto membranes. Blots were hybridized as described in Figure 1. Tobacco *EF1α* gene served as a loading control.
(b) Effects of calcium channel blockers on the expression of five HR marker genes. Leaf disks were treated with La^{3+} (0.5 mM) or Gd^{3+} (0.5 mM) for 1 h prior to Spm treatment (0.5 mM) and further incubated for 12 h. RNA separation and hybridization were performed as in (a).

Prevention of opening of the mitochondrial permeability transition pore suppresses the upregulation of HR marker genes triggered by Spm

Spm triggers mitochondrial malfunction, subsequently activating two MAPKs, SIPK and WIPK (Takahashi *et al.*, 2003). To determine whether mitochondria are involved in Spm-induced gene expression, an inhibitor of the mitochondrial permeability transition (PT) pore opening, bongkrekic acid (BK) (Narita *et al.*, 1998), was utilized. BK almost completely inhibited Spm-mediated induction of the five HR marker genes (Figure 4a). Previously we showed that the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), mimetically activated phosphorylation activity of the 46 kDa protein kinase, as well as expression of *WIPK* and alternative oxidase (*AOX*) genes (Takahashi *et al.*, 2003). In this study, we examine the effect of CCCP on the expression of the five HR marker genes by treating tobacco leaf disks with 0.1 mM uncoupler for specific periods of time. At 3 h after CCCP addition, all five HR marker genes were significantly induced. Interestingly, the induction kinetics of *HIN1*, *HSR203J*, and *HMGR* genes in the presence of CCCP were distinct from those of *HSR201* and *HSR515*. Higher mRNA levels of the former three genes were observed, whereas

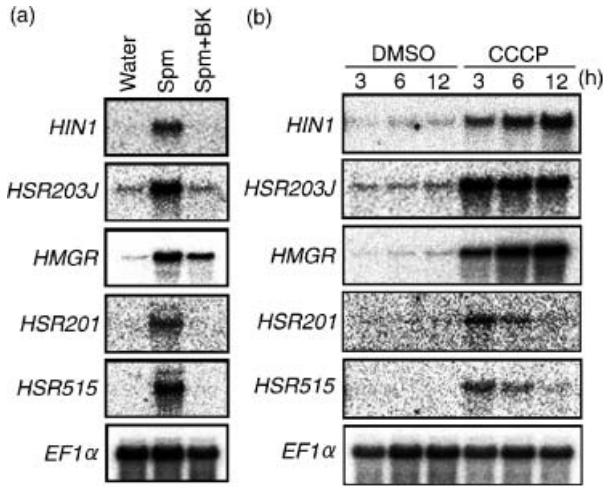

Figure 4. Effects of mitochondrial permeability transition (PT) pore opening blocker and uncoupler on expression of *HIN*, *HSR203J*, *HMGR*, *HSR201*, and *HSR515* genes.
(a) Effects of mitochondrial PT pore opening blocker on expression of *HIN*, *HSR203J*, *HMGR*, *HSR201*, and *HSR515* genes induced by spermine (Spm). Leaf disks were treated with bongkrekic acid (50 μM) 1 h before Spm treatment (0.5 mM) and incubated for 12 h. Total RNA (10 μg) was separated on a formaldehyde–1% agarose gel and transferred onto membranes. Blots were hybridized as described in Figure 1. The tobacco *EF1α* gene served as a loading control.
(b) Total RNA was extracted at the indicated times from control and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 0.1 mM)-treated tobacco leaf disks. RNA separation and hybridization were performed as in (a).

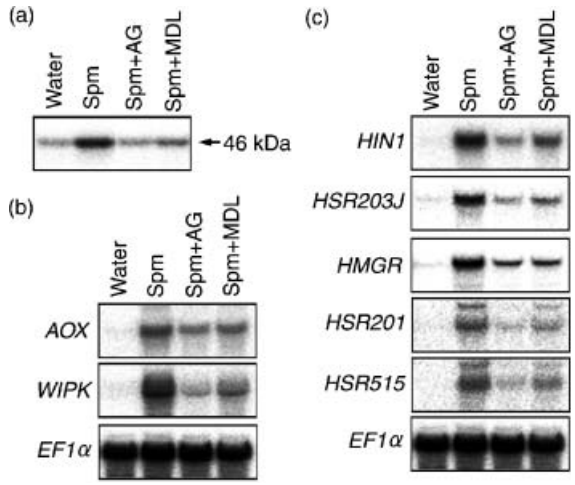

Figure 5. Effects of amine oxidase (AO) and polyamine oxidase (PAO) inhibitors on mitogen-activated protein kinase activation and spermine (Spm)-induced expression of *AOX*, *HIN1*, *HSR203J*, *HMGR*, *HSR201*, and *HSR515* genes.
(a) Effects of AO and PAO inhibitors on in-gel kinase activity. Leaf disks were treated with aminoguanidine (AG; 2 mM) or MDL72527 (10 mM) for 1 h prior to Spm treatment (0.5 mM) and further incubated for 6 h. An in-gel kinase assay was subsequently performed as described in the text.
Effects of amine and polyamine oxidase inhibitors on Spm-induced expression of *AOX* and *WIPK* genes (b), and *HIN1*, *HSR203J*, *HMGR*, *HSR201*, and *HSR515* genes (c). In (b) and (c), leaf disks were pre-treated as described for (a), except that incubation was performed for 12 h. Total RNA (10 μg) was separated on a formaldehyde–1% agarose gel and transferred onto membranes. Blots were hybridized as described in Figure 1.

expression of the latter two genes peaked at 3 h, followed by a gradual decrease (Figure 4b). These results suggest that despite different expression profiles following CCCP application, upregulation of the five HR marker genes reflects the malfunctional state of mitochondria.

Inhibitors of amine oxidase and polyamine oxidase suppress induction of the five HR marker genes by Spm

To date, we have shown that ROS induced by Spm action is a prerequisite, not only for the upregulation of HR marker genes, but also for the activation of SIPK and WIPK (Takahashi *et al.*, 2003). The next issue to solve is the source of ROS involved in Spm signal transduction. One candidate is H_2O_2 derived from the degradation of Spm catalyzed by either amine oxidase (AO) or polyamine oxidase (PAO). AO and PAO catalyze the deamination of biologically important amines oxidatively with the formation of the corresponding aldehyde, ammonia, and H_2O_2 (Agostinelli *et al.*, 1998). We employed specific inhibitors, such as aminoguanidine (AG) for AO and MDL72527 (kindly provided by Aventis Pharma Co. Ltd, Bridgewater, NJ, USA) for PAO. Pre-treatment of tobacco leaf disks with both AG and MDL72527 suppressed the activation of 46 kDa MAPK, and induction of *WIPK* as well as the five HR marker genes by Spm (Figure 5). Under similar conditions, accumulation of *AOX* mRNA in the presence of Spm was also disrupted (Figure 5b). The data indicate that the main source of ROS is H_2O_2 originating from Spm catabolism. Moreover, ROS generation occurs upstream of mitochondrial malfunction.

Positional relationship between SIPK/WIPK activation and induction of the five HR marker genes by Spm

Recently, Yang *et al.* (2001) identified a tobacco gene, *NtMEK2*, encoding a kinase (MAPKK) upstream of both SIPK and WIPK. The group further established an elegant system in which a constitutive active mutant of NtMEK2, NtMEK2^{DD}, was produced in a steroid hormone-inducible manner. To determine the positional relationship between activation of MAPKs and induction of the five HR marker genes in the Spm-signaling pathway, we employed a similar experimental system in which NtMEK2^{DD} was transiently produced in tobacco leaves with an *Agrobacterium* infiltration method to activate endogenous SIPK and WIPK. In tobacco leaves producing constitutively active NtMEK2^{DD} protein, *HIN1*, *HMGR*, and *HSR203J* genes were upregulated, but *HSR201* and *HSR515* levels remained unchanged, compared with those in NtMEK2-producing tobacco leaves (Figure 6). Kim and Zhang (2004) have reported the stimulation of *HIN1* and *HMGR* expression because of NtMEK2^{DD} expression.

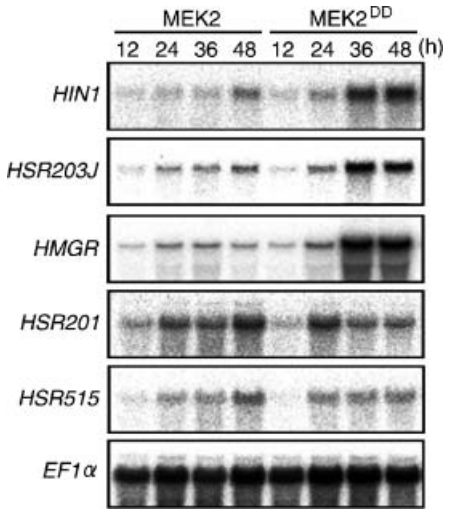

Figure 6. Effects of the overexpression of NtMEK2^{DD} on transcription of *HIN1*, *HSR203J*, *HMGR*, *HSR201*, and *HSR515* genes.
Tobacco leaves were infiltrated with *Agrobacterium tumefaciens* harboring NtMEK2^{DD} or NtMEK2 (control) under control of the 35S promoter of CaMV. Total RNA samples were isolated from leaves at the indicated times after agroinfiltration. Aliquots (10 μg each) were separated on formaldehyde–1% agarose gels and transferred onto membranes. Blots were hybridized as described in Figure 1.

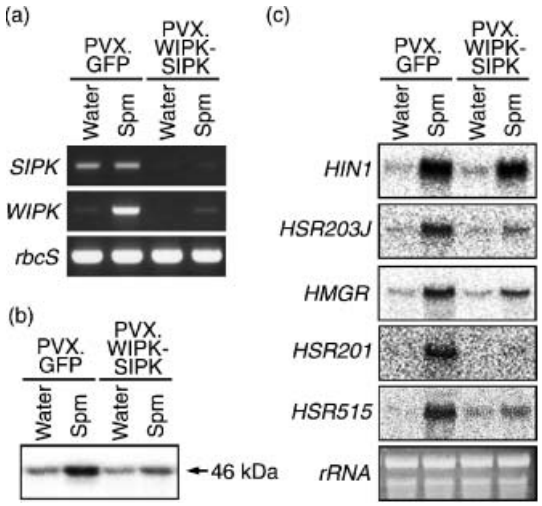

Figure 7. Effects of *SIPK/WIPK* silencing plants on Spm-induced mitogen-activated protein kinase (MAPK) activation and expression of *HIN1*, *HSR203J*, *HMGR*, *HSR201*, and *HSR515* genes.
(a) RT-PCR analysis of *SIPK* and *WIPK* genes by Spm. cDNA samples derived from the control or *SIPK/WIPK*-silenced leaf disks treated for 6 h with water or Spm (0.5 mM) were subjected to PCR cycling (see Experimental procedures).
(b) Effects of *SIPK/WIPK*-silenced leaf disks on MAPK activation by Spm. An in-gel kinase assay was subsequently performed as described in the text.
(c) Effects of *SIPK/WIPK*-silenced plants on Spm-induced expression of *HIN1*, *HSR203J*, *HMGR*, *HSR201*, and *HSR515* genes. Control or *SIPK/WIPK*-silenced leaf disks were treated for 6 h with water or Spm. Total RNA (10 μg) was separated on a formaldehyde–1% agarose gel and transferred onto membranes. Blots were hybridized as described in Figure 1.

Next, we performed a loss-of-function experiment by employing the virus-induced gene silencing (VIGS) method. The potato virus X (PVX)-derived vector (Baulcombe, 1999) was used to concurrently silence SIPK and WIPK genes in *Nicotiana benthamiana* leaves (Sharma *et al.*, 2003). Levels of *NbWIPK* and *NbSIPK* mRNA in leaves of PVX.WIPK-SIPK-inoculated plants were determined by RT-PCR using gene-specific primers that anneal to regions outside the partial cDNA fragments cloned into the PVX vector. Negligible levels of *NbSIPK* and *NbWIPK* transcripts were detected in plants inoculated with PVX.WIPK-SIPK. In contrast, higher levels of these transcripts were observed in control tobacco leaves treated with PVX.GFP (Figure 7a). Moreover, activation of the 46 kDa MAPK by Spm was suppressed in PVX.WIPK-SIPK-inoculated tobacco leaf disks (Figure 7b). Under these conditions, accumulation of *HSR203J*, *HMGR*, *HSR201*, and *HSR515* mRNA induced by Spm was reduced, whereas the level of *HIN1* mRNA was comparable to that following PVX.GFP inoculation (Figure 7c). Inconsistent results obtained from the gain-of-function and the loss-of-function experiments are also discussed.

Discussion

In this study, we show that exogenously applied Spm specifically activates the expression of four HR marker genes in tobacco leaves (Figures 1 and 2a). This induction requires ROS production and Ca^{2+} influx, and is mediated through mitochondrial malfunction (Figures 3, and 4), although distinct in terms of involvement of SA signaling (Figure 2b). Our data imply that the induction of *HIN1*, *HSR203J*, and *HMGR* genes by Spm is independent of the SA-signaling pathway, whereas that of *HSR201* and *HSR515* genes partially depends on this pathway. In contrast, Czernic *et al.* (1996) demonstrated that *HSR201* and *HSR515* expression during bacterial pathogen-induced HR is not affected in *NahG*-transgenic tobacco plants. The reason for this discrepancy between their data and our findings on SA involvement is currently unclear.

ROS production is one of the earliest responses in plants attacked by pathogens (Lamb and Dixon, 1997). ROS inhibit pathogen growth directly by microbicidal action, stimulate cross-linking of the cell wall, resulting in restricting penetration of pathogens, and enhance phytoalexin production and the expression of defense-related genes. Recent extensive studies disclosed various sources of ROS, including NADPH-dependent oxidase located in the plasma membrane, peroxidase linked to cell wall, germin-like oxalate oxidase in the extracellular space, and AO and PAO-type enzymes in the apoplasmic space (Allan and Fluhr, 1997; Lamb and Dixon, 1997; Zhou *et al.*, 1998). Of these, AO catalyzes the oxidative deamination of

biologically important amines, forming the corresponding aldehyde, ammonia, and H_2O_2 (Agostinelli *et al.*, 1998). In mammals, Spm induces mitochondrial uncoupling and cytochrome *c* release from the organelle. These phenomena are prevented by AO inhibitors (Maccarrone *et al.*, 2001). Thus, it is suggested that during apoptosis of animal cells, oxidative degradation of Spm results in H_2O_2 production, which directly induces cell death or indirectly transmits signals (Maccarrone *et al.*, 2001). In plants, infiltration of Spm into healthy tobacco leaf induced H_2O_2 generation (Yoda *et al.*, 2003). In a recent work on the hypersensitivity of barley to the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* by Cowley and Walters (2002), enhanced activities of PA biosynthetic enzymes such as ODC, ADC and SAMDC, and PA catabolic enzymes, including diamine oxidase and PAO were observed. In a current review, Walters (2003a) has discussed the involvement of Spm in a signal pathway during HR to pathogen infection, and pointed out the importance of PA catabolism in such processes. Here, we report that activation of MAPKs and expression of five HR marker genes by Spm are suppressed in the presence of specific inhibitors of AO and PAO (Figure 5). Our data indicate that H_2O_2 generated by Spm degradation is a major source of ROS. These AO/PAO inhibitors additionally suppressed the induction of the *AOX* gene by Spm, implying that mitochondrial dysfunction (reflected by *AOX* gene upregulation) is preceded by ROS production. It is known that the oxidative burst after pathogen infection occurs in a biphasic manner. A rapid response within a few to several minutes is termed phase I, and the later phase occurring several hours after pathogen attack is designated phase II. ROS production in phase I is not subjected to attack by avirulent pathogens, while that in phase II is associated with the expression of *Avr* genes, and plays a critical role in programmed cell death (PCD) (Chandra *et al.*, 1996). The oxidative burst after a temperature shift in TMV-infected tobacco leaves consists of two phases (Allan *et al.*, 2001). During TMV-induced HR, expression of genes involved in PA biosynthesis and concomitant accumulation of PAs were observed in the middle to late stages (Yamakawa *et al.*, 1998; Yoda *et al.*, 2003). Therefore, it is likely that H_2O_2 production derived from Spm catabolism contributes to the second phase of ROS production during TMV-induced HR. In relation to PA catabolism, it is worth to mention that a novel flavin-containing spermine oxidase (SMO) has been identified in mammalian cells including human and mouse (Vujcic *et al.*, 2002; Wang *et al.*, 2001). In general, Spm and Spd are first acetylated by Spd/Spm N^1-acetyltransferase and then oxidized by PAO to produce Spd and Put, respectively. Interestingly, newly identified SMO favors Spm over N^1-acetyl Spm but fails to act on N^1-acetyl Spd and Spd (Vujcic *et al.*, 2002). Although the SMO-like enzyme activity is not detected in higher plants yet, Spm-specific MAPK activation and gene expression that we observed may be explained by such an activity.

Ca^{2+} influx is additionally involved in various defense responses. Pathogen-induced Ca^{2+} influx occurs both before (Blume *et al.*, 2000; Schwacke and Hager, 1992) and after ROS production (Kawano and Muto, 2000; Price *et al.*, 1994), indicating that the two distinct plasma membrane calcium channels function during different phases of the response. Ca^{2+} influx into the cytosol is an important step in abiotic and biotic stress-signaling pathways. The data presented in this report support the idea that Ca^{2+} influx is an important component of the early phase of the Spm-signaling pathway in tobacco (Figure 3b).

It should be emphasized that mitochondria are involved in transduction of the Spm signal. Results using BK suggest that the opening/closing status of the PT pore in the mitochondrial membrane may be transmitted to the nucleus. Our findings also imply that the mitochondrion acts as a central depot within the cell where diverse stress stimuli are integrated (Lam *et al.*, 1999). The opening of the mitochondrial PT pore by Spm may result in collapse of the electrochemical transmembrane potential and allow efflux of putative death signal components. The downstream reaction of the malfunction of this organelle is transcriptional activation of HR marker genes that differ with respect to involvement of SIPK/WIPK activation.

Both Spm-induced MAPK activation (Takahashi *et al.*, 2003) and expression of the HR marker genes in this study are similarly suppressed by various chemicals, including antioxidants, calcium channel blockers, mitochondrial PT pore blocker, and inhibitors of AO and PAO. In addition, the uncoupler CCCP mimics Spm activity. In view of the above evidence, we conclude that activation of the two MAPKs and upregulation of the five HR marker genes by Spm are commonly preceded by ROS production, Ca^{2+} influx, and mitochondrial dysfunction (Figure 8).

MAPK cascades are major pathways by which extracellular stimuli are transduced into intracellular responses in plants (Asai *et al.*, 2002). In tobacco, two MAPKs, SIPK and WIPK, have been implicated in plant defense signaling during pathogen attack. Recently, Yang *et al.* (2001) identified the upstream kinase, NtMEK2, of SIPK and WIPK in a cascade. Moreover, PVX-mediated silencing of *NtMEK2*, *SIPK* or *WIPK* led to attenuation of *N* gene-mediated resistance to TMV in *N. benthamiana* (Jin *et al.*, 2003). Yang *et al.* (2001) have further established a sophisticated system in which the constitutively active mutant, $NtMEK2^{DD}$, activates endogenous SIPK and WIPK, and leads to HR-like cell death and defense gene expression in tobacco leaves. In this study, we adopt a similar system to transiently express $NtMEK2^{DD}$ in tobacco leaves. Gain-of-function data suggest that *HIN1*, *HSR203J*, and *HMGR* genes are induced downstream of SIPK/WIPK activation, but the induction of *HSR201* and *HSR515*

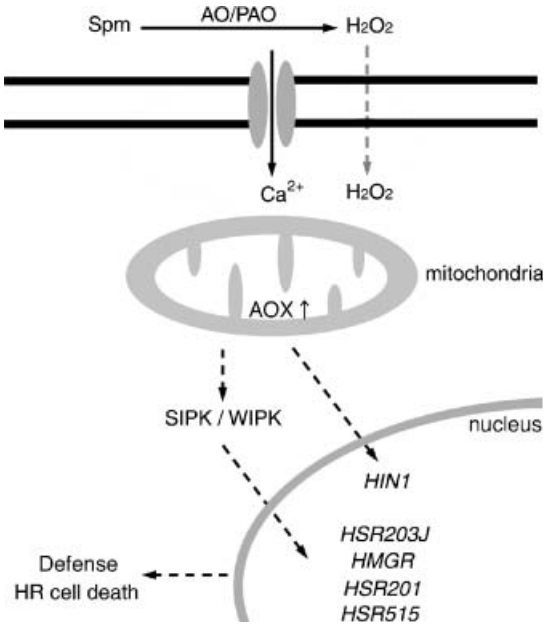

Figure 8. A proposed model of spermine (Spm) signal transduction pathways in tobacco. Free Spm is accumulated in intercellular spaces during *Tobacco mosaic virus* (TMV)-triggered hypersensitive response (HR). This polyamine is catabolized by amine oxidase (AO) and/or polyamine oxidase (PAO) localized in apoplastic spaces, resulting in the production of H_2O_2 and initiation of Ca^{2+} flux into the cytosol. The combined events cause mitochondrial malfunction. The downstream reaction of malfunction of this organelle is the upregulation of a subset of HR marker genes by pathways separated into at least two branches; one independent of and the other dependent on the activation of salicylic acid-induced protein kinase (SIPK)/wound-induced protein kinase (WIPK). Activation of these HR marker genes may contribute to defense against secondary pathogen attack and lead to programmed cell death during HR.

genes is independent of the MAPK pathways (Figure 6). Kim and Zhang (2004) observed that *HIN1*, *HMGR*, and several *PR* genes were upregulated in a similar system using NtMEK2^{DD}. Although analysis of plants expressing constitutively active components of the MAPK cascade is highly informative, the data must be interpreted with caution, as sustained activation of the cascade components may lead to pleiotrophic effects. Thus, we used a loss-of-function approach to investigate the roles of SIPK and WIPK in Spm signal transduction pathways. Interestingly, loss-of-function data were indicative of two branches downstream of MAPK activation; one dependent on and another independent of SIPK/WIPK activation. The former branch includes *HSR203J*, *HMGR*, *HSR201*, and *HSR515*, while the second contains *HIN1* (Figure 7c). In yeast and animal systems, MAPK cascades play central roles in converting signals generated from receptors/sensors into nuclear/cellular responses. A single MAPK phosphorylates and activates multiple downstream substrates in both cytoplasmic and nuclear compartments, therefore controlling multiple responses (Chang and Karin, 2001; Davis, 2000; Widmann *et al.*, 1999). In addition to playing roles in biotic stress responses, these MAPKs are implicated in plant responses to various abiotic stress conditions, such as wounding, high/low osmolarity, drought, salinity, ozone, and UV irradiation (Zhang and Klessig, 2001). These findings suggest that events downstream of MAPK activation differ, depending on the upstream stress factors. Using a specific kinase inhibitor, Lee *et al.* (2001) concluded that *HIN1* expression is regulated by SIPK in tobacco cells treated with harpin$_{Psph}$, a bacterial non-race-specific elicitor from *Pseudomonas syringae* pv. *phaseolicola*. This discrepancy with regard to the involvement of SIPK activation in *HIN1* induction may be explained with the above hypothesis. The kinetics of *HSR201* and *HSR515* induction by Spm was slower than that of *HIN1*, *HSR203J*, and *HMGR*. *HSR201* and *HSR515* genes may be induced under control of WIPK, as the activation kinetics of the WIPK was slower than that of SIPK, and SIPK was involved in regulating WIPK gene expression (Liu *et al.*, 2003). We additionally examined the expression of HR marker genes by Spm using SIPK- or WIPK-silenced plants. However, no consistent results were obtained. Activation of SIPK is elicited by ozone in tobacco plants. Notably, strong and stable activation of WIPK was evident in SIPK-suppressed tobacco plants (Samuel and Ellis, 2002). Moreover, Liu *et al.* (2003) reported slightly elevated levels of WIPK protein in SIPK-silenced plants. We also observed a similar phenomenon. In SIPK or WIPK-silenced plants, it is possible that non-silenced counter MAPK is more strongly activated to compensate for the activity of silenced MAPK, and its activation spuriously transduces the signal to the downstream pathways.

Based on previous reports and the data presented in this study, we propose the following model for Spm signal transduction pathways in tobacco (Figure 8): free Spm is accumulated in intercellular spaces during HR triggered by TMV infection. Spm is catabolized by AO and/or PAO localized in the apoplastic spaces, which generates H_2O_2 and concomitantly enhances Ca^{2+} flux into the cytoplasm. The merged events induce mitochondrial malfunction via an unknown mechanism, as reflected by *AOX* gene induction. The downstream reaction of the malfunction of this organelle involves transcriptional activation of a subset of HR marker genes, which differ with respect to involvement of SIPK/WIPK activation. Tentatively, we conclude that the SIPK/WIPK-independent pathway directs *HIN1* expression, while the MAPK-dependent pathway directs the expression of *HSR203J* and *HMGR*, and possibly switches on *HSR201* and *HSR515* (Figure 8). Upregulation of the downstream HR genes may contribute to defense against secondary pathogen attack and/or lead to PCD during HR. In keeping with the latter hypothesis, Pontier *et al.* (1998) highlighted that activation of *HSR203* is correlated with PCD. Recently, an Spm-deficient *Arabidopsis* mutant was isolated, and it is alive (Imai *et al.*, 2004). This finding suggests that Spm is not essentially required for survival, but may exert specific effects under certain stress conditions. Analysis using an

Spm-deficient mutant in biotic stress responses is required to elucidate the defensive role of Spm.

Experimental procedures

Plant materials and treatment

Tobacco (*Nicotiana tabacum* L. cv. Xanthi nc and *NahG* transgenic) plants were incubated in a growth chamber at 25°C with a 14 h light/10 h dark photocycle. *NahG*-transgenic tobacco seeds were provided by Syngenta Corporation (Wilmington, DE, USA) Six- to 8-week-old plants were used for experiments. For chemical treatment, leaf disks from healthy tobacco plants were floated on half-strength MS solution (pH 7.0) and incubated for 12 h at 25°C to minimize the wounding effect. Where necessary, chemicals were added to media and further incubated for specific periods.

Preparation of protein extracts

The following procedure was performed at 4°C. Frozen plant materials were ground with a mortar and pestle in liquid nitrogen. Powdered tissues were quickly transferred to microcentrifuge tubes and homogenized by vortexing with extraction buffer [100 mM Hepes–KOH pH7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μg ml^{-1} antipain, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin, and 10% (v/v) glycerol]. Homogenates were centrifuged at 15 000 × ***g*** for 15 min at 4°C. Aliquots of supernatants were frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with BSA as a standard.

RNA gel blot analysis

Total RNA was isolated according to the method of Nagy *et al.* (1988). Aliquots (10 μg) were separated by electrophoresis on formaldehyde–1.0% agarose gels (v/w), and blotted onto Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ, USA) in 20 × SSC. Hybridization was performed as described previously (Berberich *et al.*, 1999).

Protein kinase activity assay

An in-gel kinase activity assay was performed as described in a previous report (Berberich *et al.*, 1999). Briefly, extracts containing 20 μg protein were loaded on 10% polyacrylamide gels embedded with 0.25 mg ml^{-1} MBP in a separation gel as a substrate for kinases. Following electrophoresis, SDS was removed by washing the gel in a solution containing 20% (v/v) 2-propanol and 50 mM Tris–HCl (pH 8.0), followed by solution (Buffer B) containing 50 mM Tris–HCl (pH 8.0) and 1 mM DTT. Proteins were denatured in Buffer B containing 6 M guanidine hydrochloride, and refolded in Buffer B containing 0.5% (v/v) Tween-20. The gel was incubated for 1 h at room temperature in a kinase reaction buffer containing 40 mM Hepes–KOH (pH 7.5), 2 mM DTT, 15 mM $MgCl_2$, 0.1 mM EGTA, 5 μM ATP, and 1.85 MBq [γ-^{32}P]-ATP. The reaction was terminated by transferring the gel to a stop solution of 5% (w/v) trichloroacetic acid and 1% (w/v) NaPPi. Unincorporated [γ-^{32}P]-ATP was removed by washing with stop solution for at least 6 h with five changes. The gel was dried onto Whatman 3 mm paper and exposed to X-ray film for autoradiography.

Cloning of NtMEK2 *cDNA and generation of a constitutively active NtMEK2 construct*

A pair of primers, 5′-GACAATCGAATAGCTTTGTCCCAT-3′ and 5′-ACGTTAAGAAGAAAAATGAGGAGG-3′, were designed to pull out *NtMEK2* cDNA (GenBank accession no. AF325168) by amplification from tobacco cDNA. PCR products were cloned into pCR 4Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA), and confirmed by direct sequencing. $NtMEK2^{DD}$, an active mutant of NtMEK2, was generated by substituting both conserved Thr 227 and Ser 233 residues between the kinase subdomains VII and VIII with Asp(D). The mutant was generated following a procedure described in a previous report (Yang *et al.*, 2001).

Agrobacterium*-mediated transient expression assay*

Agrobacterium-mediated transient expression assays were performed as described by Yang *et al.* (2001). The respective fragments encompassing the open reading frame regions of NtMEK2 and the constitutive active mutant, $NtMEK2^{DD}$ were inserted into the *Sma*I–*Sac*I sites of the pBI121 binary vector (Clontech, Palo Alto, CA, USA). Open reading frames of NtMEK2 and $NtMEK2^{DD}$ were placed under control of the Ω sequence from TMV (Gallie *et al.*, 1987). *Agrobacterium tumefaciens* LBA4404 cells, including the above constructs, were grown overnight in YPE medium containing 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} kanamycin, and 100 μM acetosyringone. Cells were collected by centrifugation (4000 × ***g***), resuspended to an OD_{600} value of 0.8 with MS medium (pH 5.9) containing 100 μM acetosyringone, and infiltrated into fully expanded tobacco leaves. Leaf samples were collected, immediately frozen in liquid nitrogen, and stored at −80°C until use.

Silencing of WIPK *and* SIPK *genes in* N. benthamiana *by VIGS using PVX*

Virus-induced gene silencing methods were performed as described previously (Sharma *et al.*, 2003). Briefly, a VIGS construct, pTXS.WIPK-SIPK, was generated by inserting a 194 bp *NbWIPK* fragment and a 200 bp *NbSIPK* fragment in tandem into the viral vector, in the antisense direction. *In vitro* run-off transcripts were synthesized from pTXS.WIPK-SIPK and pTXS.GFP, and inoculated into *N. benthamiana* plants. Plants displayed mild mosaic symptoms 3 weeks after inoculation of leaves with each transcript. The third or fourth leaf above the inoculated one, where the silencing is most consistently established, was used for further analysis.

RT-PCR

Total RNA fractions extracted using an RNA extraction kit (Invitrogen) were treated with DNase I, and converted into cDNA by reverse transcription (RT). The cDNA fragments were used as templates in PCR using ready-to-go RT-PCR beads (Amersham Biosciences). RT was initiated in the presence of oligo (dT) primers (42°C, 30 min), and after heat inactivation of reverse transcriptase at 95°C for 5 min, the appropriate primer pairs were added for PCR cycling. The following conditions were employed: 25 cycles for SIPK and WIPK, and 20 cycles for *rbcS* (each cycle performed for 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C). The following primer pairs were used: *SIPK* (forward: 5′-TATAATTCCACCACCACAGA-3′; reverse: 5′-CTTCATCTGTTCCTCCGTAA-3′) and *WIPK* (forward: 5′-CAATTCCCTGATTTTCCTTCGG-3′; reverse: 5′-GGAAAGTAGATACTCCAGATC-3′). Constitutively expressed *rbcS* cDNA was additionally

amplified by PCR using the following primer pairs (forward, 5′-CCTCTGCAGCAGTTGCCACC-3′; reverse, 5′-CCTGTGGGTATGCCTTCTTC-3′) and served as internal controls of the assays.

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