Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco

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Hypersensitive response (HR), a form of programmed cell death, is frequently associated with plant disease resistance. It has been proposed that mitogen-activated protein kinase (MAPK) cascades regulate HR cell death based on pharmacological studies by using kinase inhibitors. However, direct evidence is lacking. Here, we demonstrate that NtMEK2, a MAPK kinase, is upstream of salicylic acid-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK), two tobacco MAPKs that are activated by various pathogens or pathogen-derived elicitors. Expression of a constitutively active mutant of _NtMEK2_ induces HR-like cell death in tobacco, which is preceded by the activation of endogenous SIPK and WIPK. In addition, NtMEK2-SIPK/WIPK cascade appears to control the expression of 3-hydroxy-3-methylglutaryl CoA reductase (_HMGR_) and ʟ-phenylalanine ammonia lyase (_PAL_), two defense genes encoding key enzymes in the phytoalexin and salicylic acid biosynthesis pathways. These results demonstrate that a plant MAPK cascade controls multiple defense responses against pathogen invasion.

The active defense mechanisms of plants against invading pathogens often include rapid cell death, known as the hypersensitive response (HR), the activation of a complex array of defense genes, and the production of antimicrobial phytoalexins (1, 2). Several lines of evidence suggest HR cell death during plant disease resistance is a form of programmed cell death, as it requires active transcription and translation and is genetically defined (3–5). Although the details for the regulation and execution of HR remain poorly understood, production of reactive oxygen species, ion fluxes, protein phosphorylation/dephosphorylation, and gene activation have been implicated in the process (5). In addition to these local responses, the uninfected portions of the plant usually develop systemic acquired resistance, which is manifested as enhanced resistance to a subsequent challenge by the initial or even unrelated pathogens (2).

Plant-defense responses are initiated by the recognition of pathogens, which is mediated either by a gene-for-gene interaction between a plant resistance (_R_) gene and a pathogen avirulence (_Avr_) gene or by the binding of nonrace-specific elicitors such as elicitins to a putative receptor (1, 6–9). Signals generated by such interactions are transduced into cellular responses via several interlinked pathways. Recent studies suggest that plant mitogen-activated protein kinase (MAPK) cascade is one of the converging points after the perception of different pathogens and pathogen-derived elicitors (10–15).

MAPK cascades are major pathways by which extracellular stimuli are transduced into intracellular responses in yeast and mammalian cells (16, 17). The basic assembly of MAPK cascade is a three-kinase module conserved in all eukaryotes. MAPK, the last kinase in the three-kinase cascade, is activated by dual phosphorylation of Thr and Tyr residues in a TXY motif by MAPK kinase (MAPKK). MAPKK is, in turn, activated by MAPKK kinase. In mammals, three of the five subgroups of the MAPK family, the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), the p38 kinase, and extracellular signal-regulated protein kinase 5, are activated in response to various stress signals, including UV and ionizing radiation, hyperosmolarity, oxidative stress, and cytokines (16, 17). The outcome of SAPK/JNK and/or p38 activation depends on the magnitude and duration of their activation. A transient activation of these MAPKs induces various defense responses and allows the cells to adapt to an adverse environment. By contrast, a persistent activation leads to apoptosis (16, 17).

The induction of MAPK-like activities has been associated with HR cell death in tobacco cells treated with xylanase, a fungal elicitor from _Trichoderma viride_ and _Arabidopsis_ cells treated with harpin, a bacterial elicitor from _Pseudomonas syringae_ pv. _Syringae_ (18, 19). Through the use of kinase inhibitors, we linked the activation of salicylic acid (SA)-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK), two tobacco MAPKs, to HR cell death induced by fungal elicitins (20). SIPK was first identified as SA-induced protein kinase (21). It is also activated by various biotic and abiotic stresses, much like mammalian JNK/SAPK (12, 16). WIPK, which was first cloned as a gene whose transcript is induced by wounding (22), is activated by pathogens and pathogen-derived elicitors as well (10, 11, 20). Its ortholog in parsley, _ERMK_, is also implicated in plant-defense response (13). However, the exact roles of these MAPKs remain unknown. Here, we report the identification of NtMEK2, a tobacco MAPKK. as the upstream kinase for both SIPK and WIPK based on _in vitro_ and _in vivo_ evidence. Expression of _NtMEK2_DD, a constitutively active mutant of NtMEK2, activates endogenous SIPK and WIPK and leads to HR-like cell death and the activation of a subset of defense genes, demonstrating that the NtMEK2-SIPK/WIPK cascade controls multiple defense responses during plant disease resistance.

Materials and Methods

Cloning of Tobacco MAPKKs. Two primers, 5′-YTIGARTWYAT-GGAYVRNGG-3′ and 5′-CKYTCNGGNSHCATRTA-3′ (where Y = T + C; I = deoxyinosine; R = A + G; W = A + T; V = A + C + G; N = A + T + C + G; K = T + G; S = C + G; and H = A + T + C), which correspond to the conserved MAPKK domains LEF/YMDG/Q/RG and YMS/APER, were used to PCR amplify the cDNA reverse transcribed from

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poly(A)$^+$ RNAs prepared from tobacco (*Nicotiana tabacum* cv. Xanthi nc). The reverse transcription–PCR products were cloned into pGEM-T vector (Promega). Four unique clones with homology to MAPKKs were identified, which were used to screen a tobacco ZapExpress cDNA library. Positive clones containing the longest insert were sequenced.

Preparation of Recombinant Proteins. An *Nde*I site was introduced in front of the ATG start codon of MAPKs or MAPKKs by PCR, which was then ligated in-frame into the pET-28a(+) vector (Novagen). Mutations were introduced by QuickChange site-directed mutagenesis (Stratagene) and confirmed by sequencing. BL21(DE3) cells transformed with pET-28a(+) constructs were induced with 0.5 mM isopropyl β-D-thiogalactoside for 3 h. His-tagged proteins were purified by using nickel columns (Amersham Pharmacia), desalted, and concentrated by using Centricon-10 (Millipore).

Antibody Preparation and Immunoblot Analysis. NtMEK2 antibody was raised in rabbit against purified recombinant protein corresponding to the N-terminal 156 amino acids of NtMEK2 that share the least homology with other tobacco MAPKKs (Zymed). The antibody was affinity purified before use.

For immunoblot analysis, 15 μg of total protein or 2 ng of recombinant protein per lane was separated on 10% SDS–polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were then probed with either Anti-Flag (1:5,000, Sigma) or Ab-NtMEK2 antibody (0.5 μg/ml), as previously described (10).

Preparation of Protein Extracts. Proteins were extracted from cells or leaf discs as previously described (10, 23). The concentration of protein extracts was determined by using the Bio-Rad protein assay kit with BSA as standard.

Kinase Assays. Autophosphorylation assay was performed by incubating equal amounts (0.5 μg) of purified recombinant NtMEK2 and its mutants in reaction buffer (25 mM Tris, pH 7.5/10 mM MnCl$_2$/1 mM EGTA/1 mM DTT) in the presence of 25 μM [γ-^{32}P]ATP (≈3,000 dpm/pmol) at 30°C for 30 min. The reaction was stopped by the addition of SDS loading buffer, and kinase activities were detected by autoradiography after SDS/PAGE.

Phosphorylation activities of NtMEK2 and its mutants were determined by using inactive mutant MAPKs (HisMAPK^R, 1 μg) as substrates under the same conditions as autophosphorylation assay, except 0.1 μg of HisNtMEK2 was used in each reaction.

MAPK activation assay was carried out by first incubating HisMAPKs (1 μg) with 0.1 μg of HisNtMEK2 or its mutant in the presence of 50 μM unlabeled ATP at 30°C for 15 min. Myelin basic protein (MBP, final concentration of 0.25 μg/μl) and [γ-^{32}P]ATP (1 μCi per reaction) were then added. The reactions were stopped by the addition of SDS loading buffer after 30 min. The phosphorylated MBP was visualized by autoradiography after being resolved on a 15% SDS–polyacrylamide gel.

Immune complex–kinase assays of NtMEK2, SIPK, WIPK, and Flag-tagged NtMEK2 were performed by using 75 μg of protein extract with 4 μg of Ab-NtMEK2, 2 μg of Ab-p48N, 2 μg of Ab-p44N, or 2 μg of anti-Flag (Sigma), respectively, as previously described (10). The in-gel kinase activity assay was performed as described previously (21).

Agrobacterium-Mediated Transient Transformation. Tobacco plants (*N. tabacum* cv. Xanthi nc [NN] and *N. tabacum* cv. Xanthi nc [NN]/NahG transgenic) were grown at 22°C in a growth room programmed for a 14-h light cycle. Seven to eight-week-old tobacco plants were used for experiments. Tobacco MAPKKs and their mutants with a Flag-epitope at their N termini were inserted into the *Xho*I/*Spe*I sites of pTA7002 vector (24). The 5′-untranslated region was replaced with the Ω sequence from tobacco mosaic virus. *Agrobacterium tumefaciens* LBA4404 carrying different constructs was grown overnight in AB medium (28) containing 100 μg/ml streptomycin, 50 μg/ml kanamycin, and 100 μM acetosyringone. Cells were collected by centrifugation (4,000 × g), resuspended to OD$_{600}$ of 0.8 in MS medium (pH 5.9; Life Technologies) with 100 μM acetosyringone, and infiltrated into the fully expanded leaves. The expression of transgene was induced by infiltration of dexamethasone (DEX, 30 μM) 40–48 h later. Samples for protein and RNA preparation were collected at indicated times, quick frozen in liquid N$_2$, and stored at −80°C until use.

Results

Identification of Upstream Kinase of SIPK and WIPK. Attempts to manipulate the activity of SIPK or WIPK by overexpressing these two genes in sense or antisense orientation were unsuccessful (S.Z. and D. Klessig, unpublished data). As a result, we turned our attention to their upstream kinase(s). By using a yeast two-hybrid screening, a MAPKK that specifically interacts with SIPK and is likely to be SIPK kinase was identified (25). To identify WIPK kinase, we cloned six tobacco MAPKKs that belong to four distinct groups on the basis of phylogenetic analysis (data not shown). One member from each of these four groups was studied in detail. Quite unexpectedly, our data demonstrated that a single MAPKK, NtMEK2, is the upstream kinase for both SIPK and WIPK. The deduced amino acid sequence of NtMEK2 is shown in Fig. 1. It is most closely related to *Arabidopsis* AtMEK4 of unknown function.

In yeast and animals, MAPKKs are activated through the phosphorylation of two Ser/Thr residues in a conserved S/TxxxS/T motif by MAPKK kinases (17). When these two Ser/Thr residues are replaced with Glu (E) or Asp (D), the mutant MAPKK becomes constitutively active (26, 27). To generate such a mutant of NtMEK2 for gain-of-function studies, we mutated several Ser/Thr residues between the kinase subdomains VII and VIII to D as single, double, or triple mutants. The activities of the His-tagged recombinant proteins were determined by an autophosphorylation assay. Fig. 2 shows that HisNtMEK2^{DD}, where the conserved Thr-227 and Ser-233 were replaced by Asp, has much higher kinase activity than the wild-type protein. Mutagenesis of other tobacco MAPKKs at corresponding positions also leads to increases in kinase activity (data not shown). These results suggest that plant MAPKKs have an activation motif with two Ser/Thr residues separated by five amino acids instead of the three in MAPKKs from yeast and animals (Fig. 1).

For a MAPKK to function as the upstream kinase of either SIPK or WIPK, this MAPKK should be able to phosphorylate and activate SIPK or WIPK. In addition, this MAPKK should be activated before or concurrently with the activation of SIPK or WIPK *in vivo*. To test whether NtMEK2 is able to phosphorylate SIPK or WIPK, inactive HisMAPK^R proteins with the catalytic essential Lys (K) residue substituted with an Arg (R) were used as substrates. HisMAPK^R proteins lack autophosphorylation activity (Fig. 2B, first five lanes) and are, therefore, ideal substrates for assaying MAPKK activity. As shown in Fig. 2B, HisNtMEK2^{DD} phosphorylated HisSIPK^R and HisWIPK^R, but not HisMPK6^R or HisMPK7^R, two other tobacco MAPKs, whereas HisNtMEK2^{WT} showed little activity. Phosphorylation of HisSIPK and HisWIPK by the active HisNtMEK2^{DD} enhanced their activities toward MBP (Fig. 2C). There is a 50- to 100-fold increase in the activity of NtMEK2^{DD} over that of NtMEK2^{WT}, which correlates with its ability to activate SIPK and WIPK (Fig. 2). That NtMEK2 might be the upstream kinase for both SIPK and WIPK is not too surprising, as SIPK and

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NtMEK2  (100.0%)  MRPLQPPP P-PAAAATTS SSTTASPMPP PPSRNRPRRRTDL
AtMEK4  ( 67.5%)  MRPIQSPP P-GVSV--- ---------- -PVKSRPRRRPDL
MEK1    ( 34.7%)  MPKKKPTPIQLNPAPDG SAVNGTSSAE TNLEALQKKLEEL

NtMEK2  TLPLPQRD PALAVPLPL PPTS----- ----APSSSSS SSSSPLPTPL---
AtMEK4  TLPLPQRD VSLAVPLPL PPTSGGSGGS SGSAPSSGGS ASSTNINSS IEAK
MEK1    ELDEQQRK R-LEAFLTQ KQKVG---- ---------- ----------ELK

                  I                    II  111        III
NtMEK2  NFSELERI NRIGSGAGG TVYKVLHRPT GRLYALKVIY GNHEDSVRLQMCR
AtMEK4  NYSDLVRG NRIGSGAGG TVYKVIHRPS SRLYALKVIY GNHEETVRRQICR
MEK1    D-DDFEKI SELGAGNGG VVFKVSHKPS GLVMARKLIH LEIKPAIRNQIIR
                                         •
                  IV                   V
NtMEK2  EIEIILRDV DNPNVVRCHDMFDHNGEIQ VLLEFMDKGS LEGI------HIPK
AtMEK4  EIEIILRDV NHPNVVKCHEMFDQNGEIQ VLLEFMDKGS LEGA------HVWK
MEK1    ELQVLHECNSPYIVGFY GAFYSDGEIS ICMEHMDGGS LDQVLKKAGRIP-

                  VIA              VIB              VII
NtMEK2  ESALSDLT RQVLSGLYY LH-RRKIVHR DIKPSNLLIN SRREVKIADFGVS
AtMEK4  EQQLADLS RQILSGLAY LH-SRHIVHR DIKPSNLLIN SAKNVKIADFGVS
MEK1    EQILGKVS IAVIKGLTY LREKHKIMHR DVKPSNILVN SRGEIKLCDFGVS

              227   233 237 VIII                 IX
NtMEK2  RVLAQIMD PCNSSVGTI AYMSPERINT DLNHGQYDGY AGDIWSLGV SILE
AtMEK4  RILAQIMD PCNSSVGTI AYMSPERINT DLNQGKYDGY AGDIWSLGV SILE
MEK1    GQLIDSMA --NSFVGTR SYMSPER---- -LQGTHY-SV QSDIWSMGL SLVE
              *      *    *         *
                                 X
NtMEK2  FYLGRFPF SVGRSGDWA SLM-CAICMS HGT-APANAS R----------
AtMEK4  FYLGRFPF PVSRQGDWA SLM-CAICMS QPPEAPATAS P----------
MEK1    MAVGRYPI PPPDAKELE LMFGCQV-EG DAAETPPRPR TPGRPLSSY GMDS

                                        XI
NtMEK2  ---------- ---------- ----------EFRDFIACCLQRDPARRWTAVQ
AtMEK4  ---------- ---------- ----------EFRHFISCCLQREPGKRR SAMQ
MEK1    RPPMAIFELLDYIVNEP PPKLPSGVFS LEFQDFVNKC LIKNPAERADLKQ

NtMEK2  LLRHPFITQNSPAATTTGNMMPLPNQVHQPAHQLLPP PPHFSS
AtMEK4  LLQHPFILRASPSQ---------NRSPQNLHQLLPP PRPLSSSSS PTT
MEK1    LMVHAFIKRSDAEEVDFAGWLCSTIGLNQPSTPTHAAGV
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Fig. 1. Alignment of tobacco NtMEK2 with *Arabidopsis* AtMEK4 (BAA28830) and human MEK1 (Q02750). Roman numerals indicate the 11 conserved subdomains of the kinase catalytic domain. Numbers in parentheses indicate the percentage of amino acid sequence identity to the NtMEK2. The conserved Ser/Thr residues (Thr-227, Ser-233, and Thr-237) between subdomains VII and VIII for MAPKKs are marked with asterisks underneath. The conserved Lys-111 that is important for the ATP binding is marked with a dot.

Fig. 2. Constitutively active NtMEK2 mutant phosphorylates and activates SIPK and WIPK. (*A*) NtMEK2^{DD}, the T227D/S233D double mutant of NtMEK2, has elevated kinase activity. Autophosphorylation activities of 0.5 μg of His-tagged wild-type (WT) NtMEK2 and its mutants were determined as described in *Materials and Methods*. (*B*) NtMEK2^{DD} preferentially phosphorylates SIPK and WIPK. Phosphorylation activities of HisNtMEK2^{WT} and HisNtMEK2^{DD} (0.1 μg) were determined by using inactive mutant MAPKs (HisMAPK^R, 1 μg) as substrates. Reactions in the absence (−) of either MAPK or MAPKK were used as controls. (*C*) Phosphorylation by NtMEK2^{DD} activates SIPK and WIPK. Wild-type MAPKs (HisMAPK, 1 μg) were incubated in the absence (−) or with 0.1 μg of HisNtMEK2^{WT} or HisNtMEK2^{DD} in the presence of 50 μM unlabeled ATP. MBP (final concentration of 0.25 μg/μl) and [γ-^{32}P]ATP (1 μCi per reaction) were then added. Phosphorylated MBP (P-MBP) that reflects the activity of MAPK was visualized by autoradiography after SDS/PAGE.

WIPK share almost 75% identity and belong to the two most closely related subfamilies of plant MAPKs (21).

NtMEK2 Is Activated Concurrently with SIPK and WIPK in Elicitin-Treated Cells. Our previous results demonstrated that the long-lasting activation of SIPK and delayed activation of WIPK are associated with the cell death induced by cryptogein, a fungal elicitin from phytopathogenic *Phytophthora* spp. (20, 23). As shown in Fig. 3*A*, treatment of tobacco cells with 25 nM cryptogein led to ≈80% cell death within 8 h, as determined by the fluorescein diacetate method (20). Within 24 h, the shrinkage of cytoplasm, a hallmark of HR cell death, was clearly visible (data not shown). To study the activation of endogenous MAPKK(s) in these cells, antibody was raised against recombinant protein corresponding to the N-terminal 156-aa unique region of NtMEK2. The specificity of Ab-NtMEK2 antibody was tested by immunoblot analysis against different full-length recombinant tobacco MAPKKs. As shown in Fig. 3*B*, Ab-NtMEK2 recognized NtMEK2 but not NtMEK1, NtMEK7, or NtMEK8. After the specificity of the Ab-NtMEK2 was established, it was used in an immune complex–kinase assay to determine whether NtMEK2 is activated during elicitin-induced HR cell death. As shown in Fig. 3*C*, NtMEK2 was activated before HR cell death and concurrently with the activation of SIPK and WIPK *in vivo* (20), indicating that NtMEK2 is the upstream kinase of SIPK and WIPK in the same pathway. The truncated NtMEK2, against which the antibody was raised, but not the corresponding regions of NtMEK1 and NtMEK7, could compete for the
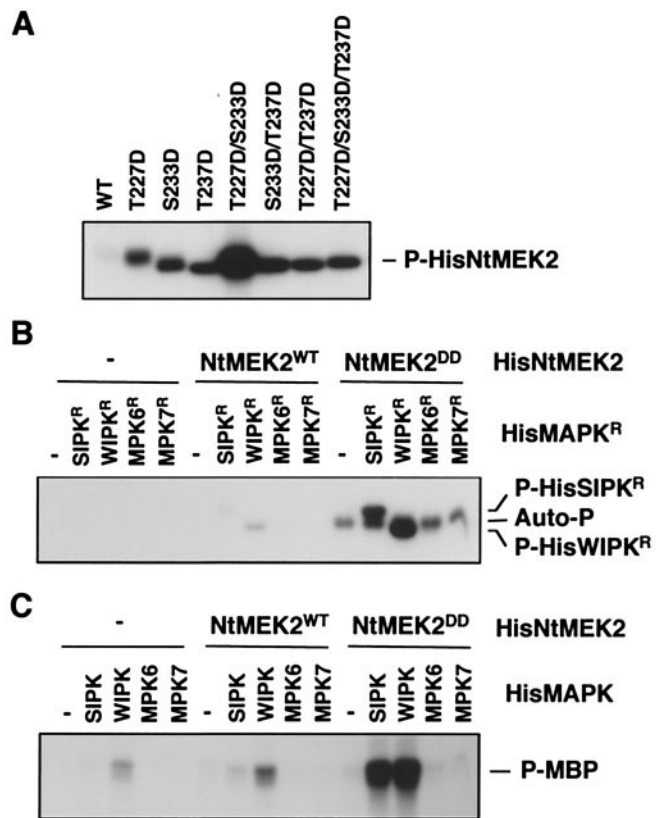
binding of the Ab-NtMEK2, demonstrating the specificity of the immune complex–kinase assay. The N-terminal portion of NtMEK8, ΔNtMEK8, is insoluble. As a result, it cannot be used as a competitor in the immune complex–kinase assay. Nonetheless, immunoblot analysis indicated that Ab-NtMEK2 does not cross-react with NtMEK8 (Fig. 3*B*).

NtMEK2 Activates SIPK and WIPK *in Vivo*. To study the function of NtMEK2 in plant disease resistance signaling, we used a steroid-inducible transient transformation system (24, 28). *Agrobacterium* cells carrying Flag-tagged *NtMEK2*^{WT} or active *NtMEK2*^{DD} constructs were infiltrated into tobacco leaves. An inactive mutant of *NtMEK2*, *NtMEK2*^{R}, was used as a negative control. Expression of transgene was induced by the application of DEX 40–48 h later. As shown in Fig. 4*A Top*, expression of all three proteins was detectable 6 h after the application of DEX, whereas no Flag-tagged protein was present before induction (0 h) or in leaves infiltrated with vector control (Fig. 4*C Upper*). Expression of neither *NtMEK2*^{WT} nor *NtMEK2*^{R} activated any MAPKs as determined by an in-gel kinase assay (Fig. 4*B Top*);
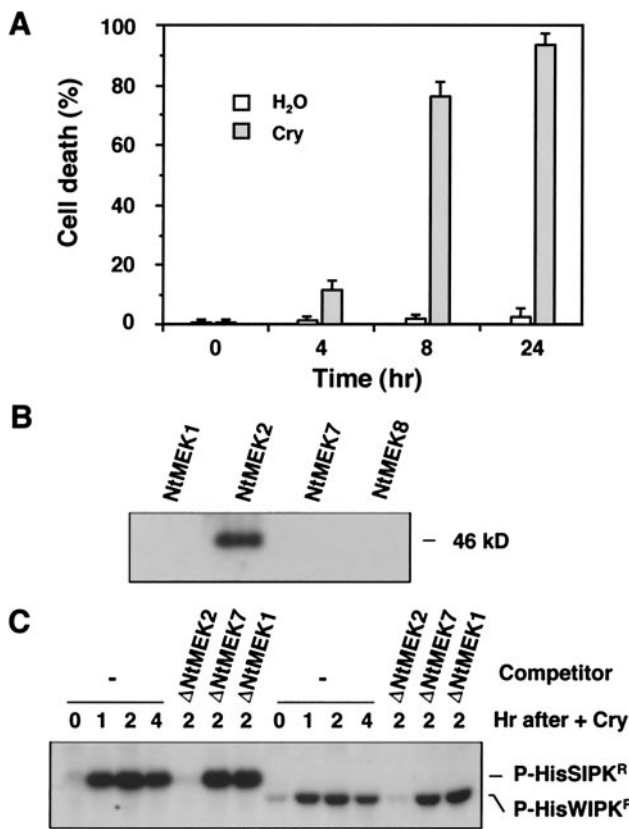
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Fig. 3. Activation of NtMEK2 during HR-like cell death initiated by fungal elicitin. (*A*) Cryptogein (Cry), a fungal elicitin from *Phytophthora cryptogea*, induces HR-like cell death in tobacco suspension cells. Cells were treated with cryptogein at a final concentration of 25 nM, and cell viability was monitored by the fluorescein diacetate method (20) at the indicated times. Data represent the mean of three replicates ±SE. (*B*) Ab-NtMEK2 specifically recognized NtMEK2 protein. Two nanograms each of the purified recombinant NtMEK1, NtMEK2, NtMEK7, and NtMEK8 were subjected to immunoblot analysis with Ab-NtMEK2. (*C*) Activation of NtMEK2 in cells treated with cryptogein. Protein extracts (75 μg) from cryptogein-treated cells were immunoprecipitated with purified Ab-NtMEK2 (4 μg) in the absence (−) or the presence of the N-terminal portion of NtMEK2 (ΔNtMEK2), NtMEK7 (ΔNtMEK7), or NtMEK1 (ΔNtMEK1) as competitor. Kinase activity of the immune complex was subsequently assayed with HisSIPK^R or HisWIPK^R as a substrate.

however, cells expressing *NtMEK2*^{DD} showed high and long-lasting activation of a 48- and a 44-kDa kinase (Fig. 4*B Top*), which is similar to the activation of SIPK and WIPK in tobacco challenged by tobacco mosaic virus or suspension cells treated with fungal elicitins (10, 20, 23). Immune complex–kinase assay by using SIPK- and WIPK-specific antibodies confirmed that they were SIPK and WIPK (Fig. 4*B Middle* and *Bottom*). Activation of SIPK and WIPK *in vivo* correlated with high MAPKK activity of Flag-NtMEK2^{DD} as demonstrated by immune complex–kinase assay by using HisSIPK^R and HisWIPK^R as substrates (Fig. 4*A Middle* and *Bottom*). The very low basal activity of NtMEK2^{WT} was not sufficient to activate either SIPK or WIPK *in vivo* (Fig. 4*B*).

Activation of SIPK and WIPK by NtMEK2^{DD} is selective, as the expression of active mutants of other tobacco MAPKKs, including NtMEK1^{DD}, NtMEK7^{DD}, and NtMEK8^{DD}, did not activate SIPK or WIPK, even though these proteins were expressed at a similar level (Fig. 4*C* and data not shown).

Induction of *NtMEK2*^{DD} Expression Activates Defense Responses. The activation of SIPK and WIPK by ectopically expressed NtMEK2^{DD}
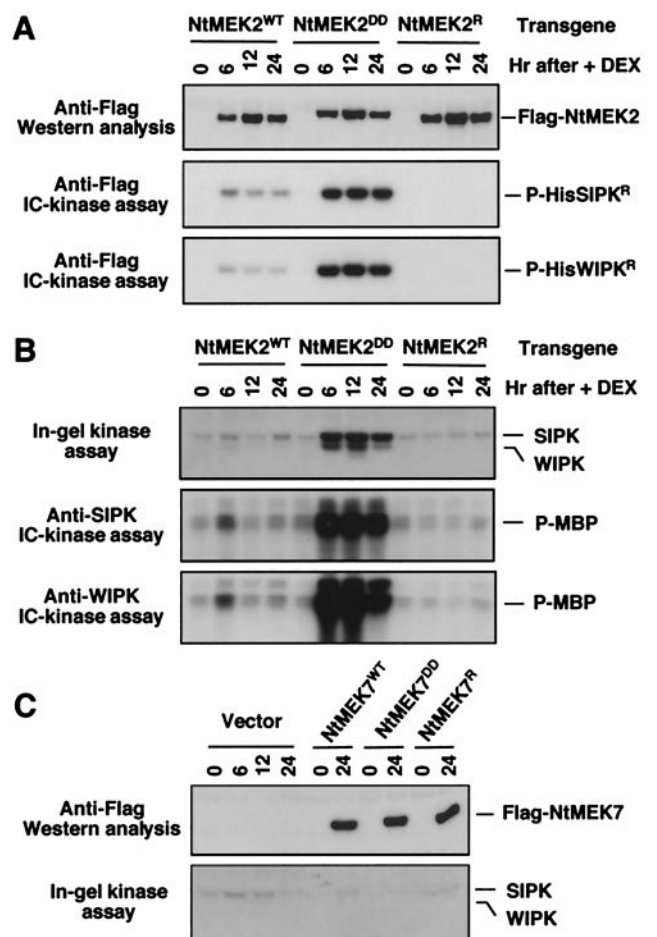

Fig. 4. Expression of *NtMEK2*^{DD} activates SIPK and WIPK in tobacco. (*A*) Induction of NtMEK2 and its mutants by steroid in *Agrobacterium*-mediated transient transformation. Tobacco leaves were infiltrated with *Agrobacterium* carrying pTA7002 constructs. DEX (30 μM) was infiltrated 40 h later, and samples were taken at indicated times. The expression of transgenes was monitored by immunoblot analysis by using anti-Flag antibody (*Top*). The kinase activities of Flag-tagged NtMEK2 and its mutants were determined by immune complex (IC)-kinase assay with HisSIPK^R (*Middle*) or HisWIPK^R (*Bottom*) as a substrate. (*B*) Induction of *NtMEK2*^{DD} expression activates SIPK and WIPK *in vivo*. The MAPK activities in cells after DEX treatment were determined by an in-gel kinase assay with MBP substrate (*Top*). The identities of the two kinases were confirmed by immune complex–kinase assays by using SIPK-specific (Ab-p48N; *Middle*) or WIPK-specific (Ab-p44N; *Bottom*) antibody. (*C*) Expression of *NtMEK7*^{DD} does not activate SIPK or WIPK. The levels of *NtMEK7* transgene expression were determined by immunoblot analysis (*Upper*). Endogenous SIPK and WIPK activities were detected by an in-gel kinase assay with MBP as a substrate (*Lower*).

leads to the activation of several defense responses. Within 8–12 h after the application of DEX, cell death in small areas could be seen. By 24–36 h, the whole area infiltrated with *Agrobacterium* carrying *NtMEK2*^{DD} collapsed, a phenotype identical to the HR elicited by various pathogens or pathogen-derived elicitors (Fig. 5). The timing for the appearance of cell death depends on the age of the leaves. In older leaves, cell death happens much later because of delayed and lower level expression of transgene after DEX application. Besides the similarity at the macroscopic level, cell death induced by NtMEk2^{DD} is associated with membrane leakage, as determined by Evens–Blue staining and shrinkage of cytoplasm (data not shown), which are diagnostic for HR cell death. These results support our hypothesis based on inhibitor studies that SIPK and WIPK are involved in regulating HR cell death (20). Active mutants of other tobacco MAPKKs, including NtMEK7^{DD}, do not

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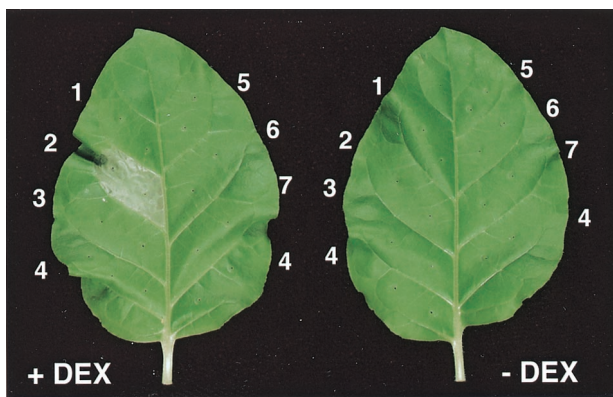
Constructs	+ DEX	-DEX
1. NtMEK2^{WT}	-	-
2. NtMEK2^{DD}	HR	-
3. NtMEK2^{R}	-	-
4. Vector	-	-
5. NtMEK7^{WT}	-	-
6. NtMEK7^{DD}	-	-
7. NtMEK7^{R}	-	-

Fig. 5. Induction of *NtMEK2*^{DD} expression leads to HR-like cell death. Different sections of a tobacco leaf were infiltrated with *Agrobacterium* carrying indicated constructs or vector control, and DEX (30 μM) was applied 40 h later. Solvent (0.1% of ethanol) was used in the control leaf (−DEX). Photo was taken 30 h after application of DEX. In the table, HR denotes the development of an HR-like necrosis in the leaf section; — indicates no visible phenotype.

Fig. 6. Induction of *NtMEK2*^{DD} expression leads to the activation of a subset of defense genes. Total RNA was isolated from samples collected at the same time as those for protein analyses (Fig. 4). Equal amounts of RNA (10 μg) were electrophoresed on a 1.2% formaldehyde-agarose gel and transferred to Zeta-Probe membrane (Bio-Rad). The transcript levels of *HMGR*, *PAL*, and *LOX* were determined by sequential probing with [α-^{32}P]CTP random primer-labeled cDNA inserts as previously described (23). An ethidium bromide-stained gel was used to show equal loading of samples.

cause HR-like cell death under the same condition (Fig. 5 and data not shown), suggesting that this phenotype is caused by a specific MAPK pathway.

SA plays important roles in the activation of several defense responses (2, 5). To test whether SA is required for NtMEK2^{DD}-induced cell death, transgenic Xanthi nc plants expressing the *NahG* gene were used. NahG encodes the SA-metabolizing enzyme salicylate hydroxylase (29). As a result, the SA-dependent responses in such transgenic plants are compromised. Comparison of the phenotype of NahG and wild-type plants indicated that SA is not required for NtMEK2^{DD}-induced cell death. Interestingly, we noticed consistently that the leaves collapse a few hours earlier in the *NahG* plants (data not shown).

In addition to the HR-like cell death, expression of *NtMEK2*^{DD} also activated a subset of defense genes. As shown in Fig. 6, the steady-state levels of 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*) and L-phenylalanine ammonia lyase (*PAL*) mRNAs were greatly elevated in leaves expressing *NtMEK2*^{DD}. Within 6 h after DEX application, the levels of *HMGR* and *PAL* transcripts nearly peaked, which was significantly earlier than cell death at 12 h in this particular experiment. This result strongly argues against the possibility that the defense gene activation is a secondary effect caused by cell death. HMGR catalyzes the rate-limiting step in the biosynthesis of all isoprenoid compounds including capsidiol, the major tobacco phytoalexin accumulated during a defense response (30), whereas PAL catalyzes the first committed step in the biosynthesis of phenylpropanoid by converting L-phenylalanine to *trans*-cinnamic acid, the precursor for lignins and SA biosynthesis (31). Activation of these two genes is prolonged and correlates with SIPK/WIPK activity in the cells. Again, expression of the active mutants of NtMEK1, NtMEK7, and NtMEK8 under the same conditions failed to give this phenotype (data not shown). Previously, we have linked fungal elicitor-induced *PAL* expression with SIPK activation by the use of kinase inhibitor (23). In contrast, the mRNA levels of lipoxygenase (*LOX*) (32), another
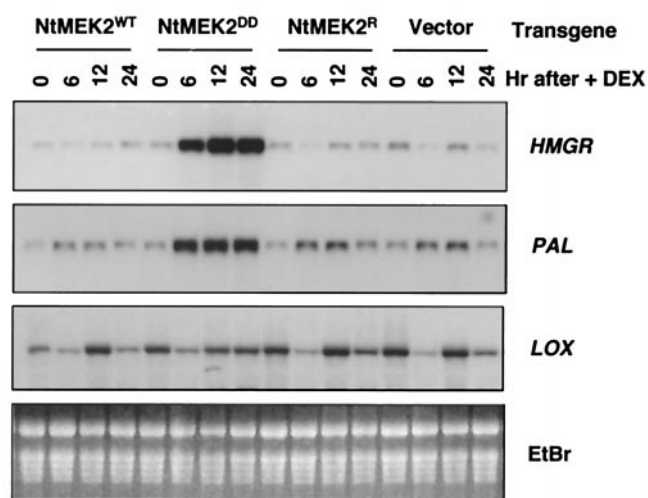
pathogen-activated defense gene, did not correlate with the SIPK/WIPK activities (Fig. 6), suggesting that *LOX* expression is not controlled by this MAPK pathway.

Discussion

Similar defense responses are elicited in plants by viral, bacterial, and fungal pathogens, suggesting shared signaling pathway(s) (1, 8). Increasing evidence demonstrated that SIPK/WIPK or their orthologs in other plant species are converging points after the perception of different pathogens (11–13). In this paper, we report that NtMEK2 is an upstream kinase for both SIPK and WIPK based on *in vitro* and *in vivo* evidence. Expression of a constitutively active mutant of *NtMEK2* induces HR-like cell death in tobacco, which is preceded by the activation of endogenous SIPK and WIPK. In addition, NtMEK2-SIPK/WIPK cascade appears to control the expression of *HMGR* and *PAL*, two defense genes encoding key enzymes in the phytoalexin and SA biosynthesis pathways. These results demonstrate that the NtMEK2-SIPK/WIPK cascade controls multiple defense responses during plant disease resistance. By the use of a conditional system, the correlation between SIPK/WIPK activation and the induction of defense responses is strengthened in this gain-of-function study. Furthermore, parallel studies of three other tobacco MAPKKs, which failed to activate SIPK/WIPK and give similar phenotypes, demonstrated the specificity of the NtMEK2-SIPK/WIPK cascade. Because MAPKs are the sole substrates of MAPKKs, the phenotypes generated by expressing *NtMEK2*^{DD} are likely the results of endogenous SIPK and WIPK activation. This greatly reduced the possibility of nonspecific effects, as the magnitude of SIPK/WIPK activation induced by expression of *NtMEK2*^{DD} is similar to that induced by pathogens or pathogen-derived elicitors (10, 20, 23).

The finding that SIPK and WIPK share a common upstream MAPKK could explain why the activation of SIPK and WIPK during stress responses is frequently associated (10, 11, 20). In tobacco suspension cells treated with fungal elicitins (20, 23) or plants infected by tobacco mosaic virus (10), there is a prolonged activation of SIPK, followed by a delayed activation of WIPK. This delay is caused by the requirement of gene activation and *de novo*

synthesis of WIPK protein (20). In contrast, SIPK is transiently activated alone in wounded tobacco leaves or suspension cells treated with fungal cell wall elicitor (23, 33). This is likely a result of the combination of a low basal level of WIPK protein in untreated tobacco plants/cells and a short duration of NtMEK2 activation. In cells treated with fungal cell wall elicitor, WIPK protein eventually accumulates to a high level (20). However, it is not accompanied by the activation of WIPK, possibly because of the absence of NtMEK2 activity. As a result, it appears that the presence or absence of WIPK protein and the duration of NtMEK2 activation could influence whether only SIPK or both SIPK and WIPK are activated in response to a stress signal.

An alternative explanation for differential activation of SIPK and WIPK is the presence of other unidentified MAPKKs that can act on SIPK or WIPK alone. By using a yeast two-hybrid screening, a tobacco MAPKK was identified as a SIPK-interacting protein (25). This MAPKK was named SIPKK, although its phosphorylation activity toward SIPK is yet to be demonstrated. NtMEK1 is identical to SIPKK except for three amino acid residues, which may represent variation of the same gene in different tobacco cultivars. NtMEK1 shares 30.7% amino acid identity with NtMEK2. Overexpression of *NtMEK1*[DD] did not activate SIPK or WIPK in tobacco (data not shown), suggesting that NtMEK1 is not the upstream kinase.

HR cell death, as well as a number of other defense responses such as the generation of reactive oxygen species and activation of defense genes, can be blocked by protein kinase inhibitors (18, 20, 23, 34, 35). Potential targets for the kinase inhibitor could be at the recognition step, as several *R* genes, including *Pto* and *Xa21*, encode protein kinases (8) or kinases immediately downstream of R protein, such as Pto-interacting kinase, Pti (36). By adding kinase inhibitor at various times after the cells were challenged with fungal elicitin, we demonstrated that a prolonged activation of a kinase and/or a kinase activated late in the signal pathway is required for plant cells to commit to the death program (20). The kinetics of SIPK and WIPK activation matches this profile. In this study, we provide more direct evidence linking SIPK and WIPK with HR cell death and defense gene activation. Activation of SIPK and WIPK by NtMEK2[DD] is not accompanied by increase in H_2O_2 production

(data not shown). This is in accord with a previous finding by using kinase inhibitor that these two MAPKs are not required for H_2O_2 production during plant-defense responses (11). Interestingly, we found that H_2O_2 can activate SIPK in tobacco cells (S.Z. and D. Klessig, unpublished data). Very recently, it was reported that AtMPK6 and AtMPK3, the SIPK and WIPK orthologs in *Arabidopsis*, can also be activated by H_2O_2 (37). It is likely that the signaling pathway leading to H_2O_2 burst separates from the pathway leading to MAPK activation after the perception of pathogens or elicitors, and the H_2O_2 generated can then feed into the MAPK pathway, forming a positive feedback loop. The potential role(s) of such a positive feedback in plant disease resistance remains to be evaluated.

In mammalian cells, activation of the SAPK/JNK and p38 kinase plays important roles in stress-induced apoptosis (16, 17). Recently, it was shown that SAPK/JNK regulates cytochrome *c* release in the mitochondrial death signaling pathway (38). How plant HR cell death, a form of programmed cell death, is regulated and how NtMEK2-SIPK/WIPK cascade is integrated in the signaling pathway(s) remain to be discovered. Nevertheless, the finding that a plant MAPK pathway may play a role similar to JNK/SAPK is very intriguing. The role(s) that SIPK and WIPK each plays in the NtMEK2[DD]-induced HR-like cell death and defense gene activation is currently unknown. On the basis of kinase inhibitor experiments, we hypothesized that SIPK is involved in the activation of defense gene expression (23), whereas the prolonged activation of SIPK, perhaps in combination with the activation of WIPK, turns on the HR cell death pathway (20, 23). Future analyses of transgenic plants or mutants in which the expression/activation of either SIPK or WIPK is altered should clarify the role of each MAPK in the signaling pathway(s).

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