Functions of protein kinases, Calcium-Dependent Protein Kinases (CDPKs) and BRI1-Associated Kinase 1 (BAK1), in wild tobacco (*Nicotiana attenuata*) immunity to herbivore and pathogen

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1. Introduction

Plant and phytohormones

Plants, due to their sessile nature, have developed sophisticated regulatory networks to modulate their development and growth, and to respond properly to environmental stresses. Within these networks, auxins, cytokines, gibberellins, brassinosteroids (BRs), jasmonates, ethylene, salicylic acid (SA), abscisic acid (ABA), and strigolactone play crucial roles to modulate almost every aspect of a plant's life (Chow and McCourt, 2006; Santner and Estelle, 2009). These hormone molecules exist in very low concentrations but alter plant physiology dramatically. Accordingly, plants have evolved elaborate systems to tightly control and sense the levels of these hormones. In recent years, new genetic and biochemical approaches have greatly advanced our understanding of the biosynthesis, transport, catabolism, perception, signal transduction, and physiological functions of these phytohormones in various plant species. Now it has become clear that although an individual hormone has specific roles in modulating growth and stress responses, complex antagonistic and synergistic interactions among them enable plants to fine-tune their cellular processes (Chow and McCourt, 2006; Grant and Jones, 2009). However, the interactions between phytohormone-mediated pathways and other cellular signaling cascades, such as Ca^{2+}, reactive oxygen species (ROS), and mitogen-activated protein kinase (MAPK), still remain largely unexplored.

Jasmonic acid biosynthesis and signaling

JA and its derivatives, collectively named as jasmonates, are involved in plant development and responses to various stresses, such as attack from herbivores and necrotrophic fungi (Wasternack, 2007; Howe and Jander, 2008; Browse, 2009; Wu and Baldwin, 2009, 2010). JA is synthesized through an oxylipin pathway in which several enzymes, including phospholipase, 13-lipoxygenase (13-LOX), allene-oxide synthase (AOS), allene-oxide cyclase (AOC), *cis*(+)-12-oxophytodienoic acid (OPDA) reductase (OPR), and acyl-coenzyme A oxidase (ACX) play pivotal roles (Liechti and Farmer, 2002; Wasternack, 2007; Browse, 2009). In chloroplasts, phospholipases release α-linolenic acid from chloroplast membranes. After an oxidization reaction catalyzed by 13-LOX, α-linolenic acid is further converted to (13*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT), which becomes thereafter OPDA after two steps of reactions catalyzed by AOS and AOC. OPDA is transported to peroxisomes, where a reduction reaction happens to the cyclopentenone ring,

catalysed by an OPR enzyme. After three steps of β-oxidation, JA is formed (Wasternack, 2007) (Figure 1).

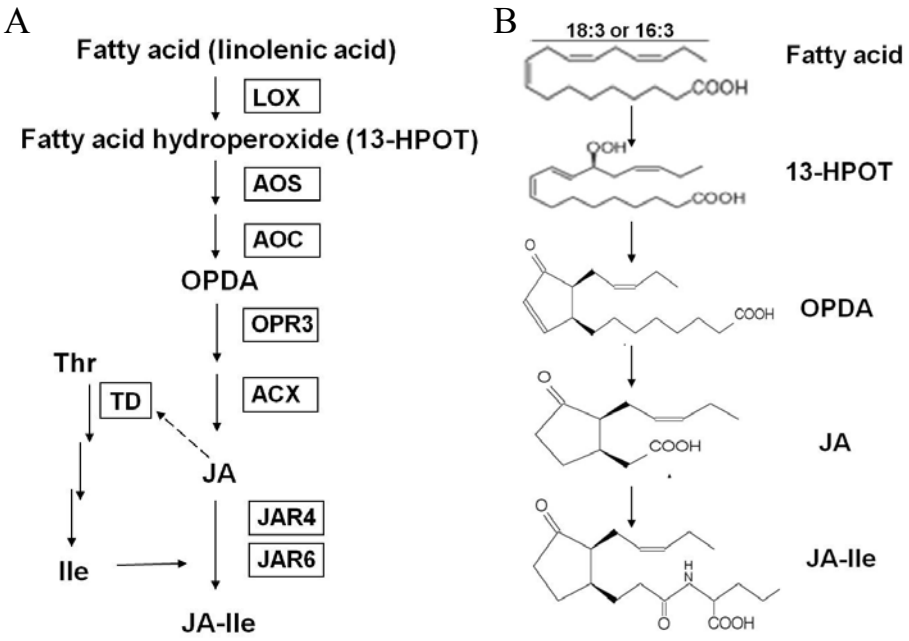

Figure 1. Biosynthesis of Jasmonic acid (JA) and JA-isoleucine (JA-Ile)

(A) the brief scheme of biosynthesis of JA and JA-Ile. Catalytic enzymes labeled in boxes. (B) chemical diagram of JA, JA-Ile, and their precursors.

After mechanical wounding or herbivory, a burst of JA is produced. Give the rapid nature of JA biosynthesis after these challenges, which happens before the transcriptional changes of JA biosynthetic genes, and the high abundance of JA biosynthetic enzymes, it is generally believed that JA biosynthesis induced by wounding and herbivory is controlled at an post-translational level. Thus far, still very little is known about how JA biosynthesis is regulated. In *Nicotiana attenuata*, two MAPKs, salicylic acid-induced protein kinase and wound-induced protein kinase play important roles in regulating wounding- and herbivory-induced JA biosynthesis (Wu et al., 2007). Chemical profiling of JA precursors revealed that these two kinases control JA biosynthesis by different mechanisms (Kallenbach et al., 2010).

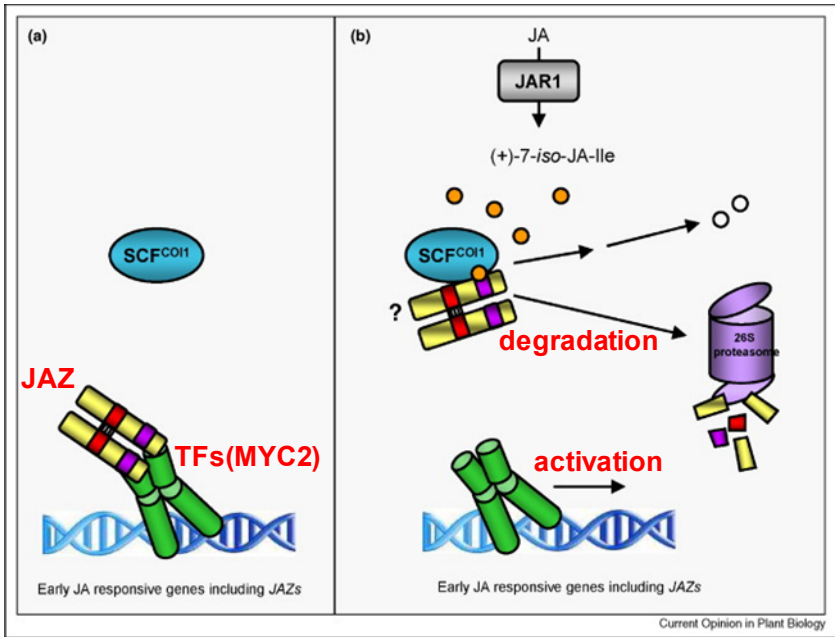

Figure 2. COI1 mediates JA signaling through promoting degradation of JAZs.
(a) In the absent of the jasmonic acid-amino acid conjugate JA-Ile, JAZ proteins (the repressor of jasmonate signaling) bind to transcription factors (such as, MYC2), which activate jasmonate responses. (b) In the presence of bio-active JA-Ile, COI1 binds to JAZs, and mediates the ubiquitination of JAZs; subsequently, JAZs are degradated by 26S proteasome. The JA-Ile induced early JA responsive genes includes *JAZ*s, and activation of transcription of *JAZ* genes provide a feedback control to limit the JA responses [modified figure from (Fonseca et al., 2009)].

JA signaling plays a critical role in conveying JA accumulation to JA-induced responses. Importantly, the Ile conjugate of JA that is catalyzed by jasmonate resistant proteins (JARs), JA-Ile (instead of JA) is responsible for eliciting most of JA-induced responses (Staswick and Tiryaki, 2004; Wang et al., 2008) An F-box protein, COI1, (coronatine insensitive1) has been found previously to be critical for JA-induced responses (Feys et al., 1994; Xie et al., 1998). However, the mechanism how COI1 transduces JA signaling was unclear until recent work identified that COI1 is the JA-Ile receptor (Chini et al., 2007; Thines et al., 2007; Yan et al., 2009). Binding of JA-Ile to COI1 promotes the interaction of COI1 with jasmonate ZIM-domain (JAZ) proteins; JAZs are subsequently ubiquitinated by SCF^{COI1} E3 ubiquitin ligase and are degraded by the 26S proteasome. The degradation of the JAZ repressors releases transcription factors MYC2, which activates JA responses in Arabidopsis (Chini et al., 2007; Thines et al., 2007; Fonseca et al., 2009; Yan et al., 2009) (Figure 2).

In *N. attenuata*, the critical role of JA biosynthesis and signaling in plant defense against herbivores has been well demonstrated.

Nicotiana attenuata: an ecological model for plant-herbivore interactions

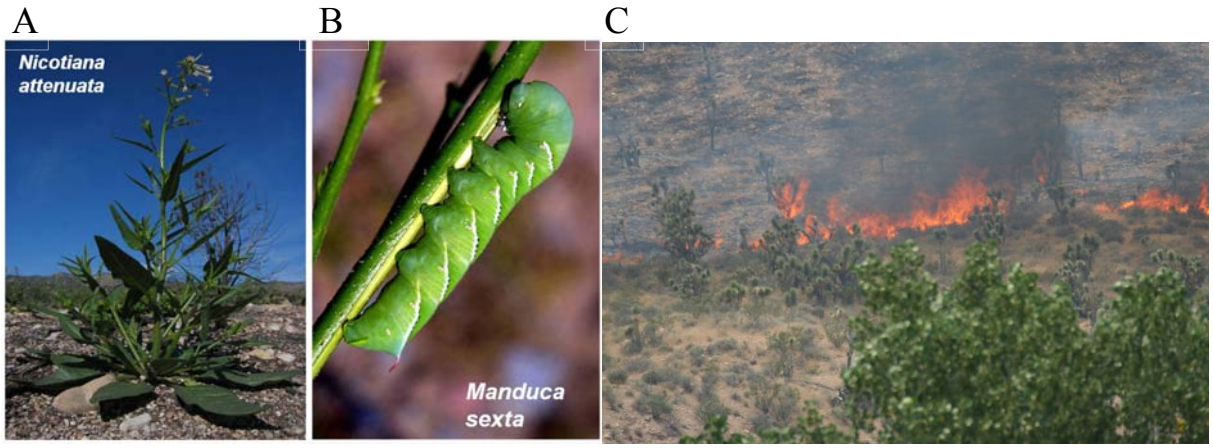

Figure 3. *Nicotiana attenuata*, *Manduca Sexta*, and *N. attenuata*'s natural habitat (photo courtesy by D. Kessler).

(A) *N. attenuata* in its natural habitat Utah. (B) *Manduca sexta* (C) Fire in Utah 2005.

N. attenuata (2n = 24), an annual wild tobacco plant, germinates and grows after sensing smoke-derived cues from fire in its desert habitats (the Great Basin Desert of North America). After fire, *N. attenuata* dominates the burned area as a pioneer plant, and the coming herbivores have to develop new populations on each new generation of plants. Among the herbivores fed on *N. attenuata* plant, the larvae of leaf-chewing insect *Manduca sexta* (Lepidoptera, Sphingidae) belong to the most damaging defoliators. Tobacco hornworm *M. sexta* is specialized on Solanaceous host plants and native to North and South America. As pollinators, the adult moths visit and feed on the nectar of *N. attenuata* flowers and lay eggs directly on *N. attenuata*. After larvae hatch from the eggs, they feed on leaves and can cause major defoliation during development of all five larval instars (Figure 3).

The long-lasting coevolution has equipped *N. attenuata* with sophisticated defense system to counteract *M. sexta* herbivore attack. Feeding of *M. sexta* larvae iduces numerous defense responses in *N. attenuata*, including kinase activation, jasmonate accumulation, production of anti-herbivore secondary metabolites, and release of volatiles. The anti-herbivore secondary metabolites, which function as toxins, repellents, and antidigestives, belong to plant direct defense. Herbivory-induced plant volatiles can attract natural enemies (predators and parasitoids) of herbivores and serve as indirect defense (Figure 4). *M. sexta* is also able to adapt to these chemical defenses. For example, *M. sexta* accumulates and uses neurotoxin nicotine as chemical defense against predators of larvae, which is important for its survival and success on host plants. In addition, the largely selfing, short generation time (2-3

months), and availability for genetic engineering also make *N. attenuata* useful for both laboratory and field studies (Wu and Baldwin, 2009, 2010).

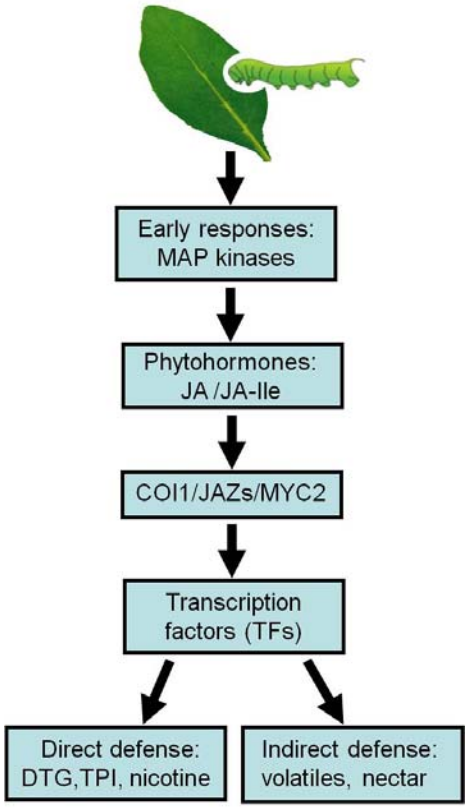

Figure 4. *N.attenuata*'s defense responses to *M. sexta*.

One of the earliest cellular events in *M. sexta*-attacked *N. attenuata* is activation of MAPKs. *N. attenuata* perceives fatty acid-amino acid conjugates (FACs) in the oral secretions (OS) of *M. sexta* during feeding and rapidly activates salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), two mitogen-activated protein kinases (MAPKs). Subsequently, enhanced MAPK activity elicits accumulation of JA, JA-Ile, and ethylene, the important phytohormones mediating plant responses to herbivores (Halitschke et al., 2001; Wu et al., 2007).

In *N. attenuata*, nicotine, trypsin proteinase inhibitors (TPIs), diterpene glycosides (DTGs), and caffeoylputrescine (CP) have all been identified to serve as direct defense to impair the mass gain of *M. sexta* feeding on *N. attenuata*: silencing *putrescine N-methyl transferase* (*PMT*) in nicotine biosynthesis (Steppuhn et al., 2004), *TPI* gene (Zavala et al., 2004), *geranylgeranyl diphosphate synthase* (*GGPPS*) in DTG biosynthesis (Jassbi et al., 2008; Heiling et al., 2010), and *NaMYB8* that regulates biosynthesis of CP (Kaur et al., 2010) diminishes herbivory-induced nicotine, TPI, DTG, and CP accumulation and thus compromise *N. attenuata*'s resistance to herbivores. Importantly, both laboratory and field

studies demonstrated that the biosynthesis of these defensive compounds is dictated mainly by JA signaling: abolishing JA biosynthesis (silencing *LOX3*) (Halitschke and Baldwin, 2003), JA-Ile accumulation (silencing *JAR4* and *JAR6*) (Wang et al., 2008), and JA signaling (silencing *COI1*) (Paschold et al., 2007) greatly impairs the accumulation of these secondary metabolites and increases plant susceptibility to *M. sexta*.

Although much is known about JA biosynthesis and its signaling, the regulatory network of JA biosynthesis in response to herbivory remains largely unknown.

Functions of calcium-dependent protein kinases in plant resistance to biotic and abiotic stresses

Both pathogen and herbivore attack elicit Ca^{2+} spikes at the attacked regions (Maffei et al., 2004; Garcia-Brugger et al., 2006; Maffei et al., 2006). Ca^{2+} signals play important roles in regulating almost all aspects of plant development and cellular responses to the environmental cues (Sanders et al., 2002; Dodd et al., 2010; Kudla et al., 2010). The information encoded by the Ca^{2+} oscillation, normally termed as Ca^{2+} signature, is decoded by a group of Ca^{2+}-binding proteins which transmit the information through protein phosphorylation or activation of other proteins (Harper et al., 2004; Harper and Harmon, 2005; DeFalco et al., 2010). Among these Ca^{2+}-binding proteins, calcium-dependent protein kinases (CDPKs) compose a unique family of calcium sensor proteins: CDPK proteins contain not only Ca^{2+}-binding motifs, but also protein kinase domain, which is lacking in other Ca^{2+}-binding proteins.

Despite large number of family members (34 CDPKs in Arabidopsis), which causes functional redundancy and restricts the study of individual CDPK protein (Cheng et al., 2002; Hrabak et al., 2003), a growing body of evidence has indicated that CDPKs play critical roles in plant responses to biotic stresses, especially in plant-pathogen interactions. *Nicotiana tabacum* CDPK2 and CDPK3 are involved in Cf-9/Avr-9 gene for gene interaction (Romeis et al., 2000; Romeis et al., 2001). Furthermore, ectopically expression of *NtCDPK2* lacking its autoinhibitory and calcium binding C-terminal domains resulted in activation of biotic stress responses upon water (osmotic) stress in *N. benthamiana* (Ludwig et al., 2005). Importantly, enhanced CDPK signaling impairs stress-elicited MAPK activation, suggesting interactions exist between CDPK and MAPK pathways in plant responses to biotic and abiotic stresses (Ludwig et al., 2004; Ludwig et al., 2005).

In Arabidopsis, *cpk1* mutant shows enhanced susceptibility to pathogen attack compared with WT plants, and over-expression of *AtCPK1* results in elevated SA levels, SA-

induced defense genes, and enhanced pathogen resistance (Coca and San Segundo, 2010). Recently Boudsocq *et al.* (2010) demonstrated that four AtCDPKs are critical for flagellin (flg22)-induced transcriptional reprogramming in plant innate immunity. Importantly, CDPK and MAPK pathways differently regulate microbe (or pathogen)-associated molecular pattern (MAMP or PAMP)-elicited gene expression. In contrast to the advance in studying CDPK's physiological functions in plant-pathogen interactions, only a few studies reported that herbivory induces expression of *CDPK* genes in *N. attenuata* and defensin gene (*AtPDF1.2*) in Arabidopsis (Wu et al., 2007; Kanchiswamy et al., 2010). Whether CDPKs are involved in plant-herbivore interactions was largely unknown.

BAK1/SERK3: a multiple functions co-receptor for plant receptor kinases

BRs are a class of phytohormones that regulate seed germination, stem and root elongation, vascular differentiation, leaf expansion, and apical dominance (Halliday, 2004). Genetic analysis revealed that BR insensitive 1 (BRI1), a leucine-rich repeat receptor-like kinase (LRR-RLK), is the BR receptor (Li and Chory, 1997; Kinoshita et al., 2005). Another LRR-RLK, the SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3), also named BRI1-ASSOCIATED KINASE 1 (BAK1), physically interacts with BRI1and plays an essential role in BR signaling (Li et al., 2002; Nam and Li, 2002; Belkhadir and Chory, 2006; Karlova and de Vries, 2006).

Apart from its function in BR signaling, BAK1/SERK3 has been shown to play an important role in plants' defense against pathogens. BAK1/SERK3 binds to FLS2, the flagellin receptor, and positively regulates FLS2-mediated innate immunity in Arabidopsis (Chinchilla et al., 2007). In *N. benthamiana*, BAK1/SERK3 is also required for multiple pathogen-associated molecular patterns (PAMPs)-elicited responses and for resistance to bacteria and oomycetes (Heese et al., 2007). BAK1 was found to rapidly form complex with AtPep1 (a damage-associated molecular pattern, DAMP) receptor 1, AtPEPR1 (Schulze et al., 2010). Furthermore, BAK1 is indispensible for full amplitudes of cell membrane depolarization induced by several Peps, flg22, and elf18, suggesting that BAK1 mediates DAMP- and pathogen-associated molecular pattern (PAMP)-triggered membrane depolarization, a convergence of DAMP and PAMP/MAMP signaling in Arabidopsis (Jeworutzki et al., 2010; Krol et al., 2010).

BAK1/SERK3 has been proposed to have multiple functions as a common co-receptor and is thought to cooperate with other receptors in signal transduction (Gendron and Wang,

2007; Boller and Felix, 2009). Whether BAK1/SERK3 is involved in plants' defense responses to herbivory has not been examined.

Research topics

The objective of this thesis is to study the function of two CDPKs, NaCDPK4 and NaCDPK5, and NaBAK1 in *N. attenuata*'s resistance to herbivores and pathogens.

Thesis Chapter I: NaCDPK4 and NaCDPK5 in plant-herbivore and -pathogen interactions

To investigate the function of CDPKs in plant-herbivore interactions, we cloned *CDPK4* and *CDPK5* in *N. attenuata* (*NaCDPK4* and *NaCDPK5*) and knocked down their transcripts levels using a RNA interference (RNAi) approach. Traits important for herbivore resistance in these plants were determined. Our results suggest that either NaCDPK5 alone or NaCDPK4 and NaCDPK5 together negatively regulate *N. attenuata*'s resistance to herbivore *M. sexta* and pathogen *Pseudomonas syringae* pv. *tomato* DC3000.

Thesis Chapter II: NaBAK1 in plant defense responses to herbivory

To investigate the function of BAK1/SERK3 in plant-herbivore interactions, we cloned *BAK1* in *N. attenuata* (*NaBAK1*) and silenced its transcripts levels using a virus-induced gene silencing (VIGS) approach. Traits important for herbivore defense in these plants were examined. Our data demonstrate that BAK1/SERK3 is involved in regulating *N. attenuata*'s defense responses, such as accumulation of JA/JA-Ile and induction of TPI activity.

2. Chapter I: NaCDPK4 and NaCDPK5 in plant resistance to herbivores and pathogens

Calcium sensor protein kinases, CDPK4 and CDPK5, modulate *Nicotiana attenuata*'s resistance to specialist herbivore *Manduca sexta* and pathogen *Pseudomonas syringae* pv. *tomato* DC3000

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Abstract

Calcium-dependent protein kinases (CDPKs) compose an unique family of kinases in plant kingdom. CDPKs comprise protein kinase and Ca^{2+}-binding domains, and are believed to decode Ca^{2+} signature in plant growth, development, and responses to environmental stimuli. A growing body of evidence has indicated the important roles of CDPKs in plant resistance to pathogens. However, whether they mediate resistance to herbivores still remained unknown. Here, we report the functional characterization of two CDPKs (NaCDPK4 and NaCDPK5) in *Nicotiana attenuata*'s responses to herbivores and pathogens. Using an RNAi approach, we co-silenced *NaCDPK4* and *NaCDPK5* in *N. attenuata* and generated IRcdpk5 plants. Under greenhouse condition, co-silencing *NaCDPK4* and *NaCDPK5* resulted in remarkably elevated levels of herbivory-induced jasmonic acid (JA) and JA-isoleucine (JA-Ile), and thus anti-herbivore secondary metabolites (diterpen glycosides and trypsin proteinase inhibitors). Consistently, highly enhanced resistance to *M. sexta* larvae was observed in IRcdpk5 plants. Reducing herbivory-elicited JA levels by ectopically expressing *jasmonate methyltransferase* or abolishing JA signaling by knock-down *COI1* in IRcdpk5 plants revealed that the highly elevated anti-herbivore secondary metabolites and resistance to herbivore of IRcdpk5 plants are completely dependent on herbivory-induced accumulation of JA and JA signaling (COI1). In addition, compared with wild-type (WT) plants, IRcdpk5 plants have elevated resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), although they did not have different salicylic acid levels from WT plants. We further specifically silenced *NaCDPK4* with an invert repeat construct using

the N-terminal region of *NaCDPK4* (IRcdpk4 plants). Compared with WT plants, similar levels of herbivory-induced JA, JA-Ile, anti-herbivore secondary metabolites, and resistance to *M. sexta* and *Pst* DC3000 were found in IRcdpk4 plants. Our data suggest that either NaCDPK5 alone or NaCDPK4 and NaCDPK5 together are negative regulators of plant resistance to herbivore *M. sexta* and pathogen *Pst* DC3000 in *N. attenuata*.

Introduction

Due to the sessile nature, plants have developed elaborate networks to regulate their development and growth, and to respond properly to the environmental stimuli. Within the regulatory networks, phytohormones play crucial roles to modulate almost every aspect in a plant's life (Chow and McCourt, 2006; Santner and Estelle, 2009). Accordingly, plants have evolved sophisticated systems to accurately control and sense the levels of these phytohormones. Using genetic and biochemical approaches, our understanding of the molecular mechanisms by which plants regulate the biosynthesis, catabolism, and perception of the phytohormones has greatly advanced in recent years. However, the interactions between phytohormone-mediated pathways and other cellular signaling cascades, such as Ca^{2+}, reactive oxygen species (ROS), and mitogen-activated protein kinase (MAPK), still remain largely unexplored.

Among these phytohormones, jasmonates and salicylic acid (SA) have been well studied for their roles in modulating plant responses to biotic (herbivore and pathogen) stresses. Jasmonic acid (JA) and its derivatives, collectively named as jasmonates, are involved in plant resistance to herbivore and necrotrophic fungus attack (Wasternack, 2007; Howe and Jander, 2008; Browse, 2009; Wu and Baldwin, 2009, 2010). JA is synthesized through an oxylipin pathway: starting from linolenic acid (18:3) released from membrane glycerolipids, this fatty acid subsequently catalyzed by 13-lipoxgenase (13-LOX), allene oxidase synthase (AOS), and allene oxide cyclase (AOC) to produce (9S,13S)-12-oxo-phytodienoic acid (OPDA) in chloroplasts; OPDA is imported into peroxisomes and is catalyzed by OPDA reducatase (OPR), carboxyl-CoA ligase (OPCL), and acyl-CoA oxidase (ACX) to form (3R, 7S)-jasmonic acid (Liechti and Farmer, 2002; Wasternack, 2007; Browse, 2009).

Importantly, instead of JA itself, the conjugate of JA and isoleucine, JA-isoleucine (JA-Ile), which is catalyzed by jasmonate resistant proteins (JARs), elicits most of jasmonate-induced responses (Staswick et al., 2002; Wang et al., 2008). JA-Ile is perceived by the F-box protein COI1, which was recently identified as the receptor of jasmonates (Yan et al., 2009).

Binding of JA-Ile to COI1 promotes the interaction of COI1 with jasmonate ZIM-domain (JAZ) proteins; JAZs are subsequently labeled with ubiquitins by SCF^{COI1} E3 ubiquitin ligase and degraded by the 26S proteasome. The degradation of JAZ repressors release transcription factor MYC2, which activates jasmonate responses in Arabidopsis (Xie et al., 1998; Chini et al., 2007; Thines et al., 2007).

In *Nicotiana attenuata*, a wild tobacco, the ecological importance of JA biosynthesis and signaling in plant-herbivore interactions has been intensively studied (Halitschke and Baldwin, 2003; Paschold et al., 2007). After herbivore attack, *N. attenuata* perceives the fatty acid-amino acid conjugates (FACs) in the oral secretions (OS) of the *M. sexta* larvae (Halitschke et al., 2003). Subsequently, MAPK activity was quickly enhances, which elicits accumulation of JA, JA-Ile, and ethylene, the important phytohormones mediating plant responses to herbivores (Wu et al., 2007). Accordingly, plants dramatically alter their transcriptomes, including elevating expression levels of transcription factors and biosynthetic genes for anti-herbivore secondary metabolites (Halitschke et al., 2003). These secondary metabolites, such as diterpene glycosides (DTGs) and trypsin proteinase inhibitors (TPIs), can serve as direct defense to impair the mass gain of *M. sexta* feeding on *N. attenuata* (Wu and Baldwin, 2009, 2010).

Besides JA, herbivore attack also elicits Ca^{2+} spike at the infested region of the leaves (Maffei et al., 2004; Maffei et al., 2006). Ca^{2+} signals play important roles in regulating almost all aspects of plant development and cellular responses to the environmental cues (Sanders et al., 2002; Dodd et al., 2010; Kudla et al., 2010). The information encoded by the Ca^{2+} oscillation, normally termed as Ca^{2+} signature, is decoded by a group of Ca^{2+}-binding proteins which transmit the information through protein phosphorylation and/or activation of other proteins (Harper et al., 2004; Harper and Harmon, 2005; DeFalco et al., 2010). Among these Ca^{2+}-binding proteins, calcium-dependent protein kinases (CDPKs) compose an unique family of calcium sensor proteins: CDPK proteins contain not only Ca^{2+}-binding motifs, but also protein kinase domains, which are lacking in other Ca^{2+}-binding proteins.

Despite large number of family members (34 CDPKs in Arabidopsis), which causes functional redundancy and restricts the study of individual CDPK protein (Cheng et al., 2002; Hrabak et al., 2003), a growing body of evidence has indicated that CDPKs play critical roles in plant's responses to biotic stresses, especially in plant-pathogen interactions. *Nicotiana tabacum* CDPK2 and CDPK3 were found to be involved in Cf-9/Avr-9 gene for gene interaction (Romeis et al., 2000; Romeis et al., 2001). Furthermore, ectopic expression of *NtCDPK2* lacking its autoinhibitory and calcium binding C-terminal domains resulted in

activation of biotic stress responses upon water (osmotic) stress in *N. benthamiana* (Ludwig et al., 2005). Importantly, enhanced CDPK signaling impaired stress-elicited MAPK activation, suggesting interactions exist between CDPK and MAPK pathways in plant's responses to biotic and abiotic stresses (Ludwig et al., 2005).

In Arabidopsis, *cpk1* mutant shows more susceptibility to pathogen attacked compared with WT plants, and over-expression of *AtCPK1* results in elevated SA levels, SA-induced defense genes, and enhanced pathogen resistance (Coca and San Segundo, 2010). Recently Boudsocq *et al.* showed four AtCDPKs are critical components for flagellin (flg22)-induced transcriptional reprogramming in plant innate immunity. Importantly, CDPKs and MAPK pathways differently regulate microbe-associated molecular patterns (MAMP)-elicited gene expression (Boudsocq et al., 2010).

In contrast to the advance in studying CDPK's physiological function in plant-pathogen interactions, only a few studies reported that herbivory induces expression of *CDPK* genes in *N. attenuata* and defensin gene (*AtPDF1.2*) in Arabidopsis (Wu et al., 2007; Kanchiswamy et al., 2010). Whether CDPKs are involved in plant-herbivore interactions was largely unknown. To investigate the function of CDPKs in plant-herbivore interactions, we cloned *CDPK4* and *CDPK5* in *N. attenuata* (*NaCDPK4* and *NaCDPK5*) and attenuated their transcripts levels using a RNA interference (RNAi) approach. Traits important for herbivore resistance in these plants were determined. Co-silencing *NaCDPK4* and *NaCDPK5* in *N. attenuata* resulted in substantially elevated wounding- and herbivory-induced levels of JA, JA-Ile, anti-herbivore secondary metabolites (such as DTGs and TPIs), and defense against specialist herbivore *M. sexta*. In addition, compared with WT plants, (*NaCDPK4* and *NaCDPK5*)-silenced plants also exhibited elevated resistance to pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Interestingly, specific silencing *NaCDPK4* did not enhance *N. attenuata*'s resistance to *M. sexta* and *Pst* DC3000. Taken together, our results suggest that either NaCDPK5 alone or NaCDPK4 and NaCDPK5 together negatively regulate *N. attenuata*'s resistance to herbivores and pathogens.

Results

Cloning of Na*CDPK4* and Na*CDPK5* in *N. attenuata*

According to the sequences of *NtCDPK4* (accession number: AF435451) and *NtCDPK5* (accession number: AY971376) (Wang et al., 2005; Zhang et al., 2005), full length open reading frames of *NaCDPK4* and *NaCDPK5* were obtained through RT-PCR and screening the *N. atteunata* cDNA library. The alignment of deduced NaCDPK4 and NaCDPK5 proteins with several plant CDPK proteins revealed that NaCDPK4 and NaCDPK5 proteins are almost identical to NtCDPK4 and NtCDPK5 (96.5 % and 97.7 % identity, respectively) (Supplemental Figure 1).

NaCDPK4 and NaCDPK5, as NtCDPK4 and NtCDPK5, comprise all characteristic structures for CDPKs: a variable N-terminal domain, followed by a kinase catalytic domain, a junction domain, and a calmodulin-like domain with four conserved Ca2+ binding EF hands (Wang et al., 2005; Zhang et al., 2005), suggesting that NaCDPK4 and NaCDPK5 are calcium-dependent protein kinases in N. attenuata. NaCDPK4 and NaCDPK5 also share high homology with potato StCDPK1 (86 % and 91.4% identity, respectively), LeCPK2 (88.2 % and 93.4% identity, respectively), MtCDPK1 (80.5% and 81.7% identity, respectively). Moreover, protein sequence of NaCDPK4 showed 89.2% homology to that of NaCDPK5, and the variable N-terminal of NaCDPK4 and NaCDPK5 protein showed the highest difference (Supplemental Figure 1).

A putative myristoylation site (MGxxxSxxx, PlantsP database) and a putative palmitoylation site (MGxCx, Cys at 4th position) was found at the beginning of N-terminal of the deduced NaCDPK5 protein (but not in NaCDPK4 protein), which frequently exist in CDPKs (Martin and Busconi, 2000; Dammann et al., 2003), suggesting a possible association of NaCDPK5 with cell membrane as the cell membrane location of NtCDPK5 (Wang et al., 2005).

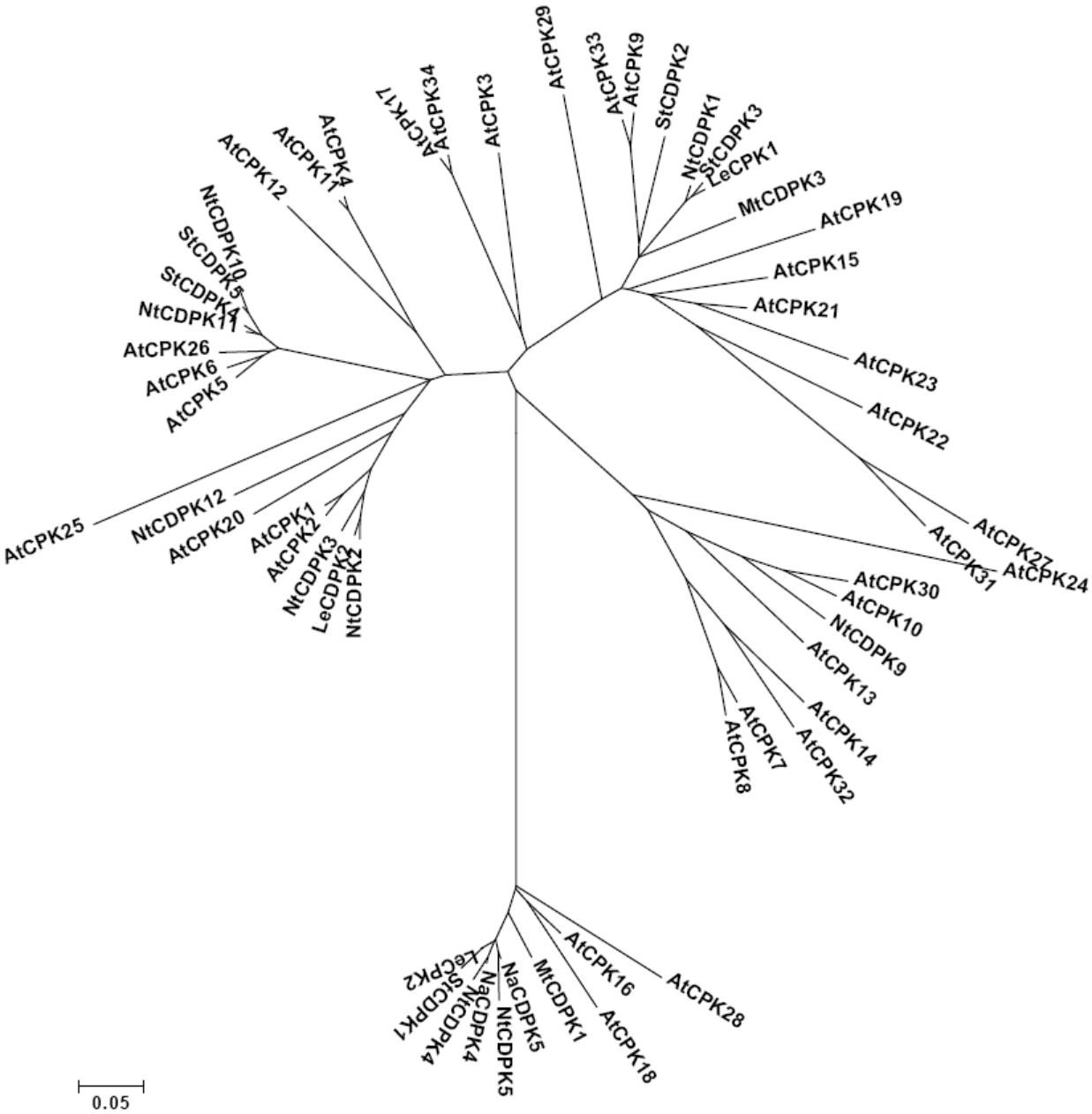


Figure 1. Phylogenetic tree of CDPK proteins

An unrooted phylogenetic tree of CDPKs in Arabidopsis and *Nicotiana* was constructed using their protein sequences. NCBI (GenBank) accession numbers of genes encoding these CDPK proteins are listed in Supplemental Table 3.

An unrooted phylogenetic tree was constructed using the deduced NaCDPK4 and NaCDPK5 protein and other plant CDPK protein sequences (Figure 1). Consistent with the amino acid sequences alignment data, NaCDPK4 and NaCDPK5 closely clustered with NtCDPK4 and NtCDPK5, respectively.

Wounding and herbivory induce transcript accumulation of *NaCDPK4* and *NaCDPK5* in *N. attenuata*

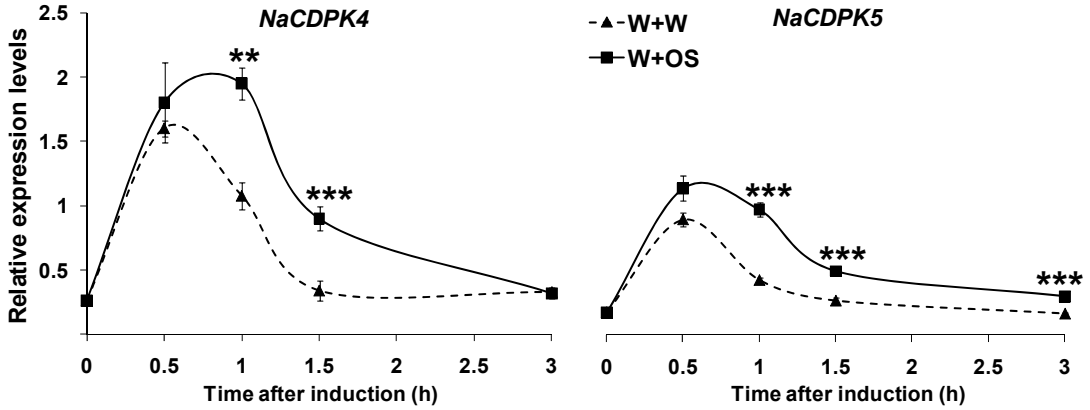


Figure 2. Wounding and herbivory induce the accumulation of *NaCDPK4* and *NaCDPK5* transcripts in *N. attenuata*.

N. attenuata leaves were wounded with a pattern wheel and 20 µl of water (W+W) or *M. sexta* oral secretions (W+OS) were immediately applied to the wounds. Samples were harvested at indicated times, and the transcript levels (± SE) of *NaCDPK4* and *NaCDPK5* were analyzed by quantitative real-time PCR. Asterisks represent significantly different transcript levels between W+W- and W+OS-treated samples (unpaired *t*-test; **, $p < 0.01$; ***, $p < 0.001$; N = 5)

To examine whether herbivory induces the accumulation of *NaCDPK4* and *NaCDPK5* transcripts, *N. attenuata* rosette leaves were wounded with a pattern wheel, and were immediately applied with 20 µl of *M. sexta* OS (W+OS), which effectively triggers herbivory-induced responses in *N. attenuata* (Halitschke et al., 2003). As a control, 20 µl of water were applied to the wounds (W+W). Quantitative real-time PCR (qPCR) was used to measure the levels of *NaCDPK4* and *NaCDPK5* transcripts at different times after treatments. Transcript levels of *NaCDPK4* and *NaCDPK5* quickly increased 0.5 h after W+W and W+OS treatments and gradually decreased to near basal levels after 3 h (Figure 2). Compared with W+W treatment, W+OS induced even higher transcript levels of *NaCDPK4* and *NaCDPK5*. These data suggest that NaCDPK4 and NaCDPK5 are likely involved in *N. attenuata*'s responses to wounding and herbivory.

Co-silencing *NaCDPK4* and *NaCDPK5* in *N. attenuata*

To study the function of *NaCDPK4* and *NaCDPK5* in *N. attenuata*'s responses to herbivory, an RNAi-based approach was employed to silence these genes. Since the high sequence similarity of *NaCDPK4* and *NaCDPK5*, the most divergence N-terminal region was

chosen to specifically silence *NaCDPK4* and *NaCDPK5* independently (Supplemental Figure 1). A 251 bp fragment of *NaCDPK5* N-terminal region was cloned into a binary vector pRESC5 in an inverted repeat (IR) fashion, and *Agrobacterium* harboring this vector was further used to transform *N. attenuata* to obtain *NaCDPK5*-silenced plants (IRcdpk5 plants hereafter) (Krugel et al., 2002). Similarly, a 201 bp fragment of Na*CDPK4* N-terminal region was used to generate *NaCDPK4*-silenced plants (IRcdpk4 plants hereafter) Several independently transformed lines of IRcdpk5 and IRcdpk4 plants which have single insertions were identified by Southern blotting (data not shown).

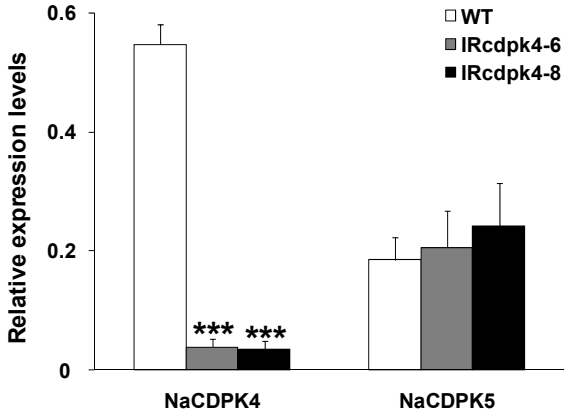


Figure 3 Specifically silencing transcripts of *NaCDPK4* but not *NaCDPK5* in IRcdpk4 plants.

N. attenuata leaves were wounded with a pattern wheel and 20 µl of *M. sexta* oral secretions (W+OS) were immediately applied to the wounds. Samples were harvested 1 h after W+OS treatment, and the transcript levels (± SE) of *NaCDPK4* and *NaCDPK5* were analyzed by quantitative real-time PCR. Asterisks represent significantly different transcript levels between W+W- and W+OS-treated samples (unpaired *t*-test; **, $p < 0.01$; ***, $p < 0.001$; N = 5)

The transcript levels of *NaCDPK4* and *NaCDPK5* in IRcdpk4 and IRcdpk5 lines were analyzed by qPCR. Two lines of IRcdpk4 plants (line IRcdpk4-6 and IRcdpk4-8) with greatly reduced W+OS-elicited transcripts of *NaCDPK4* (7.1% and 6.2% of those in wild-type (WT) plants, respectively) and comparable transcript levels of *NaCDPK5* (Figure 3) were selected for further study.

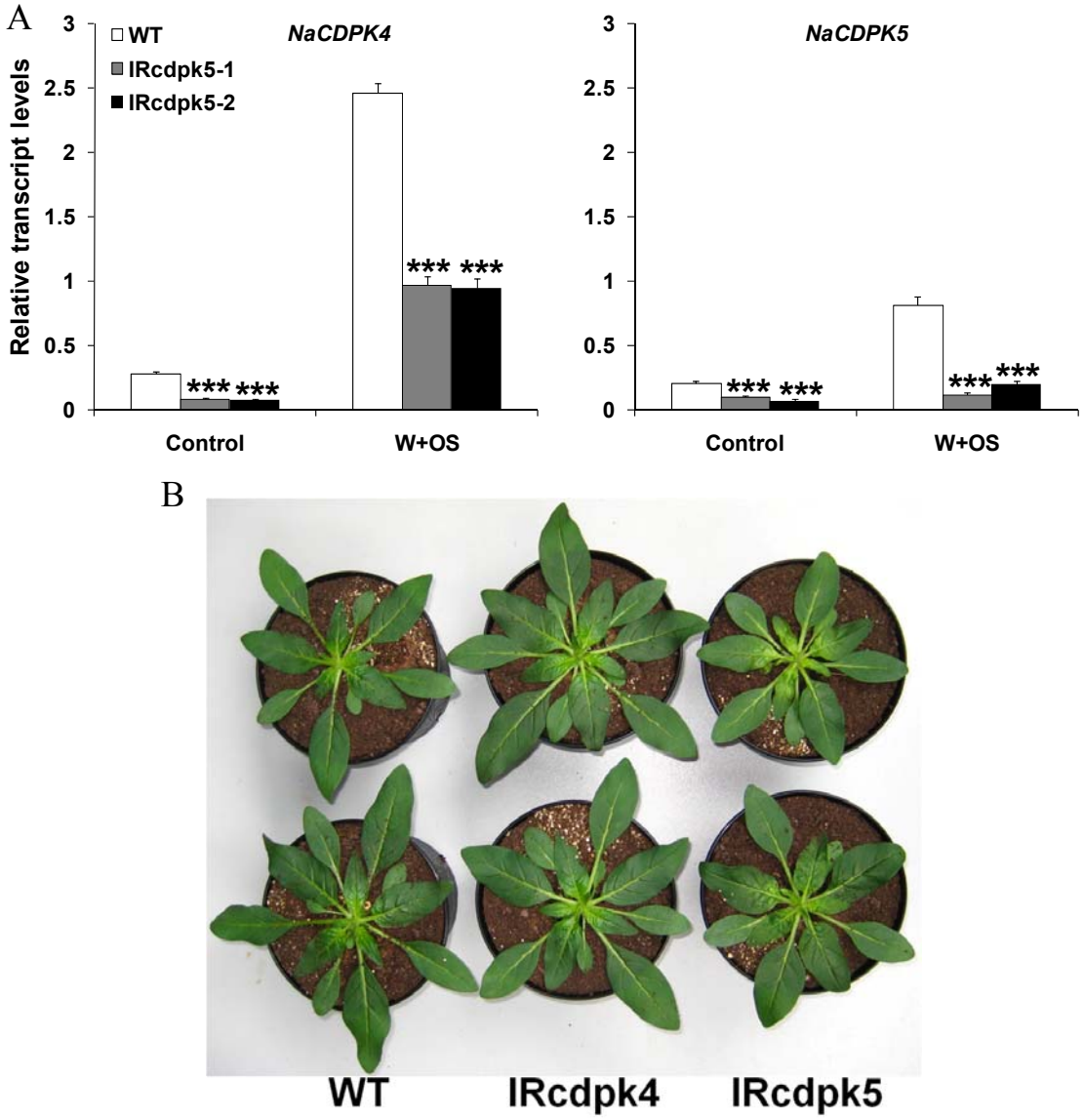


Figure 4 Silencing efficiency and morphology of IRcdpk5 plants

(A) IRcdpk5 plants were generated using an RNAi approach. *N. attenuata* leaves were wounded with a pattern wheel and 20 µl *M. sexta* oral secretions (W+OS) were immediately applied to the wounds. Mean (± SE) levels of *NaCDPK4* and *NaCDPK5* transcripts in WT and IRcdpk5 plants were measured with qPCR in samples harvested before (as untreated control) and 1 h after W+OS treatment. Asterisks represent significantly different transcript levels between wild-type (WT) and IRcdpk5 plants (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5). (B) Morphology of WT, IRcdpk4, and IRcdpk5 rosette plants. Photos were taken 30 days after plants were germinated.

Two line of IRcdpk5 plants (line IRcdpk5-1 and IRcdpk5-2) showed highly reduced *NaCDPK5* transcripts before (48.4% and 31.9% of those in WT plants, respectively) and 1 h after W+OS treatment (14.3% and 24.5% of those in WT plants, respectively). However, the transcript levels of *NaCDPK4* in IRcdpk5 plants were only 30% and 40% of those in WT

plants before and 1 h after W+OS treatment, respectively (Figure 4A). These data suggest that RNAi using N-terminal region of *NaCDPK4* can specifically silence transcript levels of *NaCDPK4* but not *NaCDPK5*; in contrast, using the N-terminal region of *NaCDPK5* for RNAi resulted in co-silencing transcripts of *NaCDPK4* and *NaCDPK5*.

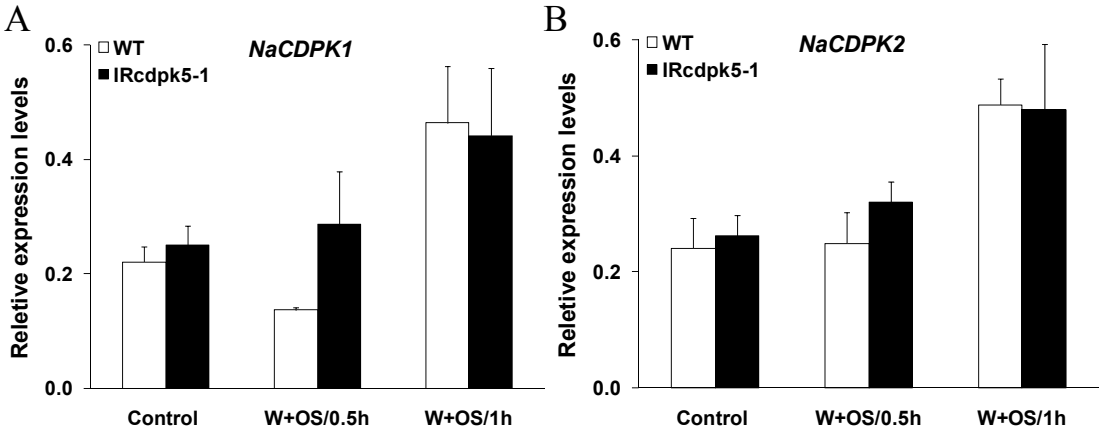


Figure 5 Transcript accumulation of *NaCDPK1* and *NaCDPK2* in IRcdpk5 plants.

Leaves of IRcdpk5-1 plants were wounded with a pattern wheel and 20 µl of *M. sexta* oral secretions (W+OS) were immediately applied to the wounds. Samples were harvested before (untreated control), 0.5 h, and 1 h after W+OS treatment, and the transcript levels (± SE) of *NaCDPK1* and *NaCDPK2*, were analyzed by quantitative real-time PCR. Asterisks represent significantly different transcript levels between W+W- and W+OS-treated samples (unpaired *t*-test; **, $p < 0.01$; ***, $p < 0.001$; N = 5)

To further examine the specificity of gene silencing, transcript levels of other known *CDPKs* (*NaCDPK1* and *NaCDPK2*) were quantified by qPCR in IRcdpk5 plants (Romeis et al., 2001; Lee et al., 2003; Ludwig et al., 2005; Ishida et al., 2008; Witte et al., 2010). *NaCDPK1* and *NaCDPK2* transcript levels in IRcdpk5 plants were not significantly different from those in WT in all conditions examined (Figure 5), which confirm the IRcdpk5 construct specifically silencing two close homologue (*NaCDPK4* and *NaCDPK5*), but not other family members (*NaCDPK1*, and *NaCDPK2*) in *N. attenuata*.

Compared with WT plants, IRcdpk4 plants are indistinguishable from WT plants. In contrast, IRcdpk5 plants have moderately curly and reduced size of rosette leaves (Figure 4B). In addition, during the elongation stage, the IRcdpk5 plants showed a dwarf stature (picture not shown), due to the co-silencing of *NaCDPK4* and *NaCDPK5*.

IRcdpk5 plants have elevated W+W- and W+OS-induced accumulation of JA, JA-Ile, and SA

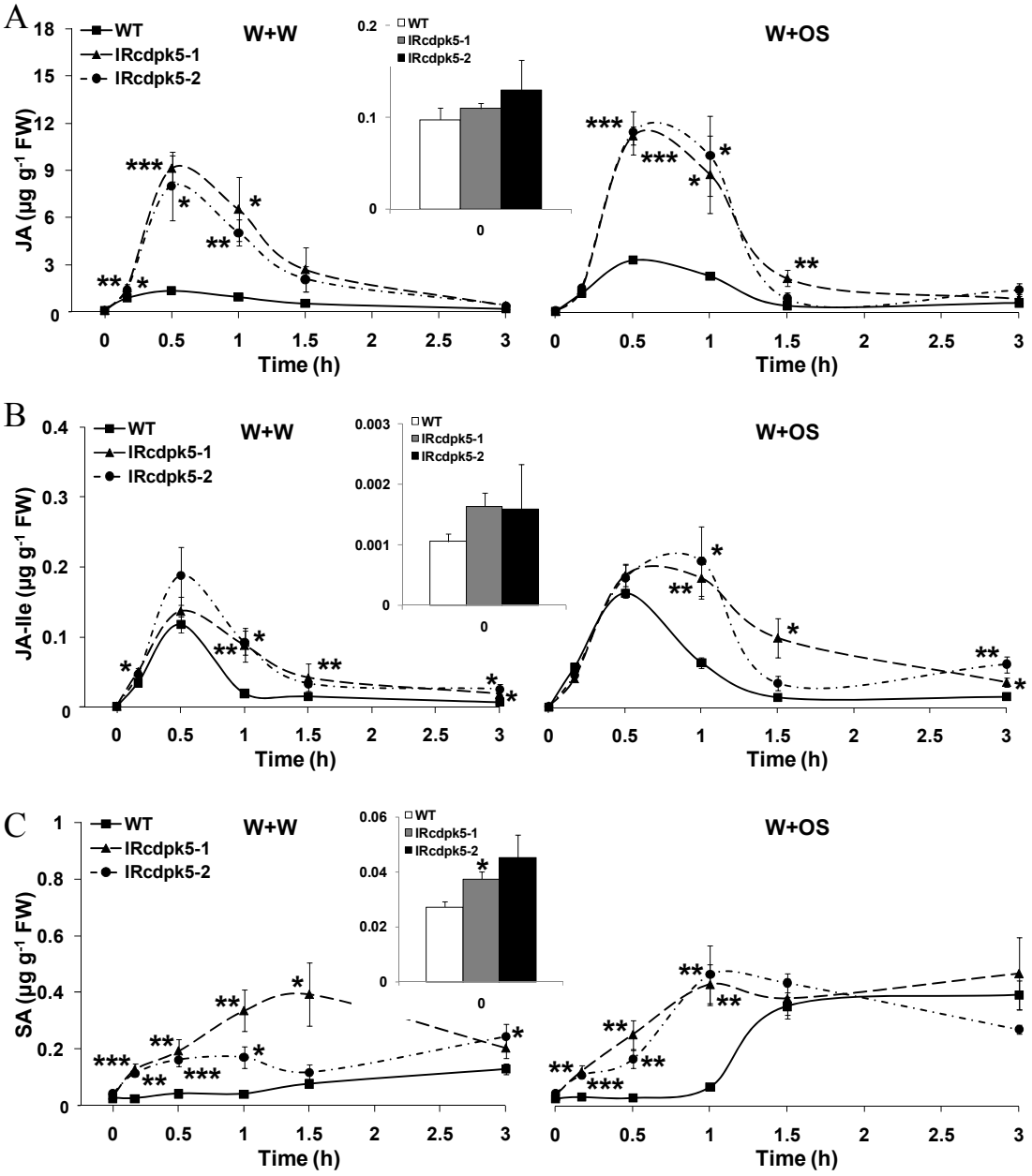
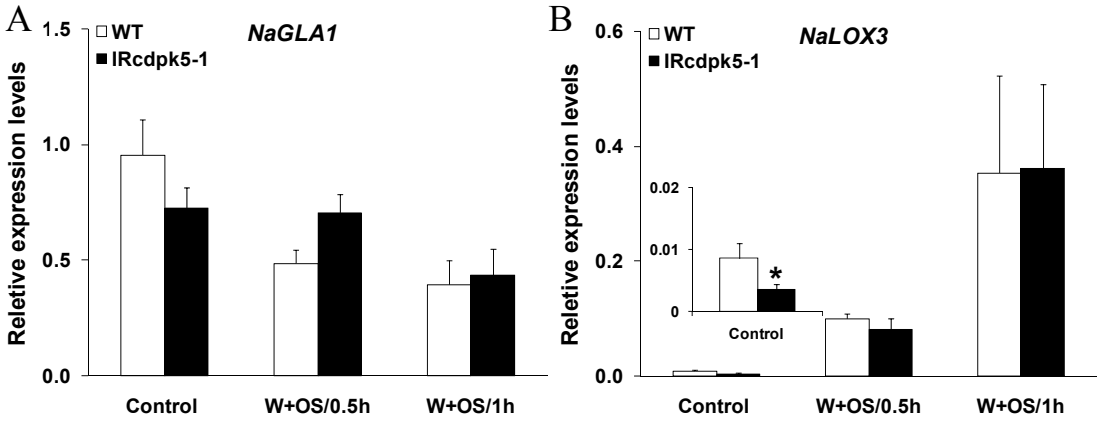


Figure 6. IRcdpk5 plants have enhanced W+W- and W+OS-induced accumulation of JA, JA-Ile, and SA.

Leaves of wild-type (WT) and IRcdpk5 plants were wounded with a pattern wheel and 20 µl of water (W+W) or *M. sexta* oral secretions (W+OS) was immediately applied to the wounds. Individual leaves from 5 replicate plants were harvested at the indicated times. Mean (± SE) concentrations of JA (A), JA-Ile (B), and SA (C) in WT and IRcdpk5 plants. Asterisks indicate significant differences between WT and IRcdpk5 plants (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5).

To examine whether NaCDPK4 and NaCDPK5 regulates wounding- and herbivory-induced accumulation of JA and JA-Ile, IRcdpk5 and WT plants were elicited with W+W and W+OS and the JA and JA-Ile levels were quantified by LC-MS/MS at different times. W+W induced up to 1.37 µg/g JA and 0.5 h after W+OS treatment greater amount of JA (3.26 µg/g) was detected in WT plants, indicating that *N. attenuata* recognizes herbivory and deploy elevated level of defense. Remarkably, 0.5 h after W+W and W+OS treatment, IRcdpk5 plants had 8.01-9.11 µg/g and 10.97-11.16 µg/g JA, respectively (Figure 6A), suggesting that NaCDPK4 and NaCDPK5 negatively regulate the accumulation of wounding- and herbivory-induced JA in *N. attenuata*. Increased levels of JA-Ile were also detected in IRcdpk5 plants after W+W and W+OS treatment (Figure 6B). Notably, the increase of induced JA-Ile levels was considerably lower than that of elicited JA in IRcdpk5 plants. It is possible that reduced JAR activity and/or limited availability of Ile impaired the accumulation of JA-Ile in IRcdpk5 plants.

It is well known that SA antagonized JA accumulation (Doares et al., 1995). Thus, the SA levels were measured in IRcdpk5 and WT plants after W+W or W+OS treatment. Elevated SA contents were found after both W+W and W+OS treatment in IRcdpk5 plants compared with those in WT plants (Figure 6C). This indicates that NaCDPK4 and NaCDPK5 also negatively regulate wounding- and herbivory- induced SA levels. Herbivory strongly elevates biosynthesis and emission of ethylene, which is correlated with enhanced defense levels in *N. attenuata* plants (von Dahl et al., 2007). We found that IRcdpk5 plants emitted the same amount of herbivory-elicited ethylene as WT plants (data not shown). Therefore NaCDPK4 and NaCDPK5 are not involved in regulating herbivory-elicited ethylene production in *N. attenuata*.



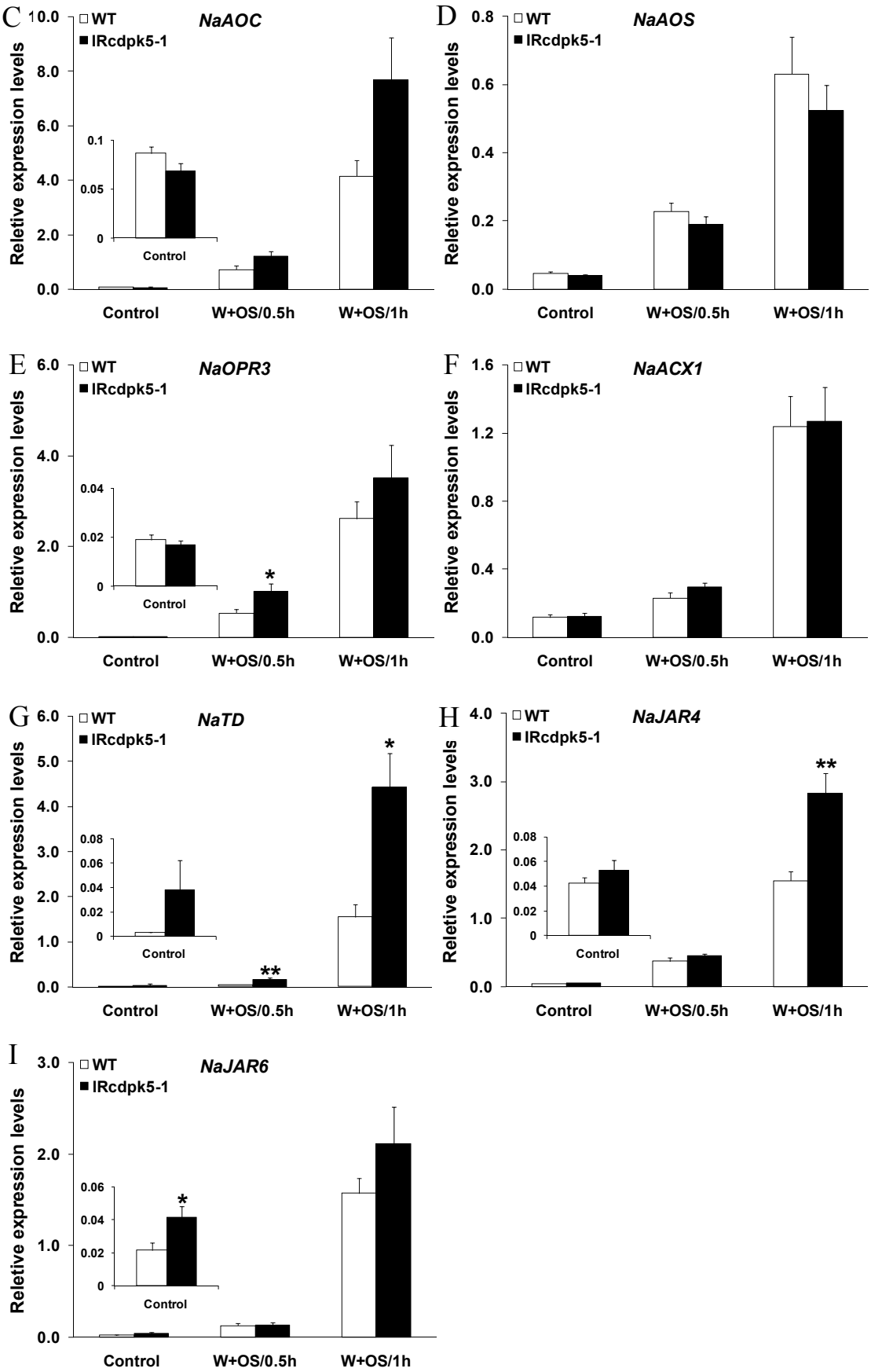


Figure 7 Transcript levels of JA and JA-Ile biosynthetic genes in WT and IRcdpk5 plants.

N. attenuata leaves were wounded with a pattern wheel and 20 µl of *M. sexta* oral secretions (W+OS) were immediately applied to the wounds. Samples were harvested before (untreated control), 0.5 h, and 1 h after W+OS treatment. Transcript levels (± SE) of *NaGLA1* (A), *NaLOX3* (B), *NaAOS* (C), *NaAOC* (D), *NaOPR3* (E), *NaACX1* (F), *NaTD* (G), *NaJAR4* (H), and *NaJAR6* (I) were analyzed by quantitative real-time PCR in WT and IRcdpk5-1 plants. Asterisks represent significantly different transcript levels between W+W- and W+OS-treated samples (unpaired *t*-test; **, $p < 0.01$; ***, $p < 0.001$; N = 5)

To further examine whether the elevated W+W- and W+OS-induced JA accumulation in IRcdpk5 plants was associated with altered transcript levels of genes in the JA biosynthesis pathway, the transcript abundance of *NaGLA1*, *NaLOX3*, *NaAOS*, *NaAOC*, *NaOPR3*, and *NaACX1* was quantified by qPCR. Compared with those in WT, IRcdpk5-1 plants showed no significantly different expression levels of these genes 0.5 h and 1 h after W+OS treatment, except that minor difference were detected in *NaLOX3* (control) and *NaOPR3* (0.5 h after W+OS) (Figure 7A, B, C, D). Thus, the large difference of W+W- and W+OS-induced accumulation of JA between IRcdpk5 and WT plants probably results from modification of certain enzymes in the JA biosynthesis pathway at the post-transcriptional level, such as protein abundance and/or enzyme activity, rather than changes at transcript level.

Threonine deaminase (NaTD) plays an important role in converting Thr to Ile, which is used by JAR4 and JAR6 to conjugate Ile with JA to produce JA-Ile in *N. attenuata* (Kang et al., 2006; Wang et al., 2008). Quantification of these JA-Ile biosynthesis genes revealed increased transcript levels of *NaTD* (4.85 and 2.87 fold, 0.5 h and 1 h after W+OS, respectively) and *NaJAR4* (1.83 fold, 1 h after W+OS) and *NaJAR6* (1.92 fold, untreated control) in IRcdpk5-1 plants compared with WT plants (Figure 7G, H, I). This suggests that compared with the dramatically elevated JA levels, the less pronounced increase of JA-Ile in IRcdpk5 plants is not due to decreased *NaJAR4* and *NaJAR6* transcript levels, but probably results from attenuated abundance/enzymatic activity of JAR proteins or insufficient supply of Ile.

Taken together, NaCDPK4 and NaCDPK5 suppress the accumulation of wounding- and herbivory-induced JA and JA-Ile at the post-transcriptional level.

Ile availability, rather than JAR activity, limits the accumulation of JA-Ile in IRcdpk5 plants

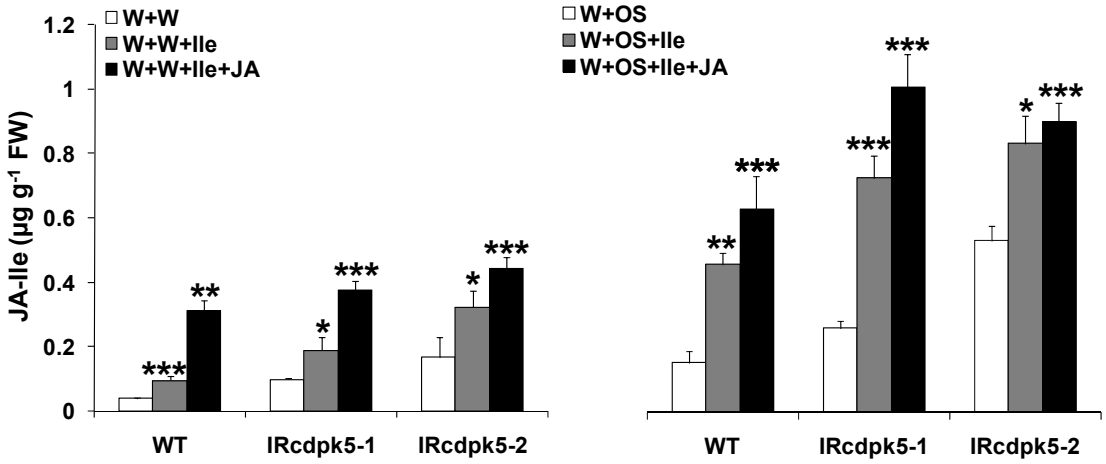


Figure 8 Ile availability, rather than JAR activity, limits the accumulation of JA-Ile in IRcdpk5 plants.

Leaves of WT and IRcdpk5 plants were wounded with a pattern wheel and treated with 20 µl of water or *M. sexta* oral secretions (OS) (W+W and W+OS, respectively) or an excess (0.625 nmol) of Ile (W+W+Ile and W+OS+Ile) or both Ile and JA (W+W+Ile+JA and W+OS+Ile+JA). Mean JA-Ile levels (± SE) were analyzed 1 h after plants were wounded or OS elicited and supplied with an excess of JA-Ile biosynthesis substrates. Asterisks represents significantly different levels between indicated treatments in WT and IRcdpk5 plants, respectively (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5).

In order to investigate whether impaired JAR activity and/or Ile availability account for the limitation for the JA-Ile levels in IRcdpk5 plants, the substrates for JA-Ile biosynthesis were supplied to the wounds of IRcdpk5 and WT plants during W+W and W+OS treatments, and the resulting JA-Ile levels were quantified (Paschold et al., 2008). The water and OS used in W+W and W+OS treatments were supplemented with either 0.625 µmol of Ile (W+W+Ile and W+OS+Ile) or both Ile and JA (W+W+Ile+JA and W+OS+Ile+JA). Compared with W+W and W+OS treatment, 1 h after application of W+W+Ile and W+OS+Ile, significantly increased JA-Ile levels were detected in IRcdpk5 and WT plants (2.44 and 3.0 fold, respectively), indicating that Ile availability is the limiting factor for JA-Ile accumulation when plants have relatively high (or sufficient) JA levels (Figure 8). Morever, elevated JA-Ile levels were detected when both Ile and JA were supplied (W+W+Ile+JA and W+OS+Ile+JA) in both IRcdpk5 and WT plants. Importantly, IRcdpk5 plants had similar or somewhat elevated JA-Ile levels compared with WT plants after applying Ile and JA together (Figure 8).

Apparently, IRcdpk5 and WT plants have similar JAR activity to synthesize wounding- and herbivory-induced JA-Ile, when substrates are sufficiently available.

Therefore, we infer that substrate (Ile) availability, rather than the enzymatic activity of JAR proteins, limits the accumulation of JA-Ile observed in IRcdpk5 plants.

IRcdpk5 plants have increased levels of W+OS-induced defensive compounds and resistance to the specialist herbivore *M. sexta*

In *N. attenuata*, herbivory attack or simulated herbivory (W+OS treatment) enhances the levels of diterpene glucosides (DTGs), trypsin proteinase inhibitors (TPIs), and caffeoylputrescine (CP), which are important plant secondary metabolites serve as direct defense against the specialist herbivore *M. sexta* (Zavala et al., 2004; Heiling et al., 2010; Kaur et al., 2010). Therefore, we examined whether elevated herbivory-induced JA and JA-Ile levels led to increased anti-herbivory defensive secondary metabolites (DTGs, CP, and TPIs) levels in IRcdpk5 plants.

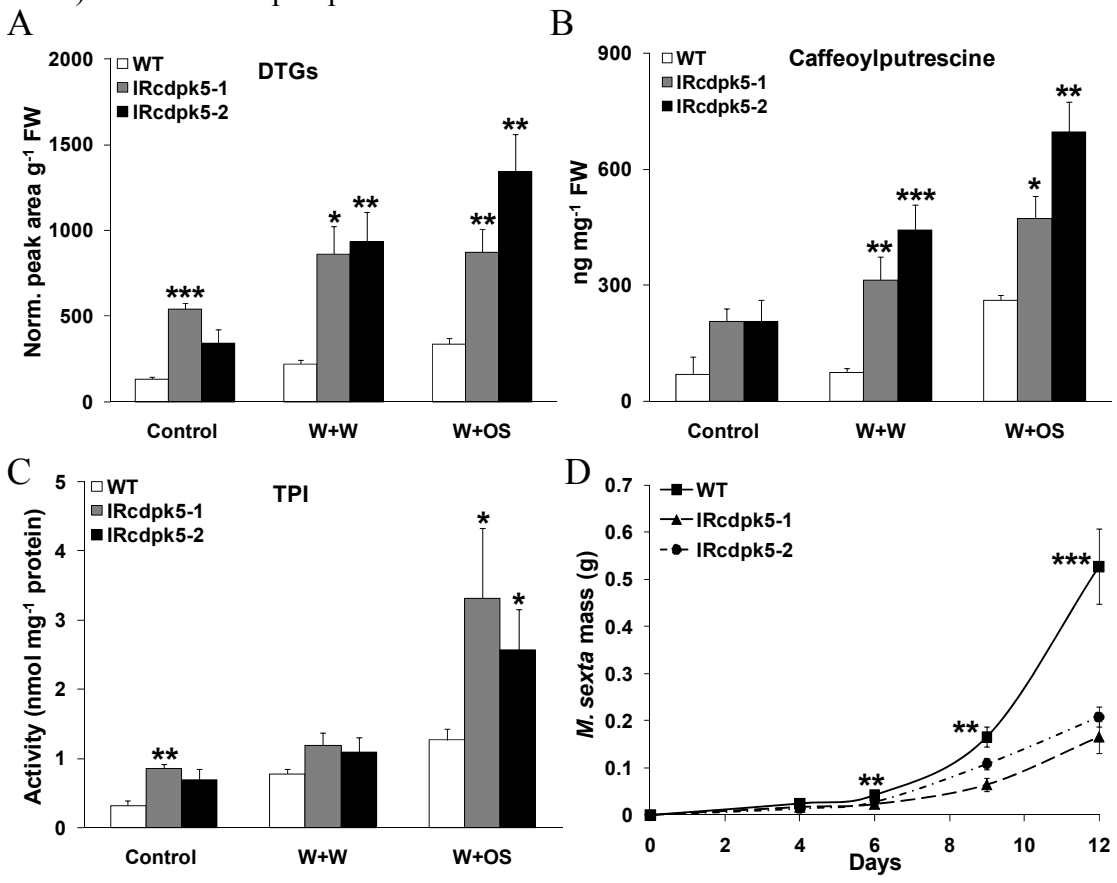


Figure. 9 Silencing *NaCDPK4* and *NaCDPK5* elevates the levels of W+W- and W+OS-induced defensive comopounds and *N. attenuata*'s resistance to the specialist herbivore *M. sexta*

Leaves of WT and IRcdpk5 plants were left untreated (control) or wounded with a pattern wheel and 20 µl of *M. sexta* oral secretions or water was immediately applied to the wounds (W+OS and W+W,

respectively). Samples were harvested 3 days after treatment, and mean (± SE) of DTG contents (A) CP concentrations (B), and TPI activity (C), were determined. (D) *M. sexta* gain less mass on IRcdpk5 plants than on WT plants. One neonate *M. sexta* larvae was placed on a fully developed leaf of each plant, and the larval mass was recorded over 12 days (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 25).

When untreated, IRcdpk5 and WT plants had similar JA and JA-Ile levels (Figure 6A, B); however, compared with those in WT plants, IRcdpk5-1 plants had increased DTG and CP levels (Figure 9A, B). After both W+W and W+OS treatments, highly elevated DTG and CP levels were also detected in IRcdpk5 plants, which is consistent with the enhanced accumulation of wounding- and herbivory-induced JA and JA-Ile in IRcdpk5 plants. Compared with WT plants, IRcdpk5-1 plants had about 165% increased basal TPI activity, and W+OS induced 1 fold higher TPI activity in both IRcdpk5 lines (Figure 9C). However, W+W treatment did not induced higher TPI activity in IRcdpk5 plants than in WT plants, which might due to the quantitative difference of W+W-induced JA-Ile between IRcdpk5 and WT plants is not as pronounce as those elicited by W+OS (Figure 6B). These results suggest that silencing *NaCDPK4* and *NaCDPK5* results in elevated herbivory-induced compounds for direct defense, and this is most likely due to increased herbivory-elicited JA and JA-Ile levels in IRcdpk5 plants.

Bioassays were performed to determine whether silencing Na*CDPK4* and Na*CDPK5* altered the performance of the specialist herbivore *M. sexta* on *N. attenuata* plants. Consistent with the enhanced accumulation of W+OS-induced defensive secondary metabolites in IRcdpk5 plants, *M. sexta* larvae gained about 60% less masses on IRcdpk5 plants than on WT plants after feeding for 12 days (Figure 9D).

Thus, NaCDPK4 and NaCDPK5 negatively control the accumulation of W+OS-induced anti-herbivore secondary metabolites (DTGs, CP, and TPIs) and thus plant resistance to specialist herbivore *M. sexta*.

Highly elevated defense levels in IRcdpk5 plants are totally depend on JA and JA signaling

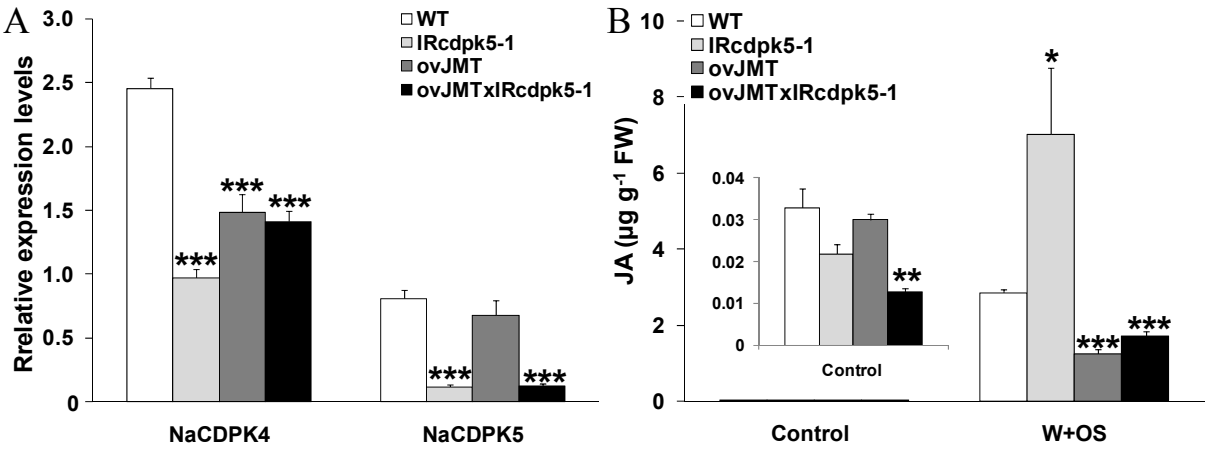


Figure 10 Overexpression of *jasmonic acid carboxyl methyltransferase* gene in WT (ovJMT) and IRcdpk5-1 (ovJMTxIRcdpk5-1) plants result in impaired W+OS-induced JA levels.

N. attenuata leaves were wounded with a pattern wheel and 20 µl of *M. sexta* oral secretions (W+OS) were immediately applied to the wounds. Samples were harvested before (untreated control) and 1 h after W+OS treatment. The 1 h W+OS-induced transcript levels (± SE) of *NaCDPK4* and *NaCDPK5* were analyzed by quantitative real-time PCR (A), and Mean (± SE) concentrations of JA before and 1 h after W+OS treatment were quantified by LC-MS/MS (B) in WT, IRcdpk5-1, ovJMT, and ovJMTxIRcdpk5-1 plants. Asterisks represent significantly different transcript levels between WT and IRcdpk5-1 or ovJMT or ovJMTxIRcdpk5-1 plants (unpaired *t*-test; **, $p < 0.01$; ***, $p < 0.001$; N = 5)

In *N. attenuata*, JA and its signaling play a central role in plant resistance to herbivores (Halitschke and Baldwin, 2003; Paschold et al., 2007). We further tested whether the elevated defense levels of IRcdpk5 plants against *M. sexta* was solely dependent on the overproduction of JA but not other JA-independent pathways, IRcdpk5 plants were crossed with a *N. attenuata* line overexpressing Arabidopsis *S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase* (ovJMT plants) to obtain ovJMTxIRcdpk5-1 plants. The ovJMT plants are able to methylate JA to form methyl jasmonic acid (MeJA) to reduce elicited JA levels (Stitz and Baldwin, unpublished data). In order to confirm successful crossing, relative transcript abundance of *NaCDPK4* and *NaCDPK5* were quantified by qPCR in WT, IRcdpk5-1, ovJMT and ovJMTxIRcdpk5-1 plants 1 h after W+OS treatment. Compares with WT plants, both IRcdpk5-1 and ovJMTxIRcdpk5-1 plants have highly diminished transcript levels of *NaCDPK4* and *NaCDPK5* after elicitation. In addition, ovJMT plants have significantly

reduced *NaCDPK4* transcript levels but not those of *NaCDPK5*, suggesting that transcript levels of *NaCDPK4* were regulated by elicited JA levels (Figure 10A).

Analysis of JA levels before and 1 h after W+OS treatment in these plants revealed that ovJMT and ovJMTxIRcdpk5-1 plants had strongly reduced W+OS-induced JA levels compared with WT plants. However, without treatments, JA levels were comparable in WT, ovJMT, and IRcdpk5-1 plants, except ovJMTxIRcdpk5-1 plants showed somewhat reduced JA contents (Figure 10B). These data confirm that ovJMTxIRcdpk5-1 plants indeed had reduced *NaCDPK4* and *NaCDPK5* transcript levels and had impaired accumulation of JA after W+OS elicitation.

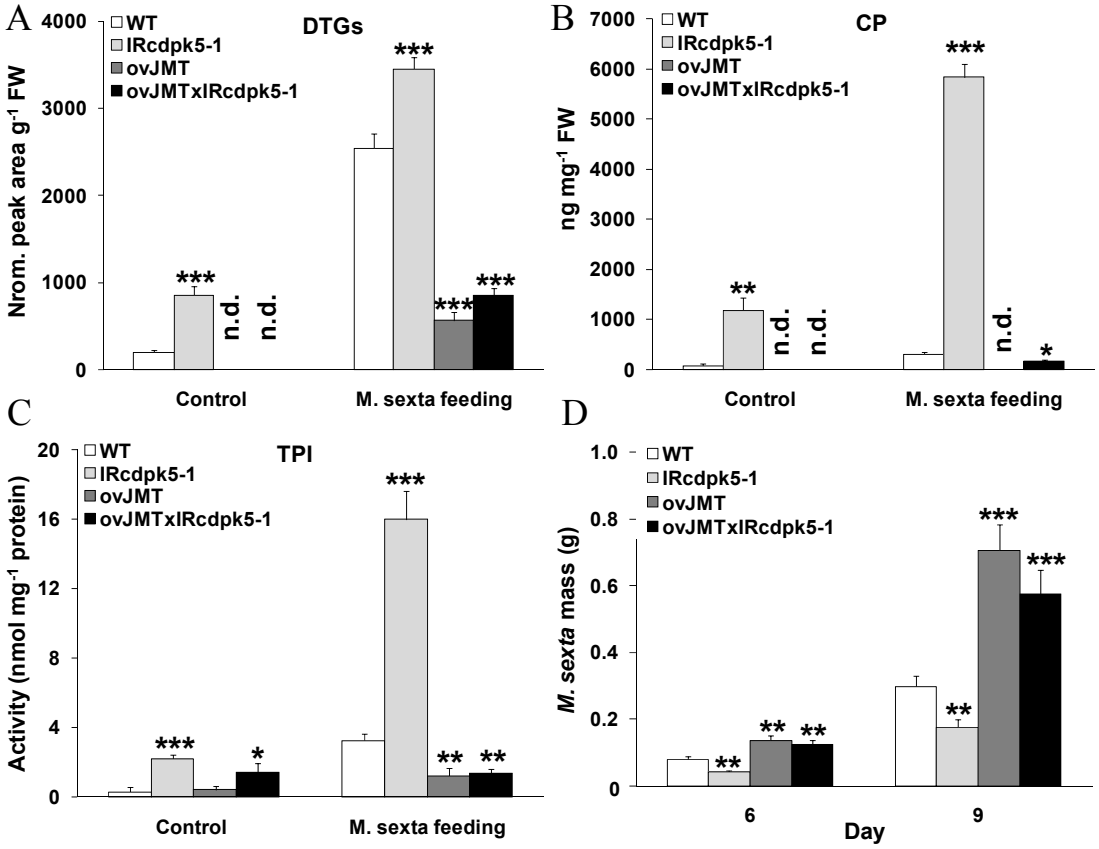


Figure 11 Depleting the accumulation of JA in IRcdpk5 plants by ectopic expression of *JMT* gene completely abolishes over-production of defensive secondary metabolites and defense against *M. sexta*

M. sexta neonates were placed on wild type (WT), IRcdpk5-1, ovJMT, and ovJMTxIRcdpk5-1 plants, and feeding were continued for 9 days. Damaged leaf tissue was harvested on day 9 and the contents (± SE) of DTGs (A), CP (B), and activity of TPIs (C) were quantified in 5 replicated plants. (D) The mass of *M. sexta* on WT, IRcdpk5-1, ovJMT, and ovJMTxIRcdpk5-1 plants (N = 20). Asterisks indicate significant differences between WT and IRcdpk5-1 or ovJMT or ovJMTxIRcdpk5-1 plants (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Compounds for direct defenses (DTGs, CP, and TPIs) were further quantified 9 days after *M. sexta* larvae feeding in WT, IRcdpk5-1, ovJMT and ovJMTxIRcdpk5-1 plants; non-infested plants were harvested as controls. In controls plants, the contents of DTGs, CP, and activity of TPIs were higher in IRcdpk5-1 plants than in WT plants. *M. sexta* feeding increased the levels of these defensive compounds substantially in WT and IRcdpk5 plants, and greater levels of these compounds were detected in IRcdpk5-1 plants than in WT plants (Figure 11A, B, C). Importantly, ovJMT and ovJMTxIRcdpk5-1 plants showed dramatically diminished accumulation of DTGs, CP, and TPIs (Figure 11A, B, C). These results confirm that these anti-herbivore secondary metabolites are mainly regulated by JA.

Consistent with the levels of herbivory-induced secondary metabolites in these plants, compared with those on WT plants, *M. sexta* larvae gained around 100% more mass on ovJMT and ovJMTxIRcdpk5-1 plants, and 100% less mass on IRcdpk5-1 plants after feeding for 6 or 9 days (Figure 11D). Therefore, diminishing the accumulation of herbivory-induced JA levels in IRcdpk5 plants completely abolished the highly elevated defense levels in these plants.

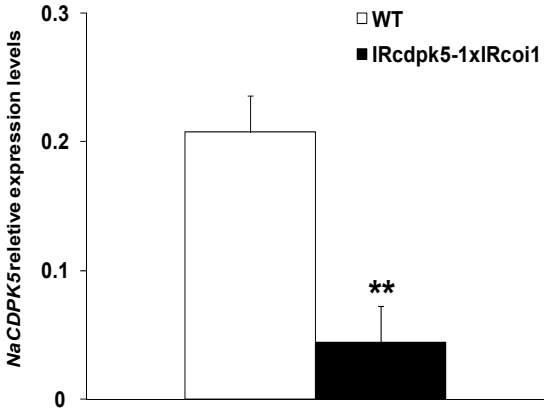


Figure 12 Transcript levels of *NaCDPK5* in WT plants and IRcdpk5-1 and IRcoi1 crossed (IRcdpk5-1xIRcoi1) plants.

Transcript levels of *NaCDPK5* were analyzed in untreated wild-type (WT) plants and IRcdpk5-1and IRcoi1crossed (IRcdpk5-1xIRcoi1) plants by qPCR.

To further confirm that JA signaling is critical for the enhanced defense of IRcdpk5 plants, next we performed *M. sexta* bioassays on IRcdpk5 plants crossed with a *N. attenuata* line silenced in *COI1* transcripts (IRcoi1 plants) (Paschold et al., 2007). The successful crossing of IRcdpk5-1xIRcoi1 plants was confirmed by quantification of transcript levels of

NaCDPK5 in untreated WT and IRcdpk5-1xIRcoi1 plants: compared with WT plants, IRcdpk5-1xIRcoi1 plants had significantly reduced *NaCDPK5* transcripts (Figure 12).

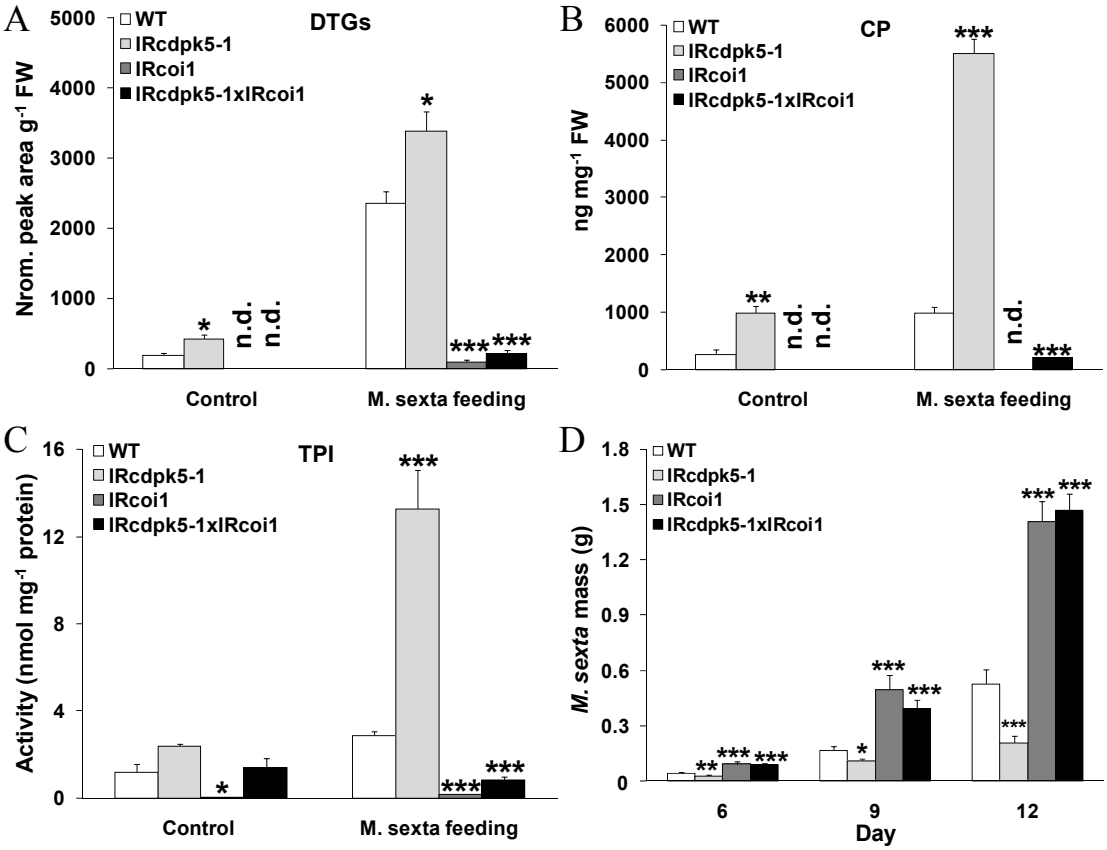


Figure 13 JA signaling is required for IRcdpk5 plants to over-produce defensive secondary metabolites and defense against *M. sexta*

M. sexta neonates were placed on wild type (WT), IRcdpk5-1, IRcoi1, and ovJMTxIRcoi1 plants, and feeding were continued for 12 days. Damaged leaf tissue was harvested on day 12 and the contents (± SE) of DTGs (A), CP (B), and activity of TPIs (C) were quantified in 5 replicated plants. (D) The mass of *M. sexta* on WT, IRcdpk5-1, IRcoi1, and IRcdpk5-1xIRcoi1 plants (N = 20). Asterisks indicate significant differences between WT and IRcdpk5-1 or IRcoi1 or IRcdpk5-1xIRcoi1 plants (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Insect feeding-elicited anti-herbivore secondary metabolites and mass gain of *M. sexta* larvae were measured in WT, IRcdpk5-1, IRcoi1, and IRcdpk5-1xIRcoi1 plants. Compared with WT plants, IRcoi1 and IRcdpk5-1xIRcoi1 plants had greatly reduced secondary metabolites (DTGs, CP, and TPIs) and highly compromised resistance to *M. sexta* feeding, while IRcdpk5-1 plants displayed markedly high levels of these secondary metabolites and resistance to the insects (Figure 13).

All together, the highly enhanced defense levels of IRcdpk5 plants against *M. sexta* are completely dependent on the elevated levels of JA and JA signaling.

Specifically silencing Na*CDPK4* but not Na*CDPK5* does not elevate *N. attenuata*'s resistance to *M. sexta*

Next we examined whether specifically silencing *NaCDPK4* in *N. attenuata* plants results in similar phenotypes observed in IRcdpk5 plants.

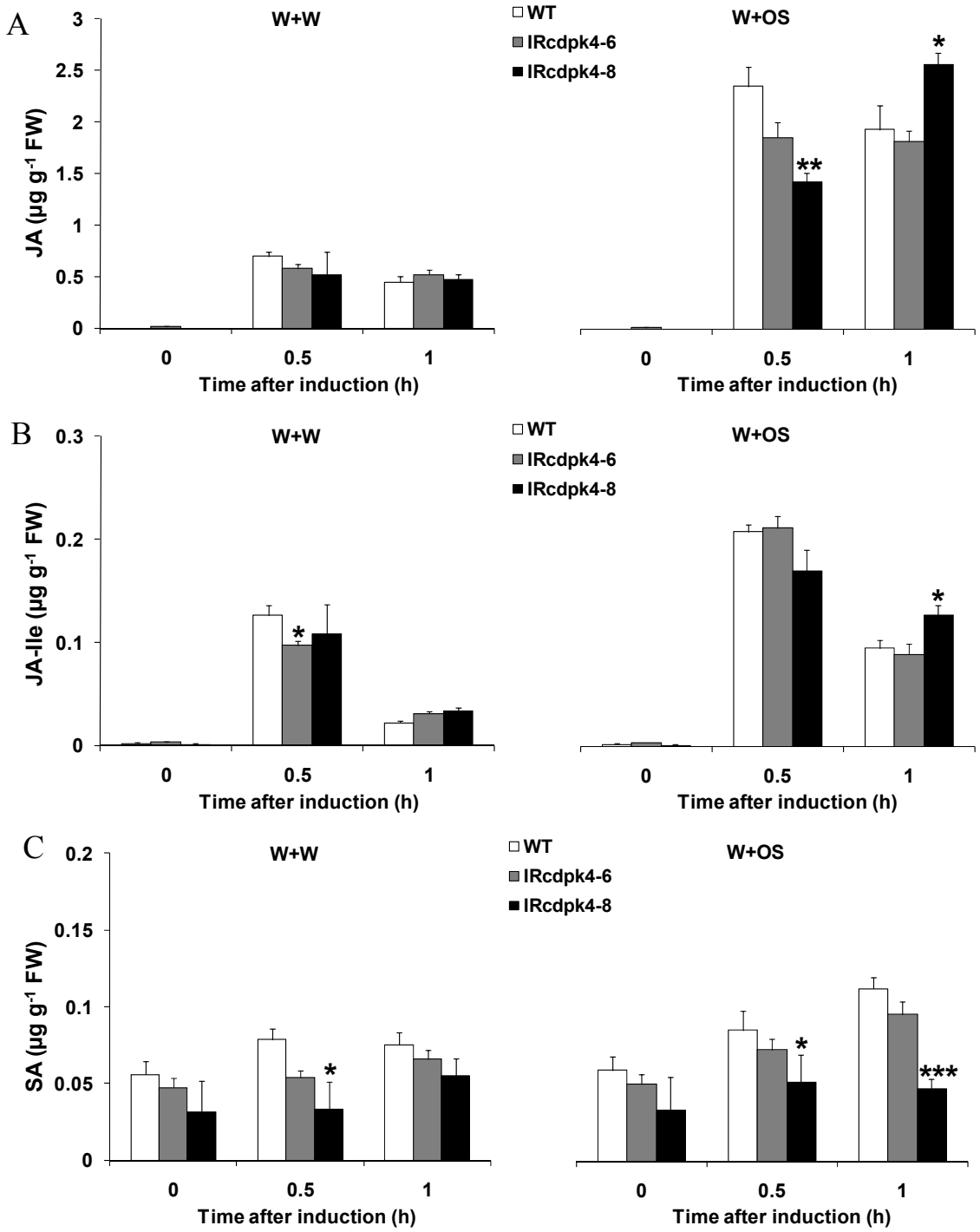


Figure 14 IRcdpk4 plants have similar W+W- and W+OS-induced accumulation of JA, JA-Ile, and SA as those in WT plants.

Leaves of wild-type (WT) and IRcdpk4 plants were wounded with a pattern wheel and 20 µl of water (W+W) or *M. sexta* oral secretions (W+OS) was immediately applied to the wounds. Individual leaves

from 5 replicate plants were harvested at the indicated times. Mean (± SE) concentrations of JA (A), JA-Ile (B), and SA (C) in WT and IRcdpk4 plants. Asterisks indicate significant differences between WT and IRcdpk4 plants (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5).

W+W- and W+OS-induced JA, JA-Ile, and defense-related secondary metabolites (DTGs, CP, and TPIs), and masses gain of *M. sexta* larvae were determined in WT and IRcdpk4 plants, which had greatly abolished transcript abundance of *NaCDPK4* but similar abundance of *NaCDPK5* (Figure 3). Compared with WT, two IRcdpk4 lines only showed minor difference in JA, JA-Ile and SA levels at several time points after W+W and W+OS treatment (Figure 14).

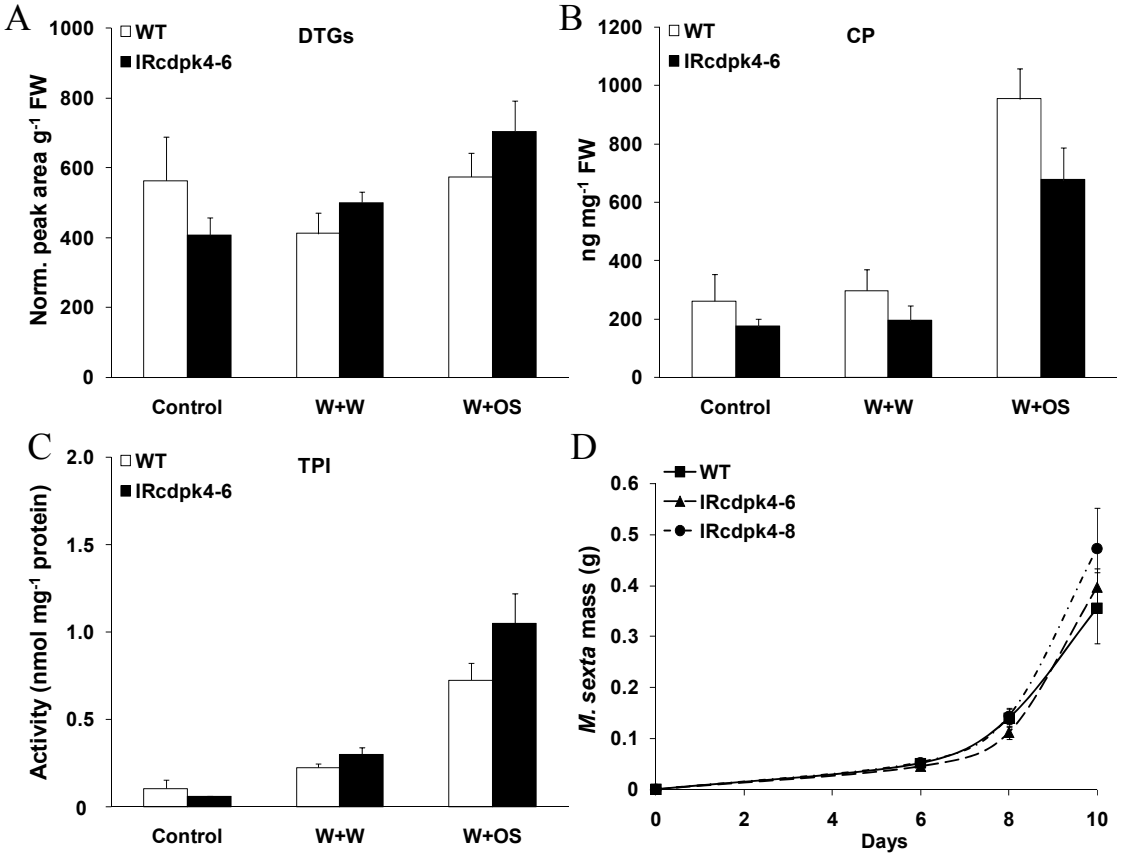


Figure 15 Specifically silencing *NaCDPK4* does not elevate the levels of W+W- and W+OS-induced defensive comopounds and *N. attenuata*'s resistance to the specialist herbivore *M. sexta*

Leaves of WT and IRcdpk4-6 plants were left untreated (control) or wounded with a pattern wheel and 20 µl of water or *M. sexta* oral secretions was immediately applied to the wounds (W+W and W+OS, respectively). Samples were harvested 3 days after treatment, and mean (± SE) of DTG contents (A) CP concentrations (B), and TPI activity (C), were determined. (D) *M. sexta* gain less mass on IRcdpk4-6 plants than on WT plants. One neonate *M. sexta* larvae was placed on a fully developed

leaf of each plant, and the larval mass was recorded over 10 days (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 25).

Furthermore, quantification of W+W- and W+OS-induced secondary metabolites revealed no differences of DTGs, CP, and TPIs in WT and IRcdpk4 plants after the elicitation (Figure 15A, B, C). Accordingly, *M. sexta* larvae gained similar masses on WT and IRcdpk4 plants (Figure 15D).

All together, specific silencing *NaCDPK4* (but not *NaCDPK5*) did not result in the phenotypes observed in IRcdpk5 plants with *NaCDPK4* and *NaCDPK5* co-silenced, suggesting that silencing *NaCDPK4* itself does not alter herbivory-induced JA, JA-Ile, SA, anti-herbivory secondary metabolites, and resistance to *M. sexta* larvae.

Silencing *NaCDPK4* and *NaCDPK5* enhances *N. attenuata*'s resistance to *Pseudomonas syringae* pv. *tomato* DC3000

Pst DC3000 is a virulent pathogen, which causes necrosis but not hypersensitive response (HR) in *N. attenuata* (Rayapuram et al., 2008). To investigate NaCDPK4 and NaCDPK5's functions in *N. attenuata*'s resistance to *Pst* Dc3000, we inoculated *Pst* DC3000 into WT and IRcdpk5 leaves, and measured the growth of pathogen.

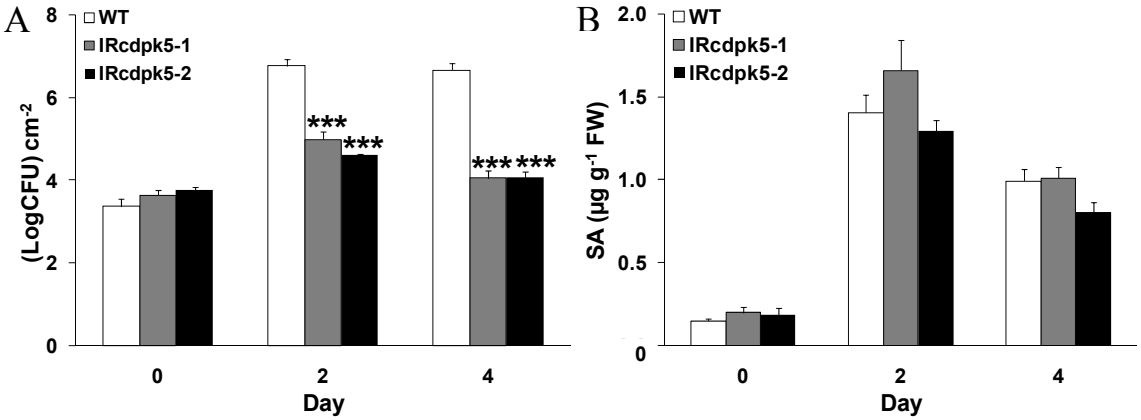


Figure 16. IRcdpk5 plants have increased resistance levels to *Pst* DC3000

(A) Basal leaves of early elongated wild type (WT) and IRcdpk5 plants were inoculated with *Pst* DC3000 (OD_{600} = 0.001), and the population of *Pst* DC3000 were determined over 4 days (N = 10). (B) SA contents in WT and IRcdpk5 plants after inoculation of *Pst* DC3000 (N = 5). Asterisks indicate significant differences between WT and IRcdpk5 plants (unpaired *t*-test; ***, $p < 0.001$)

Compared with WT plants, IRcdpk5 plants exhibited greatly impaired amplification of *Pst* DC3000 2 and 4 days after inoculation (Figure 16A). Give the important role of SA in plant-pathogen interactions (Vernooij et al., 1994), we determined the levels of SA 2 and 4

days after pathogen infiltration. Similar SA levels were found in WT and IRcdpk5 plants (Figure 16B), suggesting that the elevated resistance to *Pst* DC3000 of IRcdpk5 plants was not due to higher levels of SA.

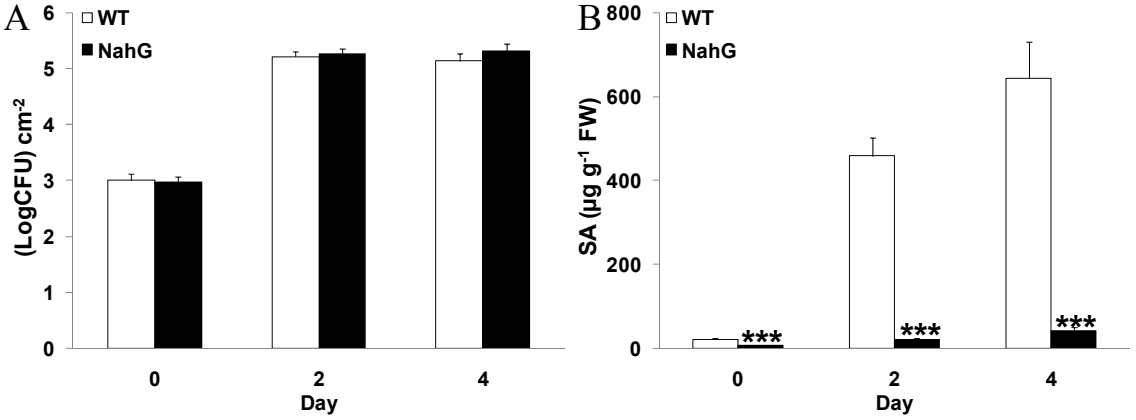


Figure 17 Overexpressing *NahG* gene in wild-type plants effectively abolishes the accumulation of SA after *Pseudomonas syringae* pv *tomato* DC3000 infection.

(A) Basal leaves of early elongated wild type (WT) and *NahG*-overexpressing (NahG) plants were inoculated with *Pst* DC3000 (OD_{600} = 0.001), and the population of *Pst* DC3000 were determined over 4 days (N = 10). (B) SA contents in WT and NahG plants after inoculation of *Pst* DC3000 (N = 5). Asterisks indicate significant differences between WT and NahG plants (unpaired *t*-test; ***, $p < 0.001$)

To further test whether *N. attenuata*'s resistance against *Pst* DC3000 depends on pathogen-elicited SA levels, we generated plants transformed with a bacterial *salicylic acid hydroxylase* gene (NahG) driven by the 35S promoter (NahG plants), which effectively abolished pathogen-induced accumulation of SA in NahG plants (Figure 17A). However, *Pst* DC3000 amplified to similar level in WT and NahG plants, confirming that resistance of *N. attenuata* to *Pst* DC3000 is independent of pathogen-induced SA levels (Figure 17B).

Specifically silencing *NaCDPK4* but not *NaCDPK5* does not elevate *N. attenuata*'s resistance to *Pst* DC3000

Co-silencing *NaCDPK4* and *NaCDPK5* resulted in elevated pathogen resistance in *N. attenuata*. Whether specifically silencing *NaCDPK4* leads to enhanced *Pst* DC3000 resistance was further examined in IRcdpk4 plants.

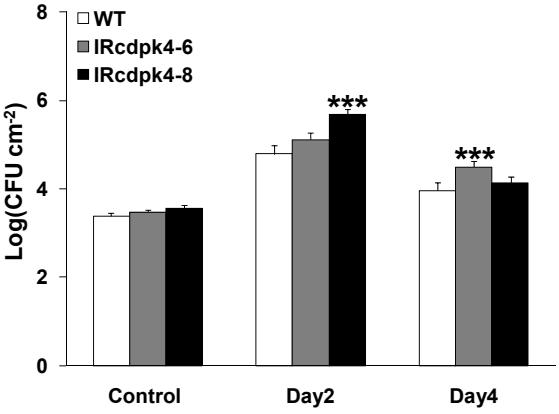


Figure 18 IRcdpk4 plants do not have increased resistance levels to *Pst* DC3000
(A) Basal leaves of early elongated wild type (WT) and IRcdpk4 plants were inoculated with *Pst* DC3000 (OD_{600} = 0.001), and the population of *Pst* DC3000 were determined over 4 days (N = 10). (B) SA contents in WT and IRcdpk4 plants after inoculation of *Pst* DC3000 (N = 5). Asterisks indicate significant differences between WT and IRcdpk4 plants (unpaired *t*-test; ***, $p < 0.001$)

Compared with WT plants, amplification of *Pst* DC3000 was similar or somewhat elevated (2 days after inoculation in IRcdpk4-8 plants and 4 days after infiltration in IRcdpk4-6 plants) in IRcdpk4 plants (Figure 18), suggest that silencing Na*CDPK4* does not enhance resistance to *Pst* DC3000 in *N. attenuata*. Thus, we infer that either silencing *NaCDPK5* alone or co-silencing both *NaCDPK4* and *NaCDPK5* results in elevated resistance to *Pst* DC3000 in *N. attenuata*.

Discussion

In *N. benthamiana*, *N. tabacum*, and Arabidopsis, CDPKs are required for plant innate immunity to pathogens (Romeis et al., 2000; Romeis et al., 2001; Ludwig et al., 2005; Boudsocq et al., 2010; Coca and San Segundo, 2010). Using a reverse genetic approach, here we show that *N. attenuata* NaCDPK4 and NaCDPK5 negatively regulate plant's resistance to both herbivore *M. sexta* and pathogen *Pst* DC3000. Silencing *NaCDPK4* and *NaCDPK5* resulted in elevated herbivory-induced accumulation of JA, JA-Ile, and anti-herbivore secondary metabolites. Moreover, NaCDPK4 and NaCDPK5 regulate herbivore resistance in a JA- and JA signaling (COI1)-dependent manner. Our results suggest that NaCDPK4 and NaCDPK5 belong to the early signaling networks that regulate *N. attenuata*'s defense responses to herbivores. Furthermore, silencing *NaCDPK4* and *NaCDPK5* in *N. attenuata* highly elevates plant resistance to *Pst* DC3000 in a SA-independent manner.

NaCDPK4 and NaCDPK5 negatively regulate herbivory-induced JA and JA-Ile levels

Ectopic expression of N-terminal *N. tabacum* CDPK2 lacking its autoinhibitory and calcium-binding domains (VK variants) in *N. bethamiana* resulted in elevated JA and OPDA levels, and impaired activation of salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) induced by osmotic stress (Ludwig et al., 2005). Furthermore, after Avr9 infiltration, compromised MAPK activity was observed in VK variants-expressed leaves compared with MAPK activity in full-length CDPK2 (VKJC variants)-expressed leaves. Importantly, expression of kinase-inactive VK $_{(D/A)}$ variants resulted in enhanced MAPK activity than those in leaves expressing full length VKJC variants in the Avr9/Cf-9 interaction (Ludwig et al., 2005). Thus it was proposed that CDPKs negatively regulate MAPK activity in plant responses to abiotic (osmotic) and biotic (Avr9) stresses.

In *N. attenuata* two MAPK, SIPK and WIPK, positively modulate wounding- and herbivory-induced accumulation of JA, JA-Ile and herbivory-elicited TPI levels (Wu et al., 2007). Given that silencing *NaCDPK4* and *NaCDPK5* resulted in elevated accumulation of herbivory-elicited JA, JA-Ile, and jasmonate-induced anti-herbivore secondary metabolites, it is possible that NaCDPK4 and NaCDPK5 negatively mediate MAPK activity, which triggers the accumulation of herbivory-induced JA and JA-Ile in *N. attenuata*. Recently, Ishida *et al.* showed that NtCDPK1 phosphorylated REPRESSION OF SHOOT GROWTH (RSG), a *N. tabaccum* transcriptional activator of gibberellin (GA) biosynthetic enzymes, in response to GAs. Phosphorylation of Ser-114 of RSG by NtCDPK1 promotes 14-3-3 protein binding to RSG, which sequesters RSG in the cytoplasm to maintain the GAs homeostasis in plants (Ishida et al., 2008). Therefore, possibly NaCDPK4 and NaCDPK5 regulate JA homeostasis in a similar way in plant-herbivore interactions. NaCDPK4 and NaCDPK5 might phosphorylate an activator of JA biosynthetic enzymes and thus deactivate this activator of JA biosynthesis. Alternatively, NaCDPK4 and NaCDPK5 might phosphorylate and therefore deactivate a suppressor of JA biosynthetic enzymes. Thus silencing *NaCDPK4* and *NaCDPK5* results in high levels of herbivory-elicited JA

Our analysis of the transcriptional profile of JA biosynthetic genes showed that JA biosynthetic enzymes do not have greatly enhanced transcript levels, suggesting that these JA biosynthesis enzymes are regulated at post-transcriptional levels in (*NaCDPK4* and *NaCDPK5*)-silenced plants. Searching substrates of NaCDPK4 and NaCDPK5, and measuring the protein abundance and catalytic activity of JA biosynthetic enzymes are particularly important for understanding how these kinases mediate JA homeostasis.

Thus far, only a handful of genes have been identified to be negative regulator of JA accumulation. Arabidopsis *cev1* mutants (defect in a cellulose synthase, CeAS3) grow smaller than do WT plants, and have stunted root length and longer root hair. *cev1* mutant accumulates anthocyanin, constitutively expresses defense-related genes, and have enhanced resistance to fungus pathogen powdery mildew (Ellis and Turner, 2001; Ellis et al., 2002). Furthermore, *cev1* plants had elevated basal JA and ethylene levels. Importantly, cellulose inhibitors-treated WT plants represented some of the *cev1* phenotypes, suggesting that cell wall signaling is involved in regulating JA and ethylene responses (Ellis et al., 2002). However, *NaCDPK4* and *NaCDPK5*-silenced plants had similar basal JA and W+OS-elicited ethylene levels, and IRcdpk5 seedlings grow similarly to WT seedlings (data not shown). Thus, it is very unlikely that NaCDPK4 and NaCDPK5 modulate herbivory-induced JA levels through regulating cellulose synthase.

Another two gain of function mutants, *dgl-D* (Hyun et al., 2008) and *fou2* (Bonaventure et al., 2007), show highly elevated JA levels after being wounded. Measuring the transcript levels of *NaGLA1*, the homologue of *DGL* gene in *N. attenuata* (Kallenbach et al., 2010), didn't reveal any changes on *NaGLA1*'s transcript levels. It is valuable to examine whether NaGLA1 and TPC [the locus of *fou2* encodes a Ca^{2+}-permeant non-selective cation channel, named Two Pore Channel 1 (TPC1)] have up-regulated protein abundance/activity, which leads to the higher JA levels in IRcdpk5 plants.

NaCDPK4 and NaCDPK5 modulate anti-herbivore secondary metabolites and resistance to *M. sexta* in a JA- and JA signaling (COI1)-dependent manner

In Arabidopsis, CEV1 (CeAS3) regulates anthocyanin accumulation and transcriptional activation of *VSP1* gene in a JA signaling (COI1)-dependent fashion. However, the phenotype of longer root hair are not controlled by COI1 in *cev1* mutant, which indicates part of the *cev1* phenotypes is depend on COI1 (Ellis and Turner, 2001).

Silencing *NaCDPK4* and *NaCDPK5* results in elevated wounding- and herbivory-induced accumulation of JA and anti-herbivore secondary metabolites (DTGs and TPIs). Consistently, *NaCDPK4* and *NaCDPK5*-silenced plants had enhanced resistance to *M. sexta* larvae. In contrast to the observation that COI1 only regulates part of phenotypes of *cev1* mutants, all the herbivore resistance traits we tested are depend on JA levels and JA signaling (NaCOI1). However, we cannot rule out the possibility that NaCDPK4 and NaCDPK5 regulate other traits (for example, pathogen resistance) in a NaCOI1-independent manner. Whether NaCDPK4 and NaCDPK5 also regulate responses to other environmental stimuli in a JA/JA signaling-independent pathway deserves further study.

Silencing *NaCDPK4* and *NaCDPK5* enhances *N. attenuata*'s resistance to *Pst* DC3000

Recently, Boudscoq *et al.* (2010) showed that AtCDPKs (AtCDPK4, 5, 6,11) positively regulate flg22-induced responses in Arabidopsis. However, transcriptional analysis revealed that CDPK and MAPK cascades independently regulate MAMP-elicited early gene responses. Given the highly increased resistance to *Pst* DC3000 in IRcdpk5 plants, whether NaCDPK4 and NaCDPK5 negatively regulate plant's resistance to *Pst* Dc3000 through MAPK cascades should be examined in future studies.

It is well known that SA plays critical roles for plant resistance to pathogens. *Pst* DC3000 induced similar SA levels in both WT and (*NaCDPK4* and *NaCDPK5*)-silenced plants. However, IRcdpk5 plants have enhanced resistance to *Pst* DC3000, suggesting that NaCDPK4 and NaCDPK5 regulate plant resistance to *Pst* DC3000 in a SA-independent manner. Furthermore, *Pst* DC3000 amplified to the similar levels in WT and *NahG*-overexpressing plants which have highly impaired pathogen-elicited accumulation of SA. This further confirms that SA is not involved in *N. attenuata*'s resistance to *Pst* DC3000.

Functional redundancy of NaCDPK4 and NaCDPK5 in plant-herbivore and plant-pathogen interactions

Arabidopsis CPK4, 5, 6, 11, which belong to the same subgroup (I) of the four subgroups of AtCPK family (Boudsocq et al., 2010). Pathogen elicitor flg22-induced responses and *Pst* DC3000 susceptibility are not different between single *cpk* (*4*, *5*, *6*, *11*) mutant and WT plant, but flg22-elicited ROS and pathogen resistance of seedling are impaired in double (*cpk5cpk6*) or triple (*cpk5cpk6cpk11*) mutants, demonstrating functional redundancy of CDPKs in plant-pathogen interactions.

NaCDPK4 and NaCDPK5 clustered with NtCDPK4, NtCDPK5, MtCDPK1, StCDPK1, and AtCDPK18, 16, 28 in the phylogenetic analysis. MtCDPK1 is involved in regulation of cell expansion or cell wall synthesis, defense genes expression, and symbiotic interactions in root (Ivashuta et al., 2005). Additionally, StCDPK1 regulates tuber development in potato (Gargantini et al., 2009). Given that (*NaCDPK4* and *NaCDPK5*)-silenced plants showed dwarf status in the elongated stage, we infer that NaCDPK4 and NaCDPK5 might be involved in regulating root or stem development in *N. attenuata*.

Only three genes, *AtCPK16*, *AtCPK18*, and *AtCPK28* exist in subgroup IV, forming the smallest subgroup of the Arabidopsis CDPK family (Cheng et al., 2002). Up to now, there are no reports on these members of subgroup IV. It would be interesting to investigate whether other CDPKs are closely related to NaCDPK4 and NaCDPK5 in *N. attenuata*, and what their physiological functions are. Furthermore, specifically silencing *NaCDPK5* is very

necessary to dissect the specific roles of NaCDPK4 and NaCDPK5 in plant-herbivore and plant-pathogen interactions.

Future work should focus on the regulation/phosphorylation, subcellular localization, and activation by calcium (or other activators) of NaCDPK proteins using genetic and biochemical approaches. Importantly, identification of NaCDPK's substrates by yeast two hybrid and/or other newly developed techniques (Bohmer and Romeis, 2007) could provide useful insight into how CDPKs regulate plant herbivore and pathogen resistance in *N. attenuata*.

Methods

Plant growth and sample treatments

Nicotiana attenuata (Solanaceae) seeds were from a line maintained in our laboratory that was originally collected in Utah (USA) and inbred for 30 generations in the greenhouse. Seed germination and plant cultivation followed Krügel *et al*.(Krugel et al., 2002). Seeds were germinated on Petri dishes to synchronize their germination, and the seedlings were transferred to soil after 10 days. Four- to five-week-old plants were used for all experiments except for growth observations. For simulated herbivory treatment, leaves were wounded with a pattern wheel and herbivore oral secretions (OS) (20 µL of 1/5 diluted OS) were immediately rubbed onto each wounded leaf (W+OS); for wounding treatment, leaves were wounded with a pattern wheel, and 20 µL of water were rubbed onto each leaf (W+W).

For the collection of OS, larvae were reared on *N. attenuata* WT plants until the third to fifth instars. OS was collected on ice as described in Roda *et al.* (2004) (Roda et al., 2004) and stored under nitrogen at -20 °C. OS was diluted 1:5 (v/v) with water prior the treatment.

Generation of transformed plants

To create *NaCDPK4*-silenced and *NaCDPK5*-silenced plants, 201 bp fragment of the *NaCDPK4* gene and 251 bp fragment of the *NaCDPK5* gene were inserted into the pRESC5 transformation vector in an inverted repeat (IR) orientation (primer sequences are listed in Supplemental Table 1) to form pRESC5-CDPK4 and pRESC5-CDPK5, respectively. These vectors were subsequently transformed into *Agrobacterium tumefaciens* to transform *N. attenuata* (Krugel et al., 2002). The number of T-DNA insertions was determined by Southern hybridization of genomic DNA using a PCR fragment of the *hygromycin phosphotransferase* (*hptII*) gene as a probe. Two T_2 homozygous lines of IRcdpk4 and two T_2 homozygous lines

of IRcdpk5, all with single T-DNA insertions, were identified and used in subsequent experiments. Crossing IRcoi1 and ovJMT with IRcdpk5-1 plants was done by removing anthers from flowers of IRcoi1 and ovJMT plants before pollen maturation and pollinating the stigmas with pollen from IRcdpk5-1 plants. The transcript levels of *NaCDPK5* were examined in the respective heterozygote descendents to confirm successful crossing.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from ground leaf samples using TRIzol reagent (Invitrogen) following the manufacturer's instructions. For qPCR analysis, 5 replicated biological samples were used. 0.5 µg of total RNA sample was reverse-transcribed with oligo(dT) and Superscript II reverse transcriptase (Invitrogen). qPCR was performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems) using qPCR Core kits (Eurogentec). A *N. attenuata actin2* gene was employed as the internal standard for normalizing cDNA concentration variations. Sequences of primers used for qPCR are listed in Supplemental Table 2.

Sequence alignment and phylogeny analysis

The protein sequences were retrieved from GenBank. Sequences were aligned in MegAlign (DNASTAR, Lasergene 8) using the Clustal W algorithm and verified manually. For phylogeny analysis, the unrooted Neighbor-Joining tree and bootstrap values were obtained using MEGA 4 software (default parameters and 1000 replications) (Tamura et al., 2007) (www.megasoftware.net). Accession numbers of genes encoding these proteins were retrieved from GenBank, which are listed in Supplemental Table 3.

Analysis of SA, JA and JA-Ile concentrations

One milliliter of ethyl acetate spiked with 200 ng of D2-JA and 40 ng of $^{13}C_6$-JA-Ile, and D4-SA, the internal standards for JA, JA-Ile, and SA, respectively, was added to each briefly crushed leaf sample (~ 150 mg). Samples were then ground on a FastPrep homogenizer (Thermo Electron). After being centrifuged at 13,000 g for 10 min at 4 ºC, supernatants were transferred to fresh Eppendorf tubes and evaporated to dryness on a vacuum concentrator (Eppendorf). Each residue was resuspended in 0.5 mL of 70% methanol (v/v) and centrifuged to remove particles. The supernatants were analyzed on a HPLC-MS/MS (1200L LC-MS system, Varian).

***Manduca sexta* growth bioassays**

Freshly hatched *M. sexta* larvae were placed on 30 replicated plants of each genotype. To compare herbivore growth rates on WT and IRcdpk5 plants, larvae were weighed on day 4, 6, 9 and 12; for the comparison of herbivore growth on WT, IRcdpk5, ovJMT, IRcoi1, and the crossed (ovJMTxIRcdpk5-1 and IRcdpk5-1xIRcoi1) plants, larval mass on day 6, 9, and 12 was recorded.

Analyses of herbivore defense-related secondary metabolites

Trypsin proteinase inhibitor activity was analyzed with a radial diffusion assay described by van Dam *et al.* (Van Dam et al., 2001). The accumulation of the direct defenses, caffeoylputrescine and diterpene glycosides, were analyzed in samples harvested 3 days after W+W and W+OS treatment using a HPLC method described in Keinanen *et al*. (Keinanen et al., 2001).

Quantification of pathogen growth

Pseudomonas syringae pv tomato DC3000 (*Pst* DC3000) was grown at 28 °C in LB liquid medium with antibiotics until OD_{600} was ~ 0.6. After 10 min centrifugation at 3000 g, the supernatant was discarded, and cells were diluted to OD_{600} = 0.001 (~ 5×10^5 cells mL^{-1}) in a 10 mM $MgCl_2$ solution. The bacterial suspension was inoculated into the abaxial sides of basal leaves in early elongated plants using 1 mL syringes. Leaf discs (1 cm^2) were ground in 1 mL of sterile water and a series of dilutions of each leaf extract were spread on LB agar plates containing antibiotics. Colonies were counted after 36 h of incubation at 28°C.

Statistical analysis

Data were analyzed by unpaired *t*-test using StatView, version 5.0 (SAS Institute).

Supplemental Data

Supplemental Figure 1 NaCDPK4 and NaCDPK5 are close homologue of NtCDPK4 and NtCDPK5, respectively.

Supplemental Table 1 Primers used for cloning of *NaCDPK4* and *NaCDPK5*, and preparation of pRESC5-NaCDPK4 and pRESC5-NaCDPK5 constructs

Supplemental Table 2 Primer sequences used for quantitative real-time PCR (SYBR Green analysis)

Supplemental Table 3 Accession numbers of genes encoding CDPK proteins retrieved from GenBank

Acknowledgements

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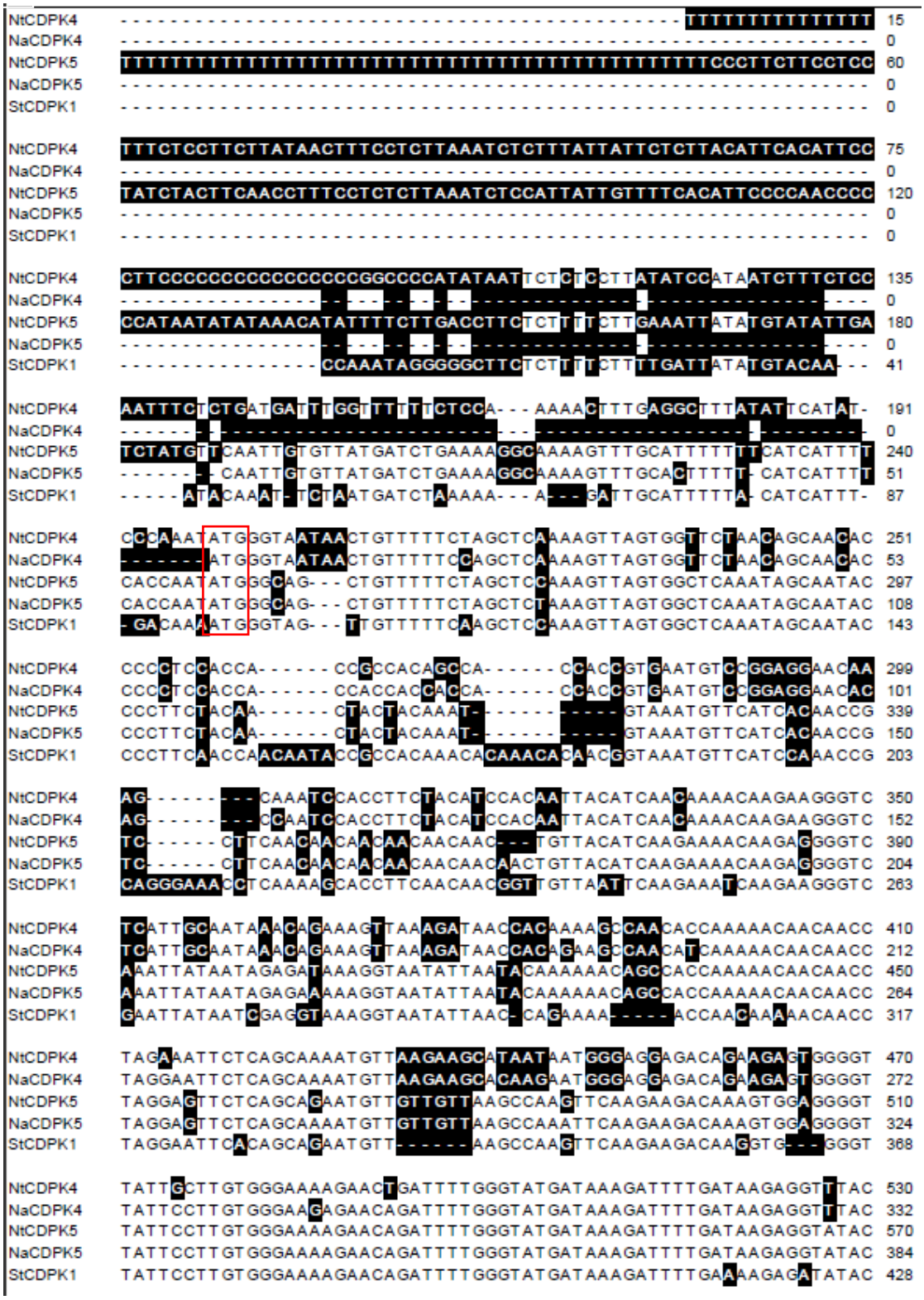
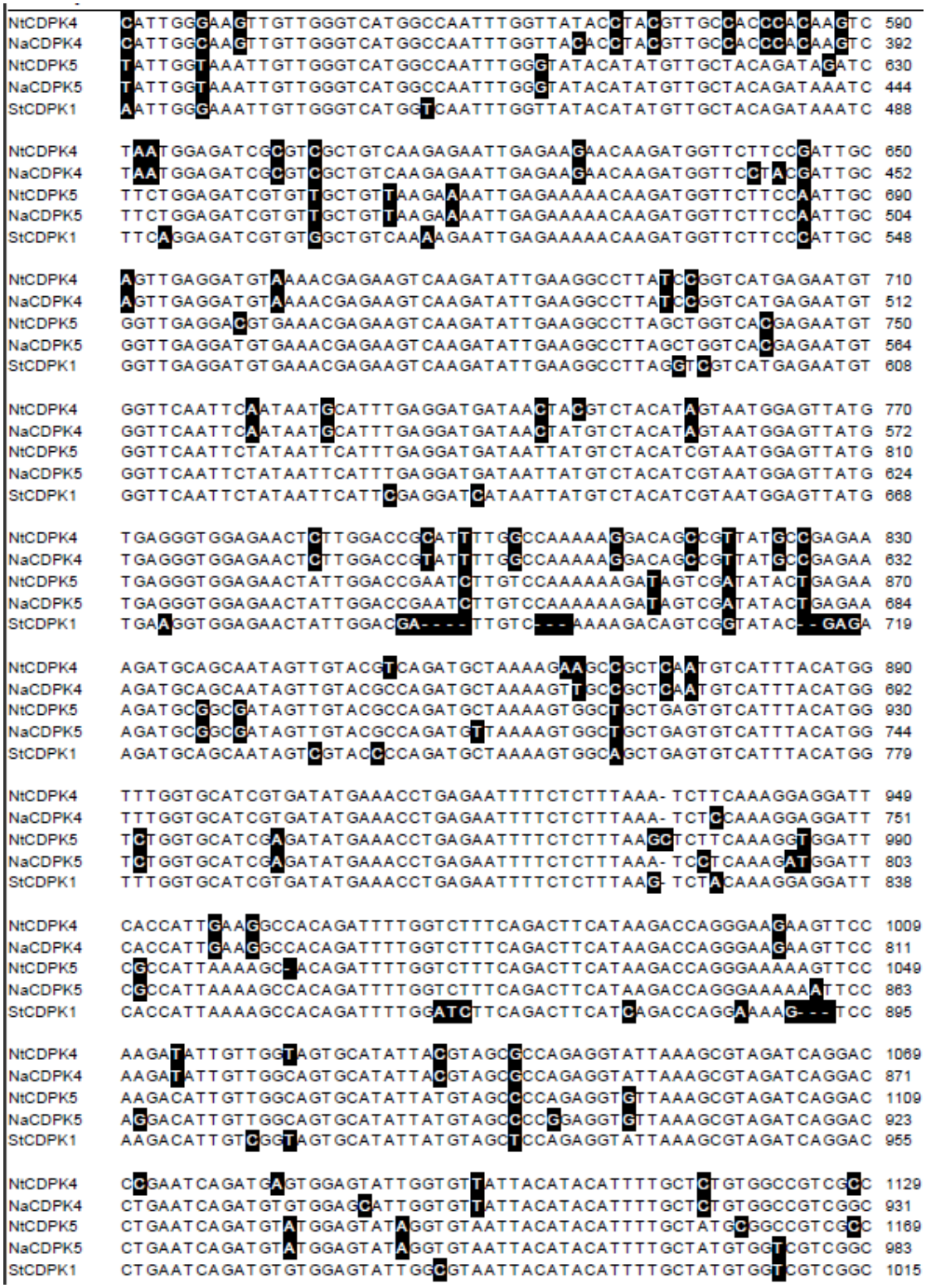
Supplemental Data

Supplemental Figure 1

A

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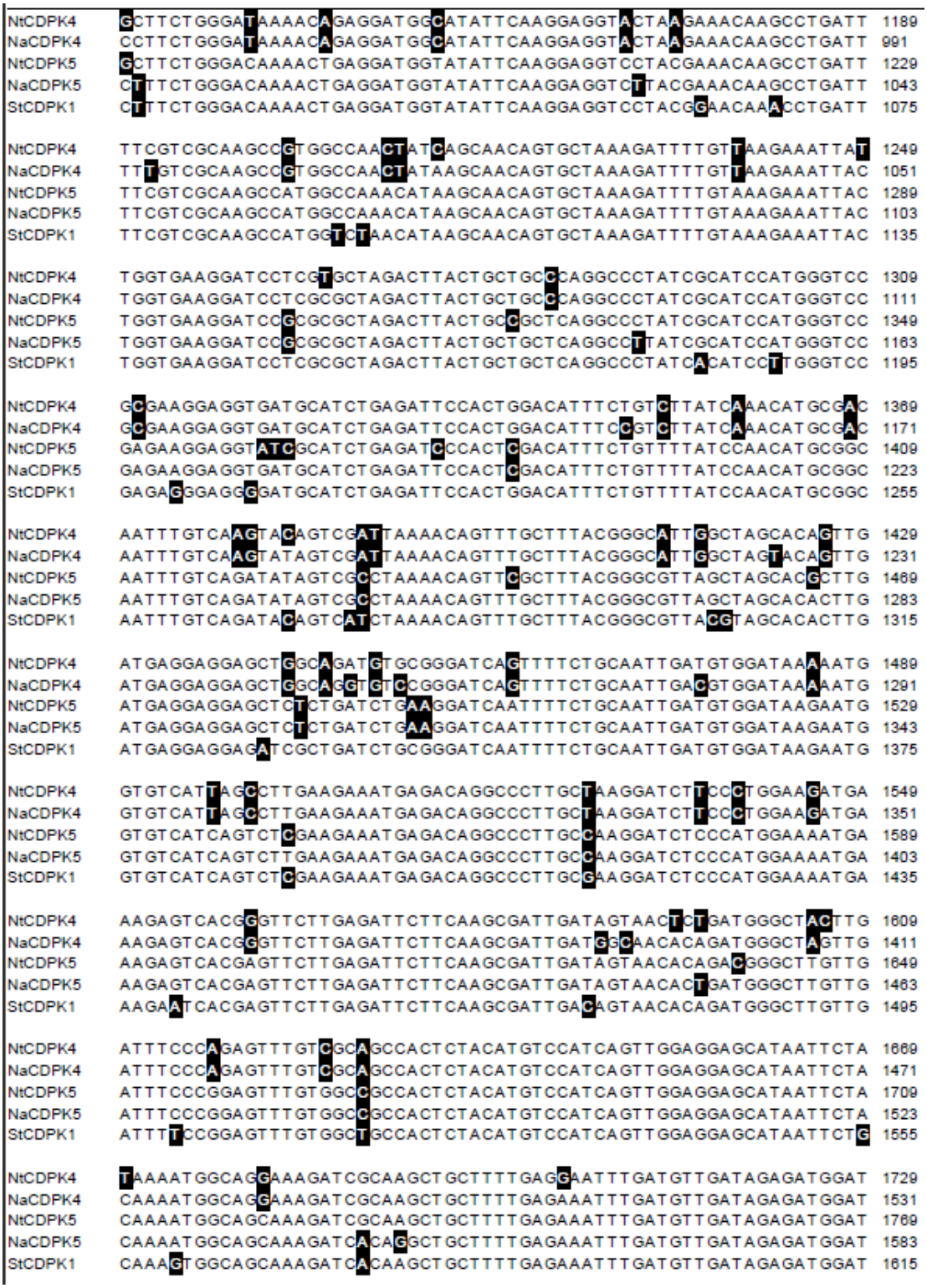
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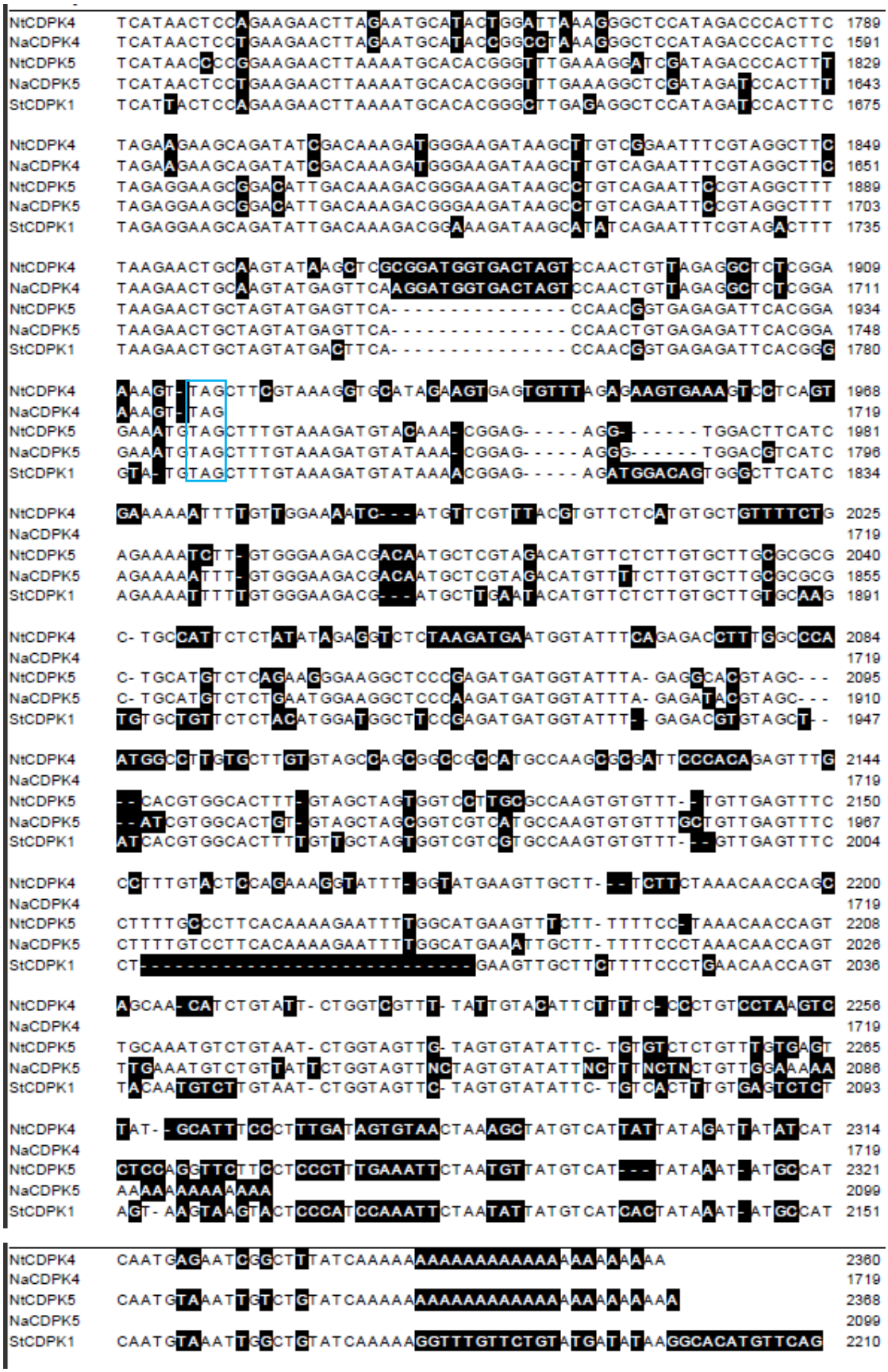
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StCDPK1 TAAGAACTGCTAGTATGACTTCA----------CCAACGGTGAGAGATTCACGGG 1780
NtCDPK4 AAAGT-TAGCTTCGTAAAGGTGCATAGAAGTGAGTGTTTAGAGAAGTGAAAGTCCTCAGT 1968
NaCDPK4 AAAGT-TAG 1719
NtCDPK5 GAAATGTAGCTTTGTAAAGATGTACAAA-CGGAG-----AGG------TGGACTTCATC 1981
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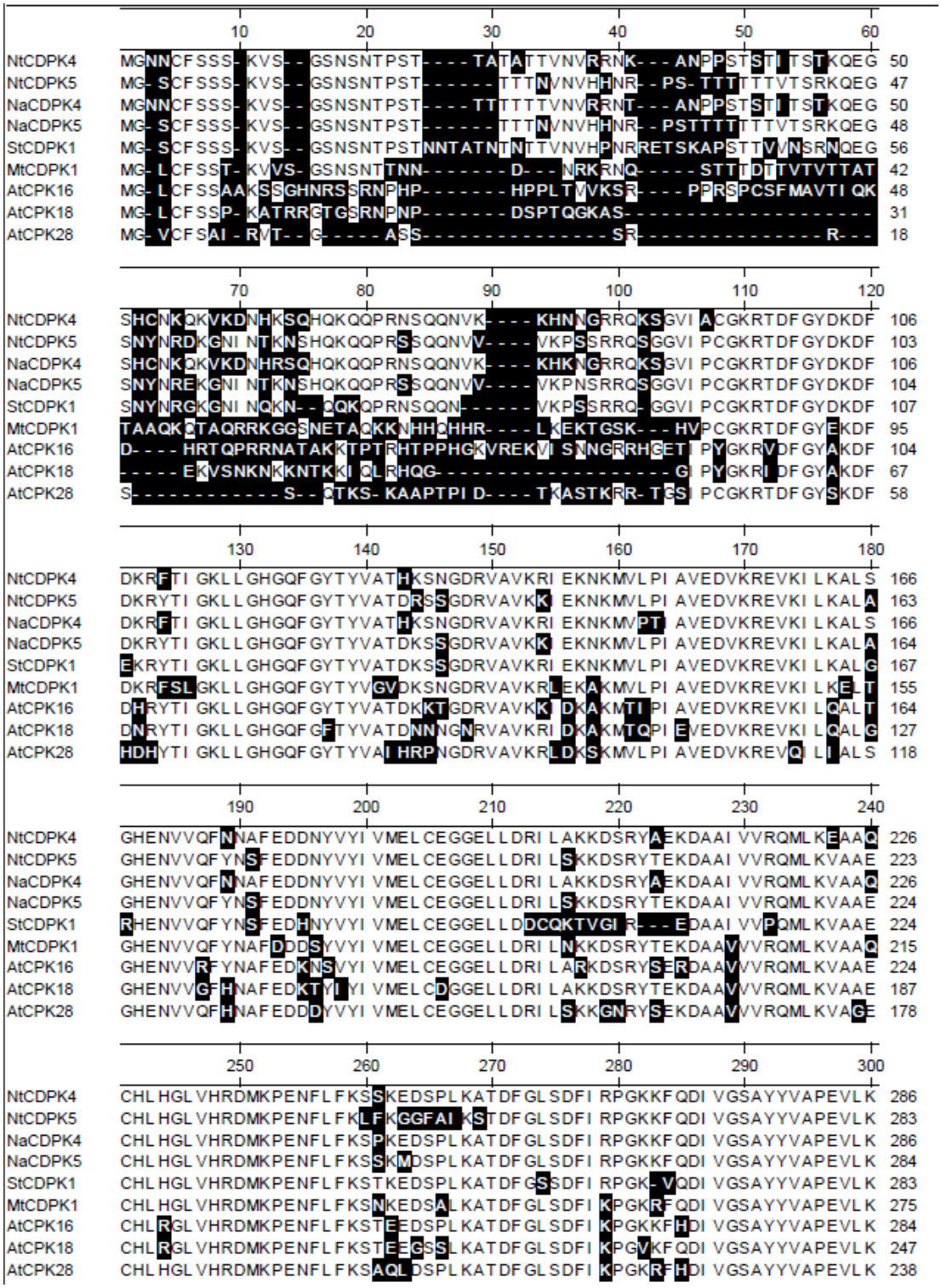
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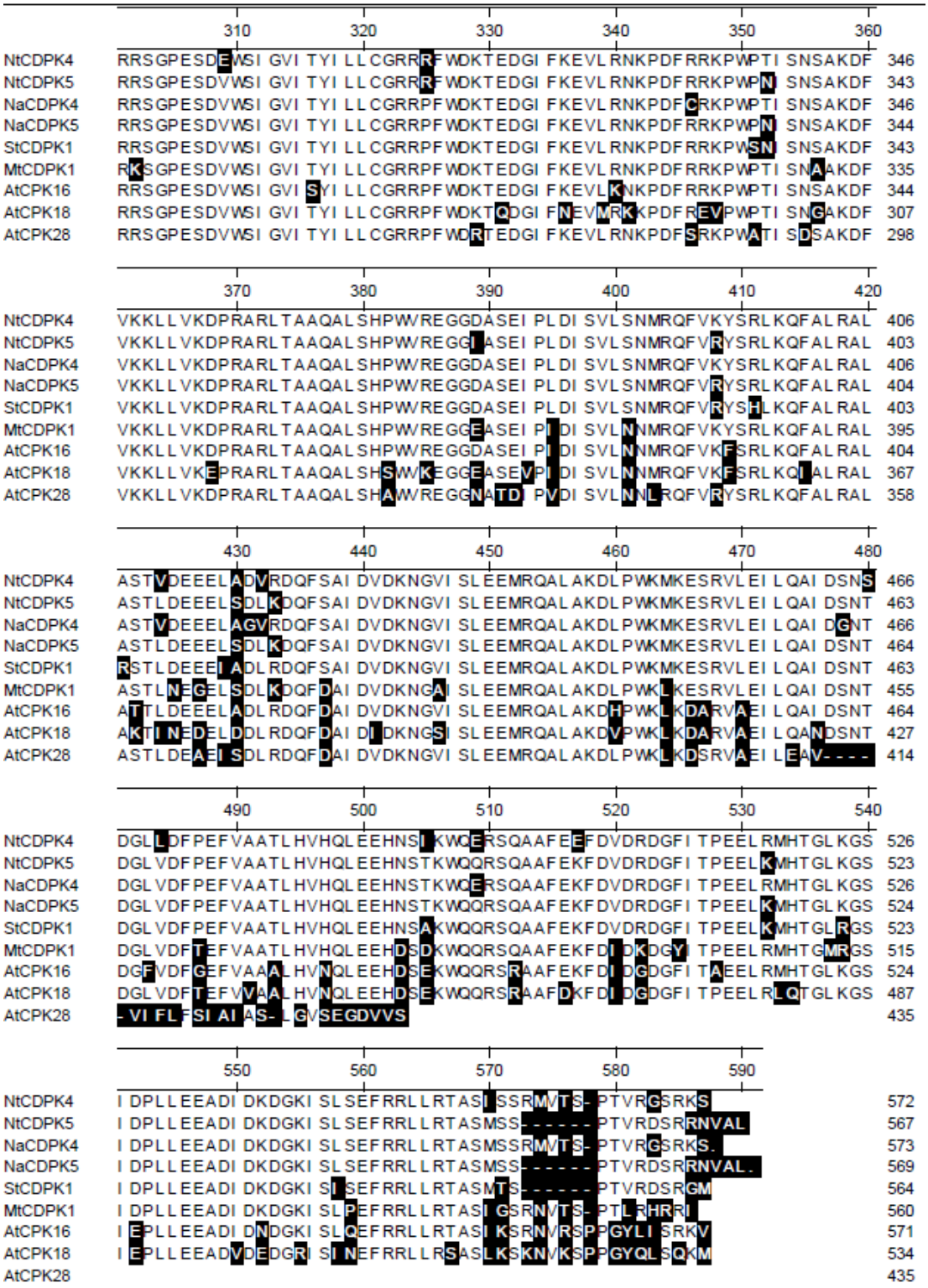

Supplemental Figure 1 NaCDPK4 and NaCDPK5 are close homologue of NtCDPK4 and NtCDPK5, respectively.

(A) Nucleotide sequence alignment of *NaCDPK4* and *NaCDPK5* with tobacco *NtCDPK4* and *NtCDPK5*, and potato *StCDPK1*. Nucleotides different from consensus sequence are shade in black. (B) Amino acid sequence of NaCDPK4 and NaCDPK5 with tobacco NtCDPK4 and NtCDPK5, and potato StCDPK1. Amino acids different from consensus sequence are shade in black.

Supplemental Table 1 Primers used for cloning of *NaCDPK4* and *NaCDPK5*, and preparation of pRESC5-NaCDPK4 and pRESC5-NaCDPK5 constructs

Genes	Primer (5'-3')	Purpose
NaCDPK4-1	ATGGGTAATAACTGTTTTTCCAG	Cloning of full length NaCDPK4
NaCDPK4-2	GATTGAAAAGGCTCTCGGAGATTGTC	Cloning of full length NaCDPK4
NaCDPK5-1	ATGGGCAGCTGTTTTTCTAGCTC	Cloning of full length NaCDPK5
NaCDPK5-2	TTACAAAGCTACATTTCTCCGTG	Cloning of full length NaCDPK5
pRESC5-C4-1	ATGGGTAATAACTGTTTTTCCAGC	Construct vector pRESC5-NaCDPK4
pRESC5-C4-2	TTGATGTTGGCTTCTGTGGTTATC	Construct vector pRESC5-NaCDPK4
pRESC5-C5-1	GTTAGTGGTTCAAATAGCAATACCCC	Construct vector pRESC5-NaCDPK5
pRESC5-C5-2	CCACAAGGAATAACCCCTCCAC	Construct vector pRESC5-NaCDPK5

Supplemental Table 2 Primer sequences used for quantitative real-time PCR (SYBR Green analysis)

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
NaActin2	GGTCGTACCACCGGTATTGTG	GTCAAGACGGAGAATGGCATG
NaCDPK1	CCGTGCAGCCGATACAAAC	TTTCCCAAGTGTATATTGCTTCCTAA
NaCDPK2	GCAATGTGGCGAACTCGG	CTGCTGTTTCACCTCTGGCAC
NaCDPK4	CGTTGCCACCCACAAGTCTAA	TACATCCTCAACTGCAATCGTAG
NaCDPK5	GGGTATACATATGTTGCTACAGAT	ACCACATTCTCGTGACCAGC
NaGLA1	AGTAGCAGATGATGTTAGTACATGTA	ACATGTGAATATGCCCATGGCATACT
NaLOX3	GGCAGTGAAATTCAAAGTAAGAGC	CCCAAAATTTGAATCCACAACA
NaAOS	GACGGCAAGAGTTTTCCCAC	TAACCGCCGGTGAGTTCAGT
NaAOC	AACTACCTAACCCTCTCATTTCTCA	AAGCGAAGATAGGCAGGGC
NaOPR3	AATGGAGTTGGAGTTTGTTT	AGGTGGTTGAAGCAGTCGTT
NaACX1	GAATGTCTGTTGCTTGTGCTCA	TACCGCAAAGCACCTCCAG
NaTD	TAAGGCATTTGATGGGAGGC	TCTCCCTGTTCACGATAATGGAA
NaJAR4	ATGCCAGTCGGTCTAACTGAA	TGCCATTGTGGAATCCTTTTAT
NaJAR6	TGGAGTAAACGTTAACCCGAAA	AGAATTTGCTTGCTCAATGCCA

Supplemental Table 3 Accession numbers of genes encoding CDPK proteins retrieved from GenBank

Genes	Accession number	Genes	Accession number
AtCPK1	NM_120569.2	*AtCPK29*	NM_202421.2
AtCPK2	NM_111902.1	*AtCPK30*	NM_106132.3
AtCPK3	NM_118496.3	*AtCPK31*	NM_148230.2
AtCPK4	NM_117025.5	*AtCPK32*	NM_115613.3
AtCPK5	NM_119697.3	*AtCPK33*	NM_103952.3
AtCPK6	NM_127284.2	*AtCPK34*	NM_121941.1
AtCPK7	NM_121286.3	*NtCDPK1*	AF072908.1
AtCPK8	NM_180522.2	*NtCDPK2*	AJ344154.1
AtCPK9	NM_112932.2	*NtCDPK3*	AJ344155.1
AtCPK10	NM_101746.4	*NtCDPK4*	AF435451.2
AtCPK11	AY050981.1	*NtCDPK5*	AY971376.1
AtCPK12	NM_122264.4	*NtCDPK9*	HQ141792.1
AtCPK13	NM_115044.4	*NtCDPK10*	HM989874
AtCPK14	NM_129750.3	*NtCDPK11*	HQ158609.1
AtCPK15	NM_118315.1	*NtCDPK12*	GQ337420.1
AtCPK16	NM_127343.1	*StCDPK1*	AF030879.1
AtCPK17	AY072126.1	*StCDPK2*	GU182404.1
AtCPK18	NM_119774.2	*StCDPK3*	AF518003.2
AtCPK19	NM_104875.2	*StCDPK4*	AB279737.1
AtCPK20	NM_129449.1	*StCDPK5*	AB279738.1
AtCPK21	NM_116710.2	*LeCPK1*	AJ308296.1
AtCPK22	NM_116709.1	*LeCPK2*	GQ205414.1
AtCPK23	NM_116712.1	*LeCDPK2*	AB530160.1
AtCPK24	NM_128707.2	*MtCDPK1*	AY821654.1
AtCPK25	NM_129148.1	*MtCDPK3*	DQ454073.1
AtCPK26	NM_119985.2	*NaCDPK4* partial	EF121310
AtCPK27	NM_116708.2	*NaCDPK5* partial	EF121305
AtCPK28	NM_001126036.1		

3. Chapter II: Role of NaBAK1 in herbivory-induced defense responses

BAK1 regulates the accumulation of jasmonic acid and the levels of trypsin proteinase inhibitors in *Nicotiana attenuata*'s responses to herbivory

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Abstract

BAK1 is a co-receptor of brassinosteroid (BR) receptor BRI1, and plays a well-characterized role in BR signaling. BAK1 also physically interacts with the flagellin receptor FLS2 and regulates pathogen resistance. Here we examined the role of BAK1 in mediating *Nicotiana attenuata*'s resistance responses to its specialist herbivore, *Manduca sexta*. A virus-induced gene silencing system was used to generate empty vector (EV) and *NaBAK1*-silenced plants. The wounding- and herbivory-induced responses were examined on EV and *NaBAK1*-silenced plants by wounding plants or simulating herbivory by treating wounds with larval oral secretions (OS). After wounding or OS elicitation, *NaBAK1*-silenced plants showed attenuated jasmonic acid (JA) and JA-isoleucine bursts, phytohormone responses important in mediating plant defenses against herbivores. However, these decreased JA and JA-Ile levels did not result from compromised MAPK activity or elevated SA levels. After simulated herbivory, *NaBAK1*-silenced plants had EV levels of defensive secondary metabolites, namely, trypsin proteinase inhibitors (TPIs), and similar levels of resistance to *Manduca sexta* larvae. Additional experiments demonstrated that decreased JA levels in NaBAK1-VIGS plants, rather than the enzymatic activity of JAR proteins or Ile levels, were responsible for the reduced JA-Ile levels observed in these plants. Methyl jasmonate application elicited higher levels of TPI activity in *NaBAK1*-silenced plants than in EV plants, suggesting that silencing *NaBAK1* enhances the accumulation of TPIs induced by a given level of JA. Thus NaBAK1 is involved in modulating herbivory-induced JA accumulation and how JA levels are transduced into TPI levels in *N. attenuata*.

Keywords:

BAK1, defense, herbivory, jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile), *Nicotiana attenuata*, SERK

Abbreviations:

ABA, abscisic acid; BR, brassinosteroid; BRI1, brassinosteroid insensitive 1; BAK1, BRI1-associated receptor kinase 1; DTG, diterpene glycoside; JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine; MAPK, mitogen-activated protein kinase; OS, oral secretions; SA, salicylic acid; SERK, somatic embryogenesis receptor kinase; TPI, trypsin proteinase inhibitor

Introduction

Plants have evolved sophisticated regulatory networks to modulate their growth and to cope with environmental stresses. Within these networks, gibberellins, auxins, cytokines, jasmonates, ethylene, brassinosteroids (BRs), salicylic acid (SA), abscisic acid (ABA), and strigolactone play roles as regulators of almost every aspect of a plant's life (Santner *et al.*, 2009; Chow and McCourt, 2006). These hormone molecules exist in very low concentrations but alter plant physiology dramatically. Accordingly, plants possess elaborate systems to tightly control both the accumulation and perception of these hormones. In recent years, new genetic and biochemical tools have greatly augmented our understanding of the biosynthesis, transport, degradation, signal transduction, and physiological functions of these phytohormones in various plant species. Now it has become clear that although an individual hormone has specific roles in modulating growth and stress responses, complex antagonistic and synergistic interactions among them enable plants to fine-tune their cellular processes (Chow and McCourt, 2006; Grant and Jones, 2009).

Among these phytohormones, jasmonic acid (JA) is one of the best studied. JA is involved in plant development and responses to various stresses, such as attack from herbivores and necrotrophic fungi (Wasternack, 2007; Howe and Jander, 2008; Browse, 2009; Wu and Baldwin, 2009). JA is synthesized by the oxylipin pathway in which several enzymes, including 13-lipoxygenase (13-LOX), allene-oxide synthase (AOS), allene-oxide cyclase (AOC), (9S,13S)-12-oxo-phytodienoic acid (OPDA) reductase (OPR), and acyl-coenzyme A oxidase (ACX) play pivotal roles (Wasternack, 2007; Liechti and Farmer, 2002).

Importantly, instead of JA, JA-Ile [the Ile conjugate of JA that is catalyzed by jasmonate resistant proteins (JARs)] is responsible for eliciting most of JA-induced responses (Staswick *et al.*, 2002; Wang *et al.*, 2008). Recent work has identified COI1, an F-box protein, as the JA-Ile receptor; JA-Ile interacts with COI1 and promotes the binding of COI1 to the jasmonate-ZIM domain (JAZ) proteins, which repress JA signaling. JAZs are subsequently ubiquitinated by SCF^{COI1} E3 ubiquitin ligase and are degraded by the 26S proteasome. The degradation of the JAZ repressors releases MYC2 transcription factors, which are responsible for activating JA response in Arabidopsis (Xie *et al.*, 1998; Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2009).

The ecological significance of JA biosynthesis and signaling in plant resistance to herbivores has been intensively studied in *Nicotiana attenuata*, a species of wild tobacco (Halitschke and Baldwin, 2003; Paschold *et al.*, 2007). *N. attenuata* perceives fatty acid-amino acid conjugates (FACs) in the oral secretions (OS) (mixtures of caterpillar saliva and regurgitants) of its specialist herbivore, *Manduca sexta*, and rapidly activates the salicylic acid-induced protein kinase (SIPK) and the wound-induced protein kinase (WIPK), two mitogen-activated protein kinases (MAPKs); subsequently, JA, JA-Ile, and ethylene biosynthesis are induced (Halitschke *et al.*, 2001; Wu *et al.*, 2007). In *N. attenuata*, silencing the expression of *LOX3*, *JAR4* and *JAR6*, and *COI1* greatly impairs the plants' direct and indirect defenses and increases the plants' susceptibility to *M. sexta*, demonstrating the central role of JA/JA-Ile biosynthesis and signaling in plant-herbivore interactions (Halitschke and Baldwin, 2003; Paschold *et al.*, 2007; Wang *et al.*, 2007; Wang *et al.*, 2008). Herbivory-induced JA accumulation and signaling finally activates the biosynthesis of anti-herbivore compounds, such as nicotine, trypsin proteinase inhibitors (TPIs), and diterpene glycosides (DTGs) (Halitschke and Baldwin, 2003; Paschold *et al.*, 2007). The important ecological roles of these defensive compounds have been demonstrated in plants that are impaired in their biosynthesis. Silencing *putrescine N-methyl transferase* (*PMT*) in nicotine biosynthesis, *TPI* gene, and geranylgeranyl diphosphate synthase (*GGPPS*) in DTG biosynthesis diminish herbivory-induced nicotine, TPI, and DTG accumulation, respectively, and thus compromise *N. attenuata*'s resistance to herbivores (Steppuhn *et al.*, 2004; Zavala *et al.*, 2004; Jassbi *et al.*, 2008; Heiling *et al.*, 2010).

BRs are a class of phytohormones that regulate seed germination, stem and root elongation, vascular differentiation, leaf expansion, and apical dominance (Halliday, 2004). Genetic analysis revealed that BR insensitive 1 (BRI1), a leucine-rich repeat receptor-like

kinase (LRR-RLK), is the BR receptor (Li and Chory, 1997; Kinoshita *et al.*, 2005). Another LRR-RLK, the Somatic Embryogenesis Receptor Kinase 3 (SERK3), also named BRI1-associated Kinase 1 (BAK1), physically interacts with BRI1 and plays an essential role in BR signaling (Li *et al.*, 2002; Nam and Li, 2002; Belkhadir and Chory, 2006; Karlova and de Vries, 2006). Apart from its function in BR signaling, SERK3/BAK1 has been shown to play an important role in plants' defense against pathogens. SERK3/BAK1 binds to FLS2, the flagellin receptor, and positively regulates FLS2-mediated innate immunity in Arabidopsis (Chinchilla *et al.*, 2007). In *N. benthamiana*, SERK3/BAK1 is required for multiple pathogen-associated molecular patterns (PAMPs)-elicited responses and for resistance to bacteria and oomycetes (Heese *et al.*, 2007). Therefore SERK3/BAK1 has been proposed to have multiple functions as a common co-receptor and is thought to cooperate with other receptors (Gendron and Wang, 2007). Whether SERK3/BAK1 is involved in plants' defense responses to herbivory has not been examined.

To investigate the function of SERK3/BAK1 in plant-herbivore interactions, we cloned *BAK1* in *N. attenuata* (*NaBAK1*) and its transcripts levels were silenced using a virus-induced gene silencing (VIGS) approach. Traits important for herbivore defense in these plants were examined. Silencing *NaBAK1* did not result in compromised wounding- or herbivory-induced MAPK activation, but impaired the accumulation of JA and JA-Ile after these stresses. However, *NaBAK1*-silenced plants still accumulated normal levels of herbivory-induced defensive compounds, TPIs. TPI activity analysis in methyl jasmonate (MeJA)-treated control and *NaBAK1*-silenced plants confirmed that NaBAK1 negatively modulates JA-induced TPI levels.

Material and Methods

Plant growth and sample treatments

Nicotiana attenuata (Solanaceae) seeds were from a line maintained in our laboratory that was originally collected in Utah (USA) and inbred for 30 generations in the glasshouse. Seed germination and plant cultivation followed Krügel *et al.* (2002). After being individually transferred into 1-liter pots, plants were grown at 20 - 22 ºC under 16 h of light. A virus-induced gene silencing (VIGS) system was used to silence the accumulation of *NaBAK1* transcripts following a VIGS procedure optimized for *N. attenuata* (Ratcliff *et al.*, 2001; Saedler and Baldwin, 2004). For wounding and water treatments (W+W), leaves were

wounded with a pattern wheel, and 20 µl of water were immediately rubbed onto the wounded leaf; for wounding and OS treatments (W+OS), 20 µl of *M. sexta* OS (one-fifth diluted) were rubbed onto the wounded leaf; for wounding and FAC treatments (W+FAC), 20 µl of *N*-linolenoyl-I-Gln (FAC A) solution (27.6 ng μl^{-1}) was rubbed onto the wounded leaf (Halitschke and Baldwin, 2003; Halitschke *et al.*, 2001). For methyl jasmonate (MeJA) treatments, MeJA was dissolved in heat-liquefied lanolin at a concentration of 7.5 mg ml^{-1}; 20 µl of the paste were applied to the base of a leaf, and 20 µl of pure lanolin were applied as a control. For the assays of JAR activity and substrate availability, we followed the procedure described in Paschold *et al.* (2008). Samples were harvested in liquid nitrogen and stored at -80 ^{o}C freezer until analyses.

Cloning of *NaBAK1* and *NaSERK1*, VIGS constructs preparation, and sequence analysis

NaBAK1 full length open reading frame (accession number HM639279) and *NaSERK1* (accession number HM639280) partial sequence were amplified by PCR from a cDNA sample prepared from *N. attenuata* leaf tissue, using primers NaBAK1-1 and NaBAK1-2, and NaSERK1-1 and NaSERK1-2, respectively (supplemental Table S1). PCR products were cloned and sequenced.

Around 300 bp fragments from *NaBAK1*'s coding sequence and 3' untranslated region (3UTR) were cloned into pTV00 vector to form pTV-NaBAK1 and pTV-NaBAK1-3UTR, respectively, using *Bam*H I and *Sal* I enzymes (supplemental Table S1). The identity of pTV-NaBAK1 and pTV-NaBAK1-3UTR was confirmed by sequencing. pTV-NaBAK1 and pTV-NaBAK1-3UTR plasmids were transformed, respectively, to an *Agrobacterium* strain for VIGS (Ratcliff *et al.*, 2001)**.**

Nucleotide and protein sequences were aligned in MegAlign (DNASTAR, Lasergene 8) using Clustal W algorithm. For phylogeny analysis, the unrooted Neighbor-Joining tree and bootstrap values were obtained using MEGA 4 software using default parameters and 1000 replications (Tamura *et al.*, 2007) (www.megasoftware.net).

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from ground leaf samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. For qPCR analysis, 5 replicated biological samples were used. 0.5 µg of total RNA sample was reverse-transcribed with oligo(dT) and Superscript II reverse transcriptase (Invitrogen). qPCR was performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA)

using qPCR Core kits (Eurogentec, Seraing, Belgium). An *N. attenuata actin2* gene was employed as the internal standard for normalizing cDNA concentration variations. The primer sequences for qPCR analysis are provided in supplemental Table S2.

Protein extraction, kinase activity assay, and SDS-PAGE

Each protein sample was extracted from pooled leaves of 5 replicated plants. Protein extraction and MAPK in-gel kinase activity assay were done following Zhang and Klessig (1997). In-gel kinase images were obtained on an FLA-3000 phosphor imager system (Fujifilm, Tokyo, Japan). SDS-PAGE was done on a Bio-Rad Mini-PROTEAN 3 Cell using 10% gels. Gel staining was done using GelCode Blue Safe Stain reagent (Thermo Scientific).

Analysis of JA, JA-Ile, and SA concentrations

JA, JA-Ile, and SA concentrations were measured in 5 biological replicates following Wu *et al*. (2007). One milliliter of ethyl acetate spiked with 200 ng of D_2-JA ,40 ng of $^{13}C_6$-JA-Ile, and 40 ng of D_4-SA, the internal standards for JA, JA-Ile, and SA, respectively, was added to each briefly ground leaf sample (~ 150 mg). Samples were then ground on a FastPrep homogenizer (Thermo Electron, Waltham, MA, USA). After being centrifuged at maximum speed for 10 min at 4 ºC, supernatants were transferred to fresh Eppendorf tubes and evaporated to dryness on a vacuum concentrator (Eppendorf, Hamburg, Germany). Each residue was resuspended in 0.5 ml of 70% methanol (v/v) and centrifuged to clarify phases. The supernatants were analyzed on a HPLC-MS/MS (Varian, Palo Alto, CA, USA).

Analysis of secondary metabolites

The concentrations of nicotine and DTGs were analyzed by HPLC as described in Keinanen *et al*. (2001). TPI activity was measured from 5 biological replicates using a radial diffusion assay described in Van Dam *et al.* (2001).

Measurement of ethylene emission

Five leaves treated with W+OS were immediately sealed in a three-neck 250 ml round bottom flask and kept in the plant growth chamber for 5 h. The headspace was flushed into a photoacoustic laser spectrometer with hydrocarbon-free clean air (INVIVO, Bonn, Germany), and the ethylene concentration was quantified by comparing ethylene peak areas with peak areas generated by a standard ethylene gas (von Dahl *et al.*, 2007). Data were obtained from 5 replicated measurements.

Herbivore growth assay

Specialist herbivore *M. sexta* was obtained from a colony maintained in our lab. Each plant was infested with one neonate larva. Larval mass was recorded on day 6, 8, and 11.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) or unpaired *t*-test using StatView, version 5.0 (SAS, Cary, NC, USA).

Results

Cloning of *NaBAK1* in *N. attenuata*

A 696 bp fragment of *NaBAK1* gene in *N. attenuata* was cloned from cDNA according to the *N. benthamiana NbSERK3/BAK1* sequence (EST accession number: CK291393) (Heese *et al.*, 2007). This partial *NaBAK1* sequence showed 95% homology to *NbSERK3/BAK1*. Using this cloned *NaBAK1* fragment as the bait, we identified *NaBAK1* full length open reading frame from an *N. attenuata* transcriptome database prepared by 454 sequencing; this full length sequence was further confirmed by cloning and sequencing (accession number: HM639279).

Arabidopsis has five *SERK* gene family members, which are involved in different signaling pathways (Albrecht *et al.*, 2008). Cloning and searching an *N. attenuata* transcriptome database prepared by 454 sequencing resulted in identification of another *SERK* gene in *N. attenuata* (accession number: HM639280), whose deduced protein sequence showed high similarity to SERK1 proteins (92%, 90%, and 87% similarity to *Solanum tuberosum* StSERK1, *Medicago truncatula* MtSERK1, and Arabidopsis AtSERK1 protein, respectively) (Figure S1). Therefore, this protein is designated NaSERK1.

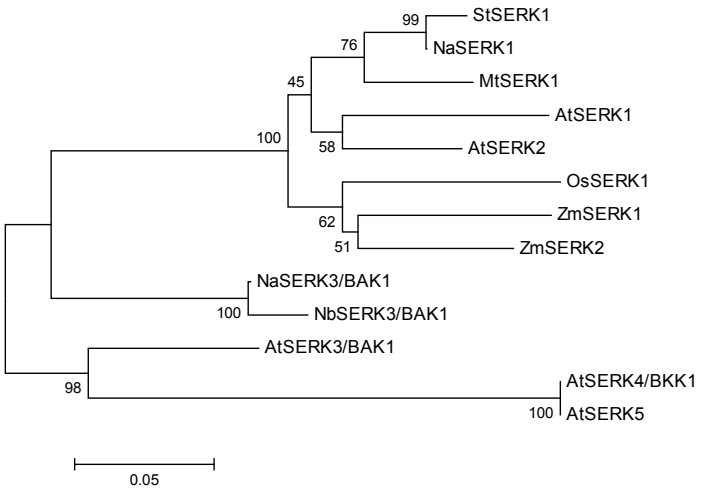


Figure 1. Phylogenetic tree of SERK proteins

Phylogenetic tree (unrooted) was constructed using protein sequences of several SERK genes from different plant species. NCBI (GenBank) accession numbers of *SERK* genes and SERK proteins included in this analysis are: *N. attenuata*: *NaBAK1* (HM639279), *NaSERK1* (HM639280); *N. benthamiana*: *NbSERK3/BAK1* (CK291393); *Arabidopsis thaliana*: AtSERK1 (NP_177328), AtSERK2 (NP_174683), AtSERK3 (NP_567920), AtSERK4 (NP_178999), AtSERK5 (NP_179000); *Solanum tuberosum*: StSERK1 (ABO14172); *Medicago truncatula*: MtSERK1 (AAN64294); *Oryza sativa*: OsSERK1 (BAD86793); *Zea mays*: ZmSERK1 (CAC37638); ZmSERK2 (CAC37639).

To further examine these SERK protein sequences, an unrooted phylogenetic tree was constructed using the deduced peptide and protein sequences (Figure 1). Consistent with the sequence similarity data, NaBAK1 and NaSERK1 closely clustered with NbSERK3/BAK1 and StSERK1, respectively.

Herbivory specifically induces transcript accumulation of *NaBAK1* in *N. attenuata*

To examine whether herbivory induces the accumulation of *NaBAK1* transcripts, we wounded *N. attenuata* rosette leaves with a pattern wheel and immediately applied 20 µl of *M. sexta* OS to each leaf (W+OS), since this treatment effectively elicits herbivory-induced responses in *N. attenuata* (Halitschke *et al.*, 2001). For comparison, 20 µl of water was applied to wounds (W+W). Using gene-specific primers, quantitative real-time PCR (qPCR) was used to measure the transcript levels of *NaBAK1* at different times (Figure 2). *NaBAK1* transcript levels quickly increased about 5 fold 1.5 h after W+OS treatment and gradually decreased to near basal levels after 6 h. In contrast, after W+W treatment, *NaBAK1* transcript

levels increased only marginally (Figure 2). These data suggest that NaBAK1 is likely involved in *N. attenuata*'s defense against herbivores.

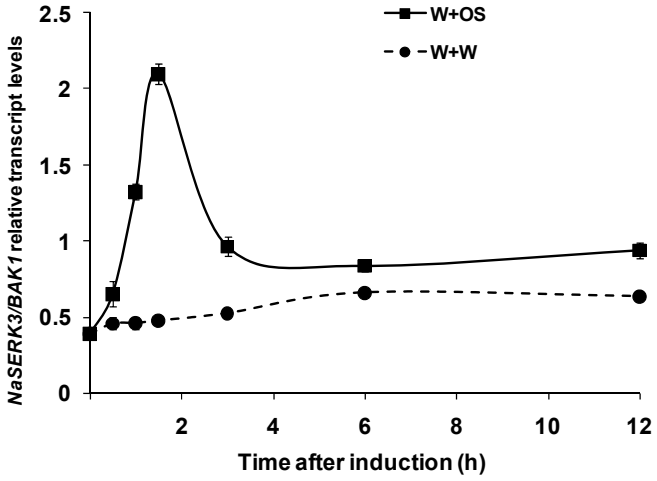


Figure 2. Herbivory specifically induces the accumulation of *NaBAK1* transcripts in *N. attenuata*.

N. attenuata leaves were wounded with a pattern wheel and 20 µl of water (W+W) or *M. sexta* oral secretions (W+OS) were immediately applied to each leaf. Samples were harvested at indicated times after treatments. The transcript levels (± SE) of *NaBAK1* were analyzed by quantitative real-time PCR.

Silencing of *NaBAK1* using VIGS

To study the function of NaBAK1 in *N. attenuata*'s responses to herbivory, we knocked down the transcript levels of *NaBAK1* gene using a VIGS approach (Saedler & Baldwin, 2004). A 283 bp fragment in the coding region of *NaBAK1* cDNA was cloned into the pTV00 vector to form pTV-NaBAK1. Plants were inoculated with *Agrobacterium* carrying pTV-NaBAK1 to generate *NaBAK1*-silenced (hereafter, NaBAK1-VIGS) plants; plants inoculated with *Agrobacterium* carrying pTV00 empty vector were used for comparison (hereafter, EV plants).

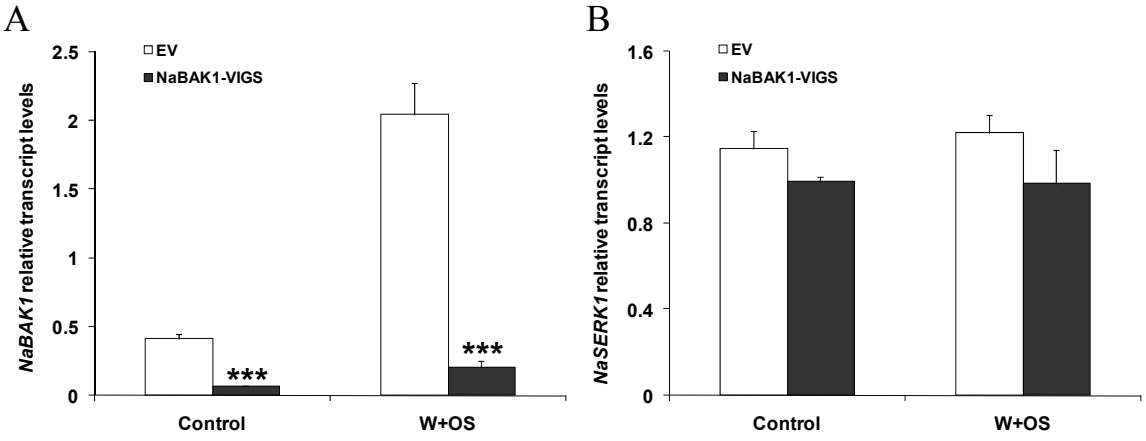


Figure 3. Silencing *NaBAK1*, but not *NaSERK1* by virus-induced gene silencing

N. attenuata plants were infiltrated with *Agrobacterium* carrying pTV00 or a pTV-NaBAK1 to generate EV and NaBAK1-VIGS plants, respectively. Mean (± SE) levels of *NaBAK1* (A) and *NaSERK1* (B) transcripts in EV and NaBAK1-VIGS plants were measured with qPCR in untreated (control) and 1 h W+OS-treated samples. Asterisks represent significantly different transcript levels between EV and NaBAK1-VIGS plants (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5).

To confirm the efficiency and specificity of gene silencing, *NaBAK1* and *NaSERK1* transcript levels were quantified in EV and NaBAK1-VIGS plants using qPCR. Transcript levels of *NaBAK1* in NaBAK1-VIGS plants were only 15% and 10% as high as those in EV plants before and 1 h after W+OS treatment, respectively (Figure 3A). In contrast, no significant difference in the transcript levels of *NaSERK1* was found between EV and NaBAK1-VIGS plants before and 1 h after W+OS treatment (unpaired *t*-test; $P = 0.1299$ for control and $P = 0.1808$ for W+OS treatment) (Figure 3B). These data suggest pTV-NaBAK1 construct efficiently and specifically silenced transcripts of *NaBAK1* without co-silencing *NaSERK1*.

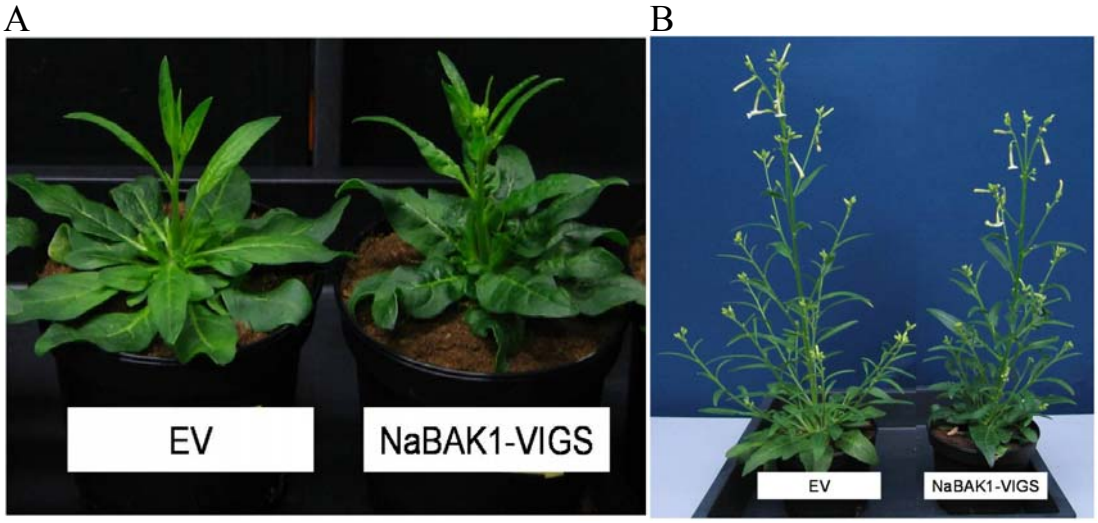


Figure 4. Morphology of EV and NaBAK1-VIGS plants

N. attenuata plants were inoculated with *Agrobacterium* carrying pTV00 empty vector or pTV-NaBAK1 to generate EV and *NaBAK1*-silenced (NaBAK1-VIGS) plants, respectively. (A) Plants at early elongation stage. (B) Plants at flowering stage.

Compared with those of EV plants, the leaves of NaBAK1-VIGS plants were somewhat darker green and moderately curly; in addition, by the late elongation stage, the NaBAK1-VIGS plants showed a semi-dwarf stature similar to that of Arabidopsis *bak1* mutants (Li *et al.*, 2002; Nam and Li, 2002) (Figure 4). All experiments were performed on the leaves of plants at the early elongation stage, when EV and NaBAK1-VIGS plants showed no substantial morphological difference (Figure 4A).

Silencing *NaBAK1* impairs the wounding- and herbivory-induced accumulation of JA and JA-Ile, but not the accumulation of SA and ethylene

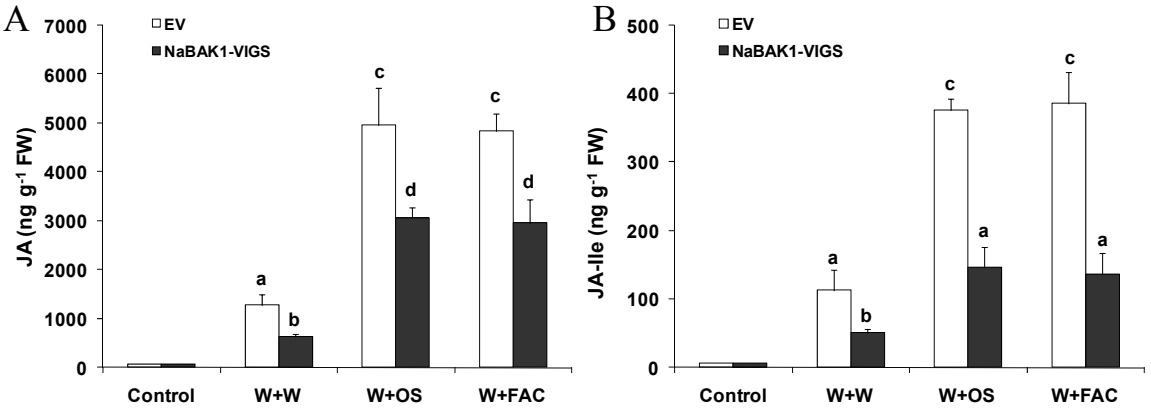
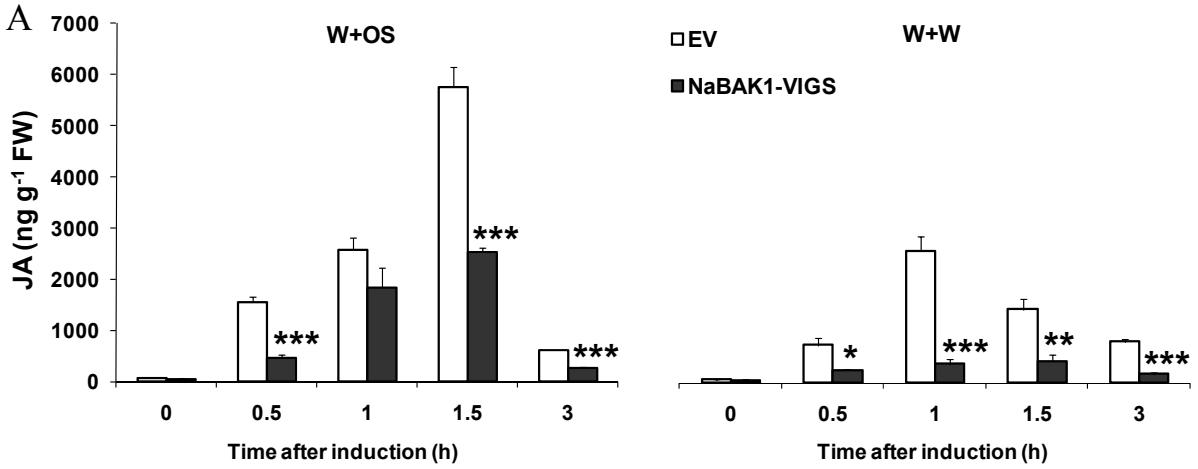


Figure 5. NaBAK1 positively regulates wounding- and herbivory-induced accumulation of JA and JA-Ile.

Leaves of EV and NaBAK1-VIGS plants were wounded with a pattern wheel and 20 µl of water (W+W), *M. sexta* oral secretions (W+OS), or FAC A solution (W+FAC) were immediately applied to each leaf. Individual leaves from 5 replicate plants were harvested after 1.5 h. Mean JA (A) and JA-Ile (B) concentrations (± SE) in EV and NaBAK1-VIGS plants were analyzed with HPLC-MS/MS. Different letters indicate significant differences (two-way ANOVA, Fisher's PLSD test; $p < 0.05$; N = 5).

To examine whether NaBAK1 regulates the wounding- and herbivory-induced accumulation of JA and JA-Ile, we elicited EV and NaBAK1-VIGS plants with W+W and W+OS, and measured their JA and JA-Ile content after 1.5 h. W+W and W+OS elicited highly increased levels of JA in EV plants, and silencing *NaBAK1* greatly diminished the accumulation of JA after these treatments (Figure 5A). Similarly, 1.5 h after either treatment the concentrations of JA-Ile in NaBAK1-VIGS plants were also highly reduced (Figure 5B). Examining the JA and JA-Ile bursts in EV and NaBAK1-VIGS plants throughout the duration of the burst revealed similar results (Figure 6). Notably, the differences of JA and JA-Ile levels in EV and NaBAK1-VIGS plants 1 h after W+W treatment were alleviated in the plants treated with W+OS (Figure 6), suggesting that NaBAK1 has somewhat different roles in modulating wounding- and herbivory-induced JA accumulation.



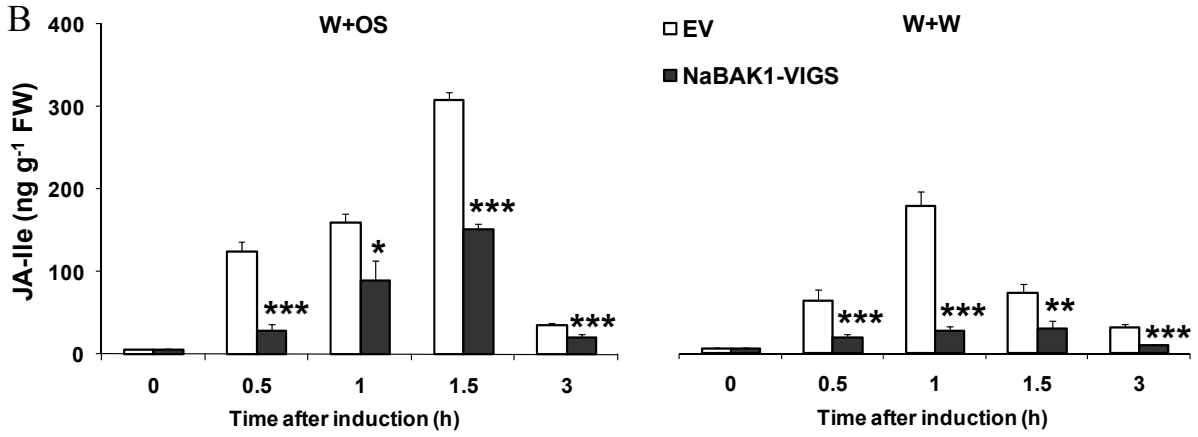


Figure 6. NaBAK1 positively regulates the W+OS- and W+W-induced accumulation of jasmonic acid (JA) and JA-isoleucine (JA-Ile).

Leaves of EV and NaBAK1-VIGS plants were wounded with a pattern wheel and 20 µl of water (W+W) or *M. sexta* oral secretions (W+OS) was immediately applied to each leaf. Individual leaves from 5 replicate plants were harvested at the indicated times. Asterisks represent significantly different levels between EV and NaBAK1-VIGS plants at indicated times (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5). (A) Mean (± SE) JA concentrations in EV and NaBAK1-VIGS plants. (B) Mean (± SE) JA-Ile concentrations in EV and NaBAK1-VIGS plants.

NaBAK1-silenced plants may have compromised perception of pathogens or pathogen-derived elicitors in *M. sexta* OS, and this could result in the decreased JA accumulation if these pathogens or pathogen-derived elicitors contribute to the elicitation of JA. To rule out this possibility, we wounded plants with a pattern wheel and applied a synthetic FAC, *N*-linolenoyl-I-Gln (18:3-Gln, I), (W+FAC), one of the most abundant FACs in *M. sexta* OS, and quantified JA and JA-Ile levels 1.5 h after W+FAC treatment. Similar to W+OS treatment, W+FAC induced the same JA and JA-Ile levels in EV plants, confirming that FACs are required and sufficient to elicit herbivory-induced JA and JA-Ile accumulation; nevertheless, W+FAC treatment also elicited significantly reduced JA and JA-Ile levels in NaBAK1-VIGS plants compared with those in EV plants (Figure 5). These data argue against the hypothesis that the impaired JA and JA-Ile accumulation was resulted from compromised pathogen- (or pathogen-derived elicitor)-induced responses in NaBAK1-VIGS plants.

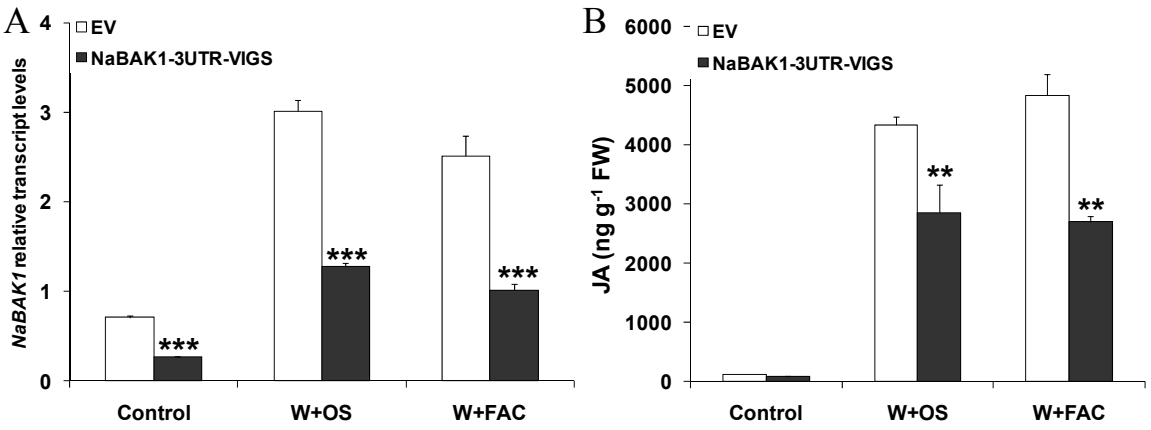


Figure 7 Specifically silencing *NaBAK1* by its 3' untranslated region (3UTR) results in impaired W+OS- and W+FAC-elicited JA levels.

N. attenuata plants were infiltrated with *Agrobacterium* carrying pTV00 or a pTV-NaBAK1-3UTR to generate EV and NaBAK1-3UTR-VIGS plants, respectively. Leaves of EV and NaBAK1-3UTR-VIGS plants were wounded with a pattern wheel and 20 µl of *M. sexta* oral secretions (W+OS) or FAC A solution (W+FAC) was immediately applied to each leaf. Individual leaves from 5 replicate plants were harvested after 1.5 h. Mean (± SE) levels of *NaBAK1* transcripts in EV and NaBAK1-3UTR-VIGS plants were measured with qPCR (A). Mean JA concentrations (± SE) in EV and NaBAK1-3UTR-VIGS plants were analyzed with HPLC-MS/MS (B). Asterisks represent significantly different transcript levels between EV and NaBAK1-3UTR-VIGS plants (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5).

To test whether NaBAK1 specifically regulates herbivory-elicited accumulation of JA, we further silenced transcripts of *NaBAK1* using its 3' untranslated region (UTR), and quantified W+OS- and W+FAC-induced JA levels in NaBAK1-3UTR-VIGS plants.

A unique 275 bp fragment of *NaBAK1*'s 3UTR was selected to specifically silence *NaBAK1* and to avoid co-silencing of other SERK family members. qPCR analysis revealed that transcripts of *NaBAK1* in NaBAK1-3UTR-VIGS plants were around 40% of those in EV plants before and 1.5 h after W+OS and W+FAC treatments (Figure 7A), suggesting that NaBAK1-3UTR-VIGS construct can successfully and significantly reduce transcript levels of *NaBAK1* in *N. attenuata*. Importantly, the W+OS- and W+FAC-elicited accumulation of JA was greatly impaired in NaBAK1-3UTR-VIGS plants (Figure 7B), which is consistent with the highly reduced herbivory-induced JA levels in NaBAK1-VIGS plants. These results suggest that silencing *NaBAK1* alone is sufficient to impair herbivory-elicited accumulation of JA.

However, the NaBAK1-VIGS construct showed higher silencing efficiency (10-15% of those in EV plants) than NaBAK1-3UTR-VIGS construct (37-42% of those in EV plants). Therefore, we used NaBAK1-VIGS plants for analyzing herbivory-elicited traits in the following experiments.

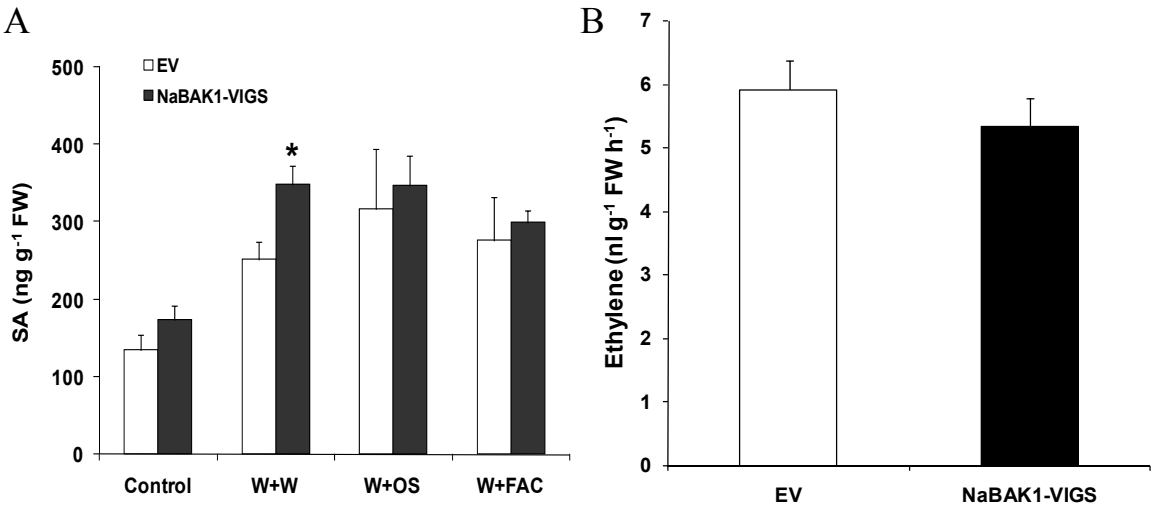
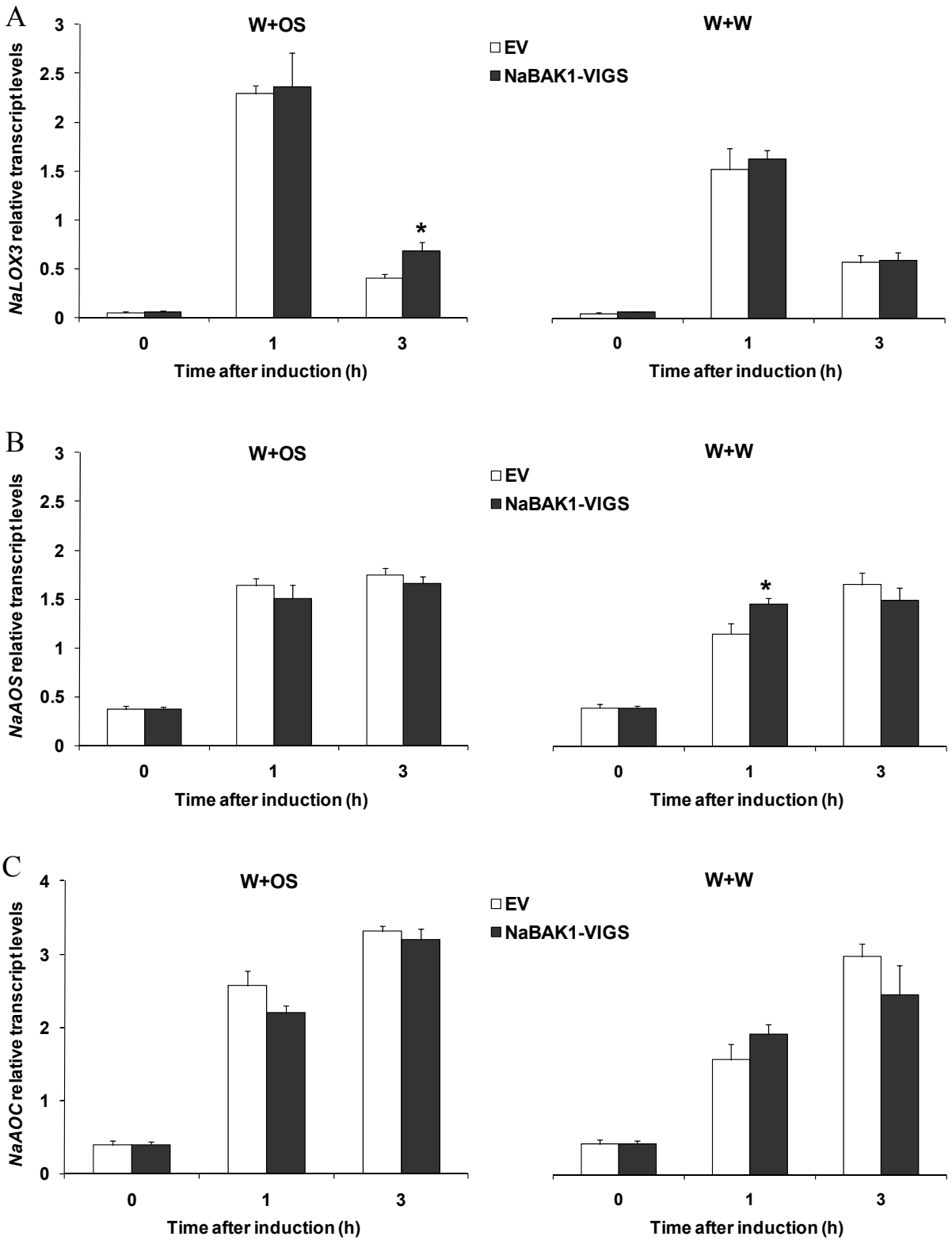


Figure 8. EV and NaBAK1-VIGS plants have the same levels of SA and ethylene production after W+OS induction.

Leaves of EV and NaBAK1-VIGS plants were wounded with a pattern wheel and 20 µl of water (W+W), *M. sexta* oral secretions (W+OS), or FAC A solution (W+FAC) was immediately applied to each leaf. Individual leaves from 5 replicate plants were harvested after 1.5 h. Asterisks represent significantly different levels between EV and NaBAK1-VIGS plants at indicated times (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5). (A) Mean SA concentrations (± SE) in EV and NaBAK1-VIGS plants. Leaves of EV and NaBAK1-VIGS plants were wounded with a pattern wheel and treated with 20 µl of *M. sexta* OS (W+OS). (B) Ethylene emission (± SE) was measured using a photoacoustic spectrometer 5 h after W+OS treatment (N = 5).

It is well known that SA antagonizes JA accumulation (Penacortes *et al.*, 1993; Doares *et al.*, 1995). Therefore, we measured the SA content in EV and NaBAK1-VIGS plants. We found no significant differences in the SA levels of EV and NaBAK1-VIGS plants after W+OS or W+FAC treatments, suggesting that the reduced herbivory-induced JA levels were not due to the antagonistic effect of SA levels in NaBAK1-VIGS plants (Figure 8A). Herbivory strongly increases ethylene biosynthesis and emissions, which is correlated with elevated defense levels in *N. attenuata* plants (von Dahl *et al.*, 2007). We determined the levels of W+OS-induced ethylene in both EV and NaBAK1-VIGS plants (Figure 8B), which,

unlike the levels of JA and JA-Ile, were not affected by *NaBAK1* silencing. Therefore, NaBAK1 is not involved in regulating herbivory-induced ethylene production in *N. attenuata*.



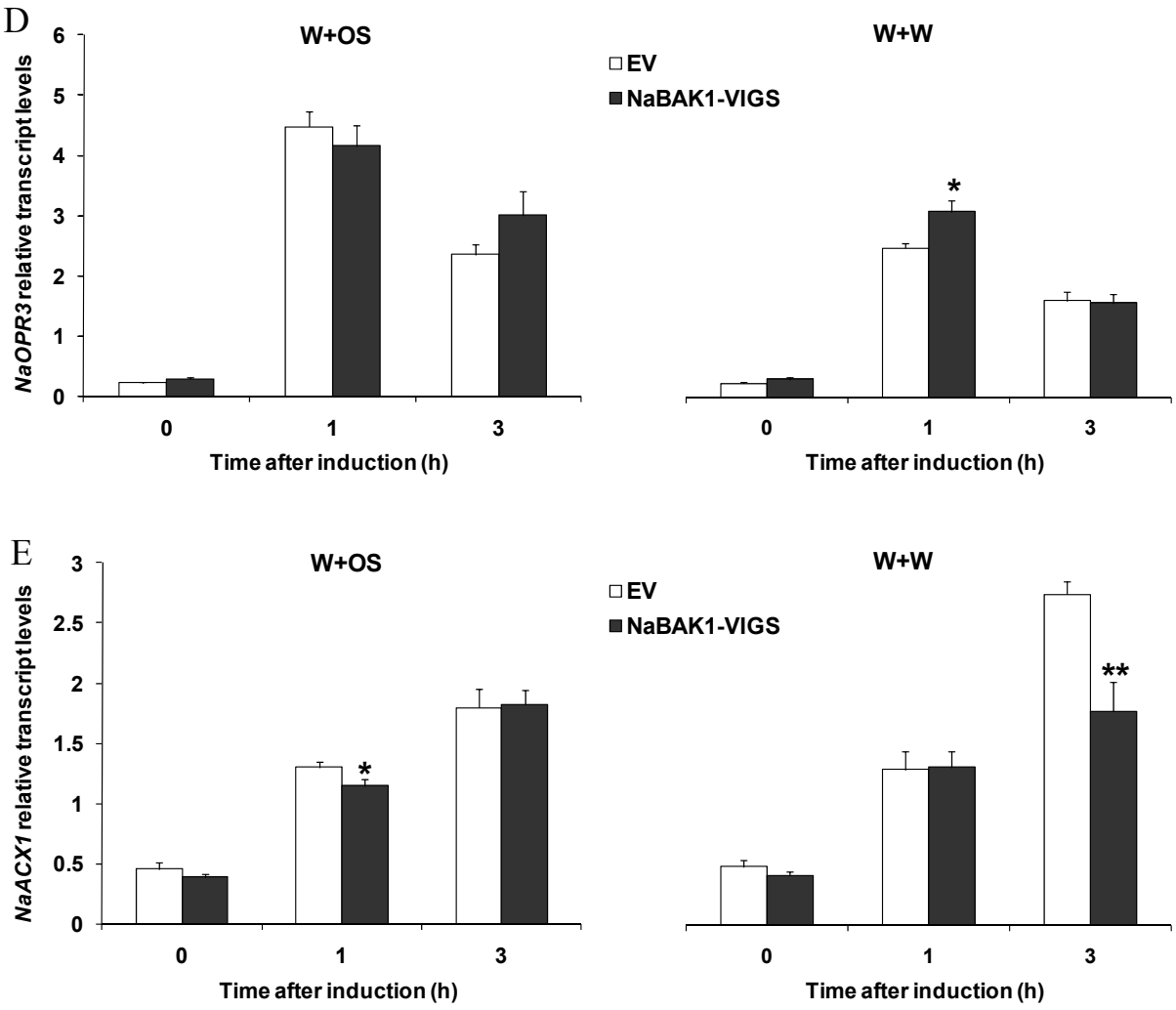


Figure 9. Transcript accumulation of JA biosynthetic genes in EV and NaBAK1-VIGS plants

Mean transcript levels (± SE) of *NaLOX3* (A), *NaAOS* (B), *NaAOC* (C), *NaOPR3* (D), and *NaACX1* (E) were measured with qPCR. Asterisks represent significantly different levels between EV and NaBAK1-VIGS plants at indicated times (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5).

To further explore whether the attenuated wounding- and herbivory-induced JA accumulation in NaBAK1-VIGS plants was associated with altered transcript levels of genes in the JA biosynthetic pathway, the transcript abundance of *NaLOX3*, *NaAOS*, *NaAOC*, *NaOPR3*, and *NaACX1,* was measured by qPCR. Comparing EV with NaBAK1-VIGS plants within 3 h, no large differences in transcript levels for any of these genes were found, except that minor differences were detected in *NaLOX3* (3 h after W+OS), *NaAOS* (1 h after W+W), *NaOPR3* (1 h after W+W), and *NaACX1* (1 h after W+OS and 3 h after W+W) (Figure 9). Therefore, the large differences of wounding- and herbivory-induced JA levels between EV and NaBAK1-VIGS plants probably resulted from posttranscriptional modifications of certain

genes/enzymes involved in JA biosynthesis, e.g. protein abundance or enzyme activity, rather than from changes in transcript abundance. Furthermore, these data suggest that NaBAK1 modulate the transcript levels of JA biosynthetic genes in a treatment- and gene-specific manner.

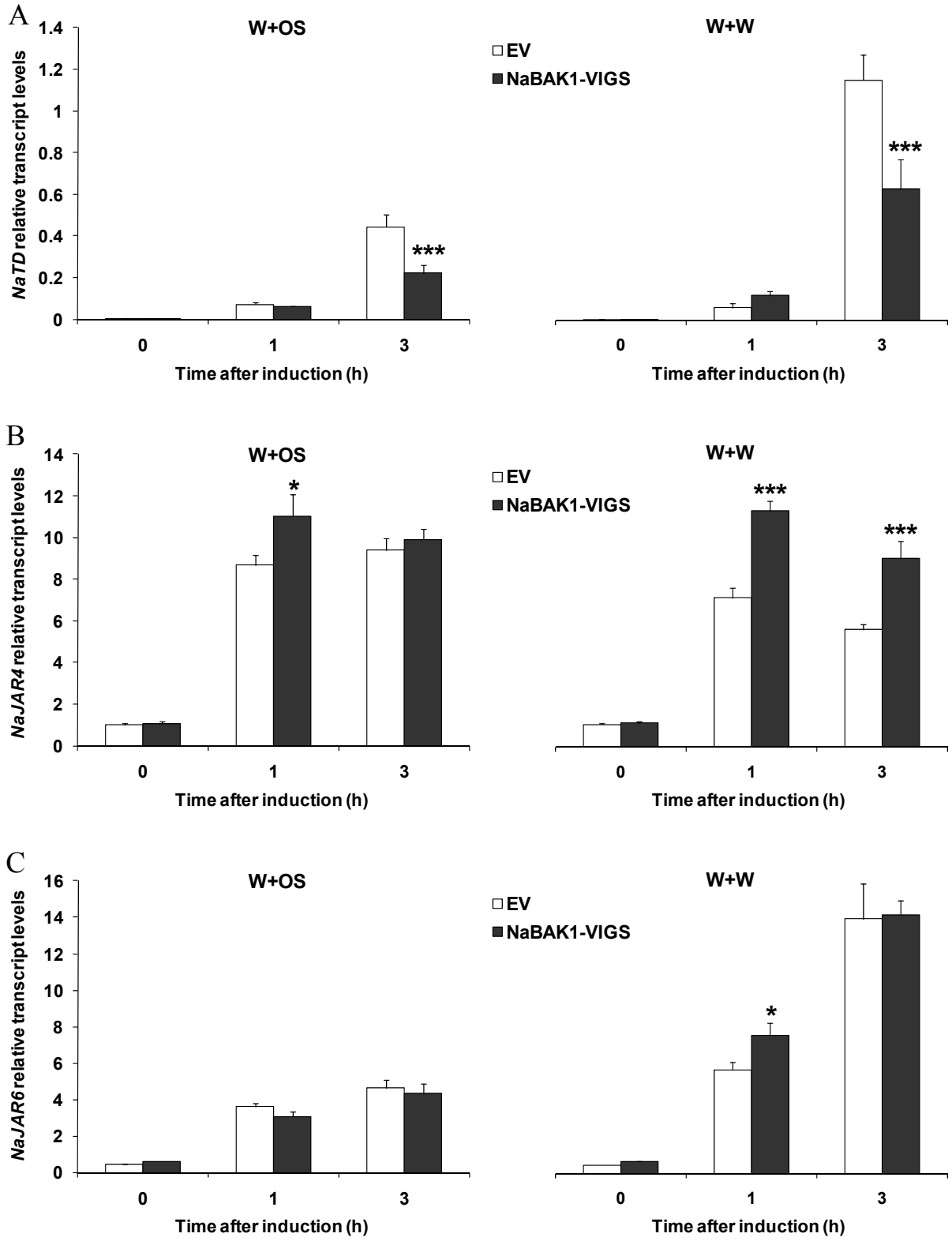


Figure 10. Transcript accumulation of *NaTD*, *NaJAR4*, and *NaJAR6* in wounding- and herbivory-induced EV and NaBAK1-VIGS plants.

Leaves of EV and NaBAK1-VIGS plants were wounded with a pattern wheel and 20 µl of water (W+W) or *M. sexta* oral secretions (W+OS) was immediately applied to each leaf. Leaves from replicate plants were harvested at the indicated times. Mean transcript levels (± SE) of *N. attenuata NaTD* (A) *NaJAR4* (B) and *NaJAR6* (C) were measured with quantitative real-time PCR. Asterisks represents significantly different levels between EV and NaBAK1-VIGS plants at indicated times (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5).

In *N. attenuata*, threonine deaminase (NaTD) plays an important role in converting Thr to Ile to supply the Ile used by NaJAR4 and NaJAR6 to conjugate Ile with JA to form JA-Ile (Kang *et al.*, 2006; Wang *et al.*, 2007; Wang *et al.*, 2008). We quantified the transcript abundance of these JA-Ile biosynthetic genes, and found that transcript levels of *NaTD* were significantly reduced in NaBAK1-VIGS plants compared with in EV plants only 3 h after treatments with both W+W and W+OS (Figure 10A). Conversely, *NaJAR4* and *NaJAR6* transcript levels were somewhat higher in NaBAK1-VIGS plants than in EV plants (Figure 10B, C). This suggests that the reduced levels of JA-Ile in NaBAK1-VIGS plants are not likely due to decreased NaJAR4 and NaJAR6 activity.

Together, these results indicate that NaBAK1 is required for the wounding- and herbivory-induced accumulation of JA and JA-Ile, and this regulation does not occur on a transcriptional level.

JA levels, rather than JAR activity, limit the accumulation of JA-Ile in NaBAK1-VIGS plants

In order to investigate whether impaired JAR activity and/or the limited availability of JA-Ile biosynthesis substrates (Ile and/or JA) account for the reduced JA-Ile levels in NaBAK1-VIGS plants, we supplied the substrates for JA-Ile biosynthesis to the wound site of EV and NaBAK1-VIGS plants during W+W and W+OS treatments and the resulted JA-Ile levels were quantified (Kang *et al.*, 2006; Paschold *et al.*, 2008).

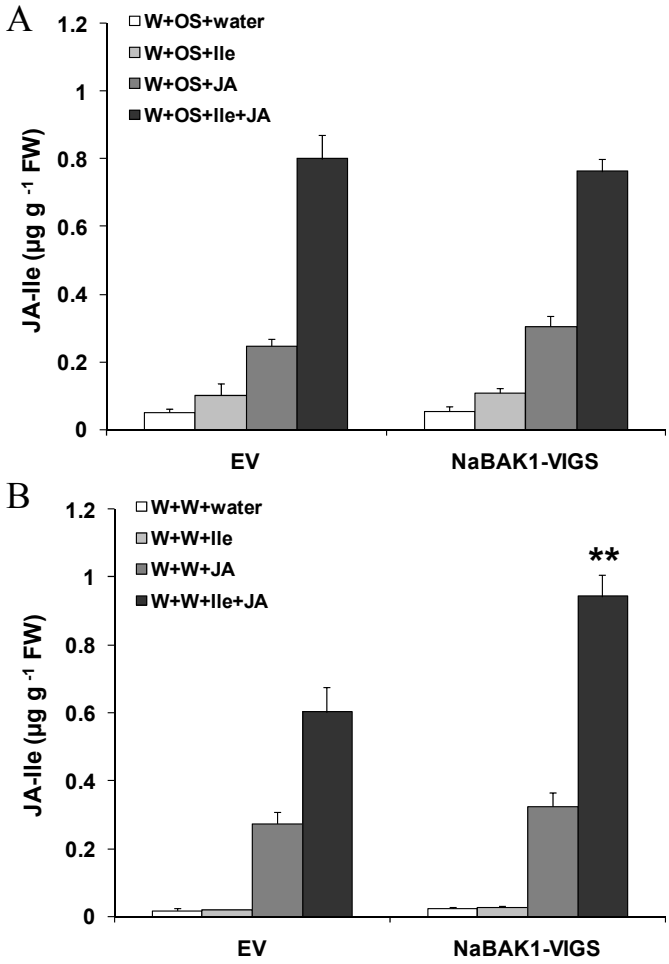


Figure 11. Ile and JA, rather than JAR activity, limit the accumulation of JA-Ile in EV and NaBAK1-VIGS plants.

Leaves of EV and NaBAK1-VIGS plants were wounded with a pattern wheel and treated with 20 µl of *M. sexta* oral secretions (OS) or water (W+OS and W+W, respectively) or an excess (0.625 nmol) of Ile or JA (W+OS+Ile, W+OS+JA, W+W+Ile, and W+W+JA) or both (W+OS+Ile+JA and W+W+Ile+JA). Mean JA-Ile levels (± SE) were analyzed 2 h after plants were OS elicited and supplied with an excess of JA-Ile biosynthesis substrates (A) and wounded and supplied with an excess of JA-Ile biosynthesis substrates (B). Asterisks represents significantly different levels between EV and NaBAK1-VIGS plants after indicated treatments (unpaired *t*-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N = 5).

The water and OS used for W+W and W+OS treatments were supplemented with either 0.625 µmol of Ile (W+W+Ile and W+OS+Ile), JA (W+W+JA and W+OS+JA), or both (W+W+Ile+JA and W+OS+Ile+JA). Compared with W+OS, treating EV plants with W+OS+Ile only slightly affect the levels of JA-Ile, whereas treating EV with W+OS+JA increased JA-Ile levels 3.8 fold (Fig. 6A). This suggests that JA but not Ile is the limited

factor for JA-Ile biosynthesis. Dramatically increased JA-Ile levels (15 fold) were detected when both Ile and JA were supplied (W+OS+Ile+JA); therefore only when excess amount of JA is applied (higher than endogenous levels of JA), Ile becomes the limited substrate (Figure 11A). Similar results were obtained from EV plants that were treated with W+W and supplied with these substrates (Figure 11B). Importantly, NaBAK1-VIGS plants had similar or somewhat elevated JA-Ile levels compared with EV plants after being supplied with Ile and JA (Figure 11). Apparently, when substrates are sufficiently available, NaBAK1-VIGS and EV plants have similar JAR activity to synthesize W+W- and W+OS-induced JA-Ile bursts.

We conclude that substrate availability, most likely the decreased JA levels in NaBAK1-VIGS plants, rather than the enzymatic activity of JAR proteins, are responsible for the reduced JA-Ile levels observed in these plants.

Silencing *NaBAK1* does not impair W+W- and W+OS-induced MAPK activity

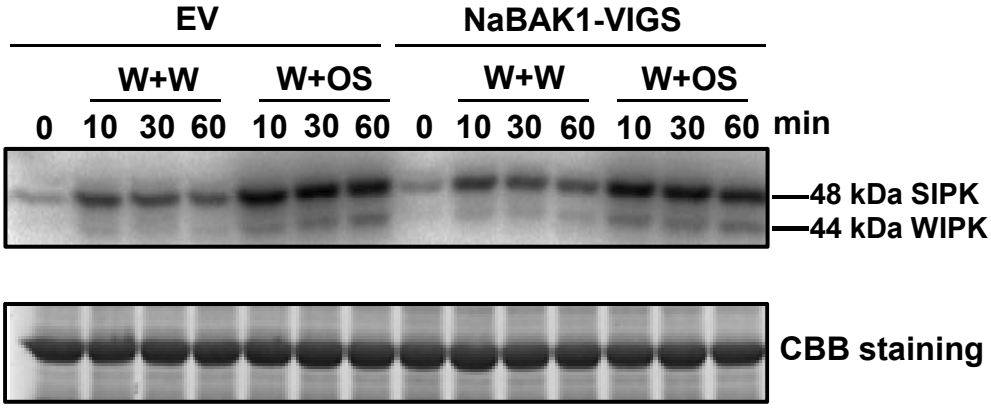


Figure 12. Silencing NaBAK1 does not impair wounding- and herbivory-induced MAPK activity in *N. attenuata* leaves.

Leaves of EV and NaBAK1-VIGS plants were treated with W+W or W+OS and harvested at the indicated times. MAPK activity was analyzed by an in-gel kinase assay using myelin basic protein as the substrate (upper panel). Same amount of each protein sample was run on another PAGE gel and stained with Coomassie Brilliant Blue (CBB) to confirm equal loading (bottom panel).

Compared with wild type plants, treating Arabidopsis *bak1* mutants with pathogen elicitor, flg22, leads to partially compromised MAPK activation (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). To test whether decreased JA levels after W+OS treatment were due to attenuated MAPK activity, we determined the MAPK activity in NaBAK1-VIGS and EV plants with an in-gel kinase assay. Both W+W and W+OS treatment induced similar levels of

SIPK and WIPK activity in EV and NaBAK1-VIGS plants (Figure 12). Therefore, we conclude that NaBAK1 mediates wounding- and herbivory-elicited JA and JA-Ile accumulation downstream of SIPK and WIPK or through a SIPK- and WIPK-independent pathway in *N. attenuata*.

***NaBAK1*-silenced plants have increased levels of basal and MeJA-induced TPI activity**

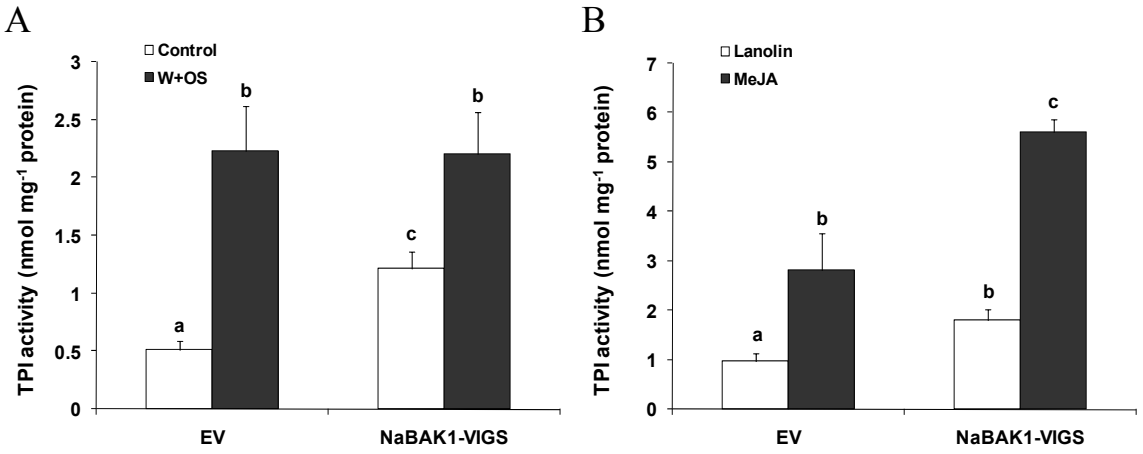


Figure 13. *NaBAK1*-silenced plants have increased levels of basal and MeJA-induced TPI activity.

Leaves from EV and NaBAK1-VIGS plants were left untreated (control) or wounded with a pattern wheel and 20 µl of *M. sexta* oral secretions was immediately applied to each leaf (W+OS). Samples were harvested three days after treatment. TPI activity (± SE) (A) was determined. Each EV and NaBAK1-VIGS plants was treated with 20 µl of lanolin pastes or lanolin pastes containing MeJA (7.5 mg ml^{-1}). After 3 days, samples were harvested, and TPI activity (± SE) (B) was analyzed. Error bars represent standard errors; different letters indicate significant differences (two-way ANOVA, Fisher's PLSD test; $p < 0.05$; N = 5).

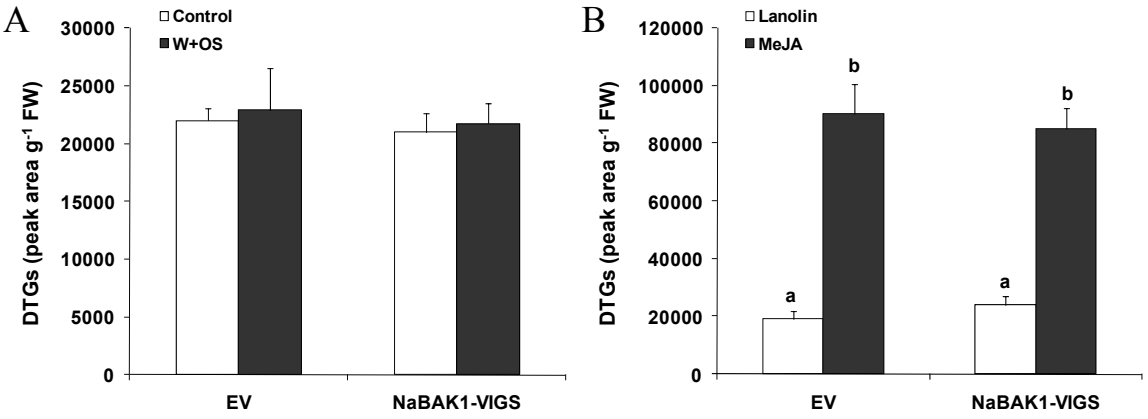


Figure 14. EV and NaBAK1-VIGS plants have similar levels of DTGs in response to W+OS and MeJA treatment

(A) Leaves from EV and NaBAK1-VIGS plants were left untreated (control) or wounded with a pattern wheel and 20 µl of *M. sexta* oral secretions were immediately applied to each leaf (W+OS). Samples were harvested three days after treatment, and DTG content were determined. (B) EV and NaBAK1-VIGS leaves were treated with 20 µl of lanolin or lanolin containing MeJA (7.5 µg μl^{-1}). Samples were harvested three days after treatment, and DTG content were determined. Error bars represent standard errors; different letters indicate significant differences (two-way ANOVA, Fisher's PLSD test; $p < 0.05$; N = 5).

We next examined whether after herbivory the impaired JA and JA-Ile accumulation in NaBAK1-VIGS plants led to reduced levels of defensive compounds against herbivores. Under glasshouse conditions, herbivore attack or W+OS treatment increases the contents of TPIs and DTGs in *N. attenuata* (Zavala *et al.*, 2004; Paschold *et al.*, 2007). However under the growth condition optimized for VIGS, neither EV nor NaBAK1-VIGS plants showed enhanced levels of DTGs after W+OS treatment; only TPIs were induced after this treatment (Figure 13A and 14A). We speculate that this was due to the relative low temperature and light intensity which were needed for the VIGS. Therefore, we focused our analyses on TPIs.

When untreated, EV and NaBAK1-VIGS had similar levels of JA and JA-Ile (Figure 5); however, compared with the basal TPI activity in EV plants, NaBAK1-VIGS plants exhibited around 100% higher levels of TPI activity; moreover, after W+OS treatment, although NaBAK1-VIGS plants had lower JA and JA-Ile than did EV plants, similar levels of TPI activity were detected in EV and NaBAK1-VIGS plants (Figure 13A). These data suggest that silencing *NaBAK1* enhances JA-induced TPI activity. To test this hypothesis, we applied 150 µg of MeJA dissolved in 20 µl of lanolin paste to one leaf of each EV and NaBAK1-VIGS plant (the same amount of lanolin was used for controls). In agreement with our hypothesis, after 3 days, MeJA induced higher levels of TPIs activity in NaBAK1-VIGS plants than in EV plants (Figure 13B). Moreover, similar MeJA-induced DTG levels were seen in EV and NaBAK1-VIGS plants (Figure 14B).

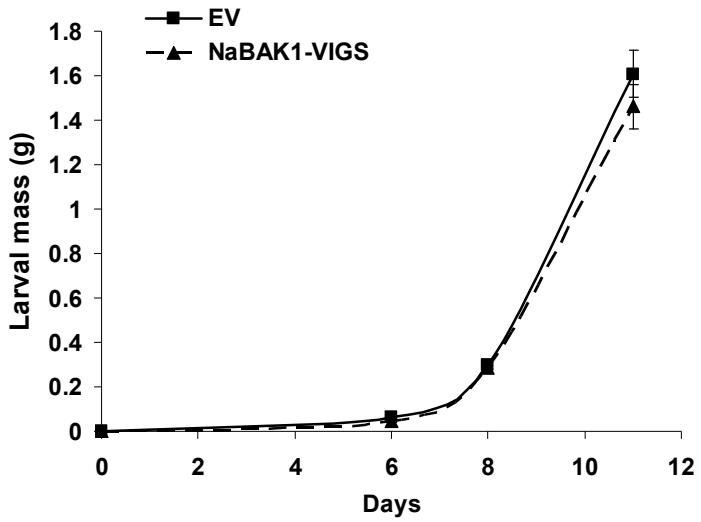


Figure 15. *M. sexta* has similar growth rates on EV and NaBAK1-VIGS plants

Each EV and NaBAK1-VIGS plant was infested with one *M. sexta* neonate. The larval mass was subsequently recorded on day 6, 8, and 11 (N = 25).

Bioassays were performed to determine whether silencing *NaBAK1* altered the performance of the specialist insect herbivore *M. sexta* on *N. attenuata* plants. Consistent with the equal levels of W+OS-induced defensive secondary metabolites in EV and NaBAK1-VIGS plants, *M. sexta* larvae gained similar masses on these plants (Figure 15).

Discussion

In both *N. benthamiana* and Arabidopsis, SERK3/BAK1 is required for maintaining plant innate immunity to pathogens (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). Here, we used a reverse genetic approach to examine the functions of SERK3/BAK1 in plants' resistance to herbivores. Our data demonstrate that SERK3/BAK1 is involved in regulating *N. attenuata*'s defense responses, such as accumulation of JA/JA-Ile and induction of TPI activity.

SERK3/BAK1 in SERK family

In Arabidopsis, *AtSERK3/BAK1* belongs to a small gene family consisting of five members. Particular combinations of SERKs, such as AtSERK1 and AtSERK2, AtSERK1 and AtSERK3/BAK1, and AtSERK3/BAK1 and AtSERK4/BKK1, play important roles in

BR-dependent and BR-independent signaling pathways, suggesting that closely related SERK members provide functional specificity (Albrecht *et al.*, 2008). AtSERK3/BAK1 has multiple functions in BR signaling (Li *et al.*, 2002; Nam and Li, 2002), innate immunity (Chinchilla *et al.*, 2007; Heese *et al.*, 2007), and cell death control (He *et al.*, 2007; Kemmerling *et al.*, 2007).

Two *SERK* genes in *N. attenuata* were cloned. Using a VIGS system, we specifically silenced *NaBAK1* (*NaSERK3*) without co-silencing its homologue, *NaSERK1*. Our cloning and searching a transcriptome database prepared by 454 sequencing did not resulted in finding other *SERK* genes. We suspect that either there are no other *SERK* genes in *N. attenuata* or they have extremely low transcript abundance. Whether other *SERK* genes exist in *N. attenuata*, and whether pTV-NaBAK1 construct attenuates transcripts of these *SERK*s remain unclear.

Function of NaBAK1 in herbivory-induced early responses in plants

In Arabidopsis, the binding of bacterial flagellin to the FLS2 receptor activates MAPK signaling (Asai *et al.*, 2002). SERK3/BAK1 as a component of plants' innate immunity has recently been identified: SERK3/BAK1 binds to FLS2 and is implicated in the activation of two MAPKs, MPK6 and MPK3, homologues of SIPK and WIPK in *Nicotiana*, respectively (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). Plants may possess receptors that perceive herbivory by binding with herbivore-derived cues, such as FACs (Truitt *et al.*, 2004); this hypothesis is further supported by the finding that FACs in herbivore OS rapidly activates MAPKs (Wu *et al.*, 2007). Unlike Arabidopsis *bak1* mutants, which have decreased flagellin-induced MAPK activity, *NaBAK1*-silenced plants have similar SIPK and WIPK activity as EV plants after W+OS treatment (Fig. 7). This suggests that NaBAK1 does not interact with the yet-to-be-identified FAC receptor. Furthermore, silencing *NaBAK1* also decreases wounding-induced JA, supporting the notion that NaBAK1 does not function at the level of perception of FACs.

Herbivory rapidly elevates MAPK activity, which is positively associated with JA biosynthesis in *N. attenuata* (Wu *et al.*, 2007). Although EV and NaBAK1-VIGS plants have similar levels of MAPK activity, *NaBAK1*-silenced plants have low levels of elicited JA, indicating that NaBAK1 modulates wounding- and herbivory-induced JA levels either downstream of MAPK signaling or in a MAPK-independent pathway. Our transcriptional analyses demonstrate that NaBAK1 is not associated with the transcriptional regulation of JA

biosynthetic genes; therefore, it is likely that NaBAK1 is involved in positively regulating the activity of certain JA-biosynthetic enzyme(s). In Arabidopsis, wounding increases the transcript levels of *AtPROPEP1* gene that codes the precursor of AtPep1 (a 23-amino acid peptide). The plant endogenous peptide elicitor AtPep1 can be perceived by a LRR-RLK, PEPR1, and thereafter activates expression of *PDF1.2* in a jasmonate-dependent manner (Huffaker et al., 2006; Yamaguchi et al., 2006). Importantly, application of *At*Pep1 to Arabidopsis cell suspension culture enhances the phosphorylation levels of BAK1 and induces the formation of heterocomplexes that consist of BAK1 and very likely PEPR1 (Schulze et al., 2010). Whether Pep1 and its receptor, PEPR1, exist in *N. attenuata*, and are also involved in wounding- and herbivory-induced JA accumulation are unknown. In addition, whether the binding of BAK1 to PEPR1 is required for Pep1-induced JA production deserves further study.

BR and JA signaling

JA signaling plays crucial roles in plant-herbivore interactions (Halitschke and Baldwin, 2003; Paschold *et al.*, 2007; Wang *et al.*, 2008). In untreated plants, which have similar levels of JA, NaBAK1-VIGS plants exhibit higher TPI activity. Although NaBAK1-VIGS plants have highly decreased JA levels after herbivory, the accumulation of herbivory-induced TPIs is not affected.

Analysis of MeJA-induced TPI levels confirmed that NaBAK1 negatively modulates levels of jasmonate-induced TPIs in *N. attenuata*. Conversely, the contents of DTGs are similar when EV and NaBAK1-VIGS are treated with MeJA. This suggests that the different MeJA-induced TPI levels in EV and *NaBAK1*-silenced plants are not due to differences in transport or metabolism of the applied MeJA, namely that MeJA diffuse more quickly into the leaves of NaBAK1-VIGS plants and MeJA is more efficiently hydrolyzed to JA (and subsequently converted to JA-Ile) in NaBAK1-VIGS plants, both of which would lead to elevated levels of MeJA-induced TPIs in NaBAK1-VIGS plants. Moreover, the distinct patterns of TPI and DTG accumulation also indicated the specificity of NaBAK1 in regulating different JA-induced defenses.

Recently, Campos *et al.* (2009) showed that the tomato BR biosynthesis mutant *dpy* has augmented levels of the anti-herbivore metabolite zingiberene, as well as elevated transcripts of the serine proteinase inhibitor *PI-I*, suggesting that BR negatively interacts with JA in the formation of anti-herbivory traits in tomato. We hypothesize that BR signaling

might also negatively regulate certain JA responses in *N. attenuata,* such as TPI accumulation, under herbivore attack. Consistent with this hypothesis, the *psc1* mutants have a leaky mutation of *DWARF4* gene that encodes a key enzyme in BR biosynthesis, and these mutants partially suppressed *coi1* mutants' insensitivity to JA inhibition of root growth in Arabidopsis seedlings (Ren *et al.*, 2009).

However, the possibility cannot be excluded that NaBAK1 regulates JA-induced TPI accumulation in a BR signaling-independent manner. In Arabidopsis SERK3/BAK1 mediates pathogen-induced programmed cell death independently of BR signaling (He *et al.*, 2007; Kemmerling *et al.*, 2007). Thus, it would be interesting to explore the dependence of SERK3/BAK1 on BR signaling in the regulation of herbivory-induced TPI responses using plants silenced in BR biosynthetic genes or *BRI1*, the BR receptor gene. Furthermore, these plants can also be used to investigate whether BR and its signaling play any roles in wounding- and herbivory-induced JA accumulation.

In summary, we show that NaBAK1 is a member of the herbivory-induced response network and plays multiple roles in plant-herbivore interactions: NaBAK1 is required for herbivory-induced JA and JA-Ile accumulation and NaBAK1 also plays a negative role in controlling TPI activity induced by herbivory-elicited jasmonates. Further study should address the molecular mechanism that NaBAK1 regulates herbivory-induced JA and TPI accumulation, and whether other SERKs are also involved in plant-herbivore interactions.

Supplementary material

Fig. S1. Alignment of SERK proteins.

Table S1 Primers used for cloning of *NaBAK1* and *NaSERK1*, and preparation of pTV-NaBAK1 construct

Table S2. Primer sequences used for quantitative real-time PCR (SYBR Green analysis)

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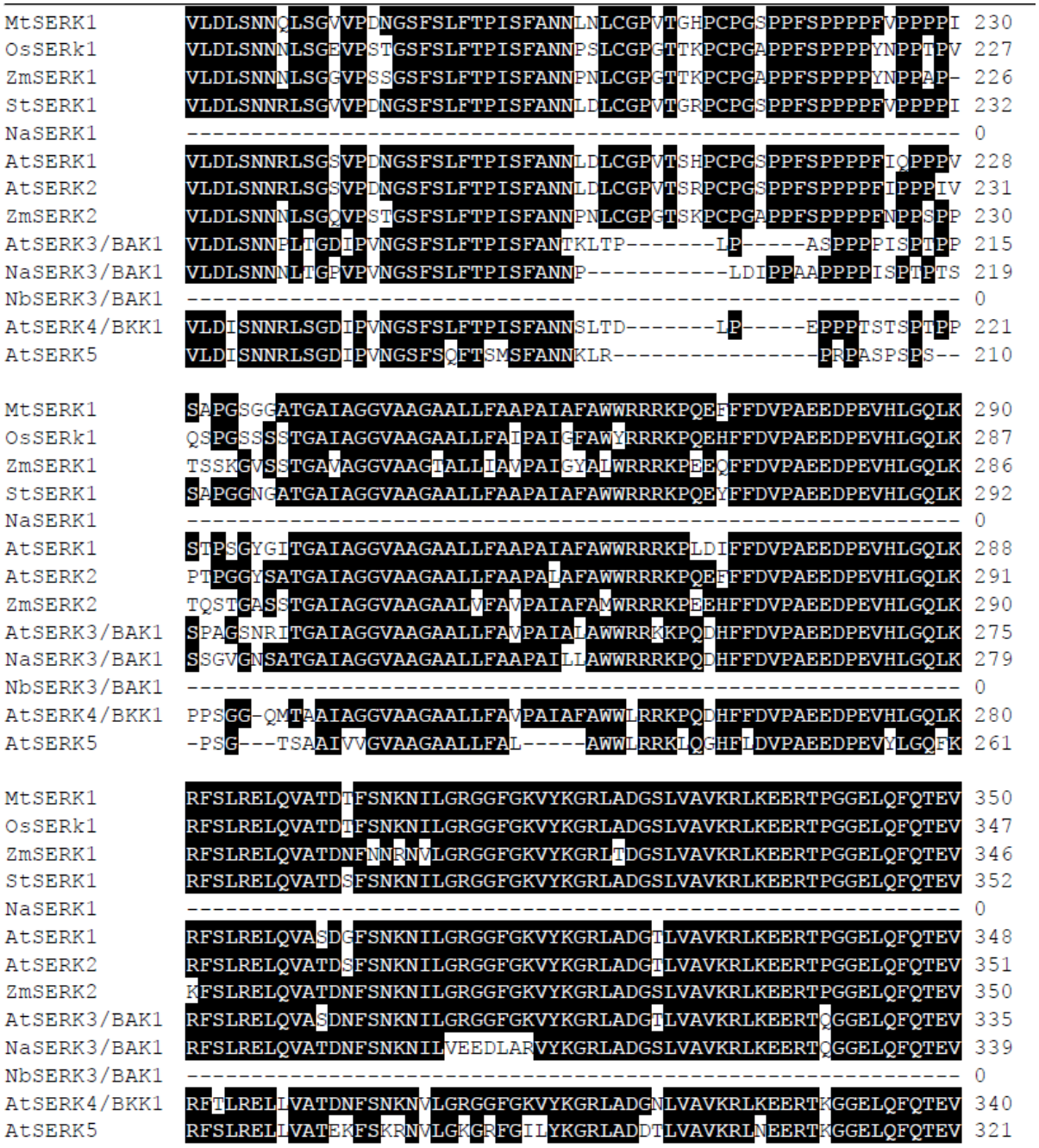
Supplementary material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AtSERK4/BKK1 RFTLRELLVATDNFSNKNVLGRGGFGKVYKGRLADGNLVAVKRLKEERTKGGELQFQTEV 340
AtSERK5 RFSLRELLVATEKFSKRNVLGKGRFGILYKGRLADDTLVAVKRLNEERTKGGELQFQTEV 321


MtSERK1 EMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPPHQEPLDWPTRKRIAL 410
OsSERk1 EMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASRLRERPPSEPPLDWRTRRRIAL 407
ZmSERK1 ELISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASRLRERAPNEPPLEWETRARIAL 406
StSERK1 EMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPPSEPPLXWPXRKRIAL 412
NaSERK1 ----------NGSVASCLRERPPSEPPLDWPTRKRIAL 28
AtSERK1 EMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPPSQPPLDWPTRKRIAL 408
AtSERK2 EMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPPSQLPLAWSIRQQIAL 411
ZmSERK2 EMISMAVHKNLLRLRGFCMTPTERLLVYPYMANGSVASRLRERQPSEPPLSWEPRRRIAL 410
AtSERK3/BAK1 EMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPESQPPLDWPKRQRIAL 395
NaSERK3/BAK1 EMISMAVHRNLLRLLGFCMTATERLLVYPYMSNGSVASRLRERPESDPPLEWSIRKRIAL 399
NbSERK3/BAK1 ---------- 0
AtSERK4/BKK1 EMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPEGNPALDWPKRKHIAL 400
AtSERK5 EMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPEGNPALDWPKRKHIAL 381
MtSERK1 GSARGLSYLHDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLMDYKDTHVTTAVRGT 470
OsSERk1 GSARGLSYLHDHCDPKIIHRDVKAANILLDEDFEAVVGDFGLAKLMDYKDTHVTTAVRGT 467
ZmSERK1 GSARGLSYLHDHCDPKIIHRDVKAANILLDEDFEAVVGDFGLAKLMDYKDTHVTTAVRGT 466
StSERK1 GSARGLSYLHDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLMDYKDTHVTTAVRGT 472
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NaSERK3/BAK1 GSARGLAYLHDHCDPKIIHRDVKAANILLDEEYEAVVGDFGLAKLMDYKDTHVTTAVRGT 459
NbSERK3/BAK1 ---------- 0
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AtSERK5 GSARGLAYLHDHCDQKIIHTDVKAANILLDEEFEAVVGDFGLAKLMNYNDSHVTTAVRGT 441
MtSERK1 IGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKE 530
OsSERk1 IGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKE 527
ZmSERK1 IGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKALLKE 526
StSERK1 IGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKE 532
NaSERK1 IGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKE 148
AtSERK1 IGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKE 528
AtSERK2 IGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKE 531
ZmSERK2 IGHIAPEYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKE 530
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NaSERK3/BAK1 IGHIAPEYLSTGKSSEKTDVFGYGVMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKD 519
NbSERK3/BAK1 ----------VFGYGVMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKD 41
AtSERK4/BKK1 IGHIAPEYLSTGKSSEKTDVFGYGVMLLELITGQKAFDLARLANDDDIMLLDWVKEVLKE 520
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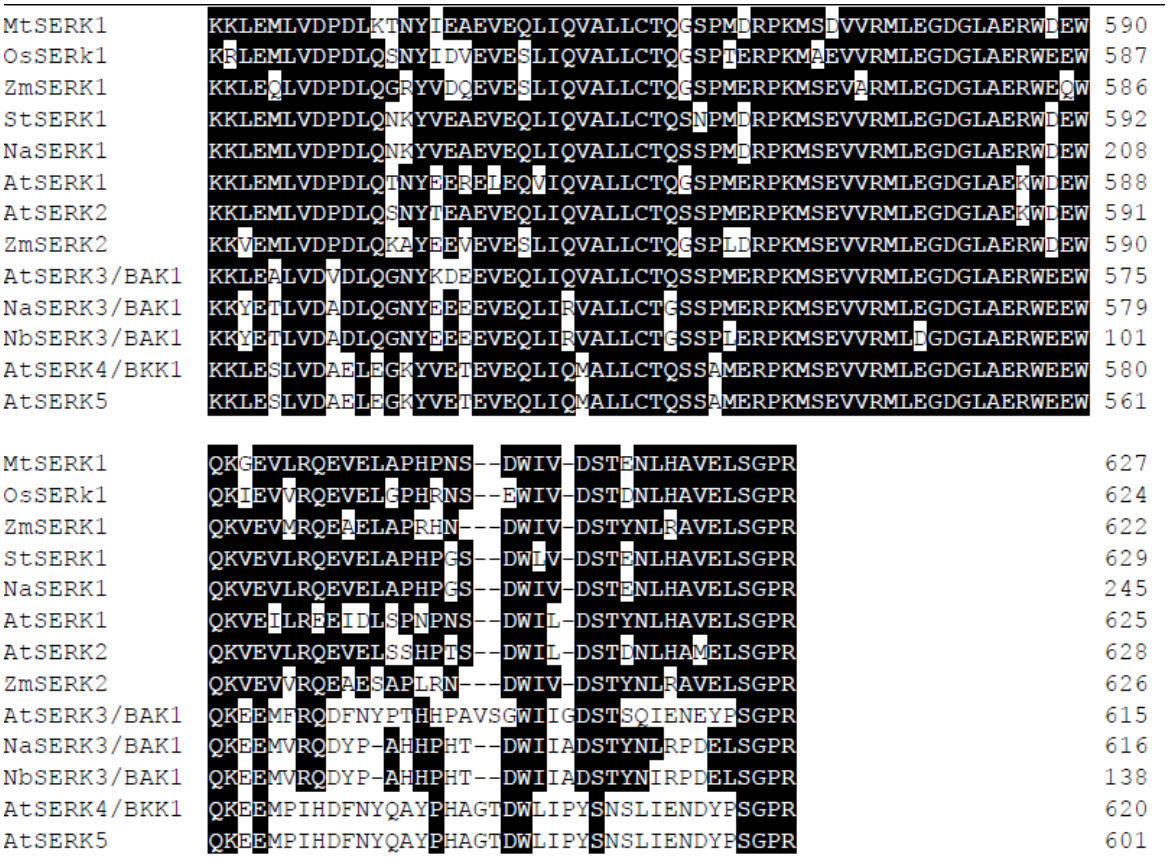

Figure S1. Alignment of SERK proteins

Amino acid sequence alignment of deduced *N. attenuata* NaBAK1, NaSERK1 (partial), *N. benthamiana* NbSERK3/BAK1 (partial), and other SERK proteins. NCBI (GenBank) accession numbers of *SERK* genes and SERK proteins included in this alignment are: *N. attenuata*: *NaBAK1* (HM639279), *NaSERK1* (HM639280); *N. benthamiana*: *NbSERK3/BAK1* (CK291393); *Arabidopsis thaliana*: AtSERK1 (NP_177328), AtSERK2 (NP_174683), AtSERK3 (NP_567920), AtSERK4 (NP_178999), AtSERK5 (NP_179000); *Solanum tuberosum*: StSERK1 (ABO14172); *Medicago truncatula*: MtSERK1 (AAN64294); *Oryza sativa*: OsSERK1 (BAD86793); *Zea mays*: ZmSERK1 (CAC37638); ZmSERK2 (CAC37639). Amino acids in white background are different from the consensus sequence.

Table S1 Primers used for cloning of NaBAK1 and NaSERK1, and preparation of pTV-NaBAK1 construct

Genes	Primer (5'-3')
***NaBAK1*-1**	ATGGATCAATGGATATTGGGGATC
***NaBAK1*-2**	GAACACCCACTATCTGATACATCCAG
***NaSERK1*-1**	AATGGAAGTGTTGCATCGTGCCTG
***NaSERK1*-2**	CGCCTATGTTCAACTTGTCAGGGCATAG
***NaBAK1*-VIGS-BamHI**	ACGT**GGATCC**AAGTGGAGTCTGCGATAATCC
***NaBAK1*-VIGS-SalI**	ACGT**GTCGAC**GATTGGGTCAAGGGACTTC
***NaBAK1*-3UTR-VIGS-BamHI**	GCGC**GGATCC**AACACCCACTATCTGATACATCC
***NaBAK1*-3UTR-VIGS-SalI**	CCGG**GTCGAC**TGATCGATCTTCCTGCAATTCTG

* Nucleotides underlined are *Bam*H I and *Sal* I sites. Stop codon (TGA) is labeled in red.

Table S2 Primer sequences used for quantitative real-time PCR (SYBR Green analysis)

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
NaActin2	GGTCGTACCACCGGTATTGTG	GTCAAGACGGAGAATGGCATG
NaBAK1	TTAAGTCTTTATATTTGTATGTCAGGAA	AAAAGAAAATACATTTGTGCTTCCAC
NaSERK1	GAACTCTAATTTTGTTGATCTTGAAAGTT	ATATAGCCACTACGCCTATGTTC
NaLOX3	GGCAGTGAAATTCAAAGTAAGAGC	CCCAAAATTTGAATCCACAACA
NaAOS	GACGGCAAGAGTTTTCCCAC	TAACCGCCGGTGAGTTCAGT
NaAOC	AACTACCTAACCCTCTCATTTCTCA	AAGCGAAGATAGGCAGGGC
NaOPR3	AATGGAGTTGGAGTTTGTTT	AGGTGGTTGAAGCAGTCGTT
NaACX1	GAATGTCTGTTGCTTGTGCTCA	TACCGCAAAGCACCTCCAG
NaTD	TAAGGCATTTGATGGGAGGC	TCTCCCTGTTCACGATAATGGAA
NaJAR4	ATGCCAGTCGGTCTAACTGAA	TGCCATTGTGGAATCCTTTTAT
NaJAR6	TGGAGTAAACGTTAACCCGAAA	AGAATTTGCTTGCTCAATGCCA

4. Discussion

JA plays a pivotal role in plant resistance to herbivores by modulating the accumulation of defense compounds (Howe and Jander, 2008; Browse, 2009; Wu and Baldwin, 2010). Almost all the enzymes involved in JA biosynthesis have been identified in various plant species. Recent advancement in jasmonate responses repressor JAZ proteins has also shed light on the mechanism of JA signaling (Katsir et al., 2008; Chini et al., 2009; Fonseca et al., 2009). However, still very little is known about how JA biosynthesis is regulated, especially after wounding and herbivory. In this thesis, two CDPKs, NaCDPK4 and NaCDPK5 and BAK1 were studied. These kinases were found to be negative and positive regulators for JA biosynthesis after both wounding and herbivory.

Protein kinases regulate JA biosynthesis in plant responses to insect herbivores and wounding

NaCDPK4 and NaCDPK5 negatively regulate herbivory-induced JA and JA-Ile levels

Ectopic expression of N-terminal *N. tabacum* CDPK2 lacking its autoinhibitory and calcium-binding domains (VK variants) in *N. bethamiana* results in elevated JA and OPDA levels, and impaired activation of salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) induced by osmotic stress (Ludwig et al., 2005). Furthermore, after Avr9 infiltration, compromised MAPK activity was observed in VK variants-expressed leaves compared with MAPK activity in full-length CDPK2 (VKJC variants)-expressed leaves. Importantly, expression of *N. tabacum* kinase-inactive VK $_{(D/A)}$ variants, which might decrease the activity of endogenous functional kinase-active *N. bethamiana* CDPKs through competing for substrates, resulted in enhanced MAPK activity than those in leaves expressing full length VKJC variants in the Avr9/Cf-9 interaction (Ludwig et al., 2005). Thus it was proposed that CDPKs negatively regulate MAPK activity in plant responses to abiotic (osmotic) and biotic (Avr9) stresses.

In *N. attenuata* two MAPK, SIPK and WIPK, positively modulate wounding- and herbivory-induced accumulation of JA, JA-Ile, and herbivory-elicited TPI levels (Wu et al., 2007). Given that silencing *NaCDPK4* and *NaCDPK5* results in elevated accumulation of herbivory-elicited JA, JA-Ile, and jasmonate-induced anti-herbivore secondary metabolites, it

is possible that NaCDPK4 and NaCDPK5 negatively mediate MAPK activity, which triggers the accumulation of herbivory-induced JA and JA-Ile in *N. attenuata*.

Recently, Ishida *et al.* (2008) showed that NtCDPK1 phosphorylated REPRESSION OF SHOOT GROWTH (RSG), a *N. tabacum* transcriptional activator of gibberellin (GA) biosynthetic enzymes, in response to GAs. Phosphorylation of Ser-114 of RSG by NtCDPK1 promotes 14-3-3 protein binding to RSG, which sequesters RSG in the cytoplasm to maintain the GA homeostasis in plants. Therefore, possibly NaCDPK4 and NaCDPK5 regulate JA homeostasis in a similar way in plant-herbivore interactions. NaCDPK4 and NaCDPK5 might phosphorylate an activator of JA biosynthetic enzymes and thus deactivate this activator of JA biosynthesis. Alternatively, NaCDPK4 and NaCDPK5 might phosphorylate and deactivate a suppressor of JA biosynthetic enzymes. Thus, silencing *NaCDPK4* and *NaCDPK5* results in high levels of herbivory-elicited JA. Our analysis of the transcriptional profile of JA biosynthetic genes showed that JA biosynthetic enzymes do not have greatly enhanced transcript levels, suggesting that these JA biosynthesis enzymes are regulated at post-transcriptional levels in (*NaCDPK4* and *NaCDPK5*)-silenced plants. Identification of substrates for NaCDPK4 and NaCDPK5, and measuring the protein abundance and catalytic activity of JA biosynthetic enzymes are particularly important for understanding how these kinases mediate JA homeostasis.

Plants with higher JA levels

Thus far, only a handful of genes are known to be negative regulators of JA accumulation. Arabidopsis *cev1* mutants (defect in a cellulose synthase, CeAS3) grow smaller than do WT plants, and have stunted root length and longer root hair. *cev1* mutant accumulates anthocyanin, constitutively expresses defense-related genes, and have enhanced resistance to fungal pathogen powdery mildew (Ellis and Turner, 2001; Ellis et al., 2002). Furthermore, *cev1* plants had elevated basal JA and ethylene levels. Importantly, cellulose inhibitors-treated WT plants represented some of the *cev1* phenotypes, suggesting that cell wall signaling is involved in regulating JA and ethylene responses (Ellis et al., 2002). However, (*NaCDPK4* and *NaCDPK5*)-silenced plants had similar basal JA and (W+OS)-elicited ethylene levels, and IRcdpk5 seedlings grow similarly to WT seedlings (data not shown). Thus, it is very unlikely that NaCDPK4 and NaCDPK5 modulate herbivory-induced JA levels through regulating cellulose synthase.

Another two gain of function mutants, *dgl-D* (Hyun et al., 2008) and *fou2* (Bonaventure et al., 2007), show highly elevated JA levels after being wounded. Measuring

the transcript levels of *NaGLA1*, the homologue of *DGL* gene in *N. attenuata* (Kallenbach et al., 2010), didn't reveal any changes on *NaGLA1*'s transcript levels. It is valuable to examine whether NaGLA1 and TPC [the locus of fou2 encodes a Ca2+-permeant non-selective cation channel, named Two Pore Channel 1 (TPC1)] have up-regulated protein abundance/activity, which leads to the higher JA levels in IRcdpk5 plants.

Function of NaBAK1 in herbivory-induced early responses in plants

In Arabidopsis, the binding of bacterial flagellin to the FLS2 receptor activates MAPK signaling (Asai et al., 2002). SERK3/BAK1 as a component of plants' innate immunity has recently been identified: SERK3/BAK1 binds to FLS2 and is implicated in the activation of two MAPKs, MPK6 and MPK3, homologues of SIPK and WIPK in *Nicotiana*, respectively (Chinchilla et al., 2007; Heese et al., 2007). Plants may possess receptors that perceive herbivory by binding with herbivore-derived cues, such as FACs (Truitt et al., 2004); this hypothesis is further supported by the finding that FACs in herbivore OS rapidly activates MAPKs (Wu et al., 2007). Unlike Arabidopsis *bak1* mutants, which have decreased flagellin-induced MAPK activity, *NaBAK1*-silenced plants have similar SIPK and WIPK activity as EV plants after W+OS treatment. This suggests that NaBAK1 does not interact with the yet-to-be-identified FAC receptor. Furthermore, silencing *NaBAK1* also decreases wounding-induced JA, supporting the notion that NaBAK1 does not function at the level of perception of FACs. However, whether NaBAK1 regulates wounding-induced responses through interaction with sensors for wounding or wounding-elicited DAMPs remain unknown.

Independently silencing *SIPK* and *WIPK* results in greatly impaired herbivory- and wounding-induced JA bursts (Wu et al., 2007). Although EV and *NaBAK1*-VIGS plants have similar levels of MAPK activity, *NaBAK1*-silenced plants have decreased levels of elicited JA, indicating that NaBAK1 modulates herbivory- and wounding-induced JA levels either downstream of MAPK signaling or in a MAPK-independent pathway.

In Arabidopsis, wounding increases the expression levels of *AtPROPEP1* gene, which codes the precursor of plant endogenous peptide elicitor AtPep1 (a 23-amino acid peptide). This AtPep1 is perceived by a cell membrane receptor (LRR-RLK), PEPR1, and thereafter activates expression of *PDF1.2* in a jasmonate-dependent manner (Huffaker et al., 2006; Yamaguchi et al., 2006). Importantly, application of AtPep1 to Arabidopsis cell suspension culture enhances the phosphorylation levels of AtBAK1 and induces the formation of heterocomplexes that consist of AtBAK1 and very likely AtPEPR1 (Schulze et al., 2010).

Whether PEPR1 is also involved in wounding- and herbivory-induced JA accumulation and binding of BAK1 to PEPR1 is required for Pep1-induced JA production deserve further study.

Finally, our transcriptional analyses demonstrate that NaBAK1 is not associated with the transcriptional regulation of JA biosynthetic genes; therefore, it is likely that NaBAK1 is involved in positively regulating the protein abundance and/or activity of certain JA-biosynthetic enzyme(s).

Protein kinases regulate JA signaling-mediated anti-herbivore secondary metabolites in plant immunity to insect herbivores

NaCDPK4 and NaCDPK5 modulate anti-herbivore secondary metabolites, which depends on JA and JA signaling (COI1).

In Arabidopsis, CEV1 (CeAS3) regulates anthocyanin accumulation and transcriptional activation of *VSP1* gene in a JA signaling (COI1)-dependent fashion. However, the phenotype of longer root hair is not controlled by COI1 in *cev1* mutant, which indicates part of the *cev1* phenotypes is depend on COI1 (Ellis and Turner, 2001). Significantly, the impaired root length of light-grown *cev1* seedling can be partially rescued by *coi1* mutant, confirmed the COI1 regulates part but not all of *cev1* phenotypes (Ellis et al., 2002).

Silencing *NaCDPK4* and *NaCDPK5* results in elevated wounding- and herbivory-induced accumulation of JA and anti-herbivore secondary metabolites (DTGs, CP, and TPIs). Consistently, (*NaCDPK4* and *NaCDPK5*)-silenced plants had enhanced resistance to *M. sexta* larvae. In contrast to the observation that COI1 only regulates part of phenotypes of *cev1* mutants, all the herbivore resistance traits we tested are depend on JA levels and JA signaling (NaCOI1). However, we cannot rule out the possibility that NaCDPK4 and NaCDPK5 regulate other traits (for example, pathogen resistance) in a NaCOI1-independent manner. Whether NaCDPK4 and NaCDPK5 also regulate responses to other environmental stimuli in a JA/JA signaling-independent pathway deserves further study.

BAK1 negatively regulates MeJA-induced TPI activity

Analysis of MeJA-induced TPI levels confirmed that NaBAK1 negatively modulates the levels of jasmonate-induced TPIs in *N. attenuata*. Conversely, the contents of DTGs are similar when EV and *NaBAK1*-VIGS are treated with MeJA. This suggests that the different MeJA-induced TPI levels in EV and *NaBAK1*-silenced plants are not due to differences in

transport or metabolism of the applied MeJA, namely that MeJA diffuse more quickly into the leaves of *NaBAK1*-VIGS plants and MeJA is more efficiently hydrolyzed to JA (and subsequently converted to JA-Ile) in *NaBAK1*-VIGS plants, both of which would lead to elevated levels of MeJA-induced TPIs in *NaBAK1*-VIGS plants. Moreover, the distinct patterns of TPI and DTG accumulation also indicated the specificity of NaBAK1 in regulating different JA-induced defenses.

Whether BAK1 regualtes herbivory-elicited TPI activity in a BR/BR signaling dependent manner?

Recently, Campos *et al.* (2009) showed that the tomato BR biosynthesis mutant *dpy* has augmented levels of the anti-herbivore metabolite zingiberene, as well as elevated transcripts of the serine proteinase inhibitor *PI-I*, suggesting that BR negatively interacts with JA in the formation of anti-herbivory traits in tomato. We hypothesize that BR signaling might also negatively regulate certain JA responses in *N. attenuata*, such as TPI accumulation, under herbivore attack. Consistent with this hypothesis, the *psc1* mutants have a leaky mutation of *DWARF4* gene that encodes a key enzyme in BR biosynthesis, and these mutants partially suppressed *coi1* mutants' insensitivity to JA inhibition of root growth in Arabidopsis seedlings (Ren et al., 2009).

However, we cannot exclude the possibility that NaBAK1 regulates JA-induced TPI accumulation in a BR signaling-independent manner. In Arabidopsis SERK3/BAK1 mediates pathogen-induced programmed cell death in a BR signaling-independent manner (He et al., 2007; Kemmerling et al., 2007). Thus, it would be interesting to explore the dependence of SERK3/BAK1 on BR signaling in the regulation of herbivory-induced TPI responses using plants silenced in BR biosynthetic genes or *BRI1*, the BR receptor gene. Furthermore, these plants can also be used to investigate whether BR and its signaling play any roles in wounding- and herbivory-induced JA accumulation.

Whether BAK1 regulates herbivory-elicited TPI activity through specific JAZ proteins?

COI1 is required for all JA-induced transcriptional changes/responses (Xie et al., 1998; Devoto et al., 2005), and was identified recently as the jasmonate receptor (Yan et al., 2009). Importantly, JAZ proteins function as repressors in COI1-mediated JA signaling. The JAZ family consists of 12 JAZ proteins, which may have overlapping functions (Chini et al., 2007; Thines et al., 2007). It is possible that individual JAZ or particular combination of certain JAZ proteins regulate specific jasmonate responses. Therefore, it would be very

interesting to investigate whether NaBAK1 specifically regulates particular NaJAZ proteins and thus controls the levels of TPIs but not other secondary metabolites.

CDPKs in plant immunity to pathogens

Silencing NaCDPK4 and NaCDPK5 enhances N. attenuata's resistance to Pst DC3000

Recently, Boudscoq *et al.* showed that AtCDPKs (AtCDPK4, 5, 6, and 11) positively regulate flg22-induced responses in Arabidopsis. However, transcriptional analysis revealed that CDPK and MAPK cascades independently regulate MAMP-elicited early gene responses. Given the highly increased resistance to *Pst* DC3000 in IRcdpk5 plants, whether NaCDPK4 and NaCDPK5 negatively regulate plant resistance to *Pst* Dc3000 through MAPK cascades should be examined in future studies.

It is well known that SA plays critical roles for plant resistance to pathogens. *Pst* DC3000 induced similar SA levels in both WT and *NaCDPK4* and *NaCDPK5*-silenced plants. However, IRcdpk5 plants have enhanced resistance to *Pst* DC3000, suggesting that NaCDPK4 and NaCDPK5 regulate plant resistance to *Pst* DC3000 in a SA-independent manner. Furthermore, *Pst* DC3000 amplified to the similar levels in WT and *NahG* overexpressing plants which have highly impaired pathogen-elicited accumulation of SA. This further confirms that SA is not involved in *N. attenuata*'s resistance to *Pst* DC3000.

Functional redundancy of protein kinases in plant-herbivore and plant-pathogen interactions

Functional redundancy of NaCDPK4 and NaCDPK5 in plant-herbivore and plant-pathogen interactions

Arabidopsis CPK4, 5, 6, 11, which belong to the same subgroup (I) of the four subgroups of AtCPK family (Boudsocq et al., 2010). Pathogen elieictor flg22-induced responses and *Pst* DC3000 susceptibility are not different between single *cpk* (4, 5, 6, and 11) mutant and WT plant, but flg22-elicited ROS and pathogen resistance of seedling are impaired in double (*cpk5 cpk6*) or triple (*cpk5 cpk6 cpk11*) mutants, demonstrating functional redundancy of CDPKs in plant-pathogen interactions.

NaCDPK4 and NaCDPK5 clustered with NtCDPK4, NtCDPK5, MtCDPK1, StCDPK1, and AtCDPK18, 16, 28 in the phylogenetic analysis. MtCDPK1 is involved in regulation of cell expansion or cell wall synthesis, defense genes expression, and symbiotic

interactions in root (Ivashuta et al., 2005). Additionally, StCDPK1 regulates tuber development in potato (Gargantini et al., 2009). Given that (*NaCDPK4* and *NaCDPK5*)-silenced plants showed dwarf status in the elongated stage, we infer that NaCDPK4 and NaCDPK5 might be involved in regulating root or stem development in *N. attenuata*.

Only three genes, AtCPK16, AtCPK18, and AtCPK28 exist in subgroup IV, forming the smallest subgroup of the Arabidopsis CDPK family (Cheng et al., 2002). Up to now, there are no reports on these members of subgroup IV. It would be interesting to investigate whether other CDPKs are closely related to NaCDPK4 and NaCDPK5 in *N. attenuata*, and what their physiological functions are. Furthermore, specifically silencing *NaCDPK5* is very necessary to dissect the specific roles of NaCDPK4 and NaCDPK5 in plant-herbivore and plant-pathogen interactions.

SERK3/BAK1 in SERK family

In Arabidopsis, AtSERK3/BAK1 belongs to a small gene family consisting of five members. Particular combinations of SERKs, such as AtSERK1 and AtSERK2, AtSERK1 and AtSERK3/BAK1, and AtSERK3/BAK1 and AtSERK4/BKK1, play important roles in BR-dependent and BR-independent signaling pathways, suggesting that closely related SERK members provide functional specificity (Albrecht et al., 2008). AtSERK3/BAK1 has multiple functions in BR signaling (Li et al., 2002; Nam and Li, 2002), innate immunity (Chinchilla et al., 2007; Heese et al., 2007), and cell death control (He et al., 2007; Kemmerling et al., 2007).

Two SERK genes in *N. attenuata* were cloned. Using a VIGS system, we specifically silenced *NaBAK1* (*NaSERK3*) without co-silencing its homologue, *NaSERK1*. Our cloning and searching a transcriptome database prepared by 454 sequencing did not result in finding other SERK genes. We suspect that either there are no other *SERK* genes in *N. attenuata* or they have extremely low transcript abundance in leaves.

5. Conclusion

Although the central roles of jasmonates (JA and JA-Ile) biosynthesis and signaling have been well studied in plant-herbivore interactions, how the early signaling components regulate biosynthesis of JA in plant responses to herbivore attack is unclear. Herbivory rapidly elicits Ca^{2+} spike in infested regions of plants, and this Ca^{2+} signature need to be sensed by Ca^{2+}-binding proteins, such as Ca^{2+}-dependent protein kinase (CDPK), to activate downstream defense responses.

Whether Ca^{2+}-dependent protein kinases (CDPKs), which can decode the herbivory-elicited Ca^{2+} signature, and the co-receptor for RLKs (BAK1), which might be required for full recognition of herbivore-derived cues by RLKs, are involved in plant-herbivore interactions were not known. In this study, we silenced the transcript levels of *NaCDPK4*, *NaCDPK5*, and *NaBAK1*, and examined their functions in *N. attenuata*'s defense responses to specialist herbivore *M. sexta* larvae. Our results suggest that these protein kinases belong to the early signaling networks in plant-herbivore interactions.

N. attenuata NaCDPK4 and NaCDPK5 negatively regulate plant resistance to both herbivore *M. sexta* and pathogen *Pst* DC3000: silencing *NaCDPK4* and *NaCDPK5* resulted in elevated herbivory-induced accumulation of JA, JA-Ile, and anti-herbivore secondary metabolites. Moreover, NaCDPK4 and NaCDPK5 regulate herbivore resistance in a JA- and JA signaling (COI1)-dependent manner. Furthermore, silencing *NaCDPK4* and *NaCDPK5* in *N. attenuata* highly elevates plant resistance to *Pst* DC3000 independent of pathogen-elicited SA. Future work should focus on the regulation/phosphorylation, subcellular localization, and activation by calcium (or other activators) of NaCDPK proteins using genetic and biochemical approaches. Importantly, identification of NaCDPKs' substrates by yeast two hybrid and/or other newly developed techniques could provide useful insight into how CDPKs regulate plant herbivore and pathogen resistance in *N. attenuata*.

NaBAK1 is a member of the herbivory-induced response network and plays multiple roles in plant-herbivore interactions: NaBAK1 is required for herbivory-induced JA and JA-Ile accumulation and NaBAK1 also plays a negative role in controlling TPI activity induced by herbivory-elicited jasmonates. Further study should address the molecular mechanism that NaBAK1 regulates herbivory-induced JA and TPI accumulation, and whether other SERKs are also involved in plant-herbivore interactions.

5. Conclusion

The advance in identification of the components in sophisticated regulatory networks of JA biosynthesis and signaling will improve our understanding of the mechanisms by which herbivore-elicited jasmonate pathways activate. Genes that controlled jasmonate biosynthesis and signaling can be selected as target genes for improving herbivore resistance traits in crop plants.

6. Zusammenfassung

Die Rolle von Jasmonsäure (JA) und deren Konjugaten in pflanzlicher Resistenz gegen Insekten ist wohl bekannt. Es gibt jedoch kaum Erkenntnisse über die Regulation der JA Biosynthese nach Herbivorenbefall. Insektenfraß aktiviert die Freisetzung von Ca^{2+} Ionen im betroffenen Pflanzengewebe. Diese Ca^{2+} Signatur wird von Signalmolekülen wahrgenommen, die bestimmte Verteidigungsreaktionen kontrollieren. Eine wichtige Rolle in diesem Prozess spielen Ca^{2+} abhängige Proteinkinasen (CDPKs). Ob CDPKs eine Rolle in der herbivor-induzierten JA Biosythese spielen ist allerdings noch nicht bekannt. In dieser Arbeit wurde daher die Rolle von CDPKs und eines Co-Rezeptors (BAK) in der Herbivor-induzierten JA Biosynthese und der JA-abhängigen Verteidigungsreaktion in *Nicotiana attenuata* untersucht.

Die Ergebnisse zeigen, dass *N. attenuata* NaCDPK4 und NaCDPK5 wichtige negative Regulatoren der Herbivor-induzierten JA und JA-Ile Biosynthese sind. Pflanzen mit genetisch verringerten Transkriptraten von *NaCDPK4* und *NaCDPK5* haben erhöhte Resistenz gegen Larven des Tabakschwärmers *Manduca sexta*. Diese erhöhte Resistenz ist abhängig von dem JA-Ile Rezeptor COI1. Die Ergebnisse zeigen zusätzlich dass *NaCDPK4* und *NaCDPK5* die Resistenz gegen pathogene Bakterien, *Pseudomonas syringae Pst* DC3000, erhöhen. Diese Reaktion ist unabhängig von der erhöhten Konzentrationen an Salizylsäure. Welche Interaktionspartner NaCDPK4 und NACDPK5 haben und welche Rolle diese in der Signaltransduktion nach Herbivorenbefall spielen ist Gegenstand zukünftiger Forschung.

Unsere Ergebnisse zeigen weiterhin, dass NaBAK1 ein wichtiger Bestandteil der herbivor-induzierten Abwehr in *N. attenuata* ist. Pflanzen mit verringerten NaBAK1 Transkriptraten haben deutlich weniger JA, JA-Ile Level nach Herbivorenbefall und geringere Aktivität von Trypsin Protease Inhibitoren, wichtige Verteidigungsmetabolite für die Insektenabwehr.

Wir können somit zeigen, dass NaCDPK4, NACDPK5 und NaBAK1 eine zentrale Rolle in der Signaltransduktion nach Herbivorenbefall in *N. attenuata* spielen. Diese Ergebnisse sind wichtig um die komplexe Aktivierung der induzierten Herbivorenabwehr in Pflanzen zu verstehen.

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Patent

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Oral presentations

Yang D. (2009). NaBAK1 positively regulates Jasmonic acid/JA-Isoleucine (JA-Ile) metabolism in *Nicotiana attenuata* in response to mechanical wounding and chewing herbivore *Manduca sexta*. **8th IMPRS Symposium**, MPI for Chemical Ecology, Dornburg, DE

Yang D. (2009). BAK1 positively regulates wounding- and herbivory-induced jasmonic acid (JA) and JA-amino acid conjugate (JA-Ile) in *Nicotiana attenuata*. **5th Plant Science Student Conference**, Halle (Saale), DE

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Poster Presentation

Yang D. (2010). Common signaling partner? BAK1 regulates particular BRI1-independent components of *Nicotiana attenuata*'s responses to its specialist herbivore *Manduca sexta*. presented at **5th EPSO Conference** "Plants for Life", European Plant Science Organization (EPSO), Kittila, FI

10. Selbständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe.

Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt.

Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten.

Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Dahai Yang

Jena, 06. January, 2011