Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Two mitogen-activated protein kinase kinases, MKK1 and MEK2, are involved in wounding- and specialist lepidopteran herbivore *Manduca sexta*-induced responses in *Nicotiana attenuata*

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Abstract

In a wild tobacco plant, *Nicotiana attenuata*, two mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), play central roles in modulating herbivory-induced phytohormone and anti-herbivore secondary metabolites. However, the identities of their upstream MAPK kinases (MAPKKs) were elusive. Ectopic overexpression studies in *N. benthamiana* and *N. tabacum* suggested that two MAPKKs, MKK1 and MEK2, may activate SIPK and WIPK. The homologues of *MKK1* and *MEK2* were cloned in *N. attenuata* (*NaMKK1* and *NaMEK2*) and a virus-induced gene silencing approach was used to knock-down the transcript levels of these *MAPKK* genes. Plants silenced in *NaMKK1* and *NaMEK2* were treated with wounding or simulated herbivory by applying the oral secretions of the specialist herbivore *Manduca sexta* to wounds. MAPK activity assay indicated that after wounding or simulated herbivory NaMKK1 is not required for the phosphorylation of NaSIPK and NaWIPK; in contrast, NaMEK2 and other unknown MAPKKs are important for simulated herbivory-elicited activation of NaSIPK and NaWIPK, and after wounding NaMEK2 probably does not activate NaWIPK but plays a minor role in activating NaSIPK. Consistently, NaMEK2 and certain other MAPKKs, but not NaMKK1, are needed for wounding- and simulated herbivory-elicited accumulation of jasmonic acid (JA), JA–isoleucine, and ethylene. Furthermore, both NaMEK2 and NaMKK1 regulate the levels of trypsin proteinase inhibitors. The findings underscore the complexity of MAPK signalling pathways and highlight the importance of MAPKKs in regulating wounding- and herbivory-induced responses.

Key words: Defence, ethylene, herbivory, jasmonic acid, mitogen-activated protein kinase kinase, trypsin proteinase inhibitors.

Introduction

Mitogen-activated protein kinase (MAPK) cascades play critical roles in regulating various cellular processes in eukaryotes (Herskowitz, 1995; Chang and Karin, 2001; MAPK Group, 2002). They are located downstream of receptors and sensors and control cell physiology in response to various intra- and extracellular stimuli. The highly conserved MAPK cascades are composed of three kinases: MAPKs are phosphorylated by MAPK

kinases (MAPKKs) at the threonine and tyrosine residues located in the activation loop (T-loop) between subdomains VII and VIII of the kinase catalytic domain; these MAPKKs are activated by the triple kinases, MAP kinase kinase kinases (MAPKKKs). Activated (phosphorylated) MAPKs can directly phosphorylate certain downstream targets, which include mainly transcription factors which, in turn, initiate stimulus-induced transcriptional changes,

enzymes, and even proteins that function in cytokinesis (Chang and Karin, 2001; Liu *et al.*, 2004; Sasabe and Machida, 2006; Beck *et al.*, 2010).

Although the functions of MAPK cascades have been intensively studied in animals and yeast, how MAPK signalling is involved in plant development and stress responses is still not well understood. In plants, MAPK signalling pathways are important for development (Bergmann *et al.*, 2004; Lukowitz *et al.*, 2004; HC Wang *et al.*, 2007; Rodriguez *et al.*, 2010), responses to abiotic stresses, such as drought and salt (Kiegerl *et al.*, 2000; Kovtun *et al.*, 2000; Cardinale *et al.*, 2002; Xiong and Yang, 2003), and resistance to viral, bacterial, and fungal pathogens (reviewed in Pedley and Martin, 2005; Rodriguez *et al.*, 2010).

Emerging evidence has also indicated the involvement of MAPK signalling in plant defence against herbivores (Kandoth *et al.*, 2007; Wu *et al.*, 2007). In *Nicotiana attenuata*, attack from its natural herbivore, *Manduca sexta*, induces a myriad of responses on transcriptomic, proteomic, and metabolomic levels (Wu and Baldwin, 2010). *Nicotiana attenuata* recognizes the fatty acid–amino acid conjugates (FACs) in *M. sexta* oral secretions (OS) that are introduced into wounds during feeding and rapidly activates two MAPKs, salicylic acid-induced kinase (NaSIPK) and wound-induced protein kinase (NaWIPK); importantly, these kinases are required for the herbivory-induced biosynthesis of jasmonic acid (JA) and ethylene (Wu *et al.*, 2007). The central role of JA in plant defence against herbivores has been well documented (reviewed in Wasternack, 2007; Howe and Jander, 2008; Wu and Baldwin, 2010). JAR (JASMONATE RESISTANT) proteins conjugate JA with isoleucine to form JA–Ile (Staswick and Tiryaki, 2004), which binds to the COI1 receptor and thus activates most of the JA-induced responses (Chini *et al.*, 2007; Thines *et al.*, 2007), including biosynthesis of various defensive compounds, such as direct defensive compounds, trypsin proteinase inhibitors (TPIs) (Zavala *et al.*, 2004), caffeoylputrescine (CP) (Kaur *et al.*, 2010), nicotine (Steppuhn *et al.*, 2004), and diterpene glycosides (DTGs) (Jassbi *et al.*, 2008; Heiling *et al.*, 2010), and indirect defensive compounds, such as *trans*-α-bergamotene (Kessler and Baldwin, 2001). In *N. attenuata*, *M. sexta* attack, but not mechanical wounding, induces a burst of ethylene; genetic analysis indicated that ethylene is important for herbivory-induced nicotine production (von Dahl *et al.*, 2007). Yet little is known about the signalling pathway that transduces FAC recognition into MAPK activation and eventually JA production in plants.

The *Arabidopsis* genome harbours ~60 MAPKKKs, 10 MAPKKS, and 20 MAPKs (MAPK Group, 2002). The small number of MAPKKs suggests that MAPKKs may have multiple MAPK targets and that interactions among different signalling pathways are concentrated at the level of MAPKKs (MAPK Group, 2002; Hamel *et al.*, 2006; Andreasson and Ellis, 2010). A growing body of evidence has revealed the important functions of MAPKKs in plant development and stress-induced responses. In tobacco, NQK1/NtMEK1 is required for cell cytokinesis (Soyano

et al., 2003). The *Arabidopsis* double mutant *mkk4 mkk5* develops densely clustered stomata and is seedling lethal, demonstrating the important role of MAPKKs in development (HC Wang *et al.*, 2007). Detached leaves of an *mkk9* mutant have delayed senescence (Zhou *et al.*, 2009). Several MAPKKs are involved in abiotic stress responses (Kiegerl *et al.*, 2000; Teige *et al.*, 2004; Gomi *et al.*, 2005; Xu *et al.*, 2008), and resistance to pathogens (Asai *et al.*, 2002; Jin *et al.*, 2003; Liu *et al.*, 2004; Meszaros *et al.*, 2006; Doczi *et al.*, 2007; Takahashi *et al.*, 2007). In *Arabidopsis*, after perception of pathogen elicitor flg22, AtMKK4 and AtMKK5 activate AtMPK3 and AtMPK6, the homologues of *Nicotiana* WIPK and SIPK, respectively (Asai *et al.*, 2002). Furthermore, overexpressing the constitutively active form of NtMEK2 (the homologue of AtMKK4/AtMKK5) in tobacco leads to activation of NtSIPK and NtWIPK (Yang *et al.*, 2001; Jin *et al.*, 2003). Overexpression of another *Arabidopsis* MAPKK, AtMKK9, activates AtMPK3 and AtMPK6 in protoplasts, and this MAPK cascade mediates the stability of EIN3 (ETHYLENE INSENSITIVE3), an important component in ethylene signalling (Yoo *et al.*, 2008). Furthermore, *Arabidopsis* plants overexpressing AtMKK9 have enhanced ethylene and camalexin levels (Xu *et al.*, 2008). In *N. benthamiana*, a close homologue of *Arabidopsis* AtMKK9, NbMKK1, interacts with NbSIPK in yeast, and ectopically overexpressing NbMKK1 activates NbSIPK (Takahashi *et al.*, 2007).

In *N. attenuata*, NaSIPK and NaWIPK are pivotal MAPKs that regulate plant responses to herbivory. However, their upstream MAPKKs involved in herbivore defence responses were unknown. Using a reverse genetic approach, the transcript levels of two *MAPKK* genes, *NaMEK2* and *NaMKK1*, were knocked down and it was found that NaMEK2 is important in mediating *M. sexta* herbivory-induced defence responses, while NaMKK1 plays only a minor role. After simulated herbivory, NaMEK2 and certain other MAPKKs, but not NaMKK1, are required for the activation of NaSIPK and NaWIPK, and thus JA and ethylene biosynthesis. The data highlight the important roles of MAPKKs in plant–herbivore interaction and the complexity of the regulation of JA and ethylene biosynthesis.

Materials and methods

Molecular cloning and virus-induced gene silencing (VIGS)

Nicotiana attenuata NaMKK1 and *NaMEK2* (GenBank accession numbers: HQ023234 and HQ023235) were amplified using Phusion DNA polymerase (Finnzymes Oy, Espoo, Finland) (primer sequences are listed in Supplementary Table S1 available at *JXB* online) and the purified PCR products were cloned into pJET1.2 vector (Fermentas GmbH, St. Leon-Rot, Germany) and sequenced. Partial *NaMEK2* and *NaMKK1* sequence were amplified using plasmids as templates and gene-specific primers (listed in Supplementary Table S2). The PCR products were digested with appropriate restriction endonucleases and were further ligated into pTV00 to obtain the constructs pTV-NaMEK2 and pTV-NaMKK1.

Agrobacterium tumefaciens carrying these constructs was inoculated into plants to obtain VIGS (virus-induced gene silencing)

plants following a procedure optimized for *N. attenuata* (Saedler and Baldwin, 2004). Plants inoculated with *A. tumefaciens* carrying pTV00 (empty vector) were used for comparisons (EV plants). Plants silenced in *NaPDS* (*phytoene desaturase*) were used to monitor the degree of VIGS visually, since these plants showed a photo-bleaching phenotype (Saedler and Baldwin, 2004). About 14 d after inoculation, when the leaves of *NaPDS*-silenced plants were completely white, experiments were performed.

Phylogenetic analysis of MAPKKs

MAPKK protein sequences were deduced from their respective nucleotide sequences (accession numbers are listed in Supplementary Table S3 at *JXB* online). Protein sequences were aligned using the Clustal W algorithm (DNAStar Inc., Madison, WI, USA). An unrooted Neighbor–Joining tree and bootstrap analysis (1000 replications) were conducted using MEGA 4 software (Tamura *et al.*, 2007).

Plant growth and treatments

Plants of the 31st generation of an *N. attenuata* inbred line were used in all experiments. Plants were grown at 22 °C under 16 h of light in a growth chamber. In all the experiments, leaves of rosette-stage (~4–5 weeks old) plants were used. Wounding was performed by rolling a fabric pattern wheel three times on each side of the midvein. The wounded leaves were immediately supplied with either 15 μl of water (W+W) or 15 μl of 1:5 diluted OS from *M. sexta*. For the collection of *M. sexta* OS, larvae were reared on *N. attenuata* wild-type plants until the third to fifth instar. OS were collected on ice as described in Roda *et al.* (2004).

Manduca sexta *growth bioassays*

Manduca sexta eggs from in-house reared populations were kept in a growth chamber (Snijders Scientific, Tilburg, The Netherlands) at 26 °C under 16 h of light, and at 24 °C in 8 h of darkness, until larvae hatched. Freshly hatched *M. sexta* neonates were placed on fully developed leaves of 30 replicated rosette-stage NaMEK2-VIGS, NaMKK1-VIGS, and EV plants (one larva per plant). The larval masses were measured on day 5, 8, and 12.

Transcriptional analysis

Total RNA was extracted from leaves using the TRIzol reagent (Invitrogen, Paisley, UK). A 0.5 μg aliquot of total RNA of each sample was reverse-transcribed using oligo(dT)$_{12–18}$ and Super-script II reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was carried out on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using qPCR Core kits (Eurogentec, Liege, Belgium). *Elongation factor 1A* (*NaEF1A*) transcript levels were used to normalize total cDNA concentration variations. The sequences of primers used for qPCR are provided in Supplementary Table S4 at *JXB* online.

Phytohormone analysis

About 100 mg of frozen plant tissue were homogenized in 2 ml microcentrifuge tubes containing two metal balls and 1 ml of ethyl acetate spiked with 200 ng of D$_2$-JA, and 40 ng of D$_4$-salicylic acid (SA) and ^{13}C$_6$-JA–Ile. Homogenization was done twice with 200 strokes min^{-1} for 1 min using a Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ, USA). Samples were centrifuged at 13 000 *g* for 20 min at 4 °C. The supernatants were dried on a vacuum concentrator (Eppendorf AG, Hamburg, Germany). The residues were resuspended in 500 μl of 70% methanol by vortexing for 5 min, and centrifuged for 10 min at 4 °C (13 000 *g*). Supernatants were transferred to crimp vials, and sample measurements were carried out as described in Wu *et al.* (2007). Ethylene emissions were measured on a photoacoustic spectrometer (INVIVO GmbH,

Sankt Augustin, Germany) as described in von Dahl *et al.* (2007). Leaves of *N. attenuata* plants were treated with W+OS or left untreated for control. Immediately after treatments three leaves were weighed and enclosed in a three-neck 250 ml round-bottom glass flask for 5 h, and then the concentration of collected ethylene was measured.

Analysis of trypsin proteinase inhibitor activity

Trypsin proteinase inhibitor (NaTPI) activity was quantified using a radial diffusion assay protocol described by van Dam *et al.* (2001).

Protein extraction and in-gel kinase activity assay

The tissue of five replicates was pooled and ground in liquid nitrogen. About 100 mg of tissue were resuspended in 300 μl of extraction buffer [100 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na$_3$VO$_4$, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulphonyl floride, 10% glycerol, and one proteinase inhibitor cocktail tablet per 10 ml of extraction buffer (Roche, Mannheim, Germany)]. Samples were then centrifuged at 4 °C, 13 000 *g* for 20 min and the supernatants were transferred to fresh tubes. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (Sigma-Aldrich, Hamburg, Germany) as a standard. A 10 μg aliquot of total protein from each sample was used for in-gel kinase activity assay according to a procedure described by Zhang and Klessig (1997). The images of in-gel kinase activity assays were obtained on a phosphorimager (FLA-3000 phosphor imager system, Fuji Photo Film, Stamford, CT, USA), and the band intensities were quantified using the AIDA software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Statistical analysis

Data were analysed by unpaired *t*-tests using SPSS Statistics Version 17.0 (www.spss.com).

Results

Phylogenetic analysis of N. attenuata NaMEK2 and NaMKK1

Using sequences of *NtMEK2* in *N. tabacum* (Yang *et al.*, 2001; Zhang and Liu, 2001) and *NbMKK1* in *N. benthamiana* (Takahashi *et al.*, 2007) as references, the open reading frames of *NaMEK2* and *NaMKK1* in *N. attenuata* were cloned. Sequence alignments indicated that the protein sequences of NaMEK2 and NaMKK1 shared 99% and 95% similarity to that of NtMEK2 and NbMKK1, respectively, and the conserved motif sequence [S/TxxxxxS/T] of MAPKKs (MAPK Group, 2002) was also found in both kinases (Supplementary Fig. S1 at *JXB* online). Moreover, phylogenetic analysis of NaMEK2, NaMKK1, and MAPKKs in *A. thaliana*, *N. tabacum*, *N. benthamiana*, and *Oryza sativa* indicated that NaMEK2 is a close homologue of AtMKK4 and AtMKK5 (group C of MAPKKs) and NaMKK1 is closely related to AtMKK7, AtMKK8, and AtMKK9 (group D of MAPKKs) (MAPK Group, 2002) (Fig. 1). It is likely that both *NaMKK1* and *NaMEK2* are single genes in *N. attenuata*, since searching the tobacco expressed sequence tag (EST) database and an *N. attenuata* transcriptome database obtained by 454 sequencing revealed no other close
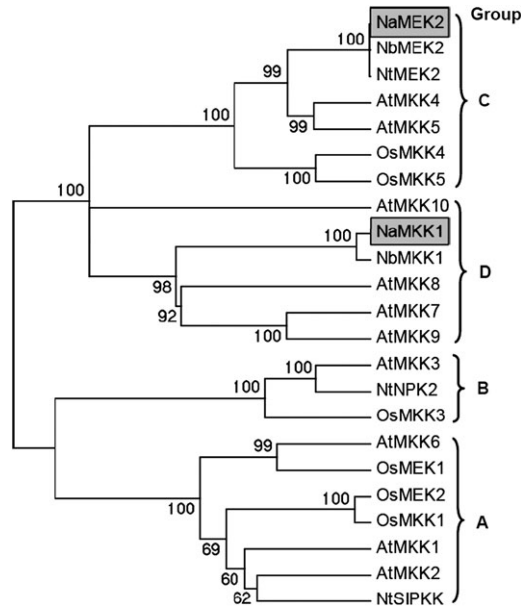
Fig. 1. Phylogenetic analysis of plant mitogen-activated protein kinase kinases (MAPKKs). Protein sequences of MAPKKs in *Arabidopsis*, *Nicotiana* spp., and rice were aligned using the Clustal W algorithm. An unrooted Neighbor–Joining tree and bootstrap analysis were performed with the MEGA 4 program. The species of origin of the MAPKKs are indicated by the abbreviation in front of the protein names: At, *Arabidopsis thaliana*; Na, *Nicotiana attenuata*; Nb, *Nicotiana benthamiana*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*. NaMKK1 and NaMEK2 are highlighted with grey backgrounds. Letters A to D represent different MAPKK groups (see MAPK Group, 2002).

homologues. However, the possibility that *NaMKK1* and *NaMEK2* have paralogues, which might have low expression levels or are expressed in specific organs or tissues, cannot be completely ruled out.

Transcriptional regulation of NaMKK1 *and* NaMEK2 *in* N. attenuata

To determine whether these two *MAPKK* genes are involved in herbivory-induced transcriptional responses, their transcriptional changes were examined in *N. attenuata* after wounding and simulated herbivory treatment. A fabric pattern wheel was rolled over *N. attenuata* leaves to generate puncture wounds; thereafter, 15 µl of water were immediately applied to wounds (W+W). To mimic herbivory, 15 µl of *M. sexta* OS were applied (W+OS) (Halitschke *et al.*, 2003). qPCR analyses indicated that the transcript levels of *NaMKK1* were elevated ~7-fold 1 h after W+W; while after W+OS treatment *NaMKK1* reached its highest level of transcription (10-fold increase) by 5 h (Fig. 2A). W+W treatment marginally enhanced the levels of *NaMEK2* transcripts, while *NaMEK2* transcript levels were elevated >25 times 1 h after W+OS (Fig. 2B). These transcript data suggest possible involvement of *NaMKK1* and *NaMEK2* in wounding and herbivore defence responses. Whether these differential transcriptional
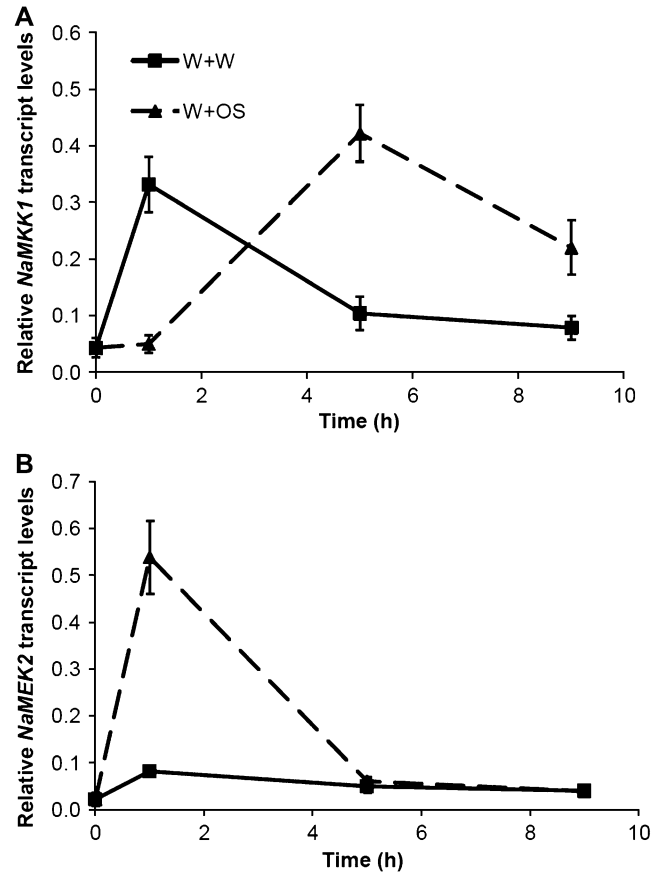
Fig. 2. Transcript levels of *NaMKK1* and *NaMEK2* after wounding and simulated herbivory. *Nicotiana attenuata* plants were wounded with a fabric pattern wheel, and 15 µl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W+W and W+OS, respectively); untreated plants served as controls. Samples were harvested after 1, 5, and 9 h and the transcript levels of (A) *NaMKK1* and (B) *NaMEK2* were analysed by qPCR.

regulations of *NaMKK1* and *NaMEK2* are further translated into different levels of NaMKK1 and NaMEK2 protein abundance/activity needs to be studied further.

NaMEK2, but not NaMKK1, is required for the phosphorylation of NaSIPK and NaWIPK after simulated herbivory

To study the function of NaMKK1 and NaMEK2 in wounding- and *M. sexta* herbivory-induced responses in *N. attenuata*, RNA interference (RNAi) constructs harbouring partial *NaMKK1* and *NaMEK2* sequences in an inverted repeat orientation were prepared and *N. attenuata* was transformed by *A. tumefaciens* carrying these constructs. Although stable RNAi lines of *NaMKK1*-silenced plants were obtained, after screening 20 independent lines transformed with the *NaMEK2*-RNAi construct, all 20 lines were found to be tetraploids. Therefore, a transient silencing approach, VIGS, was employed to knock-down the transcript levels of these two *MAPKK* genes. Plants inoculated with *A. tumefaciens* carrying pTV00, pTV-NaMKK1, and pTV-NaMEK2 formed EV,
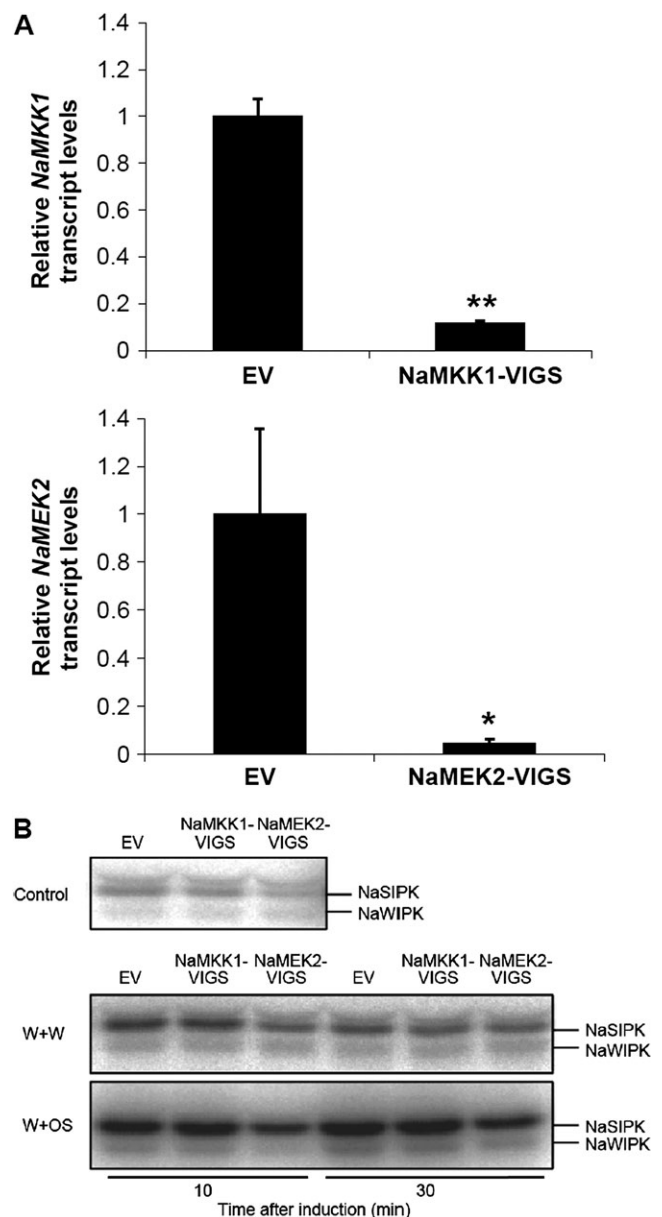
NaMKK1-VIGS, and NaMEK2-VIGS plants, respectively. qPCR analyses indicated that compared with those in EV plants, *NaMKK1* and *NaMEK2* transcript levels were reduced 89% and 95% in NaMKK1-VIGS and NaMEK2-VIGS plants (Fig. 3A). Silencing either gene using VIGS did not result in obvious developmental abnormalities in *N. attenuata* (data not shown).

An in-gel kinase activity assay was performed to determine whether NaMKK1 and NaMEK2 are the upstream MAPKKs for NaSIPK and NaWIPK when plants are challenged with wounding and herbivory. Plants were treated with W+W and W+OS and samples were collected after 10 min and 30 min. When untreated, no obvious different levels of MAPK activity were detected among EV, NaMKK1-VIGS, and NaMEK2-VIGS plants (Fig. 3B, top panel). In all plants, W+W and W+OS treatment rapidly enhanced the activity of NaSIPK and NaWIPK (as early as 10 min), indicating that *N. attenuata* recognized *M. sexta* OS and responded with higher MAPK activity levels than those induced by just wounding (Wu *et al.*, 2007). Importantly, compared with EV, 10 min after W+W and 10 min and 30 min after W+OS, NaMEK2-VIGS plants showed ~50% reduced NaSIPK activity levels (Fig. 3B, middle panel; for quantification of band intensities see Supplementary Fig. S2A at *JXB* online). In-gel kinase assays revealed only weak NaWIPK activity even after inductions. NaMEK2-VIGS plants seemed to have decreased levels of NaWIPK activity after W+OS, but not after W+W (Fig. 3B, bottom panel); in contrast, silencing *NaMKK1* had no detectable effect on the activity levels of NaSIPK (and probably also NaWIPK) after either treatment.

Therefore, after mechanical wounding and simulated herbivory, NaMEK2 is important for the activation of NaSIPK, while NaWIPK seems to require NaMEK2 for phosphorylation only after simulated herbivory. Consistent results were obtained from an independently repeated experiment (Supplementary Fig. S2B at *JXB* online). These data suggest that very probably some other MAPKKs are also involved in the regulation of NaSIPK and NaWIPK in *N. attenuata*'s responses to wounding and herbivory, since silencing neither *NaMEK2* nor *NaMKK1* greatly compromises MAPK activity.

NaMEK2 but not NaMKK1 regulates wounding- and herbivory-induced accumulation of phytohormones in N. attenuata

Given that NaSIPK and NaWIPK are important regulators of wounding- and *M. sexta* herbivory-induced JA and JA–Ile accumulation and these phytohormones play a central role in mediating resistance to herbivores, whether silencing *NaMKK1* and *NaMEK2* alters the levels of JA and JA–Ile after these treatments was examined next. In EV plants, 1.5 h after W+W treatment JA reached 800 ng g^{-1} fresh mass (FM); consistent with the more highly augmented NaSIPK and NaWIPK activity levels after W+OS, EV plants accumulated up to 3600 ng g^{-1} FM of JA (Fig. 4A). The


Fig. 3. MAPK activity in wounding- or simulated herbivory-induced responses in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants. (A) *NaMKK1* and *NaMEK2* transcript levels are highly suppressed in NaMKK1-VIGS and NaMEK2-VIGS plants. Rosette leaves of EV, NaMKK1-VIGS, and NaMEK2-VIGS plants were harvested and the transcript levels of *NaMKK1* and *NaMEK2* were analysed by qPCR. The transcript levels of *NaMKK1* and *NaMEK2* in EV plants were designated as 1. Asterisks indicate significant differences between EV and NaMKK1-VIGS or NaMEK2-VIGS plants (*t*-test; *P <0.05; **P <0.01; n=5). (B) MAPK activity in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants after wounding and simulated herbivory treatment. Plants were wounded with a fabric pattern wheel, and 15 μl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds [W+W (middle panel) and W+OS (bottom panel), respectively]; untreated plants served as controls (top panel). Samples were harvested after 0, 10, and 30 min and immediately frozen in liquid nitrogen. MAPK activity was detected with an in-gel kinase assay.

JA–Ile contents in EV plants followed similar patterns: 1 h after treatments, W+OS induced 4-fold higher JA–Ile levels than did W+W treatment (Fig. 4B). Congruent with their MAPK activity levels, NaMKK1-VIGS plants had no different JA and JA–Ile levels compared with EV plants after W+W and W+OS treatment, whereas NaMEK2-VIGS exhibited ~50% lower levels of JA and JA–Ile at most of the time points examined after either treatment (Fig. 4).

Since wounding barely elicits ethylene biosynthesis, ethylene production was only measured in W+OS-elicited plants (von Dahl *et al.*, 2007). After W+OS treatment, the same amount of ethylene was detected in NaMKK1-VIGS and EV plants, and NaMEK2-VIGS plants had 40% reduced ethylene emissions (Fig. 4C).

Many studies have indicated that SA suppresses JA accumulation (Spoel *et al.*, 2003; Cipollini *et al.*, 2004; Leon-Reyes *et al.*, 2010). To rule out the possibility that the decreased JA levels in NaMEK2-VIGS plants resulted from augmented SA levels in these plants, SA contents were quantified. EV, NaMKK1-VIGS, and NaMEK2-VIGS plants showed no difference in basal and W+W-induced SA levels (Supplementary Fig. S3 at *JXB* online). After W+OS treatment, SA levels in *NaMKK1*- and *NaMEK2*-silenced plants were not higher than those of EV plants and tended to
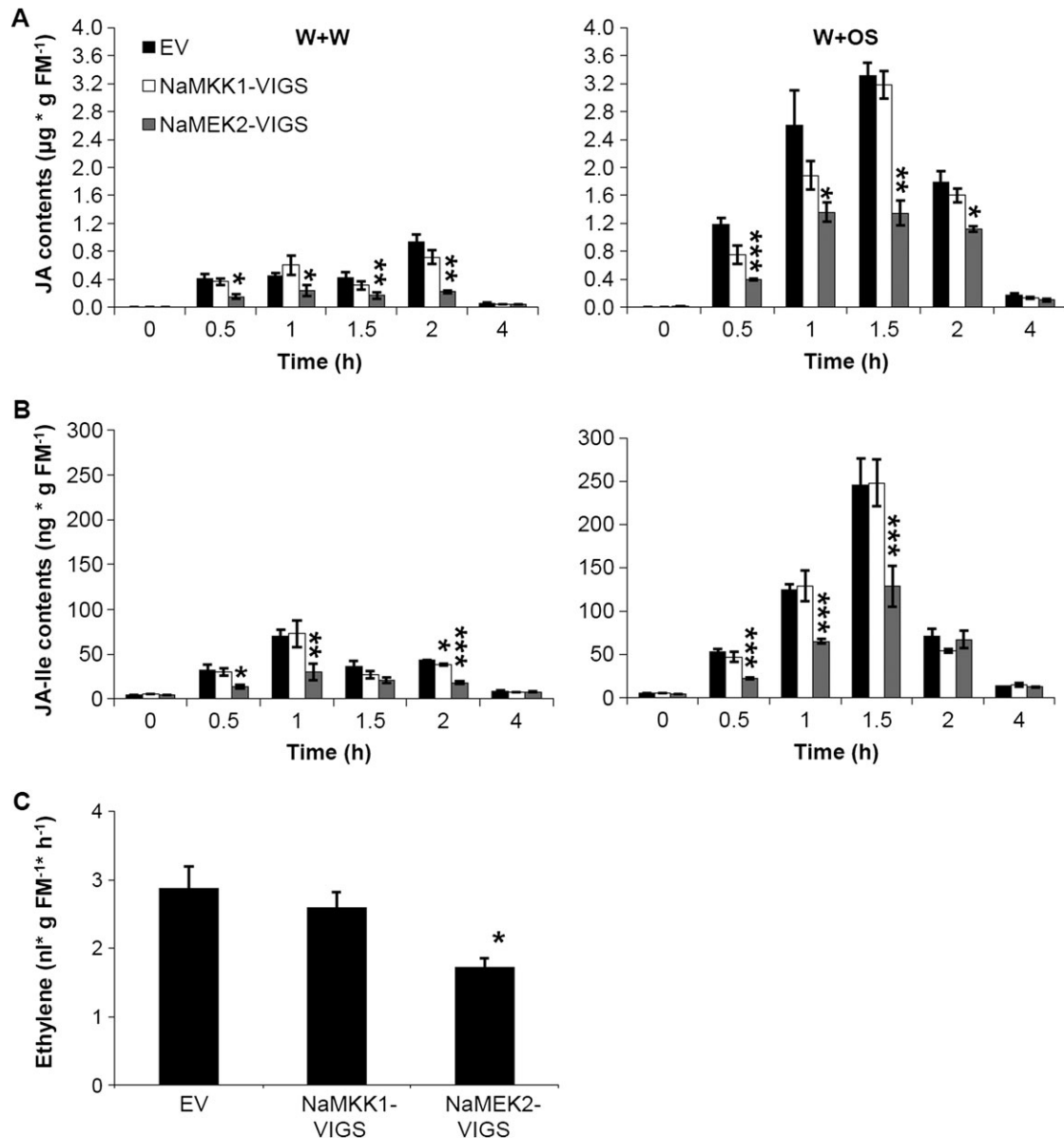
Fig. 4. Silencing *NaMKK1* and *NaMEK2* decreases wounding- or simulated herbivory-induced levels of phytohormones. (A) and (B) EV, NaMKK1-VIGS, and NaMEK2-VIGS plants were wounded with a fabric pattern wheel, and 15 μl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W+W and W+OS, respectively). Samples were harvested after the indicated times. Contents (mean ±SE) of JA (A) and JA–Ile (B) were measured with HPLC-MS/MS. (C) EV, NaMKK1-VIGS and NaMEK2-VIGS plants were treated with W+OS, and ethylene accumulated in 5 h was collected and analysed. Asterisks indicate significant differences between EV and NaMKK1-VIGS or NaMEK2-VIGS plants (*t*-test; $*P <0.05$; $**P <0.01$; $***P <0.001$; $n=5$).

be lower by 50% in *NaMEK2*-silenced plants 1 h after W+OS (Supplementary Fig. S3).

Thus, NaMEK2 but not NaMKK1 is important for the regulation of wounding- and *M. sexta* herbivory-induced defence-related phytohormones.

Silencing NaMKK1 and NaMEK2 in N. attenuata compromises NaTPI activity

TPIs are important anti-herbivore compounds in Solanaceae, including *N. attenuata* (Ryan, 1989; Haq *et al.*, 2004; Zavala *et al.*, 2004). To analyse the function of NaMKK1 and NaMEK2 in regulating NaTPI, the activity of NaTPI was determined in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants 3 d after they were treated with W+W or W+OS (non-treated plants served as controls). No significant differences were found among the non-treated samples (Fig. 5A). However, after W+W treatment, compared with EV, NaMKK1-VIGS plants showed ~60% decreased NaTPI activity levels, while NaMEK2-VIGS plants had similar levels. Furthermore, W+OS-treated NaMKK1-VIGS and NaMEK2-VIGS exhibited ~40% and 50% lower levels of NaTPI activity, respectively (Fig. 5A).

In *N. attenuata*, nicotine, CP, and DTGs are also important defensive compounds against *M. sexta* larvae. Neither W+W nor W+OS treatment elevated the contents of these compounds even in EV plants (data not shown). Very probably this was caused by the low temperature required for efficient VIGS (Kaplan *et al.*, 2004; Shohael *et al.*, 2006).

Bioassays were performed to examine whether knocking down *NaMKK1* and *NaMEK2* alters the performance of the specialist herbivore *M. sexta*. Freshly hatched neonates were placed on EV, NaMKK1-VIGS, and NaMEK2-VIGS plants, and their masses were recorded over 12 d. Despite the decreased NaTPI activity in NaMKK1-VIGS and NaMEK2-VIGS, *M. sexta* larvae gained similar mass on all plants (Fig. 5B).

Discussion

MAPKs play central roles in the activation of plant defence responses against abiotic and biotic stresses (Bergmann *et al.*, 2004; Lukowitz *et al.*, 2004; Pedley and Martin, 2005; Andreasson and Ellis, 2010; Rodriguez *et al.*, 2010; HC Wang *et al.*, 2007). At least two MAPKs, SIPK and WIPK, are important for plant resistance to herbivores (Wu *et al.*, 2007). However, the identities of their upstream MAPKKs in plant–herbivore interactions were largely unknown. Using a reverse genetic approach, it is shown here that two MAPKKs, NaMKK1 and NaMEK2, are involved in wounding- and *M. sexta* feeding-induced responses in *N. attenuata*.

NaMEK2, but not NaMKK1, is upstream of NaSIPK and NaWIPK

Thus far, only two MAPKs, NaSIPK and NaWIPK and their homologues in tomato, are known to play a role in resistance to herbivore attack (Kandoth *et al.*, 2007; Wu *et al.*, 2007). Whether AtMPK6 and AtMPK3 (homologues
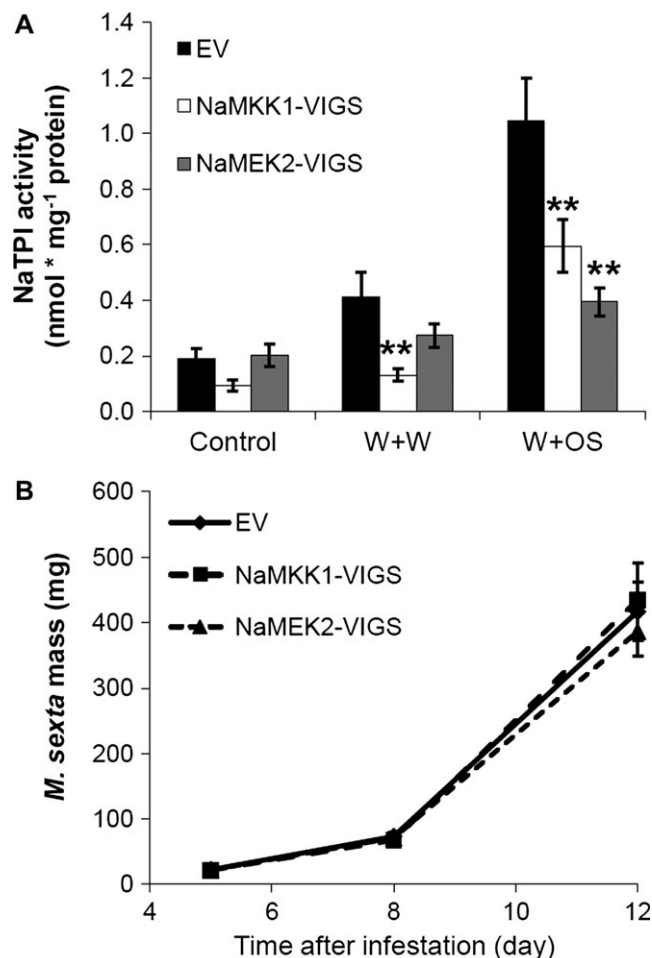
Fig. 5. NaMKK1-VIGS and NaMEK2-VIGS plants have decreased NaTPI activity, but do not exhibit compromised resistance to *M. sexta*. (A) NaTPI activity in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants. Plants were wounded with a fabric pattern wheel, and 15 μl of water or *M. sexta* oral secretions (OS) were applied immediately to wounds (W+W and W+OS, respectively); untreated plants served as controls. Three days after treatments, samples were collected and NaTPI activity was analysed. Asterisks indicate significant differences between EV and NaMKK1-VIGS or NaMEK2-VIGS plants (*t*-test; *$P <0.05$; **$P <0.01$; $n=5$). (B) *Manduca sexta* mass gain on EV, NaMKK1-VIGS, and NaMEK2-VIGS plants. Each type of plant was infested with 30 *M. sexta* neonates (one larva per plant) and larval masses (mean ±SE) were measured after 5, 8, and 12 d.

of NaSIPK and NaWIPK in *Arabidopsis*) are also important for herbivory-induced responses is still unclear.

Using ectopic overexpression systems, a few studies have demonstrated that in *Arabidopsis* AtMKK4 and AtMKK5 phosphorylate AtMPK6 and AtMPK3 (Asai *et al.*, 2002), and their close homologue in tobacco, NtMEK2, activates NtSIPK and NtWIPK (Yang *et al.*, 2001; Zhang and Liu, 2001). Consistent with an AtMKK4/AtMKK5–AtMPK6/AtMPK3 and NtMEK2–NtSIPK/NtWIPK cascade, NaMEK2 was identified to be located upstream of NaSIPK and NaWIPK in the herbivory-induced signalling pathway using a knock-down approach. Importantly,

silencing *NaMEK2* only reduced NaSIPK activity levels after W+OS treatment by ~50%, although qPCR analysis indicated that only 5% of *NaMEK2* transcript levels were detected in NaMEK2-VIGS plants. It is speculated that one or more other unknown MAPKKs also phosphorylate NaSIPK when *N. attenuata* is challenged by *M. sexta* feeding. The possibility that NaMEK2-VIGS plants had substantially greater levels of NaMEK2 protein than the levels of *NaMEK2* transcripts cannot be ruled out. In the wounding-activated signalling pathway, NaWIPK seems not to be located downstream of NaMEK2, given that *NaMEK2*-silenced plants did not have noticeably altered NaWIPK activity after wounding. Given the low activity of NaWIPK in in-gel kinase assays, this should be confirmed with immunocomplex kinase activity assays using an NaWIPK-specific antibody. Furthermore, in contrast to its reduced activity after simulated herbivory (at least by 30 min), the activity of NaSIPK in *NaMEK2*-silenced plants decreased only shortly after wounding (10 min) but regained the levels found in EV plants by 30 min. These data suggest that wounding and herbivory may activate overlapping but distinct MAPK pathways involving different MAPKKs, given that NaMEK2 is important for herbivory-induced activation of NaSIPK (and probably NaWIPK), but in response to wounding it only plays a minor role. Identification of the other MAPKK (or MAPKKs) that activate NaSIPK and NaWIPK will provide valuable insight into the mechanism by which plants distinguish mechanical wounding and herbivory and thus deploy appropriate defences.

Overexpression of *NbMKK1* results in phosphorylation of NbSIPK in *N. benthamiana* (Takahashi *et al.*, 2007). Transiently expressing AtMKK9 (a close homologue of NaMKK1) in tobacco and *Arabidopsis* leads to SIPK/AtMPK6 and WIPK/AtMPK3 activation (Xu *et al.*, 2008), and transforming *Arabidopsis* protoplasts with a constitutively active form of AtMKK9 results in phosphorylation of AtMPK6 and AtMPK3 (Yoo *et al.*, 2008). However, silencing experiments indicated that NaMKK1 is not required for the phosphorylation of NaSIPK and NaWIPK after wounding and simulated herbivory. It is very unlikely that the silencing of *NaMKK1* was not sufficient to knock-down the protein levels of NaMKK1 since, after wounding and simulated herbivory treatment, *NaMKK1*-silenced plants exhibited compromised NaTPI activity. It is speculated that this was because ectopic overexpression may have led to very high levels of protein and thus produced non-physiological interactions. It is also possible that NaMKK1 activates NaSIPK and NaWIPK in other stimulus-activated signalling cascades, such as those elicited by pathogens (Takahashi *et al.*, 2007).

Function of NaMEK2 in wounding- and herbivory-induced biosynthesis of phytohormones

SIPK and WIPK are important regulators of wounding- and herbivory-induced JA (and JA–Ile) accumulation (Kandoth *et al.*, 2007; Wu *et al.*, 2007). Consistent with this, *NaMEK2*-silenced plants had nearly 50% decreased JA

and JA–Ile levels after both wounding and simulated herbivory. NaMKK1 is not required for the phosphorylation of NaSIPK and NaWIPK after wounding and herbivory; congruently, no changes of JA and JA–Ile levels were detected in *NaMKK1*-silenced plants. Moreover, compared with those in EV, no large differences in SA levels were found in *NaMKK1*- and *NaMEK2*-silenced plants. Kobayashi *et al.* (2010) examined the function of SIPK and WIPK in modulating the levels of JA and SA after *N* gene-carrying tobacco plants were challenged with tobacco mosaic virus (TMV): simultaneously silencing *SIPK* and *WIPK* highly compromises TMV viral accumulation, and this is associated with increased SA and decreased JA contents. It is speculated that silencing *MKK1* in tobacco may not affect viral amplification and the accumulation of JA and SA, but *MEK2*-silenced plants could somewhat resemble plants silenced in both *SIPK* and *WIPK* (TMV amplification and the accumulation of JA and SA).

An elegant study revealed that *Arabidopsis* AtMPK6 phosphorylates 1-aminocyclopropane-1-carboxylic acid synthases (AtACS2 and AtACS6) and thus stabilizes these enzymes and greatly enhances ethylene biosynthesis (Liu and Zhang, 2004). However, after application of a pathogen elicitor, an *mpk6* null mutant still produces 50% of the amount of ethylene synthesized by wild-type plants (Liu and Zhang, 2004). Similarly, silencing *NaSIPK* in *N. attenuata* results in 40% reduced ethylene production after simulated herbivory treatment (Wu *et al.*, 2007). These facts suggest that certain AtMPK6/NaSIPK-independent pathways regulate the other 50% of ethylene production after pathogen/herbivory elicitation. Notably, even though the activity of NaSIPK in NaMEK2-VIGS plants is not decreased to the same extent as that in NaSIPK-VIGS plants or in *mpk6* null mutants (Liu and Zhang, 2004; Wu *et al.*, 2007), ethylene production is still 40% reduced. Either full activation of NaSIPK is critical for herbivory-induced ethylene biosynthesis or, in addition to NaSIPK, NaMEK2 also phosphorylates another MAPK, which also regulates ethylene production. Identification of this NaSIPK-independent ethylene regulation pathway will provide valuable insight into the mechanism by which plants control the biosynthesis of this important hormone.

NaMKK1 and NaMEK2 regulate the defence metabolite NaTPI

TPIs play an important role as direct defences against herbivores in solanaceous plants (Ryan, 1989; Haq *et al.*, 2004). Many studies have indicated that JA signalling plays a major role in regulating the levels of TPIs (Koiwa *et al.*, 1997; Paschold *et al.*, 2007; L Wang *et al.*, 2007). Despite the unaltered JA and JA–Ile levels in *NaMKK1*-silenced plants after wounding and simulated herbivory, NaTPI activity was reduced by 50%. Similar inconsistency between JA–Ile and NaTPI activity levels were also seen in NaMEK2-VIGS plants: after wounding, NaMEK2-VIGS plants did not have lower levels of NaTPI activity than did EV plants, although wounding-induced JA–Ile levels were

reduced. These results reveal a complex regulatory network, including MAPK cascades (more specifically, certain transcription factors that are probably controlled directly by MAPK cascades) and JA signalling, in modulating TPI defence in responses to wounding and herbivory. Probably due to the low temperatures required for VIGS, other defence-related secondary metabolites (nicotine, CP, and DTGs) were not elevated after either W+W or W+OS, even in EV plants. Given the critical role of JA signalling in regulating CP and DTGs (Paschold *et al.*, 2007), it is expected that the contents of these compounds in *NaMEK2*-silenced plants are also decreased, and NaMKK1 may also control the levels of these compounds in a largely JA signalling-independent manner. This hypothesis should be tested in plants whose *NaMKK1* and *NaMEK2* are stably silenced with an RNAi approach.

Although NaTPI activity levels were decreased in both NaMKK1-VIGS and NaMEK2-VIGS plants, *M. sexta* gained similar masses on these plants compared with those on EV plants. One possibility is that the decrease of NaTPI activity in these plants was not sufficient to weaken plant defence. Moreover, green leave volatiles (GLVs) are released from wounded leaves during insect feeding and these C6 compounds are thought to function as indirect defence, but also feeding stimulants or herbivore attractants (Meldau *et al.*, 2009; Allmann and Baldwin., 2010; Dicke and Baldwin, 2010). In *N. attenuata*, GLVs stimulate *M. sexta* feeding, and silencing *NaSIPK* and *NaWIPK* impairs GLV emission and results in similar larval growth to those fed on wild-type plants, despite their decreased contents of direct defensive compounds (Meldau *et al.*, 2009). This might also account for the normal growth of *M. sexta* on NaMEK2-VIGS plants. Whether NaMKK1 also controls GLV emission requires further investigation.

In *Arabidopsis* and rice, two close homologues of *MEK2* exist (*AtMKK4* and *AtMKK5* in *Arabidopsis* and *OsMKK4* and *OsMKK5* in rice). However, only one *MEK2* was found in the EST database of *N. tabacum* and *N. benthamiana* and in an *N. attenuata* transcriptome database prepared by 454 sequencing. It is possible that one of the two paralogues of *MEK2* was deleted from the genomes of *Nicotiana* spp. or it is not expressed. More sequence information and phylogeny analyses will provide valuable information about the evolution of *MEK2*—whether *MEK2* is an ancient *MAPKK* that appeared before the divergence of monocots and dicots or the gene duplication of *MEK2* in *Arabidopsis* and rice is completely independent. Assuming that another copy of *MEK2* does exist in *Nicotiana* spp., perhaps it is only expressed in specific organs or tissues. Alternatively, its transcript levels might be very low, and, if so, the possibility of co-silencing by the pTV-NaMEK2 construct cannot be ruled out, given the high sequence similarity between AtMKK4 and AtMKK5. A similar scenario may also apply to *NaMKK1*, which could also have one or more paralogues in *N. attenuata*, although current data point to the likelihood that *NaMEK2* and *NaMKK1* are single-copy genes.

Taken together, the present analyses indicate the involvement of two MAPKKs, NaMKK1 and NaMEK2, in plant responses to herbivores. Gene silencing revealed that these MAPKKs are upstream of different MAPKs and play overlapping but distinct roles in wounding- and herbivory-induced defence. Identification of other important components in wounding- and herbivory-specific signalling pathways, such as MAPKKKs, MAPKKs, MAPKs, and transcription factors, will greatly facilitate our understanding of how plants have evolved to cope with these stresses. Field studies will further reveal the ecological significance of these regulators in plant interactions with various herbivore feeding guilds.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Alignment of the protein sequences of *N. attenuata* NaMKK1 and NaMEK2 with *N. benthamiana* NbMKK1 and *N. tabacum* NtMEK2.

Figure S2 Wounding- and simulated herbivory-induced MAPK activity in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants.

Figure S3 Salicylic acid accumulation in EV, NaMKK1-VIGS, and NaMEK2-VIGS plants after W+W and W+OS treatment.

Table S1. Primers used to clone the open reading frames of *NaMKK1* and *NaMEK2* in *N. attenuata*.

Table S2. Primers used to clone partial *NaMKK1* and *NaMEK2* into pTV00 to obtain VIGS constructs.

Table S3. GenBank accession numbers or Swiss-Prot accession numbers of the MAPKKs for phylogenetic analysis.

Table S4. Primers used for qPCR.

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