Molecular study of the trypsin proteinase inhibitor defense mechanism and early herbivory-induced signaling in *Nicotiana*

Dissertation

Zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.)

Vorgelegt dem Rat der biologisch-Phamazeutischen Fakultät

der Friedrich-Schiller Universität Jena

Von Master of Science in Analytical Chemistry

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geboren am 20. Oktober 1973 in Hebei, China

Gutachter

1. _____
2. _____
3. _____

Tag der Doktoprüfung: _____

Tag der öffentlichen verteidigung: _____

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Manuscript overview

Manuscript I

Herbivory Rapidly Activates MAPK Signaling in Attacked and Unattacked Leaf Regions but Not between Leaves of *Nicotiana attenuata*

In this manuscript, we investigated the early signaling events happen after herbivory in *N. attenuata*. We found that fatty acid-amino acid conjugates in *Manduca sexta* oral secretions (OS) are the elicitors responsible for eliciting herbivory-specific responses. Two MAPKs, salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) are quickly activated after herbivory. Using a virus-induced gene silencing (VIGS) system, we investigated the functions of SIPK and WIPK in *N. attenuata*. SIPK and WIPK both regulate levels of OS-elicited jasmonic acid (JA), salicylic acid, and JA-isoleucine conjugate; moreover, SIPK mediates ethylene production. Using quantitative-PCR analyses, we found that both kinases regulate a wide array of defense-related gene expressions. Applying OS to wounds created in one portion of a leaf, SIPK is activated in both wounded and specific unwounded regions of the leaf but not in phylotactically connected adjacent leaves. We propose that *M. sexta* attack elicits a mobile signal that travels to nonwounded regions of the attacked leaf where it activates MAPK signaling and, thus, downstream responses; subsequently, a different signal is transported by the vascular system to systemic leaves to initiate defense responses without activating MAPKs in systemic leaves.

I isolated all the all MAPK, CDPK, and two WRKY genes. Baldwin I. T. and I designed all the experiments to characterize the function of both SIPK and WIPK. Together with Hettenhausen C. and Meldau S., we generated VIGS plants and analyzed their phytohormone phenotypes. I and Hettenhausen C. analyzed defense-related gene expressions. I and Meldau S. did in-gel kinase activity assays.

Manuscript overview

Manuscript II

The evolution of proteinase inhibitor defenses during allopolyploidy speciation in *Nicotiana* native to North America

In this manuscript, we investigated the evolution of a trypsin proteinase inhibitor (TPI) gene in two allopolyploid species: *N. clevelandii* and *N. quadrivalvis*. Using TPI as a marker gene, we constructed a gene tree which showed that *N. obtusifolia* was one of the parental species involved in the polyploidy speciation event. Southern blotting analysis indicated that TPI gene from *N. attenuata*, the other parental species, was lost in both *N. clevelandii* and *N. quadrivalvis* genomes during evolution. We show that compared with wounded plants, TPI activity levels are higher after herbivory in *N. attenuata*, *N. clevelandii* and *N. quadrivalvis*, suggesting an herbivory-specific recognition mechanism is retained in both tetraploid, although they both possess only TPI gene from *N. obtusifolia*, whose TPI gene doesn't show any herbivory-specific responses.

I cloned all the TPI cDNA and genomic sequences. I performed the Southern and gene tree analyses. I and Hettenhausen C. did the TPI expression measurement and JA analysis. Baldwin I.T. and I designed all the experiments.

Manuscript overview

Manuscript III

A deletion mutation in a trypsin proteinase inhibitor gene in *Nicotiana attenuata* Arizona ecotype triggers nonsense-mediated mRNA decay

In this manuscript, we investigated how nonsense-mediated mRNA decay (NMD) influenced the stability of a premature termination codon (PTC)-harboring trypsin proteinase inhibitor mRNA. Using virus-induced gene silencing system, we demonstrated that UPF1, 2, and 3 genes are all involved in NMD process in plants. I prepared various constructs expressing different forms of TPI genes and transformed in cell suspension cultures. We show that relative positions of introns to PTCs are important for initiating NMD in plants; intronless genes having different PTC positions have different stabilities. Using translation inhibitor, we demonstrate that translation is an important step to initiate NMD.

I cloned the TPI gene from Arizona ecotype and prepared all the transformation constructs with different forms of TPIs. Kang J. H. set up all the cell lines. I and Hettenhausen C. did expression analyses. Baldwin I. T. and I designed all the experiments.

1. Introduction

Nicotiana attenuata and Herbivore Interaction

The wild tobacco, *N. attenuata* Torr. Ex Watson (synonymous to *Nicotiana torreyana* Nelson & Macbr.), is an annual plant growing in disturbed desert in south-western USA. *N. attenuata* plants germinate in nitrogen-rich soil after seeds being exposed to cues derived from smoke (Figure 1A) (Baldwin and Morse, 1994; Baldwin et al., 1994). *N. attenuata* plants are diploid (2n = 24) and mainly inbreeding, although they also maintained features of outcrossing. *N. attenuata*'s short generation time and selfing properties made it a good system for both molecular and genetic studies.

In nature, *N. attenuata* plants are attacked by herbivores from more than 20 taxa, including mammalian browsers which can consume entire plants, intracellular sucking insects, and leaf-chewing insects, among which *Manduca sexta* and *M. quinquemaculata* are the major defoliators of *N. attenuata* (Figure 1B and 1C).

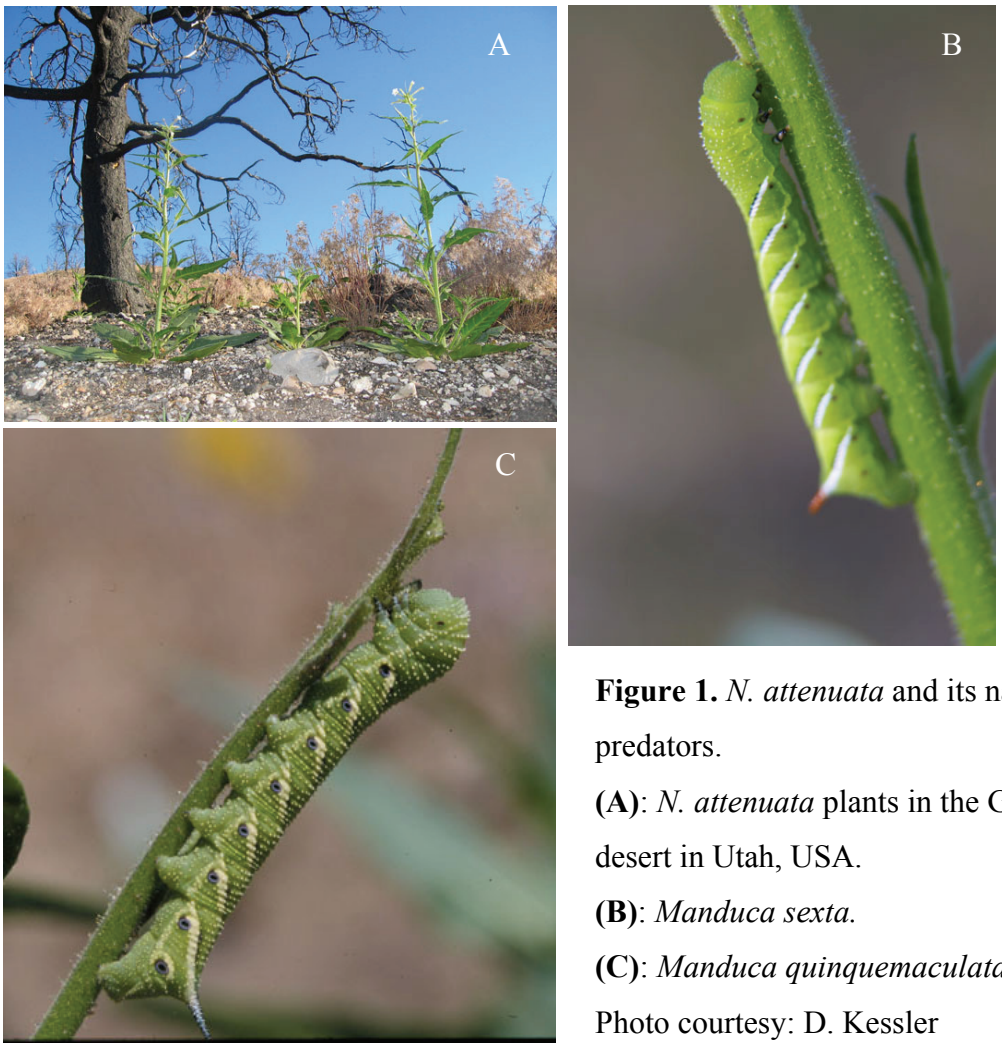

Figure 1. *N. attenuata* and its natural predators.
(A): *N. attenuata* plants in the Great Basin desert in Utah, USA.
(B): *Manduca sexta.*
(C): *Manduca quinquemaculata.*
Photo courtesy: D. Kessler

Several studies have revealed the importance of fatty acid-amino acid conjugates (FACs) from herbivore oral secretions (OS) in eliciting herbivory-specific responses in plants (Alborn et al., 1997; Alborn et al., 2003; Halitschke et al., 2003). During herbivory, OS are introduced into wounds and elicit 1) jasmonic acid (JA) and ethylene bursts which are greater than those elicited by mechanical wounding (Kahl et al., 2000); 2) high levels of trypsin proteinase inhibitor (TPI), an important direct defense compound (Zavala et al., 2004a); and 3) the release of volatile organic compounds (VOCs), which function as indirect defenses by attracting predators to feed on herbivores (Kessler and Baldwin, 2001). The functions of FACs are also demonstrated by the fact that removing FACs from OS by ion-exchange chromatography abolished *N. attenuata*'s herbivory-specific responses, i.e. *cis*-*α*-bergamotene emission, JA bursts, and extensive OS-specific transcript accumulation; moreover, adding synthetic FACs back to FAC-free OS restored all of the OS-elicited responses, and treating wounds with aqueous FAC solutions mimicked the effects of OS (Alborn et al., 1997; Halitschke et al., 2001; Halitschke et al., 2003). Through a largely unknown signal transduction network, plants perceive FACs and rapidly initiate a suite of defense-related responses.

Trypsin Proteinase Inhibitor Defenses

Proteinase inhibitors are a family of important compounds involved in direct defense {Ryan, 1990 #53; Koiwa, 1997 #55}. In genus *Nicotiana*, trypsin proteinase inhibitors (TPI) play a central role in plant-herbivore interactions (Zavala et al., 2004b). As a direct defense, they bind to proteinases in *M. sexta* larvae midguts and thus slow the growth and increase their mortality in their larval stage (Glawe et al., 2003). Herbivore-elicited TPI activity is a polygenic trait under both transcriptional and post-transcriptional control. JA, ethylene, and abscisic acid (ABA) are all known to be involved in the signaling network regulating TPI transcription (Pena-Cortes et al., 1995; O'Donnell et al., 1996; Koiwa et al., 1997; Halitschke and Baldwin, 2003); several TPI-specific proteases, which have been recently characterized, modulate post-translational processing of the pre-TPI protein (Horn et al., 2005).

Polyploidy Speciation and Evolution of Herbivory-induced Plant Defense Mechanisms

Polyploidy is a common phenomenon in the plant kingdom. It is estimated that about 70% of all angiosperms and 95% of ferns have experienced at least one episode of chromosome duplication in their evolutionary histories (Masterson, 1994; Leitch and Bennett, 1997; Otto and Whitton, 2000). Large-scale chromosome duplication also shaped the evolutionary histories of yeast and vertebrates (Sidow, 1996; Kellis et al., 2004). A

remarkable number of what were classically considered typical diploid plants, e.g. Arabidopsis and maize, are now regarded as paleopolyploids (Helentjaris et al., 1988; The Arabidopsis Genome Initiative, 2000; Wolfe, 2001). Because most polyploids can adapt to a wide range of habitats and survive under unfavorable conditions (Levin, 1983; Soltis and Soltis, 2000), polyploidization is thought to provide evolutionary and ecological advantages over taxa that retain their original chromosome number. A commonly proposed explanation is that polyploidy, by increasing gene redundancy, releases the selective constraints on the redundant genes, allowing them to evolve novel functions that benefit species ecologically (Otto and Whitton, 2000; Soltis and Soltis, 2000).

Cytological and morphological evidence suggested that N. section Bigelovianae, *N. quadrivalvis* (syn: *N. bigelovii* (Torr.) Wats.) and *N. clevelandii*, were tetraploids derived from amphidiploidy involving *N. attenuata* and an unknown "alatoid" ancestor (Goodspeed, 1954). Recently, plastid DNA (Clarkson et al., 2004) and glutamine synthetase (Qu et al., 2004) phylogenetic analyses all revealed that *N. obtusifolia* (syn: *N. trigonophylla* Dunal) was the missing alatoid progenitor and source of the maternal lineage for this polyploidy event. Lou and Baldwin (2003) investigated several herbivory-related traits in *N. attenuata*, *N. quadrivalvis*, and *N. clevelandii.* They show that most of *N. attenuata*'s herbivory-specific responses were kept in *N. quadrivalvis*, but lost in *N. clevelandii*, demonstrating the dynamic nature of polyploidy speciation and evolution. As one of the most important defense-related traits, how TPI defense mechanism evolved after polyploidy speciation in tetraploids, *N. clevelandii and N. quadrivalvis*, was unknown.

Nonsense-mediated mRNA Decay

Nonsense-mediated mRNA decay (NMD) is a conserved mRNA surveillance pathway in all eukaryotes; the pathway rapidly degrades mRNA containing premature termination codons (PTCs), which can lead to the synthesis of truncated proteins and have dominant negative effects (Hentze and Kulozik, 1999; Hilleren and Parker, 1999; Conti and Izaurralde, 2005). In humans, approximately 30% of inherited genetic disorders are due to genes with frameshifts or nonsense mutations that result in PTCs (Culbertson, 1999; Frischmeyer and Dietz, 1999; Holbrook et al., 2004). Through the NMD pathway, eukaryotes identify and degrade aberrant mRNA to eliminate genomic noise and ensure the fidelity of gene transcription. Although a lot has been known in both mammals and yeast, very little is known about NMD in plants.

In *N. attenuata*, TPI activity increases after FACs in larval OS are introduced into wounds during caterpillar feeding, which requires a functional jasmonate signaling cascade

(Halitschke and Baldwin, 2003; Roda et al., 2004). However not all ecotypes of *N. attenuata* increase TPI activity after herbivore attack or jasmonate elicitation. In an *N. attenuata* ecotype collected near Flagstaff Arizona (AZ) in 1996 and again in 2004, no detectable levels of TPI activity and substantially decreased levels of TPI mRNA are found (Glawe et al., 2003). Sequencing of AZ TPI indicated that it contains a PTC which initiates NMD and finally leads to decreased levels of TPI mRNA.

Herbivory-induced Early Signaling in *N. attenuata*

JA has long been recognized as the main signaling molecule mediating a plant's defense system against herbivores (Creelman and Mullet, 1997; Reymond and Farmer, 1998; Halitschke et al., 2003). In *N. attenuata*, applying OS to mechanically generated wounds induces a rapid JA burst with higher levels than does wounding itself (Kahl et al., 2000). The function of JA in *N. attenuata*'s defense against herbivory has been demonstrated by silencing lipoxygenase3 (LOX3), which leads to greatly reduced levels of herbivory-induced JA, and therefore, lower levels of nicotine and TPI activity than those in wild type (WT) plants (Halitschke and Baldwin, 2003).

JA-isoleucine (JA-Ile) is emerging as an important signal compound activating defense responses to herbivores (Staswick and Tiryaki, 2004; Kang et al., 2006). After mechanical wounding or applying OS to wounds, JA-Ile is rapidly produced in wounded leaves, closely following the kinetics of JA burst. In both Arabidopsis and *N. attenuata*, the conjugation between JA and amino acids are mediated by Jasmonate Resistance (JAR) genes (Staswick and Tiryaki, 2004; Kang et al., 2006), which are rapidly induced after both wounding and herbivory. In *N. attenuata*, silencing Jasmonate Resistance4 (JAR4) and JAR6 genes results in reduced levels of JA-Ile and in turn TPI activity; nevertheless, nicotine biosynthesis is not regulated by JARs (Wang et al., 2007).

Although a lot has been known about how phytohormones mediate herbivory-induced plants' responses, the early steps after plants' perception of herbivory, namely, how it senses herbivore-specific elicitors and uses signal transduction and regulatory networks to regulate defenses is poorly understood.

The mitogen-activated protein kinase (MAPK) cascade is a conserved pathway involved in modulating a large number of cellular responses in all eukaryotes (Herskowitz, 1995; Chang and Karin, 2001; Group, 2002). MAPKs phosphorylate their substrates, which are mainly transcription factors and which in turn trigger downstream reactions (Hill and Treisman, 1995; Karin and Hunter, 1995; Hazzalin and Mahadevan, 2002). In plants, MAPKs also play essential signaling roles, mediating responses to various stress stimuli (reviewed in

Hirt, 1997; Romeis, 2001; Zhang and Klessig, 2001). A growing body of evidence has revealed that in plants, MAPKs also regulate transcription factors at both transcription and protein activity levels (Kim and Zhang, 2004; Andreasson et al., 2005; Menke et al., 2005; Yap et al., 2005), and thus potentially mediate downstream stress-related responses.

Salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), two tobacco MAPKs and their orthologues in other plant species, are rapidly activated after various challenges (Romeis et al., 1999; Droillard et al., 2000; Samuel et al., 2000; Asai et al., 2002; Link et al., 2002). Both SIPK and WIPK are activated after wounding. Using transgenic cultivated tobacco, Seo et al. (1995, 1999) demonstrated that WIPK regulates wound-induced JA biosynthesis and is systemically activated after stems are cut. Similarly, in wounded tomato plants, a 48 kDa MAPK is activated both locally and systemically (Stratmann and Ryan, 1997). Biochemical analysis has demonstrated that SIPK is also highly activated by wounding (Zhang and Klessig, 1998). Although MAPKs are clearly involved in the wound response, it is unknown how they are involved in responses elicited by herbivore attack, particularly in plant species such as *N. attenuata*, where the wound response is known to be reconfigured during herbivore attack.

The objective of this thesis is to study 1) how MAPKs are involved in plant-herbivore interaction, by characterizing SIPK- and WIPK-silenced plants. 2) how TPI defense mechanism evolved after polyploidy speciation and evolution, and thus demonstrate the complexity of polygenetic trait evolution; and how NMD process influenced TPI mRNA levels in *N. attenuata* AZ ecotype plants.

Manuscript I describe how MAPKs are involved in early signaling responses in *N. attenuata* after being challenged with herbivory. Using a virus-induced gene silencing system, we show that they are important signaling components modulating OS-elicited hormonal responses and transcriptional regulation of defense-related genes.

Manuscript II describes that after polyploidy speciation, tetraploids, *N. clevelandii* and *N. quadrivalvis*, lost the TPI gene inherited from *N. attenuata*, the paternal ancestor. Although both tetraploids only possess the TPI gene from *N. obtusifolia*, which doesn't have herbivory-specific responses, they both inherited the signaling system from N. attenuata, thus show herbivory-specific TPI and JA responses after *M. sexta* attack.

In manuscript III, we studied how NMD decreased the stability of TPI mRNA in AZ. Using a virus-induced gene silencing system, functions of genes involved in NMD were investigated. Gene features, e.g. intron, intron positions, and positions of PTCs, which influence NMD in plants, were also examined.

Manuscript I

Plant Cell (2007)

Running title: MAPKs in a Plant–Herbivore Interaction

Herbivory Rapidly Activates MAPK Signaling in Attacked and Unattacked Leaf Regions but Not between Leaves of *Nicotiana attenuata*

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Abstract

Mitogen-activated protein kinase (MAPK) signaling plays a central role in transducing extracellular stimuli into intracellular responses, but its role in mediating plants' responses to herbivore attack remains largely unexplored. When *Manduca sexta* larvae attack their host plant, *Nicotiana attenuata*, the plant's wound response is reconfigured at transcriptional, phytohormone, and defensive levels, due to the introduction of oral secretions (OS) into wounds during feeding. We show that OS dramatically amplify wound-induced MAPK activity and that fatty acid-amino acid conjugates (FACs) in *M. sexta* OS are the elicitors. Virus-induced gene silencing of *salicylic acid-induced protein kinase* (*SIPK*) and *wound-induced protein kinase* (*WIPK*) revealed their importance in reconfiguring the wound response. Silencing *SIPK* and *WIPK:* 1) decreases transcript levels of *WRKY6*, known to orchestrate OS-elicited transcriptional responses, and of 2 additional WRKYs, 4 CDPKs, and 3 MAPKs, and each others' transcript levels; and 2) attenuates levels of OS-elicited jasmonic acid (JA), salicylic acid (SA), JA-isoleucine/leucine conjugates (JA-Ile/JA-Leu), and ethylene, and their biosynthetic transcripts. We found that after applying OS to wounds created in one portion of a leaf, SIPK is activated in both wounded and specific unwounded regions of the leaf, but not in phylotactically connected adjacent leaves. We propose that *M. sexta* attack elicits a mobile signal that travels to non-wounded regions of the attacked leaf where it activates MAPK signaling and thus downstream responses; subsequently, a different signal is transported by the vascular system to systemic leaves to initiate defense responses without activating MAPKs in systemic leaves.

Introduction

Over time plants have acquired sophisticated defense mechanisms to cope with herbivory. Plants react to herbivore attack with finely tuned transcriptional changes (Hui et al., 2003; Reymond et al., 2004), elevated phytohormone biosyntheses (Reymond and Farmer, 1998), and finally, the production of direct and indirect defense compounds (Kessler and Baldwin, 2001; Zavala et al., 2004). In *Nicotiana attenuata*, the application of *Manduca sexta*'s oral secretions and regurgitants (OS) to mechanical wounds elicits: 1) jasmonic acid (JA) and ethylene bursts which are greater than those elicited by mechanical wounding (Kahl et al., 2000); 2) high levels of trypsin proteinase inhibitor (TPI), an important direct defense compound (Halitschke and Baldwin, 2003; Zavala et al., 2004); and 3) the release of volatile organic compounds (VOCs), which function as indirect defenses by attracting predators to feeding herbivores (Kessler and Baldwin, 2001).

Several lines of evidence point to the importance of fatty acid-amino acid conjugates (FACs) in herbivore OS in eliciting herbivory-specific responses (Alborn et al., 1997; Alborn et al., 2003). Removing FACs from OS by ion-exchange chromatography abolished *N. attenuata*'s herbivory-specific responses, i.e. *cis*-α-bergamotene emission, JA bursts, and extensive OS-specific transcript accumulation. Moreover, adding synthetic FACs back to FAC-free OS restored all of the OS-elicited responses, and treating wounds with aqueous FAC solutions mimicked the effects of OS (Alborn et al., 1997; Halitschke et al., 2001; Halitschke et al., 2003). These facts demonstrate that when a plant perceives FACs, it activates its anti-herbivore defenses. Ethylene and more importantly JA have long been recognized as the main signaling molecules mediating a plant's defense system against herbivores (Creelman and Mullet, 1997; Reymond and Farmer, 1998; Halitschke and Baldwin, 2003). However, the early steps in a plant's response to herbivore attack, namely, how it senses herbivore-specific elicitors and uses signal transduction and regulatory networks to regulate defenses is poorly understood.

The mitogen-activated protein kinase (MAPK) cascade is a conserved pathway involved in modulating a large number of cellular responses in all eukaryotes (Herskowitz, 1995; Chang and Karin, 2001; MAPK Group, 2002). MAPKs are activated by the dual phosphorylation of threonine and tyrosine residues in a TXY motif located in the activation loop between subdomains VII and VIII by their upstream MAPK kinases (MAPKKs). Subsequently, MAPKs phosphorylate their substrates, which are mainly transcription factors and which in turn trigger downstream reactions (Hill and Treisman, 1995; Karin and Hunter, 1995; Hazzalin and Mahadevan, 2002). In plants, MAPKs also play essential signaling roles,

mediating responses to various stress stimuli (reviewed in Hirt, 1997; Romeis, 2001; Zhang and Klessig, 2001). A growing body of evidence has revealed that in plants, MAPKs also regulate transcription factors at both transcription and protein activity levels (Andreasson et al., 2005; Kim and Zhang, 2004; Menke et al., 2005; Yap et al., 2005), and thus potentially mediate downstream stress-related responses.

Salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), two tobacco MAPKs and their orthologues in other plant species, are rapidly activated after various challenges, e.g., ozone (Samuel et al., 2000; Ahlfors et al., 2004), temperature (Jonak et al., 1996; Link et al., 2002; Sangwan et al., 2002), pathogens (Romeis et al., 1999; Asai et al., 2002; Jin et al., 2003), osmotic stress (Droillard et al., 2000; Mikolajczyk et al., 2000). Both SIPK and WIPK are activated after wounding. Using transgenic cultivated tobacco, Seo et al. (1995, 1999) demonstrated that WIPK regulates wound-induced JA biosynthesis and is systemically activated after stems are cut. Similarly, in wounded tomato plants, a 48 kDa MAPK is activated both locally and systemically (Stratmann and Ryan, 1997). MMK4, the orthologue of WIPK in alfalfa, is also quickly activated after wounding (Bogre et al., 1997). Biochemical analysis has demonstrated that SIPK and MPK4 are highly activated by wounding (Zhang and Klessig, 1998a; Ichimura et al., 2000). Although MAPKs are clearly involved in the wound response, it is unknown how they are involved in responses elicited by herbivore attack, particularly in plant species such as *N. attenuata*, where the wound response is known to be reconfigured during herbivore attack.

Here we demonstrate that in *N. attenuata*, applying *M. sexta* OS to puncture wounds in leaves dramatically amplifies the wound-induced increase in SIPK activity and *WIPK* transcripts and that FACs in *M. sexta* OS are the responsible elicitors. We show that both SIPK and WIPK are upstream signaling components regulating wound- and OS-elicited JA, salicylic acid (SA), JA-isoleucine/leucine conjugate (JA-Ile/JA-Leu), and ethylene biosynthesis. Transcriptional analyses revealed that SIPK and WIPK mediate the wounding- and OS-elicited accumulation of many defense-related genes, including 3 MAPKs and 4 calcium-dependent protein kinases (CDPKs); moreover, they even regulate each other's transcript accumulation, highlighting the complicated transcriptional cross-talk that occurs among protein kinases. After OS elicitation, a mobile signal quickly moves to particular undamaged regions of the elicited leaf and activates MAPK signaling and downstream responses. In contrast to the signaling observed within attacked leaves, a distinct signal travels to systemic distal leaves and activates defense-related responses without activating MAPKs.

Results

Herbivory activates MAPKs in *N. attenuata*

MAPKs are rapidly activated after mechanical wounding (Bogre et al., 1997; Zhang and Klessig, 1998a). To determine if MAPKs are involved in activating defense responses to herbivore attack, *N. attenuata* leaves were wounded with a pattern wheel; immediately thereafter puncture wounds were treated with water (W+W) or 5-fold diluted *M. sexta* OS (W+OS), and MAPK activity was analyzed by an in-gel kinase assay using myelin basic protein as a substrate (Figure 1A, top panel). After mechanical wounding, a 48 kDa protein kinase was activated which reached a maximum at 10 min and rapidly declined to a basal level within 1 h; in comparison, leaves wounded with W+OS dramatically increased the activity of 48 kDa-kinase within 10 min and sustained elevated levels for 2 h. The in-gel activity assay also showed that a 44 kDa kinase, although characterized by significantly less activity than the 48 kDa protein kinase, was also activated, but this activity was not enhanced by the W+OS treatment. An unidentified 40 kDa protein kinase having rather low levels of in-gel kinase activity was also activated 10 min after both W+W and W+OS treatments. Notably, W+W-treated plants showed higher activity levels of 40 kDa protein kinase for as long as 2 h than did W+OS-treated plants. Schittko et al. (2000) demonstrated that applying highly diluted *M. sexta* OS (up to 1000 fold) to *N. attenuata* elicits the same level of JA burst as using undiluted OS. We also tested the ability of diluted OS to activate MAPKs (Figure 1A, bottom panel). Consistent with the JA analysis (Schittko et al., 2000), the in-gel kinase activity assay showed that even a 1000-fold diluted OS significantly increased the levels of SIPK activity above those of the W+W treatment.

Since the SIPK and WIPK from cultivated tobacco (Seo et al., 1995; Zhang and Klessig, 1998a) are also 48 kDa and 44 kDa kinases, we silenced transcript levels of *N. attenuata*'s *SIPK* and *WIPK* by virus-induced gene silencing (VIGS) to confirm their identities (Ratcliff et al., 2001; Saedler and Baldwin, 2004). *Agrobacterium* was transformed with pTV-SIPK and pTV-WIPK constructs carrying cDNA fragments of *N. attenuata*'s *SIPK* and *WIPK*. Although these 2 fragments share certain homology, the longest perfect match is 16 nucleotides (nt) long. Given that a 22 or 23 nt perfect match is the minimum size capable of initiating RNAi (Thomas et al., 2001; Xu et al., 2006), neither construct had a co-silencing effect on the other's transcripts. Young *N. attenuata* leaves were inoculated with these construct-transformed *Agrobacterium*, generating *SIPK*-silenced (SIPK-VIGS) and *WIPK*-silenced (WIPK-VIGS) plants, respectively; plants inoculated with *Agrobacterium*, which carried empty vector pTV00, were used for comparison (EV). No morphological differences

associated with SIPK- or WIPK-silencing were observed. Eighteen days after inoculation, transcript levels of *SIPK* and *WIPK* were reduced by about 90% and 80%, respectively (Figure 1B). An in-gel kinase assay was performed to examine activity levels in various kinase-silenced plants (Figure 1C). Compared with EV plants, SIPK-VIGS plants had greatly reduced 48 kDa protein kinase levels; the 44 kDa kinase bands apparent in EV plants were not detected in WIPK-VIGS plants, indicating that the 48 kDa and 44 kDa protein kinases are SIPK and WIPK, respectively. Repeated kinase assay showed the same results (data not shown). Notably, levels of WIPK activity were higher in *SIPK*-silenced plants than in EV plants. This increase was likely a result of decreased competition between SIPK and WIPK for their common upstream kinase, MEK2 (Yang et al., 2001), which was also demonstrated in cultivated tobacco plants: after ozone exposure, *SIPK*-silenced plants had higher levels of WIPK activity than did WT plants, a difference which could be attributed to decreased competition for the binding to MEK2 (Samuel and Ellis, 2002).

A recent study of the cultivated tobacco *Ntf4* gene, a MAPK gene having high homology to *SIPK*, indicated that *Ntf4* is also involved in stress responses (Ren et al., 2006). The VIGS construct used for silencing *SIPK* contained a 35 nt perfect match with the *Naf4*, the orthologue of *Ntf4* in *N. attenuata*. Reportedly a match as small as 22 or 23 nt can trigger RNA interference (RNAi) (Thomas et al., 2001; Xu et al., 2006). We determined whether levels of *Naf4* transcripts in SIPK-VIGS plants differed from those in EV plants (Figure 1D). Even in untreated SIPK-VIGS plants, transcript levels of *Naf4* were 50% lower than in EV plants and even after both treatments, *Naf4* transcript levels remained several times lower than those in EV plants. This could have resulted from the co-silencing of *Naf4* by the pTV-SIPK construct. Strikingly, although levels of *Naf4* transcripts in untreated WIPK-VIGS plants did not differ from those in untreated EV plants, levels of *Naf4* transcripts in WIPK-VIGS plants were lower than those in EV plants in both treatments (Figure 1D). It is unlikely that silencing of *WIPK* co-silenced *Naf4*, given that pTV-WIPK and *Naf4* sequences have no matches longer than 20 nt (Thomas et al., 2001; Xu et al., 2006).

Wounding is known to elevate *WIPK* transcript levels in cultivated tobacco (Seo et al., 1995; Zhang and Klessig, 1998a). In *N. attenuata*, 0.5 h after wounding *WIPK* transcript levels were at their highest and when OS were added to wounds, this wound-induced maximum increased 1.5-fold ($P = 0.01$, unpaired *t*-test) (Figure 1E). Quantitative RT-PCR (q-PCR) measurement also showed that 1.5 h after W+W treatment, *SIPK* transcript levels were slightly elevated ($P = 0.011$, unpaired *t*-test) compared to those in untreated plants. W+OS increased SIPK transcript levels 2-fold after 1.5 h ($P = 0.0018$, unpaired *t*-test); afterward

these levels gradually decreased to those found in W+W treated plants (Figure 1F). We also measured transcript levels of *Naf4* after W+W and W+OS treatments (Figure 1G). Both treatments enhanced the levels of *Naf4* transcripts: 1.5 h after W+W treatment a significant increase in the transcript levels of *Naf4* was detected (P = 0.0083, unpaired t-test); and treatment with W+OS elicited even higher levels, which lasted 3 h. These data demonstrate that SIPK, WIPK, and Naf4 are involved in this plant-herbivore interaction.

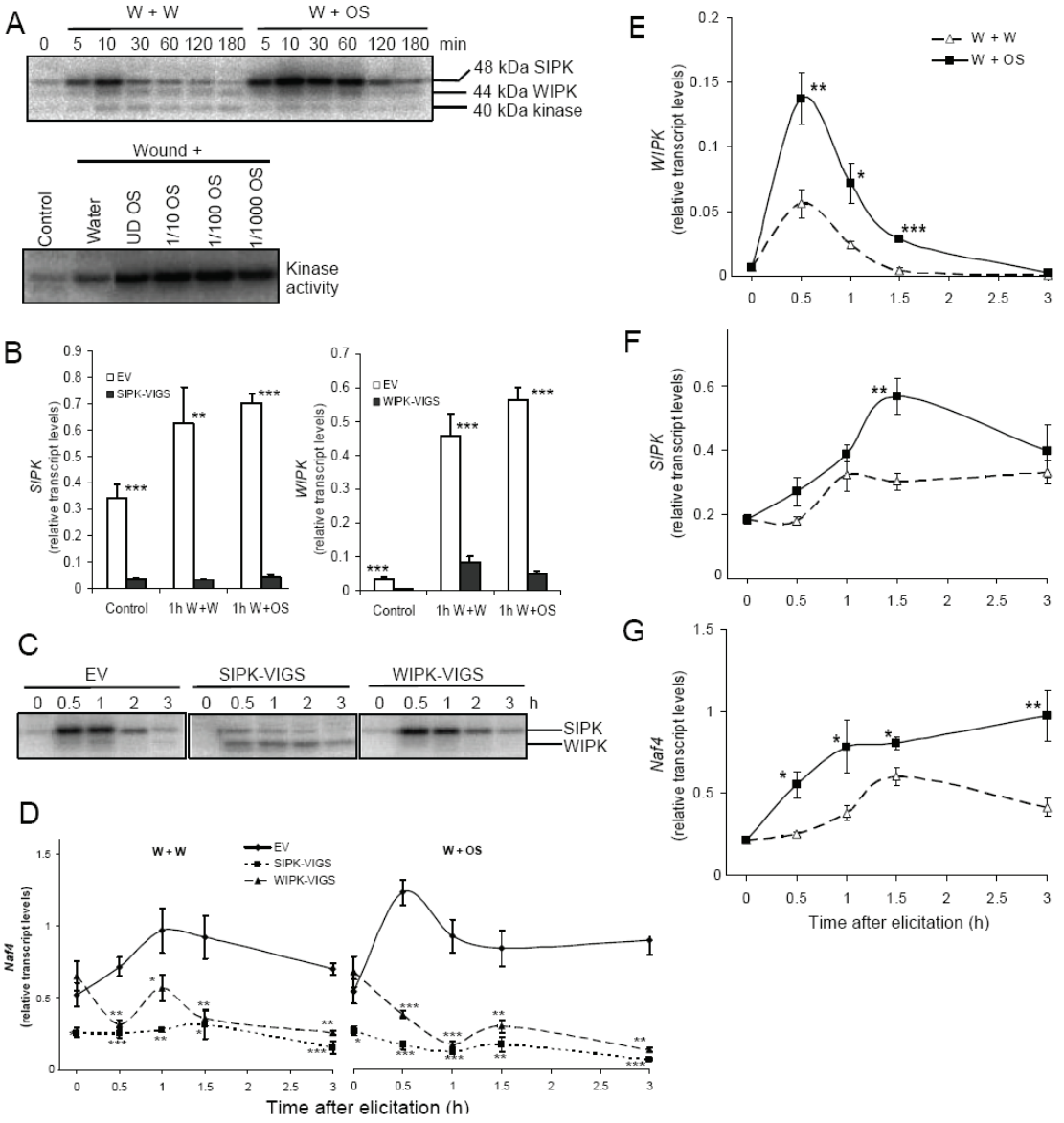

Figure 1. Applying *M. sexta* OS to *N. attenuata* leaves activates MAPKs.
(A) Top panel: *N. attenuata* leaves were wounded with a pattern wheel; 20 µL of water (W+W) or *M. sexta* oral secretions (OS, 1/5 diluted) (W+OS) was applied to the wounds and leaves from 4 replicate plants were harvested at the indicated times. Bottom panel: 20 µL of water, undiluted OS (UD OS), 1/10, 1/100, and 1/1000 diluted OS was applied to wounds and leaves from 4 replicate plants were harvested after 30min. Kinase activity was analyzed by an in-gel kinase assay using MBP as the substrate.
(B) *N. attenuata* plants were infiltrated with *Agrobacterium* carrying pTV00 empty vector, or constructs harboring a fragment of SIPK or WIPK to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Levels of SIPK and WIPK transcripts in SIPK-VIGS and WIPK-VIGS plants were measured with q-PCR (+ SE) using 5 replicated untreated (control), 1 h W+W- and W+OS-treated samples.

(C) EV, SIPK-VIGS, and WIPK-VIGS plants were treated with W+OS and collected at indicated times; 5 replicated samples were pooled and MAPK activity was detected with an in-gel kinase assay.
(D) Mean transcript levels (± SE) of *Naf4* in EV, SIPK-VIGS, and WIPK-VIGS plants after W+W and W+OS treatments as measured with q-PCR. Stars represent significantly different transcript levels between EV and VIGS plants at the indicated times (N = 5, two-way ANOVA, Fisher's PLSD *, P < 0.05; **, P < 0.01; ***, P < 0.001).
(E) to **(G)** Mean transcript levels (± SE) of *WIPK*, *SIPK*, and *Naf4* after W+W and W+OS treatments as measured with q-PCR in wild-type N. attenuata. Stars represent significant differences between transcript levels in samples treated with W+OS and W+W at the indicated times (N = 5, unpaired t-test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

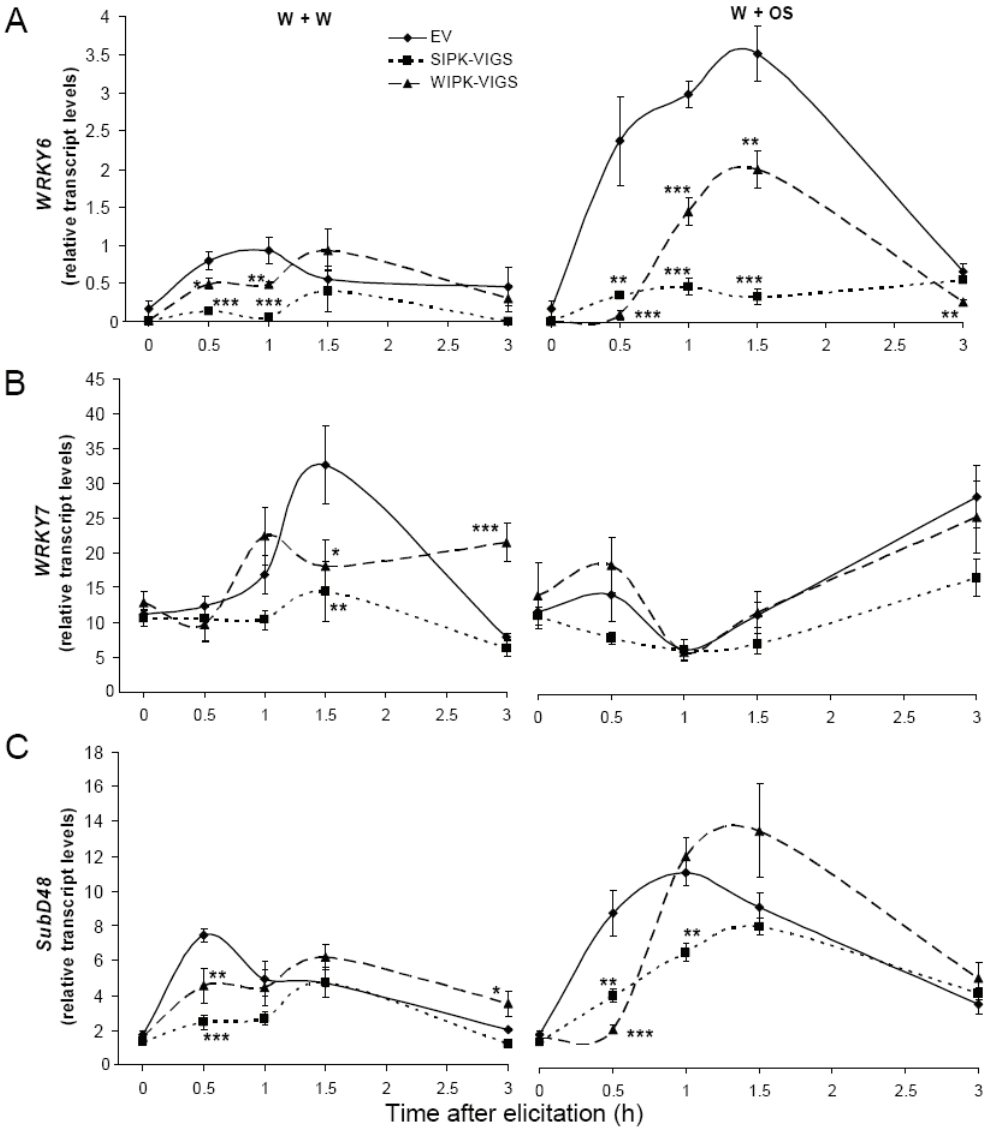

Figure 2. SIPK and WIPK regulate transcript accumulation of *WRKY* genes.
N. attenuata plants were inoculated with *Agrobacterium* carrying the pTV00 empty vector, or constructs harboring a fragment of *SIPK* or *WIPK* to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Leaves were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* oral secretions and regurgitants (OS) (W+OS) was applied to the wounds and harvested at the indicated times. Mean levels (± SE) from 5 replicate plants of *WRKY6* **(A)**, WRKY7 **(B)**, and SubD48 **(C)** transcripts in EV, SIPK-VIGS, and WIPK-VIGS plants after W+W and W+OS treatments were measured with q-PCR. Stars represent significantly different transcript levels between EV and SIPK-VIGS or WIPK-VIGS plants after the indicated times (N = 5, two-way ANOVA, Fisher's PLSD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

SIPK and WIPK regulate transcript levels of 3 *WRKY* transcription factors

WRKYs are an important family of transcription factors in plants which modulate both developmental and defense responses (Eulgem et al., 2000; Ulker and Somssich, 2004). MAPKs are known to be upstream regulators modulating the transcript accumulation of WRKY transcription factors (Kim and Zhang, 2004). In *N. attenuata*, we have identified WRKY6 as playing a central role in orchestrating FAC- and OS-elicited responses (Qu and Baldwin, in review). Here we determine whether OS-elicited MAPKs regulate the transcript levels of *WRKY6*, as well as of two additional *N. attenuata WRKY*s: *WRKY7* and *SubD48* (Figure 2A, B, C). In EV plants, OS elicitation increased *WRKY6* and *SubD48* transcript levels, which peaked after 1.5 h, but tended to suppress the wounding-induced increases in *WRKY7* transcripts. W+OS-treated SIPK-VIGS and WIPK-VIGS plants had significantly attenuated *WRKY6* and *SubD48* levels. Transcript levels of *WRKY6*, which are known to be FAC-elicited (Qu and Baldwin, in review), were greatly reduced in SIPK-VIGS and WIPK-VIGS plants; after 1.5 h, their levels were 10 and 50%, respectively, of the levels in EV plants. Similar reductions were found when these plants were treated with W+W, but the responses were smaller than with OS elicitation. Interestingly, in WIPK-VIGS plants, transcript levels tended to rebound after the initial suppression of the elicited responses, eventually attaining values higher (among *WRKY7* and *SubD48* in W+W treated plants) than those found in EV plants (Figure 2).

SIPK and WIPK mediate transcript accumulation of 3 MAPKs and 4 CDPKs

Liu et al. (2003) has elegantly demonstrated that SIPK mediates pathogen-elicited increases in *WIPK* transcript levels in cultivated tobacco. To determine if OS-elicited *WIPK* transcript levels are also mediated by SIPK, we measured transcript levels of *WIPK* in *SIPK*-silenced plants (Figure 3A). Untreated SIPK-VIGS plants had similar *WIPK* transcript levels compared to EV plants and transcript levels in EV plants increased almost 20 and 80 times 1.5 h after W+W and W+OS treatments, respectively. In SIPK-VIGS plants, *WIPK* transcript levels were only about 50 and 25% of those in EV plants after W+W and W+OS treatments. We conclude that OS-elicited activation of SIPK enhances levels of *WIPK* transcript in *N. attenuata*. *SIPK* transcript levels in WIPK-VIGS plants were significantly reduced compared with EV plants, even when untreated; both treatments slightly increased *SIPK* transcript levels in EV plants but not in WIPK-VIGS plants (Figure 3B).

To determine if other MAPKs are also transcriptionally regulated by SIPK and WIPK, transcript levels of *N. attenuata MPK4*, an orthologue of Arabidopsis *MPK4*, were measured

with q-PCR (Figure 3C). Levels of *MPK4* transcripts were about three times as high in untreated EV plants as in untreated SIPK- and WIPK-VIGS plants. Both W+W and W+OS treatments increased *MPK4* transcript levels in EV plant but not in SIPK- and WIPK-VIGS plants, except that 3 h after OS elicitation, transcript levels in SIPK-VIGS rebounded to those in EV plants.

Two *N. attenuata* MAPKs, *Naf3* and *Naf6*, which are orthologous to *Ntf3* and *Ntf6* of cultivated tobacco, were cloned and their transcript levels were measured (Figure 3D, E). In EV plants, *Naf3* levels increased significantly 0.5 h after W+W treatment (P = 0.010, unpaired t-test), then quickly declined before rebounding again by 1.5 h. OS treatment dramatically amplified the wound-induced 0.5 h peak in *Naf3* transcript levels, which rapidly declined. When untreated, EV and SIPK-VIGS plants showed similar levels of *Naf3* transcript, while levels in WIPK-VIGS plants were significantly reduced. After W+W treatment, lower levels of *Naf3* transcripts were detected in SIPK-VIGS and WIPK-VIGS plants compared with EV plants, except after 1 h when *Naf3* levels in EV plants transiently declined. W+OS-treated WIPK-VIGS plants had lower levels of *Naf3* transcripts than did EV plants, except after 3 h; levels in SIPK-VIGS plants were lower than in EV plants only at 0.5 h and significantly higher at 1.5 h.

In contrast to the response in *Naf3* transcripts, *Naf6* transcripts were not elevated. W+W and W+OS treatments gradually decreased *Naf6* transcript levels in EV plants. When untreated, SIPK-VIGS plants had significantly lower levels of *Naf6* transcripts, but WIPK-VIGS and EV plants had the same. Following W+W treatment, SIPK-VIGS and WIPK-VIGS plants had lower levels of *Naf6* transcripts than did EV plants before 3 h, when SIPK-VIGS plants still had low levels but levels in WIPK-VIGS plants returned to those found in EV plants. W+OS treatment significantly reduced levels in both SIPK-VIGS and WIPK-VIGS plants for up to 1 h.

In addition to MAPKs, CDPKs are also known to be involved in plant development and defense reactions (Romeis et al., 2000; Romeis et al., 2001; Ivashuta et al., 2005; Yoon et al., 2006). CDPKs are largely plant-specific, calcium-dependent, and calmodulin-independent protein kinases (reviewed in Cheng et al., 2002). We cloned *CDPK2*, *CDPK4*, *CDPK5,* and *CDPK8* from *N. attenuata* and measured the transcript accumulation profiles of these genes in EV, SIPK-VIGS, and WIPK-VIGS plants.

While *CDPK2* transcript levels were strongly suppressed in SIPK-VIGS plants (to 40% of those in EV plants), levels in WIPK-VIGS plants did not differ significantly from those in EV controls (Figure 4A). After W+W treatment *CDPK2* transcripts in SIPK-VIGS

plants remained low throughout the 3 h time course, but in OS-treated plants, levels returned to those found in EV plants by 3h.

In EV plants, OS elicitation increased levels of *CDPK4* transcripts; these reached maximum levels after 1.5 h whereas in W+W-treated plants, levels of *CDPK4* transcripts peaked as early as 0.5 h and after 1.5 h attained similar levels to those in OS-elicited plants (Figure 4B). Compared with EV plants, untreated SIPK-VIGS and WIPK-VIGS plants showed significantly lower levels of *CDPK4* transcripts. *CDPK4* transcript levels were lower in SIPK-VIGS plants than in EV plants for up to 3 h after both treatments. WIPK-VIGS plants, in contrast, showed a different profile: following W+W treatment their levels of *CDPK4* transcripts were lower than those of EV plants before 1.5 h but no difference was observed thereafter; whereas in W+OS-treated WIPK-VIGS and in EV plants, levels of *CDPK4* transcripts were similar as early as 0.5 h.

We also measured the transcript accumulation profiles of *CDPK5* (Figure 4C). After both treatments, EV plants dramatically increased the levels of *CDPK5* transcripts; W+OS treatment in particular led to an almost 20-fold increase after 1.5 h, while W+W treatment resulted in an almost 10-fold increase after 1 h. When untreated, both SIPK-VIGS and WIPK-VIGS plants showed similar levels of *CDPK5* transcripts compared to those in EV plants. However, after either elicitation, levels of *CDPK5* transcripts in both SIPK-VIGS and WIPK-VIGS plants were significantly lower than in EV plants for up to 3 h. After this time, *CDPK5* transcript levels were similar in all plants, except that W+W-treated SIPK-VIGS plants still showed slightly but significantly lower levels of *CDPK5* transcripts.

Similarly, substantially lower levels of *CDPK8* transcripts before and after either treatment were detected in SIPK-VIGS and WIPK-VIGS plants, compared to transcript levels in EV plants (Figure 4D). Following W+W treatment, EV plants had increased *CDPK8* transcript levels after 0.5 and 1.5 h, while following W+OS treatment, levels of transcripts significantly increased only after 0.5 h ($P = 0.0092$, unpaired *t*-test) and then quickly declined, as was seen in *Naf3* transcripts following the same treatment (Figure 3D). *CDPK8* transcript levels in SIPK-VIGS and WIPK-VIGS plants were significantly repressed throughout the time course, except that 3 h after W+OS treatment, *CDPK8* transcript levels in SIPK-VIGS plants rebounded to those in EV plants.

In summary, both SIPK and WIPK regulate the transcript levels of *MPK4*, *Naf3*, *Naf6*, *CDPK4*, *CDPK5*, and *CDPK8*. The transcript level of *CDPK2* is mediated by SIPK but not WIPK. Furthermore, SIPK and WIPK regulate each other's transcript levels.

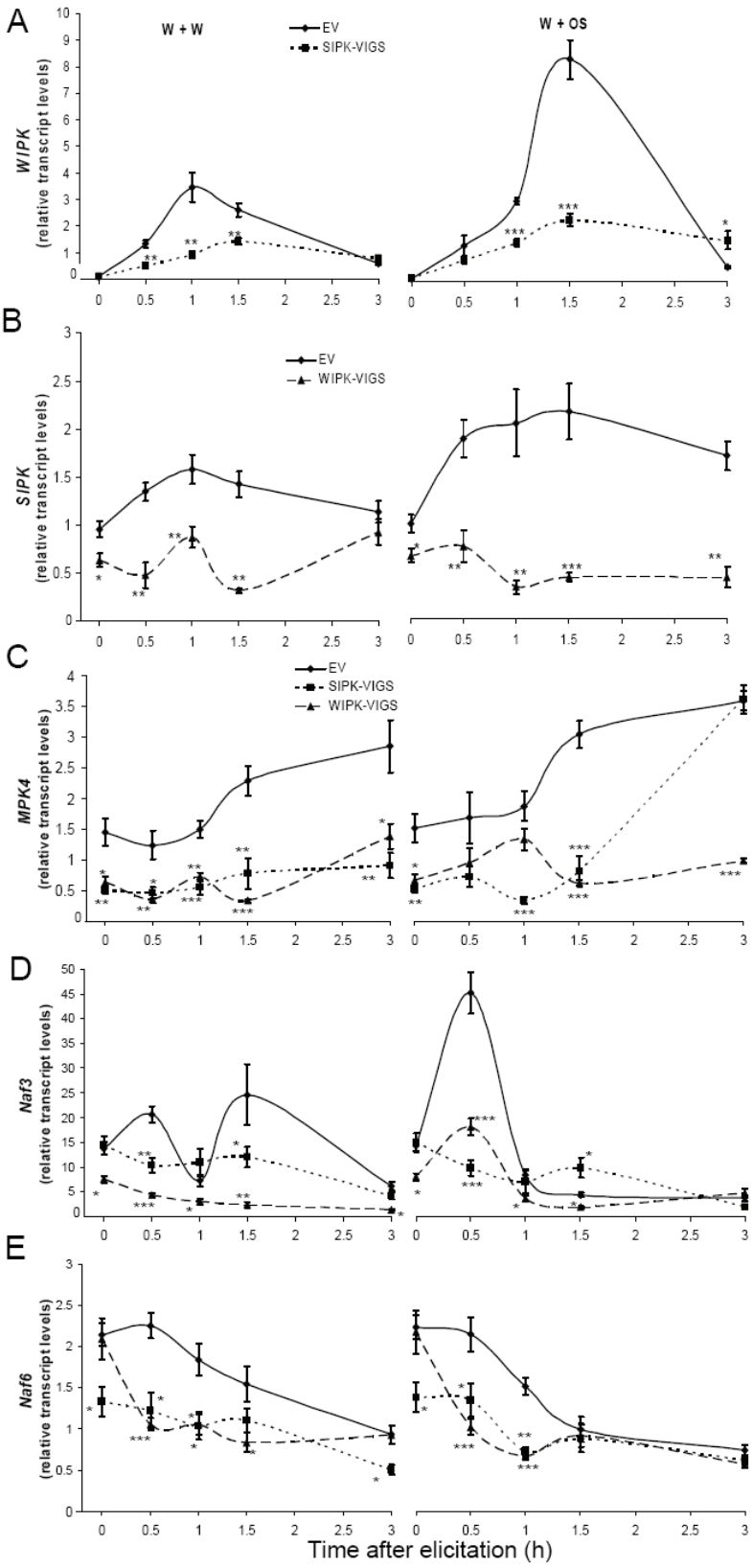

Figure 3. SIPK and WIPK regulate transcript accumulation of MAPKs.
N. attenuata plants were inoculated with *Agrobacterium* carrying the pTV00 empty vector, or constructs harboring a fragment of *SIPK* or *WIPK* to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Leaves were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* oral secretions (W+OS) was applied to the wounds. Individual leaves from 5 replicate plants were harvested at the indicated times. Stars represent significantly different transcript levels between EV and VIGS plants at the indicated times (A, B: unpaired *t*-test; C to F: two-way ANOVA, Fisher's PLSD *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(A) Mean *WIPK* transcript levels (± SE) in EV and SIPK-VIGS plants after W+W and W+OS treatments as measured with q-PCR.
(B) Mean transcript levels (± SE) of S*IPK* in EV and WIPK-VIGS plants after W+W and W+OS treatments as measured with q-PCR.
(C) to **(E)** Mean transcript levels (± SE) of *MPK4*, *Naf3*, and *Naf6* in EV, SIPK-VIGS, and WIPK-VIGS plants after W+W and W+OS treatments, respectively, as measured with q-PCR.

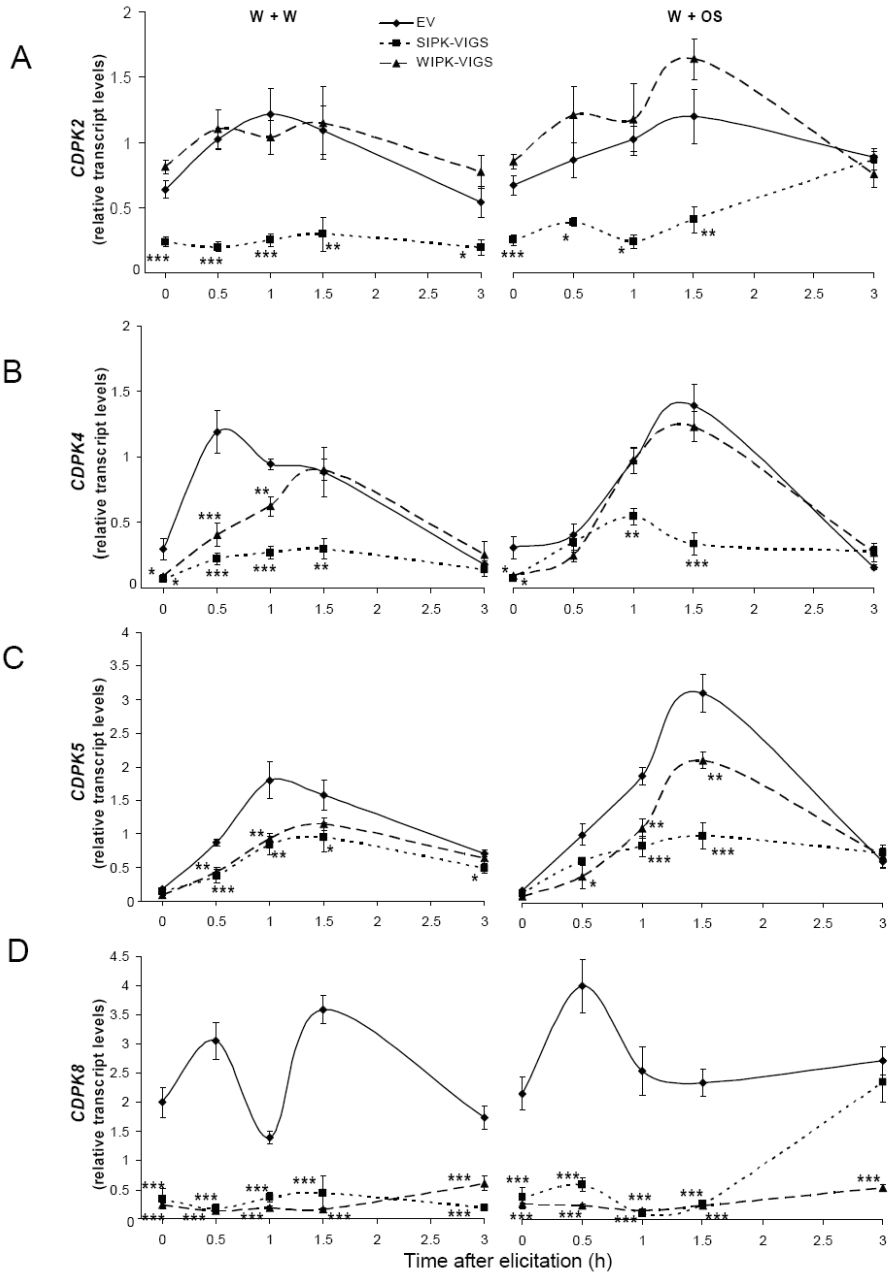

Figure 4. SIPK and WIPK mediate transcript accumulation of CDPKs.
N. attenuata plants were inoculated with *Agrobacterium* carrying the pTV00 empty vector, or constructs harboring a fragment of *SIPK* or *WIPK* to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Leaves were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* oral secretions (W+OS) was applied to the wounds. Individual leaves from 5 replicate plants were harvested at the indicated times.
(A) to **(D)** Mean (± SE) transcript levels in 5 replicate plants of *CDPK2*, *CDPK4*, *CDPK5,* and *CDPK8* in EV, SIPK-VIGS, and WIPK-VIGS plants after W+W and W+OS treatments were measured with q-PCR. Stars represent significantly different transcript levels between EV and SIPK-VIGS or WIPK-VIGS plants after the indicated times (two-way ANOVA, Fisher's PLSD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Silencing *SIPK* and *WIPK* attenuates OS-elicited JA, SA, JA-Ile/JA-Leu, and ethylene biosynthesis

JA, SA, JA-Ile, and ethylene are known to regulate a large number of genes related to plant defense responses (Dong, 1998; Reymond and Farmer, 1998; Farmer et al., 2003; Kang et al., 2006). Several studies have described an association between MAPK activation and stress-related phytohormone biosynthesis (Seo et al., 1995; Seo et al., 1999; Yang et al., 2001; Kim et al., 2003; Kim and Zhang, 2004). To determine whether wounding- and OS-activated MAPKs mediate the biosyntheses of wounding- and OS-elicited phytohormones, we measured the accumulation of JA, SA, JA-Ile/JA-Leu, and ethylene in EV and SIPK-VIGS and WIPK-VIGS plants.

W+OS treatment elicited higher JA levels than did W+W and these were remarkably reduced in SIPK-VIGS and WIPK-VIGS plants compared to EV plants. After either treatment in SIPK-VIGS plants, JA levels in particular were reduced by almost 80%; in contrast, JA levels in WIPK-VIGS plants were reduced by only about 40% (Figure 5A). In *N. attenuata*, *lipoxygenase 3* (*LOX3*) supplies the fatty acid hydroperoxides required for JA biosynthesis. The patterns of *LOX3* transcript accumulation were largely consistent with those of JA accumulation: levels of *LOX3* transcript were dramatically lower in both SIPK-VIGS and WIPK-VIGS plants compared to EV plants, with SIPK-VIGS plants showing the greater reductions (Figure 5B). Transcripts of *allene oxide synthase* (*AOS*), which is involved in a later step in JA synthesis, responded more gradually to elicitation. SIPK-VIGS plants had significantly lower levels of *AOS* transcripts than did EV plants, but levels in WIPK-VIGS plants were the same as those in EV plants or higher at 3 h (Figure 5C).

Seo et al. (1999) demonstrated that WIPK of cultivated tobacco is involved in wound-induced SA accumulation. To determine if this is also the case in *N. attenuata*, we measured SA levels in SIPK-VIGS, WIPK-VIGS, and EV plants. When untreated, SIPK-VIGS plants showed significantly lower levels of SA than did EV plants, whereas WIPK-VIGS plants and EV plants had similar levels (Figure 6A). One hour after W+W treatment, EV plants had slightly increased SA levels, but these quickly decreased to basal levels; both SIPK- and WIPK-VIGS plants had lower levels of SA than did EV plants up to 1 h; after that, levels of SA in both plants gradually reached the same as those in EV plants. W+OS elicitation dramatically increased SA levels by the 3 h harvest in EV plants, but this increase was suppressed in SIPK- and WIPK-VIGS plants. Notably, W+W and W+OS treatments didn't elicit significantly different SA levels in EV plants for up to 1.5 h ($P > 0.12$, unpaired *t*-test). *Isochorismate synthase* (*ICS*) is one of the key enzymes involved in SA biosynthesis.

Following either treatment, levels of *ICS* transcripts were lower in SIPK- and WIPK-VIGS plants compared with those of EV plants, except at the 3 h harvest, when levels of *ICS* transcripts in SIPK-VIGS plants rebounded to those in EV plants (Figure 6B).

JA-Ile is emerging as an important signal compound activating defense responses to herbivores (Kang et al., 2006; Staswick and Tiryaki, 2004). The elicited dynamics in JA-Ile/JA-Leu levels (Figure 7A) closely followed those of JA (Figure 6A), with the exception that JA-Ile/JA-Leu levels were not as reduced as the JA levels in WIPK-VIGS plants. In *N. attenuata*, JAR4 and JAR6 are enzymes conjugating JA, Ile and Leu to form JA-Ile and JA-Leu (Wang et al., 2007; Kang et al., 2006). q-PCR analyses revealed that transcripts of both genes were strongly elicited by either treatment in EV plants; SIPK-VIGS and WIPK-VIGS plants had significantly lower levels of *JAR4* and *JAR6* transcripts than did EV plants (Figure 7B). Although higher levels of JA-Ile/JA-Leu were found in WIPK-VIGS plants than in SIPK-VIGS plants after either elicitation, both had the same levels of *JAR4* and *JAR6* transcripts. A *threonine deaminase* (*TD*) gene in *N. attenuata* also plays important roles in JA-Ile biosynthesis, converting threonine to Ile at the wound site and thus supplying the amino acid for this conjugation reaction (Kang et al., 2006). After either treatment, *TD* transcripts were dramatically increased in EV plants and these increases were suppressed in SIPK-VIGS and WIPK-VIGS plants (Figure 7C). These data suggest that both SIPK and WIPK regulate the levels of *JAR4*, *JAR6*, and *TD* transcripts.

Manduca sexta herbivory and OS elicitation, but not wounding, increase ethylene biosynthesis and emissions in *N. attenuata* (Kahl et al., 2000). To determine if OS-elicited ethylene production correlates with the activation of MAPKs, we analyzed ethylene production in SIPK-VIGS, WIPK-VIGS, and EV plants. Leaves cut from petioles and subsequently treated with W+W or W+OS were sealed in 250 mL flasks for 5 h; the accumulated ethylene was measured with a photoacoustic laser spectrometer (Figure 8A). No detectable amount of ethylene was observed in any plant treated with W+W. After W+OS treatment both EV and WIPK-VIGS plants accumulated similar levels of ethylene, but SIPK-VIGS plants only accumulated about half as much, indicating that SIPK, but not WIPK, plays an important role in regulating ethylene biosynthesis after herbivory. In cultivated tobacco, elegant gain-of-function analyses have demonstrated that SIPK/WIPK activation mediates transcript accumulations of *ACC synthase* (*ACS*) and *ACC oxidase* (*ACO*) genes (Kim et al., 2003). In EV plants, both treatments strongly increased levels of *ACS3* to a similar degree*;* although OS treatment amplified the wound response for *ACO1* transcripts, *ACO3* transcripts did not respond (Figure 8B, C, D). When untreated, both WIPK-VIGS and SIPK-VIGS plants

had lower levels of *ACS3* transcripts (Figure 8B) and SIPK-VIGS plants had lower *ACO3* transcripts (Figure 8C) than did EV plants. After both treatments, SIPK-VIGS plants had significantly repressed transcript levels of both *ACO* genes for the duration of the time course, and for *ACS3* transcript, suppressed levels were found at all harvests with the exception of the 3 h harvest, when levels returned to those in EV plants. In contrast, WIPK-VIGS plants had only transiently lower *ACS3* transcript levels after OS elicitation (Figure 8B); all other ethylene biosynthetic transcripts were at levels comparable to (for W+OS elicitations) or higher than (for W+W elicitations) those found in EV plants. For example, *ACO1* transcript levels in WIPK-VIGS plants were 1.8 times higher 3 h after wounding than those in EV plant (P = 0.024, unpaired t-test), but OS elicitation abolished the difference between EV and WIPK-VIGS plants (Figure 8D). These data indicate that both SIPK and WIPK positively regulate OS-elicited JA, SA, JA-Ile, and ethylene biosynthesis.

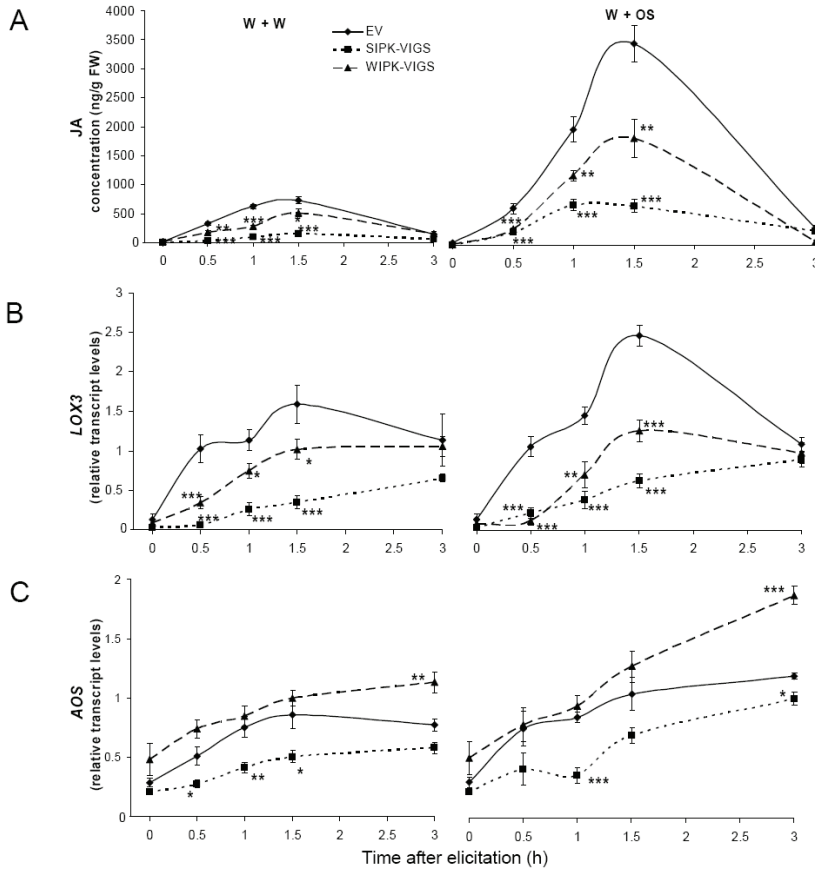

Figure 5. SIPK and WIPK mediate levels of JA and transcript accumulation of JA biosynthetic genes.
N. attenuata plants were inoculated with *Agrobacterium* carrying the pTV00 empty vector, or constructs harboring a fragment of *SIPK* or *WIPK* to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Leaves were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* oral secretions (W+OS) was applied to the wounds. Individual leaves from 5 replicate plants were harvested at the indicated times. Stars represent significantly different levels between EV and SIPK-VIGS or WIPK-VIGS plants after the indicated times (two-way ANOVA, Fisher's PLSD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
(A) Mean (± SE) JA concentrations were measured using HPLC-MS/MS.
(B) and **(C)** Mean transcript levels (± SE) of *LOX3* and *AOS* as measured with q-PCR.

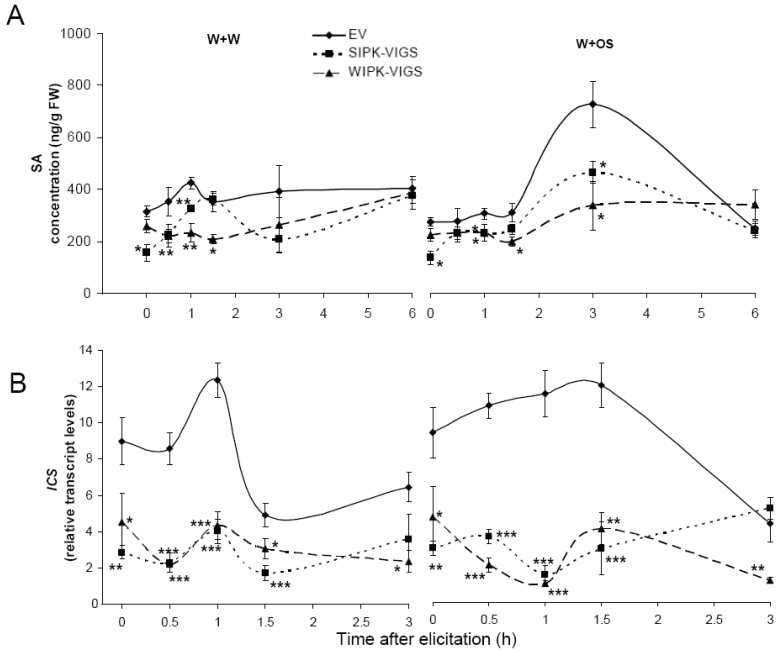

Figure 6. SIPK and WIPK mediate levels of SA and transcripts levels of *isochorismate synthase* (*ICS*).
N. attenuata plants were inoculated with *Agrobacterium* carrying the pTV00 empty vector, or constructs harboring a fragment of *SIPK* or *WIPK* to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Leaves were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* oral secretions (OS) (W+OS) was applied to the wounds. Individual leaves from 5 replicated plants were harvested at the indicated times after elicitation. Stars represent significantly different levels between EV and SIPK-VIGS or WIPK-VIGS plants at the indicated times (two-way ANOVA, Fisher's PLSD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **(A)** Mean (± SE) SA concentrations were measured using HPLC-MS/MS. **(B)** Mean transcript levels (± SE) of *ICS* in EV, SIPK-VIGS, and WIPK-VIGS plants after W+W and W+OS treatments were measured with q-PCR.

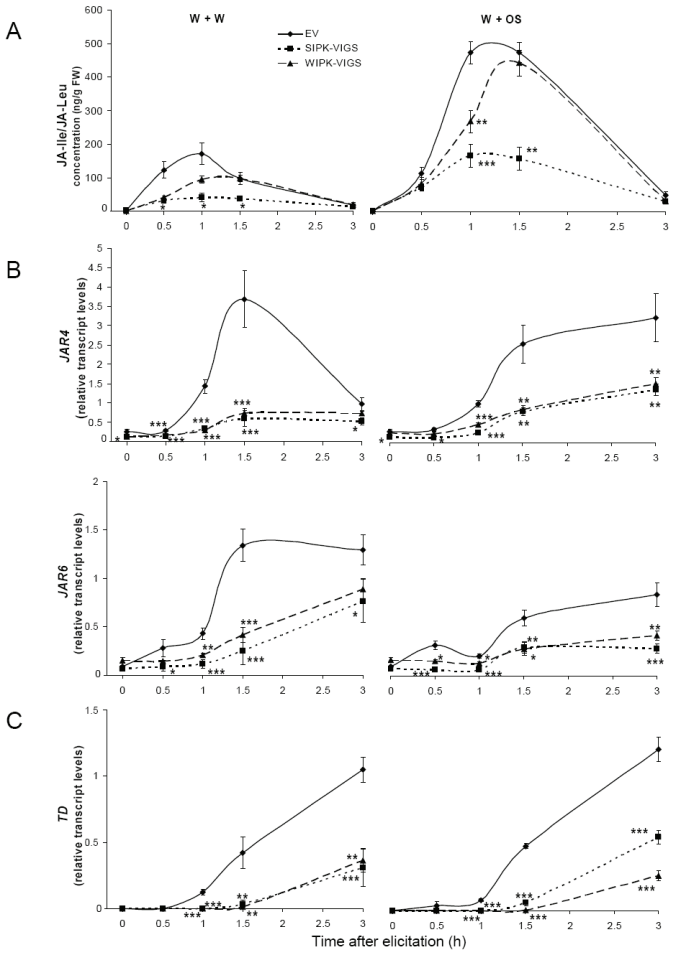

Figure 7. SIPK and WIPK mediate levels of JA-Ile/JA-Leu and transcript levels of genes involved in JA-Ile/JA-Leu biosynthesis.
N. attenuata plants were inoculated with *Agrobacterium* carrying the pTV00 empty vector, or constructs harboring a fragment of *SIPK* or *WIPK* to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Leaves were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* oral secretions (OS) (W+OS) was applied to the wounds. Individual leaves from 5 replicated plants were harvested at the indicated times after elicitation. Stars represent significantly different transcript levels between EV and SIPK-VIGS or WIPK-VIGS plants at the indicated times (two-way ANOVA, Fisher's PLSD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **(A)** Mean (± SE) JA-Ile and JA-Leu concentrations were measured using HPLC-MS/MS. **(B)** to **(C)** Mean transcript levels (± SE) of *JAR4*, *JAR6*, and *TD* in EV, SIPK-VIGS, and WIPK-VIGS plants after W+W and W+OS treatments as measured with q-PCR.

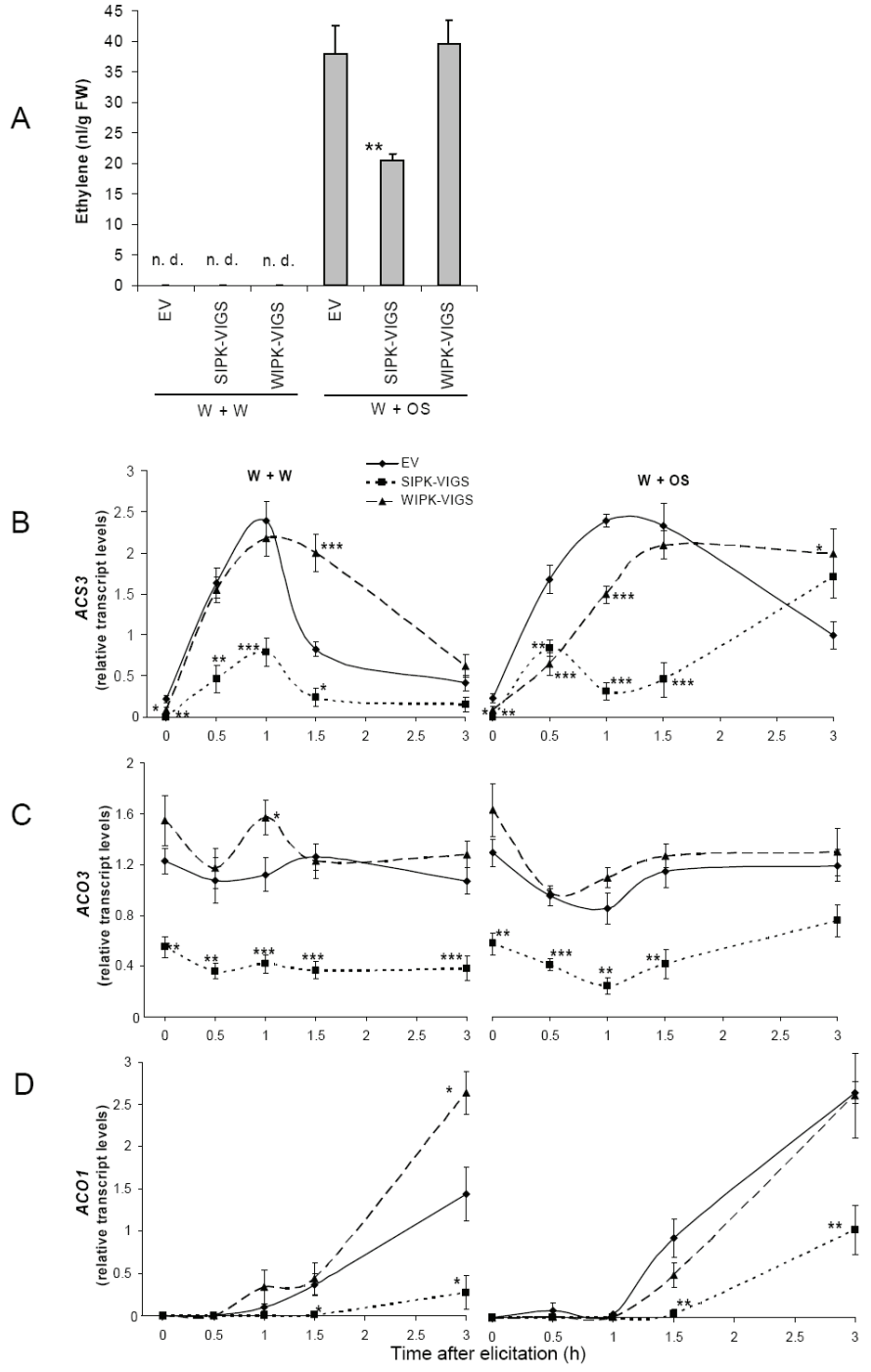

Figure 8. SIPK but not WIPK mediates ethylene biosynthesis.
N. attenuata plants were infiltrated with *Agrobacterium* carrying the pTV00 empty vector, or constructs harboring a fragment of *SIPK* or *WIPK* to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Stars represent significant differences between EV and SIPK-VIGS or WIPK-VIGS plants (two-way ANOVA, Fisher's PLSD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **(A)** Mean (± SE) ethylene accumulated. Leaves were wounded with a pattern wheel; 20 µL of water (W+W) or *M. sexta* oral secretions (W+OS) was applied to the wounds and leaf samples were collected in 250 mL flasks. After 5 h, ethylene produced in 4 replicate plants was measured with a photoacoustic laser spectrometer. **(B)** to **(D)** Leaves were wounded with a pattern wheel; 20 µL of water (W+W) or *M. sexta* oral secretions (W+OS) was applied to the wounds. Individual leaf samples from 5 replicate plants were harvested at indicated times. Average transcript levels (± SE) of *ACS3, ACO3* and *ACO1* in EV, SIPK-VIGS, and WIPK-VIGS plants after W+W and W+OS treatments were measured with q-PCR.

JA and ethylene signaling do not mediate OS-elicited MAPK activity

Research with cell suspension cultures and in-gel kinase assays has shown that treatment with methyl jasmonate (MeJA) or ethylene activates kinases (Kumar and Klessig, 2000; Ouaked et al., 2003). To determine whether this is also the case with intact plants, we analyzed kinase activity and *WIPK* transcript accumulation in *N. attenuata* plants which had been treated with MeJA and ethephon; both of these readily diffuse into plants to release JA and ethylene. Up to 1 h after treatment, slightly increased levels of *WIPK* transcripts and SIPK activity were detected in MeJA- and ethephon-treated plants; however, these levels were not higher than those in lanolin- and MES-treated plants, their respective controls. These increased levels are likely to have resulted from the plants being touched when these chemicals were applied. Even after 6 h, MeJA and ethephon treatments didn't elevate SIPK or WIPK activity or *WIPK* transcript levels more than their respective controls (Supplemental Figure 1A), although applying MeJA dramatically elicited the JA-responsive gene *TPI*, and ethephon effectively released a large quantity of ethylene as measured by a photoacoustic spectrometer (Supplemental Figures 1A, 2). MAPK activity was also analyzed in transgenic plants impaired in their JA and ethylene signaling. We used an antisense-*LOX3* line (asLOX3) that elicited only 50% of the JA produced by W+OS elicitation in WT *N. attenuata* plants (Halitschke and Baldwin, 2003), and a *COI1*-silenced line (irCOI1), whose ability to perceive JA was remarkably reduced (Paschold et al., 2007). To study the impact of ethylene production and perception on MAPK activation, we used an *ACC oxidase*-silenced line (irACO) of *N. attenuata,* which produced substantially less ethylene after OS elicitation than WT plants did, and a line overexpressing a mutant Arabidopsis *ETR1* gene, whose ethylene perception was highly attenuated (ETR1) (von Dahl, et al., 2007). After these transgenic plants were challenged with W+OS, in-gel kinase activity assays revealed that their levels of MAPK activity were the same as those of WT plants (Supplemental Figure 1B). All these data suggest that JA and ethylene signaling do not mediate OS-elicited MAPK activity levels in *N. attenuata* plants.

Silencing *SIPK* and *WIPK* leads to decreased levels of TPI activity and *phenylalanine ammonia-lyase* mRNA

TPI is an anti-digestive protein which is strongly OS-elicited in *N. attenuata* and functions as a direct defense by slowing *M. sexta* growth and increasing its mortality (Ryan, 1990; Glawe et al., 2003; Zavala et al., 2004). Knowing that JA and ethylene strongly modulate TPI levels in numerous solanecous taxa (O'Donnell et al., 1996; Koiwa et al., 1997;

Halitschke and Baldwin, 2003), and that silencing MAPK expression attenuates OS-elicited JA and ethylene, led us to hypothesize that TPIs would be impaired in SIPK- and WIPK-VIGS plants. W+OS-treated EV plants significantly increased TPI levels compared to W+W and untreated EV plants. As predicted, W+OS-treated SIPK-VIGS and WIPK-VIGS plants showed only about 50% of the TPI activity found in W+OS-treated EV plants, but no significant differences were found among any control or W+W-treated plants (Figure 9A).

Phenylalanine ammonia-lyase (*PAL*) is another gene important for plant defense (Bennett and Wallsgrove, 1994). We investigated *PAL* transcript levels in EV, SIPK-VIGS, and WIPK-VIGS plants (Figure 9B) and found they were suppressed in SIPK-VIGS plants but not in WIPK-VIGS plants. In SIPK-VIGS plants, suppression lasted for the duration of the experiment, except in OS-elicited plants, levels of *PAL* transcripts returned to those of EV plants by 3h. WIPK-VIGS plants had significantly higher levels of *PAL* transcripts than did EV plants 3 h after either elicitation, suggesting that WIPK negatively regulates *PAL* transcript levels.

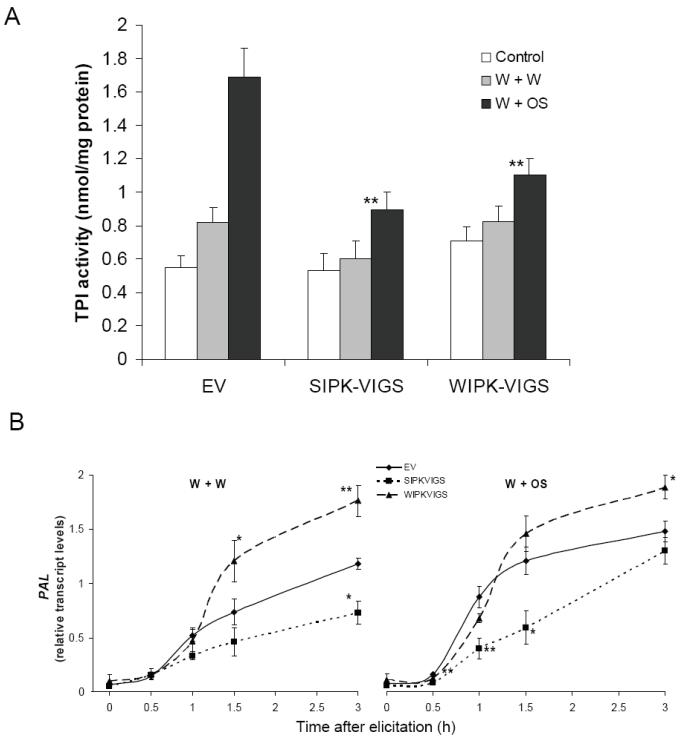

Figure 9. SIPK and WIPK mediate levels of TPI activity and transcript levels of *Phenylalanine ammonia-lyase* (*PAL*).
N. attenuata plants were inoculated with *Agrobacterium* carrying the pTV00 empty vector, or constructs harboring a fragment of *SIPK* or *WIPK* to generate EV, SIPK-VIGS, and WIPK-VIGS plants, respectively. Stars represent significant differences between EV and SIPK-VIGS or WIPK-VIGS plants after the specific treatments (N = 5, two-way ANOVA, Fisher's PLSD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **(A)** Mean (+ SE) TPI activity in EV, SIPK-VIGS, and WIPK-VIGS plants. Leaves were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* oral secretions (W+OS) was applied to the wounds and leaves from 5 replicated plants were harvested individually 3 days after treatments, untreated plants served as controls. **(B)** Mean transcript levels (± SE) of *PAL* in EV, SIPK-VIGS, and WIPK-VIGS plants after W+W and W+OS treatments as measured with q-PCR. Leaves were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* OS (W+OS) was applied to the wounds.

FACs in *M. sexta* OS are elicitors that activate herbivory-specific MAPK signaling

Microarray and biochemical analyses indicated that the FACs in *M. sexta* OS are responsible for eliciting OS-specific JA, VOCs, and transcriptional responses in *N. attenuata* (Halitschke et al., 2001; Halitschke et al., 2003). To determine if FACs activate MAPKs in *N. attenuata*, synthetic FACs were applied to wounded *N. attenuata* leaves. N-linolenoyl-l-Gln (FAC A), N-linolenoyl-l-Glu (FAC B), N-linoleoyl-l-Gln (FAC C), and N-linoleoyl-l-Glu (FAC D), the 4 most abundant FACs in OS which are found at concentrations of 0.6 to 1.2 mM in *M. sexta* OS, potently elicit herbivory-specific responses in *N. attenuata* (Halitschke et al., 2001). In-gel kinase activity assays demonstrated that all FACs at concentrations of 0.2 mM, which are similar to the concentrations of FACs in the 5-fold diluted OS we used for W+OS treatments, elicited levels of SIPK activity that were several times those elicited by W+W or 10% DMSO, the solvent for the FAC treatments (Figure 10A). Furthermore, removing FACs by ion-exchange chromatography made the OS no more able than water to elicit SIPK. The activity assay also revealed that all FACs were similarly able to activate SIPK. Consistent with the finding that W+OS does not elicit higher levels of WIPK activity compared to W+W, plants treated with wounding and various FACs showed levels of WIPK activity similar to those in plants treated with wounding and OS, 10% DMSO, FAC-free OS, or water (Figure 10A).

Consistent with the activity assay, northern blotting analysis revealed that levels of *WIPK* transcript were highly amplified when different FACs were applied to puncture wounds compared to when both 10% DMSO, the solvent for FACs, and the pure water treatments were used (Figure 10B). Similar levels of *WIPK* transcripts were found when leaves were treated with FAC-free OS and with W+W. Northern blotting also demonstrated that FAC-treated and W+OS-treated samples accumulated comparable levels of *WIPK* transcripts. Furthermore, we measured the JA bursts elicited by different FAC treatments. One hour after elicitation, all plants treated with OS or with different FACs had accumulated similarly elevated levels of JA ($P = 0.69$, ANOVA), levels that were about 1.5 times higher than those in water-, 10% DMSO-, and FAC-free-OS-treated plants (Figure 10C). These data strongly support the idea that FACs are important elicitors in *M. sexta* OS; FACs are recognized by *N. attenuata* and in turn trigger downstream responses by activating MAPKs.

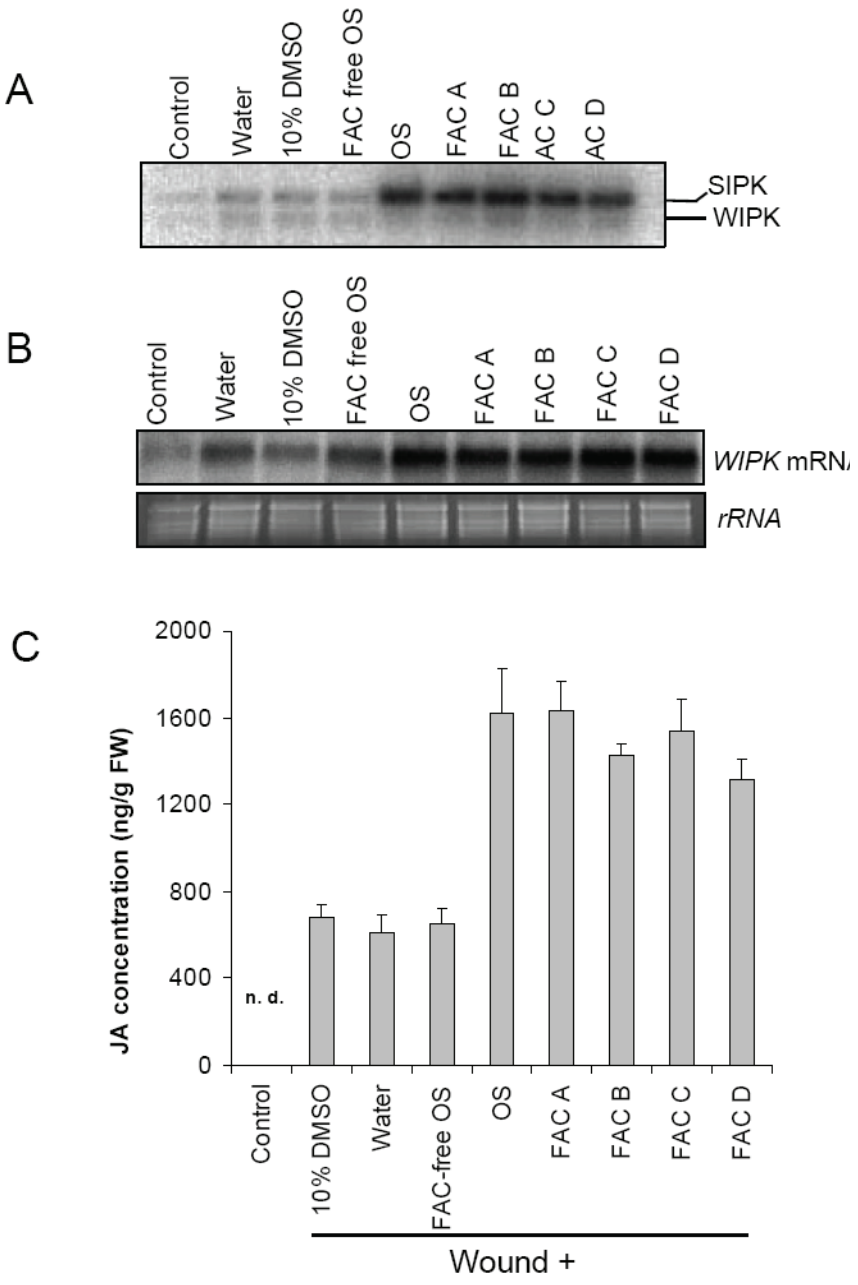

Figure 10. Fatty acid-amino acid conjugates (FACs) in *M. sexta* OS elicit MAPK activity and jasmonic acid burst.
N. attenuata leaves were wounded with a pattern wheel and 20 µL of the following solutions was immediately applied to the puncture wounds: water, 10% DMSO, FAC-free OS, OS, FAC A, B, C, and D dissolved in 10% DMSO; untreated and unwounded plants were used as controls. **(A)** Four replicate samples were harvested 30 min after each treatment and pooled; an in-gel kinase assay was performed to test each treatment's ability to activate MAPKs. (**B)** Total RNA was extracted from samples 1 h after elicitation; *WIPK* transcript accumulation was determined by northern blotting from pooled 4 replicates. **(C)** Mean (+ SE) JA concentrations in 5 replicate leaf samples 1 h after elicitation were measured by HPLC-MS/MS (n.d. = not detected).

OS elicitation of MAPKs at the wound site and in systemic tissues within a leaf

M. sexta larvae typically spend hours feeding at the same location before moving on to other regions of the leaf on which they were oviposited (for the first two instars), or to other leaves (in later instars) (McFadden, 1968; Kessler and Baldwin, 2002b). Herbivory triggers defense responses in adjoining attacked tissues as well as in unattacked leaves sharing vascular connections with the attacked leaves (Schittko and Baldwin, 2003; Orians, 2005). Yet, how the responses are conveyed to non-attacked regions within an attacked leaf has not been well studied (Schittko et al., 2000). We divided fully expanded S0 leaves from bolting plants into 4 regions, along each leaf's midrib vein and perpendicularly at the middle of each midrib vein; the 4 regions were designated 0, 1, 2, and 3 (Figure 11A). In 4 replicated plants, either regions 0 or 1 were treated with W+OS, and all regions were harvested at different times.

Remarkably, W+OS application in regions 0 and 1 elicited SIPK activity in distinct regions of the leaf (Figure 11B). When region 0 was elicited, high levels of SIPK activity were observed in treated region 0; slightly lower levels were detected in region 1; SIPK in region 3 were also activated, although levels were much lower than those in regions 0 and 1; in region 2, no elevation of SIPK activity was observed. Over time, SIPK activity levels in all regions waned, and region 2 remained unelicited throughout. Eliciting region 1 produced a distinct distribution of kinase activity: SIPK activity levels increased greatly in region 1 but only slightly in region 0. However, SIPK activity levels in region 3 also highly increased, reaching those of region 1. Similarly, region 2 did not increase SIPK activity levels when region 1 was treated with OS. These findings suggest that certain mobile signal(s) move(s) to various regions of the herbivore-attacked leaf and elicit SIPK activity.

The duration of the response was also influenced by the region that was elicited. Elicited kinase levels lasted longer when leaves were treated in region 1. Even in leaves that had been elicited 1.5 h earlier in region 1, SIPK activity levels were higher than in leaves elicited in region 0 (Figure 11B, kinase activity panels). These results suggest that there might be less negative SIPK-regulator activity at the tip than at the base of a leaf. The pattern of *WIPK* transcript accumulation followed the pattern of SIPK activity: regions 0 and 1 showed greatly elevated *WIPK* transcript levels when leaves were elicited in 0, and regions 1 and 3 showed highly elevated *WIPK* transcript levels when region 1 was elicited (Figure 11B), confirming the idea that *WIPK* transcript accumulation is regulated by SIPK. Moreover, in undamaged regions of the herbivore-attacked leaf, WIPK is also likely involved in herbivore resistance.

To examine how fast the mobile signal(s) move(s) across the leaf, we analyzed the spatial distribution of SIPK activity shortly after elicitation in S0 leaves (Figure 11C). When leaves were treated in region 0, region 1 showed elevated SIPK activity levels as soon as 5 min afterwards. Similarly, when leaves were treated in region 1, region 3 also showed increased levels of SIPK activity after only 5 min. After 10 min, SIPK activity levels in leaves treated in either region 0 or 1 were similar to those harvested after 30 min.

We compared SIPK activity levels in leaves at different developmental stages (Figure 11D). When OS were applied to region 0, after 30 min both younger (S3) and older (S0) leaves had elevated SIPK activity levels in both regions 0 and 1. Notably, eliciting region 1 in older leaves rapidly activated SIPK activity levels in 1 and 3. Region 3 of younger leaves showed only slightly enhanced SIPK activity levels after 30 min, suggesting that the maturation of the vascular system that comes with age allows the OS-elicited responses to be propagated more vigorously within an elicited leaf.

JA, SA, and ethylene are known to regulate the transcript levels of defense genes in many plant species (Dong, 1998; Reymond and Farmer, 1998). The data obtained from SIPK-VIGS and WIPK-VIGS plants reveal a correlation between OS-elicited MAPK activation and the accumulation of phytohormones. Thus, northern blotting was carried out to examine the spatial patterns of OS-elicited transcript accumulation for *LOX3*, *PAL*, and *ACO1,* genes important in JA, SA, and ethylene biosynthesis, respectively (Figure 11B). After OS were applied, the transcript accumulation patterns of *LOX3*, *PAL,* and *ACO1* resembled those of kinase activation albeit with different kinetics. Elicitation of region 0 quickly increased *LOX3* and *PAL* transcript levels. As with the kinase activity, the levels of both genes were highest in elicited region 0 and adjacent region 1; region 3 only showed transiently enhanced levels of both transcripts. In contrast, the elicitation of region 1 resulted in similar transcript levels of induced *LOX3* and *PAL* in regions 1 and 3 but only slightly enhanced levels in region 0, a pattern that resembled the distribution of SIPK activity. Neither treatment affected *LOX3* or *PAL* transcript levels in region 2 at any time. Long-lasting kinase activity obtained by applying OS to region 1 prolonged the levels of *LOX3* and *PAL* transcripts as well. *ACO1* showed a slower kinetic than the other 2 genes, which reached maximum levels at 3 h; nevertheless, the pattern of its transcript accumulation in different regions resembled that of SIPK activity.

To investigate if the spatial pattern of JA levels also matched the distribution of *LOX3* accumulation, JA levels produced in different regions were examined 1 h after treating either region 0 or 1 with W+OS, which is when WT plants accumulate the most JA (Figure 11E).

Leaves in which region 0 had been elicited showed elevated JA levels in regions 0 and 1; remarkably, region 0 showed almost 7 times more JA than adjacent region 1, though such a dramatic difference did not characterize levels of SIPK activity and *LOX3* transcripts in these 2 regions. When leaves were treated in region 1, JA was detected only in regions 1 and 3, which was also consistent with the spatial distribution of SIPK activity and *LOX3* transcript levels. Similarly, region 3 had about 1.5 times less JA than region 1. However, JA levels in OS-treated region 1 were 3.5 times lower than in OS-treated region 0. JA levels in regions 2 and 3 in leaves with OS-treated region 0, and in regions 0 and 2 in leaves with OS-treated region 1, were not detectable.

TD and *TPI* are two genes involved in plants' direct defenses against herbivores (Zavala et al., 2004; Chen et al., 2005; Kang et al. 2006) and both are regulated at least in part by JA (Hermsmeier et al., 2001; Glawe et al., 2003). After regions 0 and 1 were elicited, transcript levels of *TD* and *TPI* genes were analyzed by northern blotting in samples harvested after 3 h and 12 h, respectively, which is when transcript levels are at their highest (Kang et al., 2006; Wu et al., 2006) (Figure 11F). The spatial distribution of *TD* transcript levels 3 h after elicitation largely resembled that of JA's, except that although JA was not detectable in region 0 of leaves treated in region 1, the level of *TD* transcripts in region 0 was comparable to *TD* transcript levels in both regions 1 and 3. When leaves were treated in region 0, high levels of *TPI* were observed in both regions 0 and 1, while regions 2 and 3 showed comparatively lower levels, albeit higher than those in non-induced samples. All regions in leaves elicited in region 1 had similar levels of *TPI* transcripts, and these levels were comparable to levels in regions 0 and 1 when leaves were elicited in region 0.

Systemic TPI elicitation doesn’t require SIPK activation in systemic leaves

M. sexta attack increases levels of *TPI* transcripts and activity in *N. attenuata*. This response is not limited to attacked leaves but spreads throughout the plant (van Dam et al., 2001; Zavala et al., 2004). In tomato, wounding has been found to activate MAPKs both locally and systemically (Stratmann and Ryan, 1997); wounding tobacco leaves with carborundum quickly increases levels of *WIPK* transcript in systemic leaves; cutting tobacco stem activates WIPK systemically as well (Seo et al., 1999). To investigate if MAPK signaling is involved in systemic *TPI* induction in *N. attenuata*, leaves at node +1 from plants at the rosette stage were elicited with W+OS. Kinase activity was assayed in both treated local and untreated systemic leaves at node -1. In contrast to the high SIPK activity levels in elicited leaves, no elevation of SIPK activity was detected in systemic leaves (Figure 12A).

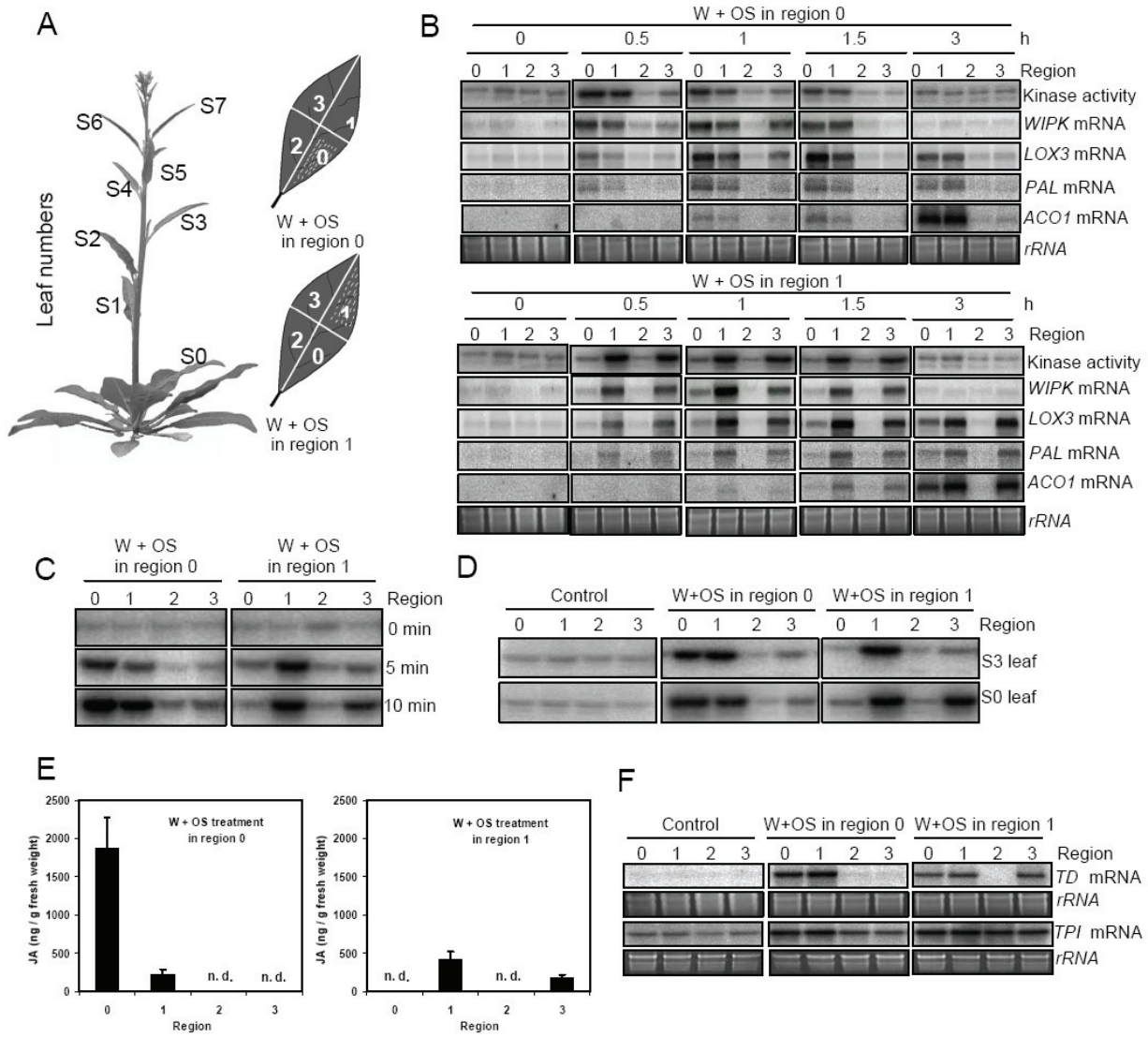

Figure 11. Spatial distribution of OS-elicited responses within single leaves growing at different nodes.
Experiments were conducted on leaves growing at either S0 or S3 nodes from 40-day-old bolting wild-type *N. attenuata* plants. Four replicate leaves from different plants were used for each treatment.
(A) Numbering of the leaves at different phyllotaxic positions (nodes) on bolting plants and illustration of treatments at different leaf regions. Wounds are illustrated with dotted lines; each leaf was wounded with a pattern wheel, and 10 µL *M. sexta* oral secretions (W+OS) was applied to either region 0 or 1. Leaves were harvested in four sections at the specified times.
(B) Spatial distribution of W+OS-elicited responses in S0 leaves. Total protein and RNA were extracted from S0 leaves. Kinase activity was determined by an in-gel kinase assay using MBP as the substrate. Transcript levels of *WIPK, LOX3, PAL,* and *ACO1* were examined with northern blotting.
(C) Spatial distribution of W+OS-elicited SIPK activity in S0 leaves shortly after elicitation. S0 leaves were treated with W+OS; after 5 min and after 10 min samples were collected. Kinase activity was analyzed using an in-gel assay.
(D) Spatial distribution of W+OS-elicited SIPK activity in S0 and S3 leaves. Both S0 and S3 leaves were treated with W+OS and harvested after 30 min. The spatial distribution of kinase activity was analyzed by an in-gel kinase assay.
(E) Mean (± SE) JA concentrations in 4 replicated samples collected 1 h after W+OS treatment (n.d. = not detected).
(F) In W+OS-treated S0 leaves, the accumulation of *TD* and *TPI* transcripts after 3 h and 12 h, respectively, was determined by northern blotting.

Northern blotting also revealed that no systemic induction of *WIPK* took place even after 96 h, although both local and systemic samples had strongly elevated levels of *TPI* transcripts (Figure 12B). To investigate whether applying OS to wounds suppresses systemic MAPK responses elicited by wounding and whether systemic leaves other than -1 leaves have any elevated MAPK activity, we treated *N. attenuata* plants with W+W and W+OS, and examined kinase activity and *WIPK* transcript accumulation in +1 (local) and -1, +2 (systemic) leaves (Supplemental Figure 3). Up to 1 h, neither activation of SIPK and WIPK nor elevation of *WIPK* transcript levels was detected in systemic leaves. These data suggest that the systemic induction of *TPI* doesn't require the activation of SIPK in systemic leaves.

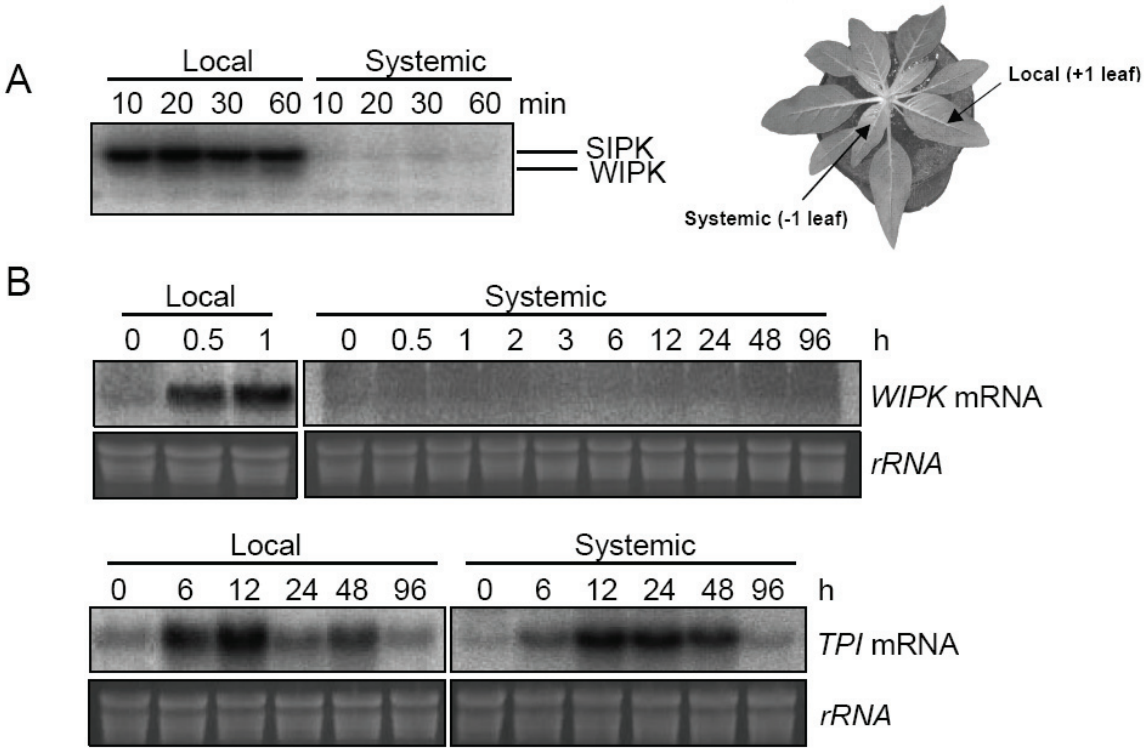

Figure 12. *TPI* transcript accumulation in systemic leaves doesn't require activating SIPK in systemic leaves.
Leaves at node +1 from rosette-stage *N. attenuata* were wounded with a pattern wheel; 20 µL of *M. sexta* oral secretions (OS) was applied to the wounds. Treated leaves (+1, local) and systemic untreated leaves (-1) were harvested at indicated times. Four replicate leaves were pooled after harvesting.
(A) Kinase activity assay in both local and systemic leaves after elicitation.
(B) Transcript accumulation analyses of *WIPK* and *TPI* in local and systemic leaves by northern blotting.

Discussion

JA and ethylene are known to play a central role in mediating a plant's responses to herbivore attack, which involves reconfiguring a plant's wound response (reviewed in Kessler and Baldwin, 2002a; Reymond and Farmer, 1998). How plants perceive herbivory and along which signaling pathways the messages are transformed into cellular responses, are poorly understood. Here we demonstrate that applying *M. sexta* OS to wounds in *N. attenuata* leaves rapidly and markedly elicits MAPKs more than wounding does. By removing FACs from OS and applying synthetic FACs, we demonstrate that FACs elicit these responses. Silencing *SIPK* and *WIPK* by VIGS revealed that both kinases regulate the transcript levels not only of *WRKY* transcription factors, MAPKs, and CDPKs but also of JA and ethylene, which highlights their central role in the OS-elicited, hormonally-mediated, signal transduction network. By applying JA and ethylene and using transgenic plants impaired in the production and perception of JA and ethylene, we show that neither OS-elicited JA nor ethylene production contributes to OS-elicited MAPK activation.

OS elicitation activates MAPKs and other defense responses not only in the damaged regions of a leaf but also in particular non-damaged regions. The spatial distribution of these responses depends on the developmental stages of the leaves and on the locations of herbivore damage, both of which implicate vascular maturation in the signaling. In addition to these responses in the attacked leaf, enhanced levels of *TPI* transcripts were found in unelicited leaves on the same plant. This between-leaf systemic signaling of defense response, however, occurs without MAPK activation in systemic leaves and suggests the existence of a mobile signal other than the one activating responses within attacked leaves.

Herbivore-specific elicitors, FACs, and plants' perception of herbivory

Volicitin, a hydroxylated FAC, has been reported to bind to *Zea mays* cell membranes with high affinity (Truitt et al., 2004), suggesting that a ligand-receptor binding mechanism may be critical for herbivory recognition. Moreover, FACs vary in their ability to elicit VOCs in *Z. mays* seedlings: 18:2-Glu (FAC D) were least active, while 18:3-Gln (FAC A) and 18:3-Glu (FAC B) were significantly more active (Alborn et al., 2003). In contrast, applying similar concentrations of different FACs to *N. attenuata* resulted in similar levels of SIPK activity and similar levels of *WIPK* transcripts and JA production. Assuming that FAC-specific receptors exist in the *N. attenuata* cell membrane, two possible scenarios may account for these results: 1) there is only one receptor gene; this gene encodes a FAC receptor protein, and different FACs bind to it with similar affinities, which in turn activates

downstream MAPKs and herbivore-specific genes; or 2) several genes encode different receptors, which bind to specific individual FACs; these receptors likely are similarly abundant and share the same downstream signaling networks.

Although no FAC-specific receptors have yet been identified, some evidence suggests that ligand-receptor interactions play important roles in plant resistance to pathogens. In wild-type Arabidopsis protoplast, flagellin binds to receptor FLS2 and initiates downstream responses through MEKK1, MKK4/MKK5, and MPK3/MPK6 signaling cascades. In the *fls2* mutant, these responses are absent, indicating the critical role of flagellin perception by receptor FLS2 (Asai et al., 2002; Zipfel et al., 2004). The *N* gene, a putative receptor involved in cultivated tobacco's resistance to the tobacco mosaic virus (TMV), is structurally similar to the toll and the interleukin-1 receptors (Whitham et al., 1994). Tobacco carrying the *N* gene activated WIPK and elevated levels of *WIPK* transcripts and proteins after TMV infection; in contrast, tobacco without the *N* gene neither activated WIPK nor altered levels of *WIPK* mRNA or protein (Zhang and Klessig, 1998b). Identifying FAC-binding proteins and FAC-specific receptors will clarify how plants perceive herbivores on the molecular level and enrich our understanding of this ubiquitous interaction.

SIPK and WIPK regulate transcript levels of defense-related genes in *N. attenuata*

Both the range and scope of genes found to be regulated by SIPK and WIPK, ranging from genes involved in JA, and ethylene biosyntheses, to transcription factors, MAPKs, and several CDPKs, were surprising. The regulation of WRKY transcription factors is likely to contribute significantly to the large scope of transcriptional responses resulting from silencing SIPK. SIPK is known to phosphorylate WRKY1 in tobacco (Menke et al., 2005), and the activation of SIPK and WIPK is known to lead to higher transcript levels of several *WRKY*s (Kim and Zhang, 2004). We propose that SIPK and WIPK regulate transcript levels of *WRKY* transcription factors and may even directly phosphorylate particular WRKYs, and thereby mediate the transcript levels of defense-related genes, including transcription factors, e.g. *WRKY*s themselves, since it is known that WRKYs can bind to the promoters of *WRKY* genes and modulate their transcriptions (Eulgem et al., 1999; Turck et al., 2004). A recent study of yeast suggests that MAPKs may physically associate with promoters and thus influence the transcription of certain genes (Pokholok et al., 2006). SIPK and WIPK may also directly associate with the promoters of transcription factors, phytohormone biosynthetic genes, and thus modulate their transcript accumulations.

CDPKs have been shown to be involved in development and stress responses (Cheng et al., 2002; Ludwig et al., 2004). CDPK2 mediates JA and ethylene biosynthesis, and also biotic stress responses (Romeis et al., 2001; Ludwig et al., 2005). Expression analysis showed that CDPK4 is involved in plant stress reactions (Zhang et al., 2005); our q-PCR analysis also suggested that CDPK2, 4, 5, and 8 are involved in herbivore resistance in *N. attenuata*. In Arabidopsis and cultivated tobacco, MPK4 was also shown to be involved in various stress responses and the regulation of phytohormone biosynthesis (Ichimura et al., 2000; Petersen et al., 2000; Droillard et al., 2004; Gomi et al., 2005). The orthologue of *Naf6* in cultivated tobacco has been shown to be involved in pathogen resistance (Liu et al., 2004), and our results suggest that *MPK4*, *Naf6,* and *Naf3* are involved in responses to herbivore attack. It is possible that in both SIPK- and WIPK-VIGS plants, the decreased transcript levels of these genes reduced their respective kinase activity, and thereby influenced downstream targets.

Using q-PCR, we found that after both WT and EV *N. attenuata* plants were treated with either W+W or W+OS, *SIPK* transcript levels were elevated (Figure 1F, 3B). In contrast, *SIPK* transcript levels in cultivated tobacco were unchanged after wounding as analyzed by RNA blotting (Zhang and Klessig, 1998). Differences in the sensitivity of the detection techniques used (q-PCR and RNA blotting), or species-specific differences are likely to account for the differences between these two studies.

SIPK and WIPK were found to influence the accumulation of each other's transcripts. An important study has demonstrated that SIPK positively regulates the accumulation of *WIPK* and also of its protein after TMV infection (Liu et al., 2003). In *N. attenuata*, after either W+W or W+OS treatment, SIPK activation increased levels of *WIPK* transcripts. However, compared with those in WT control plants, levels of *WIPK* transcripts in *SIPK*-silenced rice and Arabidopsis were higher and unchanged after plants were inoculated with fungal elicitors and pathogens, respectively (Menke et al., 2004; Lieberherr et al., 2005). These data reflect the treatment- or species-specific effects of SIPK regulation on *WIPK* transcript levels. Although silencing *WIPK* was shown to reduce *SIPK* transcript levels, SIPK activity was not affected according to the in-gel kinase activity assay, suggesting its independence from transcriptional regulation. Moreover, silencing *WIPK* also reduces levels of *Naf4* after W+W and W+OS treatments. Since pTV-WIPK and *Naf4* sequences have no matches longer than 20 nt, it is not likely that silencing of *WIPK* also co-silenced *Naf4* (Thomas et al., 2001; Xu et al., 2006). Our transcriptional analyses suggest that the transcriptional interactions among different MAPKs, even CDPKs, form a complicated cross-

talking network, and the interactions that occur at the level of protein activity may be even more complicated.

In SIPK-VIGS plants, we detected highly decreased transcript levels of *Naf4*, a closely related homologue of *SIPK* (Ren et al., 2006). The VIGS construct pTV-SIPK, which shares a 35 nt perfect match with *Naf4*, may have co-silenced *Naf4*. Alternatively, *Naf4* may be transcriptionally regulated by *SIPK*; that is, silencing *SIPK* may reduce levels of *Naf4* transcripts, as we demonstrate happens between *SIPK* and *WIPK* and among other kinases. Naf4 may have contributed at least partly to the phenotypes we observed in SIPK-VIGS plants. Both proteins have more than 90% identity, which suggests a short history of divergence; whether they have similar functions remains unknown. In cultivated tobacco, where long-lasting Naf4 activity causes hypersensitive-like responses such as cell death, a comparable phenotype was obtained when SIPK was constitutively activated (Ren et al., 2006; Zhang and Liu, 2001), suggesting both kinases may have similar functions. Using gene-specific VIGS constructs or transforming plants with specific RNAi constructs to individually silence *Naf4* and *SIPK* will elucidate the nature, redundant or distinct, of these genes' functions.

The transcript levels of three genes directly related to resistance to herbivores in *N. attenuata*, *TPI*, *PAL,* and *TD*, were found to be regulated by SIPK and WIPK. As an anti-digestive protein, TPI plays an important role in *N. attenuata*'s resistance to herbivory (Glawe et al., 2003; Zavala et al., 2004). PAL is important in the biosyntheses of phenolic compounds which are implicated in resistance to abiotic and biotic stresses (Bennett and Wallsgrove, 1994). TD has been shown to play an anti-nutritive defense role by decreasing the level of threonine in *M. sexta*'s midgut and causing nutritional deficiency (Chen et al., 2005). Both SIPK and WIPK positively regulate transcript levels of *TPI* and *TD*; in contrast, levels of *PAL* transcripts are regulated positively by SIPK but negatively by WIPK, demonstrating overlapping yet distinct functions of SIPK and WIPK. Studies have suggested that PAL is activated by CDPKs (Allwood et al., 1999; Cheng et al., 2001); whether OS-elicited CDPK activity or even MAPK activity is involved in activating PAL in addition to regulating its transcriptional levels is unknown. The ecological significance of SIPK and WIPK in relation to herbivore resistance in *N. attenuata* needs to be studied further.

Activation of SIPK and WIPK and accumulation of phytohormones

The OS elicitation of SIPK and WIPK regulates levels of JA, SA, JA-Ile, and ethylene in *N. attenuata*. Both SIPK-VIGS and WIPK-VIGS plants had highly reduced levels of JA

after either wounding or herbivory, strongly suggesting the idea that most elevated JA levels are due to either wounding- or OS-elicited SIPK and WIPK activation. Other kinases, e.g. CDPKs, are probably involved in regulating JA levels, as shown by the activation of CDPK2, which also elevates JA levels (Ludwig et al., 2005). In cultivated tobacco, genetic analyses of *WIPK* indicated that WIPK is located upstream of JA biosynthesis (Seo et al., 1995; Seo et al., 1999). Conversely, applying dexamethasone (DEX) to transgenic tobacco plants carrying a DEX-inducible promoter-active mutant $MEK2^{DD}$ construct highly elevated SIPK and WIPK activity levels; however, levels of JA remained the same (Kim et al., 2003). In addition to the MAPKs, several lines of evidence suggest the importance of calcium in regulating JA biosynthesis. After wounding or herbivore attack, leaves quickly increase cytosolic calcium concentrations and depolarize cell membranes (Fisahn et al., 2004; Maffei et al., 2004); leaves and cell suspension cultures to which calcium channel blockers were applied, lowered their elevated levels of JA after heat and oligogalacturonide treatment, respectively (Fisahn et al., 2004; Moscatiello et al., 2006). In humans, calcium is necessary for the activity of LOX and phospholipase A_2, which are enzymes involved in the production of leukotrienes and lipoxins. Although no indisputable evidence exists, it is likely that calcium signaling partly modulates JA metabolism (Creelman and Mullet, 1997). Wounding or herbivory probably triggers not only MAPKs but calcium and other signaling pathways, and this likelihood may also be reflected in the possible involvement of CDPK2 in wounding/osmotic stress responses (Ludwig et al., 2005).

The transcript levels of genes involved in JA biosynthesis change after W+OS and W+W treatments, yet W+OS leads to a more dramatic increase in JA production than does W+W treatment. Moreover, the OS-elicited JA burst comes well before the OS-elicited increase in *LOX3* and *AOS* transcripts in WT *N. attenuata* plants (Ziegler et al., 2001; Halitschke and Baldwin, 2003). This implies that post-translational processes are important for the JA burst. That much higher activity levels of SIPK (and likely other kinases) are found in W+OS-treated plants than in W+W-treated plants might result in better phosphorylation of enzymes involved in JA biosynthesis, and thus higher levels of enzyme activity, is consistent with this view.

After wounding, *WIPK*-silenced cultivated tobacco plants produce higher levels of SA than do WT plants (Seo et al., 1995). The opposite happened in *N. attenuata*: after W+OS treatment, both SIPK-VIGS and WIPK-VIGS plants had lower levels of SA than did EV plants. Although the interaction between the SA- and JA-signaling pathways appears to be positive and negative, it is primarily negative (reviewed in Kunkel and Brooks, 2002). The

lower levels of JA in SIPK- and WIPK-VIGS plants did not correlate with higher levels of SA. Furthermore, *N. attenuata* plants had substantially higher levels of JA and SA after W+OS treatment compared to those treated with W+W, suggesting that herbivory-elicited JA and SA may interact synergistically. The responses of *N. attenuata* and cultivated tobacco in wound- and probably also OS-induced SA production are likely species-specific.

Evidence has emerged that the conjugation reaction between JA and Ile, catalyzed by JAR, plays an important role in activating plant defenses (Staswick and Tiryaki, 2004; Kang, et al., 2006; Wang et al., 2007). After wounding and OS elicitation, dramatically lower levels of JA-Ile/JA-Leu in SIPK-VIGS plants were detected compared to EV plants for up to 3 h. WIPK-VIGS plants, on the other hand, showed decreased levels of JA-Ile/JA-Leu only up to 1.5 h, although levels of one of the substrates for JARs, JA, were still about 50% lower than levels in EV plants. The decreased levels of JA-Ile/JA-Leu in SIPK- and WIPK-VIGS plants may have resulted from the decreased levels of JA, one of the substrates for conjugating JA-Ile/JA-Leu. It is also likely that the lower transcript levels of *JAR*s and *TD* in SIPK- and WIPK-VIGS plants also contributed to their JA-Ile/JA-Leu phenotype.

ACS phosphorylation by SIPK has been demonstrated in Arabidopsis to be a key step in ethylene biosynthesis (Kim et al., 2003; Liu and Zhang, 2004). Herbivore-elicited ethylene production also likely depends on the phosphorylation of ACS enzymes by SIPK; however, since substantially lower levels of *ACS3*, *ACO1* and *ACO3* were detected in SIPK-VIGS plants, the transcript accumulation of these genes may also contribute to the regulation of ethylene biosynthesis. Compared with the very low levels of JA and JA-Ile, ethylene levels in SIPK-VIGS plants were only reduced by 50%, which is similar to ethylene levels produced in Arabidopsis *SIPK* mutants after flagellin treatment, suggesting that there may be other signaling pathways leading to ethylene biosynthesis, e.g. CDPKs (Tatsuki and Mori, 2001; Liu and Zhang, 2004; Ludwig et al., 2005). The remarkable difference between ethylene production in *N. attenuata* after wounding and after OS elicitation may also result from the substantially different abilities of these two forms of elicitation to activate kinases. Compared to W+OS, wounding alone is much less able to activate SIPK and probably certain CDPKs involved in ethylene biosynthesis as well. Moreover, both flagellin and W+OS treatments activate SIPK and in turn trigger ethylene biosynthesis, confirming the hypothesis that various stress stimuli converge onto MAPK pathways (Zhang and Klessig, 2001).

Genetic and biochemical studies have indicated that MAPKs are located upstream of JA and ethylene production (Seo et al., 1999; Kim et al., 2003; Liu and Zhang, 2004). Our loss-of-function analysis provides additional evidence. Our experience with intact plants

rather than cell suspension cultures leads us to posit that neither JA nor ethylene has any feedback effect on MAPKs, as these compounds are located downstream of MAPKs. We propose that the differences observed in suspension cells and intact plants are due to the specificity of MAPK signaling in different tissues, although these differences may also reflect the easily perturbed physiology of cell suspension culture, for example, an easily changed cytosolic pH (Tena and Renaudin, 1998).

Functions of SIPK and WIPK

Both transcriptional analyses and phytohormone levels point to overlapping functions of SIPK and WIPK. Silencing both kinases reduces JA and JA-Ile accumulation after W+OS treatment, a reduction that is also reflected in their transcript levels of phytohormone biosynthetic genes. In addition, both SIPK and WIPK modulate the transcript accumulation of WRKY transcription factors; more strikingly, they both influence the transcript accumulation of several other MAPKs and CDPKs, although with different specificity and at different levels. It is likely that SIPK and WIPK share common substrates, including transcription factors, and thus both mediate the transcript levels of the same target genes. Consistent with this idea, using a protein-microarray approach, Feilner et al. (2005) demonstrated that SIPK and WIPK share many common substrates. Intriguingly, SIPK and WIPK transcriptionally regulate each other, suggesting that one (or a few) transcription factor(s), involved in the regulation of transcript levels of both *SIPK* and *WIPK* is (are) their common substrate(s).

Transcriptional analyses also revealed different functions of SIPK and WIPK. Although in most cases SIPK and WIPK positively regulate the transcript levels of many defense-related genes, WIPK was found to negatively regulate a few genes, namely, *AOS*, *ACO1*, and *PAL*. The substantially decreased levels of the phytohormones JA, JA-Ile, and ethylene in SIPK- and WIPK-silenced plants led us to hypothesize that both kinases may have many more phosphorylation targets in phytohormone biosynthetic pathways than the known ACS in ethylene synthesis. Identifying the substrates for SIPK and WIPK and the ability of these two kinases to directly phosphorylate phytohormone biosynthetic enzymes will help us to better understand how SIPK and WIPK regulate phytohormone biosynthesis.

Mobile signals and defense response activation in systemic tissues and leaves

In this study we demonstrate that applying OS to a specific region on a single leaf effected a specific distribution of MAPK activity and, subsequently, of defense-related gene transcripts. Kinetic data showed that even after only 5 min the kinase activity had spread to

adjacent regions in fully developed leaves, indicating that certain signals were being quickly transmitted. This mobile signal may be elicited by FACs and located downstream of FAC receptors and upstream of MAPKs; after FACs bind to receptors, it may be quickly activated or released and transported to adjacent regions. To our knowledge, neither the nature of this signal nor the mechanism of transport has been studied. In Lima bean, *Spodoptera littoralis* feeding rapidly and strongly depolarizes membrane potential throughout the entire attacked leaf (Maffei et al., 2004). In animal neurons, the changes of membrane potential are known to be critical for sending signals long distances; however, the study of plant electrophysiology is still in its infancy (Brenner et al., 2006). Given how rapidly MAPKs are activated in undamaged regions, it may be that the signal is carried by electricity or rapidly propagated hydraulic signals; another hypothesis has rather small molecules, such as FACs or FAC-elicited signal compounds, entering the xylem vessel through wounds and being carried to specific regions of the leaf (Malone et al., 1994). Whether electric signaling or xylem transport is involved in activating MAPKs in undamaged regions needs further investigation.

Clearly, plants regulate the direction and extent of signal transport within a leaf. In fully developed leaves, when region 0 was treated with W+OS, SIPK was quickly and markedly activated in both regions 0 and 1, and a slight increase of activity was also observed in region 3. The application of W+OS to 1 led to substantially increased kinase activity in regions 1 and 3, as well as to marginally elevated activity levels in region 0. No sample from region 2 showed any elevated SIPK activity. The specific spatial distribution of MAPK activation and subsequent accumulation of defense-related genes may directly influence how the mobile signal molecules are transported or moved. The significant difference in MAPK activity levels among regions 1 and 3 and regions 3 and 0, after regions 0 and 1 were treated with OS, suggests that the transport of the mobile signal molecules is also controlled quantitatively. Using leaves at different developmental stages, we showed that applying W+OS to a specific region in a single leaf results in a pattern of activity distribution that is also developmentally regulated: in younger sink leaves: applying W+OS to region 1 activates SIPK; adjacent region 3 showed low activity levels, however, which contrasted with high activity levels in older source leaves. Studies have shown that during the development of a leaf, there is a gradient in maturation from the base to the tip which allows veins to load or unload sugar (reviewed in Turgeon, 1989); in not fully developed leaves, some parts of a leaf still import sugar but other parts export sugar. This suggests that leaves at different developmental stages develop different transport systems. Whether this holds true for the

transport of the signal eliciting MAPK activity in adjacent regions in the herbivore-attacked leaf needs to be studied further.

The spatial distribution of accumulated JA in these 4 regions after W+OS treatment in either region 0 or region 1 largely resembles the distribution of *LOX3*. Remarkably, when region 0 was treated with OS, JA was highly induced and levels were much higher than those in region 1, or those in regions 1 and 3 which were elicited when leaves were treated with W+OS in region 1. The complex interplay of mechanisms modulating JA biosynthesis includes MAPK activation, JA biosynthetic gene transcript accumulation, and even the particular region of a leaf attacked by herbivores. Both *TD* and *TPI* are known to be involved in eliciting and producing direct defenses (Zavala et al., 2004; Chen et al., 2005; Kang et al., 2006) and are largely regulated by JA. Intriguingly, the spatial distribution of *TPI* transcripts doesn't match the distribution of JA levels: *TPI* transcripts were elevated in all regions of the leaf wherever OS was applied to the base or the tip of the leaf. Perhaps other mobile signals are activated by JA and move to all parts of the herbivore-attacked leaf before finally eliciting *TPI*.

The patterns of spatial distribution among MAPK activity, JA levels, and direct-defense gene transcript accumulations within the damaged leaf suggest that various intercellular signal cascades are involved: different mobile signals induce or activate different downstream elements on signal transduction pathways; and the directions and extent of their movements are probably tightly regulated by certain transport systems. Finally, complex interactions among the signaling pathways may also account for the spatial regulation of these defense-related responses. Our results demonstrate that the responses depend on both the ages of the attacked leaves and the position of the herbivore on the leaves. However, the ecological significance of these different responses has not been established.

Herbivory has long been known to enhance proteinase inhibitor (PI) activity in intact systemic leaves (Green and Ryan, 1972), although the mechanism of this long-distance signal transduction is still not known. Several studies have found that systemic leaves orthostichous to the wounded leaf accumulate markedly higher levels of *PI* transcripts and activity than do non-orthostichous leaves, suggesting the signal is transported by the vascular system (Jones et al., 1993; Orians et al., 2000; Schittko and Baldwin, 2003; Orians, 2005). The involvement of electric and hydraulic signals in mediating the systemic responses has also been proposed (Alarcon and Malone, 1994; Stankovic and Davies, 1997). More important, an elegant genetic study strongly argues that JA plays important roles in mediating systemic PI response: either JA or a JA-elicited mobile signal is responsible for systemic PI induction (Li et al., 2002).

In tomato, wounding activates MAPK both locally and systemically (Stratmann and Ryan, 1997). Infecting cultivated tobacco plants with the tobacco mosaic virus leads to the systemic accumulation of *WIPK* mRNA and in turn the protein (Zhang and Klessig, 1998b). Cutting cultivated tobacco stems also activates WIPK activity in systemic leaves (Seo et al., 1999). These studies implicate MAPKs in systemic signaling. However, *N. attenuata* plants treated with either W+W or W+OS didn't show systemic SIPK activity or *WIPK* transcript accumulation, suggesting that different plant species have specific mechanisms for modulating systemic MAPK signaling; such mechanisms are not conserved even in closely related species such as *N. attenuata*, cultivated tobacco, and tomato. Whether these discrepancies are due to differences among treatments or the morphology of the experimental plants still needs to be investigated. It is very likely that the mobile signal which activates SIPK in undamaged regions on a single leaf and the signal which moves to distal undamaged leaves and induces TPI are distinct molecules, since the former is located upstream of *SIPK* but the latter clearly is not.

In conclusion, in this study we examine temporally and spatially how plants deploy defense reactions which are modulated by SIPK and WIPK in response to herbivore attack, and summarize the results in a model (Figure 13). Plants recognize FAC components in herbivores' OS and quickly activate SIPK and WIPK. Both kinases in turn regulate a broad array of gene transcript levels and likely even modify specific enzyme activity by phosphorylation, for example, ACS; together with the subsequently elevated levels of JA, ethylene, and SA, SIPK and WIPK further modulate the transcript accumulation of other defense-related genes. Spatially, when an herbivore chews on a leaf, a quickly elicited mobile signal conveys the "attack alert" message to particular unattacked regions of the leaf, thus activating MAPKs and the transcript accumulation of defense-related genes. Then a signal compound, either JA itself or something downstream of JA, transmits the "attack alert" message to distal leaves without activating SIPK and WIPK in those leaves. Identifying substrates for SIPK and WIPK, and understanding how they interact with other components of the signaling pathway will shed light on the complicated signaling pathways that have evolved during the long history of the plant-herbivore arms race.

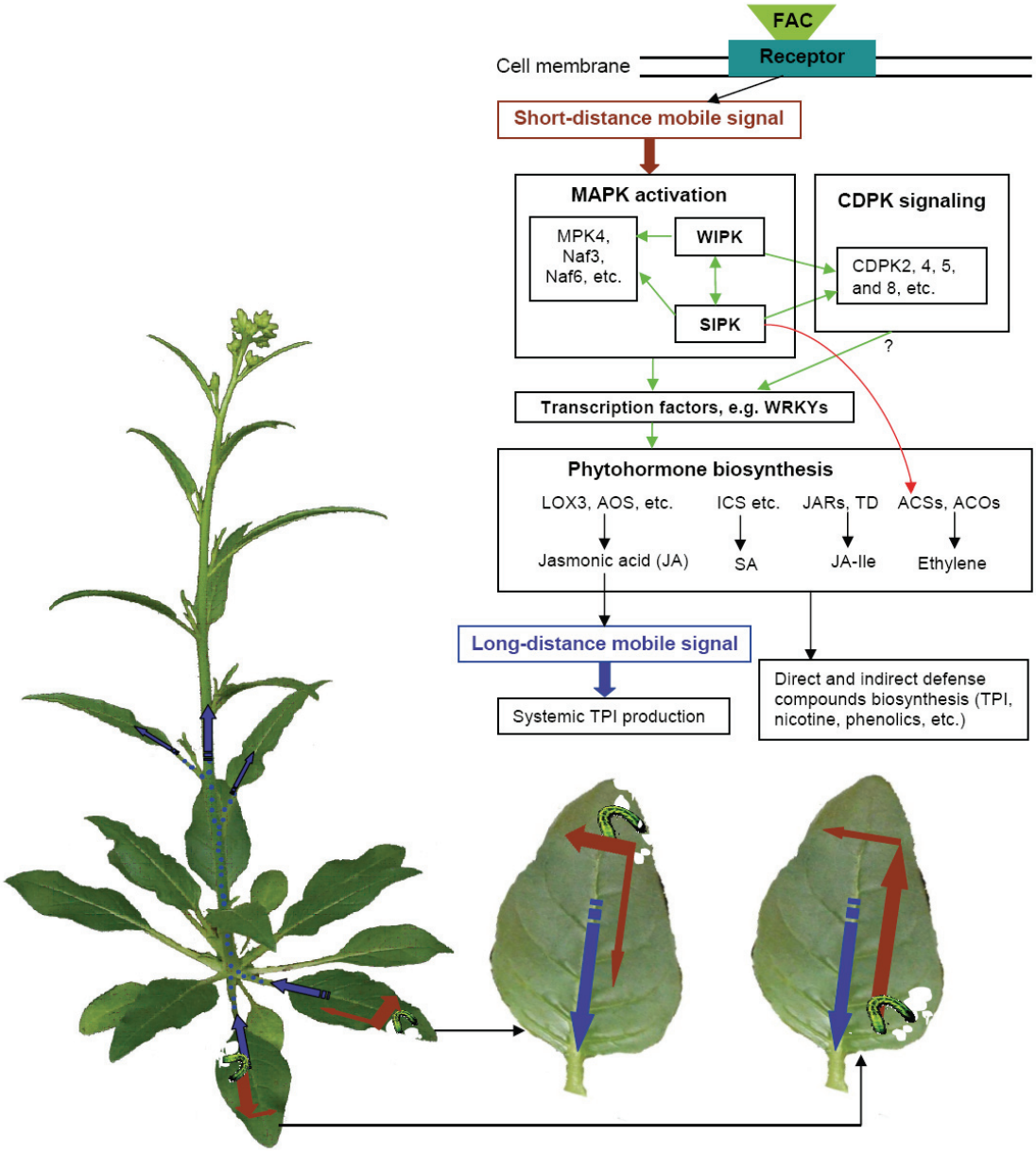

Figure 13. Model summarizing how OS-elicited responses activate defenses in local and systemic leaves of *Nicotiana attenuata*.
After attack from *Manduca sexta* larvae, FACs in the larvae's oral secretions bind to hypothetical receptors in the cell membranes and activate a short-distance mobile signal which enhances SIPK and WIPK activity in both wounded regions and, in particular, non-wounded adjacent regions in the leaf. Afterwards, activating SIPK and WIPK leads to the transcriptional regulation of other MAPKs, CDPKs, and transcription factors, such as WRKYs. Through WRKY and other transcription factors, both kinases subsequently enhance transcript levels of genes involved in jasmonic acid (JA), salicylic acid (SA), JA-isoleucine conjugate (JA-Ile), and ethylene biosynthesis, which in turn enhance levels of JA, SA, JA-Ile and ethylene. SIPK may also directly phosphorylate some of these genes' protein products --for example, ACS -- and thus enhance their activity. A long-distance mobile signal such as JA or a JA-elicited substance moves through the vascular system to distal leaves and enhances both local and systemic levels of trypsin proteinase inhibitor (TPI) activity. Green arrows represent regulation at transcriptional levels; red arrows represent direct phosphorylation; arrows in brown and blue represent short- and long-distance mobile signals, respectively.

Methods

Plant growth and sample treatments

N. attenuata Torr. ex Watts. (Solanaceae) seeds were from a line maintained in our laboratory that was originally collected in Utah (USA); seeds were germinated on agar with Gamborg B5 media (Duchefa, Harlem, the Netherlands). *N. attenuata* plants of the same genotype and generation as the WT plants were transformed with a construct carrying an antisense *lipoxygenase 3* gene to obtain asLOX3 plants (Halitschke and Baldwin, 2003); constructs carrying fragments in an inverted repeat orientation of *COI1*, which mediates JA perception (Paschold et al., 2007), and *ACC oxidase*, which mediates ethylene production (von Dahl et al., 2007), were transformed into *N. attenuata* to form irCOI1 and irACO lines, respectively. The ETR1 line was prepared by expressing the Arabidopsis mutant ethylene receptor gene, *ETR1*, to inhibit ethylene perception (von Dahl et al., 2007). All transformed plants were homozygous for a single transgene insertion, diploid as determined by flow cytometry, and used in the T_2-T_3 generation.

All plants except those used for virus-induced gene silencing (VIGS) were grown in the greenhouse in 1 L individual pots at 26-28 ^{o}C under 16 h of light supplied by Philips Sun-T Agro 400- or 600-W sodium lights. All treatments were performed on +1 leaves, unless otherwise noted. For W+W treatments, leaves were wounded with a pattern wheel and 20 µL of water was rubbed onto each leaf; for W+OS treatments, 20 µL of *M. sexta* OS (1/5 diluted, unless otherwise noted) was rubbed into wounds. MeJA was dissolved in heat-liquefied lanolin at a concentration of 7.5 µg/µL; 20 µL of the resulting lanolin paste was applied to leaves, pure lanolin was applied as a control. Ethephon was dissolved in 5 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 at 5 µg/µL, and 20 µL was applied to each leaf; MES buffer solution was used as a control. N-linolenoyl-l-Gln (FAC A), N-linolenoyl-l-Glu (FAC B), N-linoleoyl-l-Gln (FAC C), and N-linoleoyl-l-Glu (FAC D) were synthesized in-house (Halitschke et al., 2001). Each FAC was dissolved in DMSO at a concentration of 2 mM, and then diluted in water to 0.2 mM. FAC-free OS were prepared by passing OS 4 times through spin columns filled with Amberlite IRA-400 resin (Sigma, Steinheim, Germany) (Halitschke et al., 2001). Twenty µL of each test solution was applied to each leaf. After specific times, leaves were excised, immediately frozen in liquid nitrogen, and stored at -80 ^{o}C until use.

Molecular Cloning

Genes were cloned by PCR, using cDNA obtained from 1 h W+OS treated wild-type *N. attenuata* leaves as the template. Products were gel-purified and cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced.

Northern blotting and quantitative RT-PCR (q-PCR)

Total RNA was extracted from leaves using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. Ten µg of RNA from pooled leaf tissue from 4 replicated leaf samples was separated on 1.2% formaldehyde-agarose gels and transferred to GeneScreen Plus Hybridization Transfer membranes (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Ten ng of DNA probes was labeled with α-^{32}P-dCTP (PerkinElmer Life and Analytical Sciences) using a random primer labeling kit (Amersham Biosciences, Uppsala, Sweden). Membranes were pre-hybridized with ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) for 1h and hybridized with individual probes overnight; after washing, imaging was conducted on a FLA-3000 Phosphorimage system (Fujifilm Fuji Photo Film Europe, Düsseldorf, Germany). Probes were PCR-amplified using plasmids carrying respective cDNA as templates, and the primers listed in Supplemental Table 1 were used.

For quantitative RT-PCR (q-PCR) analysis, 5 replicated biological samples were used. To minimize errors generated by the preparation of cDNA, all total RNA samples were diluted to 0.5 µg/µL; in 96-well PCR plates and using the same master enzyme mix, 2 µL of each diluted RNA sample were reverse-transcribed using oligo(dT) and Superscript II reverse transcriptase (Invitrogen) in a total volume of 20 µL. cDNA samples were further diluted with water to 40 µL. q-PCR was carried out on a ABI PRISM 7700 Sequence Detection System (Applied Biosystems), using qPCR™ Core kits (Eurogentec, Seraing, Belgium). For each analysis, a linear standard curve, threshold cycle number (Ct) vs. Log (designated transcript level), was constructed using a series dilutions of a specific cDNA standard; the levels of the transcript in all unknown samples were determined according to the standard curve. A *N. attenuata sulfite reductase* (*ECI*), which is a house-keeping gene involved in plant sulfur metabolism and has been shown to have constant levels of transcript by both northern blotting and q-PCR, after W+W and W+OS treatments (Bubner, et al., unpublished data), was used as an internal standard for normalizing cDNA concentration variations. The primers, Taqman® probe sequences used for Taqman® q-PCR, and primer sequences for SYBR Green based q-PCR are provided in Supplemental Tables 2 and 3, following the PCR conditions recommended by the manufacturer.

Protein extraction

Leaf tissue pooled from 4 replicate leaves was crushed in liquid nitrogen and 250 µL extraction buffer was used for every 100 mg tissue (100 mM Hepes pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl floride (PMSF), 10% glycerol, complete proteinase inhibitor cocktail tablets (Roche, Mannheim, Germany)). Leaf tissue was then completely suspended by vortexing. After being centrifuged at 4 ^{o}C for 20 min, supernatant was transferred to a fresh tube. Protein concentration was measured using a Bio-Rad protein assay kit with BSA as a standard.

In-gel kinase activity assay

Kinase activity assay was performed according to Zhang and Klessig (1997), using myelin basic protein (MBP) as a substrate. In brief, protein samples containing 10 µg of total protein were separated on 10% SDS-polyacrylamide gels embedded with 0.2 mg/mL MBP in the separation gel. After electrophoresis, gels were washed 3 times for 30 min each in washing buffer (25 mM Tris pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg/mL BSA, 0.1% Triton X-100 (v/v)) at room temperature. Then kinases in the gel were renatured in 25 mM Tris pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, and 5 mM NaF at 4 ^{o}C overnight, with 3 changes of buffer. At room temperature, the gels were then incubated in reaction buffer (25 mM Tris pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO4) with 0.2 mM ATP plus 1.6 µCi/mL γ-^{32}P-ATP (3000 Ci/mmol) for 1h. The reactions were stopped by a fixation solution (5% trichloroacetic acid and 1% sodium pyrophosphate (w/v)). After 6 h of washing with 5 changes, the gels were dried on a gel dryer (Bio-Rad) and images were obtained on a FLA-3000 Phosphorimage system.

TPI activity analysis

The TPI activities were quantified using a radial diffusion assay protocol described by van Dam et al. (2001).

Analysis of JA and JA-Ile/JA-Leu accumulation

Five replicated leaf samples were used. About 300 mg of each tissue sample was collected in FastPrep tubes containing 0.9 g of FastPrep matrix, flash-frozen in liquid nitrogen, and stored at -80 ^{o}C until use. One mL of ethyl acetate spiked with 200 ng of $^{13}C_2$-JA, D_4-SA,

and *para*-coumaric acid (pCA), used as the internal standards for JA, SA and JA-Ile/JA-Leu, respectively, was added to each sample and then homogenized on a FastPrep homogenizer (Thermo Electron). After centrifugation at maximum speed for 10 min at 4 ^{o}C, supernatants were transferred to fresh 2 mL Eppendorf tubes. Each pellet was re-extracted with 0.5 mL of ethyl acetate and centrifuged; supernatants were combined and then evaporated to dryness on a vacuum concentrator (Eppendorf). The residue was resuspended in 0.5 mL of 70% methanol (v/v) and centrifuged to clarify phases. The supernatants were pipetted to glass vials and then analyzed by HPLC-MS/MS.

Measurements were conducted on a 1200L LC/MS system (Varian, Palo Alto, CA, USA). At a flow rate of 0.1 mL/min, 15 µL of each sample was injected onto a Pursuit C8 column (3 µm, 150 × 2 mm) (Varian). A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) was used in a gradient mode for the separation. A negative ESI mode was used for detection. An ion with a specific m/z generated from each endogenous phytohormone or internal standard (the parent ion) was selected and fragmented to obtain its daughter ions; a specific daughter ion was used for generating the corresponding compound's chromatogram. The parent ions, daughter ions, and collision energies used in these analyses are listed in Supplemental Table 4. Each phytohormone was quantified by comparing its peak area with the peak area of its respective internal standard. As JA-Leu and JA-Ile have the same molecular weight and retention time, these two compounds could not be separated with the method we developed, which is described in detail in Wang et al., 2007.

Measuring ethylene accumulation

Four replicated measurements were used to quantify ethylene production in *N. attenuata*. Three leaves were treated either with W+W or W+OS and immediately afterward sealed in a 3-neck 250 mL round bottom flask and kept in the greenhouse for 5 h. The headspace was flashed into a photoacoustic laser spectrometer with hydrocarbon-free clean air (Invivo GmbH, Saint Augustin, Germany) and the ethylene concentration was quantified by comparing ethylene peak areas with peak areas generated by a standard ethylene gas.

Virus-induced gene silencing

A VIGS system based on tobacco rattle virus was used (Ratcliff et al., 2001). Fragments of both *SIPK* and *WIPK* were amplified by PCR using plasmids carrying *SIPK* and *WIPK* cDNA sequences and the following primers: for *SIPK*: SIPK1-31 (5'-

GCGGCGGTCGACGAGCCACGGTGGCAGGTTC), SIPK2-32 (5'-GCGGCGGGATCCGTATCCATAAGCTCATACGC); for *WIPK*: WIPK1-32 (5'-GCGGCGGTCGACGAGCTGAATGAGATGGTTGC), WIPK2-31 (5'-GCGGCGGGATCCTCTATGAAGAACATTCGCG). PCR products were digested with *BamH*I and *Sal*I and gel purified, then cloned into the binary pTV00 vector which was digested with same restriction enzymes to form pTV-SIPK and pTV-WIPK constructs. The fragments of *SIPK* and *WIPK* used for the preparation of pTV-SIPK and pTV-WIPK are shown in Supplemental Table 5. *Agrobacterium* strain GV3101 carrying either construct was co-inoculated with *Agrobacterium* carrying pBINTRA6 into young *N. attenuata* plants following VIGS procedures optimized for *N. attenuata* (Saedler and Baldwin, 2004).

GeneBank accession numbers

Sequence data from this article can be found in GenBank/EMBL database under accession numbers: DQ991135, DQ991136, EF121304, EF121305, EF121306, EF121307, EF121308, EF121309, EF121310, EF121311, EF152550, EF152551 and DQ768747.

ACKNOWLEDGMENTS

We thank Dr. Albrecht Berg and Eva Rothe for their help with the phytohormone analysis, Dr. Klaus Gase and Antje Wissgott for the preparation of VIGS constructs, Susan Kutschbach for transforming the plants, Caroline von Dahl and Anja Paschold for screening and characterizing transformed lines, and Emily Wheeler for editorial assistance.

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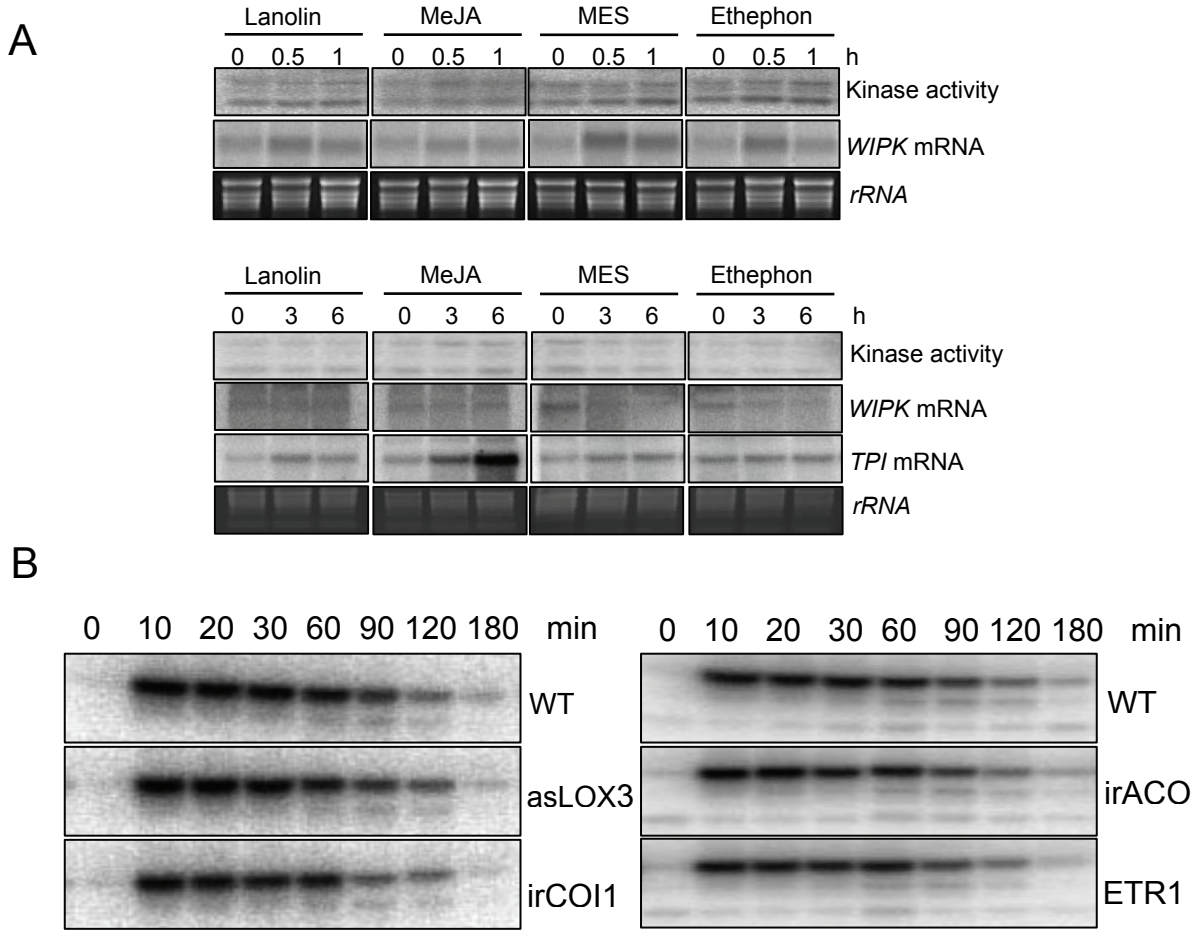
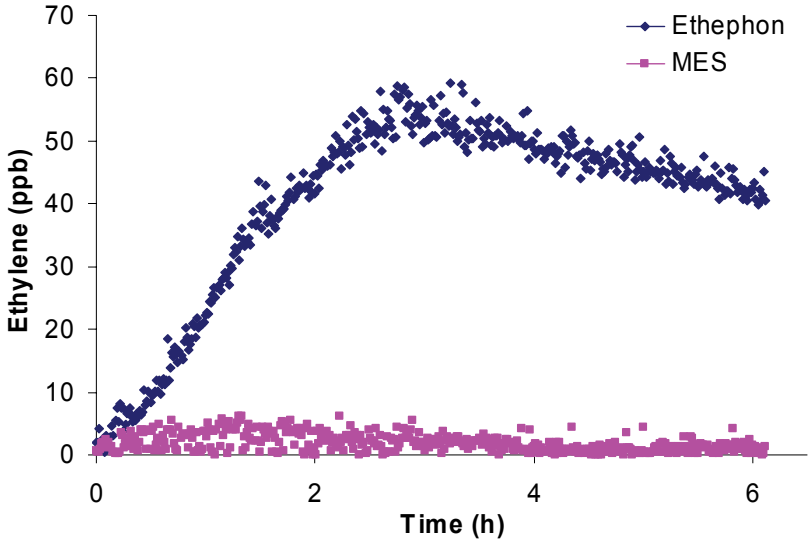
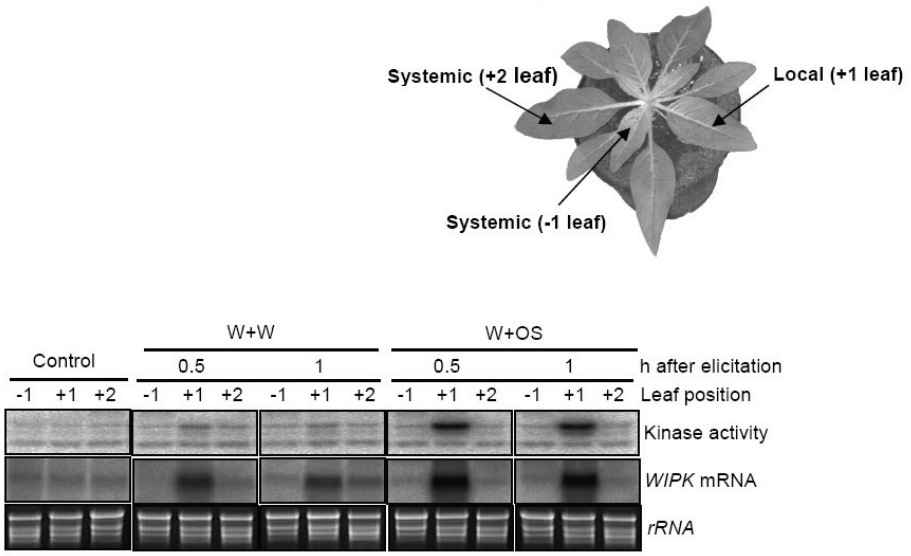
Supplemental Data



Supplemental Figure 1. OS-elicited SIPK and WIPK activity is not mediated by JA and ethylene.
(A) Treatment with exogenous JA and ethylene elicits neither SIPK activity nor the transcript accumulation of *WIPK*. Wild-type *N. attenuata* leaves were treated with 150 µg of MeJA in 20 µL of lanolin or 100 µg of ethephon in 20 µL of 5mM MES buffer (pH 5.5); lanolin and the MES buffer were used as controls. Samples were harvested at 0, 0.5, 1, 3, and 6 h; 4 individual leaves from replicate plants were pooled and in-gel kinase assays using MBP as a substrate and northern blotting were performed to measure kinase activity and the accumulation of *WIPK* and *TPI* transcripts. **(B)** MAPK activity in *N. attenuata* wild-type and transgenic lines. Wild-type (WT), asLOX3, and irCOI1 lines impaired in JA production and perception, respectively, as well as irACO and ETR1 lines impaired in ethylene production and perception, respectively, were all treated with W+OS; 4 replicate plants were harvested at the indicated times and pooled. In-gel kinase activity assays were conducted to detect MAPK activity.



Supplemental Figure 2. Ethephon application to leaves generates ethylene.
One *N. attenuata* leaf was treated with either 100 µg of ethephon dissolved in 20 µL of 2-(N-morpholino)ethanesulfonic acid (MES) buffer (5 mM, pH 5.5), or with 20 µL of MES buffer as control. Each leaf was subsequently placed in a 250 mL flask and the ethylene released was monitored by a photoacoustic laser spectrometer.



Supplemental Figure 3. SIPK activity and *WIPK* transcript levels do not change in systemic leaves of *N. attenuata* after wounding or applying *M. sexta* OS to wounds.
Leaves at node +1 from rosette-stage *N. attenuata* were wounded with a pattern wheel; 20 µL of water (W+W) or *M. sexta* oral secretions (W+OS) was applied to the wounds. Treated leaves (local) and systemic untreated leaves (-1 & +2) were harvested at indicated times. Four replicate leaves were pooled after harvesting. Kinase activity and transcript accumulation of *WIPK* in local and systemic leaves were analyzed by an in-gel kinase assay and northern blotting, respectively.

Supplemental Table 1. Primers used for preparation of northern-blotting probes.

Genes	Forward primer (5'-3')	Reverse Primer (5'-3')
WIPK	GTGGAGGTCAATTCCCTGATTTTC	GTGCCCTGTACCATCTGGTTACA
LOX3	GTCAACCGACAGTTTCAAGG	GAGCACCGCTTTAAGCCTTC
PAL	CCATGGCATCAAATGGTCAT	GTTGCAGAGCGAATGACTT
ACO1	GATGGAGAATTTCCCAATTATCAAC	GCAAATAATGTATGTCACTTGTCTT
TD	CAATGCCTGTTCCAGCGTCT	CTGACTAAGACCATTCCGTCT
TPI	GGCTGTTCACAGAGTTAGCTTCCTTG	GCTCCACTGCCATATTACAGATTAC

Supplemental Table 2. Primers and probes used for Taqman based q-PCR.

Genes	Primer 1 (5'-3')	Primer 2 (5'-3')	Taqman probe (5'-3')	Reporter	Quencher
WIPK	GGTTTATCAGAGGATCACTGCCA	AACATTCGCGGAATGTATGTATTTT	CTTCATGTATCAGCTCCTCCGTGGCC	FAM	BHQI
MPK4	TAGGAGCAACTCCGGTGCC	GCAAGGACAACATCTGAGACAGAT	CCACCGTGTTACGACATACTCCGTCATG	FAM	BHQI
CDPK2	GCAATGTGGCGAACTCGG	CTGCTGTTTCACCTCTGGCAC	CGCCAGATGATTCTCGTTCCACCAC	FAM	BHQI
CDPK4	GAATAACCCCACTCTTCTGTCTCC	CAACATCAAAAACAACAACCTAGGAA	CCCATTCTTGTGCTTCTTAACATTTTGCTGAGA	FAM	BHQI
CDPK5	TGCACCAGACCATGTAAATGACA	GAGAAAGATGCGGCGATAGTTG	TCAGCAGCCACTTTTAACATCTGGCGT	FAM	BHQI
TD	TAAGGCATTTGATGGGAGGC	TCTCCCTGTTCACGATAATGGAA	TTTTTAGATGCTTTCAGCCCTCGTTGGAA	FAM	TAMRA
PAL	ACTTGTTCGCCTACGCTGATG	TCTTCGAAAGCTCCAATCTTTTG	TCCTTGCAGTTGCAACTACCCTTTAATGCA	FAM	TAMRA
LOX3	GGCAGTGAAATTCAAAGTAAGAGC	CCCAAAATTTGAATCCACAACA	CAGTGAGGAACAAGAACAAGGAAGATCTGAAG	FAM	TAMRA
ACO3	AAGCAATGAAGGCTGTGGAAA	ACCCAAGTGGCATAAAACAAGAA	CAACTCTGCCCCAATAGCAACTGTTTGAGA	FAM	TAMRA
ACO1	CTATTGAATCTGATGTCAAGCTG	TATGTAGTAGGGACACACGCTT	CAACTGCATAGATCCAAATTCAAGAGTACTAAAG	FAM	BHQI
ACS3	ATCCTTCAAATCCATTAGGCAC	AACACTGATGAATTTCGGCTG	TGCGACGAAATTTATGCTGCTACTGTC	FAM	BHQI
WRKY6	ACAAAACAAAGATGAAGTTCCAAAG	GGAGAAGCTGGTGATGAAGATG	AAGTCATTTCCACCTTGTTCTTTGCCA	FAM	TAMRA
AOS	GACGGCAAGAGTTTTCCCAC	TAACCGCCGGTGAGTTCAGT	CTTCACCGGAACTTTCATGCCGTCG	FAM	TAMRA
JAR4	ATGCCAGTCGGTCTAACTGAA	TGCCATTGTGGAATCCTTTTAT	CAGGTCTGTATCGCTATAGGCTCGGTGATGT	FAM	BHQI
JAR6	TGGAGTAAACGTTAACCCGAAA	AGAATTTGCTTGCTCAATGCCA	TGCCCCCTGAGCTAGTCACTTATGCA	FAM	BHQI
ECI	AGAAACTGCAGGGTACTGTTGG	CAAGGAGGTATAACTGGTGCCC	CGTCAAAATTCTCCACTTGTTTCAACTGT	Yakima Yellow	BHQI

Supplemental Table 3. Primers used for SYBR Green based q-PCR.

Genes	Primer 1 (5'-3')	Primer 2 (5'-3')
SIPK	GTTGACGAATTTTCCAAAACAAAGT	CCGGAATATTATCCATACCGGCC
WRKY7	ACAGACTGCTCGGAAGTTCGA	GTTGGGACCTGGTCAGAACG
SubD48	AGCCGTAGCTGATGTTGCTGT	TAGAAATTGGGCCTTTACGAAATC
Naf4	CACATACAGCAACTGAATACAAAAAG	CCGGAATGTTATCGATTCCGGAA
Naf3	ATCCTTGGCAGTCAGCGAGA	GAAAAGGGTGTTCCGGGAGA
Naf6	ACATCAAAGTACATCCCTCCTATTCA	GCACAACAGACCATGCCGT
CDPK8	AGTGCACATTGTGATGGAACTGT	TCCTCAAAATTCCAGCAGCTG

Supplemental Table 4. Parent ions, daughter ions and collision energy used for JA, SA and JA-Ile/JA-Leu quantification by HPLC-MS/MS.

Compound	Parent ion (m/z)	Daughter ion (m/z)	Collision energy (V)
JA	209	59	12.0
$^{13}C_2$-JA	211	61	12.0
SA	137	93	15.0
D_4-SA	140	97	15.0
pCA	163	119	12.0
JA-Ile/JA-Leu	322	130	19.0

Supplemental Table 5. Sequences of partial *SIPK* and *WIPK* used for the preparation of virus-induced gene silenced constructs, pTV-SIPK and pTV-WIPK.

Genes	Sequences (5'-3')
SIPK	GAGCCACGGTGGCAGGTTCATTCAATACAATATATTTGGTAATATATTTGAAG TTACTGCTAAGTATAAGCCTCCTATTTTGCCTATTGGTAAAGGTGCTTACGGC ATCGTTTGTTCTGCTTTGAACTCGGAGACAATTGAGAATGTAGCGATAAAGAA AATCGCGAATGCTTTTGATAACAAGATTGATGCCAAGAGGACTTTGAGAGAGA TCAAGCTTCTTCGGCATATGGATCATGAAAACATTGTTGCGATCAGAGATATA ATTCCACCACCGCAGAGAGAAGCCTTTAATGATGTTTATATTGCGTATGAGCT TATGGATACGGATCC
WIPK	GAGCTGAATGAGATGGTTGCAGTTAAGAAAATCGCGAATGCGTTTGACAATTA CATGGATGCTAAGAGGACTCTCCGTGAGATTAAGCTTCTTCGCCATTTAGATC ATGAAAATGTAATTGGTTTAAGAGACGTGATTCCTCCACCGTTACGAAGGGAG TTTTCTGATGTTTACATTGCTACTGAACTCATGGATACTGATCTTCACCAAATA ATTAGATCCAACCAAGGTTTATCAGAGGATCACTGCCAGTACTTCATGTATCA GCTCCTCCGTGGCCTAAAATACATACATTCCGCGAATGTTCTTCATAGAG

Manuscript II

Planta (2006)

Evolution of proteinase inhibitor defenses in North American allopolyploid species of *Nicotiana*

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Abstract

We studied the jasmonate (JA)-elicited trypsin proteinase inhibitor (TPI) anti-herbivore defense system in North American *Nicotiana* to understand how complex polygenetic traits evolve after allopolyploidy speciation. *N. quadrivalvis* (*Nq*) and *N. clevelandii* (*Nc*) are allotetraploid descendant species of the ancestors of the diploid species *N. attenuata* (*Na*) and *N. obtusifolia* (*No*). From cDNA, intron and promoter sequence analysis, and Southern blotting, we deduced that only the maternally derived *No* TPI genes were retained in the tetraploid genomes (*Nq*, *Nc*), whereas the sequences of the paternal *Na* ancestor were deleted. The number of TPI repeats in different *Nicotiana* taxa was independent of phylogenetic associations. In *Na*, TPI activity and mRNA transcript accumulation as well as JA levels increased dramatically above wound-induced levels when the oral secretions (OS) from *Manduca sexta* larvae were introduced into wounds. This OS-mediated amplification of defense signaling and downstream response was also found in the tetraploid genomes but was absent from *No*; in *No,* OS treatment suppresses TPI mRNA accumulation and activity and does not increase JA accumulation. Hence the tetraploids retained components of *Na*'s signaling system, but lost *Na*'s TPI genes and used *No*'s TPI genes to retain a functional TPI defense system, underscoring the genomic flexibility that enables complex polygenic traits to be retained in allopolyploid species.

Keywords: *Nicotiana* · polyploidy · proteinase inhibitor · polygenic adaptation · plant-herbivore interaction · Solanaceae

Abbreviations: HPLC-MS/MS: high-performance liquid chromatography coupled tandem mass spectrometry · ANOVA: analysis of variance · ITS: internal transcribed spacer OS: oral secretions

Introduction

Polyploidy is a common phenomenon in the plant kingdom. It is estimated that about 70% of all angiosperms and 95% of ferns have experienced at least one episode of chromosome duplication in their evolutionary histories (Soltis and Soltis, 1993; Masterson, 1994; Leitch and Bennett, 1997; Otto and Whitton, 2000). Large-scale chromosome duplication also shaped the evolutionary histories of yeast and vertebrates (Sidow, 1996; Kellis et al., 2004). A remarkable number of what were classically considered typical diploid plants, e.g. *Arabidopsis* and maize, are now regarded as paleopolyploids (Helentjaris et al. 1988; The *Arabidopsis* Genome Initiative 2000; Wolfe 2001).

Because most polyploids can adapt to a wide range of habitats and survive under unfavorable conditions (Levin, 1983; Soltis and Soltis, 2000), polyploidization is thought to provide evolutionary and ecological advantages over taxa that retain their original chromosome number. A commonly proposed explanation is that polyploidy, by increasing gene redundancy, releases the selective constraints on the redundant genes, allowing them to evolve novel functions that benefit species ecologically (Ohno, 1970; Otto and Whitton, 2000; Soltis and Soltis, 2000). Several studies have shown that that polyploidization entails dramatic genetic and genomic changes, including genome rearrangements, epigenetic changes, and increases in transposon activity, etc. (Soltis and Soltis, 1995; Wendel, 2000; Soltis et al., 2004; Adams and Wendel, 2005). Given this genomic activity, it is unclear how polygenetic traits survived the merger of two distinct genomes. Only the changes of only a few polygenic traits in polyploids have been studied: specifically, the number of days to flowering in newly synthesized tetraploid *Brassican napus* (Schranz and Osborn, 2000) and the morphological responses to wind in *Brassica* allopolyploids (Murren and Pigliucci, 2005). In both studies, allopolyploids displayed a greater range of responses compared to their parents.

In the genus *Nicotiana*, trypsin proteinase inhibitors (TPI) play a central role in plant-herbivore interactions. As a direct defense, they slow the growth and increase the mortality of the solanaceous lepidopteran herbivore *Manduca sexta* in its larval stage (Glawe et al., 2003; Zavala et al., 2004a). Herbivore-elicited TPI activity is a polygenic trait under both transcriptional and post-transcriptional control. Jasmonic acid (JA), ethylene, and abscisic acid (ABA) are all known to be involved in the signaling network regulating TPI transcription (Halitschke and Baldwin 2003; Koiwa et al. 1997; O'Donnell et al. 1996; Pena-Cortes et al. 1995); several TPI-specific proteases, which have been recently characterized, modulate pos-ttranslational processing of the pre-TPI protein (Horn et al. 2005). That many species in the

genus *Nicotiana* are allopolyploids makes it an ideal system in which to study how TPI defense responses fared after allopolyploidization. Cytological and morphological evidence led Goodspeed to propose that *N.* section *Bigelovianae*, *N. quadrivalvis* (syn: *N. bigelovii* (Torr.) Wats.) (*Nq*) and *N. clevelandii* (*Nc*), were tetraploids derived from amphidiploidy involving *N. attenuata* (*Na*) and an unknown "alatoid" ancestor (Goodspeed, 1954). *Na* is found in the Great Basin Desert and north along the Sierras into California and Oregon; *Nb* is found in sandy washes along the California coast, and *Nc* grows in drier habitats throughout Baja California and southern California; whereas *No* can be found in Mexico and southwestern United States (Goodspeed 1954). Recently, plastid DNA (Clarkson et al., 2004) and glutamine synthetase (Qu et al., 2004) phylogenetic analyses all revealed that *N. obtusifolia* (syn: *N. trigonophylla* Dunal) (*No*) was the missing alatoid progenitor and source of the maternal lineage for this polyploidy event. Here we examine how TPI-mediated anti-herbivore defenses compare in extant *Na*, *No*, *Nq*, and *Nc* to infer the evolutionary dynamics that occurred in the polyploid species.

Materials and methods

Plant growth

Na Torr. ex Watts. (Solanaceae) seeds were from a line maintained in our laboratory that were originally collected in Utah (USA); all other species used in this study were kindly supplied by Dr. Verne A. Sisson (Oxford Tobacco Research Station, Oxford, NC), which were originally collected by Goodspeed (1954). Seeds were germinated on agar with Gamborg B5 media (Duchefa, Harrlem, The Netherlands) (*Na* seeds were treated with smoke beforehand (Krügel et al. 2002)). Plants were grown in the greenhouse in 1 l individual pots at 26^{o}C-28^{o}C under 16 h of light supplied by Philips Sun-T Agro 400- or 600-W sodium lights.

Plant treatment and sample harvest

Plant treatments were conducted as described by Lou and Baldwin (2003), except that in this study 20 µl of *M. sexta* oral secretions (diluted 1:5 (v/v) with deionized water) and 20 µl of deionized water were applied to elicit *M. sexta*-specific responses, and only leaves at node 1 (local leaf) were analyzed. At specific times after treatment, the treated leaves were excised, immediately frozen in liquid nitrogen, and stored at -80 ^{o}C until analysis.

Northern and Southern blotting

Samples were ground in liquid nitrogen, and Trizol reagent (Invitrogen, Carlsbad, CA) was used for RNA isolation according to manufacturer's instructions. For Northern blotting, 10 μg of RNA from each sample was denatured in loading buffer at 65 ^{o}C, separated on a 1.2% formaldehyde-agarose gel, and blotted onto a GeneScreen Plus Hybridization Transfer membrane (PerkinElmer Life and Analytical Sciences, Boston, MA) using the capillary transfer method. RNA was UV-cross-linked with membranes (Stratagene, La Jolla, CA). Equal loading was controlled by comparing ethidium bromide images.

Genomic DNA samples were extracted from young leaf tissue using the CTAB method (Joly et al., 2004). After overnight digestion with various endonucleases (Fermentas, Ontario, Canada), 8 μg of each digested DNA was loaded onto a 1% agarose gel and separated in TAE buffer. After being Southern-blotted onto a GeneScreen Plus Hybridization Transfer membrane (PerkinElmer Life and Analytical Sciences), DNA was subsequently immobilized by UV cross-linking.

10 ng of DNA probe was labeled with α-^{32}P-dCTP (PerkinElmer Life and Analytical Sciences) using a random primer labeling kit (Amershambiosciences, Uppsala, Sweden), followed by purification through Probequant G-50 spin columns (Amershambiosciences).

Membranes were prehybridized with ULTRAhyb hybridization buffer (Ambion, Austin, TX) for 1 h. Radioactive labeled probes were denatured at 95 ^{o}C for 5 min and quickly chilled on ice for another 5min before being added to the buffer. After overnight hybridization, membranes were washed at 62 ^{o}C once with 2 × SSC, 0.1% SDS, and three times with 0.1 × SSC, 0.1% SDS. Imaging and band intensity quantifications were conducted on a FLA-3000 Phosphorimage system (Fujifilm Fuji Photo Film Europe, Düsseldorf, Germany).

Probes for TPI detection were prepared by PCR using primers Repeats-1 (5'-CATGCAGATGCCAAGGCTTGTC) and PI-04 (5'-GCTCCACTGCCATATTACAGATTACAGGC). Plasmids carrying TPI cDNA inserts from *Na* were applied as templates.

Probes for lipoxygenase 3 (LOX3) were prepared by PCR using primers LOX1-34 (5'-GGAACAAGAACAAGGAAGATC), LOX2-32 (5'- CTACATGTTACTCCAGGGCC) and a plasmid carrying *Na* LOX3 partial cDNA as a template.

TPI activity assay

TPI activities were quantified using a radial diffusion assay protocol described by van Dam et al. (2001).

JA analysis using HPLC-MS/MS

For JA analysis, approximately 300 mg of each frozen leaf tissue sample were crushed and transferred to a FastPrep tube containing 0.9 g of FastPrep matrix and 1 ml of extraction buffer [acetone: 50 mM citric acid = 7:3 (v/v), containing 200 ng/ml of isotope labeled JA (1,2-^{13}C-JA) as an internal standard]. On a FastPrep homogenizer (Thermo Electron) samples were homogenized for 45 seconds at speed 6.5. After centrifugation at maximum speed for 10 minutes at 4 ^{o}C, supernatants were transferred to fresh tubes. Acetone was subsequently removed from samples with a vacuum concentrator (Eppendorf, Hamburg, Germany). The residues were extracted twice with 2 ml of ether. The ether phases of each sample were combined and evaporated to dryness on the vacuum concentrator. The pellets were resuspended with 70 µl of 70% methanol and analyzed by HPLC-MS/MS.

HPLC-MS/MS analysis was carried out on an Agilent HP1100/Thermo Electron LCQ-DecaXP LC-MS instrument (Thermo Electron). From each sample 50 µl was injected into a Phenomenex Luna C18(2) column (5µ, 250 x 4.6 mm, Phenomenex, Torrance, CA). Mobile phases A (0.5% acetic acid in water) and B (0.5% acetic acid and 1% acetone in methanol) at 0.7 ml/min were used in a gradient mode for the separation. Endogenous JA and 1,2-^{13}C-JA were ionized in an APCI source. In the positive mode ions with m/z at 211 and 213 generated from endogenous JA and the internal standard were both fragmented; total daughter ions with m/z ranging from 50 to 200 were used for peak area integration.

Molecular cloning

Using a first strand cDNA synthesis kit (Invitrogen) and following manufacturer's instructions, 1µg of total RNA from each sample was subjected to reverse transcription using oligo(dT) as primer. TPI partial cDNA was obtained by PCR using primers PI-03 (5'-GGCTGTTCACAGAGTTAGCTTCCTTG) and PI-04 (5'-GCTCCACTGCCATATTACAGATTACAGGC) and cloning the PCR product into pGEM-T Easy vectors (Promega, Madison, WI). A GeneJumper Primer Insertion Kit (Invitrogen) was

employed for sequencing TPI genes with more than six repeats. 5' sequences of *Nq, Nc* and *No* TPI cDNA were obtained using the 5' RACE PCR method (GeneRacer Kit, Invitrogen) following manufacturer's instructions and employing PI-5RACE-5 (5'-GCCTTGGCATCTGCATGCTCCACAAT) and PI-5RACE-6 (5'-GCATGCTCCACAATGCTTACAAGCA) as primary and nested primers. The *Nq* full-length TPI sequence was obtained by screening a cDNA library (Uni-Zap XR, Stratagene) prepared from *Nq* shoot and root tissue exposed to *M. sexta* feeding for 24 h. Using a *Na* TPI probe, around 150,000 plaques were screened, and five positive clones were *in vivo* excised following the kit's instructions and subsequently sequenced.

Promoters were isolated using a Universal GenomeWalker kit (Clontech Laboratories, Mountain View, CA) following manufacturer's instructions with modifications. Genomic DNA from *Na*, *Nq*, *NC*, and *No* was digested with *EcoR* V, *Dra* I, and *Ssp* I overnight; after purification DNA was ligated with GenomeWalker adaptors. Primers PIProm-2 (5'-GAGGAGAGCAAGGAAGCTAACTCTGTGA) and PIProm-3 (5'-CAAGGAAGCTAACTCTGTGAACAGCCA) were used as primary and nested PCR primers. PCR products were cloned into pGEM-T Easy vectors (Promega) and then sequenced.

TPI intron sequences were obtained by PCR using respective genomic DNA as templates with primers listed in supplemental Table 1.

Sequencing was conducted on an ABI PRISM 3700 sequencer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). For each gene at least two clones were fully sequenced.

All the sequences have been deposited in the GeneBank database under accession DQ158181 to DQ158203.

Phylogeny analysis

TPI cDNA sequences were translated into protein sequences and aligned using MegAlign (DNASTAR, Madison, WI) employing Clustal W algorithm; then sequences were reversed back to nucleotide sequences. *Capsicum annum* Pin II gene (accession AF221097) was used as an outgroup. Using MEGA 3 (Molecular Evolutionary Genetics Analysis; Kumar et al. 2004), a Neighbor-Joining tree was built with a Nucleotide Kimura 2-parameters model. Support for the groups was evaluated with 1000 bootstrap replicates.

Statistical analysis

The data from each group of comparisons at a specific time point after wounding and OS elicitations were analyzed by ANOVA after having been transformed (arcsine or log) if they did not meet the assumption of normality (StatView software, SAS Institute).

Results

Southern blot analysis

Several studies have observed that in polyploids, genome rearrangement seems to be a common phenomenon (Song et al., 1995; Feldman et al., 1997). To investigate genomic changes in the TPI genes after polyploid speciation, Southern blotting analyses were carried out on both ancestor and descendant species. A probe derived from the second exon of *Na* TPI was applied in the Southern blot. Strong hybridization signals were obtained in all blots (Fig. 1).

Na showed a single band in all lanes except one: the *EcoR* I digestion gave two strong bands, indicating that two TPI genes in the *Na* genome were tandem duplicated genes. Indeed, another TPI-like gene with a putatively intact ORF was cloned from the *Na* genome. However, despite sequencing more than 10 cDNA clones obtained by screening a cDNA library and attempting to produce PCR clones directly, we failed to find this gene in *Na* cDNA pools, suggesting that the second TPI-like gene might be a pseudogene lacking a functional promoter. The banding pattern of *No* genomic DNA gel blots was more complex: all lanes showed at least two bands, indicating the presence of two or more copies of TPI genes in *No*. As in *Na*, in the *No* cDNA pool only one functional copy of TPI was found. Conversely, only a single band was observed in the genomic DNA gel blots of both tetraploids, confirming the existence of only a single copy of the TPI gene in *Nq* and *Nc* genomes.

TPI gene cloning and phylogeny analysis

To investigate the evolution of TPI sequences on a molecular level, we used RT-PCR cloning and cDNA library screening to isolate TPI cDNA sequences. In addition to the TPI gene isolated from *Na* (Zavala et al., 2004b), one TPI gene was cloned from *No*, *Nq*, and *Nc*, respectively. Protein sequence analyses of the genes revealed that they all had typical Pin II gene structures, similar to published *N. alata*, *N. tabacum,* and *N. glutinosa* TPI protein

sequences (Atkinson et al., 1993; Choi et al., 2000; Huffaker et al., 2006) (Fig. 2). The ancestor species, *Na* and *No*, had 7 and 8 repeats, respectively; both descendant tetraploid species had only 6 repeats.

To elucidate the orthology of TPI genes in *Nq* and *Nc*, several TPI genes were cloned from several closely related *Nicotiana* species and a Neighbor-Joining gene tree was inferred from these cDNA sequences (Fig. 3). The analysis clarified that the published double copies of TPIs found in *N. alata* and *N. glutinosa* (Atkinson et al., 1993; Choi et al., 2000; Miller et al., 2000) both originated from recent gene duplication events, as indicated by their short branch lengths. *Nc* and *Nq* formed sister species that grouped together with *No* as supported by a high bootstrap percentage (100), suggesting that both *Nc* and *Nq* but not *Na* TPI genes were inherited from *No*, and that both were from a common tetraploid ancestor. The promoter and intron sequences were also cloned from *Na*, *No*, *Nq,* and *Nc*; their sequence alignments showed great similarities among *No*, *Nq,* and *Nc* (supplemental Tables 2 and 3), consistent with the orthologous relationships revealed by the cDNA gene tree.

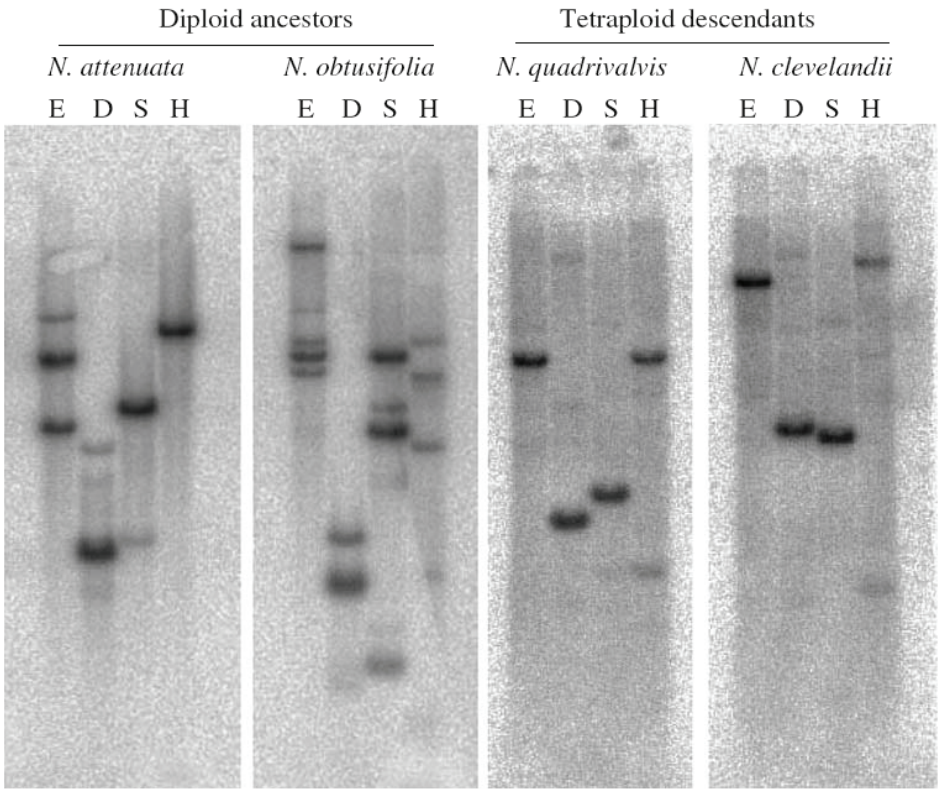

Fig. 1 Southern bloting analysis of trypsin inhibitor genes in both parental diploid and descendant tetraploid species. Genomic DNA was digested with *EcoR* I (E), *Dra* I (D), *Ssp* I (S) and *Hind* III (H), Southern-blotted onto nylon membranes, and subsequently hybridized with a TPI-specific probe

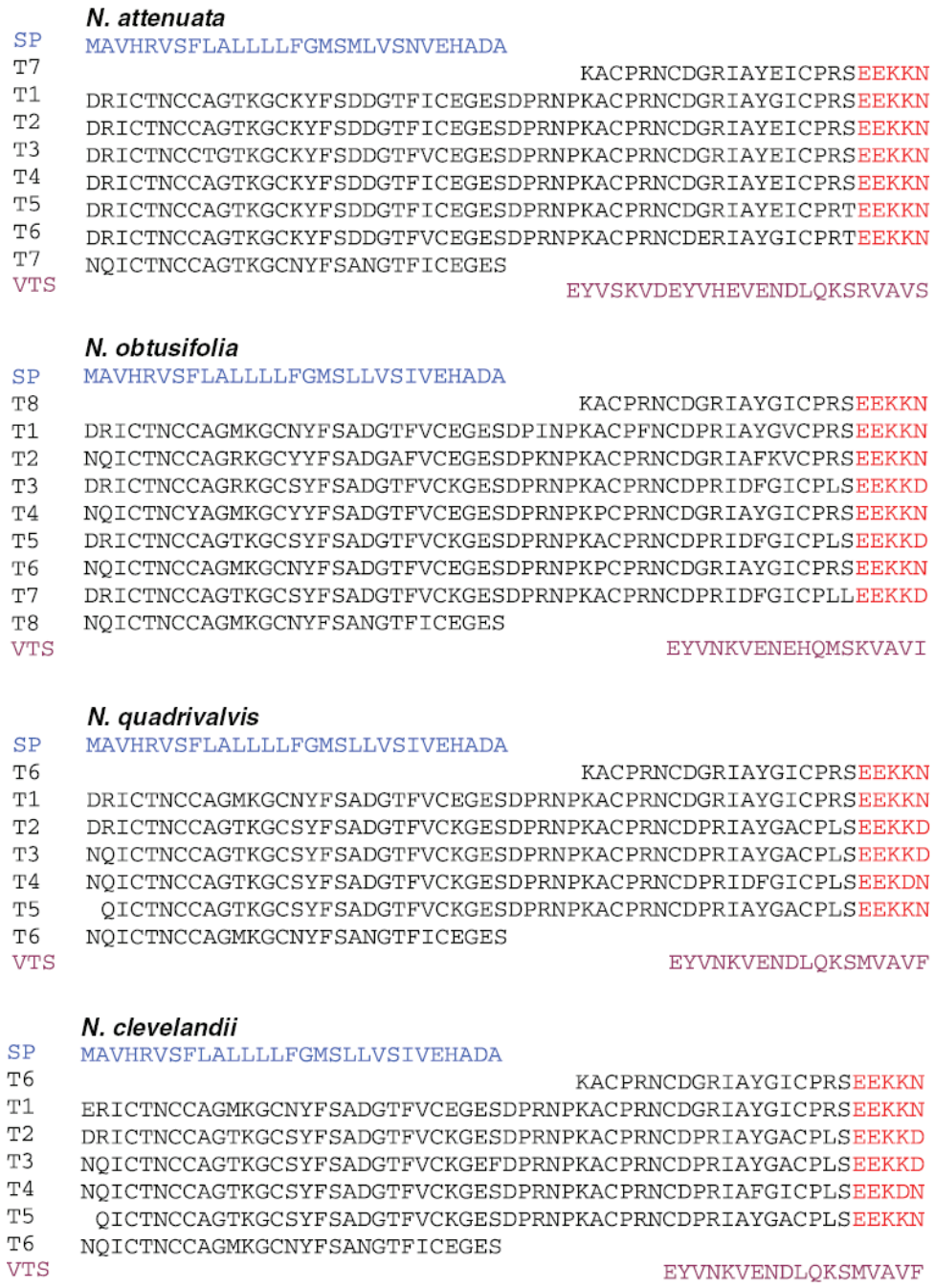

Fig. 2 Amino acid sequences of TPIs in *Na*, *No*, *Nq*, and *Nc*. Each gene contains a signal peptide at N-terminal (SP, in blue letters) followed by repetitive trypsin inhibitor domains (T1-T8), and a vacuolar targeting sequence at C-terminal (VTS, in purple letters); linkers that connect the repetitive domains are in red letters

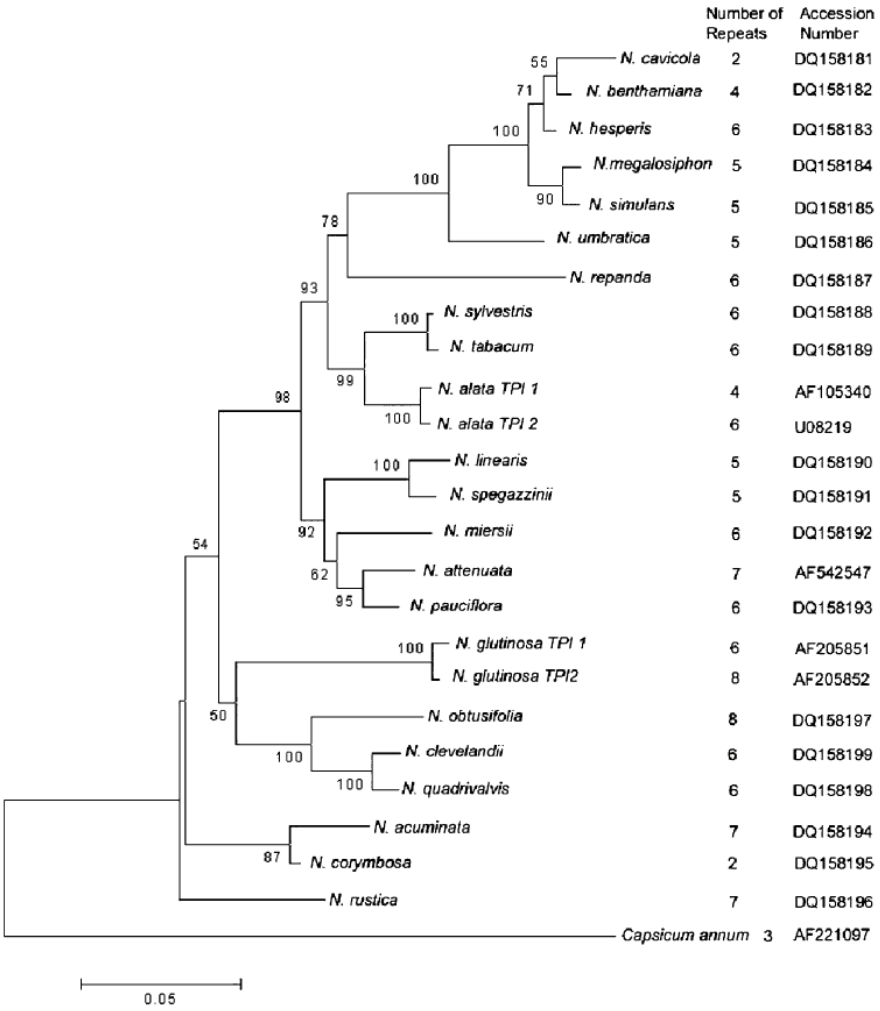

Fig. 3 Neighbor-Joining tree of *Nicotiana* TPIs based on cDNA sequences. Nucleotide Kimura 2-parameters model and 1000 bootstrap replicates were employed for tree construction. Bootstrap values less than 50% were not labeled. *N. glutinosa* (AF205851, AF205852), *N. alata* (AF105340, U08219), *N. attenuata* (AF542547) and *Capsicum annum* (AF221097) TPI genes were retrieved from GeneBank database

Herbivore-specific responses in *Na*, *Nq,* and *Nc*

Nicotiana plants are known to accumulate higher levels of TPI mRNA and activity in response to herbivore attack (Choi et al., 2000; Glawe et al., 2003; Zavala et al., 2004b). To investigate the changes that occurred after the polyploid speciation, we examined TPI mRNA expression and protein activity after wounding and treatment of the wounds with either water (W+W) or *M. sexta* OS (W+OS); the latter treatment mimics the responses elicited by *M. sexta* attack (Halitschke et al. 2001; Halitschke and Baldwin 2003, and references therein).

TPI transcript accumulation in *Na*, *Nq,* and *Nc* showed comparable patterns with only minor differences in kinetics (Fig. 4). After elicitation, TPI mRNA reached maximum levels in all species within 12-24 h and decreased to normal levels in 3-4 days. *Na* retained a high level of expression through day 2, whereas in *Nc* transcript levels started to decrease after 24 h. The responses waned more quickly in *Nq*; there TPI mRNA declined to uninduced levels within 24 h. Nevertheless, both *Na* and the tetraploids responded with higher levels of TPI transcripts to W+OS treatments than to W+W treatments, suggesting that both tetraploids

inherited components of the TPI signaling system from *Na* which enabled them to recognize attack from *M. sexta* larvae.

TPI protein activity levels largely reflected their mRNA transcription profiles (Fig. 5). All plants elicited with either W+OS or W+W enhanced their TPI activities. *Na*, *Nq,* and *Nc* showed maximum TPI activity 24 h after wounding and sustained levels even after 96 h. No activity differences between OS and wounding treatments were observed in *Na* and *Nq* before 24 h. However, differences were apparent in *Nc* before 24 h. After 48 h of OS elicitation, TPI activity in *Nc* quickly declined to levels found in the W+W treatment, suggesting that *Nc* acquired new post-transcriptional TPI regulation mechanisms. This result was consistent with Lou and Baldwin's previous findings (Lou and Baldwin, 2003), in which they observed that *Nc* did not recognize OS elicitation and 4 days after elicitation expressed similar levels of TPI activity after W+OS and W+W treatments.

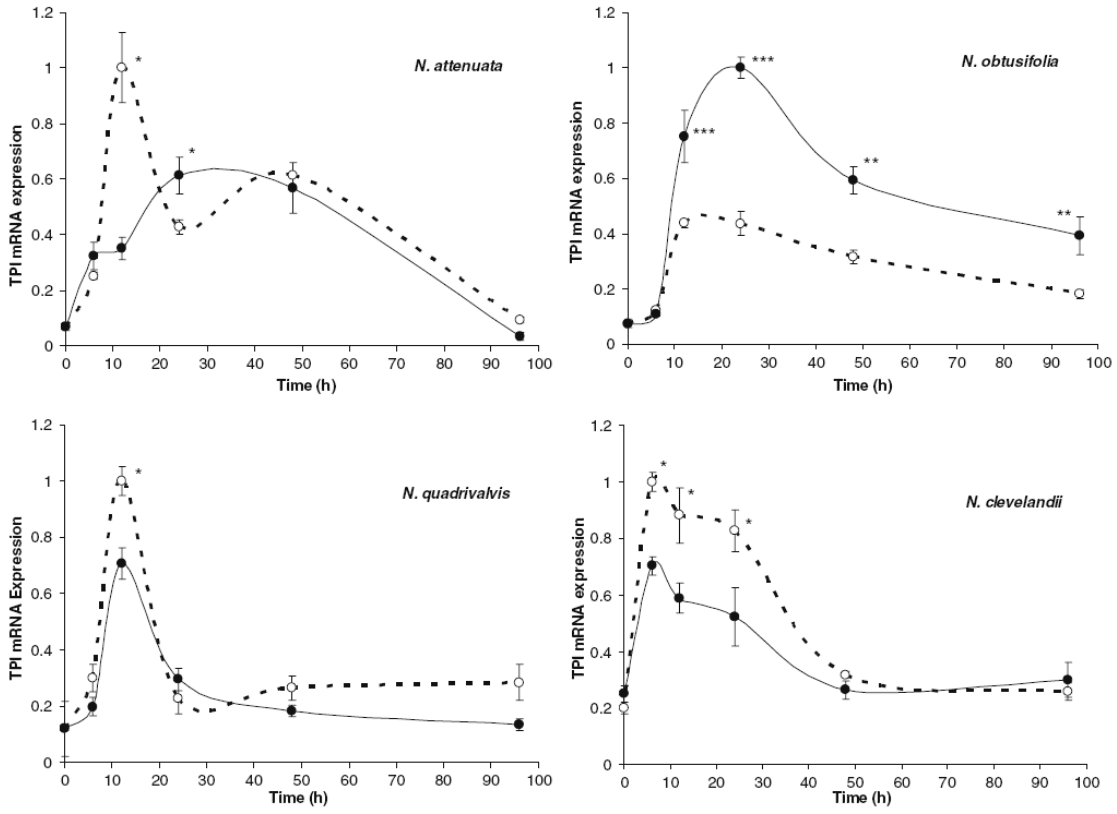

Fig. 4 Expression of TPI mRNA in *Na*, *No*, *Nq,* and *Nc* after wounding and simulated *M. sexta* attack. Accumulation of TPI mRNA was analyzed by Northern blotting. Total RNA was extracted from four replicate plants at each harvest time. A single leaf from each plant was wounded with a fabric pattern wheel and the wounds immediately treated with either 20 µl of deionized water (closed circles, solid line) or 20 µl of *M. sexta* oral secretions (open circles, dotted line). All values were normalized with the highest expression as 1. Asterisks indicate levels of significant difference between members of a pair (N = 4; *, $P < 0.05$; **, $P < 0.01$)

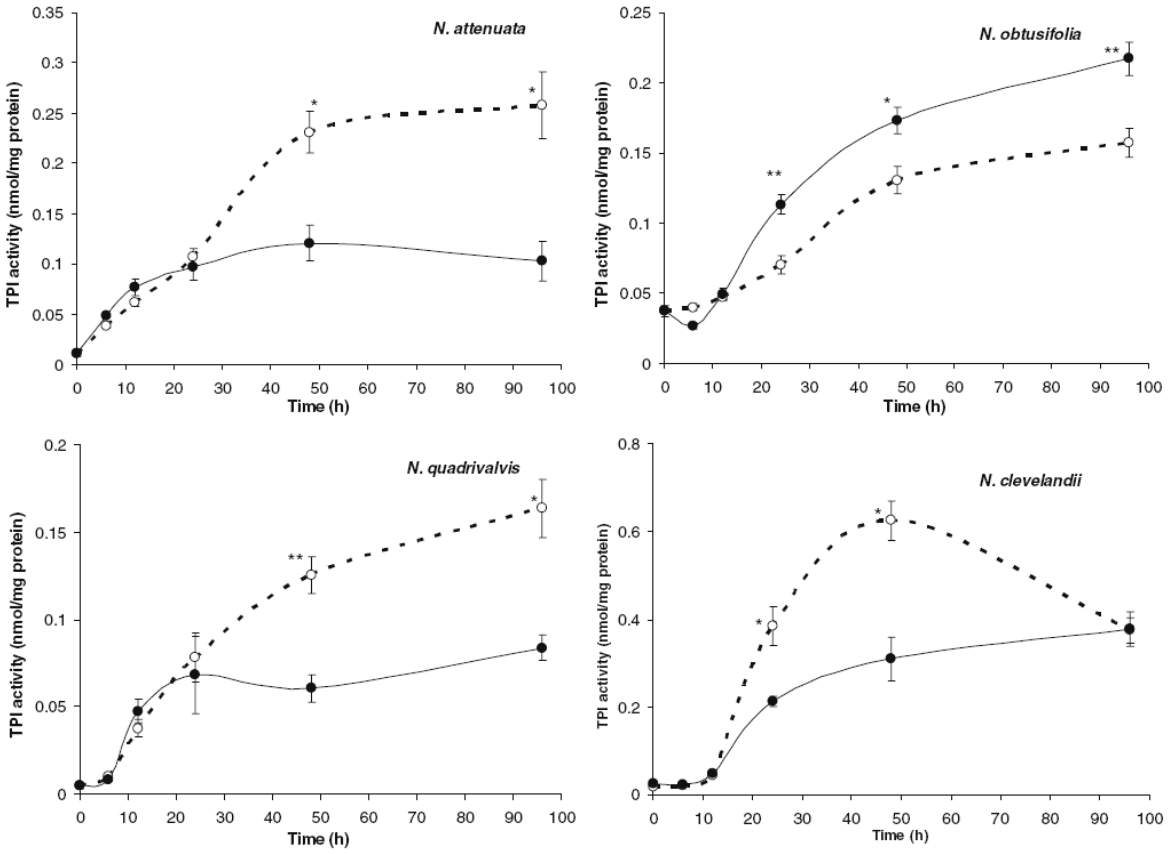

Fig. 5 TPI activity in *Na*, *No*, *Nq* and *Nc* after wounding and simulated *M. sexta* attack. TPI activity was measured in four replicate plants at each harvest time. A single leaf on each plant was wounded with a fabric pattern wheel and the wounds immediately treated with either 20 µl of deionized water (closed circles, solid line) or 20 µl of *M. sexta* oral secretions (open circles, dotted line). All values were normalized with the highest expression as 1. Asterisks indicate levels of significant difference between members of a pair (N = 4; *, $P < 0.05$; **, $P < 0.01$)

JA plays an important role in mediating TPI responses in *Na* as demonstrated by silencing the specific lypoxygenase (NaLOX3), which supplies hydroperoxide substrates for JA biosynthesis (Halitschke and Baldwin, 2003). Lou and Baldwin (2003) found that *Na* and the two tetraploid descendants, *Nq* and *Nc*, all responded to OS elicitation with higher amount of JA than was elicited by W+W treatments. We used Northern blotting analyses of LOX3 transcripts to understand the basis of the elicited JA responses (Porta and Rocha-Sosa, 2002). W+OS treatment transiently elicited higher levels of LOX3 gene expression in the paternal ancestor, *Na,* and in both tetraploid species than did W+W treatments, suggesting a correlation between elevated JA levels and increased LOX3 expressions (Supplemental Fig. 1).

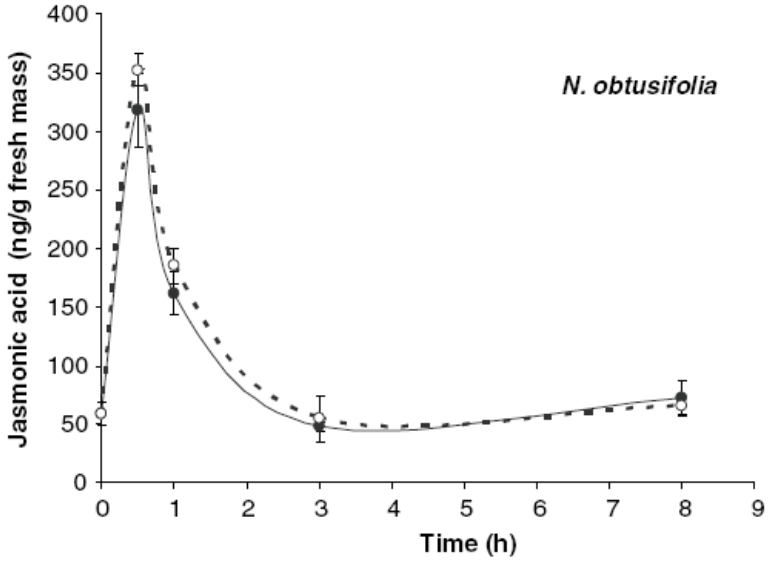

Fig. 6 Jasmonic acid (JA) concentrations in *N. obtusifolia* leaves after being elicited by wounding with a fabric pattern wheel and immediately treating the puncture wounds with either 20 µl of deionized water (closed circles, solid line) or 20 µl of *M. sexta* oral secretions (open circles, dotted line) (N = 4, $P > 0.35$)

Herbivore-specific responses in *No*

No revealed responses that were distinct from the other species. *No* responded to both elicitations with higher levels of TPI transcript (Fig. 4), but plants treated with W+OS produced significant fewer TPI transcripts than did W+W treated plants. The profile of TPI activity in *No* was similar to the profile of its mRNA expression (Fig. 5); W+W treatment resulted in significantly higher levels of TPI activity than did W+OS treatment.

JA analysis also demonstrated that *No* had a different induction pattern. JA levels in *No* were not elevated in response to OS elicitation (Fig. 6). Consistent with the results from JA analysis, LOX3 gene expression in W+OS-treated *No* plants did not differ significantly from its expression in plants treated with W+W (Supplemental Fig. 1).

Discussion

Results revealed by our phylogeny analysis using TPI cDNA sequence were consistent with glutamine synthetase gene tree (Qu et al., 2004), in which *No* and *Na* were involved in the allopolyploid speciation event that led to the formation of the common ancestor of *Nq* and *Nc*. However, in the analysis using plastid DNA and ITS genes (Chase et al. 2003; Clarkson et al. 2004), *Nc* and *Nq* were successive sister species to *No*, implying they were from two

independent polyploidy events. The discrepancy may be caused by selection pressures on these functional genes and concerted evolution on ITS genes, which might generate biased gene trees. More phylogeny analyses based on other genes may clarify the evolution history of this speciation event.

In addition, the high degree of similarity among the *No*, *Nq,* and *Nc* TPI sequences reveals their orthology and suggests that the history of divergence since speciation has been brief. The Southern blotting analysis, which revealed similar digestion patterns between *Nq* and *Nc* (Fig. 1), confirms this pattern. Southern blotting and phylogenetic analysis also revealed that the *Na* TPI orthologous genes had been completely deleted from the tetraploid genomes and that only the *No* orthologous genes were retained in the polyploid species. This gene deletion event may have happened before *Nq* and *Nc* diverged from their common ancestor, since two independent gene deletion events in *Nq* and *Nc* would be unlikely. Given the unlikeness of two independent deletion events, the genes were probably deleted before *Nq* and *Nc* diverged from their common ancestor. Moreover, according to the same logic, *Nq* and *Nc* probably descended from a common ancestor rather than from two independent speciation events. Several studies of artificially generated tetraploids have demonstrated that gene deletion is a common consequence of genome rearrangement after polyploidy hybridization (Song et al., 1995; Liu et al., 1998a; Liu et al., 1998b; Kashkush et al., 2002; Han et al., 2003; Madlung et al., 2005), and similar conclusions have been drawn from studies of natural polyploids (Lai et al., 2004; Wang et al., 2005). Gene deletions from polyploids appear to be regulated and are not completely random; genes involved in defense appear to have a higher probability of being deleted (Feldman et al., 1997; Blanc et al., 2000; Langkjaer et al., 2003). Consistent with this empirically determined pattern, the TPI gene returned to a single-copy state in both tetraploid species we analyzed. Particular gene-cytoplasm interactions may allow more fragments of the maternal genome to be retained in polyploids (Song et al., 1995). We found that TPIs in *Nq* and *Nc* were all orthologous to the *No* TPI, the maternal ancestor.

The repetitive domain structure, which appears to be a unique characteristic of *Solanaceae* Pin II genes (Kertesz et al., 2006), is thought to derive from a single domain common ancestor. Unequal crossing-over is presumed responsible for the expansion of the repeated domain. With the exception of genes with 3 repeats, *Nicotiana* TPI genes display a wide range of repeat numbers, ranging from 2 to 8. In *N. cavicola* and *N. corymbosa,* the TPI genes have only 2 repeats; one perfect domain and another imperfect domain, in which the N- and C-terminals are joined by disulfide-bonds (Lee et al., 1999) (Supplemental Fig. 2). We propose that these two genes remained in their original state, e.g., that no further repeat

expansions took place following the expansion event from the single repeat ancestor. The repeat-expansion events appear to have been haphazard, since plants with close phylogenetic relationships can have very different repeat numbers, e.g., *N. acuminata* and *N. corymbosa* with 7 and 2 repeats, respectively (Fig. 3). If expansion of the repetitive domains is the common trend for TPI gene evolution in *Solanaceae* (Kertesz et al., 2006), this random expansion phenomenon can be seen by comparing *No*, *Nq,* and *Nc* sequences. *No* has 8 repetitive domains; *Nq* and *Nc* both have 6 domains. This suggests that in *No*, two domain expansion events occurred after speciation. The evolutionary advantages of repetitive TPI domain expansion have not been established, but it is reasonable to assume that repetitive domains provide plants with a more efficient use of transcription, translation, and cell compartment targeting (Heath et al., 1995).

A growing body of evidence suggests that the evolution of signal perception, transduction, and transcriptional regulators contribute more to the evolution of the phenotype than does the evolution of coding genes (Doebley and Lukens 1998). JA plays a important role in regulating the expression of TPI genes (Pena-Cortes et al., 1995; Koiwa et al., 1997; Halitschke and Baldwin, 2003; Heidel and Baldwin, 2004). *Na*, *Nq,* and *Nc* all expressed both TPI mRNA and activity at high levels perhaps due to the higher amount of JA in their leaves after OS elicitation. Surprisingly, in *No*, the levels of JA didn't change after OS inductions, which was not consistent with TPI induction, as TPI transcript levels were suppressed by OS treatments. Several studies have revealed that another signaling compound, ethylene, also plays a critical role in TPI regulation. After blocking ethylene's effect, tomato plants do not accumulate detectable amounts of TPI transcripts (O'Donnell et al., 1996). In cultivated tobacco, increasing the activity of a MAP kinase promotes ethylene biosynthesis and elevates levels of TPI transcripts without altering JA responses (Kim et al., 2003). Moreover, abscisic acid (ABA) has also been shown to be involved in the signaling network controlling TPI inductions (Pena-Cortes et al., 1991). In *No,* differences among r specific elements in the signaling network, e.g., ethylene or ABA, have likely contributed to the OS-elicited decrease in TPI transcripts and activity levels.

OS recognition clearly plays a central role in the TPI-mediated defense against herbivore attack. This recognition is probably regulated by a network composed of an upstream signaling system, downstream interactions between *cis* and *trans*-elements, and post-transcriptional regulations. Compared to their orthologous ancestor *No*, TPI promoter sequences in both *Nq* and *Nc* have diverged little, suggesting that they may have the same *cis*-elements. However, with regard to both transcripts and activity levels, the tetraploid

descendants resemble their paternal ancestor *Na* more than their maternal ancestor, *No*. Both tetraploids have likely retained the upstream signaling network and *trans*-regulating elements from *Na* but abandoned those from *No*. In another scenario, the signaling system and *trans*-regulation elements involved in OS recognition genetically dominate those inherited from *No*; in other words, although both systems might coexist, tetraploids still possess the ability to recognize attack from *M. sexta* larvae.

As a consequence of polyploid speciation, *Nq* and *Nc* both inherited the ability to recognize herbivore attack and raise levels of TPI activity accordingly. This might have endowed tetraploids with ecological and evolutionary advantages that have helped them to survive the ongoing plant-herbivore arms race. This study illustrates the plasticity of polygenic traits following polyploidization. More detailed molecular and genetic studies of the evolution of TPI-related signaling networks and post-transcriptional processing in this polyploid species complex will provide new insights into the molecular events that have shaped the evolution of TPI phenotypes.

Acknowledgments

We thank Max-Planck Gesellschaft and the Deutsche Forschungsgemeinschaft (SPP1152) for providing funding for this work.

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Supplemental Data

Supplemental Table 1 Primers used for the cloning of TPI introns

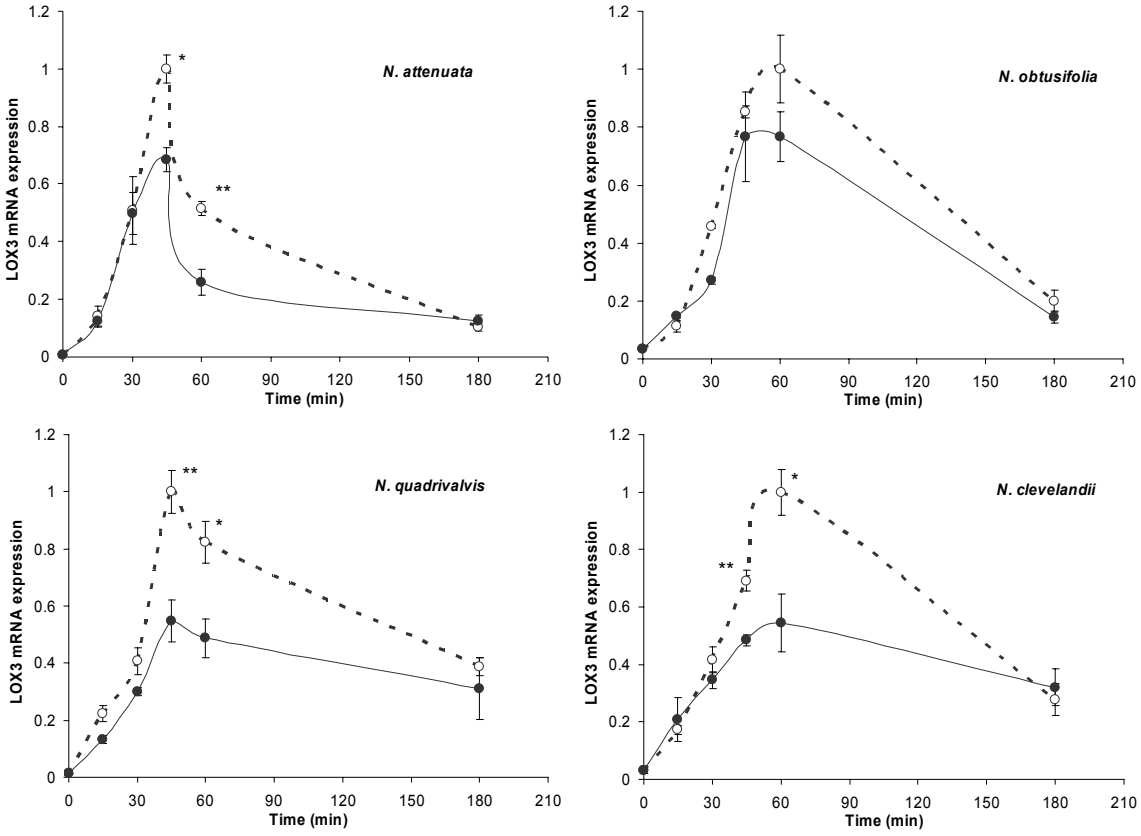
Speicies	Primer1	Primer2
N. attenuata	5' - GGCATCTGCATGTTCCACATTGCTTAC	5'- TCATGGGCAATAGGAAGTGAAAACCACG
N. obtusifolia	5’-GTTATGGCTGTTCACAGAGTTAG	5’-GCTCCACAATGCTTACAAGCAGA
N. clevelandii	5’-GTTATGGCTGTTCACAGAGTTAG	5’-GCTCCACAATGCTTACAAGCAGA
N. quadrivalvis	5’-GTTATGGCTGTTCACAGAGTTAG	5’-GCTCCACAATGCTTACAAGCAGA

Supplemental Table 2 Intron similarities among *Na*, *No*, *Nq,* and *Nc*; genomic sequences are deposited in GeneBank database under accession numbers DQ158200 to DQ158203

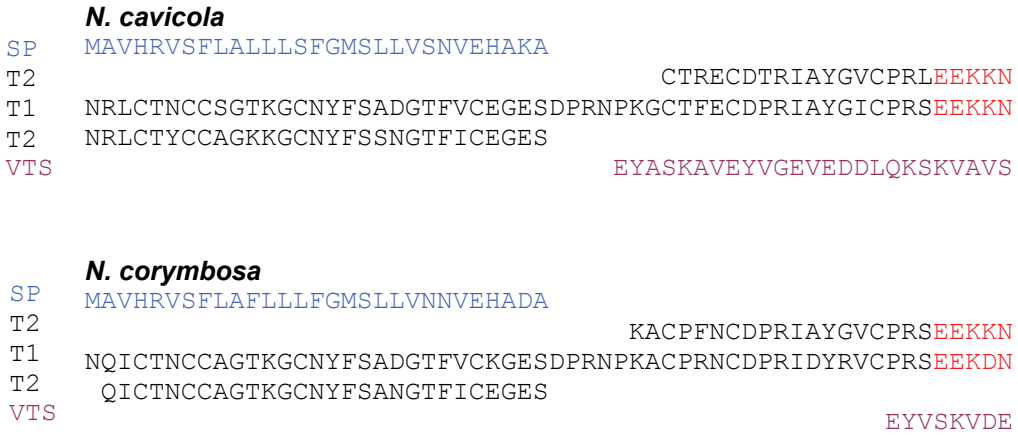
	N. attenuata	*N. obtusifolia*	*N. clevelandii*	*N. quadrivalvis*
N. attenuata		11.9%	13.5%	15%
N. obtusifolia			85.7%	80.5%
N. clevelandii				82.8%
N. quadrivalvis				

Supplemental Table 3 Promoter similarities among *Na*, *No*, *Nq* and *Nc*; genomic sequences are deposited in GeneBank database under accession numbers DQ158200 to DQ158203

	N. attenuata	*N. obtusifolia*	*N. clevelandii*	*N. quadrivalvis*
N. attenuata		38.2%	25.5%	26.6%
N. obtusifolia			87.8%	88.8%
N. clevelandii				95.4%
N. quadrivalvis				



Supplemental Fig. 1 Transcript accumulation of lipoxygenase 3 (LOX3) genes in *Na*, *No*, *Nq* and *Nc* after wounding and herbivore elicitation. The expression level of LOX3 mRNA was analyzed by northern blotting. Total RNA was extracted from three replicate plants harvested at indicated time points. A single leaf on each plant was either wounded with a fabric pattern wheel and the wounds immediately treated with 20 µl of deionized water (closed circles, solid line) or with 20 µl of *M. sexta* oral secretions (open circles, dotted line). All values were normalized with the highest expression as 1. Asterisks indicate levels of significant difference between members of a pair (N = 4; *, $P < 0.05$; **, $P < 0.01$)



Supplemental Fig. 2 Amino acid sequences of TPI in *N. cavicola* and *N. corymbosa*. Each gene contains a signal peptide at N-terminal (SP, in blue letters) followed by repetitive trypsin inhibitor domains (T1-T2) and a vacuolar targeting sequence at C-terminal (VTS, in purple letters); linkers that connect the repetitive domains are in red letters. Each gene has only one perfect domain (T1) and one imperfect domain composed of half from N-terminal and half from C-terminal (T2)

Manuscript III

Plant Journal (2007)

A deletion mutation in a trypsin proteinase inhibitor gene in *Nicotiana attenuata* Arizona ecotype triggers nonsense-mediated mRNA decay

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Running title: NMD suppresses mRNA levels of mutated *TPI* gene

Key words: nonsense-mediated mRNA decay, NMD, mRNA surveillance, premature termination codon, trypsin proteinase inhibitor, *Nicotiana attenuata*

Sequence data from this article can be found in GenBank/EMBL database under accession numbers: EF187725, EF187726, EF187727, EF187728 and EF187729

Summary

In eukaryotes, genes carrying premature termination codons (PTCs) often correlate with decreased mRNA levels compared to their counterparts without PTCs. PTC-harboring mRNA is rapidly degraded through the nonsense-mediated mRNA decay (NMD) pathway to prevent the accumulation of potentially detrimental truncated proteins. In a native ecotype of *Nicotiana attenuata* collected from Arizona (AZ), the mRNA levels of a *trypsin proteinase inhibitor* (*TPI*) gene are substantially lower than in plants collected from Utah (UT). Cloning the AZ *TPI* gene revealed that a 6 bp deletion mutation in exon 2 results in a PTC and decreases mRNA levels through NMD. Silencing *UPF1*, *2,* and *3* in *N. attenuata* AZ plants by virus-induced gene silencing (VIGS) enhanced the levels of PTC-harboring *TPI* mRNA, demonstrating a conserved role for *UPF* genes in plants. Furthermore, using cell suspension cultures that express different *TPI* construct variants, we demonstrate that both intron-containing and intronless genes are subject to NMD in plants; unlike PTCs in mammals, PTCs downstream of introns activate NMD in plants. However, when a PTC is only 4 bp upstream of an intron, the NMD surveillance mechanism is abrogated, demonstrating the complexity of the NMD activation mechanism in plants. We also demonstrate that in an intronless *TPI* gene, a PTC located at the beginning or the end of the coding sequence triggers NMD less efficiently than do PTCs located at the middle of the coding sequence.

Introduction

Nonsense-mediated mRNA decay (NMD) is a conserved mRNA surveillance pathway in all eukaryotes; the pathway rapidly degrades mRNA containing premature termination codons (PTCs), which can lead to the synthesis of truncated proteins and have dominant negative effects (Conti and Izaurralde, 2005; Hentze and Kulozik, 1999; Hilleren and Parker, 1999). In humans, approximately 30% of inherited genetic disorders are due to genes with frameshifts or nonsense mutations that result in PTCs (Culbertson, 1999; Frischmeyer and Dietz, 1999; Holbrook *et al.*, 2004). PTCs may also arise in the normally programmed rearrangement events that occur in immunoglobulin and T-cell receptor genes (Carter *et al.*, 1996; Li and Wilkinson, 1998); more important, alternative splicing, which plays a fundamental role in diversifying transcriptomes, generates a substantial number of PTC-harboring mRNA

species (Lejeune and Maquat, 2005; Lewis *et al.*, 2003). Through the NMD pathway, eukaryotes identify and degrade aberrant mRNA to eliminate genomic noise and ensure the fidelity of gene transcription.

Numerous studies have revealed the important roles of three proteins involved in NMD, namely, UPF1, UPF2, and UPF3. As core components of the surveillance complex, they are conserved in all eukaryotes (reviewed in Conti and Izaurralde, 2005; Culbertson and Leeds, 2003; Maquat, 2004). Silencing or knocking out *UPF* genes dramatically stabilizes PTC-containing mRNA species in all organisms studied to date (Arciga-Reyes *et al.*, 2006; Cali and Anderson, 1998; Gatfield *et al.*, 2003; Leeds *et al.*, 1991; Leeds *et al.*, 1992; Mendell *et al.*, 2002). Although UPF proteins occur in all eukaryotes, how PTCs are recognized and how NMD is triggered vary among different organisms (Conti and Izaurralde, 2005; Rehwinkel *et al.*, 2006). In mammalian cells, NMD is activated if a PTC is located at least 55 nucleotides upstream of an exon-exon junction (Nagy and Maquat, 1998). Budding yeast, *Saccharomyces cerevisiae*, discriminates premature from normal stop codons using particular downstream sequence elements (DSE) (Gonzalez *et al.*, 2001; Ruiz-Echevarria *et al.*, 1998; Zhang *et al.*, 1995). In yeast, the distance between a stop codon and the poly(A) tail was also shown to play an important role in NMD activation (Amrani *et al.*, 2004).

Besides the conserved function of the NMD pathway in degrading aberrant mRNA, recent studies have revealed that this pathway is also involved in regulating various cellular activities. Microarray analyses indicate that in mammalian cells, NMD is involved in regulating genes that encode key regulators in amino acid metabolism (Mendell *et al.*, 2004). In yeast, at least 10% of the transcriptome is regulated by NMD (He *et al.*, 2003). NMD may also play a role in the preservation of intron-encoded small nucleolar RNA in *Caenorhabditis elegans* (Mitrovich and Anderson, 2005). Another study in *C. elegans* demonstrated a connection between NMD and RNA interference (Domeier *et al.*, 2000). In mice, the mutant *Rent1* gene (a *UPF1* orthologue in mammals) revealed a role of *Rent1* in embryonic development (Medghalchi *et al.*, 2001). NMD also has been shown to regulate a wide range of cellular functions in *Drosophila* (Rehwinkel *et al.*, 2005b).

Although much is known about NMD mechanisms in yeast and mammals, little is known about NMD in plants. In some cultivars of soybean, a frameshift mutation causes PTCs, leading to highly attenuated levels of *Kunita trypsin inhibitor*

mRNA in embryos (Jofuku *et al.*, 1989). Transgenic tobacco plants expressing the PTC-containing *patatin* gene have lower mRNA levels than do plants expressing the wild-type (WT) *patatin* gene (Vancanneyt *et al.*, 1990). In tobacco, the *ferredoxin-1* mRNA harboring a nonsense codon is quickly degraded through the NMD pathway in light-treated tissues (Dickey *et al.*, 1994; Petracek *et al.*, 2000). In some cultivars of barley, nonsense mutations in the waxy gene decrease the amylose content of starch (Domon *et al.*, 2002). Several recent studies using Arabidopsis mutants point to the conserved roles of *UPF* genes in plants. In Arabidopsis *upf1* and *upf3* mutants, PTC-harboring mRNA species are more abundant than in WT plants; moreover, morphological changes in these mutant suggest that NMD is involved in plant development (Arciga-Reyes *et al.*, 2006; Hori and Watanabe, 2005; Yoine *et al.*, 2006).

In *Nicotiana attenuata*, a *trypsin proteinase inhibitor* (*TPI*) gene plays a central role in the plants' defense against herbivory. When insect herbivores ingest TPI-containing leaf material, the TPIs bind to proteases in the herbivore's midguts and impair protein digestion, slowing growth and increasing the mortality of herbivore larvae (Anderson *et al.*, 1997; Broadway, 1995; Zavala *et al.*, 2004). TPI activity in *N. attenuata* plants collected from Utah (UT) is dramatically increased after attack from larvae of the Solanaceous specialist, *Manduca sexta*, due to increases in both transcriptional and post-transcriptional processing of the 7-domain *TPI* (Horn *et al*. 2005; Zavala *et al*. 2004). These increases are elicited when fatty acid amino acid conjugates found in larval oral secretions are introduced into wounds during feeding and require a functional jasmonate signaling cascade (Halitschke *et al*, 2003; Roda *et al*. 2004). However not all ecotypes of *N. attenuata* increase TPI activity after herbivore attack or jasmonate elicitation. In an *N. attenuata* ecotype collected near Flagstaff Arizona (AZ) in 1996 and again in 2004, no detectable levels of TPI activity and substantially decreased levels of *TPI* mRNA are found (Glawe *et al.*, 2003).

Here we show that *TPI* in AZ plants harbors a deletion mutation at the beginning of the second exon. Because this mutation leads to a PTC in the *TPI* gene, its transcripts are rapidly degraded through the NMD pathway. We examined the function of all three *UPF* genes, *UPF1*, *2*, and *3*: silencing each *UPF* gene in AZ plants elevated PTC-harboring *TPI* mRNA levels, indicating their conserved functions in plants. Using cell suspension cultures to express different *TPI* constructs, we show that in contrast to the important role played by introns in PTC recognition in

mammals, introns are largely unnecessary for activating PTCs in plants. Nevertheless, when the PTC is located only 4 bp upstream of an intron, NMD is abrogated. The dependency of NMD on translation and the positional effects of PTCs in a coding region were also investigated.

Results

A deletion mutation in AZ TPI *decreases its mRNA levels*

To compare the levels of *TPI* mRNA in UT and AZ plants, we treated both plants with methyl jasmonate (MJ), a potent inducer of *TPI* transcription (Koiwa *et al.*, 1997; Glawe *et al.*, 2003), in a lanolin paste, and with lanolin paste alone as controls. After 24 h, when the plants have the highest induced *TPI* levels, the levels of *TPI* mRNA in UT and AZ plants were measured using northern blotting. AZ plants accumulated only 14% of the UT *TPI* mRNA levels; similarly, *TPI* mRNA in lanolin-treated AZ plants was around 30 times less abundant than in lanolin-treated UT plants (Figure 1a). To investigate the molecular mechanism underlying the *TPI*-deficient phenotype in AZ plants, we cloned both the cDNA and genomic DNA of AZ *TPI*, including a 1.3 kb promoter. Sequence alignment between cDNA and genomic sequences revealed that the *TPI* gene has a short, 69 bp first exon, and a 1.6 kb intron, followed by a 1.5 kb exon. Previously cloned UT *TPI* (Wu *et al.*, 2006) and AZ *TPI* have very high similarity; even the noncoding regions, i.e. promoter, intron, and UTRs, showed few mutations, consistent with their recent divergence from a common ancestor (Figure S1). A notable difference is that the UT *TPI* exon 2 encodes 7 repeated trypsin inhibitor domains, whereas the AZ *TPI* exon 2 has 6 repeat domains, which supports the idea that *TPI* repeat domains evolve haphazardly, as inferred from a phylogenic analysis of the number of repeats in closely related *Nicotiana* species (Wu *et al.*, 2006). More germane to the lack of TPI activity in AZ plants was the observation that AZ *TPI* harbors a 6 bp deletion at the beginning of exon 2, which results in a PTC (Figure S1); this PTC was confirmed by sequencing AZ *TPI* cDNA (Figure 1b).

To determine if the decreased *TPI* mRNA levels in AZ plants are due to mutations in promoter *cis*-elements or to an impaired *trans*-activation system, promoters of UT and AZ *TPI* genes were fused with *β-glucuronidase* (*gusA*) in a binary vector pCAMBIA1301. Callus cultures were produced from UT and AZ

hypocotyls infected with *Agrobacterium* transformed with each construct. Approximately 20 calli from each genotype and transformation were used to produce the following polyclonal cell suspension cultures: UT cells carrying *UT-TPI promoter-gusA* or *AZ-TPI promoter-gusA*; and AZ cells carrying *UT-TPI promoter-gusA* or *AZ-TPI promoter-gusA*. Analyses of GUS activity using X-Gluc as a substrate were carried out in these cells to compare promoter activity and thereby the intactness of the *trans*-activation system in AZ plants (Figure 1c). Neither WT UT nor AZ cells were stained, indicating an absence of background activity. UT cells transformed with either *UT-TPI promoter-gusA* or *AZ-TPI promoter-gusA* showed similar GUS staining efficiency; similar activity was also found in AZ cells transformed with either construct. These data suggest that both promoter and *trans*-activation systems remain intact in AZ plants.

The PTC in the AZ *TPI* gene sequence and *TPI* promoter analyses both strongly suggest that *TPI* mRNA is rapidly degraded through the NMD pathway. To characterize *TPI* mRNA stability, actinomycin D, a transcription inhibitor, was applied to UT and AZ cell suspension culture and levels of *TPI* mRNA were measured in cells collected at different times using RT-PCR (Figure 1d). In UT cells, *TPI* mRNA was very stable: even 5 h after the addition of actinomycin D, only a slight decline of *TPI* mRNA levels was observed; conversely, in AZ cells *TPI* mRNA levels were undetectable after 2 h, demonstrating that in these cells the nonsense stop codon mutation causes rapid degradation of *TPI* mRNA.

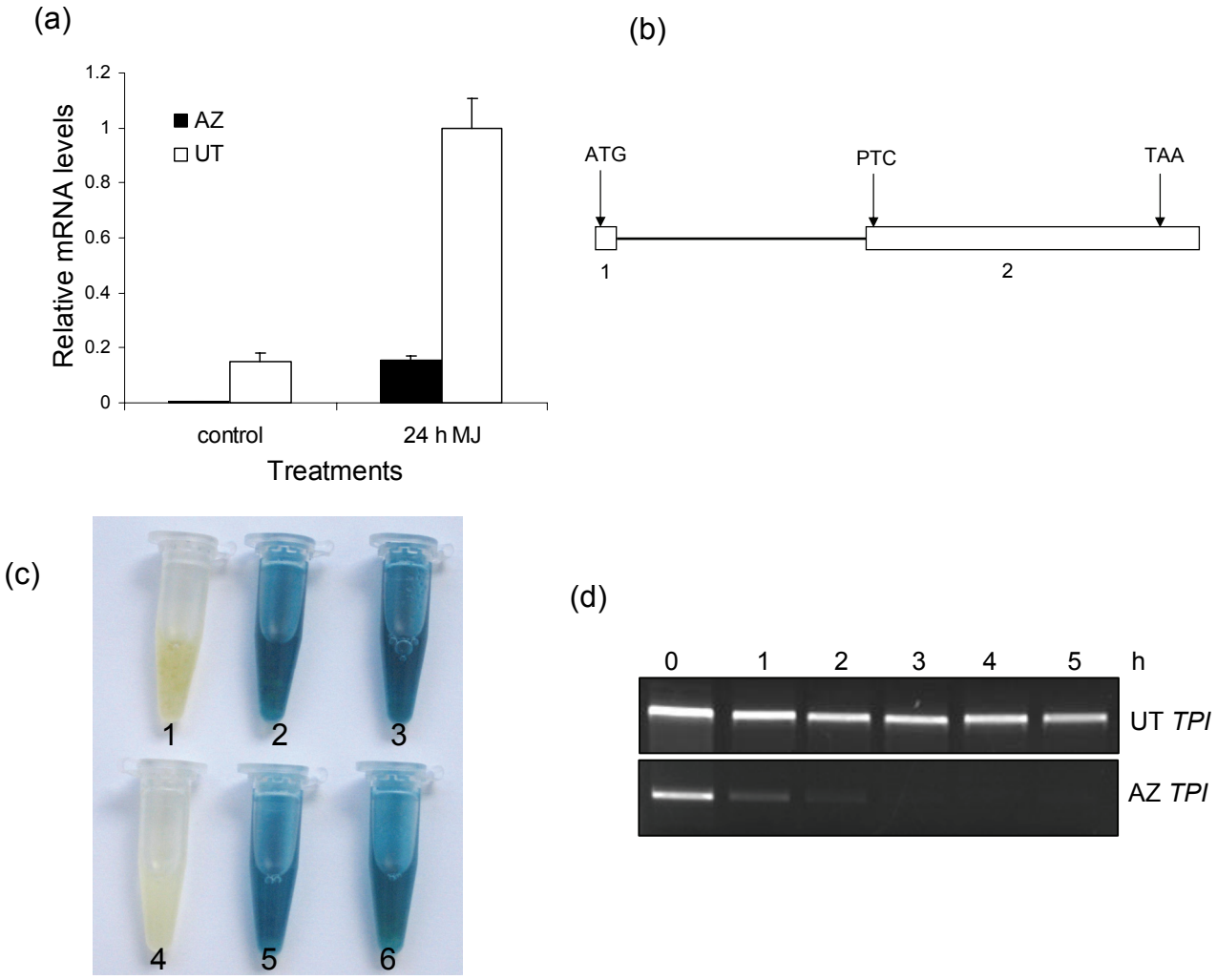

Figure 1. AZ *TPI* gene harbors a PTC and has a low level of mRNA stability.

(a) mRNA levels (mean + SE) of *TPI* in UT (white bars) and AZ (black bars) plants measured from 4 replicate plants. RNA samples were extracted 24 h after plants were treated with pure lanolin (control) or methyl jasmonate-containing lanolin (24 h MJ); *TPI* mRNA levels were quantified with northern blotting. All values were normalized to the highest value as 1.

(b) Schematic structure of *TPI* gene in AZ plants. Boxes represent the 2 exons; the intron is shown by a line. The positions of start codon (ATG), stop codon (TAA), and premature termination codon (PTC) mutation are indicated by arrows.

(c) Histochemical staining of wild-type (WT) suspension cells or suspension cells expressing various *TPI-promoter-gusA* constructs. 1: UT WT cells; 2: UT cells expressing *UT-TPI promoter-gusA*; 3: UT cells expressing *AZ-TPI promoter-gusA*; 4: AZ WT cells; 5: AZ cells expressing *UT-TPI promoter-gusA*; 6: AZ cells expressing *AZ-TPI promoter-gusA*.

(d) Comparison of *TPI* mRNA stability in UT and AZ cells. Suspension cells derived from UT and AZ WT hypocotyls were treated with 100 μg ml^{-1} actinomycin D and rapidly harvested at indicated times. One μg of total RNA from each sample was used for reverse transcription. PCR reactions were carried out under the same conditions except that 25 cycles were used for UT samples, 28 cycles for AZ. The PCR products were run on a 1% agarose gel containing ethidium bromide.

UPF *genes are required for NMD in* N. attenuata

Many studies have demonstrated the conserved roles of *UPF1*, *2*, and *3* genes in various organisms. In Arabidopsis *UPF1* and *UPF3* mutants, various PTC-harboring mRNA species have higher abundance than do they in WT Arabidopsis

(Hori and Watanabe, 2005; Arciga-Reyes et al., 2006). Nevertheless, the function of *UPF2* in plants has not been investigated. To investigate if *UPF* genes mediate NMD in *N. attenuata*, we used the virus-induced gene silencing (VIGS) system optimized for *N. attenuata* (Saedler and Baldwin, 2004) to silence the levels of all three *UPF* gene transcripts in AZ plants, and analyzed the mRNA levels of the PTC-harboring *TPI* gene.

Partial cDNA sequences of *UPF1*, *UPF2,* and *UPF3* from *N. attenuata* were isolated by PCR. Sequence alignments showed that they have 92, 68, and 50% similarities to their respective orthologues in Arabidopsis. Using these sequences, 3 constructs based on a tobacco rattle virus vector, pTV00, were prepared: pTV-UPF1, pTV-UPF2, and pTV-UPF3. *N. attenuata* AZ plants were inoculated with *Agrobacterium* transformed with constructs for silencing *UPF1*, *UPF2*, and *UPF3* genes by VIGS (Ratcliff *et al.*, 2001; Saedler and Baldwin, 2004), and UPF1-, UPF2-, and UPF3-VIGS plants were formed. Plants inoculated with *Agrobacterium* carrying empty vector (EV), pTV00, were used for comparison. After plants had been systemically infected with the virus, as indicated by the appearance of the bleaching phenotype after the virus had spread in plants inoculated with *Agrobacterium* carrying a *phytoene desaturase* construct, the degree to which *UPF* genes had been silenced in individual plants was determined using RT-PCR (Figure 2a). Compared with those in EV plants, significantly lower levels of *UPF1*, *UPF2*, and *UPF3* mRNA were detected in UPF1-, UPF2-, and UPF3-VIGS plants, respectively.

EV, UPF1-, UPF2-, and UPF3-VIGS plants were treated with either MJ in a lanolin paste or pure lanolin paste as a control. After 24 h, samples were collected and the levels of *TPI* mRNA in these plants were determined by northern blotting and quantified according to the intensity of the signal (Figure 2b). Both UPF1- and UPF2-VIGS plants showed remarkably elevated *TPI* mRNA levels: compared with MJ-treated EV plants, UPF1- and UPF2-VIGS plants showed 5.8- and 4.0-fold higher levels of *TPI* mRNA after MJ treatments; similarly, lanolin-treated UPF1- and UPF2-VIGS plants showed 5.3- and 3.3-fold higher levels of *TPI* mRNA compared to lanolin-treated EV plants. In contrast, MJ-treated UPF3-VIGS plants showed only 1.6-fold higher levels of *TPI* mRNA compared to MJ-treated EV plants, and levels of *TPI* mRNA in lanolin-treated UPF3-VIGS plants did not differ significantly from those in lanolin-treated EV plants. In order to rule out the possibility that gene duplication events may have led to redundant *UPF3* genes in *N. attenuata* and VIGS

only reduced the mRNA levels of only one of the *UPF3* paralogues, Southern blotting was performed to examine the copy number of *UPF3* (Figure 2C): a single band was shown in all lanes, consistent with the presence of only one *UPF3* in the *N. attenuata* genome.

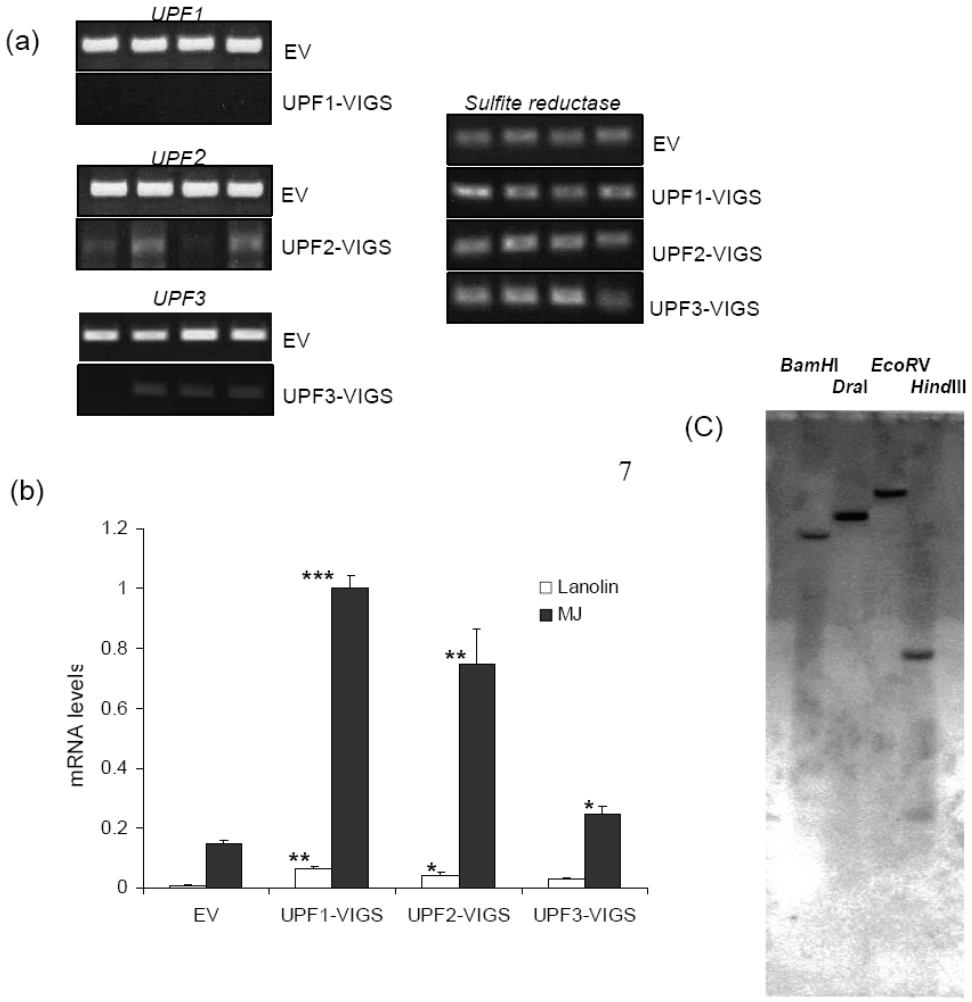

Figure 2. Silencing *UPF* genes elevates the levels of PTC-containing *TPI* mRNA in *N. attenuata*. *N. attenuata* AZ plants were inoculated with *Agrobacterium* carrying virus-induced gene silencing constructs to reduce levels of *UPF1*, *2*, and *3*, respectively; plants inoculated with empty vector (EV) were used for comparison. (a) RT-PCR was used to confirm that *UPF* genes had been silenced in UPF1-, UPF2- and UPF3-VIGS plants. Gene-specific primers and cDNA samples from 4 replicate plants were used for amplifications; a *N. attenuata sulfite reductase* gene was used to ensure that an equal amount of cDNA was used. (b) Levels (mean + SE) of *TPI* mRNA in EV, UPF1-, UPF2-, and UPF3-VIGS plants. Plants were treated with either 20 µl of 7.5 µg ul^{-1} methyl jasmonate (MJ) in lanolin or 20 µl of lanolin as a control. After 24 h, treated leaves were harvested and *TPI* mRNA levels were analyzed by northern blotting. All values were normalized with the highest value as 1. Stars represent significant differences between *TPI* mRNA levels in identically treated EV and UPF1-, UPF2-, or UPF3-VIGS plants (N = 4, ANOVA, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (c) Southern blotting analysis of *N. attenuata UPF3* gene. Genomic DNA was extracted from *N. attenuata* AZ ecotype plants. After being digested with various endonucleases, DNA was separated on a gel and blotted onto a nylon membrane. The membrane was subsequently hybridized with a *UPF3* probe.

Positions of PTCs relative to introns influence NMD in plants

In mammalian cells, the location of an intron relative to the PTC is important in evoking NMD. A nonsense codon at least 50 to 55 bp upstream of the last intron is required for NMD initiation (Le Hir *et al.*, 2001; Nagy and Maquat, 1998). In plants, several studies have shown that PTC-harboring genes with or without introns can trigger NMD (Domon *et al.*, 2002; Isshiki *et al.*, 2001; Petracek *et al.*, 2000; van Hoof and Green, 1996; Vancanneyt *et al.*, 1990; Voelker *et al.*, 1990), but the importance of intron location relative to the nonsense codon in NMD has not been studied in detail.

To address this question, several *TPI* constructs with different gene structures were prepared. These constructs were obtained by replacing the coding region of *gusA* in binary vector pCAMBIA1301 with the following genes (Figure 3a): 1) construct A: UT *TPI* genomic coding region, containing one intron; 2) construct B: AZ *TPI* genomic coding region, containing an intron and a PTC at 5' of exon 2; 3) construct C: UT *TPI* cDNA sequence; 4) construct D: cDNA form of construct B, harboring a PTC located at 14% position of the coding sequence from 5'; 5) construct E: derived from construct A with a PTC located 4 bp upstream of the intron obtained by site-directed mutagenesis, which is at the 87% position of exon 1 from 5'; 6) construct F: intronless form of construct E, obtained by site-directed mutagenesis of UT *TPI* cDNA and containing a PTC at the 3.6% position of the coding sequence from 5'. These constructs represent *TPI* genes with or without introns, and *TPI* genes possessing PTCs up- or downstream of the *TPI* intron.

Cell suspension cultures derived from WT UT and AZ plants have low levels of *TPI* mRNA; in AZ cells, the level of *TPI* mRNA is undetectable by northern blotting analysis, since AZ *TPI* mRNA is rapidly degraded by NMD (Figure 3b). Taking advantage of the extremely low level of *TPI* mRNA transcribed from endogenous AZ *TPI* gene, AZ hypocotyls were transformed with constructs A to F and were further used for callus induction; subsequently, polyclonal cell lines of the AZ genotype were generated from 20 independently transformed calli expressing each specific construct. After stable cell lines were established, *TPI* mRNA levels in these cell lines were determined by northern blotting. In order to rule out insertion-position effects, the mRNA levels of hygromycin resistance gene, *hpt II*, which served as a transformation selection marker, were compared in all cell lines, since *hpt II* is always co-transformed with the transgenes to the same loci in the host genomes.

In all cell lines, *hpt II* mRNA levels were similar, indicating that overall transcription activity of the constitutive promoter *CaMV 35S* was not affected by transgene position. Conversely, cell lines carrying different *TPI* constructs showed distinct levels of *TPI* mRNA (Figure 3c). Constructs A and C, which have intact *TPI* coding sequences with and without the intron, respectively, showed high levels of *TPI* mRNA; however, PTC-harboring constructs B and D showed substantially lower levels of *TPI* mRNA. Surprisingly, construct E maintained its high levels of *TPI* mRNA, although it harbors a PTC at the end of exon 1, only 4 bp from the intron. In contrast, the cell line expressing its cDNA form, construct F, showed remarkably lower levels of *TPI* mRNA. These results were repeatedly obtained in independently prepared polyclonal cell lines. These observations suggest that in plants, the relative positions of nonsense codons to introns influence PTC recognition; when the nonsense codon is located upstream and very close to the exon-exon junction in the *TPI* gene, the PTC recognition is abrogated.

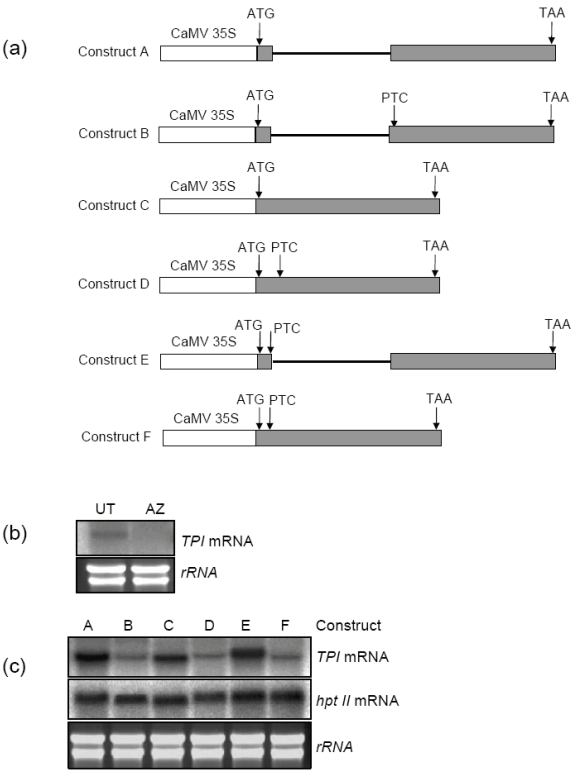

Figure 3. Intron location influences the recognition of PTCs in plants.

(a) Schematic description of constructs carrying different *TPI* genes with various gene structures and PTC locations. All *TPI* genes were placed downstream of a duplicated *CaMV 35S* promoter (white box) and upstream of a *nopaline synthase* (*nos*) 3'-UTR (not shown). Gray boxes indicate exons; positions of start (ATG) and stop (TAA) codon and nonsense codon (PTC) positions are shown by arrows.

(b) *TPI* mRNA levels are undetectable in wild-type (WT) AZ cell suspension culture. Total RNA samples were extracted from WT UT and AZ cell suspension cultures; and levels of *TPI* mRNA were examined by northern blotting.

(c) *TPI* mRNA levels in AZ cell suspension cultures expressing constructs A to F. Total RNA samples were extracted from cell suspension cultures, and levels of *TPI* mRNA were determined by northern blotting; the blot was subsequently stripped and hybridized with a probe to detect *hpt II*, which served as a selection marker and was co-transformed with *TPI* genes. The gel stained with ethidium bromide served as a loading control.

NMD in* N. attenuata *is mutation-position dependent

Several studies have demonstrated that the positions of nonsense codons affect the efficiency of NMD. In both mammalian and yeast cells, nonsense codons located more 5' induce stronger NMD than do 3' nonsense codons (Cao and Parker, 2003; Wang *et al.*, 2002). PTC-harboring *phytohemagglutinin* genes expressed in tobacco suspension culture also showed positional effects on NMD efficiency (van Hoof and Green, 1996).

In order to test if nonsense-codon positions activate NMD with different degrees of efficiency in the *TPI* gene, an intronless *one-repeat TPI* was generated by PCR; since natural *TPI* have several repetitive domains, they are difficult to use in mutagenesis studies (Barta *et al.*, 2002; Wu *et al*., 2006). Using this *one-repeat TPI* as a template, PTC mutations located at 12, 44, 56, 68, 80, and 92% positions relative to the whole coding region from the 5' were created. Subsequently, both the *one-repeat TPI* and the nonsense codon-containing derivatives were subcloned into the pCAMBIA1301 vector to replace the *gusA* gene. The construct harboring *one-repeat TPI* was designated PI-1R; those carrying *one-repeat TPI* with PTCs were named PI-1R-12% to PI-1R-92%, according to their respective PTC positions (Figure 4a).

N. attenuata AZ hypocotyls were transformed with these constructs and used to generate polyclonal suspension cell cultures. After cell cultures attained stable growth with homogeneous cultures, cells were harvested and *one-repeat TPI* mRNA levels were determined by northern blotting (Figure 4b). All cell lines showed similar levels of *hpt II* mRNA, indicating there was no insertion-position effect on the transgenes in any lines. *One-repeat TPI* mRNA levels in different lines were remarkably different: construct PI-1R, which carries an intact reading frame, showed a high level of mRNA; but when a PTC was introduced at the 12% position, a roughly 1.5-fold decrease in the *one-repeat TPI* mRNA level was observed. Moreover, when the PTC was located at the 44% position, the *one-repeat TPI* mRNA level was so negligible that the signal was barely discernible; cells lines carrying constructs PI-1R-56% and PI-1R-68% had slightly higher levels of *one-repeat TPI* mRNA levels that did PI-1R-44%, but these were still approximately 4.5-fold lower than those of the PI-1R line. In comparison, cell lines transformed with constructs PI-1R-80% and PI-1R-92% showed equal levels of *one-repeat TPI* mRNA compared to the PI-1R line. These results are consistent with the idea that NMD efficiency correlates with the positions of nonsense codons in plants, as is true in mammals and yeast.

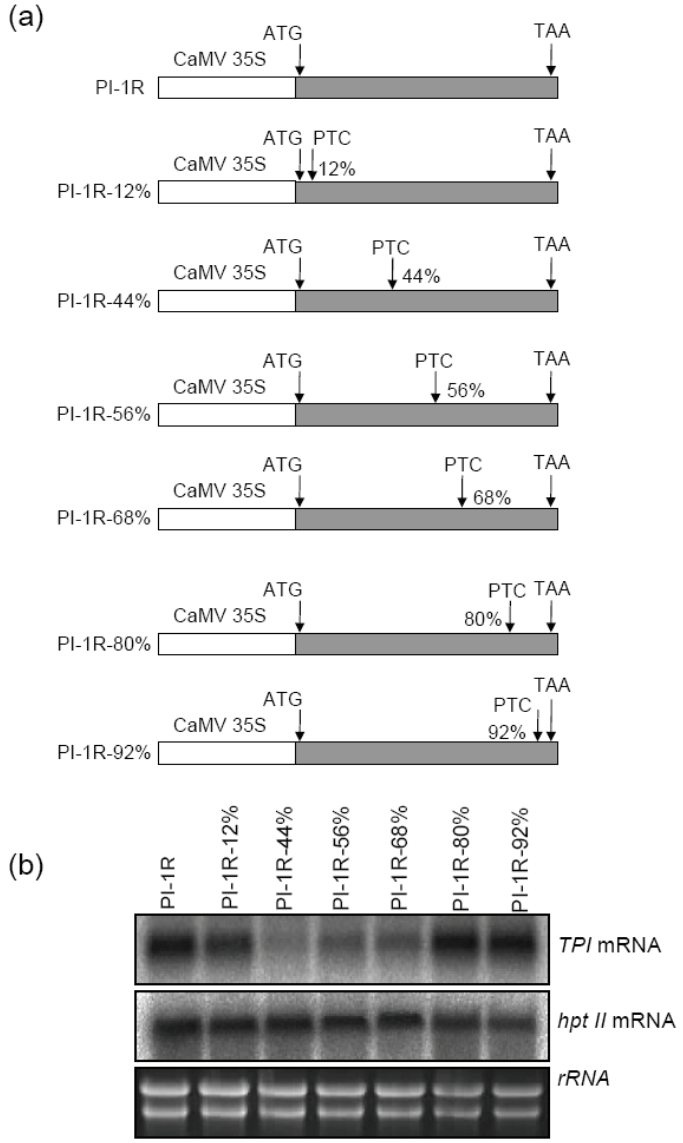

Figure 4. NMD in *N. attenuata* is PTC-position dependent.

(a) Introduction of different mutation positions into the coding region (gray boxes) of a *one-repeat TPI* gene (PI-1R). Nonsense codons were generated at 12, 44, 56, 68, 80, and 92% positions relative to the whole coding region from 5' using site-directed mutagenesis in PI-IR; all these genes were located downstream of the duplicated *CaMV 35S* promoter (white box) in a pCAMBIA1301 vector. Positions of the start codons (ATG), stop codons (TAA), and nonsense codons (PTC) are shown with arrows.

(b) RNA samples were extracted from cells lines expressing constructs PI-1R to PI-1R-92%; mRNA levels expressed from these constructs in cell lines were determined with northern blotting. The same blot was stripped and subsequently hybridized with an *hpt II* probe. The gel stained with ethidium bromide was used as an equal total RNA loading control.

***Translation is necessary for initiating NMD in* N. attenuata**

Many lines of evidence indicate that in yeast and mammals, an initial round of mRNA translation is needed for PTC-harboring genes to be degraded through the NMD pathway (Carter *et al.*, 1995; Hilleren and Parker, 1999; Ishigaki *et al.*, 2001; Zhang *et al.*, 1997). To determine whether this mechanism is conserved in *N. attenuata*, cell suspension cultures transformed with the aforementioned constructs A to F were treated with cycloheximide (CHX), a translation inhibitor. One hundred μg ml^{-1} CHX has been shown to be sufficient to suppress translation activity in mammalian cell cultures (Carter *et al.*, 1995; Wilkinson and MacLeod, 1988); thus 100, 200, and 300 μg ml^{-1} CHX, respectively, were applied to exclude the possibility that *N. attenuata* cells are less sensitive to CHX than mammalian cells are. After 2 h of CHX treatment, cells were harvested. The resulting levels of *TPI* mRNA in these cell lines were determined using northern blotting (Figure 5). Cells expressing constructs A and C, which contain the UT genomic coding sequence and cDNA sequence, respectively, and have intact reading frames, showed high levels of *TPI* mRNA; CHX treatment didn't change the levels of *TPI* mRNA. Similar to the finding in mammal cells that inhibiting translation stabilizes genes containing PTCs, cells transformed with constructs B and D, which were PTC-containing forms of constructs A and C, respectively, have substantially increased levels of *TPI* mRNA after CHX treatment, suggesting that translation is important for initiating NMD. Although having a nonsense codon in exon 1, cells transformed with construct E showed high levels of *TPI* mRNA; furthermore, CHX treatment had no impact on its mRNA levels. In contrast, cells expressing construct F, the intronless form of construct E, had remarkably lower levels of *TPI* mRNA than did cells expressing construct E; notably, even after CHX treatment, *TPI* mRNA levels were not greatly affected.

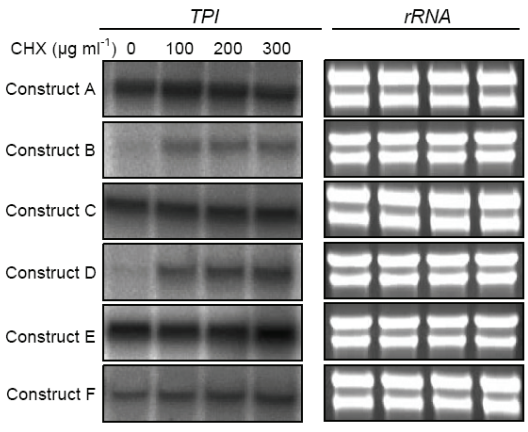

Figure 5. Translation is necessary for initiating NMD in *N. attenuata*. Polyclonal *N. attenuata* AZ cell suspension culture generated from calli harboring constructs A to F were treated with 100, 200, and 300 μg ml^{-1} cycloheximide (CHX); solvent for CHX was added as control, as indicated as 0 μg ml^{-1}. After 2 h, cells were quickly collected and flash-frozen in liquid nitrogen. Total RNA was extracted from all samples and *TPI* mRNA levels in these cell lines were analyzed by northern blotting. Right panel shows the image of the gel stained with ethidium bromide for loading control.

Discussion

NMD in genes with or without introns

By expressing several constructs carrying *TPI* genes in various forms, we show that plants can readily degrade mRNA species transcribed from PTC-harboring intronless genes; however, in intron-containing genes, the position of the intron may play a role in NMD. The function of introns in NMD has been well studied in mammalian cells. Generally, a PTC that leads to NMD is consistently located at least 50 to 55 bp upstream of the last intron (Le Hir *et al.*, 2001; Nagy and Maquat, 1998). This rule is supported by the fact that intronless genes are immune to NMD in mammals (Brocke *et al.*, 2002; Maquat and Li, 2001). Studies have revealed that exon junction complexes (EJCs), located around 20 to 24 bp upstream of each exon-exon junction, play important roles in NMD in mammalian cells (reviewed in Lejeune and Maquat, 2005; Maquat, 2004).

In plants, intron-containing genes have been shown to be subject to NMD surveillance (Domon *et al.*, 2002; Isshiki *et al.*, 2001; Vancanneyt *et al.*, 1990), but whether the relative positions of PTCs to introns influence NMD in plants has not been known. We investigated the *TPI* mRNA levels in cells expressing a series of constructs which possess introns and have PTCs located up- and downstream of these introns. In cell lines expressing a construct which has a nonsense-codon mutation downstream of the single intron, *TPI* mRNA is quickly degraded and results in low mRNA levels. However, when the mutation is located 4 bp upstream of the intron, the mRNA is as stable as that in the cell line carrying a construct expressing a normal *TPI* genomic coding sequence, implying that the distance between PTC and its downstream exon-exon junction is important. In contrast, the intronless form of this construct is readily degraded. Another study of the *waxy* gene in rice showed that upstream intron splicing is necessary for triggering NMD (Isshiki *et al.*, 2001). These facts highlight the complexity of the mechanism underlying how plants recognize PTCs. In intron-containing genes in plants, splicing which deposits EJCs on the exon-exon junctions may be important for NMD. We hypothesize that in plant cells, the distance between PTC and the last downstream EJC should also exceed a certain value; when a PTC is not located upstream of the last EJC, mRNA is recognized by NMD surveillance machinery. Whether this is a general rule for plants still needs to be investigated.

Compared with studies of the machinery of transcription and post-transcription in mammals, little is known about plants. The presence of most of the genes involved in the processes of capping, splicing, polyadenylating, exporting, and surveilling in the Arabidopsis genome suggests that plants and mammals share conserved RNA synthesis and maturation mechanisms; however, several studies have revealed differences (Belostotsky and Rose, 2005; Pendle *et al.*, 2005). These differences may lead to distinct mRNA biogenesis and quality control processes. Whether EJCs are also involved in the PTC-recognition process in plants' intron-containing genes is still unknown.

Yeast recognizes PTCs in an intronless gene by the occurrence of a downstream sequence element or by a 3'-UTR with an inappropriate configuration (Amrani *et al.*, 2004; Peltz *et al.*, 1993; Zhang *et al.*, 1995). *Drosophila* and *Caenorabditis* seem to have mechanisms similar to those in yeast, since NMD can target intronless genes in these organisms (reviewed in Conti and Izaurralde, 2005). Similarly, several studies have shown that in plants, intronless genes are also substrates of NMD (Dickey *et al.*, 1994; Petracek *et al.*, 2000; van Hoof and Green, 1996; Voelker *et al.*, 1990). Although *cis*-acting sequence elements and *trans*-acting protein factors that mediate NMD in intronless genes have been intensively studied in yeast (Gonzalez *et al.*, 2001), whether plants have inherited a similar system is unknown. By introducing PTCs to different positions of an intronless *one-repeat TPI* gene, we observed that NMD efficiency correlates with PTC location in intronless genes: a *one-repeat TPI* gene harboring a PTC at the 44% position showed the highest degree of efficiency; whereas PTCs at the 56 and 68% positions activated NMD slightly less efficiently; at the 12% position, NMD efficiency was even lower; starting at 80%, NMD was abrogated. Nonsense mutations in a *phytohemagglutinin* gene in tobacco cells consistently showed that PTC positions greater than 80% of the gene don't activate NMD (van Hoof and Green, 1996). Similar positional effects were also shown in human and yeast genes. Wang et al. (2002) found that PTCs located farther from the downstream terminal intron triggered stronger NMD than did proximal ones in a T-cell receptor-β gene. In yeast, 5' PTCs trigger faster rates of decapping mRNA than do 3' PTCs, enhancing the efficiency of NMD when PTCs are closer to 5' (Cao and Parker, 2003). Inserting stop codons into different regions of yeast *PGK1* gene also demonstrated the positional effects (Hagan *et al.*, 1995). Intriguingly, the PTC at 12% of the *TPI* gene activated NMD less efficiently than did the PTCs at 44, 56, and

68% positions. It may be that certain elements with functions similar to the stabilizer elements (STEs) and downstream sequence elements (DSEs) of yeast (Gonzalez *et al.*, 2001) occur in the *one-repeat TPI* mRNA, which determine its stability. Alternatively, in plants a PTC results in a different 3'-UTR than a normal stop codon does. Because the terminating ribosome is not able to interact with protein complexes which bind to the normal 3'-UTR, a NMD complex may be recruited which in turn degrades the PTC-harboring mRNA (Amrani *et al.*, 2004). PTCs at different regions of a gene may lead to different abnormal 3'-UTRs, resulting in distinct degrees of NMD efficiency. In summary, plants appear to share similar mRNA surveillance mechanisms with yeast and mammals in intronless and intron-containing genes, respectively, but over time, have evolved unique features to cope with plants' distinct mRNA biogenesis (Belostotsky and Rose, 2005).

Translation is necessary for NMD in plants

In both mammalian and yeast cells, translation has been shown to be necessary for PTC-containing genes to initiate NMD (Carter *et al.*, 1995; Hilleren and Parker, 1999; Ishigaki *et al.*, 2001; Zhang *et al.*, 1997). Using cell suspension cultures, we show that CHX can partially stabilize PTC-containing mRNA species, indicating the need for translation in eliciting NMD. Consistent with this observation, the application of CHX to WT Arabidopsis increased the ratio between the levels of PTC-containing mRNA, which are derived from alternative splicing, and their PTC-free counterparts (Hori and Watanabe, 2005). Construct E, having a PTC 4 bp upstream of the intron, is immune to NMD and in turn has the same level of TPI mRNA before and after CHX treatment. Notably, construct F, which is the intronless form of construct E and has a PTC located at the 3.6% position of the coding sequence, is subject to NMD but did not have elevated mRNA levels after CHX treatment. These data suggest that inhibiting translation may deactivate NMD in different genes with different degrees of efficiency, presumably depending on the positions of PTCs or certain intrinsic properties of an individual gene.

In summary, as in other organisms, NMD in plants depends on translation. Whatever the PTC-recognition mechanisms are in different species, results obtained from all eukaryotes support the idea that a critical step in the recognition of aberrant mRNA is the nature of the translation termination event.

Role of* UPF *genes in plants

UPF1, *2*, and *3* are important components of the NMD complex initially identified in yeast (Leeds *et al.*, 1991; Leeds *et al.*, 1992). With the increasing completion of genome projects, *UPF* genes have been found in ever-growing numbers of fungal, plant, and animal species (Culbertson and Leeds, 2003). Many lines of evidence have demonstrated the important roles of *UPF* genes in NMD pathways in various organisms (reviewed in Conti and Izaurralde, 2005; Culbertson and Leeds, 2003; Gonzalez *et al.*, 2001).

Sequence comparisons of *UPF* genes from different species have shown that on the amino acid sequence level, UPF1 has 40−56% similarity among distantly related organisms. Humans and Arabidopsis have 56% identity; whereas UPF2 has 17−35% and UPF3 has only 13−26% (Culbertson and Leeds, 2003). Consistently, *N. attenuata* UPF1's amino acid sequence shows 92% identity to its Arabidopsis orthologues, whereas *N. attenuata* UPF2 and 3 have only 68 and 50% identity to Arabidopsis UPF2 and 3, respectively. This is consistent with the ideas that: 1) UPF1 is the central player in NMD; 2) UPF2 and UPF3 are responsible for controlling substrate specificity by marking species-specific PTC-containing mRNA for degradation; the large differences among UPF2 and UPF3 homologues may be the result of the evolutionary divergence among mRNA biogenesis and maturation mechanisms in different organisms (Culbertson and Leeds, 2003).

In mammals, UPF1 plays a critical role in NMD (Kashima *et al.*, 2006; Ohnishi *et al.*, 2003). We show that reducing the mRNA level of *UPF1* led to large increases in PTC-harboring *TPI* mRNA levels in *N. attenuata*, demonstrating its crucial role in NMD in plants. Similarly, in Arabidopsis *UPF1* mutant plants, several PTC-containing mRNA species, including intron-containing and intronless mRNA, were shown to have higher levels of mRNA than they do in WT plants (Arciga-Reyes *et al.*, 2006). Another important component of the NMD protein complex is UPF2. Although the involvement of UPF2 in NMD has not yet been studied in other plant species, *UPF2*-silenced *N. attenuata* showed elevated levels of PTC-containing *TPI* mRNA, indicating that in plants, as in yeast and mammals, UPF2 forms part of the mRNA surveillance complex and regulates the mRNA decay process.

UPF3 is also an important component of the NMD protein complex in yeast and mammals (Conti and Izaurralde, 2005; Culbertson and Leeds, 2003). Arabidopsis *UPF3* mutants show increased levels of PTC-containing mRNA compared to WT

plants (Hori and Watanabe, 2005). Although the VIGS approach removed many *UPF3* transcripts, PTC-harboring *TPI* mRNA was only slightly stabilized. Perhaps because the UPF3 protein is rather stable, even reduced levels of *UPF3* mRNA don't substantially lower the protein levels. Alternatively, silencing *UPF3* may lead to lower levels of *TPI* mRNA, due to an unknown mechanism: as shown in the Arabidopsis *UPF3* mutant, the At1g51340 gene transcript has a higher ratio of the PTC-harboring form to the PTC-free form than WT plants do; however, the levels of mRNA of both forms are lower than those in WT plants (Hori and Watanabe, 2005). Given that NMD also plays important roles in regulating the transcriptome (He *et al.*, 2003; Mendell *et al.*, 2004), this is perhaps not surprising. The investigation into more PTC-harboring genes in *UPF3*-silenced *N. attenuata* will clarify the extent to which UPF3 is involved in the NMD process.

In *C. elegans*, NMD is also related to RNA interference (RNAi) (Domeier *et al.*, 2000; Kim *et al.*, 2005), but this was not observed in *Drosophila* (Rehwinkel *et al.*, 2005a). In plants, Arabidopsis UPF1 seems to be involved in the RNAi process, given that silencing an *AP3* gene by RNAi was not successful in the *UPF1* mutant plants (Arciga-Reyes *et al.*, 2006). However, silencing *UPF* genes in *N. attenuata* by the VIGS approach resulted in highly attenuated *UPF* mRNA levels, suggesting that NMD might not be involved in the RNAi process in *N. attenuata*. It is also probable that NMD does have an impact on RNAi in *N. attenuata*, but only a low level of NMD activity is required; thus removing most *UPF* gene transcripts is sufficient to deactivate NMD but not RNAi.

Recent molecular and genetic studies in mammals and yeast have broadened NMD's physiological functions other than mRNA-quality surveillance (Domeier *et al.*, 2000; He *et al.*, 2003; Mendell *et al.*, 2004; Mitrovich and Anderson, 2005; Rehwinkel *et al.*, 2005b; Rehwinkel *et al.*, 2006). A growing body of evidence suggests NMD plays a role in plant developmental processes (Arciga-Reyes *et al.*, 2006; Yoine *et al.*, 2006). Further research on the NMD process in plants will address how PTCs are recognized, how NMD is activated after PTC recognition, how PTC-containing mRNA is degraded, and other functions of NMD in plants in addition to mRNA quality surveillance. Comparing NMD's roles in yeast, plants, and animals will greatly facilitate our understanding of how this most complicated mRNA-processing mechanism evolved.

Experimental procedures

Plant materials and growth conditions

N. attenuata Torr. ex Watts. (Solanaceae) UT and AZ seeds were from lines maintained in our laboratory that were originally collected in Utah and Arizona (USA). Seeds were germinated on agar with Gamborg B5 media. All plants except those used for VIGS were grown in the greenhouse under 16 h of light supplied by Philips Sun-T Agro 400- or 600-W sodium lights.

Plant treatments and sample harvest

For methyl jasmonate (MJ) treatment, MJ was diluted in melted lanolin at 7.5 $\mu g\ \mu l^{-1}$, 20 µl of the solidified mixture were applied to each leaf with a small spatula. The same procedure was conducted for controls except pure lanolin was used. To rule out developmental differences among leaves, all experiments were performed with +1 leaves. Leaves were cut from petioles, and then collected in Eppendorf tubes, flash-frozen in liquid nitrogen, and stored at -80 ^{o}C until use.

Molecular cloning and construct preparations

Both UT *TPI* cDNA and genomic sequences were cloned previously (Wu *et al.*, 2006; Zavala *et al.*, 2004) (accession number: AF542547 and DQ158200). AZ *TPI* cDNA sequence was amplified by PCR using primers PI-AZ-1 (5'-CTCAGGAGATAGTAAATATGG) and PI-AZ-2 (5'-GCTCCACTGCCATATTACAG). The PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and subsequently sequenced. AZ *TPI* intron was directly PCR cloned using AZ genomic DNA as the template with primers AZIntron-1 (5'- TCATGGGCAATAGGAAGTGAAAACCACG) and AZIntron-2 (5'-GGCATCTGCATGTTCCACATTGCTTAC). AZ promoter sequence was isolated using a Universal GenomeWalker kit (Clontech, Mountain View, CA, USA) following the method described by Wu et al. (2006).

Both *TPI* promoters in UT and AZ were PCR amplified using genomic DNA as template with primer pair 1 (Table S1) and cloned into pCAMBIA1301 at *Xba*I and *Nco*I sites to form *TPI promoter-gusA* constructs.

Construct A (pUTPI-Full): the UT *TPI* genomic coding sequence carried in a plasmid was PCR amplified using primer pair 2 listed in Table S1, and subcloned into pCAMBIA1301 vector at *Nco*I and *BstE*II sites.

Construct B (pAZPI-Full): same preparation as A except a plasmid carrying the AZ *TPI* genomic sequence served as the PCR template.

Construct C (pUTPI): a plasmid carrying UT *TPI* cDNA was used as a template and PCR amplified with primer pair 2, and subsequently subcloned into pCAMBIA1301 vector at *Nco*I and *BstE*II sites.

Construct D (pAZPI): same preparation as C, except a plasmid carrying the *AZ TPI* cDNA sequence was used.

Construct E (pUTPIFull-EX1Mut): using construct A as a template, *TPI* genomic coding region was PCR amplified and further subcloned into a pUC18 vector at *Hind*III and *BamH*I sites using primer pair 3 (Table S1), and formed pUC-PIFull. This plasmid was further digested with *Sal*I and *Hind*III, a 0.7 kb fragment containing UT *TPI*'s first exon and partial intron was subcloned into pUC18 to form pUC-PIPartial. A stop codon was introduced into the *TPI* first exon carried in pUC-PIPartial using a QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), employing primer pair 4 (Table S1). The mutated fragment was excised with *Sal*I and *Hind*III and subsequently cloned into pUC-PIFull, to form pUC-PIFullEx1Mut. Employing the same method described above to prepare construct A, the *TPI* genomic coding sequence carrying a PTC in exon1 was subcloned into pCAMBIA1301 and formed pUTPIFull-EX1Mut.

Construct F (pUTPI-3.6%): using primer pair 4, site-directed mutagenesis was done on a plasmid carrying a UT *TPI* cDNA sequence to generate a PTC at 3.6% position of the coding sequence from 5'. The procedure for preparing construct C was used to subclone this fragment to pCAMBIA1301 and form pUTPI-3.6%.

To prepare the *one-repeat TPI*, PCR was performed using a plasmid containing UT *TPI* cDNA as a template with primers, PI-03-EcoRI (5'-GGAATTCCATGGCTGTTCACAGAGTTAG) and PI-04-BamHI (5'-CGGGATCCCGGCTCCACTGCCATATTAC); due to *TPI*'s repetitive structure, a ladder-like pattern of PCR products is apparent on the gel. The smallest band at a size of 0.4 kb was cut from gel and DNA was eluted. After being digested with *Eco*RI and *BamH*I, DNA was cloned into pBluescript II SK (-) vector to form pPI-1R.

pPI-1R was further used as a template for site-directed mutagenesis, using primers 4 to 9 listed in Table S1. After mutagenesis, each plasmid was PCR amplified with primer pair 2 and further subcloned into pCAMBIA1301 at *Nco*I and *BstE*II sites to generate constructs PI-1R-12%, PI-1R-44%, PI-1R-56%, PI-1R-68%, PI-1R-80%, and PI-1R-92%, respectively.

All constructs were sequenced and transformed into *Agrobacterium* strain LBA 4404 and used for plant transformation.

Cell suspension, culture preparation, and inhibitor applications

Calli were induced and transformed with *Agrobacterium* carrying specific constructs using the method described in Krugel et al. (2002). For making polyclonal cell lines, 20 calli transformed with each construct were cut into small pieces and suspended in a media containing 4.3 g l^{-1} Murashige & Skoog medium, 30 g l^{-1} sucrose, 1mg l^{-1} thiamine, 0.1 g l^{-1} myo-inositol, 0.18 g l^{-1} KH_2PO_4, 0.22 mg l^{-1} 2, 4-D, and 0.5 g l^{-1} MES at pH 5.7. Hygromycin (30 mg l^{-1}) was included in the media for the purpose of selection. After the cultures became homogeneous, cells were used for further characterization.

For inhibitor treatments, actinomycin D (5 mg ml^{-1} in methanol) was added to cell cultures at a concentration of 100 μg ml^{-1}. Similarly, cycloheximide (10 mg ml^{-1} in ethanol) was added to cell cultures at a final concentration of 100 μg ml^{-1}. Cells treated with same amount of solvents were used as controls. Cells from each line were quickly vacuum filtered through a 41 μm nylon membrane (Millipore, Billerica, MA, USA) and collected into Eppendorf tubes; after being flash-frozen in liquid nitrogen, samples were stored at -80 ^{o}C until use.

Virus-induced gene silencing

A virus-induced gene silencing system (VIGS) based on tobacco rattle virus was used (Ratcliff *et al.*, 2001). Fragments of *UPF1*, *UPF2,* and *UPF3* were amplified by PCR using primer pairs 1, 2, and 3 listed in Table S2, and further digested with *BamH*I and *Sal*I and gel purified; subsequently they were cloned into the binary pTV00 vector's *BamH*I and *Sal*I sites to form pTV-UPF1, pTV-UPF2, and pTV-UPF3 constructs, respectively. *Agrobacterium* strain GV3101 carrying these constructs were co-inoculated with *Agrobacterium* carrying pBINTRA6 into young *N.*

attenuata plants following VIGS procedures optimized for *N. attenuata* (Saedler and Baldwin, 2004).

Northern blotting and RT-PCR

Total RNA was extracted from 100 mg of each sample using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantified on a UV-photometer. Ten microgram of each total RNA sample was separated in a 1.2% denaturing agarose gel; after being transferred to a nylon membrane (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA), RNA was immobilized by UV cross-linking. *TPI*, *one-repeat TPI*, *hpt II* probes were PCR amplified from plasmids carrying respective cDNA as templates with primer pairs 10, 11, and 12 in Table S1. DNA probes were labeled with α-^{32}P-dCTP (PerkinElmer Life and Analytical Sciences) using a random primer labeling kit (Amersham Biosciences, Uppsala, Sweden). Each membrane was prehybridized with ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) for 1 h and hybridized with a probe overnight; after washing, imaging was conducted on a FLA-3000 Phosphorimage system (Fujifilm Fuji Photo Film Europe, Düsseldorf, Germany).

One microgram of total RNA obtained from each sample was reverse transcribed using oligo(dT) and Superscript II reverse transcriptase (Invitrogen) following manufacturer's instructions. For semi-quantitative RT-PCR analyses, samples with same amount of cDNA were amplified with primer pairs listed in Table S3; and 20 µl of products were separated on agarose gels. A *Nicotiana attenuata sulfite reductase* gene which is known not to be regulated by all treatments was used as an internal standard.

Southern blotting

Genomic DNA was extracted from young leaf tissue using the CTAB method (Doyle and Doyle, 1987). After overnight digestion with various endonucleases (Fermentas, Ontario, Canada), 7 µg of each digested DNA was separated on a 1% agarose gel. After being blotted onto a nylon membrane, DNA was subsequently immobilized by UV cross-linking. *UPF3* probe was prepared by PCR using a plasmid carrying partial *N. attenuata UPF3* gene and primer pair 13 listed in Table S1. Probe labeling and hybridization were carried out following the same procedures described for northern blotting.

GUS activity analysis by histochemical staining

GUS activity analysis was done following Kang and Baldwin (2006). Cells harvested from cell suspension culture were incubated in a GUS-staining solution containing 100 mM sodium phosphate buffer (pH 7.0), 0.4 mM potassium ferricyanide, 0.4 mM potassium ferrocyanide, 8 mM EDTA, 0.05% Triton X-100, 0.8 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Gluc), and 20% methanol. After being incubated at 37 ^{o}C for 4 h, staining solution was replaced with ethanol and used for photography.

Acknowledgments

We thank Dr. Klaus Gase for the preparation of VIGS constructs, and the Max Planck Society for financial support. We also thank Emily Wheeler for editorial assistance.

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Supplementary Material

Figure S1. Alignment of *TPI* genomic regions isolated from *Nicotiana attenuata* Utah (UT) and Arizona (AZ) ecotypes. Promoter regions are shown in blue boxes, intron borders are indicated by two blue vertical bars, start codons (ATG), stop codons (including premature termination codons) are marked with red boxes. UT TPI has 7 repetitive domains, AZ has 6 domains, the missing domain is shown in a green box; a 6 bp deletion which leads to a PTC is indicated by a yellow box.

Table S1 Sequences of primers used in gene cloning, preparation of constructs and probes

Table S2 Sequences of primers used for the preparation of VIGS construct

Table S3 Sequences of primers used for semi-quantitative RT-PCR

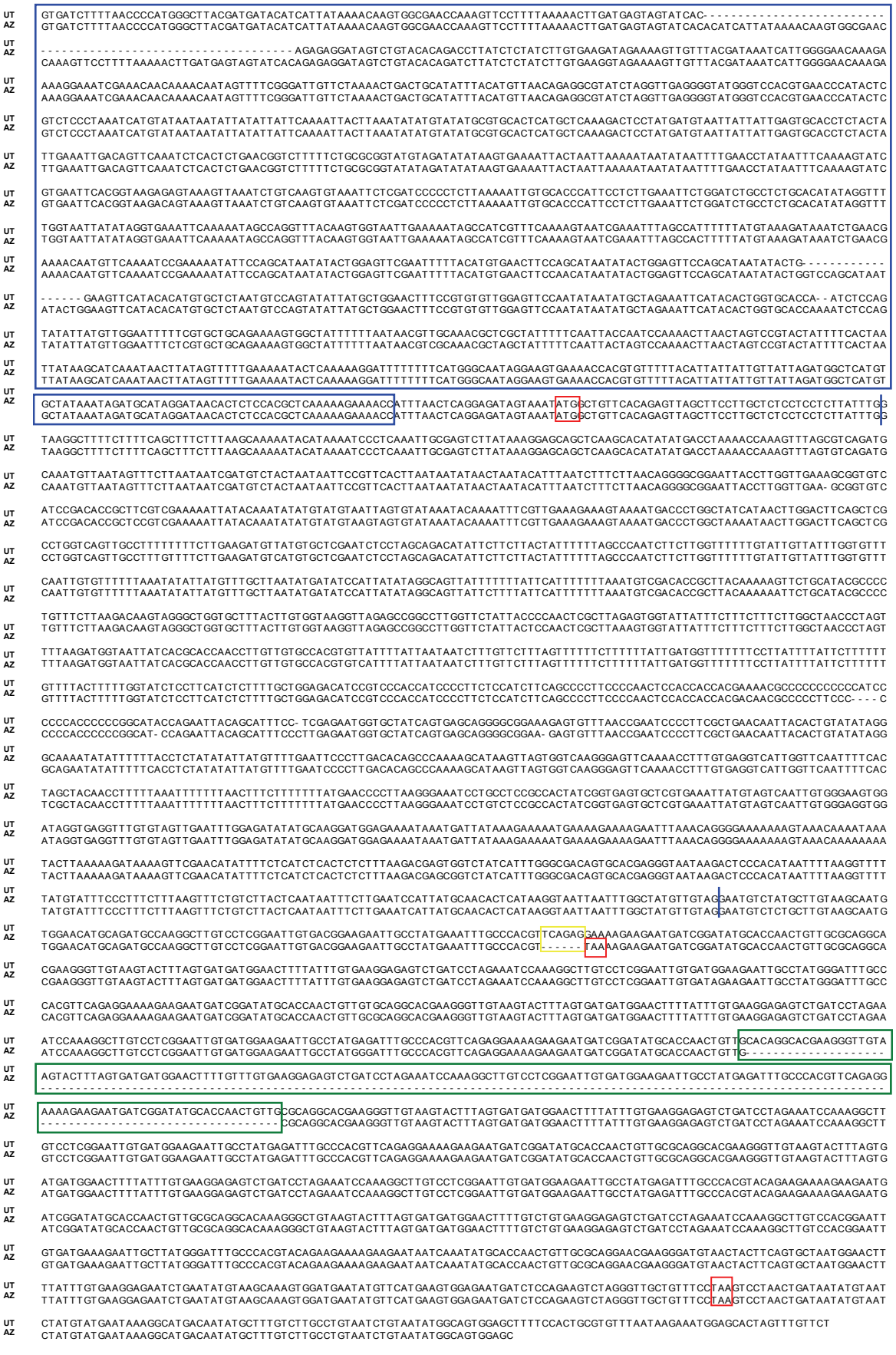

Figure S1

Table S1

Primer Pair	Primer sequence (5'-3')	Primer sequence (5'-3')
1	CGTCTAGAATGGGCTTACGATGATACATCATTATA	GTACCCATGGATTTACTATCTCCTGAGTTAAATGGTT
2	CATGCCATGGCTGTTCACAGAGTTAGCTTC	CATGGGTGACCTTAGGAAACAGCAACCCTAGACT
3	GTCAAGCTTGGCTGTTCACAGAGTTAGCT	CGGGATCCCGGCTCCACTGCCATATTACAG
4	CCTTGCTCTCCTCCTCTAATTTGGTAAGGC	GCCTTACCAAATTAGAGGAGGAGAGCAAGG
5	AATTTGCCCACGTTAAGGGGAAAAGAAGAATG	CATTCTTCTTTTCCCCTTAACGTGGGCAAATT
6	GCACCAACTGTTGAGCAGGCACGAAGGGATG	CATCCCTTCGTGCCTGCTCAACAGTTGGTGC
7	CTACTTCAGTGCTAATTGAACTTTTATTTGTG	CACAAATAAAAGTTCAATTAGCACTGAAGTAG
8	CTGAATATGTAAGCTAAGTGGATGAATATG	CATATTCATCCACTTAGCTTACATATTCAG
9	GTGGAGAATGATCTCTAGAAGTCTAGGGTT	AACCCTAGACTTCTAGAGATCATTCTCCAC
10	GGCTGTTCACAGAGTTAGCTTCCTTG	GCTCCACTGCCATATTACAGATTACAGGC
11	CACAGAGTTAGCTTCCTTG	CCATATTACAGATTACAGGC
12	GTACTTCTACACAGCCATCG	TTATCGGCACTTTGCATCGG
13	AAAGCACTGGTCCAAGAAGG	ACTATCAGCAGTTACAGAACG

Table S2

Primer Pair	Primer sequence (5'-3')	Primer sequence (5'-3')
1	GCGGCGGTCGACGGTGGTCAGGCTCTGTGCC	GCGGCGGGATCCGGCTCAGTAGCCTGAGTGG
2	GCGGCGGTCGACACCAAGTCTTGTTCAAGGCC	GCGGCGGGATCCAGTTGTCACATTAAGGGCC
3	GCGGCGGTCGACGCCTGTTGAGAATCTTCCAAG	GCGGCGGGATCCGATCCAGGAGCAGAGGTTCC

Table S3

Gene	Primer 1 (5'-3')	Primer 2 (5'-3')
TPI	GGCTGTTCACAGAGTTAGCTTCCTTG	GCTCCACTGCCATATTACAGATTACAGGC
UPF1	CCAAGGACAGGTTTTGGTC	CTGCTTTGCGCCAAGAACC
UPF2	GACGCAGCCGTAGAACTTC	CTGCAGCAGTCTGAGCTTC
UPF3	GCACTGGTCCAAGAAGGAT	CCAATTTCCCCTTCCACTAAAT
Sulfite reductase	AGAAACTGCAGGGTACTGTTGG	CAAGGAGGTATAACTGGTGCCC

3. Discussion

MAPK Signaling in Plant Defense Responses

It has been known that MAPKs play important roles in stress responses in both animal and yeast (Herskowitz, 1995; Chang and Karin, 2001). In plants, MAPKs are shown to be rapidly activated after being challenged with ozone, temperature, pathogen, osmotic pressure, and wounding (Zhang and Klessig, 1998; Romeis et al., 1999; Samuel et al., 2000; Asai et al., 2002; Droillard et al., 2004). However, whether MAPKs are involved in plants' defense responses against herbivory was unknown.

Using an in-gel kinase assay system, we show that within a few minutes, at least both SIPK and WIPK, two MAPKs, are activated. Furthermore, using a VIGS system, we demonstrate that SIPK and WIPK are upstream regulators mediating plants' defense responses: herbivory-elicited phytohormone production, and transcriptional regulation of defense-related genes. Using synthetic FACs, we found that *N. attenuata* deploy a suite of defense responses against herbivory through perception of FACs derived from OS of *M. sexta*.

Although there are no FAC-specific receptors have been identified, a report that volicitin, a hydroxylated FAC, binds to *Zea mays* cell membranes with high affinity, supports the hypothesis that plants have certain unidentified FAC-specific receptors (Truitt et al., 2004). Identifying FAC binding proteins and FAC-specific receptors will greatly enrich our understanding of how plants perceive herbivory. We found that quickly after herbivory, not only the attacked regions, but specific non-attacked regions activate SIPK, WIPK, and thus downstream defense reactions. This suggests that a mobile signal, probably downstream of receptor but upstream of MAPK kinase kinase, rapidly moves to other regions of the leaf and activates kinase signaling. The patterns of MAPK activation after herbivory are correlated with the regions being attacked and the age of the attacked leaf. Some evidence has suggested that electric and/or hydraulic signals play a role in transmitting wounding elicitation to long-distance leaves(Malone et al., 1994); whether they are also involved in this short-distance signal transmission within a leaf is still unknown.

It has long been know that herbivory enhances proteinase inhibitor (PI) activity in intact systemic leaves (Green and Ryan, 1972), although the mechanism of this long-distance signal transduction is still unclear. An elegant genetic study

demonstrated that in tomato JA plays an important role: either JA or a JA-elicited mobile signal is responsible for systemic PI induction (Li et al., 2002). In agreement with this finding, we show that after herbivory MAPKs are not activated in systemic leaves, although TPI is highly induced.

However, whether MAPKs are involved in systemic defense responses is not conserved among different species. In tomato, wounding leads to activation of MAPKs in both local and systemic leaves (Stratmann and Ryan, 1997). Cutting tobacco stems also activate WIPK activity in systemic leaves (Seo et al., 1999). Whether these discrepancies are due to differences among treatments or the morphology of the model plants needs to be investigated.

SIPK and WIPK are two MAPKs that have been intensively studied in plants, which are involved in all stress-related responses. Based on a phylogeny analysis, SIPK and WIPK belong to MAPK group II and I, respectively (Zhang and Klessig, 2001). Both transcriptional analyses and phytohormone levels in SIPK- and WIPK-silenced plants point to overlapping functions of SIPK and WIPK. Silencing either gene reduces JA and JA-Ile accumulation after herbivory. Both genes also regulate transcript levels of WRKYs, important transcription factors in plants. Moreover, SIPK and WIPK both regulate levels of several other important MAPKs and even CDPKs, although with different specificity and at different levels. It is likely that SIPK and WIPK share numbers of common substrates, including transcription factors. Consistent with this idea, using a protein-microarray approach, Feilner et al. (2005) demonstrated that SIPK and WIPK share many common substrates. Nevertheless, transcriptional analyses also revealed that they have different functions. SIPK positively regulates levels of *AOS*, *ACO1* and *PAL*; in contrast, WIPK regulates them negatively.

It has been shown that in Arabidopsis, SIPK directly phosphorylates and thus stabilizes ACS, which leads to the production of ethylene (Liu and Zhang, 2004). Similarly, in *N. attenuata*, silencing SIPK leads to reduced level of W+OS-elicited ethylene. It is very likely that phosphorylation of ACS in *N. attenuata* is also a critical step in ethylene biosynthesis, although transcriptional regulation may be also involved (von Dahl et al., 2007). Importantly, silencing SIPK only reduced 50% of the ethylene production. This is in consistent with the study in Arabidopsis: flagellin-treated *mpk6* mutants still produce 50% of ethylene as wild type plants do (Liu and Zhang, 2004). All these facts strongly suggest that both herbivory- and pathogen-elicited ethylene

biosyntheses are regulated by both SIPK and another unknown regulator, which might be a CDPK (Tatsuki and Mori, 2001; Ludwig et al., 2005).

Silencing SIPK and WIPK reduces W+OS-elicited JA by ~80% and ~40%, respectively, indicating the important function of SIPK and WIPK in regulating herbivory-elicited JA biosynthesis. After W+OS treatment, JA bust earlier than OS-elicited increase of *LOX3* and *AOS* transcription (Ziegler et al., 2001; Halitschke and Baldwin, 2003). These data imply that posttranslational processes are important for the JA burst; SIPK/WIPK may directly phosphorylate certain enzymes in JA biosynthesis and quickly enhances their activity. However, compared with ethylene biosynthesis, JA biosynthesis is contributed by several more enzymes which locate in different subcellular locations (Creelman and Mullet, 1997; Delker et al., 2006). Analyzing activities of enzyme involved in JA biosynthesis in SIPK- and WIPK-VIGS plants and compare with those in WT plants will help us to understand which enzymes might be directly regulated by SIPK and WIPK.

The ecological significance of both kinases has not yet been established. Since the greatly reduced JA, ethylene, SA levels in these kinase-silenced plants, we expect that both plants have highly reduced levels of defense against herbivory. Both greenhouse and filed characterization of SIPK- and WIPK-silenced plants will demonstrate the ecological significance of SIPK and WIPK: changes of herbivore community, larvae growth and indirect defenses.

Polyploidy Speciation and Evolution of Plants' Resistance to Herbivory

A growing body of evidence has demonstrated that polyploidy entails dramatic genome arrangements, epigenetic changes, and increased transposon activities in the neopolyploids (Soltis and Soltis, 1995; Wendel, 2000; Soltis et al., 2004; Adams and Wendel, 2005). Moreover, allopolyploidization provides plants with higher gene redundancy, which releases selection pressure on the redundant genes, thus allowing them to evolve novel functions and benefit plants ecologically (Otto and Whitton, 2000; Soltis and Soltis, 2000).

We investigated how TPI, an important herbivory-induced defense trait, evolved after polyploidization in *Nicotiana* polyploid species. Southern blotting and sequence analyses showed that both tetraploids inherited only TPI gene from the

maternal ancestor, *N. obtusifolia*; the TPI gene from *N. attenuata*, the paternal ancestor, was completely deleted from their genomes. This is not surprising since several studies using artificially synthesized polyploids have showed that after polyploidization, gene deletion is a common consequence of genome duplication (Song et al., 1995; Liu et al., 1998a; Liu et al., 1998b; Kashkush et al., 2002; Han et al., 2003). In contrast, analyses on the expression and activity of TPI demonstrated that both tetraploids response to herbivory elicitation similarly to *N. attenuata*, in which levels of both TPI expression and activity are further enhanced after applying *M. sexta* OS to wounds, suggesting that both tetraploids inherited the herbivory-related signaling components from *N. attenuata*.

It is very likely that *N. clevelandii* and *N. quadrivalvis* derived from one common tetraploid ancestor, since it is very unlikely that two independent gene deletion events happened in both tetraploids after their speciation. It is unknown when *N. attenuata* TPI gene was deleted from the ancestral tetraploid for *N. clevelandii* and *N. quadrivalvis*. Several studies have revealed that in the first a few generations of neopolyploids, gene deletion is a common consequence of genome duplication (Feldman et al., 1997; Shaked et al., 2001; Han et al., 2003; Wang et al., 2005), thus it is possible that *N. attenuata* TPI gene is deleted soon after the polyploidization event.

The levels of TPI expression and activity are primarily correlated with JA levels in *Nicotiana* (Koiwa et al., 1997; Halitschke and Baldwin, 2003). Compared with plants being elicited with wounding, *N. attenuata*, *N. clevelandii*, and *N. quadrivalvis* all have higher levels of JA after being elicited with OS (Lou and Baldwin, 2003); nevertheless, *N. obtusifolia* doesn't have herbivory-specific JA responses. Assuming the recognition of herbivory is dependent on OS-specific receptors, both tetraploids may have inherited the receptor genes from *N. attenuata*; whereas it is possible that *N. obtusifolia* have lost this receptor gene or this gene lost its specificity to OS over time. Having inherited the herbivory-specific signaling system might have provided both tetraploids with higher ecological advantages under herbivory selection pressure (Zavala and Baldwin, 2004; Zavala et al., 2004a). Molecular cloning of OS-specific receptor and studies on how these genes evolve under different herbivory selection pressure in different species and population will greatly facilitate our understanding of the long-lasting plant herbivore arms race.

Nonsense-mediated mRNA Decay and TPI-deficiency Phenotype in *N. attenuata* Arizona Ecotype

NMD has been studied intensively in yeast and mammalian cells (Hentze and Kulozik, 1999; Hilleren and Parker, 1999; Conti and Izaurralde, 2005). In plants, a grow body of evidence has shown that NMD also plays an important role in lowering levels of PTC-harboring mRNA (Jofuku et al., 1989; Vancanneyt et al., 1990; Dickey et al., 1994; Petracek et al., 2000; Arciga-Reyes et al., 2006), however, the mechanism of NMD in plants is poorly understood. Using TPI gene in *N. attenuata* as a model gene, we compared NMD process in plants with those in mammals and yeast.

We show that silencing all UPF genes, namely, UPF1, UPF2, and UPF3 stabilizes the PTC-harboring TPI mRNA in *N. attenuata*. In Arabidopsis UPF1 and UPF3 mutants, various PTC-harboring genes have higher levels of mRNA than in wild type plants (Hori and Watanabe, 2005; Arciga-Reyes et al., 2006). These facts suggest the conserved roles of UPF genes in plants. In mammalian cells, NMD process is carried out by NMD surveillance complexes, which consist of numerous proteins, including all the UPF proteins. To date, only functions of UPF proteins have been studied in plants. Compared with mammalian genomes, genome sequencing revealed that most of the genes involved in the processes of capping, splicing, polyadenylating, exporting, and surveilling are present in Arabidopsis (Belostotsky and Rose, 2005). However, whether the other components of NMD surveillance complex found in mammalian and yeast are also conserved in plants is unknown.

In mammals, intron plays a critical role in initiating NMD: a PTC that leads to NMD is consistently located at least 50-55 bp upstream of the last intron (Nagy and Maquat, 1998; Le Hir et al., 2001), and intronless PTC-harboring genes are immune to NMD (Maquat and Li, 2001; Brocke et al., 2002). In plants, both intron-containing and intronless genes are subject to NMD (Voelker et al., 1990; Dickey et al., 1994; van Hoof and Green, 1996; Isshiki et al., 2001; Domon et al., 2002). Distinct from NMD in mammals, in *N. attenuata*, when a PTC locates downstream of the TPI intron, it activates NMD; whereas when it is only 4 bp upstream of the TPI intron, NMD is not activated. This suggests that plants have distinct NMD mechanism from mammals: when PTCs are downstream of introns, NMD is activated; whereas when a PTC is only a few bp upstream of an intron, it initiates NMD. If this is a general rule for plant NMD need further investigation.

Recent molecular and genetic studies in mammals and yeast have revealed NMD's physiological functions other than mRNA-quality surveillance (Domeier et al., 2000; He et al., 2003; Mendell et al., 2004; Mitrovich and Anderson, 2005; Rehwinkel et al., 2006). A growing body of evidence suggests NMD plays a role in plant developmental processes as well (Arciga-Reyes et al., 2006; Yoine et al., 2006). Further research on the NMD process in plants will address how PTCs are recognized, how NMD is activated after PTC recognition, how PTC-containing mRNA is degraded, and other functions of NMD in plants in addition to mRNA quality surveillance. Comparing NMD's roles in yeast, plants, and animals will greatly facilitate our understanding of how this most complicated mRNA-processing mechanism evolved.

4. Conclusion

Over time, plants have acquired sophisticated defense systems to cope with herbivory. Plants react to herbivory with finely tuned transcriptional changes, elevated phytohormone levels, and finally production of direct and indirect defense compounds. Mitogen-activated protein kinase (MAPK) signaling plays an important role in transducing extracellular stimuli into intracellular responses. In plants, salicylic-acid induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) have been shown to be involved in various stress responses.

In this study, we demonstrate that both SIPK and WIPK are quickly activated after herbivory. Using a virus-induced gene silencing (VIGS) system, we obtained SIPK- and WIPK-silenced plants, which have greatly reduced SIPK and WIPK activity, respectively. We show that SIPK and WIPK regulate herbivory-induced jasmonic acid (JA), JA-isoleucine, salicylic acid levels; SIPK regulates ethylene biosynthesis. Furthermore, gene expression analyses indicated that both kinases mediate transcript levels of phytohormone biosynthesis genes, several other MAPKs and even CDPKs, and genes involved in direct defense, such as trypsin proteinase inhibitor (TPI) and phenylalanine ammonia-lyase (PAL). Thus both SIPK and WIPK are important upstream signaling components mediating plants' resistance to herbivory.

In response to herbivory, the herbivore-attacked leaf activates MAPK and downstream responses not only in the wounded regions but specific regions in the same leaf, this suggest a short-distance mobile signal moves to the specific regions of the attacked leaf and activates MAPK signaling. Subsequently, a long-distance signal, most probably JA or JA-elicited compounds, moves to distal intact leaves and initiate TPI defense.

TPI plays an important role in defending plants against herbivory. *N. clevelandii* and *N. quadrivalvis* are two tetraploids derived from *N. attenuata* and *N. obtusifolia*. After polyploidization, TPI gene from *N. attenuata* was completely deleted tetraploid genomes. However, both tetraploids inherited the defense signaling from *N. attenuata*, which enables them to respond to herbivory with enhanced levels of TPI mRNA expression and activity than responding to mechanical wounding. This

study illustrated the plasticity of polyploid evolution which might endow them with ecological and evolutionary advantages to survive in nature.

In *N. attenuata* Arizona ecotype plants, TPI mRNA has very low expression levels and TPI activity is not detectable. Sequencing revealed that a premature termination codon (PTC) in TPI coding region, which activates nonsense-mediated mRNA decay (NMD) process and thus rapidly degrades PTC-containing TPI transcripts. UPF1, UPF2, and UPF3 are conserved in all eukaryotes and are also critical components of NMD surveillance complex in plants. Plants recognize PTCs in both intron-containing and intronless genes. Moreover, somewhat similar to mammals, when a PTC is closely upstream of an intron, NMD is abrogated. Analyses using translation inhibitor show that without translation PTC-containing mRNA is much more stabilized.

5. zusammenfassung

Im Laufe der Evolution haben Pflanzen ein ausgereiftes Verteidigungssystem entwickelt um sich gegen Fraßfeinde zu wehren. Pflanzen reagieren mit fein abgestimmten transkriptionellen Veränderungen, erhöhten Phytohormonkonzentrationen und daraus resultierend, mit der Produktion von direkt und indirekt wirkenden chemischen Komponenten auf Herbivorebefall. Mitogen-aktivierte Proteinkinasen (MAPK) spielen eine entscheidende Rolle bei der Signalübertragung extrazellulärer Stimuli und der darauf folgenden Aktivierung intrazellulärer Reaktionen. Vorausgegangene Forschungen haben gezeigt, dass in Pflanzen insbesondere zwei Kinasen, die salicylsäureinduzierte Proteinkinase und die verwundungsinduzierte Proteinkinase (WIPK), bei der Reaktion auf verschiedene Stressignale eine große Rolle spielen.

In dieser Arbeit demonstrieren wir das SIPK und WIPK kurze Zeit nach Raupenfraß aktiviert werden. Durch Anwendung einer virusbasierten Methode zur selektiven Ausschaltung spezifischer Gene (VIGS) erhielten wir Pflanzen mit stark reduzierter SIPK- und WIPK Aktivität. Wir zeigen das SIPK und WIPK die nach Pflanzenfraß induzierten Hormone Jasmonsäure (JA), JA-Isoleucin und die Salicysäurekonzentration regulieren und SIPK zusätzlich auch noch die Ethylenbiosynthese kontrolliert. Weiterhin demonstrierten Expressionsanalysen das beide Kinasen sowohl die Transkriptinslevels von Genen an der Phytohormonbiosynthese beteiligter Enzyme beieinflussen, die Transkription andere MAPK, auch Calcium-abhängiger Proteinkinasen (CDPKs) transkriptionell regulieren als auch Gene kontrollieren, die in der direkten Verteidigung involviert sind, so unter anderem Trypsinproteinase Hemmer (TPI) und Phenylalanin-amonium Lyase. Daraus schließen wir, dass beide Kinasen, SIPK und WIPK wichtige vorgeschaltete Signalkomponenten darstellen, welche die Verteidigung der Pflanze gegen Fraßfeinde vermitteln.

Als Antwort auf eine Attacke werden im betroffenen Blatt MAPK und nachgeschaltete Expressionsveränderungen aktiviert; dies geschieht jedoch nicht ausschließlich in den verwundeten Bereichen, sondern zusätzlich in spezifischen unverwundeten Bereichen des gleichen Blattes. Wir postulieren ein mobiles Signal, dass auf kurze Distanz in bestimmte Bereiche des Blattes gelangt und dort die MAPK

aktiviert. Anschließend wandert ein anderes Signal, höchstwahrscheinlich JA oder von eine von JA aktivierte Substanz in abgelegene, unverwundete Blätter und induziert dort die TPI abhängige Verteidigung.

TPI spielen eine wichtige Rolle bei der Verteidigung von Pflanzen gegen Herbivoren. *N. clevelandii* und *N. quadrivalvis* sind zwei tetraploide Pflanzenspezies die von den Diploiden *N. attenuata* und *N. obtusifolia* abstammen. Nach der Polyploidisierung wurde das TPI Gen von *N. attenuata* komplett gelöscht und kommt in beiden tetraploiden Genomen nicht mehr vor. Wie dem auch sei, beide Tetraploide besitzen weiterhin die für die Verteidigung notwendige Signalkaskade von *N. attenuata*, welche ihnen die Möglichkeit gibt spezifisch auf Raupenfraß zu reagieren und die TPI Expression in stärkerem Maße zu aktivieren als durch Verwundung allein. Diese Studie illustriert die Platstizität in der Evolution der Polyploiden Pflanzen, welche sie unter Umständen mit ökologischen und evolutionären Vorteilen Vorteilen ausstattet, die für das Überleben in der Natur von Vorteil sein können.

Der Arizona-Ökotyp von *N. attenuata* besitzt sehr geringe Mengen von TPI mRNA und es ist auch keine TPI Aktivität messbar. Die Ergebnisse der Gensequenzierung zeigen ein vorzeitiges Stopcodon in der für TPI codierenden Sequenz, welches einen verfrühten Kettenabbruch bei der Translation verursacht und den darauf folgenden Abbau der durch die Veränderung sinnlosen RNA. UPF1, UPF2 und UPF3 sind konservierte Elemente des RNA-Abbaukomplexes in allen Eukaryoten. In Pflanzen ist dieser Mechanismus noch nicht eingehend erforscht, jedoch weiss man das UPF1, UPF2 und UPF3 auch hier eine wichtige Rolle spielen. Pflanzen realisieren falsche Stopcodons sowohl in Genen mit, als auch in Genen ohne Intronsequenzen, wobei, ähnlich zu Säugetieren, dieser Mechanismus aufgehoben wird, sobald das Stopcodon nahe dem 5'-Ende eines Introns lokalisiert ist. Bei Behandlung mit Translationsinhibitoren ist die betreffende RNA deutlich stabiler, was einen Zusammenhang zwischen Translation und dem RNA Abbau nahelegt.

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7. Acknowledgements

I would like to thank Prof. Ian T. Baldwin for his supervision, his support all along my Ph.D., and for all the inspiring discussions.

I specially thank Stefan Meldau, Christian Hettenhausen, Dr. Jin-Ho Kang, Dahai Yang, Jinsong Wu, Dr. Lei Wang, and Michelle Lim for their great help and discussions, and for their friendship. Many thanks to my labmates: Beatrice Berger, Meredith Shuman, and Samir Anssour for heir help and making a nice lab atmosphere.

I deeply appreciate my family's support during my many years of study.

Many thanks to:

Our molecular supporting team: Dr. Klaus Gase, Susan Kutschbach, Antje Wissgott, Thomas Hann, Wibke Kröber, and Silke Allmann.

Our analytical team and also my officemates: Dr. Matthias Schöttner, Eva Rothe, Dr. Nicolas Heinzel, Dr. Emmanuel Gaquerel, and Dr. Bernd Krock.

Dr. Tamara Krügel and all the gardeners for providing me with a lot of plants.

Emily Wheeler for her great help with my manuscript writing.

Evelyn Claußen for her help with administrative issues.

Max-Planck Society for financial support.

8. Curriculum vitae

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9. Selbständigkeitserklärung

Entsprechend der Promotionsordung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Personen, die an der Durchführung und Auswertung des Materials und bei der herstellung der Manuskripte beteiligt waren sind am Beginn der Arbeit ("Manuscript Overview") und jedes ManusKriptes angegeben.

Die hilfe eines Promotionsberaters wurde nich in Anspruch genommen.

Die vorgelegte Arbeit Wurde weder an der Friedrich-Schiller-Universität Jena, noch an einer anderen Hochschule als Dissertation eingereicht.

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