Silencing of WIPK and SIPK Mitogen-Activated Protein Kinases Reduces *Tobacco mosaic virus* Accumulation But Permits Systemic Viral Movement in Tobacco Possessing the *N* Resistance Gene

Michie Kobayashi,¹ Shigemi Seo,¹ Katsuyuki Hirai,^{1,2} Ayako Yamamoto-Katou,¹ Shinpei Katou,¹ Hideharu Seto,³ Tetsuo Meshi,¹ Ichiro Mitsuhashi,¹ and Yuko Ohashi¹

¹Plant-Microbe Interactions Research Unit, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan;

²Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan; ³RIKEN, Wako, Saitama 351-0198, Japan

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Infection of tobacco cultivars possessing the *N* resistance gene with *Tobacco mosaic virus* (TMV) results in confinement of the virus by necrotic lesions at the infection site. Although the mitogen-activated protein kinases WIPK and SIPK have been implicated in TMV resistance, evidence linking them directly to disease resistance is, as yet, insufficient. Viral multiplication was reduced slightly in *WIPK*- or *SIPK*-silenced plants but substantially in *WIPK/SIPK*-silenced plants, and was correlated with an increase in salicylic acid (SA) and a decrease in jasmonic acid (JA). Silencing of *WIPK* and *SIPK* in a tobacco cultivar lacking the *N* gene did not inhibit viral accumulation. The reduction in viral accumulation was attenuated by expressing a gene for an SA-degrading enzyme or by exogenously applying JA. Inoculation of lower leaves resulted in the systemic spread of TMV and formation of necrotic lesions in uninoculated upper leaves. These results suggested that WIPK and SIPK function to negatively regulate local resistance to TMV accumulation, partially through modulating accumulation of SA and JA in an *N*-dependent manner, but positively regulate systemic resistance.

One of the induced disease resistance responses that plants use to defend themselves against pathogens is regulated by plant resistance (*R*) genes whose products directly or indirectly recognize products of the corresponding pathogen avirulence (*Avr*) genes (Flor 1971; Dangl and Jones 2001). The absence of either an *R* gene or the corresponding *Avr* gene can allow the pathogen to spread beyond the initial point of infection, resulting in the development of systemic disease.

In many plant-pathogen interactions, host recognition of *Avr* gene products triggers rapid and localized cell death at the site of pathogen invasion, known as a hypersensitive response

(HR), usually resulting in the formation of necrotic lesions. HR is characterized by a set of defense responses, such as the generation of reactive oxygen species, accumulation of antimicrobial compounds, and induction of defense-related genes (Hammond-Kosack and Jones 1996). HR is thought to limit pathogens to the initial site of infection, thereby inhibiting their multiplication and spread.

Increasing evidence indicates that mitogen-activated protein kinase (MAPK) cascades play an important role in *R* gene-mediated disease resistance. MAPK are components of signaling pathways that transduce diverse extracellular stimuli to multiple intracellular responses. In response to these stimuli, MAPK are enzymatically activated via phosphorylation by an upstream MAPK kinase (MAPKK), which, in turn, is activated by an upstream MAPKK kinase (MAPKKK) (MAPK Group 2002; Nakagami et al. 2005; Widmann et al. 1999). Avirulent pathogens induce the activation of specific plant MAPK (Romeis et al. 1999; Zhang and Klessig 1998). Genetic studies have identified components of MAPK cascades involved in *R* gene-mediated resistance in several plant species, such as *Arabidopsis*, tomato, tobacco, and rice (Brader et al. 2007; del Pozo et al. 2004; Ekengren et al. 2003; Menke et al. 2004; Li et al. 2006; Sharma et al. 2003; Stulemeijer et al. 2007; Xiong and Yang 2003). Furthermore, specific MAPK have been shown to be required for full induction of defense responses during induced resistance to environmental stresses, including pathogen attack (Beckers et al. 2009).

The interaction between tobacco possessing the *N* gene and *Tobacco mosaic virus* (TMV) is a classic model system for studying *R*-gene-mediated disease resistance in plants. The *N* gene confers resistance to TMV (Holmes 1938; Whitham et al. 1994), and recognition of a 50-kDa helicase domain (p50) of the viral replicase by the *N* gene product induces HR at the site of virus invasion, resulting in the formation of necrotic lesions (Erickson et al. 1999). Several lines of evidence suggest that MAPK cascades play a role in *N*-gene-mediated resistance. First, WIPK and SIPK, which are two of the best-characterized plant MAPK, are activated during an early stage of TMV-induced HR in *Nicotiana tabacum* cv. Xanthi nc, a tobacco cultivar possessing the *N* gene (Zhang and Klessig 1998). Second, a gain-of-function analysis indicated that overexpression of NtMEK2^{DD}, a constitutively active form of a tobacco MAPKK upstream of WIPK and SIPK, induces HR-like cell death in tobacco (Yang et al. 2001). Third, a transient loss-of-

M. Kobayashi and S. Seo contributed equally to this work.

Corresponding author: S. Seo; Telephone: +81 (0)29 838 7440; Fax: +81 (0)29 838 7469; E-mail: sseo71@affrc.go.jp

Current address for S. Katou: International Young Researchers Empowerment Center, Shinshu University, Minamiminowa 8304, Nagano 399-4598, Japan.

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function analysis indicated that virus-induced gene silencing (VIGS) of NPK1, a MAPKKK, results in systemic spread and enhanced multiplication of TMV and compromises HR-like cell death in transgenic *N. benthamiana* plants transformed with the *N* gene (Jin et al. 2002). A similar VIGS approach was also used to show that suppression of *WIPK*, *SIPK*, and *NtMEK2* also compromises resistance to TMV (Jin et al. 2003). Furthermore, using a combination of VIGS and *N. benthamiana*, the NPK1-MEK/NQK1-Ntf6/NRK1 pathway was identified as another MAPK cascade involved in TMV resistance (Liu et al. 2004). These results suggest that at least two MAPK cascades are positively involved in resistance to TMV infection, possibly by regulating HR cell death. However, no loss-of-function studies on the roles of MAPK cascades in TMV resistance using tobacco cultivars possessing the *N* gene have been reported. To define the exact role of MAPK cascades in *N*-gene-mediated TMV resistance, stable transgenic tobacco plants in which *WIPK*, *SIPK*, or both were silenced were used.

RESULTS

Silencing of *WIPK* and *SIPK* inhibits TMV accumulation in tobacco cv. Samsun NN possessing the *N* gene.

We first examined whether *N. benthamiana* expressing the *N* gene is suitable for studying the roles of *WIPK* and *SIPK* in resistance to TMV infection and HR cell death. Leaves of transgenic *N. benthamiana* plants expressing the *N* gene were inoculated with TMV-OM, a common strain of TMV, and incubated at 20°C. Necrotic lesions were occasionally observed 2 to 3 days postinoculation (dpi) but almost all of them were a lesion type without clear margins. In addition, it was difficult to distinguish such lesions from wounds caused by rubbing the leaf surface for inoculation. In contrast, *N. tabacum* cv. Samsun NN, a TMV-resistant tobacco cultivar possessing the *N* gene, always produced necrotic lesions with clear margins in response to infection with TMV (Supplementary Fig. S1). Therefore, we used *N. tabacum* cv. Samsun NN as the cultivar for studying the roles of *WIPK* and *SIPK* in resistance to TMV infection.

We have previously generated transgenic tobacco plants in which *WIPK*, *SIPK*, or both were silenced by introducing inverted repeat (IR) constructs, including 3'-untranslated regions from *WIPK* and *SIPK* cDNA clones, into *N. tabacum* cv. Samsun NN (Seo et al. 2007). We have shown that the *SIPK* protein and the *WIPK* protein are specifically reduced in *SIPK*-silenced (lines *SIPKIR*-3 and *SIPKIR*-4) and *WIPK*-silenced (lines *WIPKIR*-2 and *WIPKIR*-6) plants, and both proteins are reduced in *WIPK*- and *SIPK*-silenced plants (lines *WIPK/SIPKIR*-2 [WS2] and *WIPK/SIPKIR*-3 [WS3]) (Seo et al. 2007). The same six lines were used in the current study.

To assay resistance to TMV, leaves of each line were inoculated with TMV-OM and incubated at 20°C. The size of local necrotic lesions on inoculated leaves of the *WIPK/SIPKIR* lines at 5 dpi, when lesion expansion had almost ceased, was smaller than in the control line (Fig. 1A; Supplementary Fig. S2). The *SIPKIR* and *WIPKIR* lines tended to produce slightly smaller necrotic lesions than the control line; however, this difference was not significant.

The amounts of TMV RNA in leaves with necrotic lesions were determined by quantitative real-time polymerase chain reaction (qRT-PCR) analysis using primers designed to amplify a region of the viral p50. The levels of TMV RNA in the *WIPK/SIPKIR* lines, except for *SIPKIR*-3, were significantly lower than those in the control line (Fig. 1B). The *WIPK/SIPKIR* lines exhibited considerably reduced accumulation of TMV RNA.

Accumulation of TMV is not inhibited in *WIPK*- and *SIPK*-silenced Samsun S tobacco plants.

The data in Figure 1 suggest that *WIPK* and *SIPK* regulate the accumulation of TMV in tobacco cultivars possessing the *N* gene. To assess whether the regulation of TMV accumulation by *WIPK* and *SIPK* occurs in a manner dependent of the *N* gene, *WIPK* and *SIPK* were silenced in *N. tabacum* cv. Samsun S, a TMV-susceptible tobacco cultivar lacking the *N* gene, by introducing the IR construct into the cultivar. Two transgenic lines (lines *WIPK/SIPKIR*-2S and *WIPK/SIPKIR*-12S) that exhibited reduced accumulation of *WIPK* and *SIPK* transcripts (Fig. 2A) were used to investigate TMV accumulation after infection. There was no major difference in the amounts of TMV RNA in inoculated leaves between the IR lines and a transgenic Samsun S line transformed with the vector alone (Fig. 2B).

Fig. 1. Formation of necrotic lesions in *Tobacco mosaic virus* (TMV)-inoculated leaves of mitogen-activated protein kinase-silenced Samsun NN plants. **A**, Diameter of local necrotic lesions at 5 days postinoculation (dpi) with TMV at 20°C. Values are the means $\pm$ standard deviation (SD) from 40 lesions. Asterisks indicate a significant difference from the control line (Student's *t* test), **P* < 0.01. **B**, Quantitative real-time polymerase chain reaction analysis of TMV RNA in TMV-inoculated leaves at 5 dpi. Values are the means $\pm$ SD from three independent measurements. Asterisks indicate significant differences from the control line (Student's *t* test); * indicates *P* < 0.02.

Effect of silencing of *WIPK* and *SIPK* on TMV infection-induced accumulation of salicylic acid, jasmonic acid, and ethylene.

Accumulation of salicylic acid (SA) and jasmonic acid (JA) induces the expression of defense-related genes involved in resistance responses and is associated with TMV-induced HR (Brederode et al. 1991; Dhondt et al. 2000; Malamy et al. 1990; Seo et al. 2001). Wound- or pathogen-induced accumulation of SA and JA are regulated by *WIPK* and *SIPK* (Samuel et al. 2005; Seo et al. 2007). To examine the temporal relationship between accumulation of SA and JA and activation of *WIPK* and *SIPK* in TMV-induced HR, the endogenous amounts of SA and JA and the enzymatic activities of *WIPK* and *SIPK* were investigated in wild-type Samsun NN plant tissues after TMV infection by taking advantage of the synchronous HR induction system. Shifting TMV-infected tissues from 30°C, a nonpermissive temperature for HR induction, to 20°C resulted in the synchronous formation of necrotic lesions in the TMV-infected region. An

increase in the kinase activities of *SIPK* and *WIPK* using myelin basic protein (MBP) as a substrate was detected 4 h after the temperature shift in TMV- but not mock-inoculated leaves (Fig. 3A). An increase in endogenous SA was detectable 2 h after temperature shifting of TMV-inoculated leaves from 30 to 20°C (Fig. 3B). JA began to accumulate 6 h after TMV-inoculated leaves were temperature shifted (Fig. 3C). Thus, accumulation of SA precedes activation of *WIPK* and *SIPK* and accumulation of JA during TMV-induced HR.

We next examined whether accumulation of SA and JA was altered in *MAPK*-silenced Samsun NN plants infected with TMV. TMV-inoculated leaf discs from each transgenic line were incubated at 30°C and transferred to 20°C, and the endogenous levels of SA and JA were determined 8 h after the temperature shift, at which time activation of *WIPK* and *SIPK* reached a peak (Zhang and Klessig 1998). Because the phytohormone ethylene is produced during TMV-induced HR (de Laat and von Loon 1982) and because stress-induced biosynthesis of ethylene is regulated by *SIPK* (Kim et al. 2003; Liu and Zhang 2004), accumulation of ethylene was also examined. Levels of SA and a sugar conjugate of SA (SAG) were approximately doubled in the *WIPK/SIPKIR* lines (Fig. 4A). The *SIPKIR*-4 line and the control lines contained almost the same levels of SA and SAG, whereas the *SIPKIR*-3 line exhibited a slightly reduced accumulation of SA and SAG. The levels of SA and SAG in the *WIPKIR* lines were slightly higher than those in the control line. *MAPK*-silenced lines and the control line contained the almost same amount of SA before the temperature shift (Fig. 4A, 0 h).

Reduced JA accumulation was observed only in the *WIPK/SIPKIR* lines after the temperature shift. WS2 plants produced 78% and WS3 plants produced 66% of the JA levels compared with controls (Fig. 4B). The levels of JA accumulated in the *SIPKIR* and *WIPKIR* lines were somewhat higher than in control plants. There were no significant differences in JA levels before the temperature shift in any of the lines (Fig. 4B, 0 h). JA stress induction is accompanied by transcriptional activation of enzymes involved in JA biosynthesis, such as lipoxygenase (LOX) and allene oxide cyclase (AOC) (Heitz et al. 1997; Stenzel et al. 2003). Consistent with reduced JA accumulation, accumulation of LOX and AOC transcripts after the temperature shift was reduced in the *WIPK/SIPKIR* lines (Supplementary Fig. S3).

Ethylene release levels from the *SIPKIR* and *WIPK/SIPKIR* lines after the temperature shift were less than half that of the control line (Fig. 4C). In contrast, the *WIPKIR* lines released approximately the same amount of ethylene as the control line. Ethylene release before the temperature shift was approximately the same for all lines (Fig. 4C, 0 h).

Effect of SA on resistance to TMV in *WIPK*- and *SIPK*-silenced Samsun NN plants.

The results in Figures 3 and 4 suggest that, in response to infection with TMV, *WIPK* and *SIPK* negatively regulate the accumulation of SA, whereas they positively regulate the accumulation of JA. Although the results also suggest that *SIPK* but not *WIPK* positively regulated TMV-induced accumulation of ethylene, TMV accumulation in *SIPK*-silenced plants was almost the same as in *WIPK*-silenced plants that produced normal amounts of ethylene, suggesting that any effect on TMV accumulation by *WIPK* and *SIPK* is independent of ethylene. Therefore, we focused on examining the contribution of SA and JA to the altered resistance in *MAPK*-silenced plants.

SA inhibits the multiplication of TMV in tobacco (Chivasa et al. 1997). To assess whether the higher concentration of SA in *WIPK*- and *SIPK*-silenced plants is responsible for the reduced TMV accumulation, generation of *WIPK*- and *SIPK*-

Fig. 2. Analysis of *WIPK*- and *SIPK*-silenced Samsun S plants. **A**, Reverse-transcriptase polymerase chain reaction (PCR) analysis of *WIPK* and *SIPK* gene expression levels in leaves. A transgenic Samsun S line transformed with the vector alone (Control) was used as a control. The number of PCR cycles is indicated on the right. **B**, RNA gel blot analysis of *Tobacco mosaic virus* (TMV) in TMV-inoculated leaves at 0 and 2 days postinoculation at 20°C. rRNA levels were used to compare gel loading for total RNA. Arrowhead indicates the position of genomic RNA.

silenced plants expressing *NahG*, a gene encoding an enzyme that converts SA to catechol (Gaffney et al. 1993), was attempted. However, an attempt to generate such transgenic plants did not succeed using *Agrobacterium* spp. Therefore, the WS3 Samsun NN line was crossed with a transgenic Xanthi nc tobacco line, which possesses the *N* gene, expressing *NahG*. Two hybrid lines, WS3/*NahG* and *NahG*/WS3, for which the

transgenic *NahG* line was used as female and pollen parents, respectively, were obtained. Reverse-transcriptase PCR analysis confirmed that the WS3/*NahG* and *NahG*/WS3 lines exhibited *NahG* transcript accumulation and reduced accumulation of *WIPK* and *SIPK* transcripts, although the level of accumulation of *NahG* transcripts in these lines was lower than that in the parental *NahG* line (Fig. 5A).

Fig. 3. Time-course analysis of mitogen-activated protein kinase activation, salicylic acid (SA) accumulation, and jasmonic acid (JA) accumulation during *Tobacco mosaic virus* (TMV)-induced hypersensitive response. Leaves of wild-type (WT) Samsun NN tobacco plants were inoculated with TMV, incubated at 30°C for 40 h, transferred to 20°C, and used for the measurement of myelin basic protein (MBP) kinase activity of **A**, SIPK and WIPK; **B**, SA and a sugar conjugate of SA (SAG); and **C**, JA. Values are the means $\pm$ standard deviation from three independent measurements.

Fig. 4. Accumulation of salicylic acid (SA), jasmonic acid (JA), and ethylene in mitogen-activated protein kinase-silenced plants after a temperature shift. *Tobacco mosaic virus* (TMV)-inoculated leaf discs prepared from each line were harvested 0 and 8 h after a shift from 30 to 20°C and used for the measurement of **A**, SA and a sugar conjugate of SA (SAG); **B**, JA; and **C**, ethylene. Values are the means $\pm$ standard deviation from three independent measurements. Asterisks indicate significant differences from the control line (Student's *t* test); * and ** indicate $P < 0.05$ and 0.001 , respectively.

Leaves of the WS3/NahG and NahG/WS3 lines were inoculated with TMV and incubated at 20°C, and the size of necrotic lesions and the level of accumulation of TMV RNA were determined at 5 dpi to evaluate resistance to TMV. The WS3/NahG and NahG/WS3 lines produced larger necrotic lesions than did the parental WS3 line (Fig. 5B). The level of TMV RNA in the hybrid lines was higher than in the parental WS3 line (Fig. 5C).

Expression of the *NahG* gene in tobacco plants inhibits accumulation of SA in response to infection with TMV (Gaffney et al. 1993). To assess whether the *NahG* gene functioned in the hybrid lines, the endogenous levels of SA and SAG in inoculated leaves were measured. NahG plants produced only small amounts of SA and SAG in response to infection; the levels of these compounds were 1% of those in the vector control line (Fig. 5D). The levels of SA and SAG in the two hybrid lines at 5 dpi were lower than in the parental WS3 line but higher than in the parental NahG line.

Effect of JA on resistance to TMV in *WIPK*- and *SIPK*-silenced Samsun NN plants.

To assess whether the lower concentration of JA in *WIPK*- and *SIPK*-silenced plants is responsible for the reduced TMV accumulation, the effect of exogenous JA on accumulation of TMV in these plants was tested. TMV-inoculated leaf discs were treated with methyl jasmonate (MeJA), a methyl ester form of JA, and incubated at 20°C, and resistance to TMV was evaluated by measuring the size of necrotic lesions and the level of accumulation of TMV RNA. Exogenously applied MeJA increased the size of local necrotic lesions in a dose-dependent manner in the vector control (Fig. 6A). In the presence of MeJA, necrotic lesions with dark-brown margins were formed and some of these lesions expanded along leaf veins (Fig. 6B, control). Such effects of MeJA on changes in the size and morphology of lesions were also observed for the WS3 line (Fig. 6A and B, WS3). Consistent with increased lesion size, accumulation of TMV RNA was enhanced by MeJA in the WS3 and vector control lines (Fig. 6C).

Silencing of *WIPK* and *SIPK* compromises resistance to systemic spread of TMV in Samsun NN tobacco plants.

It has been reported that silencing of *WIPK* and *SIPK* causes systemic spread of TMV in transgenic *N. benthamiana* expressing the *N* gene (Jin et al. 2003). To examine whether TMV spreads systemically in *MAPK*-silenced Samsun NN plants, lower leaves were inoculated with TMV and the plants were incubated at 20°C. *WIPK*- and *SIPK*-silenced plants developed systemic HR-like lesions along their veins at 10 to 15 dpi, whereas control plants did not exhibit any symptoms of lesion formation (Fig. 7A). It was confirmed that inoculation of lower

Fig. 5. Analysis of *WIPK*- and *SIPK*-silenced and NahG plants and their hybrids. The vector control line (Cont.) was used as a control. **A**, Reverse-transcriptase polymerase chain reaction (PCR) analysis of the *WIPK*, *SIPK*, and *NahG* genes in leaves. The number of PCR cycles is indicated on the right. **B**, Diameter of local necrotic lesions in *Tobacco mosaic virus* (TMV)-inoculated leaves at 5 days postinoculation (dpi). Values are the means $\pm$ standard deviation (SD) from 40 lesions. **C**, Quantitative real-time PCR analysis of TMV RNA in TMV-inoculated leaves at 5 dpi. Values are the means $\pm$ SD from three independent measurements. The inset shows a magnification of the ordinate scale for the relative level of TMV RNA. For B and C, asterisks indicate significant differences from the control line (* indicates $P < 0.02$, Student's *t* test) and the WS3 line (** indicates $P < 0.02$, Student's *t* test). **D**, salicylic acid (SA) and a sugar conjugate of SA (SAG) concentrations in leaves before (0 dpi) and after (5 dpi) inoculation with TMV at 20°C. Values are the means $\pm$ SD from three independent measurements.

leaves of *WIPK*- and *SIPK*-silenced lines other than the WS2 and WS3 lines with TMV resulted in formation of small lesions in size on inoculated leaves (Supplementary Fig. S4) and caused systemic HR-like lesions in upper leaves (Supplementary Fig. S5). Systemic HR-like lesions were also observed in *SIPK*- or *WIPK*-silenced plants (Supplementary Fig. S6).

The occurrence of systemic HR suggested that TMV spread systemically from TMV-inoculated lower leaves to the upper leaves. Different approaches were employed to examine the possibility of systemic viral spread using the WS2 or WS3 lines. RNA blot analysis of TMV in symptomatic upper leaves was performed to check for the presence of the virus. TMV RNA was detected in the *WIPK*/*SIPK* lines, whereas it was undetectable in the control line (Fig. 7B).

TMV translocates systemically in vascular bundles of tobacco cultivars lacking the *N* gene. If systemic spread of TMV occurs in *WIPK*- and *SIPK*-silenced plants, the pathogen would be detected in the vascular bundles of symptomatic upper leaves. The vascular movement of TMV was investigated by immunohistochemical analysis using petioles of upper leaves containing necrotic lesions. Strong blue signals corresponding to TMV were detected in vascular bundles in the petiole of symptomatic upper leaves of the WS3 line but not in those of the control line (Fig. 8). Brown necrotic lesions were also observed around the blue signals.

DISCUSSION

Here, we demonstrate that *WIPK* and *SIPK* negatively regulate local resistance to TMV accumulation, whereas they positively regulate resistance to systemic viral spread. A combination of VIGS and transgenic *N. benthamiana* plants transformed with the *N* gene was used to show that suppression of *WIPK*, *SIPK*, and *NtMEK2* compromises local resistance to TMV multiplication (Jin et al. 2003). Thus, our result on local resistance to TMV is different from the result reported by Jin and associates (2003). However, it should be noticed that our assay system used a native tobacco cultivar possessing the *N* gene and did not involve *Agrobacterium* spp. for silencing. We believe that our results reflect the exact roles of *WIPK* and *SIPK* in *N*-gene-mediated TMV resistance.

Inoculation of *WIPK*- and *SIPK*-silenced Samsun NN plants with TMV resulted in the formation of relatively small necrotic lesions accompanied by reduced TMV accumulation in inoculated leaves (Fig. 1). There are two possibilities for the cause of the reduction in lesion size. One is that multiplication of TMV at the infection site was inhibited, resulting in the formation of small lesions. The other is that TMV multiplication occurs normally but a resistance response that leads to a decrease in viral loads at the infection site was enhanced, resulting in the appearance of small lesions. Further studies to investigate these possibilities are required.

Because silencing of *WIPK* and *SIPK* in Samsun S tobacco, a tobacco cultivar lacking the *N* gene, did not inhibit the accumulation of TMV (Fig. 2), it is possible that any effect of TMV accumulation by *WIPK* and *SIPK* is dependent on the *N* gene. This is reasonable because TMV-induced activation of *WIPK* and *SIPK* does not occur in tobacco cultivars lacking the *N* gene (Zhang and Klessig 1998), suggesting that the function of *WIPK* and *SIPK* in resistance response of tobacco plants to TMV is regulated by the *N* gene.

Several lines of evidence indicate that SA and JA are involved in the regulation of TMV accumulation mediated by *WIPK* and *SIPK*. First, the hybrids of *WIPK*- and *SIPK*-silenced and NahG plants exhibited higher levels of TMV accumulation than did the parental *WIPK*- and *SIPK*-silenced line (Fig. 5C), suggesting that the higher concentration of SA in *WIPK*- and *SIPK*-silenced

plants contributed to the reduced TMV accumulation. However, because there was only a slight difference in the levels of TMV accumulation between the hybrid lines and the parental *WIPK*- and *SIPK*-silenced line, the contribution of SA may be minor. Second, exogenous MeJA increased the level of TMV accumulation in *WIPK*- and *SIPK*-silenced plants (Fig. 6C), suggesting that the lower concentration of JA in these plants also contributes to the reduced TMV accumulation. These results indicate the involvement of SA and JA in reduced TMV accumulation in *WIPK*- and *SIPK*-silenced plants. However, we also observed that accumulation of TMV was slightly reduced in *WIPK*- or *SIPK*-silenced plants in which production of SA and JA was less affected, suggesting that a factor other than SA and JA is involved in the reduction in TMV accumulation. Therefore, it is possible that, in wild-type tobacco cultivars possessing the *N* gene, *WIPK* and *SIPK* function to regulate TMV accumulation at the initial site of infection by modulating accumulation of SA and JA and the unknown factor.

The observation of reduced TMV accumulation is in contrast with a previous report showing that silencing of *WIPK* and *SIPK* enhances TMV multiplication in inoculated leaves of *N. benthamiana* expressing the *N* gene (Jin et al. 2003). One possible explanation for these different results is that the functions of *WIPK* and *SIPK* or *N* protein or both in response to TMV are different between *N. tabacum* and *N. benthamiana*. Another possibility is due to differences in the experimental systems used. The infection of plants with *Agrobacterium* spp. is known to alter expression of various host genes (Ditt et al. 2001). *N. benthamiana* plants infected with *Agrobacterium* spp. may express host genes involved in enhancing TMV multiplication. The present results indicated that *WIPK* and *SIPK* are involved in negatively regulating local resistance to TMV accumulation at the infection site in tobacco. MAPK have been found to negatively regulate resistance to pathogen multiplication in several other plant-pathogen interactions (Petersen et al. 2000; Xiong and Yang 2003; Yuan et al. 2007).

VIGS of COI1, a receptor necessary for JA signaling, in *N. benthamiana* has been shown to attenuate *N*-mediated TMV resistance (Liu et al. 2004), although the authors did not examine the involvement of JA biosynthesis in resistance to TMV. Therefore, it is possible that JA biosynthesis and JA signaling are negatively and positively, respectively, involved in resistance to TMV. However, because, as described above, there is the possibility that the resistance response of *N. tabacum* to TMV is partly different from that of *N. benthamiana* to TMV, analysis using tobacco plants possessing the *N* gene will be necessary to define the exact role of JA signaling.

The temporal analysis of signaling events revealed that accumulation of SA preceded activation of *WIPK* and *SIPK* and accumulation of JA (Fig. 3). This is partly supported by a previous report showing that SA biosynthesis precedes JA biosynthesis during *Pseudomonas syringae*-induced HR in tobacco (Kenton et al. 1999). Given the fact that *WIPK*- and *SIPK*-silenced Samsun NN plants exhibited enhanced SA accumulation (Fig. 4A), it is likely that activation of *WIPK* and *SIPK* is not involved in triggering SA biosynthesis but is necessary for the negative regulation of SA accumulation.

Because TMV was present in the upper leaves or petioles, forming lesions (Figs. 7 and 8), HR-like lesions in such organs are likely to be caused by the virus from inoculated leaves that has translocated through the vascular system. Thus, in *WIPK*- and *SIPK*-silenced Samsun NN plants, TMV accumulation at the infection site is inhibited but systemic viral spread is permitted, suggesting that local resistance to TMV can be uncoupled from that to systemic viral spread. The results were unexpected because inhibition of systemic spread and multiplication has been generally thought to be a hallmark of the resistance re-

sponse in TMV-resistant tobacco cultivars infected with TMV. Although it is difficult to explain the two apparently contradictory phenomena, one possible explanation is that an extremely small amount of TMV escapes from the initial site of infection before all viruses are completely limited to the site and moves systemically. A phenomenon similar to the finding in the current study has been reported for tobacco expressing deleted clones of

the *N* gene (Dinesh-Kumar and Baker 2000). It has been shown that inoculation of lower leaves of tobacco expressing deleted *N* transcripts with TMV results in the induction of systemic HR in upper leaves, although the degree of TMV multiplication at the infection site has not been determined.

On the basis of the results from the current study, we propose the following mechanism of WIPK- and SIPK-mediated TMV

Fig. 6. Effect of exogenous methyl jasmonate (MeJA) on lesion development and *Tobacco mosaic virus* (TMV) accumulation. TMV-inoculated leaf discs prepared from the WS3 and vector control lines were floated on a solution containing the indicated concentrations of MeJA or 0.1% ethanol (Control) at 20°C for 5 days. Treated discs were **A**, used for measurement of the diameter of local necrotic lesions; **B**, photographed; or **C**, subjected to quantitative real-time polymerase chain reaction analysis of TMV RNA. **A**, Values are the means from 40 lesions. **B**, Bar indicates 5 mm. **C**, Values are the means $\pm$ standard deviation from three independent measurements.

resistance in tobacco possessing the *N* gene. In response to infection with TMV, WIPK and SIPK are activated following the induction of SA biosynthesis. Activated WIPK and activated SIPK function to negatively regulate local resistance to TMV accumulation at the initial site of infection, partially through negatively regulating accumulation of the produced SA and positively regulating the accumulation of JA. On the other hand, activated WIPK and activated SIPK function to inhibit systemic spread of the virus from the infection site, although the mechanism of the inhibition remains unknown.

We have shown previously that silencing of either *WIPK* or *SIPK* reduces JA accumulation in response to wounding (Seo et al. 2007). Such nonredundant roles of WIPK and SIPK in the

induction of JA accumulation after wounding have been shown for orthologs of WIPK and SIPK in other plant species (Kandath et al. 2007; Wu et al. 2007). Interestingly, reduced JA accumulation in response to infection with TMV was observed only in *WIPK*- and *SIPK*-silenced plants (Fig. 4B), suggesting that WIPK and SIPK play redundant roles in the induction of JA accumulation after infection. These results suggested that the requirement of WIPK and SIPK for JA biosynthesis induction differs depending on the kind of stresses the plant is exposed to. Despite their substantial reduced accumulation of *LOX* and *AOC* transcripts, *WIPK*- and *SIPK*-silenced plants exhibited only slightly reduced JA accumulation in response to infection (Fig. 4B). Because the mechanism of induction of JA biosynthesis is

Fig. 7. Systemic hypersensitive response (HR) on the upper leaves of *WIPK*- and *SIPK*-silenced plants after inoculation of their lower leaves with *Tobacco mosaic virus* (TMV). **A**, Photograph of upper leaves of the WS3 and control lines 14 days after inoculation of their lower leaves with TMV. White dashed lines indicate the places at which HR-like lesions appeared. Bars indicate 2 cm. **B**, RNA gel blot analysis of TMV in upper leaves of the *WIPK*/*SIPKIR* and control lines 14 days after inoculation of their lower leaves with TMV. rRNA levels were used to compare gel loading for total RNA. Arrowhead indicates the position of genomic RNA.

Fig. 8. Viral localization in the petiole of a leaf exhibiting necrosis of *WIPK*- and *SIPK*-silenced plants. Immunohistochemical detection of *Tobacco mosaic virus* (TMV) in petioles prepared from a symptomatic upper leaf of the WS3 line and from an upper leaf of the vector control (Control) 14 days after inoculation (dpi) of their lower leaves with TMV. Middle panels are close-up views of regions bounded by rectangles in the left-hand panels, and the right-hand panels are close-up views of the middle panels. Bars indicate 250 μ m. V, vascular bundles.

not fully understood, it is difficult to explain these observations. However, one possibility is that MAPK-mediated transcriptional regulation of JA biosynthetic genes is not the sole regulator of JA biosynthesis. It has been proposed that JA biosynthesis is also regulated at the post-translational level (Schaller and Stintzi 2009).

At least two MAPK cascades are thought to be involved in TMV resistance in tobacco. One is the MEK2-SIPK/WIPK pathway (Jin et al. 2003) and the other is the NPK1-MEK/NQK1-Ntf6/NRK1 pathway (Jin et al. 2002; Liu et al. 2004). However, because the involvement of the latter pathway was examined using a combination of VIGS and *N. benthamiana*, loss-of-function studies using stable transgenic tobacco plants will be necessary to define the exact role of this pathway. In addition, the relationship and any interaction between these two MAPK cascades remains to be clarified.

MATERIALS AND METHODS

Plant materials and plant growth conditions.

The generation of *N. benthamiana* expressing the *N* gene has been described previously (Takabatake et al. 2007). The generation of *SIPK*-, *WIPK*-, and *WIPK*-and-*SIPK*-silenced Samsun NN tobacco plants has been described previously (Seo et al. 2007). Transgenic Xanthi nc tobacco plants expressing *NahG* (Gaffney et al. 1993) were used. The WS3/*NahG* and *NahG*/WS3 lines were obtained by crossing the *WIPK*/*SIPKIR*-3 line with the *NahG* line. The IR construct for silencing of *WIPK* and *SIPK* (Seo et al. 2007) was used to transform *N. tabacum* cv. Samsun S. Transformation and screening of transformants were performed as described previously (Seo et al. 2007). Kanamycin-resistant seedlings from the transgenic lines were transferred to pots containing vermiculite and grown in a chamber maintained at 25°C with 16 h of light (120 $\mu\text{mol m}^{-2} \text{s}^{-1}$). For each analysis, 1-month-old *N. benthamiana* and 2-month-old tobacco plants were used.

TMV inoculation.

The leaf surfaces of intact plants were gently rubbed with Carborundum and TMV-OM at 0.4 $\mu\text{g ml}^{-1}$ suspended in 10 mM phosphate buffer (pH 7.0). After rinsing under a gentle stream of tap water, inoculated plants were incubated in a chamber maintained at 20 or 30°C.

Immunocomplex kinase assays.

MBP kinase activity of *SIPK* and *WIPK* was measured as described previously (Seo et al. 1999).

Phytohormone measurements.

Extraction and quantification of JA was performed essentially as described previously (Baldwin et al. 1997), except that [$^2\text{H}_2$]($\pm$)-JA was used as an internal standard (Nojiri et al. 1992). Quantification of SA and SAG was performed as described previously (Seo et al. 1995). Measurement of ethylene was performed as described previously (Ohtsubo et al. 1999).

RNA extraction, RNA blotting, and reverse transcriptase and qRT-PCR analyses.

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) in accordance with the manufacturer's instructions. Blotting and hybridization were performed as described previously (Seo et al. 1999). ^{32}P -labeled cDNA fragments for TMV, *NtLOX* (National Center for Biotechnology Information [NCBI] accession number X84040), and *NtAOC* (NCBI accession number CAC83765) were used as probes.

For RT-PCR analysis in Figures 2A and 5A, wounded leaves 1 h after wounding were used as the samples, because expres-

sion of the *WIPK* gene whose transcripts accumulate in response to wounding is undetectable in healthy leaves (Seo et al. 1995). RT-PCR was performed using an RT-PCR High-Plus kit (Toyobo, Japan) and the following primers: for *WIPK*, 5'-CG GTGGAGGTCAATTCCCTG-3' and 5'-CATTTACCAAAAG GTTGCTC-3'; for *SIPK*, 5'-GATGATGTCTGATGCGGGG CGG-3' and 5'-GACAGTGCTCCTCAGATAAA-3'; for *NahG*, 5'-ATCATCAACGTGGTGGCTTTTCATCT-3' and 5'-CAACT CGTATAACTCGCCGGTCTC-3'; and for *actin*, 5'-CAGGGT TTGCTGGAGATGATGCTC-3' and 5'-TGAATGCCTGCAG CTTCCATTCC-3'.

qRT-PCR analysis was performed in a two-step reaction using a SYBR Green kit (Bio-Rad, Hercules, CA, U.S.A.) and the following primers; for *p50*, 5'-GACATGCGATGATTGGAGA AGAG-3' and 5'-GGCTGGACAACGGAGAGTAG-3', and for *actin*, 5'-GGGTTTGCTGGAGATGATGCT-3' and 5'-GCTTC GTCACCAACATATGCAT-3'. All data were normalized for expression of the *actin* gene used as an internal control.

MeJA treatment.

MeJA was purchased from Wako (Osaka, Japan), dissolved in ethanol to 100 mM, and diluted with H₂O. Leaf discs were floated on a solution containing MeJA at adequate concentrations; 0.1% ethanol was used as a control.

Immunohistochemical analysis.

Lateral cross-sections were prepared from petioles, fixed in 4% paraformaldehyde, and subjected to immunohistochemical analysis with anti-TMV antibody as described previously (Hirai et al. 2008).

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