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ORIGINAL PAPER

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Virus-induced silencing of *WIPK* and *SIPK* genes reduces resistance to a bacterial pathogen, but has no effect on the INF1-induced hypersensitive response (HR) in *Nicotiana benthamiana*

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Abstract Activation of two mitogen-activated protein kinases (MAPKs), wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK), is one of the earliest responses that occur in tobacco plants that have been wounded, treated with pathogen-derived elicitors or challenged with avirulent pathogens. We isolated cDNAs for these MAPKs (*NbWIPK* and *NbSIPK*) from *Nicotiana benthamiana*. The function of *NbWIPK* and *NbSIPK* in mediating the hypersensitive response (HR) triggered by infiltration with INF1 protein (the major elicitin secreted by *Phytophthora infestans*), and the defense response to an incompatible bacterial pathogen (*Pseudomonas cichorii*), was investigated by employing virus-induced gene silencing (VIGS) to inhibit expression of the *WIPK* and *SIPK* genes in *N. benthamiana*. Silencing of *WIPK* or *SIPK*, or both genes simultaneously, resulted in reduced resistance to *P. cichorii*, but no change was observed in the timing or extent of HR development after treatment with INF1.

Keywords *Nicotiana benthamiana* · WIPK/SIPK · INF1 · *Pseudomonas cichorii* · Virus-induced gene silencing

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Introduction

Mitogen-activated protein kinase (MAPK) cascades are major components that act downstream of receptors or sensors to transduce extracellular stimuli into intracellular responses in yeast and animal cells (Widman et al. 1999; Davis 2000). The basic MAPK cascade is a three-kinase module that is conserved in all eukaryotes. MAPK, the ultimate kinase in the cascade, is activated by dual phosphorylation of a tripeptide motif (TXY) located in the activation loop (T-loop) between subdomains VII and VIII of the kinase catalytic domain. Two MAPKs from tobacco, wound-induced protein kinase (WIPK) and salicylic acid (SA)-induced protein kinase (SIPK), and their orthologs in other plant species, have been implicated in signaling of plant defense reactions (Zhang and Klessig 2001 and references therein). WIPK, first identified as wound-induced protein kinase, transduces the wounding signal into the synthesis of jasmonic acid (Seo et al. 1995, 1999). SIPK, first identified as SA-induced protein kinase, is activated by pathogens, pathogen-derived elicitors, wounding, and osmotic and oxidative stresses (Zhang and Klessig 1997, 1998; Zhang et al. 1998; Romeis et al. 1999; Droillard et al. 2000; Mikolajczyk et al. 2000; Samuel et al. 2000; Lee et al. 2001; Samuel and Ellis 2002). Some of these treatments activate WIPK as well as SIPK. Yang et al. (2001) showed that transient overexpression of a constitutively active MAPK kinase ($NtMEK^{DD}$) induces the activation of WIPK and SIPK, followed by hypersensitive response

(HR)-like cell death in tobacco. Furthermore, Zhang and Liu (2001) demonstrated that overexpression of SIPK alone could induce HR in tobacco. These overexpression studies suggested a vital role for SIPK activation in the development of the HR. However, overexpression of signaling elements is known to sometimes drastically disturb signaling cascades, and therefore the results must be interpreted with some caution. It is advisable that such gain-of-function analysis should be complemented by a loss-of-function analysis. Therefore, in the present study, we silenced *WIPK* and *SIPK* genes in *Nicotiana benthamiana* by virus-induced gene silencing (VIGS) using potato virus X (PVX) (Baulcombe 1999; Burton et al. 2000; Voinnet 2001; Saitoh and Terauchi 2002). Plants in which *WIPK*, *SIPK*, or *WIPK* and *SIPK* together, had been silenced were evaluated for the activation of WIPK and SIPK in response to wounding, for HR development triggered by the fungal elicitor INF1—a major secreted protein of *Phytophthora infestans*, and for their defense response against an avirulent bacterial pathogen, *Pseudomonas cichorii*. In the plants in which *WIPK* and *SIPK* were both silenced, resistance to *P. cichorii* was compromised. Surprisingly, however, silencing of *SIPK* and *WIPK* did not affect the development of HR after treatment with INF1 or inoculation with *P. cichorii*.

Materials and methods

Plasmid construction

Total RNA from *N. benthamiana* leaves was reverse-transcribed using an oligo(dT)$_{20}$ primer (Toyobo, Tokyo). Using this cDNA as template, a cDNA containing the entire *WIPK* ORF was amplified by PCR with forward (WIPK-ORFU: 5′-ATGGCTGATGCAAATATGGG-3′) and reverse (WIPK1302R: 5′-GGAAAGTAGATACTCCAGATC-3′) primers designed from the DNA sequence of *WIPK* from *N. tabacum* (Seo et al. 1995). The PCR-amplified product (1195 bp) was ligated to the pT7Blue T-Vector (Novagen, Madison, Wis.) to generate pT7-NbWIPK. Similarly, a cDNA containing the entire *SIPK* ORF was amplified using forward (SIPK-ORFU: 5′-ATGGATGGTTCTGGTCAGCAG-3′) and reverse (SIPK-L1: 5′-GACAACAATCTTACCAGCAA-3′) primers based on the DNA sequence of *SIPK* of *N. tabacum* (Zhang and Klessig 1997). The PCR product (1206 bp) was cloned into the pT7Blue T-Vector to generate pT7-NbSIPK. A 273-bp fragment of *NbWIPK* was amplified from the plasmid pT7-NbWIPK by PCR with the forward primer WIPK-U/SalI (5′-CGGTCGACCCGTTACGAAGGGAGTTTTC-3′; *Sal* I site underlined) and the reverse primer WIPK-R/EcoRV (5′-CCGATATCTCCGTCATATTCTCGTTCTC-3′; *Eco* RV site underlined). The PCR product was digested with *Sal* I and *Eco* RV, and ligated into the corresponding sites in the vector pPC2S PVX (Baulcombe et al. 1995), to generate pTXS.WIPK. A 255-bp fragment of *NbSIPK* was amplified from the plasmid pT7-NbSIPK by PCR with forward (SIPK-U/SalI: 5′-CGGTCGACTTCTGCACAGGGACTTGA-3′; *Sal* I site underlined) and reverse (SIPK-R/EcoRV: 5′-CCGATATCCGTGATCTCTACCAGGAA-3′; *Eco* RV site underlined) primers. The PCR product was digested with *Sal* I and *Eco* RV, and ligated to the same restriction sites in pPC2S to generate pTXS.SIPK. A 194-bp fragment of *NbWIPK* was amplified from pT7-NbWIPK with the forward primer WIPK-U/SphI (5′-CCGCATGCAACCAAGGTTTATCAGAGGA-3′, *Sph* I site is underlined) and the reverse primer WIPK-R/EcoRV, then digested with *Eco* RV and *Sph* I. A 200-bp fragment of *NbSIPK* was obtained from pT7-NbSIPK by digestion with *Sal* I and *Sph* I using internal *Sal* I and *Sph* I sites located 909 and 1106 nt from the 5′ end of the ORF, respectively. The 194-bp *NbWIPK* fragment and the 200-bp *NbSIPK* fragment were ligated in tandem to the *Eco* RV/*Sal*I-digested pPC2S to generate pTXS.WIPK-SIPK.

In vitro transcription and plant inoculation

The plasmids pTXS.WIPK, pTXS.SIPK, pTXS.WIPK-SIPK and pTXS.GFP (Baulcombe et al. 1995) were linearized with *Spe* I, and used for in vitro transcription using a mMessage mMachine T7 kit (Ambion, Austin, Tex.) according to the manufacturer's instructions. The transcripts were inoculated into *N. benthamiana* leaves as described elsewhere (Saitoh et al. 2001). One leaf per plant was inoculated with 30 μl of RNA solution (transcript concentration 50 ng/μl).

Wounding of leaves, preparation of INF1 protein and infiltration into leaves

N. benthamiana leaves were wounded by gently rubbing the upper surface with wet carborundum. INF1 was prepared as previously described (Kamoun et al. 1997). A solution of INF1 (100 nM) was infiltrated with a needleless syringe through small incisions made on the lower leaf surface with a razor blade. At a given time point, a disk (15 mm diameter) was punched out of each infiltrated leaf with a cork borer, immediately frozen in liquid nitrogen, and stored at −70°C prior to further analysis.

Confirmation of *NbWIPK* and *NbSIPK* silencing by RT-PCR

RT-PCR analysis was performed as described by Saitoh and Terauchi (2002). For the detection of *NbWIPK* mRNA, we used WIPK147F (5′-CAATTCCCTGATTTTCCTTCGG-3′) as the forward primer and WIPK1302R as the reverse primer. For the detection of *NbSIPK* mRNA, the primers SIPK-U1 (5′-TATAATTCCACCACCACAGA-3′) and SIPK-L2 (5′-CTTCATCTGTTCCTCCGTAA-3′) were used.

Preparation of protein extracts and assay for kinase activity

Leaf disks (15 mm diameter) were ground in liquid nitrogen, thawed in 200 μl of extraction buffer [100 mM HEPES pH 7.4, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM NaF, 10 mM Na_3VO_4, 50 mM β-glycerophosphate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 5 μg/ml antipain, 5 μg/ml aprotinin and 5 μg/ml leupeptin], and centrifuged at 15,000 × *g* for 20 min at 4°C in a microcentrifuge. The crude extracts were stored at −70°C. The protein concentration of each sample was determined by the Bradford (1976) method, using BSA as a standard. The in-gel kinase assay to check the WIPK/SIPK activity was performed as described elsewhere (Matsumura et al. 2003).

Immunodetection of SIPK protein

Immunoblot analysis was performed as described by Saitoh and Terauchi (2002). Antiserum was raised against a synthetic peptide corresponding to the N-terminal sequence of SIPK (MDGSGQQTDTMMSDAGAEQ) of *N. tabacum*.

Inoculation with *Pseudomonas cichorii* and determination of growth kinetics

P. cichorii SPC9001 (Hikichi et al. 1998) was grown at 28°C in Nutrient Broth medium (Difco, Detroit, Mich.) containing

ampicillin (10 μg/ml) overnight. After centrifugation, bacterial cells were resuspended in 10 mM $MgCl_2$ (OD_{600} = 0.01). Bacterial suspensions were inoculated on to leaves using a needleless syringe. The increase in the numbers of bacteria was estimated in leaf disks (5 mm diameter) taken from infiltrated areas of three different plants at the indicated time points post-infection. The discs were homogenized in 785 μl of 10 mM $MgCl_2$, thoroughly mixed, and serial dilutions of the slurry were plated out on LB medium containing ampicillin (10 μg/ml). After incubation at 28°C for 24 h, the colonies were counted.

Results

Isolation of *NbWIPK* and *NbSIPK* cDNAs and silencing of the *WIPK* and *SIPK* genes

Complementary DNAs containing *WIPK* (GenBank Accession No. AB098729) and *SIPK* (GenBank Accession No. AB098730) ORFs were amplified from *N. benthamiana* leaves by RT-PCR. The degree of amino acid sequence homology between NbWIPK and *N. tabacum* WIPK was 97.6%, and that between NbSIPK and *N. tabacum* SIPK was 98.5%. Both NbWIPK and NbSIPK have the MAP kinase signature phosphorylation motif TEY (Fig. 1A, B). To examine the biological function of WIPK and SIPK in planta, we employed the VIGS method using PVX to specifically silence the genes in *N. benthamiana* (Baulcombe 1999; Burton et al. 2000; Voinnet 2001; Saitoh and Terauchi 2002). The VIGS constructs pTXS.WIPK and pTXS.SIPK were generated by separately inserting a 273-bp *NbWIPK* and a 255-bp *NbSIPK* fragment, each derived from the middle of the respective ORF, into the PVX vector in the antisense direction (Fig. 2). 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 anti-sense direction (Fig. 2). In vitro runoff transcripts were synthesized from pTXS.WIPK, pTXS.SIPK, pTXS.WIPK-SIPK and pTXS.GFP, and inoculated into *N. benthamiana* plants. The inoculated plants showed mild mosaic symptoms 3 weeks after the inoculation of leaves with each transcript (data not shown). The third or fourth leaf above the inoculated one, where the silencing is most consistently established (Romeis et al. 2001; Saitoh and Terauchi 2002), was used for further analysis of plants inoculated with PVX.GFP, PVX.WIPK, PVX.SIPK, and PVX.WIPK-SIPK. The levels of *NbWIPK* and *NbSIPK* mRNAs in the inoculated plants were determined by RT-PCR using gene-specific primers that anneal to regions outside the partial cDNA fragment cloned into the PVX vector (Fig. 3). *NbWIPK* transcripts could be detected in plants inoculated with PVX.GFP and PVX.SIPK, but not in those treated with PVX.WIPK or PVX.WIPK-SIPK. Conversely, *NbSIPK* transcripts could be detected in plants infiltrated with PVX.GFP and PVX.WIPK, but not in those treated with PVX.SIPK or PVX.WIPK-SIPK. Hence the VIGS constructs effectively silence the corresponding genes.

Response of *WIPK* - and *SIPK* -silenced plants to wounding and INF1 treatment

Wounding and treatment with elicitors are known to activate plant MAPKs. INF1, a protein secreted by *P. infestans*, is a well-characterized elicitor that causes HR in incompatible hosts like *N. benthamiana* (Kamoun et al. 1997, 1998). We therefore studied the responses of *WIPK* -, *SIPK* -, and *WIPK* - *SIPK* -silenced *N. benthamiana* plants to wounding and INF1, and compared them with those in control *N. benthamiana* plants. At selected times following wounding or INF1 treatment, leaf disks were punched out, and the levels of *WIPK* and *SIPK* transcripts, and the kinase activity of WIPK and SIPK, were measured. The amounts of SIPK protein present were also studied by Western analysis using anti-SIPK antibody. We also tried to detect the WIPK protein as well using anti-WIPK antibody raised against a synthetic peptide (MADANMGAGGGQFPDFPS) derived from the N-terminus of WIPK of *N. tabacum* (Seo et al. 1999). But these attempts failed, presumably because the amino acid sequence of the N-terminus of *N. benthamiana* WIPK (MADANMGGGGGGQFPDFPS; Fig. 1A) differs from that of the *N. tabacum* homologue and could not be recognized by the antibody.

Wounding caused a transient accumulation of *WIPK* transcripts in the control (PVX.GFP) as well as in PVX.SIPK-infected plants (Fig. 4A). The amount of *WIPK* transcript increased within 15 min after wounding, peaked at 60 min, and then gradually decreased over the next 3 h. In PVX.WIPK- and PVX.WIPK-SIPK-infected plants, transcript levels were much reduced as compared to the control, although some *WIPK* transcripts were still detectable. In contrast to *WIPK*, the level of *SIPK* mRNA in the PVX.GFP- as well as PVX.WIPK-infected plants was unaltered after wounding, and was almost nil in PVX.SIPK- and PVX.WIPK-SIPK-infected plants (Fig. 4A).

To determine the effect of silencing of *WIPK* and *SIPK* on the kinase activity of the corresponding proteins, we investigated whether the level of protein phosphorylation changed after wounding, using an in-gel kinase assay with Myelin Basic Protein (MBP) as substrate (Fig. 5A). Leaves of the PVX.GFP- and PVX.WIPK-infected plants that had been wounded with carborundum exhibited a rapid and transient activation of SIPK, but little activation was observed in PVX.SIPK- and PVX.WIPK-SIPK-infected plants. In addition, the results of immunoanalysis using anti-SIPK antibody demonstrated that the reduction in the SIPK activity in PVX.SIPK- and PVX.WIPK-SIPK-infected plants actually resulted from a reduction in the amount of SIPK protein present (Fig. 5A). On the other hand, WIPK activity in the leaves of PVX.GFP- and PVX.SIPK-infected plants increased until 60 min after wounding, but was only slightly enhanced in PVX.WIPK- and PVX.WIPK-SIPK-infected plants in response to wounding (Fig. 5A).

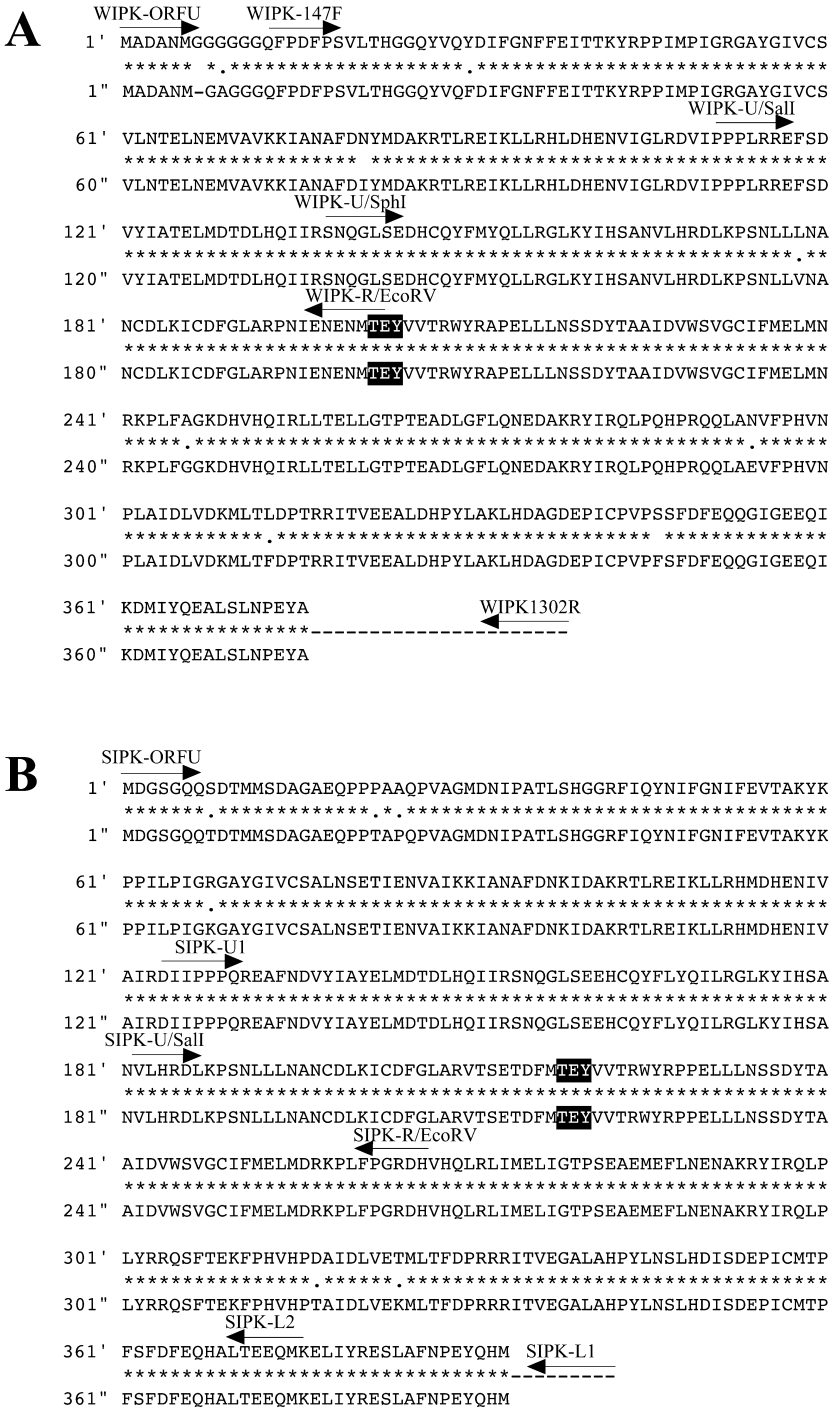

Fig. 1A, B Alignment of the predicted amino acid sequences of NbWIPK (**A**) and NbSIPK (**B**) (*upper lines*) with tobacco WIPK and SIPK (*lower lines*; Genbank Accession Nos. D61377 and U94192), respectively. The conserved phosphorylation motifs (TEY) for MAP kinase are *shaded*. The relative positions of PCR primers used in the experiments are indicated at the corresponding amino acid sequences

Next, we studied the response of *WIPK* - and *SIPK* - silenced plants to INF1 treatment. The level of *WIPK* RNA increased for 60 min after the infiltration of leaves with 100 nM INF1 protein in PVX.GFP- as well as PVX.SIPK-infected plants (Fig. 4B). The timing of *WIPK* induction following INF1 treatment was somewhat delayed in comparison to its response to wounding treatment (Fig. 4A, B). In the PVX.WIPK- and PVX.WIPK-SIPK infected plants, the levels of *WIPK* mRNA were reduced (Fig. 4B). *SIPK* transcripts were present in PVX.GFP- and PVX.WIPK-infected plants; however, *SIPK* expression was not affected by treatment with INF1 (Fig. 4B). In the PVX.SIPK- and PVX.WIPK-SIPK-plants, *SIPK* transcript levels were very low (Fig. 4B).

The kinase activity and concentration of SIPK protein were also investigated in the plants treated with INF1 (Fig. 5B). SIPK was activated in PVX.GFP- and PVX.WIPK-infected plants, reaching its maximum activity 15 min after treatment with INF1. WIPK activation was not observed in any of the INF1-treated plants, including the PVX.GFP control. In

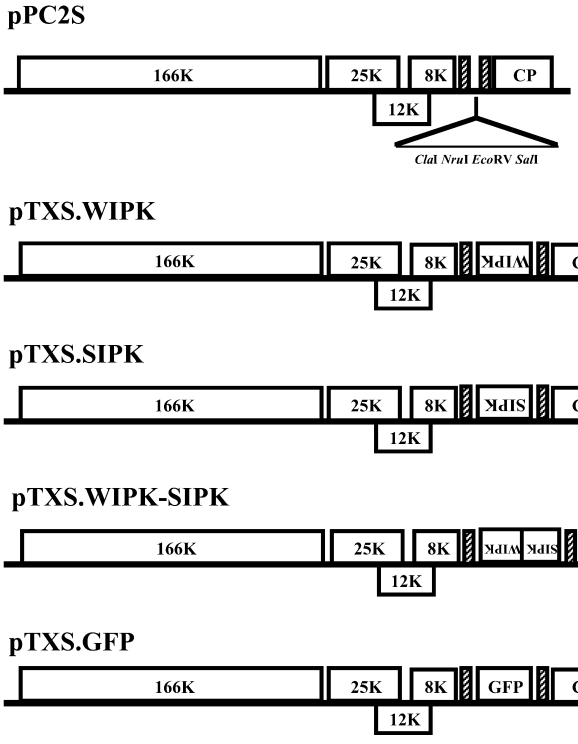

Fig. 2 Organization of the pPC2S, pTXS.WIPK, pTXS.SIPK, pTXS.WIPK-SIPK and pTXS.GFP genomes. The *boxes* represent PVX coding regions and the antisense partial cDNA fragments of *WIPK* and *SIPK*; the *horizontal lines* indicate untranslated sequences. pPC2S carries a duplication of the subgenomic coat protein RNA promoter (*dashed boxes*) and a multiple cloning site. Sizes of virus-encoded proteins are given in kDa. CP, coat protein; WIPK and SIPK indicate partial cDNA fragments of *WIPK* and *SIPK*, respectively, and are shown *upside-down* to indicate that the sequences are inserted in the antisense direction; GFP, green fluorescent protein

PVX.SIPK- and PVX.WIPK-SIPK-infected plants, the activation of SIPK was impaired. Western analysis using anti-SIPK antibody showed that the reduced SIPK activity in PVX.SIPK- and PVX.WIPK-SIPK-infected plants was caused by a reduction in the amount of the protein itself (Fig. 5B).

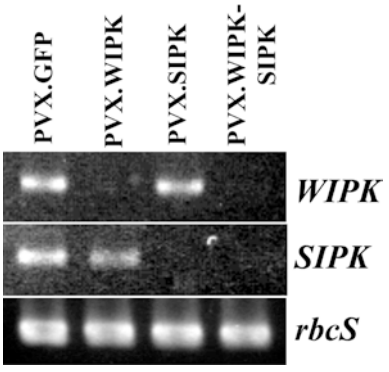

Fig. 3 RT-PCR analysis of *NbWIPK* and *NbSIPK* expression in leaves of PVX.GFP, PVX.WIPK, PVX.SIPK and PVX.WIPK-SIPK-infected *N. benthamiana* plants. Three weeks after inoculation, leaf samples were harvested from the third or fourth leaf above the inoculation site, and total RNA was isolated and used for RT-PCR as described by Saitoh and Terauchi (2002); amplification of first-strand cDNA was carried out for 25 cycles using primers specific for *NbWIPK* and *NbSIPK*, respectively. Equal input of cDNA template for PCR was demonstrated by amplification of the constitutively expressed *rbcS* gene (22 cycles)

Following infiltration of 100 nM INF1 into the leaves, a normal hypersensitivity response developed in PVX.GFP-, PVX.WIPK-, PVX.SIPK- and PVX.WIPK-SIPK-infected leaves, and no consistent difference was observed in the timing and extent of HR in *WIPK*-, *SIPK*- and *WIPK*- *SIPK*-silenced plants as compared to the control plants (Fig. 6).

VIGS of *WIPK* and *SIPK* results in enhanced susceptibility to disease

N. benthamiana leaves inoculated with *P. cichorii* exhibit resistance responses and eventually develop HR (Y. Hikichi, personal communication). To address the question of whether WIPK and SIPK are involved in the resistance responses and in the development of HR against an avirulent incompatible pathogen, we inoculated *P. cichorii* onto *N. benthamiana* leaves that had

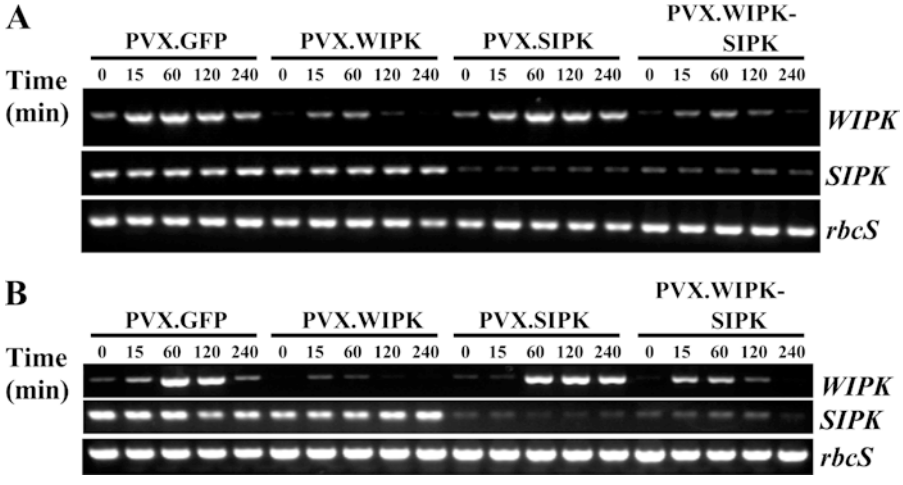

Fig. 4A, B Amounts of *WIPK*, *SIPK* and *rbcS* mRNAs present at various times after wounding (**A**) and treatment with the elicitor INF1 (**B**) as revealed by RT-PCR

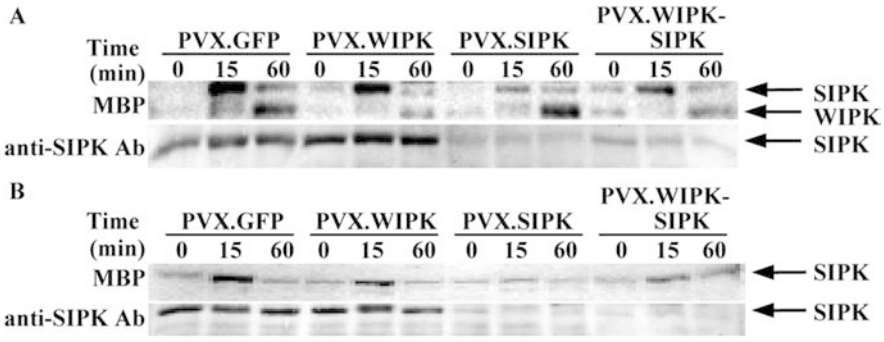

Fig. 5A, B MBP kinase activities and SIPK protein levels (detected with anti-SIPK antibody) at various times after wounding (**A**) and treatment with INF1 (**B**)

Fig. 6 Development of the hypersensitive response on leaves of *N. benthamiana* plants infected with PVX.GFP, PVX.WIPK, PVX.SIPK or PVX.WIPK-SIPK, 7 days after infiltration with 100 nM INF1

previously been inoculated with PVX.GFP, PVX.WIPK, PVX.SIPK or PVX.WIPK-SIPK. The rate of growth of *P. cichorii* in *WIPK* -, *SIPK* - and *WIPK* - *SIPK* -silenced plants was approximately 3–8-fold, 3–20-fold and more than 20-fold, respectively, greater than in plants infected with PVX.GFP during the first 3 days post inoculation (Fig. 7A). However, no marked alteration in the timing and development of HR was seen in *WIPK*/*SIPK* -silenced plants in comparison to the control, as indicated by photographs taken 4 days after infiltration with *P. cichorii* (Fig. 7B).

Discussion

To examine the functional roles of two MAPKs, WIPK and SIPK, in the development of HR and disease resistance in *N. benthamiana*, we used virus-induced gene silencing (VIGS) as a rapid gene knockout method. *WIPK* and *SIPK* in *N. benthamiana* plants were silenced individually (by inoculation with PVX.WIPK or PVX.SIPK, respectively) or simultaneously (by inoculation with PVX.WIPK-SIPK), and the responses of these gene-silenced plants were compared with those of control plants inoculated with PVX.GFP. Wounding is one of the most effective ways of activating WIPK and SIPK in tobacco plants (T. Romeis, personal communication). Therefore, we first used wounding as a stimulus to evaluate the effect of silencing of *WIPK* and *SIPK* genes on the kinase activity of WIPK and SIPK in a time-course experiment. By RT-PCR, we confirmed that the basal levels of *WIPK* and *SIPK* gene transcription were effectively reduced to a very low level in PVX.WIPK- and PVX.SIPK-infected plants, respectively, and that transcription of both *WIPK* and *SIPK* was silenced in PVX.WIPK-SIPK-infected plants. Furthermore, in the case of WIPK, de novo transcription of *WIPK* in response to wounding was also reduced to a very low level in PVX.WIPK- and PVX.WIPK-SIPK-infected plants (Fig. 4A). In agreement with the reduced levels of transcription, the MBP kinase assay showed that wounding-induced activation of SIPK was abolished in PVX.SIPK- and PVX.WIPK-SIPK-infected plants. The absence of SIPK activation resulted from the reduction in the amount of SIPK protein present, as confirmed by immunoblot analysis (Fig. 5A). Similarly, WIPK activation by wounding was reduced in PVX.WIPK- and PVX.WIPK-SIPK-infected plants (Fig. 5A). These results clearly demonstrate that PVX-mediated VIGS is effective in silencing the kinase activity of WIPK, SIPK or WIPK and SIPK together. Next, using the *WIPK*-, *SIPK*- and *WIPK*- *SIPK*-silenced plants, we examined the functional role of WIPK and SIPK in INF1-mediated HR development and in resistance to the incompatible bacterial pathogen *P. cichorii*. Most species of the genus *Phytophthora* produce 10-kDa extracellular protein elicitors, called elicitins (Pernollet et al. 1993; Kamoun et al. 1994). Elicitins induce defense responses including HR and systemic acquired resistance (SAR) on a restricted set of plant species, including tobacco (Kamoun et al. 1993; Keller et al. 1996). Zhang et al. (1998, 2000) reported that two purified elicitins, parasiticein and cryptogein, activate WIPK and SIPK in tobacco cells grown in suspension culture. Kamoun et al. (1997) identified another elicitin, INF1, that induces HR on tobacco leaves. INF1 also induces HR in *N. benthamiana* (Kamoun et al. 1998; Peart et al. 2002b; Qutob et al. 2002). In the present study, we examined in planta the effect of silencing of *WIPK* and *SIPK* on HR development following treatment with INF1. The results showed that SIPK was activated within 15 min after treatment with INF1 in the leaves of PVX.GFP- and PVX.WIPK-infected plants, but was only slightly activated in PVX.SIPK- and PVX.WIPK-SIPK-infected plants

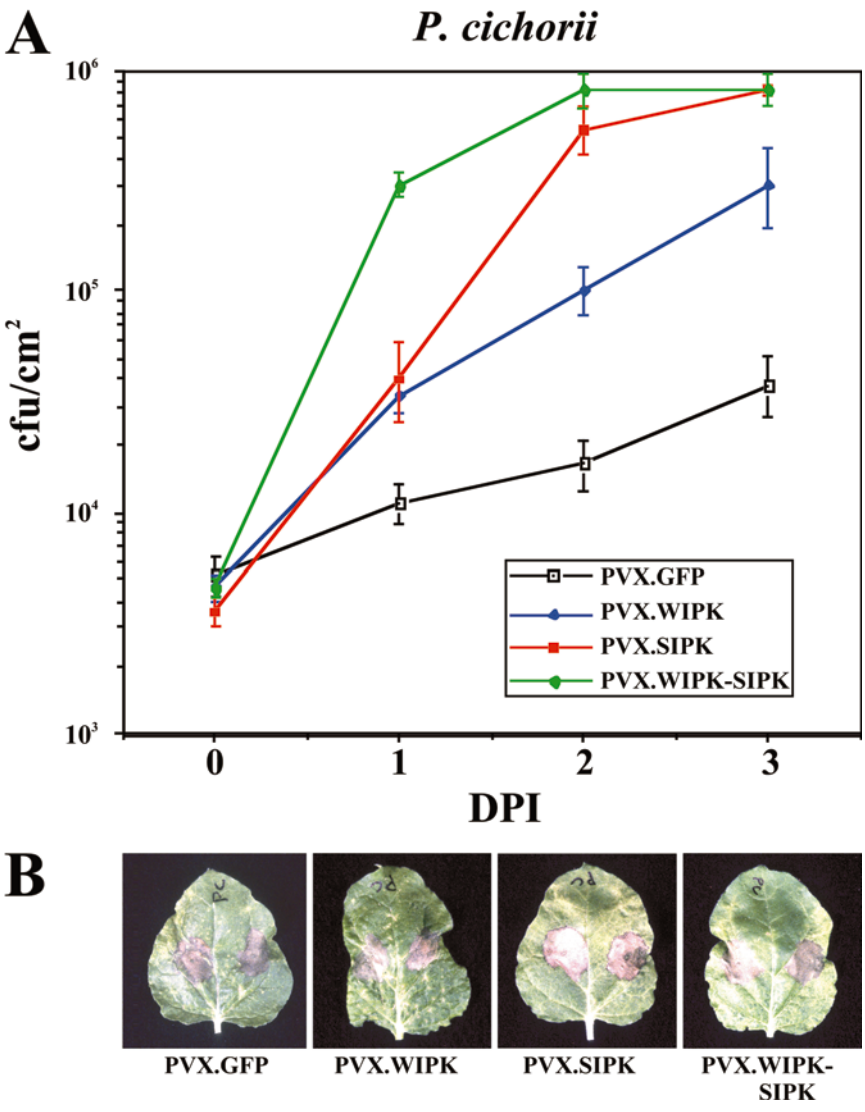

Fig. 7A, B Growth of *P. cichorii* (**A**) and development of the hypersensitive response (**B**) on leaves of *N. benthamiana* plants infected with PVX.GFP, PVX.WIPK, PVX.SIPK or PVX.WIPK-SIPK. **A** Leaves were infiltrated with *P. cichorii* ($OD_{600} = 0.01$), and bacterial growth was measured as colony forming unit per cm^2 on three consecutive days. The averages and standard errors of data from three plants are given. **B** HR-like disease symptoms on PVX.GFP-, PVX.WIPK-, PVX.SIPK- and PVX.WIPK-SIPK-infected plants 4 days after infiltration with *P. cichorii* ($OD_{600} = 0.01$)

(Fig. 5B). *WIPK* silencing in PVX.WIPK- and PVX.WIPK-SIPK-infected plants was confirmed by RT-PCR (Fig. 4B). No activation of WIPK was detected after INF1 treatment, regardless of whether the gene was silenced or not (Fig. 5B). Unexpectedly, *WIPK* -, *SIPK* - and *WIPK* - *SIPK* -silenced leaves treated with INF1 developed a normal HR like that observed in PVX.GFP-infected leaves that were treated with INF1 (Fig. 6). These results suggest that activation of WIPK and SIPK is not required to allow INF1 to induce a hypersensitive response in *N. benthamiana*. Yang et al. (2001) have shown that overexpression of a constitutively active MAPKK, NtMEK2 ^{DD}, leads to activation of WIPK and SIPK followed by HR-like cell death in tobacco. Likewise, Zhang and Liu (2001) reported that overexpression of SIPK alone caused HR-like cell death in tobacco. From these observations, the authors hypothesized that HR in plant defense is mediated by activation of SIPK (Yang et al. 2001; Zhang and Liu. 2001). Our result shows that SIPK activation can be triggered by INF1 treatment, but is not necessary for the subsequent development of HR. Therefore, we conclude that SIPK activation does not play a major role in INF1-mediated HR. WIPK and SIPK can be activated in transgenic *Cf9* tobacco leaves and cells by treatment with the elicitor protein Avr9 from *Cladosporium fulvum* (Romeis et al. 1999). In the same incompatible interaction between Avr9 and Cf9, calcium-dependent protein kinases (CDPKs) were also activated (Romeis et al. 2000). Moreover, Romeis et al. (2001) demonstrated that two tobacco CDPKs, NtCDPK2 and NtCDPK3, were indispensable in causing HR in interactions between Avr9 and Cf9, and Avr4 and Cf4. These reports raise the possibility that INF1-induced HR in *N. benthamiana* results from the activation of a CDPK cascade (Romeis 2001; Xing et al. 2002).

Recently, a simple method has been established for studying disease resistance based on the measurement of bacterial growth in *N. benthamiana* (Peart et al. 2002a, 2002b). We employed this method to examine the effect of *WIPK* and *SIPK* silencing on bacterial growth and HR in *N. benthamiana* leaves inoculated with the

avirulent pathogen *P. cichorii*. The results showed that growth of *P. cichorii* was significantly enhanced on *WIPK*-, *SIPK*- and *WIPK-SIPK* -silenced plants relative to PVX.GFP-infected plants. The reduction in resistance caused by simultaneous silencing of WIPK and SIPK is greater than that caused by silencing of either *WIPK* or *SIPK* alone (Fig. 7A). This observation suggests that WIPK and SIPK are involved in conferring resistance to pathogens on plants, and that their effects are cumulative. In agreement with the results obtained after INF1 treatment of gene-silenced plants, no perceptible difference in HR development was observed between *WIPK* -, *SIPK* - or *WIPK* - *SIPK* -silenced plants and the control plants (Fig. 7B). Based on these results, we propose that WIPK and SIPK play an important role in the control of pathogen growth in planta, but are not directly involved in regulating the development of HR caused by INF1 and *P. cichorii*.

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References

Baulcombe DC (1999) Fast forward genetics based on virus-induced gene silencing. Curr Opin Plant Biol 2:109–113

Baulcombe DC, Chapman S, Santa Cruz S (1995) Jellyfish green fluorescent protein as a reporter for virus infections. Plant J 7:1045–1053

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. J Biochem 72:248–254

Burton RA, Bibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB (2000) Virus-induced silencing of a plant cellulose synthase gene. Plant Cell 12:691–705

Davis R (2000) Signal transduction by the JNK group of MAP kinases. Cell 103:239-252

Droillard MJ, Thibivilliers S, Cazalé AC, Barbier-Brygoo H, Lauriére C (2000) Protein kinases induced by osmotic stresses and elicitor molecules in tobacco cell suspensions: two crossroad MAP kinases and one osmoregulation-specific protein kinase. FEBS Lett 474:217–222

Hikichi Y, Suzuki K, Toyoda K, Horikoshi M, Hirooka T, Okuno T (1998) Successive observation of growth and movement of genetically *lux* -marked *Pseudomonas cichorii* and the response of host tissues in the same lettuce leaf. Ann Phytopathol Soc Jpn 64:519–525

Kamoun S, Young M, Glascock C, Tyler BM (1993) Extracellular protein elicitors from *Phytophthora*: host-specificity and induction of resistance to fungal and bacterial phytopathogens. Mol Plant-Microbe Interact 6:15–25

Kamoun S, Young M, Forster H, Coffey MD, Tyler BM (1994) Potential role of elicitins in the interaction between *Pytophthora* species and tobacco. Appl Environ Microbiol 60:1593–1598

Kamoun S, van West P, de Jong AJ, de Groot KE, Vleeshouwers VGAA, Govers F (1997) A gene encoding a protein elicitor of *Phytophthora infestans*. Mol Plant-Microbe Interact 10:13–20

Kamoun S, van West P, Vleeshouwers VGAA, de Groot KE, Govers F (1998) Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. Plant Cell 10:1413–1425

Keller H, Blein JP, Bonnet P, Ricci P (1996) Physiological and molecular characteristics of elicitin-induced systemic acquired resistance in tobacco. Plant Physiol 110:365–376

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

Matsumura H, Nirasawa S, Kiba A, Urasaki N, Saitoh H, Ito M, Kawai-Yamada M, Uchimiya H, Terauchi R (2003) Overexpression of Bax inhibitor suppresses fungal elicitor-induced cell death in rice (*Oryza sativa* L.) cells. Plant J 33:425–434

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

Peart JR, Cook G, Feys BJ, Parker JE, Baulcombe DC (2002a) An *EDS1* orthologue is required for *N* -mediated resistance against tobacco mosaic virus. Plant J 29:569–579

Peart JR, Lu R, Sadanandom A, Malcuit I, Moffett P, Brice DC, Schauser L, Jaggard DAW, Xiao S, Coleman M, Dow M, Jones JDG, Shirasu K, Baulcombe DC (2002b) Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc Natl Acad Sci USA 99:10865–10869

Pernollet JC, Sallantin M, Salle-Tourne M, Huet JC (1993) Elicitin isoforms from seven *Phytophthora* species: comparison of their physico-chemical properties and toxicity to tobacco and other plant species. Physiol Mol Plant Pathol 42:53–67

Qutob D, Kamoun S, Gijzen M (2002) Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. Plant J 32:361–373

Romeis T (2001) Protein kinases in the plant defence response. Curr Opin Plant Biol 4:407–414

Romeis T, Piedras P, Zhang S, Klessig DF, Hirt H, Jones JDG (1999) Rapid Avr9- and Cf9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. Plant Cell 11:273–287

Romeis T, Piedras P, Jones JDG (2000) Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. Plant Cell 12:803–815

Romeis T, Ludwig AA, Martin R, Jones JDG (2001) Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J 20:5556–5567

Saitoh H, Terauchi R (2002) Virus-induced silencing of *FtsH* gene in *Nicotiana benthamiana* causes a striking bleached leaf phenotype. Genes Genet Syst 77:335–340

Saitoh H, Kiba A, Nishihara M, Yamamura S, Suzuki K, Terauchi R (2001) Production of antimicrobial defensin in *Nicotiana benthamiana* with a potato virus X vector. Mol Plant-Microbe Interact 14:111–115

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

Samuel MA, Miles GP, Ellis BE (2000) Ozone treatment rapidly activates MAP kinase signalling in plants. Plant J 22:367–376

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

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

Voinnet O (2001) RNA silencing as a plant immune system against viruses. Trends Genet 17:449–459

Widman C, Gibson S, Jarpe MB, Johnson GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 79:143–180

Xing T, Quellet T, Miki BL (2002) Towards genomic and proteomic studies of protein phosphorylation in plant-pathogen interactions. Trends Plant Sci 7:224–230

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

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

Zhang S, Klessig DF (1998) Resistance gene N-mediated de novo synthesis and activation of a tobacco mitogen-activated protein kinase by tobacco mosaic virus infection. Proc Natl Acad Sci USA 95:7433–7438

Zhang S, Klessig DF (2001) MAPK cascades in plant defense signaling. Trends Plant Sci 6:520–527

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

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

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