

Involvement of PPS3 Phosphorylated by Elicitor-Responsive Mitogen-Activated Protein Kinases in the Regulation of Plant Cell Death¹

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Mitogen-activated protein kinase (MAPK) cascades play pivotal roles in plant innate immunity. Overexpression of StMEK1^{DD}, a constitutively active MAPK kinase that activates salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), provokes hypersensitive response-like cell death in *Nicotiana benthamiana*. Here we purified a 51-kD MAPK, which was activated in potato (*Solanum tuberosum*) tubers treated with hyphal wall elicitor of a plant pathogen, and isolated the cDNA designated StMPK1. The deduced amino acid sequence of the StMPK1 showed strong similarity to stress-responsive MAPKs, such as tobacco (*Nicotiana tabacum*) SIPK and Arabidopsis (*Arabidopsis thaliana*) AtMPK6. To investigate the downstream signaling of StMPK1, we identified several proteins phosphorylated by StMPK1 (PPSs) using an in vitro expression cloning method. To dissect the biological function of PPSs in the plant defense, we employed virus-induced gene silencing (VIGS) in *N. benthamiana*. VIGS of NbPPS3 significantly delayed cell death induced by the transient expression of StMEK1^{DD} and treatment with hyphal wall elicitor. Furthermore, the mobility shift of NbPPS3 on SDS-polyacrylamide gel was induced by transient expression of StMEK1^{DD}. The mobility shift of NbPPS3 induced by StMEK1^{DD} was not compromised by VIGS of WIPK or SIPK alone, but drastically reduced by the silencing of both WIPK and SIPK. This work strongly supports the idea that PPS3 is a physiological substrate of StMPK1 and is involved in cell death activated by a MAPK cascade.

The mitogen-activated protein kinase (MAPK) cascade is one of the major and evolutionally conserved signaling pathways utilized to transduce extracellular stimuli into intracellular responses (MAPK Group, 2002). In the MAPK signal transduction cascade, a MAPK kinase (MAPKK) is activated by an upstream MAPKK kinase and in turn activates a MAPK. There are many reports showing that MAPKs are activated by pathogen stimuli in a wide range of plant species, either positively or negatively (Ligterink et al., 1997; Lebrun-Garcia et al., 1998; Zhang et al., 1998; Suzuki

et al., 1999; Cardinale et al., 2000; Petersen et al., 2000; Asai et al., 2002; Ekengren et al., 2003; del Pozo et al., 2004; Lee et al., 2004; Nakagami et al., 2004). Treatment of tobacco (*Nicotiana tabacum*) plants expressing the Cf-9 resistance gene with a fungal Avr factor Avr9 from *Cladosporium fulvum* leads to the activation of salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK; Romeis et al., 1999; Rivas et al., 2004). In addition, both MAPKs are activated by plant species-specific elicitors such as elicitors (Zhang et al., 2000) or tobacco mosaic virus infection (Zhang and Klessig, 1998), suggesting that SIPK and WIPK are convergent points in the signaling pathway of pathogens and pathogen-derived elicitors. In fact, the loss of function of either SIPK (or its Arabidopsis [*Arabidopsis thaliana*] ortholog AtMPK6) or WIPK compromised *N* gene-mediated gene-for-gene resistance to tobacco mosaic virus infection (Jin et al., 2003) and resistance to bacterial pathogens (Sharma et al., 2003; Menke et al., 2004).

Recently, it was reported that NtMEK2, a tobacco MAPKK, is upstream of both SIPK and WIPK (Yang et al., 2001). Expression of NtMEK2^{DD}, a constitutively active mutant of NtMEK2, induced hypersensitive response (HR)-like cell death, defense gene expression, and generation of reactive oxygen species, which were preceded by the activation of endogenous SIPK and WIPK (Yang et al., 2001; Ren et al., 2002). We also

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showed that the gain-of-function mutant of potato (*Solanum tuberosum*) NtMEK2 ortholog StMEK1 (StMEK1^{DD}) induced SIPK and WIPK activities followed by induction of *rboh* (respiratory burst oxidase homolog) gene expression, which is implicated in reactive oxygen species production, in *Nicotiana benthamiana* (Yoshioka et al., 2003). Moreover, Zhang and Liu (2001) showed that transient expression of SIPK was sufficient to induce HR-like cell death and expression of *3-hydroxy-3-methylglutaryl CoA reductase*, a gene encoding a key enzyme in phytoalexin biosynthesis pathway. These results strongly indicate that SIPK plays a central role in multiple defense responses. However, how SIPK is integrated into signaling pathways leading to defense responses remains unclear.

In mammals, MAPKs phosphorylate a wide range of proteins, including transcription factors, protein kinases, and phospholipase A₂ (Davis, 1993; Sharrocks et al., 2000; Kyriakis and Avruch, 2001). Extracellular signal-regulated kinase1/2, one subfamily of mammalian MAPK involved in cell proliferation, differentiation, and development, shares its activation motif Thr-Glu-Tyr with most plant MAPKs. It is well known that upon stimulation with growth factors, extracellular signal-regulated kinase1/2 translocates into the nucleus and phosphorylates transcription factors to promote the activation of immediate early genes (Treisman, 1996). Yeast two-hybrid screening was employed to identify target proteins of SIPK and blast- and wound-induced MAPK 1 (Cheong et al., 2003; Kanzaki et al., 2003). They showed that Hsp90 and a transcription factor, rice (*Oryza sativa*) ethylene-responsive element-binding protein 1, interact with the MAPKs. Recently, it was reported that Arabidopsis AtMPK6 phosphorylates 1-aminocyclopropane-1-carboxylic acid synthase and induces ethylene biosynthesis (Liu and Zhang, 2004). However, downstream signaling of the plant defense-related MAPKs is not well elucidated. The identification of the downstream MAPK substrate(s) will also help us understand how SIPK and WIPK regulate multiple defense responses.

In a previous study, we reported that a 51-kD protein kinase (p51-PK) with the characteristics common to MAPKs was activated in potato tubers treated with hyphal wall components (HWC) elicitor from the late blight pathogen *Phytophthora infestans* (Katou et al., 1999). Here we purified the p51-PK through sequential column chromatography and isolated its cDNA, designated StMPK1, from the amino acid sequences of internal tryptic peptides. The deduced amino acid sequence of StMPK1 cDNA showed strong similarity to stress-responsive MAPKs, such as tobacco SIPK and alfalfa (*Medicago sativa*) SIMK. To reveal downstream signaling of StMPK1, we screened proteins phosphorylated by StMPK1 using in vitro expression cloning (IVEC). IVEC has been successfully used to identify *Xenopus* proteins that undergo mitosis-specific phosphorylation (Stukenberg et al., 1997) or degradation (McGarry and Kirschner, 1998), as well as novel apoptotic protease substrates (Cryns et al., 1997; Kothakota

et al., 1997), sequence-specific DNA-binding proteins (Mead et al., 1998), and uracil-DNA glycosylases (Haushalter et al., 1999). In this study, eight positive clones designated protein phosphorylated by StMPK1 (PPS) in vitro were identified from more than 3,000 clones. We examined their function by employing virus-induced gene silencing (VIGS) technology in *N. benthamiana*. We report here that VIGS of NbPPS3 significantly reduced and delayed StMEK1^{DD}- and HWC-induced cell death. Furthermore, VIGS of SIPK and WIPK compromised the gel mobility shift of phosphorylated NbPPS3 proteins induced by StMEK1^{DD}.

RESULTS

Purification of p51-PK

We reported that p51-PK activity peaked 3 h after treatment of potato tubers with hyphal wall elicitor and seemed to be inactivated by protein phosphatase(s) (Katou et al., 1999). Thus, p51-PK was purified from potato tubers treated with elicitor for 3 h in the presence of phosphatase inhibitors. At each step, the p51-PK activity was assayed with in-gel kinase assay using myelin basic protein (MBP) as a substrate. The soluble supernatant fraction was initially fractionated on a Q-Sepharose anion-exchange column. The p51-PK was eluted at 0.25 M NaCl from the column. In addition to p51-PK, activity of a 46-kD protein kinase (p46-PK) was detected in this fraction. In subsequent column chromatography, both protein kinases were copurified. It was shown that p51-PK was phosphorylated on a Tyr residue (Katou et al., 1999). Therefore, the active fraction from Mono Q column was applied to a PY20-Sepharose column, in which antiphosphotyrosine antibody PY20 was coupled to *N*-hydroxysuccinimide-activated Sepharose (Pharmacia). After extensive washing, p51-PK and p46-PK were eluted with excess amounts of phosphotyrosine. The native molecular masses of p51-PK and p46-PK, as calculated from gel filtration on a Superdex 75-pg column, were 52 kD and 40 kD, respectively (data not shown). These are in good agreement with the 51 kD and 46 kD estimated by in-gel kinase assay (Fig. 1). This result indicates that p51-PK and p46-PK are monomeric enzymes, as all the other MAPKs. Purification of p51-PK and p46-PK were about 6,800- and 10,000-fold (summarized in Table I) with an approximate recovery of 1.1% and 4.2%, respectively. Recovery of p46-PK raised in the course of purification (Table I) may be due to the partial degradation of p51-PK by contaminating proteases.

Amino Acid Sequence Analysis of p51-PK and p46-PK

Figure 1B shows the SDS-PAGE analysis followed by Coomassie Blue staining of purification steps. The pooled fraction from the PY20-Sepharose column contained three major bands (p47-1, p47-2, and p43). The p47-1 and p47-2 likely correspond to p51-PK, whereas p43 likely corresponds to p46-PK. Peptide

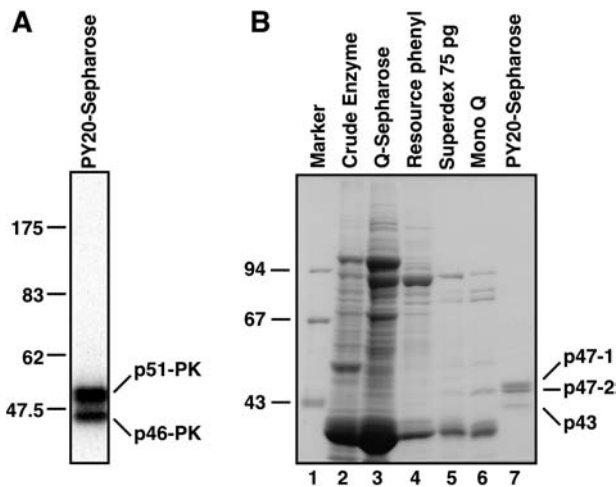


Figure 1. In-gel kinase assay and SDS-PAGE analysis of p51-PK and p46-PK. A, In-gel kinase assay of purified protein kinases. An aliquot of active fraction from PY20-Sepharose column was assayed for protein kinase activity with in-gel kinase assay using MBP as a substrate. B, Protein aliquot from each purification step was separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue; lane 1, molecular mass markers in kilodaltons; lane 2, soluble protein fraction (18 μ g); lane 3, pooled fraction from Q-Sepharose column (25 μ g); lane 4, pooled fraction from Resource phenyl column (3.7 μ g); lane 5, pooled fraction from Superdex 75-pg column (2.3 μ g); lane 6, pooled fraction from Mono Q column (1.5 μ g); lane 7, pooled fraction from PY20-Sepharose column.

mapping analyses of p47-1 and p47-2 were almost identical (data not shown), suggesting that they represent the same protein modified differently. N-terminal amino acid sequence of several internal tryptic peptides of p47-1 and p43 were identical (Fig. 2). These results suggest that p46-PK is a partial degradative product of p51-PK. However, we cannot rule out the possibility that they are distinct but highly related protein kinases.

cDNA Cloning of p51-PK Gene

Primers were designed from sequences of internal tryptic peptides 1 and 4, and a fragment of *p51-PK* cDNA was directly amplified from a potato cDNA library. A full-length cDNA clone, which contains all of the peptide sequences obtained by microsequencing of the purified protein, was obtained by screening of a cDNA library (Fig. 2). Analysis of the deduced protein sequence of the *p51-PK* cDNA indicates that it belongs to the MAPK family, so it was designated StMPK1. The calculated molecular mass and pI of StMPK1 are 45.6 kD and 5.42, respectively. The deduced amino acid sequence of *StMPK1* shows strong similarity with other plant MAPKs, such as tobacco SIPK, alfalfa SIMK, and Arabidopsis AtMPK6, which are known to be activated in response to various stresses (Zhang et al., 1998; Cardinale et al., 2000; Ichimura et al., 2000).

Posttranslational Activation of StMPK1

In addition to posttranslational activation of MAPKs in yeast, plants, and mammals, several plant MAPKs have been reported to be induced at the transcriptional level (Seo et al., 1995; Jonak et al., 1996; Mizoguchi et al., 1996). To investigate whether the activation of StMPK1 is associated with accumulation of the transcripts, we examined the mRNA levels of StMPK1 by RNA gel-blot analysis. As shown in Figure 3A, the mRNA levels of *StMPK1* remained constant after activation by hyphal wall elicitor. To investigate the protein levels of StMPK1, antibody against the N-terminal peptide of StMPK1 (StMPK1-N, MDA-SAPQMDTMMPDVAAPAV) was raised in rabbits and purified by antigen-affinity chromatography. The specificity of this antibody was assessed by immunoblot analysis against StMPK2, which shows 94% identity on the amino acid level to StMPK1 and seems to be potato ortholog of tobacco Ntf4. The immunoblot analysis showed that anti-StMPK1-N antibody recognized only StMPK1 (Fig. 3B, left), indicating that it is highly specific. We then investigated the protein level and MBP phosphorylation activity of StMPK1 by immunoblot analysis and immunocomplex kinase assay, respectively. In contrast to the steady-state levels of StMPK1 protein (Fig. 3B, right), the kinase activity of StMPK1 increased within 1 h, reached a maximal level 3 h after treatment with hyphal wall elicitor, and then declined (Fig. 3C, right). These results suggest that post-translational modification is required for activation of StMPK1. Specificity of the reaction was confirmed using preimmune serum (Fig. 3C, left, lane 1) or excess amounts of StMPK1-N peptide, which competed immunoprecipitation of MBP phosphorylation activity (Fig. 3C, left, lane 3).

In Vitro Activation of StMPK1 by StMEK1^{DD}

A specific MAPKK, which phosphorylates and activates StMPK1, is likely required for activation of StMPK1. StMPK1 shares 94% amino acid sequences with tobacco SIPK (Fig. 2). The enzymatic activation profile of StMPK1 is also similar to that of SIPK, which

Table 1. Purification of p51-PK and p46-PK from potato tubers

Protein	Recovery		Purification	
	p46-PK	p51-PK	p46-PK	p51-PK
	mg	%	fold	
Soluble fraction ^a	4,740	100.0	1.0	1.0
Q-Sepharose	1,267	100.0	1.0	3.2
Resource phenyl	38.5	135.1	39.2	44.5
Superdex 75 pg	4.28	46.8	12.1	139
Mono Q	0.987	18.1	4.2	232
PY20-Sepharose	0.005 ^b	4.2	1.1	10,594

^aThe starting soluble fraction was prepared from 1.2 kg of potato tuber slices treated with fungal cell wall elicitor for 3 h. ^bThe amount of protein was estimated by comparison with bovine serum albumin on a Coomassie-stained SDS-polyacrylamide gel.

StMPK1	1:	MDAS--APQMDTMPDVAAAPAVQPPPPQPLPGMDNIPATLSHGGRFIQYNIFGNIFEV
SIPK	1:	..G.--GQ.T....S.AG.E---..TA...VA.....
Ntf4	1:	..GP--.H.T..V.S.A.GQ---.A..S..VA.I.....
MMK1	1:	..EGGG-.PA..V.S.A.PAP-----..QM.IE...V.....
AtMPK6	1:	..GGSGQ.AA..E.TEAPGGFPAAA.S.--QM.IE.....
WIPK	1:	MA.AN.GAGGGQ-----F--PDF.SV.T...QYV.FD...F..I
peptide 1 p43: HMD		
StMPK1	59:	TAKYKPPIMPIGKAYGIVCSALNSETNENVAIKKIANAFDNKIDAKRTLREIKLLRHMD
SIPK	56:L.....I.....
Ntf4	56:H.....
MMK1	51:H.....H..V.....
AtMPK6	59:M.....S.....
WIPK	39:	.T..R.....R.....V..T.L..M..V.....IYM.....L.
peptide 2 p47-1:DIIPPPQ		
StMPK1	119:	HENIVAIR HENIVAIRDIIPPPQREAFNDVYIAYELMDTDLHQIIRSNQGLSEEHCQYFLYQILRGLK
SIPK	116:
Ntf4	116:
MMK1	111:	..V.....V.....V.....A.....
AtMPK6	119:L.N.....A.....
WIPK	99:	..VIGL..V....L.RE.S....T.....D....M..L.....
peptide 3 p47-1:YIHSANV		
StMPK1	179:	YIHSANVLRDLKPSNLLLNCANCDLKICDFGLARVTSETDFMTEYVVT
SIPK	176:
Ntf4	176:A.....
MMK1	171:A.....
AtMPK6	179:S.....A.....
WIPK	159:V.....PNI.NEN.....A.....
peptide 4 p47-1:VTSETDF		
StMPK1	179:	YIHSANVLRDLKPSNLLLNCANCDLKICDFGLARVTSETDFMTEYVVT
SIPK	176:
Ntf4	176:A.....
MMK1	171:A.....
AtMPK6	179:S.....A.....
WIPK	159:V.....PNI.NEN.....A.....
peptide 5 p47-1:KPLFPGR		
StMPK1	239:	SDYTAADVWSVGCIFMELMDRKLFPGRDHVHQLRLIMELIGTPSEAEMEFLN-ENAKR
SIPK	236:
Ntf4	236:L.....
MMK1	231:L.....DDLG.....
AtMPK6	239:L.....E.L.....
WIPK	219:N.....G.K.....I..LT..L...T..DLG..QN.D...
peptide 6 p47-1:FPHVNPA		
StMPK1	298:	YIRQLPLYRRQSFTTEKFPHVNPAAIDLVEKMLTFDPRRRITVEDALAHPYLASLHDISDE
SIPK	295:H.T.....G.....N.....
Ntf4	295:V.....T.....
MMK1	290:P.....Q.....H.E.....K.....T.....
AtMPK6	298:P.P...I.D...T.H.L...I.....L.....N.....
WIPK	279:QHP..QLA.V.....L.....D.....T.....E..D....K...AG..
peptide 7 p47-1:ESIAFNP		
StMPK1	358:	PICMTPFNDFEQHALTEEQMKELIYRESIAFNPYQRM
SIPK	355:S.....L.....H. (93.9%)
Ntf4	355:	..V.....GL.....H. (93.4%)
MMK1	350:	..V.....S.....AL.....Q (87.2%)
AtMPK6	358:	..E.TI.....N...S.....AL.....Q (86.2%)
WIPK	339:	..PV..S.....QGIG...I.DM..Q.ALSL....A (68.8%)

Figure 2. Alignment of deduced amino acid sequence of StMPK1 with plant MAPKs. The amino acid sequences of SIPK, Ntf4, and WIPK from tobacco, MMK1 from alfalfa, and AtMPK6 from Arabidopsis were deduced from cDNA sequences. Dots represent amino acid residues that match StMPK1, and dashes indicate gaps introduced to maximize alignment. The conserved TxY phosphorylation motif for MAPKK is underlined. Numbers within parentheses indicate percentage of identity to the StMPK1.

is also not associated with an increase in transcript and protein upon elicitation (Zhang et al., 1998). These results suggest that StMPK1 is a potato ortholog of SIPK. Yang et al. (2001) showed that NtMEK2^{DD}, a constitutively active NtMEK2, phosphorylated and activated SIPK. In a previous study, we cloned StMEK1, a potato ortholog of tobacco NtMEK2, and generated a constitutively active form (Katou et al., 2003). Thus, we tested whether StMEK1^{DD} activates StMPK1.

Recombinant StMPK1 and StMEK1^{DD} proteins were expressed in *Escherichia coli* and purified by

nickel-affinity column chromatography. SDS-PAGE analysis followed by Coomassie staining of purified fractions showed that they were homogeneous (Fig. 4A). Phosphorylation of StMPK1 by StMEK1^{DD} enhanced the activity of StMPK1 toward MBP about 50- to 100-fold (Fig. 4B). The specific activity of activated StMPK1 toward MBP was 400 to 600 nmol ATP min⁻¹ mg⁻¹. This value is comparable with that of SIPK purified from salicylic acid-treated tobacco suspension cultured cells (Zhang and Klessig, 1997).

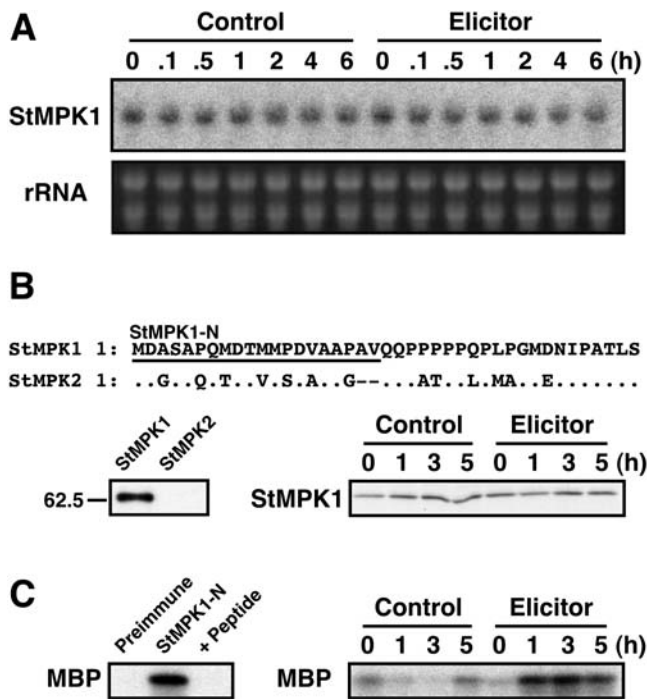


Figure 3. Posttranslational activation of StMPK1. **A**, The transcript level of *StMPK1* gene remained constant during activation by HWC elicitor. Ten micrograms of total RNA were separated on a 1.2% formaldehyde-agarose gel, transferred to a Hybond-N⁺ nylon membrane, and hybridized with 3'-UTR of *StMPK1* cDNA. The rRNA band stained with acridine orange is shown to verify that similar amount of RNA was loaded per lane. **B**, The StMPK1 protein level remains unchanged during activation by HWC elicitor. Ten nanograms each of recombinant StMPK1 and StMPK2 (left) or 20 μ g each of protein extract from potato tubers (right) were separated on a 10% SDS-polyacrylamide gel. After transfer to nitrocellulose membrane, StMPK1 was detected with anti-StMPK1-N antibody. **C**, Activation of StMPK1 by HWC elicitor. Protein extracts (400 μ g) from the elicitor-treated potato tubers were immunoprecipitated with preimmune serum or anti-StMPK1-N antibody in the absence or presence of excess amount of StMPK1-N peptide. Kinase activity of immune complex was assayed using MBP as a substrate as described in "Materials and Methods" (left). Protein extracts (400 μ g) from elicitor- or buffer-treated potato tubers were immunoprecipitated with anti-StMPK1-N antibody, and kinase activity of immune complex was assayed using MBP as a substrate (right).

Identification of Proteins Phosphorylated by StMPK1 in Vitro

To identify the possible substrates of StMPK1, we screened proteins phosphorylated by StMPK1 in vitro using IVEC. The cDNA library, constructed from poly(A)⁺ RNAs of potato tubers treated with hyphal wall elicitor for 4 h (Yoshioka et al., 2001), were excised directly in vivo into pBluescript SK(-) phagemid according to the manufacturer's instructions (Stratagene). The cDNA pools containing 30 cDNAs of each were transcribed and translated in vitro in the presence of [³⁵S]-Met. The labeled proteins were divided into two aliquots and incubated with either buffer only or with active StMPK1. Then, the reaction mixture was analyzed by SDS-PAGE followed by autoradiography.

Candidate proteins phosphorylated by StMPK1 were seen as the bands of decreased mobility on SDS-PAGE. From 111 cDNA pools screened, we obtained eight bands, which showed decreased mobility after incubation with the active StMPK1. We designated these clones as PPS. The cDNA pools, in which positive bands were recognized, were progressively subdivided into smaller pools, and a single cDNA encoding a protein with the above-noted decreased mobility was isolated. Their deduced amino acid sequences showed similarity to proteins containing a ZIM domain, pre-mRNA splicing factor, a putative transcription factor with a MYB domain and coiled-coil domain, a short-chain dehydrogenase/reductase, a TATA-binding protein-associated factor, a WRKY-type transcription factor, and unknown proteins (Table II). Here, we describe the isolation and characterization of PPS3 because the silencing of its *N. benthamiana* homolog delayed the constitutively active MAPKK-induced cell death (see below).

Incubation of ³⁵S-labeled protein transcribed and translated from PPS3 cDNA with the active StMPK1 resulted in the decreased mobility of protein band (Fig. 5A). Decreased mobility of the PPS3 is slight, suggesting that only few sites are phosphorylated by StMPK1. The Ser or Thr followed by Pro (S/TP) is a minimal consensus motif for MAPK phosphorylation (Sharrocks et al., 2000). Deduced amino acid sequence analysis of the PPS3 cDNA revealed 13 potential MAPK phosphorylation sites (Fig. 5C). To verify that PPS3 is indeed phosphorylated by StMPK1 in vitro, recombinant PPS3, expressed in *E. coli*, was incubated with active StMPK1 in the presence of [γ -³²P]ATP. As shown in Figure 5B, the thioredoxin (Trx)-fused PPS3 was highly phosphorylated by StMPK1 compared with Trx only. PPS3 was predicted to encode a 390-amino acid protein with a calculated molecular mass of 41.1 kD

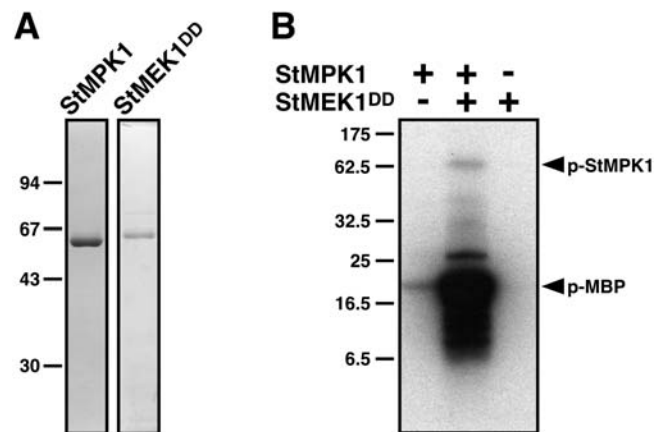


Figure 4. Activation of StMPK1 by StMEK1^{DD} in vitro. **A**, SDS-PAGE analysis of purified StMPK1 and StMEK1^{DD}. Recombinant StMPK1 and StMEK1^{DD} were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. **B**, Activation of StMPK1 by StMEK1^{DD}. Purified StMPK1 was incubated in the presence (+) or absence (-) of StMEK1^{DD} as described in "Materials and Methods." Activity of StMPK1 was then assayed using MBP as a substrate.

Table II. PPS clones obtained by IVEC

PPS	SP/TP ^a	Homologous to	GenBank Accession No.
1	4/2	Hypothetical protein	AB192878
2	9/5	Pre-mRNA splicing factor	AB192879
3	10/3	Hypothetical protein	AB111942
4	3/4	MYB-CC-type transfactor	AB192880
5	6/0	Hypothetical protein	AB192881
6	2/1	Short-chain dehydrogenase/reductase	AB192882
7	7/2	TATA-binding protein-associated factor	AB192883
8	6/1	Double WRKY-type transfactor	AB192884

^aThe number of potential MAPK phosphorylation sequence: SP or TP.

(Fig. 5C). A BLASTP search revealed that PPS3 contains a putative ZIM motif, which is found in a variety of plant transcription factors that include TIFF/YxG sequences (Fig. 5C), and may be involved in DNA binding (Nishii et al., 2000). An Arabidopsis hypothetical protein At3g17860 with 31% identity and 41% similarity to PPS3 also has TIFF/YxG sequences (Fig. 5C). RNA gel-blot analysis showed that the mRNA levels of *PPS3* remained constant after elicitation by the hyphal wall elicitor (Fig. 5D). Genomic Southern-blot analysis indicated that *PPS3* is single copy in the potato genome (data not shown).

VIGS of *NbPPS3* Compromised *StMEK1^{DD}*-Induced Cell Death

Recently, we reported that the expression of *StMEK1^{DD}* induced HR-like cell death in *N. benthamiana* (Katou et al., 2003). We examined the effect of VIGS of *NbPPS3* on *StMEK1^{DD}*-induced cell death in *N. benthamiana*. A 353-bp cDNA fragment of *NbPPS3* was inserted into pGR106 (*Potato virus X* [PVX]:*NbPPS3*). *N. benthamiana* seedlings were infected with PVX or PVX:*NbPPS3* by agroinfiltration. *N. benthamiana* infected with PVX:*NbPPS3* showed a normal developmental appearance compared with plants infected with PVX (data not shown). Three to 4 weeks after infection, the upper leaves of infected plants were infiltrated with *Agrobacterium tumefaciens* carrying *StMEK1^{DD}*. As previously reported (Katou et al., 2003), the area of leaf infiltrated with *A. tumefaciens* carrying *StMEK1^{DD}* was collapsed within 60 h in the PVX-infected plants, whereas no cell death was observed in the plants infected with PVX:*NbPPS3* (Fig. 6A). This result indicates that *NbPPS3* is required for constitutively active MAPKK-induced cell death. However, a delayed *StMEK1^{DD}*-induced cell death also appeared in the leaves of PVX:*NbPPS3*-infected plants 24 to 48 h later. PPS3 encodes a potential transcription factor as described above. To rule out the possibility that silencing of *NbPPS3* interfered with *StMEK1^{DD}* transgene expression by agroinfiltration, we performed reverse transcription-PCR and confirmed that VIGS

of *NbPPS3* did not reduce the accumulation of *StMEK1^{DD}* transcripts (data not shown).

The reduction of *NbPPS3* mRNA was confirmed by RNA gel-blot analysis in triplicate samples (Fig. 6B). The other seven PPSs were also silenced by VIGS, but HR-like cell death caused by *StMEK1^{DD}* was not compromised (data not shown).

VIGS of *SIPK/WIPK* Compromised Mobility Shift of *NbPPS3* on SDS-Polyacrylamide Gel

A retardation of mobility on SDS-PAGE gel was observed when the PPS3 was phosphorylated by *StMPK1* in vitro (Fig. 5A). To investigate the phosphorylation of PPS3 by *StMPK1* in planta, we examined whether transient expression of *StMEK1^{DD}* induced a mobility shift of *NbPPS3* in *N. benthamiana*. We cloned a cDNA fragment of a *N. benthamiana* homolog of PPS3 (*NbPPS3*), of which the deduced amino acid sequence shows 78% identity and 83% similarity to PPS3 (Fig. 5C). The calculated molecular mass and pI of *NbPPS3* are 40.3 kD and 9.28, respectively. Immunoblot analysis using anti-*NbPPS3* antibody, which was raised against peptide corresponding from 111th to 129th amino acids of *NbPPS3* (Fig. 5C), revealed that the mobility of *NbPPS3* protein on a SDS-polyacrylamide gel was retarded by the transient expression of *StMEK1^{DD}* (Fig. 7A). The mobility shift of *NbPPS3* was accompanied by the activation of 48- and 44-kD kinases (Fig. 7B), which have been confirmed to be *SIPK* and *WIPK*, respectively, by immunocomplex kinase assays (Katou et al., 2003), suggesting that *NbPPS3* may be phosphorylated by either *WIPK* or *SIPK*.

Previous studies indicated that agroinfiltration alone scarcely induced *WIPK* and *SIPK* activities (Katou et al., 2003), suggesting that the mobility shift was caused by *StMEK1^{DD}* activity. To confirm that the mobility shift of *NbPPS3* resulted from phosphorylation by *WIPK* or *SIPK*, we investigated the effect of VIGS of *WIPK*, *SIPK*, and *NbMEK2* on the *StMEK1^{DD}*-induced mobility shift of *NbPPS3*. Complementary DNA fragments corresponding to *WIPK*, *SIPK*, and *NbMEK2* were isolated from *N. benthamiana* and cloned into PVX binary vector pGR106 (PVX:*WIPK*, PVX:*SIPK*, PVX:*WIPK/SIPK*, or PVX:*NbMEK2*). The mobility shift of *NbPPS3* in PVX control-inoculated leaves was induced by expression of *StMEK1^{DD}* (Fig. 7A). VIGS of *WIPK* or *SIPK* specifically reduced the corresponding activity of these MAPK activities (Fig. 7B, top section), however, did not compromise the mobility shift of *NbPPS3* (Fig. 7A). On the other hand, double silencing of *WIPK/SIPK* reduced both MAPK activities and compromised the mobility shift of *NbPPS3*, indicating that *NbPPS3* may be phosphorylated by both *WIPK* and *SIPK* in vivo. However, the faint band was observed in the lane of double silencing (Fig. 7A) probably because of incomplete silencing of both kinases (Fig. 7B). To further confirm the possibility, *StMEK1^{DD}* itself in addition to *NbMEK2* were

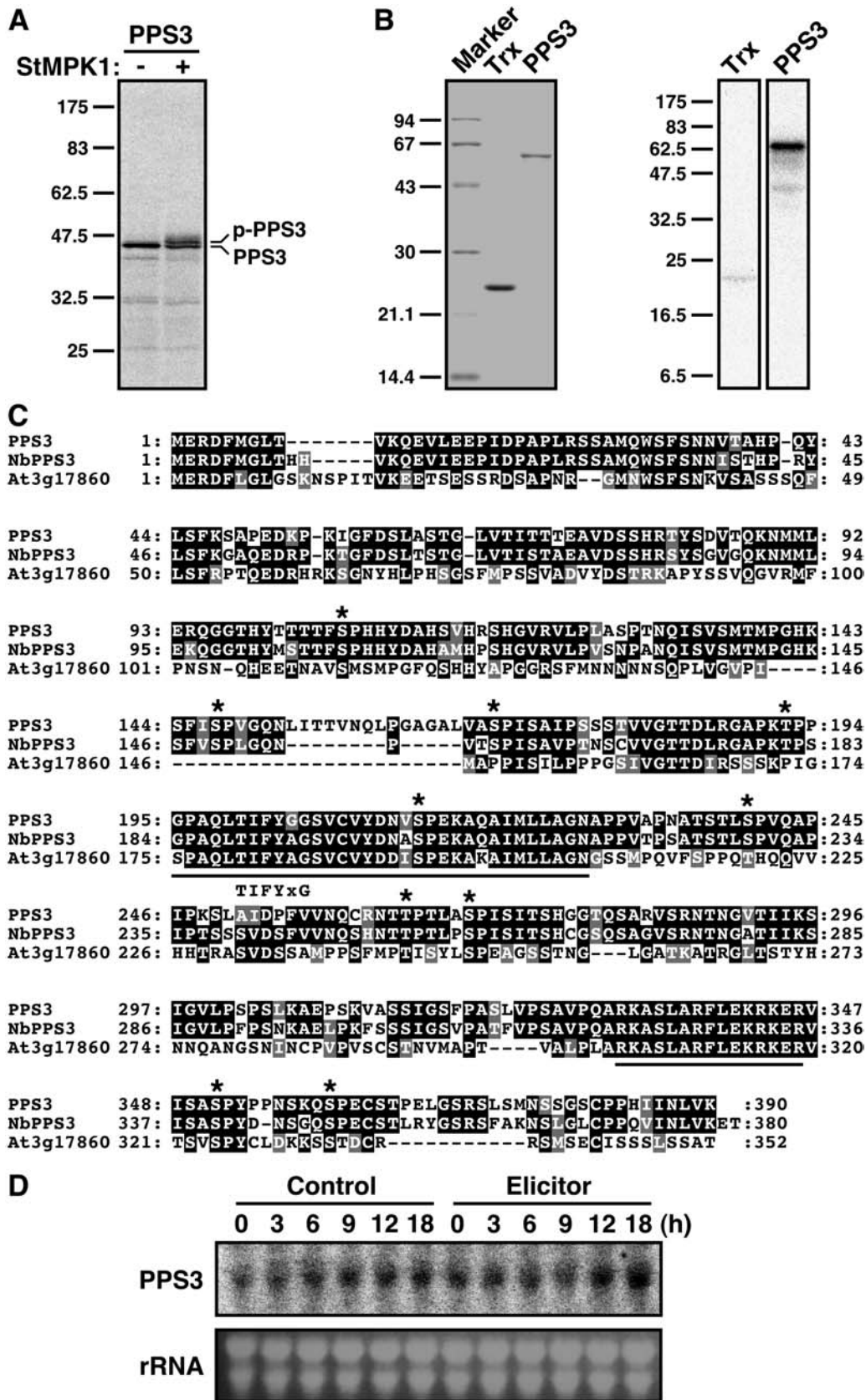


Figure 5. PPS3 is phosphorylated by StMPK1. A, The cDNA for PPS3 was subjected to in vitro transcription/translation coupled reaction in the presence of [³⁵S]-Met. Translated products were incubated with (+) or without (–) StMPK1. Reaction mixtures

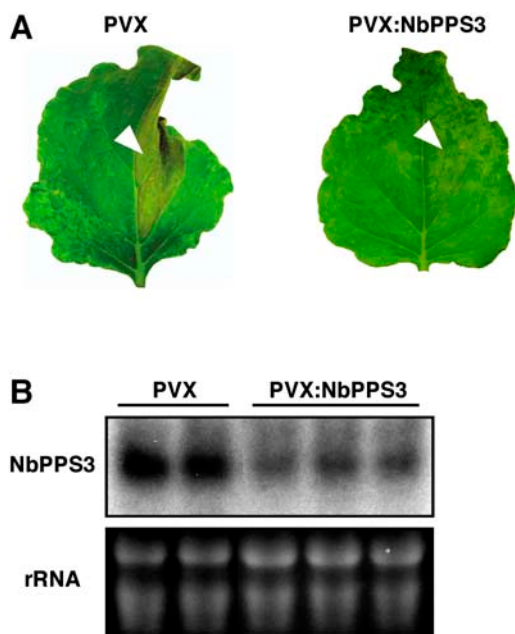


Figure 6. VIGS of *NbPPS3* delays *StMEK1^{DD}*-induced HR-like cell death. **A**, *N. benthamiana* seedlings (3 weeks old) were infected with PVX or PVX:*NbPPS3* by agroinfiltration. After 3 to 4 weeks, the upper leaves of infected plants were infiltrated with *A. tumefaciens* carrying *StMEK1^{DD}* (OD₆₀₀ of 0.5). Photographs were taken 60 h after infiltration of *A. tumefaciens*. **B**, The reduction of *NbPPS3* transcript by VIGS. Total RNAs were extracted from *N. benthamiana* leaves infected with PVX or PVX:*NbPPS3*. Ten micrograms of total RNA were separated on a 1.2% formaldehyde-agarose gel, transferred to a Hybond-N⁺ nylon membrane, and hybridized with the 3' part of *NbPPS3* cDNA that does not overlap with the nucleotide sequences used for VIGS. The rRNA band stained with acridine orange is shown to verify that similar amount of RNA was loaded per lane.

silenced by infection with PVX:*NbMEK2*. The *NbMEK2* cDNA fragment used for VIGS showed 90% nucleotide identity to the corresponding region of *StMEK1^{DD}*. The reduction of mRNA for *StMEK1^{DD}* and *NbMEK2* was confirmed by reverse transcription-PCR (data not shown). VIGS of *NbMEK2* drastically reduced the mobility shift of *NbPPS3* as well as WIPK and SIPK activities (Fig. 7, A and B). This result supports the idea that both SIPK and WIPK phosphorylate *NbPPS3*, and rules out the possible side effects of agroinfiltration and PVX infection on mobil-

ity shift of *NbPPS3*. Moreover, we conducted an in-gel kinase assay using recombinant *NbPPS3* protein as a substrate (Fig. 7B, bottom section). The phosphorylation profile of the in-gel kinase assay is almost identical to the pattern of kinase activities obtained by using MBP as a substrate (Fig. 7B, top section), indicating that WIPK and SIPK also phosphorylate *NbPPS3*. In-gel kinase assay detected only weak *NbWIPK* activity. A likely explanation for this is that basal levels of SIPK are much higher than those of WIPK (Zhang and Klessig, 1998). Alternatively, WIPK proteins may be unstable under denaturing conditions in SDS-polyacrylamide gel.

VIGS of *NbPPS3* Compromised HWC-Induced HR Cell Death

To examine the effect of VIGS of *NbPPS3* on HWC-induced cell death, *N. benthamiana* seedlings were infected with PVX or PVX:*NbPPS3* by agroinfiltration in the same way as in Figure 6. The area of leaf infiltrated with HWC elicitor was collapsed within 3 d in the PVX-infected plants, whereas no cell death was observed in the plants infected with PVX:*NbPPS3* (Fig. 8). This result indicates that *NbPPS3* is involved in HWC-induced HR cell death.

DISCUSSION

IVEC Is Useful Tool to Identify the Substrate of Protein Kinase

MAPKs play pivotal roles in induced defense responses of plants. However, the regulatory mechanisms by which MAPKs induce defense responses are unclear. To obtain candidate substrates of *StMPK1*, we employed IVEC, which has been successfully used for identification of substrate proteins in *Xenopus* and human (Cryns et al., 1997; Kothakota et al., 1997; Stukenberg et al., 1997; McGarry and Kirschner, 1998; Mead et al., 1998; Haushalter et al., 1999). Here we identified eight candidate substrates of *StMPK1*, showing that IVEC is an efficient tool to obtain candidate substrates of protein kinases in plants. IVEC has several advantages over other methods, such as yeast two-hybrid screening, peptide library

Figure 5. (Continued.)

were separated on a 12% SDS-polyacrylamide gel and exposed to x-ray film. **B**, PPS3 is phosphorylated by *StMPK1* in vitro. Purified Trx and Trx-fused PPS3 (PPS3) were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Blue (left). Purified Trx and PPS3 were used as substrates for *StMPK1*. Phosphorylation of PPS3 by *StMPK1* was visualized by autoradiography (right). **C**, Alignment of deduced amino acid sequence of PPS3 with its homologs. The amino acid sequence of PPS3 and its homologs from *N. benthamiana* and Arabidopsis were deduced from cDNA sequences. Amino acid residues identical to or similar to PPS3 are in black boxes or in gray boxes, respectively. Dashes indicate gaps introduced to maximize alignment. The potential MAPK phosphorylation sites (SP and TP), which are conserved in PPS3 and *NbPPS3*, are indicated with asterisks. The putative ZIM motif, which contains TIFF/YxG and a basic amino acid-rich region, are underlined. **D**, The accumulation of *PPS3* mRNA in hyphal wall elicitor-treated potato tubers. Ten micrograms of total RNA were separated on a 1.2% formaldehyde-agarose gel, transferred to a Hybond-N⁺ nylon membrane, and hybridized with cDNA for *PPS3*. The rRNA band stained with acridine orange is shown to verify that similar amount of RNA was loaded per lane.

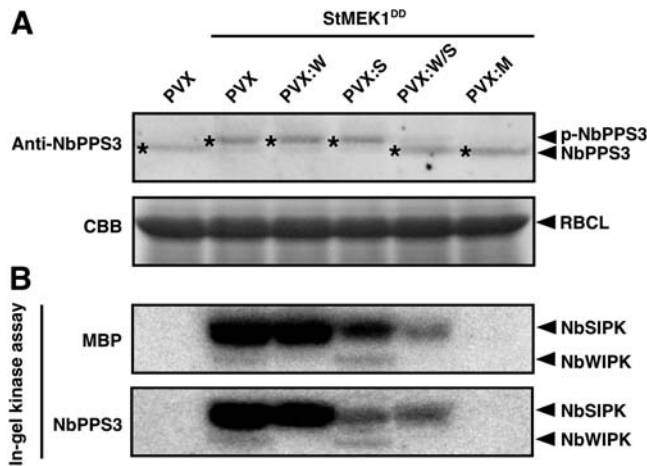


Figure 7. Mobility shift of NbPPS3 on SDS gel caused by StMEK1^{DD} is compromised by VIGS of MAPKs and MAPKK. *A*, *N. benthamiana* seedlings (3 weeks old) were infected with PVX, PVX:WIPK (PVX:W), PVX:SIPK (PVX:S), PVX:WIPK/SIPK (PVX:W/S), or PVX:NbMEK2 (PVX:M) by agroinfiltration. After 3 to 4 weeks, the upper leaves of infected plants were infiltrated with *A. tumefaciens* carrying StMEK1^{DD} (OD₆₀₀ of 0.5). Soluble protein fractions of *N. benthamiana* leaves 48 h after the agroinfiltration were analyzed by immunoblotting with anti-NbPPS3 antibody. Phosphorylated (p-NbPPS3) and nonphosphorylated NbPPS3 (NbPPS3) are shown with arrowheads. The asterisks indicate NbPPS3-specific bands. The same samples were stained with Coomassie Brilliant Blue (CBB), and the bands corresponding to ribulose-1,5-bisphosphate carboxylase large subunit (RBCL) are shown. *B*, In-gel kinase assays of the protein fractions used in immunoblot analysis were carried out using MBP and NbPPS3 as substrates.

screening, and in vivo labeling for substrate identification. IVEC does not require special equipment and identifies substrate proteins directly, whereas two-hybrid screening detects the artifactual interactions as well as interactions other than enzyme-substrate relationships (e.g. regulatory proteins or subunits, anchor proteins, and modification enzymes). Moreover, the affinity of protein-protein interaction between a protein kinase and its substrates may not be sensitive enough to be detected by the two-hybrid screening, since in principle a protein kinase can interact with a substrate in a hit-and-run manner via a short-lived intermediate complex. One limitation of this technology is its reliance on the alternation of electrophoretic mobility to detect a substrate. Kinase substrates whose mobility does not significantly shift upon phosphorylation will not be detected by this method. In vivo-labeling method will help to identify such a protein (Peck et al., 2001).

Both SIPK and WIPK Phosphorylate PPS3 Protein

Among eight candidates identified by IVEC, we focused on PPS3 because its silencing in *N. benthamiana* significantly delayed StMEK1^{DD}-induced HR-like cell death (Fig. 6). The S/TP is minimal consensus motif for MAPK phosphorylation. PPS3 and its *N. benthamiana* homolog NbPPS3 have more than 10

potential MAPK phosphorylation sequences (Fig. 5C), and recombinant protein of PPS3 is phosphorylated by StMPK1 in vitro (Fig. 5B). The mobility shift of NbPPS3 was also induced by transient expression of StMEK1^{DD} in vivo (Fig. 7A). Interestingly, the mobility shift of NbPPS3 was not compromised by VIGS of WIPK or SIPK alone, but drastically reduced by the silencing of WIPK/SIPK or NbMEK2 (Fig. 7). SIPK and WIPK share same upstream MAPKK NtMEK2 (Yang et al., 2001). A recent study using RNA interference revealed that the suppression of SIPK leads to activation of WIPK and higher sensitivity to ozone, similar to the overexpression of SIPK (Samuel and Ellis, 2002). They speculated that loss of SIPK activity in the stable SIPK-RNA interference plants was complemented by WIPK activity, leading to the ozone-induced oxidative burst and cell death. These results suggest that WIPK and SIPK have a redundant function and both phosphorylate NbPPS3 in vivo. However, the phosphorylation sites of the NbPPS3 protein are currently unknown. It is important to identify the phosphorylation sites to investigate the function of PPS3.

Several docking domains including δ domain in c-Jun and LxL motif neighbored with basic and hydrophobic regions in many transcription factors have been identified in mammalian MAPK substrates (Sharrocks et al., 2000). However, we found no such domains in the deduced amino acid sequence of PPS3. There are also many substrate proteins with no obvious docking domains (Sharrocks et al., 2000). It is therefore likely that novel docking domains exist for the MAPKs. Multiple alignment analysis of PPS3 and its homologs revealed the conservation of the ZIM motif (Fig. 5C). The ZIM motif is found in a variety of GATA-type plant transcription factors and is suggested to be involved in DNA binding (Nishii et al., 2000). Main targets of mammalian MAPKs are transcription factors (Davis, 1993; Sharrocks et al., 2000; Kyriakis and Avruch, 2001). It was reported that activation of plant MAPK cascades also resulted in induction of many genes (Yang et al., 2001; Asai et al., 2002; Katou et al., 2003; Ouaked et al., 2003; Kim and Zhang, 2004). Thus, plant MAPKs may target proteins involved in transcriptional regulation, similar to mammalian system.



Figure 8. VIGS of NbPPS3 compromised HWC-induced HR cell death. *N. benthamiana* seedlings (3 weeks old) were infected with PVX or PVX:NbPPS3 by agroinfiltration. After 3 to 4 weeks, the upper leaves of infected plants were infiltrated with HWC elicitor from *P. infestans* (1 mg mL⁻¹). Photographs were taken 3 d after treatment with HWC.

PPS3 Is Involved in StMEK1^{DD}- and HWC-Induced Cell Death

We assessed the function of *PPS* genes by employing VIGS in *N. benthamiana* and found that VIGS of *NbPPS3* significantly delayed HWC-mediated HR cell death as well as StMEK1^{DD}-induced HR-like cell death (Figs. 6 and 8). It was reported that SIPK is activated in tobacco leaves by HWC elicitor (Zhang and Klessig, 1998). Here we showed that HWC elicitor induces long-lasting StMPK1 activity in potato tissues using anti-StMPK1-N antibody (Fig. 3C). These results suggest that PPS3 is one of the downstream components of StMPK1-mediated signaling pathway leading to HR cell death. However, StMEK1^{DD}- and HWC-induced cell death never disappeared completely; it was delayed by 1 or 2 d in *NbPPS3*-silenced leaves. There are several possible explanations for the incomplete loss of cell death. First, it is well known that VIGS does not silence target genes completely (Ruiz et al., 1998). The reduction of *NbPPS3* mRNA is partial (Fig. 6B). The remainder of *NbPPS3* transcripts may be sufficient for the induction of cell death. Second, PPS3 is one of the components that transduce signals leading to HR-like cell death. In animal cells, activated MAPK phosphorylates numerous substrate proteins. In budding yeast, Hog1 MAPK is essential for the survival of yeast in high osmolarity environments (Boguslawski, 1992; Brewster et al., 1993). Hog1 phosphorylates and activates Rck2 protein kinase (Bilsland-Marchesan et al., 2000). However, *rck2* deletion mutant showed no increased osmosensitivity compared to the wild type, suggesting the presence of multiple redundant downstream targets for the Hog1 MAPK (Bilsland-Marchesan et al., 2000). Third, PPS3 is not essential for induction of cell death, but it may assist or enhance cell death. HR-assisting protein was purified from sweet pepper (*Capsicum annuum* cv ECW) as a factor to intensify harpin_{PSS}-induced HR in harpin_{PSS}-insensitive plants (Chen et al., 1998). Transgenic tobacco plants overexpressing HR-assisting protein under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter did not show constitutive HR, but exhibited high sensitivity to harpin_{PSS}-induced HR. We also found that the transient expression of *NbPPS3* under the control of CaMV 35S promoter by agro-infiltration did not induce cell death in *N. benthamiana* (data not shown). How the PPS3 regulates HR-like cell death presently is unknown, but the target genes of PPS3 are an obvious option that is under investigation.

MATERIALS AND METHODS

Plant Materials, Elicitors, and Treatment Protocol

Tubers of the potato cultivar Irish cobbler (*Solanum tuberosum*) were harvested at Nagoya University farm and stored at 4°C in the dark until use. *Nicotiana benthamiana* were grown in environmentally controlled growth cabinets under a 16-h photoperiod and an 8-h dark period at 25°C. HWC elicitor was isolated from cultured mycelia of *Phytophthora infestans* according to a method previously described (Doke and Tomiyama, 1980). Potato tuber

discs (20 mm in diameter, 2 mm thick) were incubated on a stainless steel mesh in a sealed plastic chamber at 20°C in the dark. After an 18-h aging period, potato tuber discs were treated with 100 µL of HWC elicitor (0.5 mg mL⁻¹) in 10 mM Tris-HCl, pH 7.4, or buffer only as a control.

In-Gel Kinase Assay

In-gel kinase assays were performed as described previously (Katou et al., 1999).

Purification of p51-PK

Purification of p51-PK was performed by monitoring its activity with an in-gel kinase assay using MBP as substrate. Twelve hundred grams of potato tuber slices (2 mm thick) treated with HWC elicitor for 3 h were ground for 2 min with a mixer in 1.2 L of homogenization buffer (50 mM MOPS-KOH, pH 7.6, 0.5 M sorbitol, 5 mM EDTA, 5 mM EGTA, 0.1 M NaF, 100 µM Na₃VO₄, 10 µM [p-amidinophenyl] methanesulfonyl fluoride, 50 mM 2-mercaptoethanol, and 5% [w/v] Polyclar VT). The homogenate was filtered through two layers of nylon gauze, and Dowex 1X-2 (100–200 mesh, Cl⁻ form, Dow Chemical) was added to a final volume of 7% (w/v). The homogenate was stirred for 15 min and filtered to remove Dowex. The homogenate was centrifuged at 14,000g for 30 min, and the resulting supernatant fraction was centrifuged at 275,000g for 30 min. The soluble supernatant fraction was applied to a Q-Sepharose high-performance column (25 mL, Pharmacia) equilibrated with buffer A (20 mM MOPS-KOH, pH 7.6, 50 mM NaF, 100 µM Na₃VO₄, and 7 mM 2-mercaptoethanol). Kinase activities were then eluted with 0.25 M NaCl in buffer A. Active fractions were pooled and (NH₄)₂SO₄ was added to a concentration of 0.8 M, and loaded on a Resource phenyl column (6 mL, Pharmacia) equilibrated with 0.8 M (NH₄)₂SO₄ in buffer A. Kinase activities were eluted with a 30-mL linear gradient of 0.8 to 0 M (NH₄)₂SO₄ in buffer A. Active fractions were pooled, concentrated using concentrator (Centriplus YM10, Millipore), and applied to a Superdex 75 prep grade column (120 mL, Pharmacia) equilibrated with buffer A containing 0.2 M NaCl. Kinase activities were eluted with the same buffer. Active fractions were concentrated as above, and the concentrate was diluted 10-fold to reduce salt concentration. They were then applied to a Mono Q column (1 mL, Pharmacia), and kinase activities were eluted with a 30-mL linear gradient of 0.1 to 0.3 M NaCl in buffer A. Active fractions were concentrated and desalted using concentrator (Centriplus YM10, Millipore), and Triton X-100 was added to a final concentration of 0.5%. The pooled fractions were then applied to a PY20-Sepharose column (1 mL; prepared by coupling PY20 anti-phosphotyrosine monoclonal antibody to *N*-hydroxysuccinimide-activated Sepharose, Pharmacia) equilibrated with 0.5% Triton X-100 in buffer B (buffer A without 2-mercaptoethanol). The column was washed with 5 mL of 0.5% Triton X-100 in buffer B and 2 mL of buffer B, respectively, and the kinase activities were eluted with 10 mM phosphotyrosine in buffer B.

Estimation of Molecular Mass by Gel Filtration

Active fractions from the Mono Q column were concentrated using a concentrator (Centriplus YM10, Millipore), mixed with molecular mass standards (Pharmacia), and then applied to Superdex 75 prep grade column equilibrated with buffer A containing 0.2 M NaCl. The column was calibrated with the following markers: Blue Dextran (2,000 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), chymotrypsinogen A (25 kD), and ribonuclease (13.7 kD).

Amino Acid Sequencing Analysis

Protein sequencing was performed as described previously (Hellman et al., 1995). The purified p51-PK and p46-PK were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. The Coomassie-stained bands were in-gel digested with trypsin to obtain polypeptide fragments. The fragments were separated by µRPC C2/C18 SC2.1/10 column using the Smart system (Pharmacia). The amino-terminal sequences of the fragments were determined (model 476A; Applied Biosystems).

cDNA Construction and Screening

A cDNA library constructed from poly(A)⁺ RNAs of potato tubers treated with HWC elicitor for 4 h (Yoshioka et al., 2001) was directly amplified by PCR

using degenerated primers 5'-CAYATGGAYCAYGARAAYATHGT-3' and 5'-ACRTAYTCNGTCATRAARTNGTYTC-3' (where H is A, T and G; N is A, T, C, and G; R is A and G; and Y is T and C), which correspond to peptides 1 and 4 (Fig. 3), respectively. The 330-bp PCR product was labeled with [α - 32 P]dCTP and used to screen a potato cDNA library under high stringency. From 8×10^5 plaque-forming units, we isolated 13 positive clones. Positive phage plaques were excised directly in vivo into pBluescript SK(-) phagemid according to the manufacturer's instructions (Stratagene). Nucleotide sequences were determined for both strands with ABI Prism BigDye Terminator cycle sequencing kits and a DNA sequencing system (model 3100; Applied Biosystems). The nucleotide and the deduced amino acid sequence were analyzed with DNA analytical software (DNASIS; Hitachi Software). The alignment of amino acid sequences was made using ClustalW multiple sequence alignment program (version 1.7, June 1997; Thompson et al., 1994).

RNA Gel-Blot Analysis

Total RNA from potato tubers and *N. benthamiana* leaves was prepared as described previously (Kingston, 1987). Ten micrograms of total RNA were separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N⁺; Amersham). The probes were labeled with [α - 32 P]dCTP using a random-primed DNA labeling kit (Megaprime; Amersham). Hybridization was performed according to the manufacturer's recommendations. The probes used in this study were a 233-bp cDNA containing the 3'-untranslated region (UTR) of *StMPK1*, a 1.1-kb cDNA containing the entire *PPS3* open reading frame, and a 0.5-kb cDNA corresponding to the 3' part of *NbPPS3* that does not overlap with the nucleotide sequences used for VIGS.

Antibody Production and Immunoblot Analysis

The peptides for StMPK1-N (MDASAPQMDTMMPDVAAPAV) corresponding to the amino terminus of StMPK1 and NbPPS3-N (HYDAHMHPSHGVRVLPVS) corresponding to amino acids 111 to 129 of the predicted NbPPS3 protein were synthesized and conjugated to keyhole limpet hemocyanin carrier. Polyclonal antiserum was raised in rabbits (Sawady) and purified by antigen-affinity chromatography.

For immunoblot analyses, 10 ng each of recombinant StMPK1 and StMPK2 and 20 μ g each of soluble protein from potato tubers and *N. benthamiana* leaves were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). After blocking overnight in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20 (TBS-T) containing 5% nonfat dry milk at 4°C, the membranes were incubated with anti-StMPK1-N or anti-NbPPS3-N antibody diluted with TBS-T (0.05 μ g mL⁻¹) at room temperature for 1 h. After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham) diluted with TBS-T (2,000-fold dilution). Antibody-antigen complex was detected using an enhanced chemiluminescence kit (Amersham).

Immunocomplex Kinase Assay

Protein extract (400 μ g) was incubated with anti-StMPK1-N antibody (2 μ g) in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 10 mM β -glycerophosphate, 1 mM Na₃VO₄, and 1% Triton X-100) at 4°C for 2 h with rotary shaking. Forty microliters of 50% protein A-Sepharose was added, and incubation was continued for another 2 h. Immunocomplex was collected by brief centrifugation and washed three times with immunoprecipitation buffer and then three times with buffer C (20 mM HEPES-KOH, pH 7.6, 10 mM MgCl₂, and 1 mM dithiothreitol). Kinase activity was assayed at 25°C for 20 min in a volume of 25 μ L buffer C containing 0.25 mg mL⁻¹ MBP, 100 μ M ATP, and 8 μ Ci mL⁻¹ [γ - 32 P]ATP. The reaction was terminated by the addition of SDS-sample buffer. After electrophoresis on a 15% SDS-polyacrylamide gel, the phosphorylated MBP was detected by autoradiography.

Production of Recombinant Proteins in *Escherichia coli*

An MscI site for StMPK1 and StMPK2, a NcoI site for StMEK1^{DD}, a BamHI site for PPS3, and an EcoRI site for NbPPS3 were introduced in front of the ATG start codons by PCR. Each of the amplified products except for NbPPS3 were ligated in frame into pET-32a(+) vector (Novagen); NbPPS3 was ligated in frame into pET-30a(+) vector (Novagen). The plasmids were transformed

into *E. coli* strain BL21 (DE3) pLysS. Induction of fusion proteins with 1 mM isopropyl- β -D-thiogalactoside and disruption of *E. coli* cells were performed according to the manufacturer's recommendations. StMPK1, StMPK2, and StMEK1^{DD} were soluble and purified with nickel-charged chelating column (1 mL, Pharmacia). PPS3 and NbPPS3 were solubilized with urea from inclusion bodies and refolded by sequential dilutions of urea by dialysis. StMPK1 and StMPK2 were tagged with Trx and His-tag, and StMEK1^{DD} and PPS3 were tagged with Trx, S-tag, and His-tag.

In Vitro Activation of StMPK1 by StMEK1^{DD}

In vitro activation of StMPK1 by StMEK1^{DD} was performed in buffer D (20 mM HEPES-KOH, pH 7.6, 10 mM MgCl₂, 1 mM dithiothreitol, 100 μ M Na₃VO₄, 5 mM NaF, 5 mM β -glycerophosphate, and 500 μ M ATP) containing 0.5 mg mL⁻¹ StMPK1 and 0.125 mg mL⁻¹ StMEK1^{DD} at 30°C for 30 min. To remove StMEK1^{DD}, S-protein-agarose (Novagen) equilibrated with buffer D was added. After 30 min incubation at room temperature with gentle shaking, agarose-protein complex was removed by brief centrifugation. The resulting supernatant containing active StMPK1 was concentrated using Microcon YM10 (Millipore) and stored at -80°C in buffer D containing 50% glycerol.

In Vitro Phosphorylation of PPS3 by StMPK1

Purified Trx-fused PPS3 or Trx only were incubated with 10 μ g mL⁻¹ StMPK1 in buffer C containing 1 mM EGTA, 50 μ M ATP, and 50 μ Ci mL⁻¹ [γ - 32 P]ATP at 30°C for 20 min. The reaction was terminated by the addition of SDS-sample buffer. After electrophoresis on a 12% SDS-polyacrylamide gel, the phosphorylated proteins were detected by autoradiography.

Identification of PPS Clones by IVEC

IVEC was performed according to Gao et al. (2000). The cDNA library, constructed from poly(A)⁺ RNAs of potato tubers treated with HWC elicitor for 4 h (Yoshioka et al., 2001), were excised directly in vivo into pBluescript SK(-) phagemid according to the manufacturer's instructions (Stratagene). The colony pools, each containing 30 independent colonies, were prepared, and plasmid DNAs were extracted from each pool. The ³⁵S-labeled protein pools were prepared using TnT-coupled wheat germ extract system (Promega) according to the manufacturer's instructions except that the reaction volume was scaled down to 10 μ L. About 0.1 to 0.5 μ g of plasmid DNA prepared from colony pools were added per reaction. Unlabeled Met was omitted from the mixture and 10 μ Ci of [³⁵S]-Met (1,000 Ci mmol⁻¹) was added. The transcription/translation reaction was incubated at 30°C for 90 min. The ³⁵S-labeled protein pools were split and mixed with either buffer D only or buffer D containing 10 μ g mL⁻¹ active StMPK1 in a volume of 15 μ L. Samples were incubated at 30°C for 1 h, and then reactions were terminated by adding a half volume of 3 \times SDS-PAGE sample buffer and boiled for 4 min. The denatured samples were separated on a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed in methanol/acetic acid/water solution (5/1/4, v/v/v) for 1 h with changing solution twice, washed with distilled water for 30 min, and vacuum dried. Labeled proteins were detected by 2 to 3 weeks exposure on an x-ray film.

cDNA Cloning of NbPPS3

To obtain the full-length cDNA of ortholog of PPS3 from *N. benthamiana*, NbPPS3 was amplified by PCR using phage DNA prepared from an oligo(dT)-primed Uni-ZAP XR library (Stratagene), which was constructed from poly(A)⁺ RNAs of *N. benthamiana* leaves treated for 6 h with HWC elicitor of *P. infestans*. Primers were designed from sequences of NbPPS3 cDNA fragment used for VIGS and the phage vector. The 3'-flanking regions of NbPPS3 were amplified using NbPPS3 (5'-CTCACGGAGTCAGAGTGCTCCC-3') and a vector primer T7 (5'-GTAATACGACTACTATAGGGC-3'). The 5' terminus of NbPPS3 was amplified using a vector primer T3 (5'-AATTAACCTCACTAAAGGG-3') and 5'-UTR primer (5'-TCTTGATCTGTCTACATTATTTGCCACC-3'). All PCR reactions were performed using a high fidelity taq polymerase (KOD+; Toyobo). Blunt-end PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen). The overlapping portions of the sequences of these PCR products were identical.

VIGS

VIGS was performed according to Ratcliff et al. (2001). The following primers were used to amplify cDNA fragments from *N. benthamiana* using a cDNA library from *N. benthamiana* leaves as a template (Yoshioka et al., 2003). Restriction sites were added to the 5'-end of forward or backward primer for cloning into pGR106 (Lu et al., 2003). Primers for NbPPS3 amplification were as follows: NbPPS3-*Clal*I (5'-ATCGATCTCATGGTGTCA-GAGTGCTCCC-3') and NbPPS3-*Sall*I (5'-GTCGACCGTGGGTATGGGCG-CCTGAAC-3'); restriction sites are underlined, which produced a 353-bp fragment. To clone the ortholog of tobacco (*Nicotiana tabacum*) *NtMEK2* (Yang et al., 2001) from *N. benthamiana*, we performed PCR with primers for NbMEK2: NbMEK2-*Clal*I (5'-ATCGATGTCACGATATGTTTCGATCACAACG-3') and NbMEK2-*Sall*I (5'-GTCGACCAAGGATCCATAGTTTGTGCCAG-3'), which produced a 225-bp fragment. The amplified cDNA fragments were subcloned into the TA cloning site of pGEM-T Easy (Promega) and sequenced. Each cDNA fragment was subcloned into pGR106. A 230-bp fragment of NbSIPK and a 178-bp fragment of NbWIPK, each starting from the ATG codon, were subcloned into pGR106. Additionally, both fragments were ligated in tandem into pGR106 for dual silencing. The constructs containing the inserts were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *N. benthamiana* seedlings (3 weeks old) were infected with *A. tumefaciens* carrying recombinant PVX constructs. Three to 4 weeks after infection, an upper leaf of the infected plants was infiltrated with 1 mg mL⁻¹ HWC elicitor or *A. tumefaciens* carrying *StMEK1^{DD}* under the control of the constitutive 35S promoter of CaMV as described previously (Katou et al., 2003).

Accession numbers for the StMPK1, StMPK2, PPS3, and NbPPS3 are AB062138, AB062139, AB111942, and AB111943, respectively. The accession numbers for the other sequences mentioned in this article are as follows: *Arabidopsis* (*Arabidopsis thaliana*) PPS3 homolog, AY085082; SIPK, AAB58396; Ntf4, Q40532; MMK1, Q07176; AtMPK6, AAB64027; and WIPK, BAB79636.

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