Early herbivory-induced responses in plants

Doctoral thesis by Stefan Meldau

Early herbivory-induced responses in plants

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1. Introduction

Sensing environmental cues and responding to them is crucial for the survival of all organisms. In contrast to mobile entities, plants have chosen a sessile lifestyle that renders them incapable of simply moving away from harmful situations. Despite this obvious disadvantage, plants provide a major contribution to our planet's biomass production. One could imagine that plants must have therefore developed efficient strategies to adjust their physiology to threatening environmental conditions. Herbivores, especially phytophagous insects, represent some of the most devastating enemies of plants. Our world would probably not be as green as it is, if plants had not developed ways to specifically detect and defend against insect herbivores (Bruetting, 2011). Studying the machinery that enables plants to sense and resist herbivore attack therefore helps to understand how plants became such vital players in the biosphere.

The research presented in this doctoral thesis is dedicated to the elucidation of perception mechanisms and the regulation of early physiological changes activated by herbivore attack. Early defense reactions to herbivory were studied in two model systems. First, I analyzed the interaction between the solanaceous plant *Nicotiana attenuata* and its specialized lepidopteran herbivore *Manduca sexta*. This system provides the advantage of testing the significance of lab-based observations under field conditions. Second, the recognition of attack by the generalist grasshopper *Schistocerca gregaria* and various lepidopteran herbivores was investigated in *Arabidopsis thaliana* (Brassicaceae). The wealth of genetic and molecular tools available for Arabidopsis enables in-depth analyses of early recognition and defense events. The following paragraphs will shortly introduce both model systems and the manuscripts this thesis is composed of.

***Nicotiana attenuata*, a model plant for studying the ecological significance of herbivory-induced defenses in nature**

The wild tobacco, *N. attenuata* (Torr. ex Watson), is an annual plant native to the Great Basin Desert in Southwestern USA (Figure 1A). *Nicotiana attenuata* seeds germinate into nitrogen-rich soils in post-fire habitats and germination is triggered by chemical cues derived from smoke in the absence of competing vegetation (Baldwin and Morse, 1994; Baldwin *et al*., 1994). After fires, *N. attenuata* plants can produce monocultures and represent one of the first food sources for herbivorous insects, among which the solanaceous specialist *Manduca sexta* is one of the major defoliators. *Nicotiana attenuata* plants have therefore evolved sophisticated defense responses to quickly detect and resist herbivores like *M. sexta* (Fig. 1).

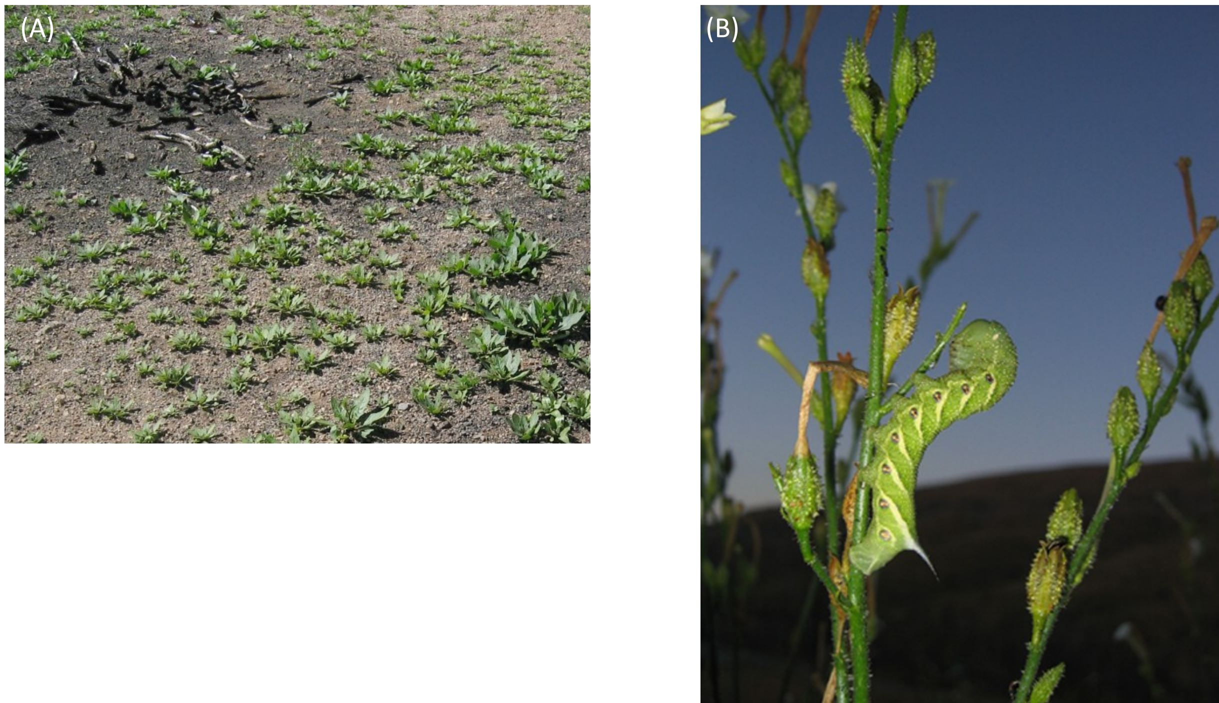

Figure 1 (A) *Nicotiana attenuata* monoculture in a post-fire habitat and (B) *Manduca sexta* feeding on *N. attenuata*. © Danny Kessler.

During *M. sexta* feeding, fatty acid-amino acid conjugates (FACs) derived from oral secretions (OS) are quickly recognized by *N. attenuata* (Halitschke *et al.* 2003). FACs amplify and modify wound-induced responses in *N. attenuata*, including the activation of protein kinases and the initiation of jasmonic acid (JA) and JA-Isoleucine (JA-Ile) biosynthesis (Wu *et al.*, 2007; Kallenbach *et al.*, 2010). Jasmonates (JA, JA-Ile and other JA derivatives) mediate the induction of defensive compounds, like nicotine, trypsin protease inhibitors, phenolic compounds and diterpene glycosides; most of these compounds were shown to be vital for the resistance to naturally occurring herbivores in the field (Zavala *et al.*, 2004, Paschold *et al.*, 2007, Wang *et al.*, 2007, Kaur *et al.*, 2010, Heiling *et al.*, 2010). The responses induced by jasmonates require the F-box protein COI1 (coronatine-insensitive 1), which is part of an Skp/Cullin/F-box complex [$SCF^{(COI1)}$], acting as a ubiquitin ligase to degrade negative transcriptional regulators of jasmonate-induced responses (Xu *et al.*, 2002; Chini *et al.*, 2007; Paschold *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008; Yan *et al.*, 2009). Hence COI1-silenced *N. attenuata* plants were found highly susceptible to herbivore attack under field conditions. One important jasmonate-elicited response to *M. sexta* attack is the emission of volatile organic compounds (VOCs), consisting primarily of 6-carbon alcohols, aldehydes and their esters (green leaf volatiles or GLVs) terpenoids, and benzenoids (Gaquerel *et al.*, 2009; Dicke and Baldwin, 2010). Field studies in Utah have shown that these volatiles act as indirect defenses,that attract natural enemies of phytophagous herbivores. But, contrarily, they may also act as feeding stimulants for insects feeding on *N. attenuata* (Kessler *et al*., 2002; Halitschke *et al*., 2004, 2008; Allmann *et al*., 2010).

Similarities between herbivory- and pathogen-induced signaling

Recent years have seen tremendous progress in the elucidation of early signaling events in herbivory-elicited plants (for review, see Wu *et al.*, 2010). Many signaling pathways important for plant-pathogen interactions have also been shown to regulate plant-herbivore interactions. Among the earliest events that are induced by pathogen or herbivore attack is the activation of mitogen-activated protein kinases (MAPKs). The MAPK cascade is a conserved pathway involved in modulating a large number of

cellular responses in all eukaryotes (Ichimura *et al.*, 2002). MAPKs activate their substrates by phosphorylation which in turn triggers downstream reactions such as phosphorylation of transcription factors (Hill and Teisman, 1995; Karin and Hunter, 1995; Hazzalin and Mahadevan, 2002). Plant adaptations to multiple (a)biotic stress stimuli require MAPK signaling (Romeis, 2001; Zhang and Klessig, 2001). Salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), two tobacco MAPKs and their orthologues in other plant species, are rapidly activated by pathogen attack (Romeis *et al.*, 1999; Asai *et al.*, 2002; Pedley and Martin, 2005). Studies of SIPK and WIPK in tobacco and their homologues in other plant species revealed that these kinases are important for regulating nitric oxide (NO), reactive oxygen species (ROS), the hypersensitive response (HR) and ethylene emissions upon pathogen attack (Asai et al, 2008; Liu et al, 2007; Asai *et al.*, 2002). Both SIPK and WIPK are also activated after wounding in N. tabacum and WIPK was shown to regulate wound-induced JA levels in this plant (Seo *et al.*, 1995, 1999, 2007; Zhang and Klessig, 1998). In *N. attenuata*, SIPK and WIPK are also activated by wounding, and FACs in *M. sexta* OS highly amplify this activation (Wu *et al.*, 2007). Both kinases regulate herbivory-induced JA and JA-Ile levels and SIPK is involved in ethylene synthesis. Although a large body of literature indicates the importance of these two MAPKs in plant resistance to abiotic and biotic stresses, no studies had examined whether plants with reduced transcript levels of SIPK or WIPK have impaired resistance to these stresses in their natural environment. The objective of the study presented in **Manuscript I** was to analyze anti-herbivore defenses in SIPK- and WIPK-silenced *N. attenuata* plants using molecular and biochemical tools and to analyze their resistance to herbivorous insects in a natural environment.

SGT1 (suppressor of G-two allele of SKP1) is a conserved protein in all eukaryotes and is crucial for resisting pathogens not only in plants, but also in humans (Muskett & Parker, 2003; Mayor *et al.*, 2007). SGT1 mediates the stability of NB-LRR (nucleotide binding-leucine rich repeat) type resistance (R) proteins that are involved in pathogen recognition in plants (Lu *et al.*, 2003; Boter *et al.*, 2007). SGT1 also interacts with components of SCF-ubiquitin ligase complexes in yeast and plants, suggesting that it functions in the proteasome-mediated protein degradation pathway (Kitagawa *et al.*,

1999; Azevedo *et al.*, 2002; Liu *et al.*, 2002). Indeed, SGT1 was identified as component of the SCF-ubiquitin ligase complexes in yeast and interaction between SGT1 and the ubiquitinylation machinery was also verified in plants (Kitagawa *et al.*, 1999; Azevedo *et al.*, 2002). In *A. thaliana*, *sgt1b* mutants are slightly insensitive to jasmonate-induced inhibition on root growth assays, which suggests a role for SGT1 in modulating jasmonate perception, a process that requires ubiquitin ligase complexes, including COI1. However, it was not known whether SGT1 is equally important for recognition and initiation of defense responses upon herbivory. The objective of the work presented in **manuscript II** is therefore to analyze the role of SGT1 in herbivory-induced signaling and JA mediated resistance in *N. attenuata*. **Manuscript III** is a mini-review that summarizes recent finding about the role of SGT1 in both, plant defense responses and regulation of growth and development.

Herbivore perception in *Arabidopsis thaliana*

In *N. attenuata*, we could show that herbivore-perception quickly triggers MAPK activation, which is crucial for herbivory-induced hormonal responses and gene expression (Wu *et al*, 2007, manuscript I). MAPK are also important for systemin-induced JA biosynthesis and herbivore resistance in tomato (Kandoth *et al*., 2007). However, in the model plant *A. thaliana*, information on how herbivore attack is perceived and which steps are involved in activation of hormonal responses is lacking, although an elicitor from grasshopper species was previously shown to quickly elicit JA and Ethylene (Schmelz *et al*. 2009). The same study demonstrated that other herbivore-derived elicitors, like FACs are unactive in *A. thaliana*. If MAPKs play a role in the activation of herbivory-induced defense responses in Arabidopsis is not clear, athough a MAPK phosphatase is thought to be involved (Schweighofer *et al*., 2009). In **manuscript IV**, we analyzed early herbivory-induced responses in *A. thaliana*, including changes in oxylipin levels, ethylene emissions, MAPK activity, intracellular Ca^{2+} release and ROS production. Our data demonstrate that lipase activity found in OS of insects presents a previously unknown elicitor of plant defense responses. In **manuscript V**, we extended our phytohormone analysis and showed that abscidic acid (ABA) and SA are induced by

grasshopper oral secretions, demonstrating that herbivory induces multiple parallel hormonal pathways.

Hormonal crosstalk regulates plant defense against herbivores

Most of the manuscripts introduced so far deal with regulation of hormonal changes after herbivore perception. Hormonal responses are connected via complex networks and signal integration leads to specific outcomes to biological inputs. However, to role of hormone-crosstalk, or hormone network interactions in mediating specificity in defense responses has only recently become an emerging theme in the field of plant-herbivore interactions. The review in **manuscript VI** presents an overview about the current knowledge on herbivore recognition and activation of different phytohormone pathways in response to herbivore perception. The paragraph drafted by me hypothesizes that integration of crosstalk between various plant hormones, including SA, Ethylene, ABA, Auxin, Gibberellins (GB), Cytokinins (CK) and Brassinosteroids (BR) regulates specific defense responses to multiple insects.

Manuscript Overview

Manuscript I

New Phytologist, 181, 161-173

Silencing two herbivory-activated MAP kinases, SIPK and WIPK, does not increase *Nicotiana attenuata*'s susceptibility to herbivores in the glasshouse and in nature

Stefan Meldau, Jianqiang Wu and Ian T. Baldwin

In this manuscript, we investigated the resistance of plants that are silenced in two MAPKs, *NaSIPK* and *NaWIPK*, to herbivores in the glasshouse and under field conditions. Surprisingly, silencing of both kinases diminished jasmonate (JA) concentration and anti-herbivory defenses, however, these plants were not particularly susceptible to herbivores. We then noticed that emission of green leaf volatile (GLV) after herbivore attack, a class of compounds previously described to alter consumption of leaf tissues by herbivorous insects, was also reduced. An application of GLVs to ir*NaSIPK* and ir*NaWIPK* plants increased their susceptibility to the specialist herbivore *M. sexta*. These effects were also found true for other JA deficient plant lines. Our results demonstrate that resistance of JA deficient plants can be partially recovered when GLV emissions are co-silenced.

I designed and performed experiments and drafted the manuscript. Wu J. isolated *NaSIPK* and *NaWIPK* cDNA sequences, designed experiments and helped with manuscript preparation. Baldwin I.T., designed experiments, helped with manuscript preparation and submitted the manuscript.

Manuscript II

New Phytologist, 189, 1143-1156

SGT1 regulates wounding- and herbivory-induced jasmonic acid accumulation and *Nicotiana attenuata*'s resistance to the specialist lepidopteran herbivore *Manduca sexta*

Stefan Meldau, Ian T. Baldwin and Jianqiang Wu

In this manuscript we investigated the role of SGT1, a conserved eukaryotic protein, in early plant defense responses to herbivores. We found that Virus-induced gene silencing (VIGS) of *NaSGT1* in *N. attenuata* resulted in reduced levels of jasmonic acids and defense compounds upon wounding and elicitation with oral secretions *M. sexta*, coupled with a decreased resistance to M. sexta larvae. Chemical profiling revealed that *NaSGT1*-silenced plants are compromised in the production of plastidic precursor molecules in the JA biosynthesis pathway, while plants had increased SA levels. However, by silencing *NaSGT1* in the 35S:*NahG* background we could rule out that reduced JA levels are caused by SA antagonism.

I designed and performed experiments and drafted the manuscript. Baldwin I.T designed experiments and helped with manuscript preparation. Wu J. isolated *NaSGT1* cDNA sequences, designed experiments, helped with manuscript preparation and submitted the manuscript.

Manuscript III

Plant Signaling and Behavior, 6(10)

For security and stability: SGT1 in plant defense and development

Stefan Meldau, Ian T. Baldwin and Jianqiang Wu

In this mini-review, we highlight recent literature on the role of SGT1 in plant defense against pathogens and herbivores and the developmental phenotypes produced by silencing SGT1. We conclude that SGT1mediates various physiological processes through its interactions with protein complexes involved in chaperone-mediated protein assembly and with the ubiquitin machinery involved in protein degradation.

I drafted the review; Baldwin I.T. provided helpful comments and Wu J. submitted the manuscript

Manuscript IV

Plant Physiology, 156, 1520-1534

Lipase activity in insect oral secretions mediates defense responses in *Arabidopsis thaliana*

Martin Schäfer, Christine Fischer, Stefan Meldau, Eileen Seebald, Ralf Oelmüller, Ian T. Baldwin

This work analysed early defense responses of *A. thaliana* plants in response to herbivory. We showed that leaves of *A. thaliana* plants respond quickly and specifically to grasshopper OS by activation of MAPKs, increased cytosolic Ca^{2+} levels and elevated oxylipin and ethylene accumulations. We further demonstrated that wound-induced ROS levels are negatively influencing oxylipin and ethylene levels. Our results revealed that lipase activity from insect OS mediate oxylipin release in *A. thaliana*, therefore providing a new mechanism of how plants can perceive herbivore attack.

I initiated the project, designed and conducted experiments, drafted and submitted the manuscript. Schäfer M. and Fischer C. designed and conducted experiments and contributed to the manuscript. Seebald E. conducted Ca^{2+} measurements and Oelmüller R. and Baldwin I.T. contributed to experimental design and manuscript preparation.

Manuscript V

Plant Signaling & Behavior, 6(9)

Grasshopper oral secretions increase salicylic acid and abscisic acid levels in wounded leaves of *Arabidopsis thaliana*

Martin Schäfer, Christine Fischer, Ian T. Baldwin and Stefan Meldau

In this addendum to manuscript IV we show that grasshopper OS applied to mechanically wounded *A. thaliana* leaves leads to higher accumulation of SA and abscidic acid levels, when compared to wounding alone. We discuss these data in the context of phytohormone crosstalk.

I designed experiments, drafted and submitted the manuscript. Schäfer M., Fischer C., conducted experiments and contributed to manuscript preparation. Baldwin I.T. provided useful comments on the manuscript.

Manuscript VI

Trends in Plants Science: submitted as invited review; under revision

From recognition to response:

The role of phytohormones in attacker-specific plant reactions

Matthias Erb, Stefan Meldau, Gregg A. Howe

In this review, we discuss the current knowledge on herbivore perception by plants in the framework of concepts that are established for pathogen-induced immunity. We argue that the paradigms of pathogen perception, namely pattern-and effector-triggered immunity can be merged with the concepts of herbivore perception where plants detect herbivore attack via herbivory- and damage-associated patterns. Various hormonal pathwys are activated in response to herbivore perception events and we developed a model where interactions between hormonal networks are integrated to shape defense responses to specific types of insect herbivores.

I drafted the paragraph about the role of various hormones in plant-herbivore interactions and designed Fig.2 as well as the hormonal network on Fig.3. Howe G. drafted the paragraph about the importance of jasmonates in mediating defense responses to herbivores and Erb M. compiled the rest of the manuscript.

New Phytologist

Research

Silencing two herbivory-activated MAP kinases, SIPK and WIPK, does not increase *Nicotiana attenuata*'s susceptibility to herbivores in the glasshouse and in nature

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Key words: green leaf volatiles, herbivores, indirect defense, mitogen-activated protein kinase, *Nicotiana attenuata*, resistance, volatile biosynthesis.

Summary

- Salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) are activated by *Manduca sexta* attack and elicitors to mediate defense signaling in *Nicotiana attenuata*. Here, the ecological consequences of *SIPK* and *WIPK* silencing for *N. attenuata*'s resistance to *M. sexta* and its other native herbivores were analyzed.
- Stably transformed plants with reduced expression of Na*SIPK* (irNa*SIPK*) and Na*WIPK*(irNa*WIPK*) were generated and characterized in field and glasshouse experiments.
- Both irNa*SIPK* and irNa*WIPK* plants had reduced direct and indirect defenses but were not particularly susceptible in nature. In the glasshouse, *M. sexta* larvae consumed less and gained the same mass on irNa*SIPK* and irNa*WIPK* as on wild-type (WT) plants. Green leaf volatile (GLV) emission was highly attenuated in irNa*SIPK* and irNa*WIPK* plants, and complementation with synthetic GLVs increased *M. sexta* performance. To test the hypothesis that reduced GLV emissions account for the lack of herbivory phenotype, GLV emissions were attenuated by silencing Na*HPL* in jasmonate-deficient plants (asNa*LOX3*), which are highly susceptible to herbivores. Reducing GLV emissions in asNa*LOX3* plants 'rescued' these plants from being heavily damaged by *M. sexta*.
- GLV emissions in irNa*SIPK* and irNa*WIPK* plants may compensate for the impaired defenses of Na*SIPK*- and Na*WIPK*-silenced plants in nature by reducing their apparency to herbivores.

Introduction

Plants respond to insect herbivory with a large-scale reconfiguration of their metabolism (Walling, 2000; Kessler & Baldwin, 2004). This reconfiguration involves the biosynthesis of compounds which directly affect the herbivores' physiology, such as toxins, anti-digestive compounds and feeding deterrents (Duffey & Stout, 1996), or indirectly in the form of volatile emissions that attract natural enemies of the herbivores (Alborn *et al.*, 1997; De Moraes *et al.*, 1998; Kessler & Baldwin, 2001). These defense responses are frequently activated by wounding or when herbivore-specific elicitors are introduced into the wounds during the feeding (Voelckel & Baldwin, 2004a; Gomez *et al.*, 2005).

One of the earliest events initiated by wounding and herbivory described so far is the activation of mitogen-activated protein kinases (MAPKs) (Seo *et al.*, 1999; Kandoth *et al.*, 2007; Wu *et al.*, 2007). The processes mediated by MAPK signaling are complex and involve transcriptional as well as translational and post-translational mechanisms (reviewed in Hirt, 1997; Romeis, 2001; Zhang & Klessig, 2001; Mishra *et al.*, 2006). Two MAPKs in tobacco, the salicylic acid-induced protein kinase (SIPK) and the wound-induced protein kinase (WIPK), and their orthologs in other plant species, are activated in response to multiple biotic and abiotic stresses, including wounding and herbivory (Zhang & Klessig, 2001; Mishra *et al.*, 2006; Kandoth *et al.*, 2007; Seo *et al.*, 2007; Wu *et al.*, 2007). In cultivated tobacco (*Nicotiana*

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tabacum), WIPK regulates wound-induced jasmonic acid (JA) biosynthesis and JA-mediated accumulation of protease inhibitors (PI) (Seo *et al.*, 2007). In tomato (*Solanum esculentum*), herbivore-induced PI accumulation has been shown to depend on the peptide systemin (Pearce *et al.*, 1991). Overexpressing prosystemin resulted in increased JA concentrations and enhanced PI accumulation, while silencing the expression of the tomato orthologs of *SIPK* and *WIPK*, namely Le*MPK1*, Le*MPK2* and Le*MPK3*, in tomato plants that ectopically express prosystemin attenuated JA accumulation, reduced PI production and increased *Manduca sexta* (Lepidoptera) larval mass gain on these plants (Kandoth *et al.*, 2007). In *N. attenuata*, fatty acid-amino acid conjugates (FACs) derived from herbivores' oral secretions (OS) rapidly activate SIPK and WIPK (hereafter, NaSIPK and NaWIPK) (Wu *et al.*, 2007). Virus-induced gene silencing (VIGS) demonstrated that NaSIPK and NaWIPK regulate herbivore-elicited JA and salicylic acid (SA) concentrations, ethylene emission, and the transcriptional responses of many genes, including transcription factors and kinases (Wu *et al.*, 2007).

Multiple aspects of *N. attenuata*'s interaction with phytophagous insects have been characterized in the past 15 yr. Many metabolites that play important roles in these plants' direct and indirect defenses, as well as chemicals which are important for *N. attenuata*'s apparency to insects, have been determined. For instance, nicotine, caffeoylputrescine, diterpene glycosides, and trypsin protease inhibitors (TPIs) are anti-herbivore defense metabolites in *N. attenuata* that are regulated by herbivore-induced JA (Kessler *et al.*, 2004, Steppuhn *et al.*, 2004, Zavala *et al.*, 2004, Jassabi *et al.*, 2008). In addition, herbivory also elicits the emission of volatile organic compounds (VOCs) such as *trans*-α-bergamotene, which function as indirect defenses by attracting predators of *M. sexta* larvae and eggs in *N. attenuata*'s native habitat (Kessler & Baldwin, 2001). However, green leaf volatiles (GLVs), which are released from damaged plants, increase *N. attenuata*'s apparency to particular herbivores in the field, demonstrating that herbivore-induced volatile emissions can be both beneficial as an indirect defense that attracts predators as well as a liability that increases herbivore loads and attack rates (Halitschke *et al.*, 2008).

To analyze the ecological function of Na*SIPK* and Na*WIPK* in *N. attenuata*'s direct and indirect defense responses to herbivore attack, we generated stably transformed plants whose transcript levels of Na*SIPK* and Na*WIPK* had been silenced by RNAi and analyzed their susceptibility to herbivores in the plants' native habitat and in the glasshouse. We show that although Na*SIPK*- and Na*WIPK*-silenced plants have impaired direct and indirect defenses, they do not suffer higher attack rates than wild-type (WT) plants do from native herbivores. Silencing Na*SIPK* and Na*WIPK* reduces GLV emissions, which in turn may compensate for the impaired direct defense in these plants. We test this hypothesis by silencing Na*HPL* in the highly herbivore-susceptible jasmonate-deficient asNa*LOX3* plants.

Materials and Methods

Generation and characterization of the transgenic lines

Partial sequences of Na*SIPK* (327 bp) and Na*WIPK* (317 bp) cDNA were inserted into the pRESC5 transformation vector to form inverted-repeat constructs (Supporting information, Fig. S1). An *Agrobacterium*-mediated transformation procedure was used to generate irNa*SIPK* and irNa*WIPK* plants (Krügel *et al.*, 2002). Lines harboring a single copy of the transgene resulting from independent transformation events were identified by Southern blotting and were further screened for homozygosity. Two independently transformed single-insert homozygous lines having strongly silenced Na*SIPK* or Na*WIPK* transcripts and reduced kinase activity (Fig. S2) were used (irNa*SIPK*, lines A-108, A-109; irNa*WIPK*, lines A-56, A-95). A *N. attenuata* line inbred for 22 generations was used for comparisons. The generation of asNa*LOX3* and asNa*HPL* plants is described elsewhere (Halitschke & Baldwin, 2003; Halitschke *et al.*, 2004). Crossing asNa*LOX3* with asNa*HPL* plants was done by removing anthers from flowers of asNa*LOX3* plants before pollen maturation and pollinating the stigmas with asNa*HPL* pollen.

Plant growth

For glasshouse experiments, *Nicotiana attenuata* Torr. ex S. Watson seed germination and plant growth were conducted as described by Krügel *et al.* (2002). In the field, seeds were germinated and plants were grown as described in Rayapuram & Baldwin (2007). A minimum of 12 plants of irNa*SIPK* and irNa*WIPK*, each paired with one size-matched WT plant, were chosen for field experiments. Plants were randomly distributed in a field plot (~700 m^2, Fig. S3). The distance between each transgenic plant and the corresponding WT plant was 0.5 m. In addition to damage caused by native herbivores, plants were repeatedly wounded and applied with *Manduca sexta* L. oral secretions (OS) during the field release to elicit defense responses and for analysis of herbivory-induced direct and indirect defense traits. The release of transgenic plants was carried out under APHIS notification (06-242-101 n). To comply with the 7CFR 340.4, the legal statute that governs the release of transgenic organisms, flowers were removed before pollen matured.

Damage quantification

Total herbivore damage was determined for 21 d, starting 15 d after plants were transplanted into the field sites. We estimated the percentage of leaf area removed (in the case of grasshoppers, flea beetles and lepidoptera), or the percentage

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of characteristic damage caused by myrid herbivores relative to the total leaf area.

Quantitative RT-PCR (qPCR)

Total RNA was extracted from leaves using TRIzol reagent (Invitrogen, Paisley, UK). A 0.5 µg quantity of total RNA of each sample was reverse-transcribed using oligo (dT) and Superscript II reverse transcriptase (Invitrogen) following manufacturer's instructions. For qPCR analysis, cDNA from five replicated biological samples was used. qPCR was carried out on a ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using qPCR Core kits (Eurogentec, Liege, Belgium); the *actin* transcript levels were used to normalize total cDNA concentration variations. The sequences of primers and probes used for qPCR are provided in Table S1.

Protein extraction and in-gel kinase activity assay

Leaf tissue pooled from four replicate leaves was ground in liquid nitrogen and 250 µl extraction buffer (100 mM Hepes pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl floride, 10% glycerol, complete proteinase inhibitor cocktail tablets (Roche, Mannheim, Germany)) was used for every 100 mg tissue. Leaf tissue was then completely suspended by vortexing. After being centrifuged at 4°C for 20 min, supernatants were transferred to fresh tubes. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) with BSA (Sigma-Aldrich, Hamburg, Germany) as a standard.

Kinase activity assay was performed as described in Wu *et al.* (2007).

Analysis of JA and JA-isoleucine/leucine conjugate accumulation

Phytohormone extraction and analysis were carried out as described in Wang *et al.* (2007).

Analysis of VOCs

To quantify OS-elicited VOC emissions, we mechanically wounded leaves with a pattern wheel and immediately applied 1 : 5 diluted *M. sexta* OS to the puncture wounds (W+OS). In the field, we enclosed plants in open-ended polystyrene transparent cups 24 h after OS elicitation and trapped VOCs at *c.* 350 ml min^{-1} for 8 h on charcoal traps (Orbo M32, Sigma-Aldrich), as described in Kessler & Baldwin (2001). After trapping, the charcoal traps were stored at −20°C until VOC elution and analysis. Before eluting the VOCs for GC-MS analysis, 40 ng tetraline was added to each trap as an internal standard and eluted with 250 µl of dichloromethane. To analyze VOCs from glasshouse-grown plants, we induced leaves with W+OS and enclosed single induced leaves in polystyrene boxes starting from 24 to 36 h after induction. Volatiles were trapped at *c.* 200 ml min^{-1} for 8 h with homemade traps containing 20 mg SuperQ (ARS, Philadelphia, PA, USA) sealed with fiberglass. GC-MS analysis and quantification were done as described in Rayapuram *et al.* (2007).

To measure GLV emissions after treatment with synthetic GLVs, rosette stage transition leaves were enclosed in plastic clip cages (6 cm diameter) and sealed with Teflon tapes; petioles of the same leaves were treated with 20 µl lanolin or 20 µl lanolin containg 4 mM of *cis*-3-hexenol (Sigma). Three hours after treatment, a small hole was punched into the plastic cage and the inlet of the ZNose® 4100 portable gas chromatograph (with a 1 m DB5 column, Electronic Sensor Technology, Newbury Park, CA, USA) was inserted into the clip cage (*c.* 1 cm above the leaf) and C_6 volatile emissions from leaves were measured. Pure *cis*-3-hexenol was used as standard to determine retention time. To determine if GLV treatment of the petiole also increased GLV emissions from wounded leaves, C_6 volatile emission was measured utilizing the same procedure as described, except that the clip cages were additionally quickly removed, leaves were wounded with a pattern wheel before clip cage was readjusted and volatiles were measured within 20 s after wounding.

Manduca sexta performance assay

Manduca sexta were obtained from in-house colonies. Freshly hatched larvae were placed on transition leaves of rosette-stage plants and enclosed in clip cages (6 cm in diameter, Fig. S8a). To measure the effect of GLVs on *M. sexta* performance, a mixture of different synthetic GLVs was applied to the petioles of the leaves: 20 µl lanolin or a mixture of GLVs (1 mM *cis*-3-hexenol, 1 mM *trans*-2-hexenol, 1 mM *cis*-3-hexenylacetate and 1 mM *cis*-3-hexenylbutyrate) dissolved in 20 µl lanolin were applied to the petioles of the leaves outside the clip cages to separate *M. sexta* larvae from the lanolin pastes (Fig. S8b). The treatment was repeated every second day during herbivore performance assay to replenish GLV emissions. Depending on their leaf consumption rates, larvae were transferred from the current leaf to the next younger leaf after 2 or 3 d of feeding. After transfer to the adjacent leaf, the treated leaves were removed from the plant, scanned, and tissue consumption was determined using SigmaScan software (Systat, San Jose, CA, USA).

Predation assay

We used a *M. sexta* egg predation assay to measure how well herbivore-induced VOCs attract the dominant predator (*Geocoris pallens*) of *N. attenuata*'s herbivores (Kessler & Baldwin,

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2002). Using a natural cellulose glue which is known to have no effect on the predation rate or the plants' VOC emissions, five *M. sexta* eggs were glued to the abaxial side of the second stem leaves of eight groups of size-matched plants (each group consisting of one irNa*SIPK*, irNa*WIPK*, and WT plant that were 0.5–1 m away from each other), 28 d after the plants were planted into the field. We elicited a rosette leaf by wounding and immediately applied *M. sexta* OS after the eggs were glued. Egg predation was examined 48 h after elicitation.

Trypsin proteinase inhibitor activity analysis

Trypsin proteinase inhibitor (TPI) activity was quantified using a radial diffusion assay protocol described by Jongsma *et al.* (1994).

Secondary metabolite analysis

Extraction and analysis of secondary metabolites were done as described in Keinänen *et al.* (2001).

Microarray analysis

We hybridized microarrays enriched with herbivore-induced ESTs from *N. attenuata*, using Cy3 (from transgenic lines) and Cy5 (from WT)-labeled cDNA. Each cDNA sample was obtained from pooled three biological replicates, 6 h after rosette-stage leaves were wounded and 20 µl of *M. sexta* OS was applied to the wounded leaves. Hybridization and analysis were carried out as described in Wang *et al.* (2007). Data were expressed as mean ratio = cy3/cy5; in cases where cy3/cy5 < 1 it is calculated as –1/(cy3/cy5).

Statistical analysis

Statistical analyses were done using StatView (SAS Institute Inc., Cary, NC, USA).

Results

Silencing Na*SIPK* and Na*WIPK* reduces OS-elicited JA and JA-Ile concentrations

Recently, Wu *et al.* (2007) showed that Na*SIPK* and Na*WIPK* play a central role in the complex herbivory-activated defense system in *N. attenuata*, and that silencing them strongly influences herbivory-induced phytohormone and transcript levels of many defense-related genes. However, the consequences of these changes for *N. attenuata*'s resistance to herbivores have not been examined. To rectify this deficit, we created *N. attenuata* lines with reduced expression levels of Na*SIPK* and Na*WIPK*, respectively, using an *Agrobacterium*-mediated transformation procedure (Fig. S1; Krügel *et al.*, 2002). T2 generation plants of irNa*SIPK* (lines A-108 and A-109) and irNa*WIPK* (lines A-56 and A-95) lines which were homozygous and each harbored a single T-DNA insertion and had silenced Na*SIPK* and Na*WIPK* transcript levels, respectively, were used. Quantitative real-time PCR (qPCR) and in-gel kinase assays verified the efficiency of the gene silencing and the reduction of kinase activity in these plants (Fig. S2). To investigate whether irNa*SIPK* and irNa*WIPK* plants had reduced concentrations of herbivore-induced JA and JA-Ile, we used a standardized elicitation of *N. attenuata* leaves that mimics *M. sexta* herbivory: source–sink transition leaves of rosette-stage plants were wounded with a pattern wheel, and 20 µl of 1 : 5 diluted *M. sexta* oral secretions were immediately applied (W+OS); 1 h after induction, leaves were harvested. Consistent with JA concentrations reported by Wu *et al.* (2007), irNa*SIPK* (A-108, A-109) and irNa*WIPK* (A-56, A-95) lines had *c.* 30 and 50% of the maximum OS-induced JA concentrations in WT plants, respectively (Fig. S4a). Both independently transformed lines of irNa*SIPK* and irNa*WIPK* showed similar degrees of silencing efficiency and of OS-induced JA and JA-Ile concentrations (Figs S2, S4), and had no other observable differences; therefore, one line each from irNa*SIPK* plants (A-109) and irNa*WIPK* plants (A-56) was used during our field studies.

Silencing Na*SIPK* and Na*WIPK* reduces direct and indirect defense metabolites

Nicotine and TPIs are potent defenses against herbivorous insects in *N. attenuata* (Steppuhn *et al.*, 2004; Zavala *et al.*, 2004). In order to analyze the ability of irNa*SIPK* and irNa*WIPK* plants to accumulate nicotine and TPI after herbivore attack, we sampled leaf tissue before and 12 d after rosette-stage plants were infested with freshly hatched *M. sexta* larvae. While the uninduced amounts of both compounds were not altered in irNa*SIPK* and irNa*WIPK* plants compared with WT plants, their induced amounts were highly diminished. Nicotine was 35 to 40% reduced, and the TPI activity attained only *c.*15–20% of the levels found in WT plants (Fig. 1a,b). In addition, the concentrations of caffeoylputrescine, a phenolic compound that is elicited by herbivory in *N. attenuata*, was also reduced to 45–50% of WT values (Fig. 1c). These data demonstrate that silencing Na*SIPK* and Na*WIPK* highly impairs the ability of *N. attenuata* to elicit direct defenses against *M. sexta*.

In response to herbivore attack, *N. attenuata* releases a bouquet of VOCs comprising terpenoids and volatile C_6 compounds, called GLVs, that attract the predators of herbivorous insects and thereby function as an indirect defense in nature (Kessler & Baldwin, 2001; Halitschke *et al.*, 2008). To analyze the herbivore-induced VOC release in irNa*SIPK* and irNa*WIPK* plants in a glasshouse condition, we elicited single leaves of rosette-stage plants with W+OS and trapped volatiles

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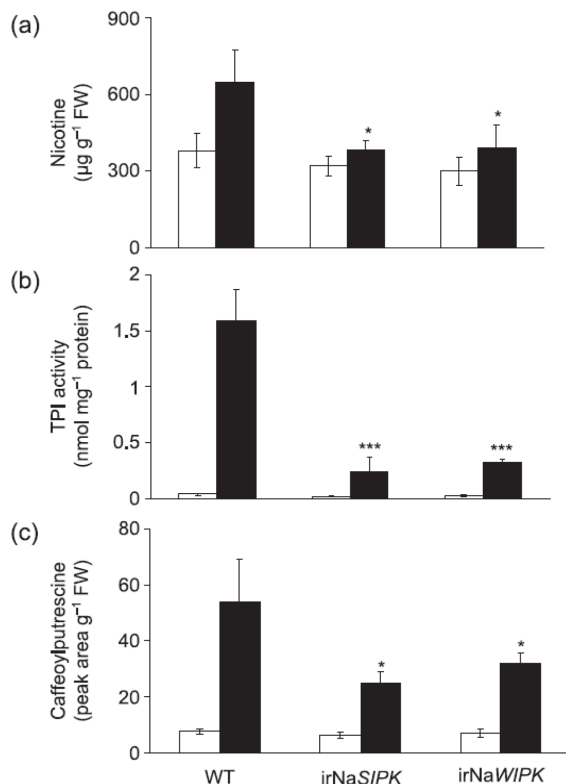

Fig. 1 Silencing Na*SIPK* and Na*WIPK* reduces amounts of direct defensive compounds in *Nicotiana attenuate*. Nicotine concentration (a), trypsin protease inhibitor (TPI) activity (b), and caffeoylputrescine concentration (c) were analyzed from eight replicates of wild-type (WT), irNa*SIPK*, and irNa*WIPK* plants. One *Manduca sexta* larva was allowed to feed for 12 d on each plant and attacked leaves were harvested for the analysis (closed bars); untreated plants served as controls (open bars). Asterisks represent significant differences between WT and irNa*SIPK* or irNa*WIPK* plants (two-way ANOVA, Fisher's PLSDs: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $n = 8$).

released from 24 to 36 h after elicitation. Two important volatile compounds whose function as indirect defenses in nature have been demonstrated are the sesquiterpene, *trans*-α-bergamotene, and the C_6 alcohol, *cis*-3-hexenol (Kessler & Baldwin, 2001; Halitschke *et al.*, 2008). Compared with those in WT plants, the amounts of these two compounds in irNa*SIPK* (A-109) and irNa*WIPK* (A-56) plants were remarkably reduced (Table 1). Two other GLVs, *trans*-2-hexenol and *cis*-3-hexenylisobutyrate, and another sesquiterpene, β-duprezianene, were also emitted at lower levels in irNa*SIPK* (A-109) and irNa*WIPK* (A-56) plants compared with those in WT plants (Table 1). Moreover, the emission of monoterpenes, α-terpineol and myrcene, was also reduced in these kinase-silenced plants. These data demonstrate that both NaSIPK and NaWIPK regulate the release and/or the biosynthesis of GLVs and terpenoids in response to herbivore attack, and suggest that these plants have strongly reduced indirect defenses against herbivorous insects.

NaSIPK and NaWIPK regulate transcript levels of genes involved in biosynthesis of alkaloids, phenolics, and volatiles

In order to analyze the effect of these kinases on *N. attenuata*'s transcriptional responses to herbivore attack, we hybridized DNA microarrays enriched in *M. sexta*-induced *N. attenuata* genes (Halitschke & Baldwin, 2003; Voelckel & Baldwin, 2004b) with cDNA prepared from pooled leaf tissue collected from three biological replicates of irNa*SIPK* (A-109), irNa*WIPK* (A-56), and WT plants 6 h after treatment with W+OS. For each analysis, two replicate microarrays were used.

Among the 136 (WT/irNa*SIPK*) and 68 (WT/irNa*WIPK*) genes that were differentially regulated, many were involved in the biosynthesis of defensive secondary metabolites. For example, the mRNA concentrations of *ornithine decarboxylase* (nicotine biosynthesis), *cinnamic acid-4-hydroxylase* (phenolic compound biosynthesis), and *hydroperoxide lyase* (GLV biosynthesis) were lower in irNa*SIPK* and irNa*WIPK* plants than in WT plants (Fig. 2). To verify these results, we analyzed the transcript levels of three genes involved in the production of VOCs by qPCR. In kinase-silenced plants, the transcript levels of all these genes, *hydroperoxide lyase* (Na*HPL*), *deoxyxylosose 5-phosphate synthase* (Na*DXS*), and *hydroxy-3-methylglutaryl reductase* (Na*HMGR*), were significantly lower 6 h after OS elicitation than those in WT plants (Fig. S5). These data are consistent with the highly reduced amounts of VOCs and other secondary metabolites in the kinase-silenced lines, demonstrating that NaSIPK and NaWIPK are important mediators of *N. attenuata*'s transcriptional responses to herbivory.

irNa*SIPK* and irNa*WIPK* plants attract fewer predators of herbivores but are not attacked more than WT plants in nature

To analyze the effect of reduced direct and indirect defenses against herbivores in nature, we planted irNa*SIPK* (A-109) irNa*WIPK* (A-56) plants in size-matched pairs with WT *N. attenuata* plants into their natural habitat, the Great Basin Desert (Utah, USA), and analyzed their interactions with the local herbivore community. *Geocoris pallens* (Lygaeidae), a native generalist lepidopteran egg and larval predator, is attracted by volatile terpenoids and GLVs released from *N. attenuata* plants that have been induced by herbivore attack or W+OS elicitation (Kessler & Baldwin, 2001; Paschold *et al.*, 2007; Rayapuram & Baldwin, 2007; Halitschke *et al.*, 2008). To determine if the decreased amounts of herbivore-induced volatiles in irNa*SIPK* (A-109) and irNa*WIPK*

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Table 1 Volatile emission from wild-type (WT), irNa*SIPK*, and irNa*WIPK* *Nicotiana attenuata* plants after being wounded and applied with *Manduca sexta* oral secretions

Compounds		WT	irNa*SIPK* (A-109)		irNa*WIPK* (A-56)	
		Relative emission[a]	Relative emission[a]	*P*-value[b]	Relative emission[a]	*P*-value[b]
GLVs						
	cis-3-hexenol	4.03 ± 0.4	1.59 ± 0.33	≤ 0.0001	1.67 ± 0.23	≤ 0.0001
	cis-3-hexenylisobutyrate	0.16 ± 0.02	0.12 ± 0.01	ns	0.09 ± 0.01	ns
	trans-2-hexen-1-ol	0.17 ± 0.02	0.13 ± 0.01	ns	0.09 ± 0.01	ns
Monoterpenes	d-limonene	2.77 ± 0.4	2.65 ± 0.44	ns	2.34 ± 0.44	ns
	myrcene	0.19 ± 0.04	0.13 ± 0.02	ns	0.07 ± 0.02	ns
	α-terpineol	3.19 ± 0.39	2.23 ± 0.22	0.024	1.49 ± 0.17	≤ 0.0001
Sesquiterpenes	*trans*-α-bergamotene	6.4 ± 0.44	1.43 ± 0.34	≤ 0.0001	1.9 ± 0.66	≤ 0.0001
	β-duprezianene	2.53 ± 0.37	1.37 ± 0.28	0.0049	1.67 ± 0.32	0.0346
Benzoids	Benzaldehyde	0.2 ± 0.04	0.23 ± 0.04	ns	0.21 ± 0.05	ns
Alkaloids	Nicotine	0.17 ± 0.02	0.13 ± 0.01	ns	0.09 ± 0.01	0.0008

[a]Volatiles were quantified by correlation to the internal standard (tetraline) as peak area ng^{-1} tetraline cm^{-2} h^{-1}.
[b]Comparisons were done between data obtained from WT and irNa*SIPK* or irNa*WIPK* plants; *P*-values were obtained from two-way ANOVA; ns, not significant.

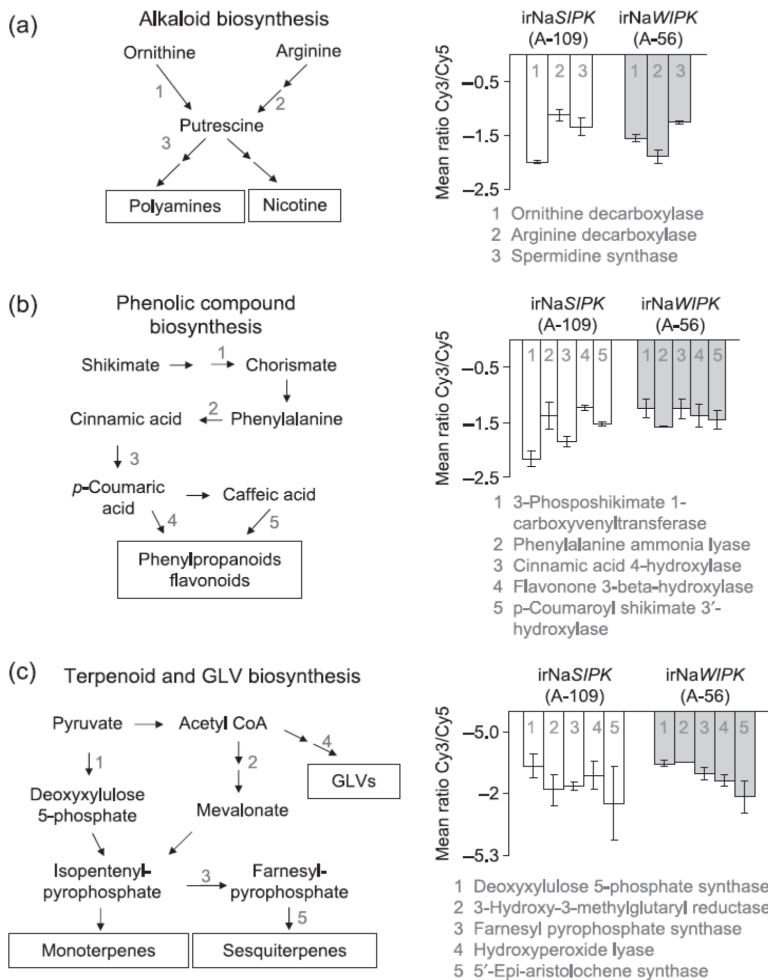

Fig. 2 NaSIPK and NaWIPK regulate expressions of genes involved in defense against herbivores. Source–sink transition leaves of rosette-stage *Nicotiana attenuata* wild-type (WT), irNa*SIPK*, and irNa*WIPK* plants were wounded with a pattern wheel and 20 μl of *Manduca sexta* oral secretions were immediately applied to the wounds. For each microarray, samples from three biological replicates were pooled 6 h after elicitation, and mRNA was extracted, converted to cDNA and labeled with Cy3 (irNa*SIPK*, irNa*WIPK*) or Cy5 (WT). Two DNA microarrays were hybridized for each comparison. Genes in alkaloid (a), phenolics (b), and terpenoid and green leaf volatile (GLV) (c) biosynthesis pathways have lower transcript levels in irNa*SIPK* and irNa*WIPK* than in WT plants.

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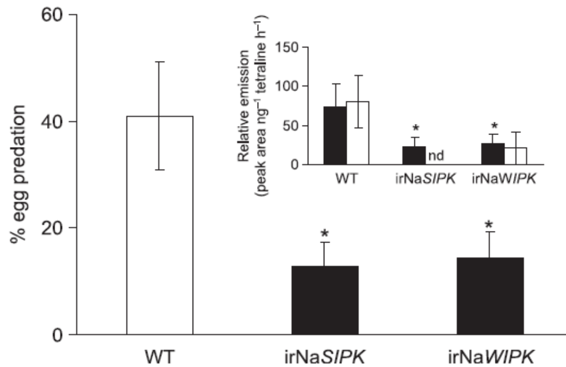

Fig. 3 irNa*SIPK* and irNa*WIPK* plants have reduced indirect defenses in nature. Mean (± SE) percentage of *Manduca sexta* eggs predated per plant 48 h after plants were wounded with a pattern wheel and applied with 20 µl of 1 : 5 diluted *M. sexta* oral secretions (W+OS). Five eggs were glued on the second stem leaf of each plant of eight groups of size-matched plants, each containing one irNa*SIPK* (A-109), irNa*WIPK* (A-56), and wild-type (WT) plant; each plant had one rosette leaf elicited with *M. sexta* OS. Inset: mean (± SE) emission of *trans*-α-bergamotene (closed bars) and *cis*-3-hexenol (open bars) 24–36 h after one rosette leaf of WT, irNa*SIPK* (A-109), and irNa*WIPK* (A-56) plants ($n = 8$) was elicited with W+OS. Asterisks indicate significant differences between WT and irNa*SIPK* or irNa*WIPK* plants (ANOVA, Fisher's PLSDs: *, $P < 0.05$; nd, not detected).

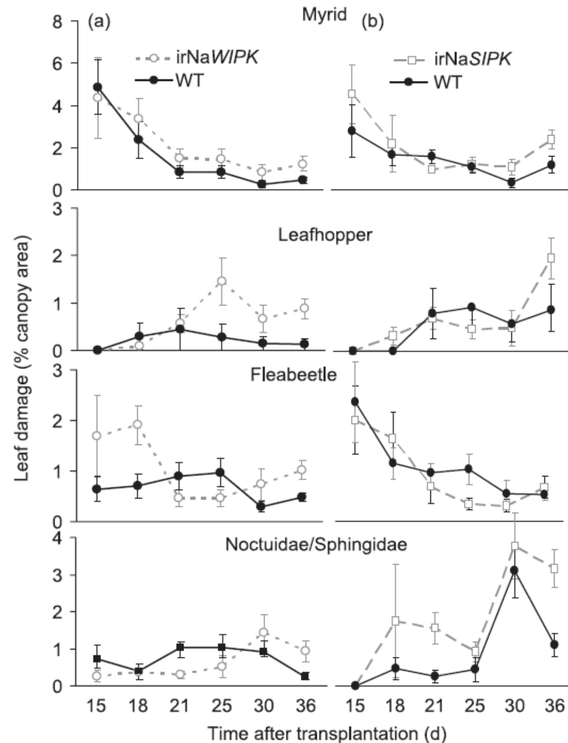

Fig. 4 irNa*SIPK* and irNa*WIPK* plants do not suffer more damage by the native herbivore community than wild-type plants. Mean (± SE) percentage of damaged leaf area caused by the native herbivore community in 12 pairs of size-matched irNa*SIPK* (A-109) (open squares)/wild-type (WT, closed circles) (a) and irNa*WIPK* (A-56) (open circles)/WT (closed circles) (b) *Nicotiana attenuata* plants growing in *N. attenuata*'s native habitat in the Great Basin Desert. The characteristic damage from myrids, leafhoppers, flea beetles and lepidoptern larvae was quantified as a percentage of damaged area to total canopy area starting 15 d after plants were transplanted into the field.

(A-56) plants resulted in less predation of *G. pallens* on *M. sexta* eggs, we performed *M. sexta* egg predation rate assays (Kessler & Baldwin, 2001). We used eight irNa*SIPK* (A-109), irNa*WIPK* (A-56), and WT plants and glued five eggs on the leaf at node S2 of each plant. Rosette leaves next to the S2 leaves were W+OS-elicited to enhance the VOC release. The predation rates were recorded 48 h after induction. Forty per cent of the glued eggs on WT plants were predated, whereas only 12% of the glued eggs on irNa*SIPK* plants (A-109) (ANOVA $F_{2,18} = 3.562$; $P = 0.028$), and on irNa*WIPK* (A-56) plants (ANOVA $F_{2,18} = 3.562$; $P = 0.0385$) 15% were predated (Fig. 3). To correlate the attraction of *G. pallens* with the release of W+OS-induced volatiles in the field, we trapped volatiles from entire plants 24–48 h after a single rosette leaf was treated with W+OS. *G. pallens* is highly responsive to *trans-α*-bergamotene and *cis*-3-hexenol (Kessler & Baldwin, 2001; Halitschke *et al.*, 2008). The amount of *trans-α*-bergamotene released from W+OS-elicited plants in the field was only 30% (irNa*SIPK*) and 33% (irNa*WIPK*), respectively, of that released from WT plants (Fig. 3, inset). Notably, *cis*-3-hexenol emission was reduced by 74% in irNa*WIPK* (A-56) plants, and in ir*NaSIPK* (A-109) plants, *cis*-3-hexenol was not detectable (Fig. 3, inset). These findings suggest that the reduced release of volatile compounds in irNa*SIPK* and irNa*WIPK* plants decreases the plants' attractiveness to native predatory insects.

In the past years, analysis of herbivore resistance of transgenic plants in the field has revealed that *N. attenuata* plants impaired in their ability to deploy JA-mediated direct defenses such as nicotine or TPIs are highly susceptible to attack from the native herbivore community (Kessler *et al.*, 2004; Steppuhn *et al.*, 2004; Paschold *et al.*, 2007). To compare the resistance of irNa*SIPK* and irNa*WIPK* plants to native insect herbivores with that of WT plants, we measured the damage inflicted by herbivores from four different feeding guilds on at least 12 irNa*SIPK*/WT and 12 irNa*WIPK*/WT pairs for 21 d, starting 15 d after plants were transplanted into the field. Surprisingly, damage caused by leafhoppers, myrids, flea beetles and lepidopteran (Noctuidae/Sphingidae) larvae on irNa*SIPK* and irNa*WIPK* plants was the same as the damage on paired WT plants, although kinase-silenced plants had reduced amounts of JA-mediated defenses, such as nicotine and TPI (Fig. 4, repeated measures ANOVA, irNa*SIPK*/

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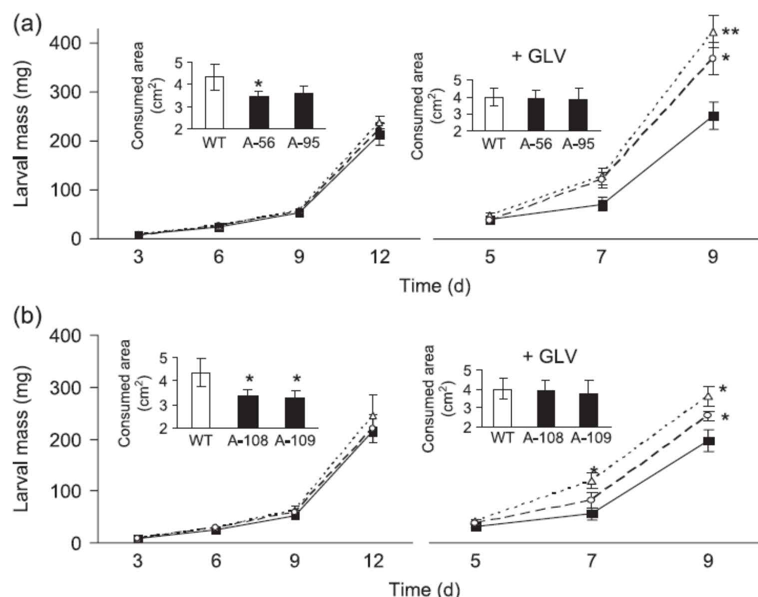

Fig. 5 Green leaf volatile (GLV) emissions influence *Manduca sexta* leaf consumption and larval mass gain on irNa*SIPK* and irNa*WIPK* plants. Mean (± SE) larval mass gain and leaf consumption (inset) of *M. sexta* larvae feeding on irNa*WIPK* (A-56, triangles; A-95, circles) (a), irNa*SIPK* (A-108, triangles; A-109, circles) (b), and wild-type (WT, squares) *Nicotiana attenuata* plants. Freshly hatched *M. sexta* larvae were placed on transition leaves of rosette-stage plants and enclosed individually in well-aerated clip cages. Twenty microlitres of lanolin or GLVs dissolved in lanolin were applied to the petioles of the leaves outside of the clip cages. For lanolin treatment, on days 3 and 6, larvae were placed on the next younger leaves and the attacked leaves were removed to analyze the consumed area; leaf tissue consumption was determined until day 6; after day 6, clip cages were removed. For GLV treatment, larvae were placed on new leaves after days 3, 5 and 7; until day 5 leaf consumption was analyzed. Consumption data were obtained from at least eight biological replicates. Asterisks indicate significant differences between larval mass gain and leaf areas consumed by larvae feeding on WT and irNa*SIPK* or irNa*WIPK* plants (two-way ANOVA, Fisher's PLSDs: *, $P < 0.05$; **, $P < 0.01$).

WT_{Myrids} $F_{1,180} = 0.93$, $P = 0.34$; irNa*SIPK*/$WT_{Leafhopper}$ $F_{1,180} = 0.09$, $P = 0.34$; irNa*SIPK*/$WT_{Fleabeetle}$ $F_{1,180} = 0.026$, $P = 0.87$; irNa*SIPK*/$WT_{Noctuidae/Sphingidae}$ $F_{1,180} = 0.46$, $P = 0.51$; irNa*WIPK*/WT_{Myrids} $F_{1,180} = 1.2$, $P = 0.28$; irNa*WIPK*/$WT_{Leafhopper}$ $F_{1,180} = 0.99$, $P = 0.32$; irNa*WIPK*/$WT_{Fleabeetle}$ $F_{1,180} = 2.178$, $P = 0.15$; irNa*WIPK*/$WT_{Noctuidae/Sphingidae}$ $F_{1,180} = 0.055$, $P = 0.82$; Fig. S6). By contrast, adjacent to our field plot, transgenic *N. attenuata* plants with silenced transcript levels of Na*WRKY3* and Na*WRKY6* transcription factors, which are also deficient in JA-mediated direct defenses, were highly susceptible to herbivores during the same field season (Skibbe *et al.*, 2008); furthermore, WT plants showed comparable herbivore damage to that observed in WT plants on our field plot (data not shown), confirming that *N. attenuata* plants with impaired JA signaling were highly vulnerable to insect herbivores during this field season, as they have been for the previous six field seasons.

To investigate the degrees of resistance of irNa*SIPK* and irNa*WIPK* plants to herbivores under controlled conditions, we performed *M. sexta* feeding assays in the glasshouse. Freshly hatched *M. sexta* larvae were placed on rosette-stage irNa*SIPK*, irNa*WIPK*, and WT plants, and the consumed leaf area and the larval weight over time were measured. To accurately measure the leaf area consumed by *M. sexta*, we enclosed the leaf and a *M. sexta* larva in a well-aerated plastic cage (Fig. S8a) to keep it from moving to other parts of the plant. After 3 d we moved each larva to an intact leaf, and after 6 d we continued the assay without cages. Total leaf area consumed for the first 6 d of feeding, and larval mass gain until 12 d were measured. We found that *M. sexta* larvae did not gain more mass on irNa*SIPK* or irNa*WIPK* plants than on WT plants, although kinase-silenced plants accumulated only low amounts of anti-herbivore defensive metabolites; importantly, the larvae that fed on irNa*SIPK* and irNa*WIPK* plants consumed significantly less leaf material than those that fed on WT plants (Fig. 5). These data suggest that irNa*SIPK* and irNa*WIPK* plants may have an altered chemistry, for example, reduced amounts of feeding stimulants or increased amounts of feeding deterrents for *M. sexta* larvae, which reduce larval consumption and slow larval mass gain.

Halitschke *et al.* (2004) reported that *M. sexta* larvae consume less leaf material on *N. attenuata* plants that have reduced Na*HPL* transcript levels and reduced GLV emissions (asNa*HPL* plants). irNa*SIPK* and irNa*WIPK* plants have highly reduced levels of W+OS-elicited Na*HPL* transcripts and GLV emissions (Fig. S5, Table 1). In order to test the hypothesis that reduced GLV emissions in irNa*SIPK*

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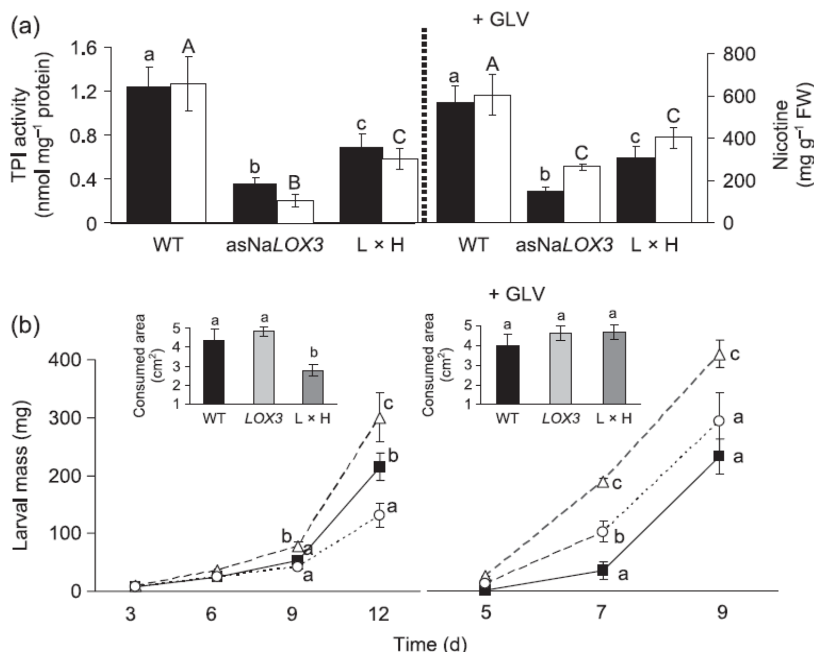

Fig. 6 Silencing Na*HPL* in asNa*LOX3* restores plants' resistance to *Manduca sexta* larvae. (a) Mean (± SE) nicotine concentration (closed bars) and trypsin protease inhibitor (TPI, open bars) activity, and (b) mean (± SE) *M. sexta* larval mass gain and leaf tissue consumption on asNa*LOX3* (triangles) asNa*LOX3* × asNa*HPL* (L × H; circles), and wild-type (WT, squares) *Nicotiana attenuata* plants. Freshly hatched *M. sexta* larvae were placed on transition leaves of rosette-stage plants and enclosed individually in well-aerated clip cages. Twenty microlitres of lanolin or green leaf volatiles (GLVs) dissolved in lanolin were applied to the petioles of the leaves outside the clip cages. For lanolin treatment, on days 3 and 6 larvae were placed on the next younger leaves and the attacked leaves were removed to analyze the consumed area; leaf tissue consumption was determined until day 6; after day 6, clip cages were removed. For GLV treatment, larvae were placed on new leaves after days 3, 5 and 7; until day 5 leaf consumption was analyzed. Consumption data were obtained from at least eight biological replicates. Nicotine concentration and TPI activity were determined from nonattacked systemic leaves of eight replicates of lanolin or GLV-treated plants, 9 d after *M. sexta* infestation. Different letters indicate significant differences of nicotine concentration, TPI activity, larval mass gain and leaf tissue consumption between WT and asNa*LOX3* or L × H (two-way ANOVA, Fisher's PLSDs).

and irNa*WIPK* plants contributed to the decreased leaf consumption by *M. sexta*, we applied a mixture of different GLVs, dissolved in lanolin, to the petioles of the leaves and performed *M. sexta* larvae growth assays. GLV application to the petioles increases basal and wound-induced GLV emission of the treated leaves (Fig. S9). After GLVs were applied, the consumption rates of *M. sexta* increased greatly (Fig. 5). Importantly, GLV treatment not only restored the consumption of *M. sexta* on irNa*SIPK*- and irNa*WIPK*-silenced plants to those on WT plants, but *M. sexta* larval mass gain on irNa*SIPK* and irNa*WIPK* plants increased significantly compared with that on WT plants (Fig. 5). Treatment with GLVs did not affect the accumulation of known defensive metabolites, such as nicotine and TPI, in irNa*SIPK* and irNa*WIPK* plants, and lanolin itself had no effect on *N. attenuata*'s defense or *M. sexta*'s leaf consumption (data not shown). These data suggest that *M. sexta* larvae do not benefit from feeding on plants with highly reduced amounts of defensive metabolites when their feeding activity is inhibited by reduced feeding stimulants, such as GLVs.

Reducing GLV emissions in asNa*LOX3* plants reduces consumption rates and larval mass gain of *M. sexta*

To further test the hypothesis that down-regulating the amount of GLVs reduces *M. sexta*'s performance on plants with impaired direct defenses, we used: plants silenced in Na*LOX3* (asNa*LOX3*), which are highly vulnerable to *M. sexta* attack because of their attenuated JA-mediated direct defenses (Halitschke & Baldwin, 2003; Kessler *et al.*, 2004); and plants produced by crossing asNa*LOX3* plants with asNa*HPL* plants that have reduced amounts of GLVs (Halitschke *et al.*, 2004). These crosses (asNa*LOX3* × asNa*HPL*, hereafter L × H) have reduced amounts of JA-mediated defenses (TPIs, nicotine), Na*LOX3*, Na*HPL* transcripts, and GLV emissions (Figs 6a, S7). After *M. sexta* feeding, L × H plants only received 45% of the damage received by asNa*LOX3* plants (ANOVA, $F_{2,42} = 9.118$, $P = 0.002$); in addition, *M. sexta* larvae that fed on L × H plants gained < 50% of the mass gained by larvae that fed on asNa*LOX3* plants (Fig. 6b). Although L × H plants

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accumulated higher amounts of nicotine and TPIs 12 d after feeding than did asNa*LOX3* plants, the nicotine and TPI concentrations in L × H plants were still much reduced compared with those in WT plants (Fig. 6a, ANOVA nicotine, $F_{5,36} = 5.783$, $P = 0.0059$; TPI, $F_{5,38} = 7.585$, $P = 0.011$). Since the L × H plants are heterozygous for the asNa*LOX3* construct, the intermediate levels of JA-mediated direct defenses were expected, as the silencing of the JA cascade is likely less than it is in homozygous asNa*LOX3* plants. Thus, reduced GLVs in L × H very likely accounted for the decreased *M. sexta* consumption and larval mass on these plants.

We tested the hypothesis that the lack of GLVs was responsible for the decrease in herbivore performance, and measured performance of *M. sexta* larvae after a mixture of different GLVs were applied to the petioles of WT, asNa*LOX3*, and L × H plants. Again, applying GLVs abrogated the differences in larval consumption among normal-emitting and lower-emitting plants: larvae on GLV-treated asNa*LOX3*, L × H and WT plants all consumed equal amounts of tissue (Fig. 6b, inset). Furthermore, applying GLVs enhanced the growth of larvae that fed on JA- and GLV-deficient plants: larvae that fed on GLV-treated L × H plants gained up to 83% as much mass as those that fed on GLV-treated asNa*LOX3* plants (Fig. 6). It is likely that the higher amounts of TPIs in L × H plants than in asNa*LOX3* plants accounted for the small difference in the mass of *M. sexta* larvae that fed on L × H and asNa*LOX3* plants.

These results demonstrate that the amount of GLV affects herbivore feeding behavior, and the decreased GLV emissions in irNa*SIPK* and irNa*WIPK* plants might offset *M. sexta* consumption and thus mass gain, although irNa*SIPK* and irNa*WIPK* have lower amounts of direct defense metabolites compared with WT plants.

Discussion

MAPK-mediated resistance of plants to pathogens has been intensively studied (reviewed in Zhang & Klessig, 2001; Mishra *et al.*, 2006), however, the role of MAPKs in plant defense against herbivores has only been recently examined (Kandoth *et al.*, 2007; Wu *et al.*, 2007). Two MAPKs in *N. attenuata*, Na*SIPK* and Na*WIPK*, are activated by herbivory and mediate herbivore-induced hormonal and transcriptional responses (Wu *et al.*, 2007). Here we investigate whether silencing Na*SIPK* and Na*WIPK* alters *N. attenuata*'s susceptibility to herbivorous insects. We show that silencing Na*SIPK* and Na*WIPK* reduces herbivore-induced direct (nicotine, caffeoylputrescine, TPI) and indirect (attraction of predators of herbivores) defenses; however, silencing does not increase *N. attenuata*'s susceptibility to herbivorous insects in both the plants' natural habitat and the glasshouse. Using a feeding assay, we found that attenuated amounts of feeding stimulants, such as GLVs in irNa*SIPK* and irNa*WIPK* plants, may account for reduced amounts of damage caused by the plants' specialist herbivore, *M. sexta*. We further demonstrate that genetically reducing GLV emissions by silencing Na*HPL* in plants that are highly susceptible to herbivores (asNa*LOX3*) restores the plants' resistance to *M. sexta*.

Several studies have demonstrated that both nicotine and TPI are potent anti-herbivore defenses (Steppuhn *et al.*, 2004; Zavala *et al.*, 2004; Steppuhn & Baldwin, 2007). The accumulation of nicotine, TPI and certain phenolic compounds in response to herbivory requires the plants' ability to rapidly increase JA pools after herbivore attack (Halitschke & Baldwin, 2003; Kessler *et al.*, 2004). Additionally, transcript levels of genes involved in the biosynthesis of nicotine, phenylpropanoids, and TPI have been shown to be reduced in *N. attenuata* plants that are impaired in JA biosynthesis or perception (Halitschke & Baldwin, 2003; Paschold *et al.*, 2007). Herbivory elicits the release of terpenes and other volatiles from *N. attenuata* and thus increases the predation rates on herbivore eggs and larvae (Kessler & Baldwin, 2001). The importance of JA-mediated signaling for the induction of these indirect defenses has been demonstrated for many plant species by JA treatment (Thaler, 1999; Gols *et al.*, 2003), by using JA-deficient plants (Howe *et al.*, 1996; Thaler *et al.*, 2002; van Poecke & Dicke, 2002; Halitschke & Baldwin, 2003; Ament *et al.*, 2004; Halitschke *et al.*, 2008), or plants with JA insensitivity (Paschold *et al.*, 2007; Rayapuram, 2007).

Silencing Na*SIPK* and Na*WIPK* reduces herbivore-induced JA concentrations; the concentrations of nicotine, TPI, caffeoylputrescine, and the transcript accumulation of genes involved in their biosyntheses are consistently lower. Moreover, highly reduced herbivore-induced volatile emissions and reduced indirect defenses in the field were also observed in irNa*SIPK* and irNa*WIPK* plants and were correlated with the decreased transcript levels of genes in the terpenoid biosynthetic pathways. These data indicate that by tuning JA concentrations, NaSIPK and NaWIPK mediate direct and indirect herbivore-resistance traits in *N. attenuata*. Silencing the homologues of Na*SIPK* (Le*MPK1* and Le*MPK2*) and Na*WIPK* (Le*MPK3*) in tomato plants that ectopically express prosystemin leads to suppressed systemin-mediated resistance to *M. sexta* (Kandoth *et al.*, 2007). Plants impaired in JA-mediated direct and indirect defenses are more frequently attacked by herbivores, and many generalist and specialist herbivores eat more and grow faster on these plants (Halitsche *et al.*, 2004). Surprisingly, the reduced JA-mediated direct and indirect defenses in irNa*SIPK* and irNa*WIPK* plants do not lead to increased amounts of damage when compared with paired WT plants in their native habitat, and in the glasshouse, and larvae of the specialist herbivore *M. sexta* do not gain more weight on these plants.

Manduca sexta larvae consume less leaf tissue on irNa*SIPK* and irNa*WIPK* plants than on WT plants, indicating that these plants have either increased amounts of feeding deterrents or decreased amounts of feeding stimulants.

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GLVs appear to stimulate feeding for lepidopteran larvae (*M. sexta*, *M. quinqemaculata*, and *Spodoptora exigua*), as they feed less on asNa*HPL* *N. attenuata* plants (Halitschke *et al.*, 2004). Na*HPL* expression is highly reduced in W+OS-elicited irNa*SIPK* and irNa*WIPK* plants; this may account for these plants' decreased GLV emissions and may contribute to reduced *M. sexta* larval consumption. Applying synthetic GLVs not only restores *M. sexta* consumption on irNa*SIPK* and irNa*WIPK* plants to the same degree as on GLV-treated WT plants but also increases *M. sexta* larval mass.

The association between GLV emission rates and herbivores' performance on plants with different amounts of anti-herbivore defensive metabolites was confirmed by silencing Na*HPL* in asNa*LOX3* plants. *M. sexta* larvae fed on L × H plants had highly decreased consumption rates and mass gains. Moreover, these were reversed after applying synthetic GLVs to L × H plants. This suggests that reducing GLV emissions could 'rescue' plants with impaired JA-mediated defenses and that the stimulatory effects of GLVs on *M. sexta* larval feeding behavior may outweigh the effects of reduced defense metabolites. GLV perception by herbivorous insects is important for host-plant location, and GLV-specific olfactory receptor neurons have been identified in several insect species (reviewed in Bruce *et al.*, 2005). If GLV emissions increase plants' apparency to herbivores and increase larval consumption, why are they produced in large amounts by most plants? We assume that the benefit of maintaining GLV emissions outweighs the cost of receiving more damage by herbivores. It is known that some insect predators also use GLVs as detection cues to find plants infested by herbivores. For example, the parasitic wasp *Cotesia glomerata* is more attracted to *A. thaliana* plants that emit higher amounts of GLVs, as a result of sense expression of *AtHPL* (Shiojiri *et al.*, 2006); the generalist predator *G. pallens* is attracted to GLVs emitted from herbivore-attacked *N. attenuata* plants (Halitschke *et al.*, 2008). Additionally, recent studies have shown that GLVs are implicated in plant resistance to pathogens: C_6 aldehydes are important constituents for defense responses in Arabidopsis against fungal pathogens (Kishimoto *et al.*, 2008) and have bactericidal effects (Croft *et al.*, 1993). Whether GLVs have anti-pathogen and signaling effects in *N. attenuata* needs to be studied further; GLV-deficient *N. attenuata* plants planted into native habitats do not appear to suffer from fungal or bacterial infections (I. T. Baldwin, unpublished). The reactive electrophiles α, β-unsaturated carbonyl groups of some GLVs may elicit stress and defense responses on plants, suggesting a role for GLVs in defense signaling (Bate & Rothstein, 1998; Stintzi *et al.*, 2001). For example, *trans*-2-hexenal, a GLV compound that is commonly emitted after herbivore wounding, elicits sesquiterpenoid phytoalexins in Arabidopsis (Bate & Rothstein, 1998). The function of volatiles as plant defense elicitors has been demonstrated for complex volatile blends in lima bean (Heil *et al.*, 2007) and for monoterpenes in Arabidopsis (Godard *et al.*, 2008).

However, volatile blends emitted from wounded *N. attenuata* plants only elicited transcriptional responses when the plant's GLV emissions are reduced due to antisense expression of NaHPL, suggesting that GLVs play a role as a negative regulator of defense responses in *N. attenuata* (Paschold *et al.*, 2006).

The glasshouse and field experiments presented here reveal an unexpected ecological function of Na*SIPK* and Na*WIPK* in *N. attenuata*'s defense against herbivores: although these MAPKs positively regulate plants' direct and indirect defenses and make silenced plants more vulnerable to herbivore attack, they also appear to decrease herbivores' consumption on plants and thus compensate for the plant's impaired resistance. We propose that reduced GLV emissions largely account for the similar herbivore damage seen in irNa*SIPK* and irNa*WIPK* plants compared with WT plants. However, we cannot rule out the possibility that other metabolic changes may also have contributed to the observed phenotypes. For example, the reduced mono- and sesquiterpenes and certain phenolic compounds in irNa*SIPK* and irNa*WIPK* plants may also affect feeding behavior of herbivores. More studies are necessary to address the effect of these compounds on different herbivores. It has been reported that certain glucopyranosides, which were isolated from different solanaceaeous species, are specifically detected by *M. sexta* larvae to distinguish between host and nonhost plants and highly stimulate *M. sexta* larval feeding (del Campo *et al.*, 2001; Haribal *et al.*, 2006). Whether these compounds are produced in *N. attenuata* and are regulated by MAPKs is still not known. It is also possible that the accumulation of certain other unknown feeding deterrents may contribute to the observed phenotypes. More detailed analyses are needed to further address the metabolic changes in these kinase-silenced plants. This study suggests that manipulating plants' GLV emissions provides a novel tool for engineering crops to reduce herbivore damage.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Partial sequences of Na*SIPK* and Na*WIPK* used for the preparation of RNAi constructs for silencing Na*SIPK* and Na*WIPK* in *Nicotiana attenuata.*

Fig. S2 irNa*SIPK* and irNa*WIPK* plants have decreased levels of Na*SIPK* and Na*WIPK* transcript and kinase activity.

Fig. S3 Arrangement of plants in the field in the Great Basin Desert, Utah.

Fig. S4 Jasmonic acid (JA) and JA-isoleucine conjugate (JA-Ile) concentrations are highly reduced in irNa*SIPK* and irNa*WIPK* plants.

Fig. S5 Silencing Na*SIPK* and Na*WIPK* impairs the transcript accumulation of genes involved in volatile biosynthesis.

Fig. S6 Silencing Na*SIPK* and Na*WIPK* impairs the accumulation of nicotine and trypsin proteinase inhibitors in *Nicotiana attenuata* in the field.

Fig. S7 asNa*LOX3* × irNa*HPL* plants have reduced transcript levels of Na*LOX3* and Na*HPL* and reduced green leaf volatile (GLV) emissions.

Fig. S8 Experimental setup of *Manduca sexta* performance measurements using clip cages and treatment of petioles of *Nicotiana attenuata* leaves with lanolin paste.

Fig. S9 Green leaf volatile (GLV) treatment of petioles of leaves leads to higher leaf GLV emission.

Table S1 Primer and probe sequences used for qPCR

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Figure S1

irNa*WIPK*

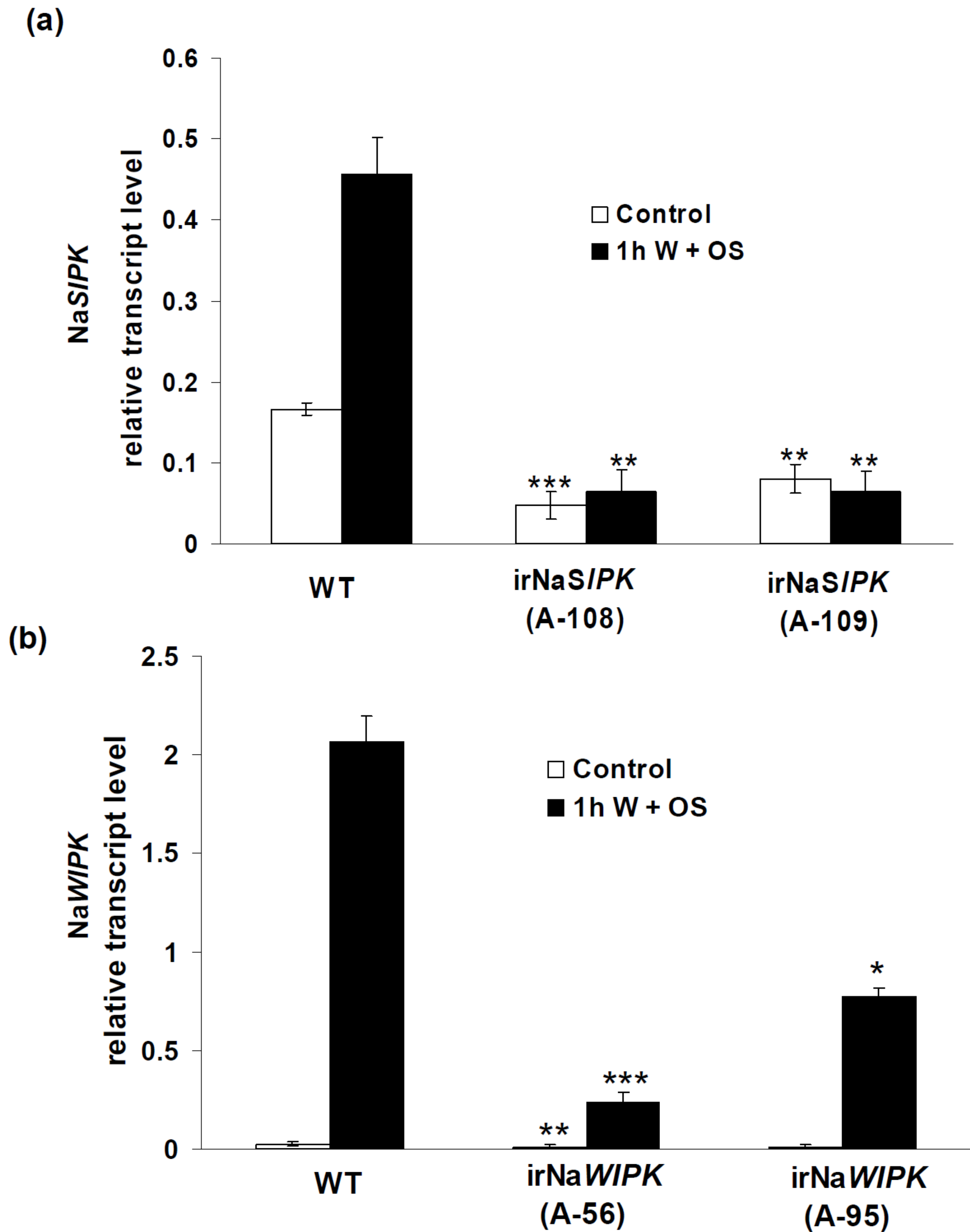
5’-
GAGCCACGGTGGCAGGTTCATTCAATACAATATATTTGGTAATATATTTGAAG
TTACTGCTAAGTATAAGCCTCCTATTTTGCCTATTGGTAAAGGTGCTTACGGC
ATCGTTTGTTCTGCTTTGAACTCGGAGACAATTGAGAATGTAGCGATAAAGAA
AATCGCGAATGCTTTTGATAACAAGATTGATGCCAAGAGGACTTTGAGAGAGA
TCAAGCTTCTTCGGCATATGGATCATGAAAACATTGTTGCGATCAGAGATATA
ATTCCACCACCGCAGAGAGAAGCCTTTAATGATGTTTATATTGCGTATGAGCT
TATGGATAC-3’

irNa*SIPK*

5’ -
GGAGCTGAATGAGATGGTTGCAGTTAAGAAAATCGCGAATGCGTTTGACAAT
TACATGGATGCTAAGAGGACTCTCCGTGAGATTAAGCTTCTTCGCCATTTAGA
TCATGAAAATGTAATTGGTTTAAGAGACGTGATTCCTCCACCGTTACGAAGGG
AGTTTTCTGATGTTTACATTGCTACTGAACTCATGGATACTGATCTTCACCAAA
TAATTAGATCCAACCAAGGTTTATCAGAGGATCACTGCCAGTACTTCATGTAT
CAGCTCCTCCGTGGCCTAAAATACATACATTCCGCGAATGTTCTTCATAGAG-
3'

Fig. S1 Partial sequences of Na*SIPK* and Na*WIPK* used for the preparation of RNAi constructs for silencing Na*SIPK* and Na*WIPK* in *Nicotiana attenuata.*

Figure S2



(c)

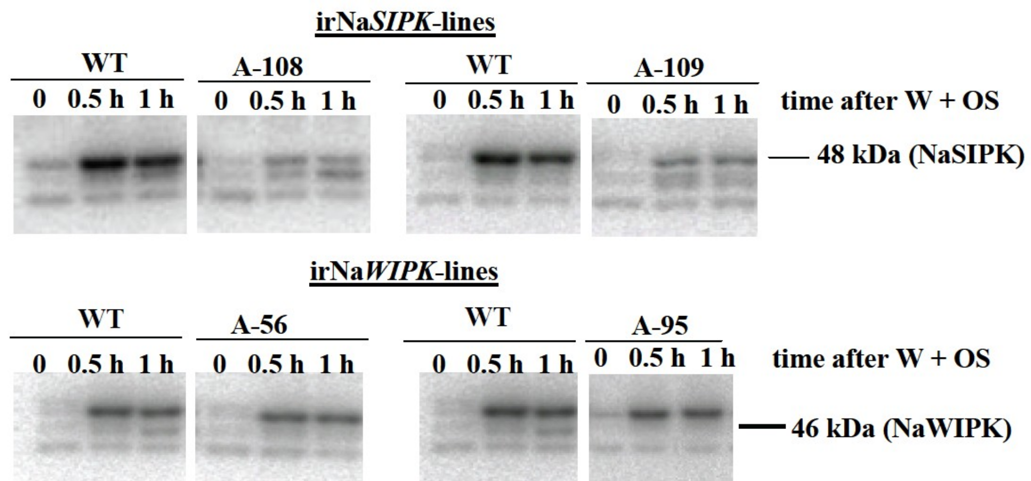


Fig. S2 irNa*SIPK* and irNa*WIPK* plants have decreased levels of Na*SIPK* and Na*WIPK* transcript and kinase activity.

Figure S3

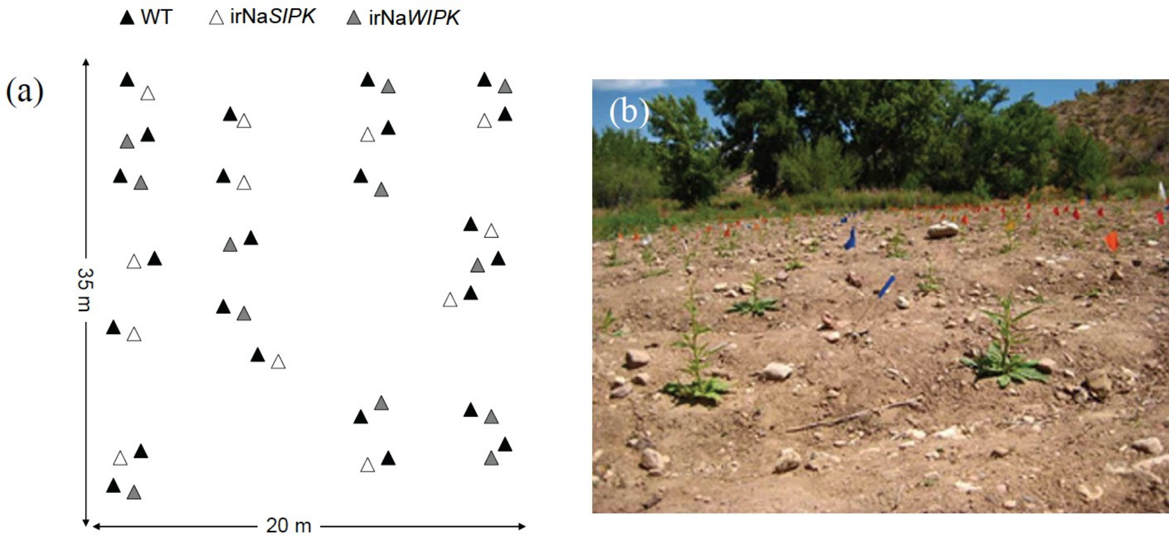


Fig. S3 Arrangement of plants in the field in the Great Basin Desert, Utah.

Figure S4

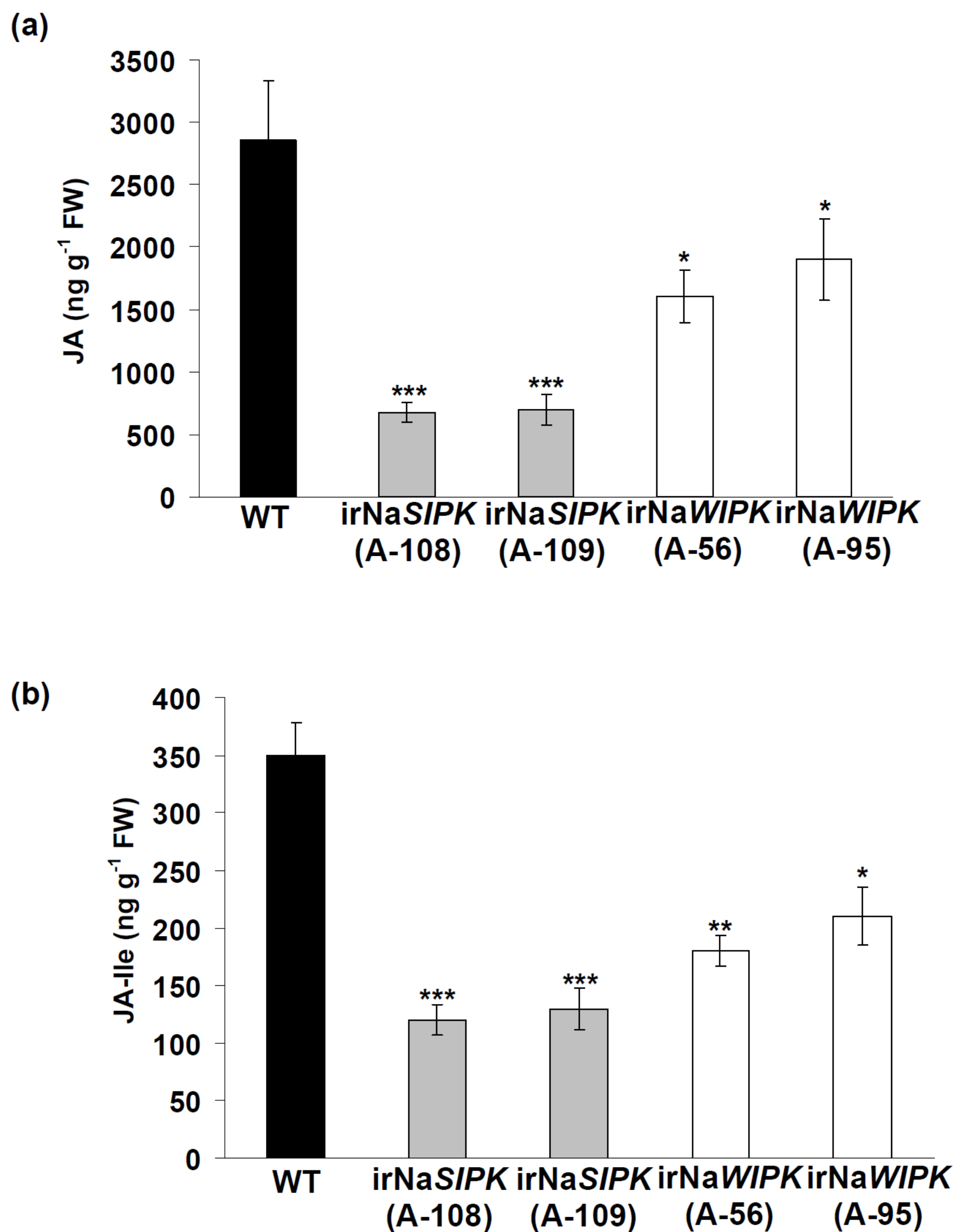


Fig. S4 Jasmonic acid (JA) and JA-isoleucine conjugate (JA-Ile) concentrations are highly reduced in irNa*SIPK* and irNa*WIPK* plants.

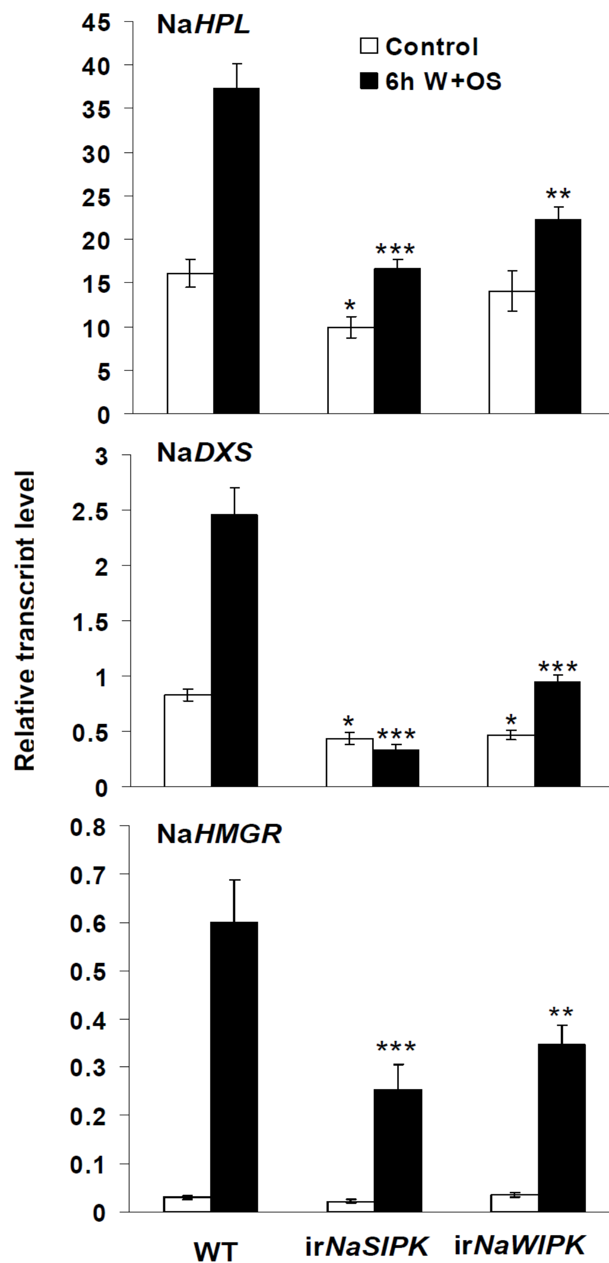


Fig. S5 Silencing Na*SIPK* and Na*WIPK* impairs the transcript accumulation of genes involved in volatile biosynthesis.

Figure S6

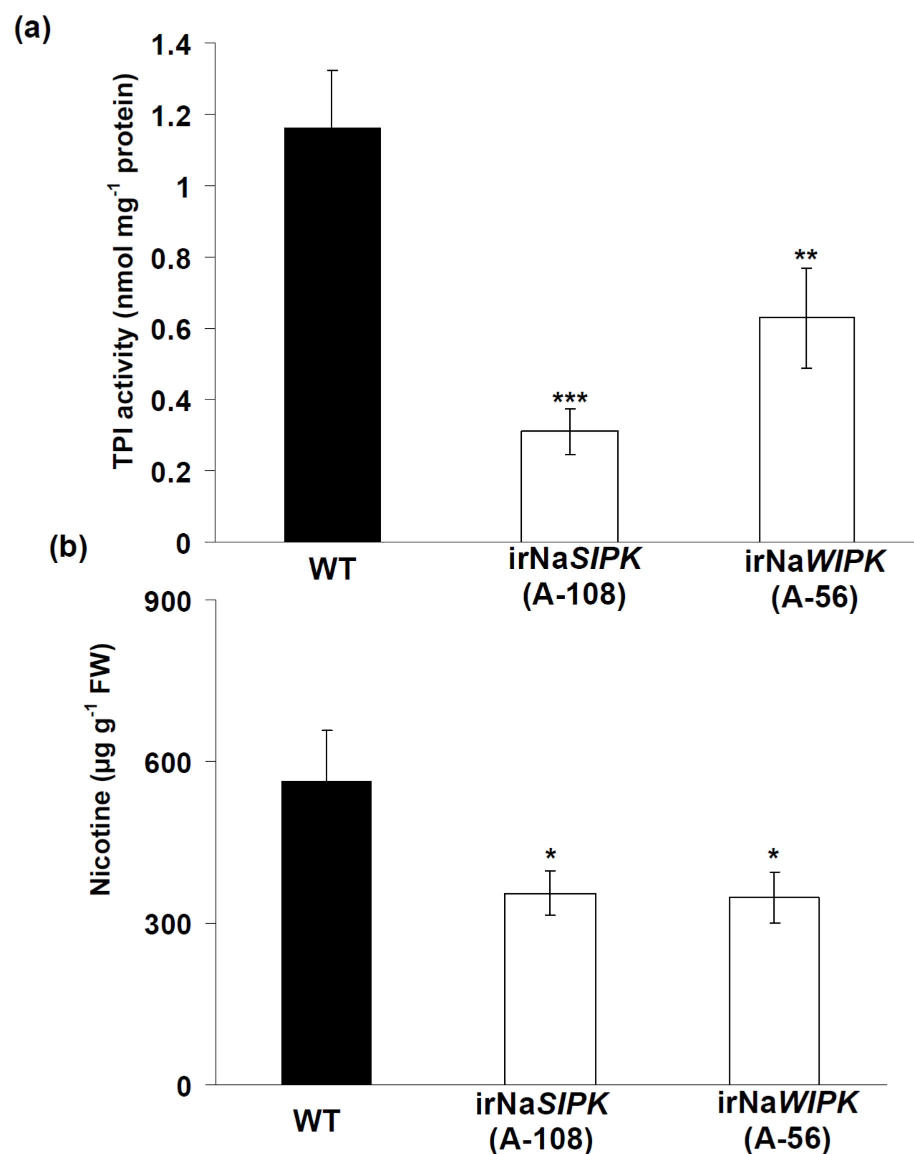


Fig. S6 Silencing Na*SIPK* and Na*WIPK* impairs the accumulation of nicotine and trypsin proteinase inhibitors in *Nicotiana attenuata* in the field.

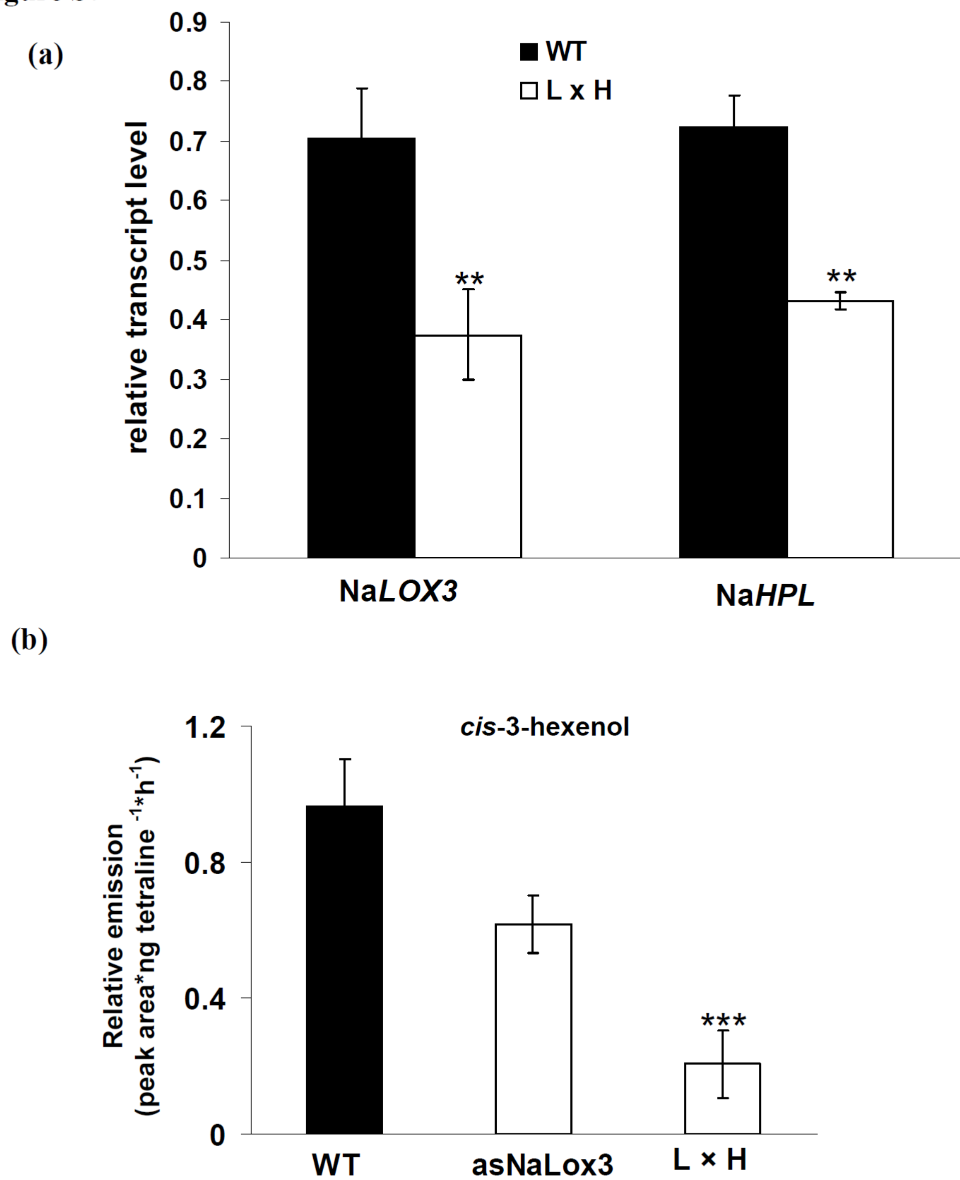


Fig. S7 asNa*LOX3* × irNa*HPL* plants have reduced transcript levels of Na*LOX3* and Na*HPL* and reduced green leaf volatile (GLV) emissions.

Figure S8

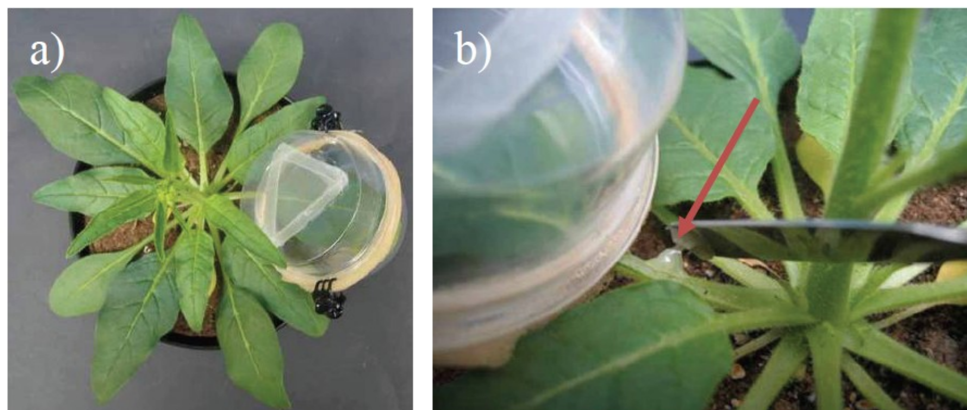


Fig. S8 Experimental setup of *Manduca sexta* performance measurements using clip cages and treatment of petioles of *Nicotiana attenuata* leaves with lanolin paste.

Figure S9

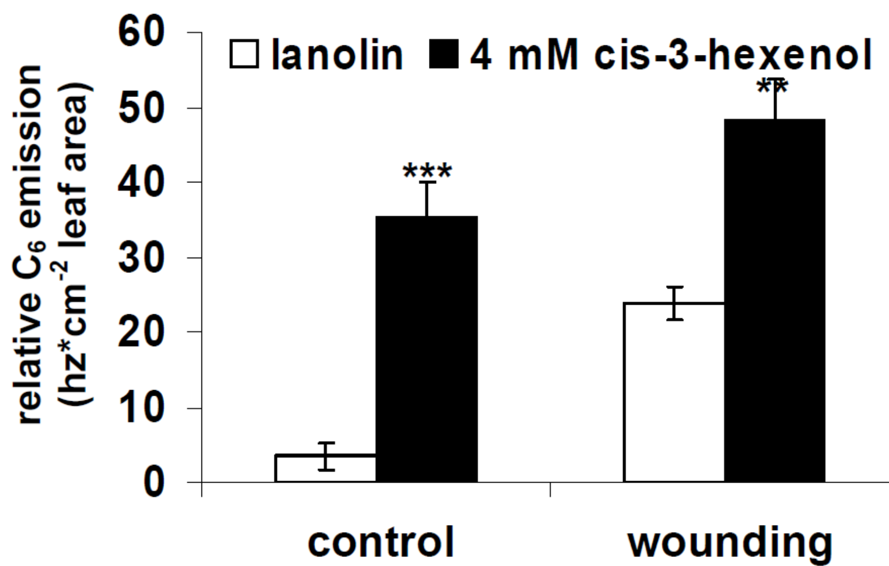


Fig. S9 Green leaf volatile (GLV) treatment of petioles of leaves leads to higher leaf GLV emission.

Table S1 Primer and probe sequences used for qPCR

Genes	Forward Primer	Reverse Primer	Taqman® Probe	SYBR
Na*SIPK*	GTTGACGAATTTTCCAAAACAAAGT	CCGGAATATTATCCATACCGGCC		x
Na*WIPK*	GGTCTTGCTAGGCCAAACATA	GACCAAACATCTATAGCAGCAG		x
Na*HPL*	CACTTAGACTTAGTCCACCTGTGC	AACACAAACTTTTCAGGATCATCA	TCAGCCATTGGTAATGAGAGATCCAAAGG	
Na*LOX3*	GGCAGTGAAATTCAAAGTAAGAGC	CCCAAAATTTGAATCCACAACA	CAGTGAGGAACAAGAACAAGGAAGATCTGAAG	
Na*DXS*	ATTGATGACAGACCAAGCTGTTT	TATCCTAGTAGAGCCACTCTC		x
Na*HMGR*	CTCAGTCAGCTTGCTTGAACTTA	ATTTCATGTGGCTCTTAACCAGC		x
Actin	GGTCGTACCACCGGTATTGTG	GTCAAGACGGAGAATGGCATG		x

New Phytologist

Research

SGT1 regulates wounding- and herbivory-induced jasmonic acid accumulation and *Nicotiana attenuata*'s resistance to the specialist lepidopteran herbivore *Manduca sexta*

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Key words: defense, herbivory, jasmonic acid, jasmonic acid-isoleucine, *Nicotiana attenuata*, SGT1.

Summary

- SGT1 (suppressor of G-two allele of SKP1) is a conserved protein in all eukaryotes and is crucial for resisting pathogens in humans and plants. We studied whether SGT1 is involved in the induced defense response of a native tobacco (*Nicotiana attenuata*) to its natural herbivore, *Manduca sexta*.
- We diminished *NaSGT1* transcription in *N. attenuata* using virus-induced gene silencing (VIGS) and analysed the induced defense responses after wounding and *M. sexta* elicitation.
- Silencing *NaSGT1* highly attenuates wounding- and herbivory-induced amounts of jasmonic acid (JA) and JA-isoleucine but elevates the concentration of salicylic acid. Chemical profiling reveals that *NaSGT1*-silenced plants are also compromised in their ability to accumulate JA precursors produced in chloroplasts. We show that the reduced JA accumulation in *NaSGT1*-silenced plants is independent of the elevated salicylic acid levels. *NaSGT1*-silenced plants have decreased contents of defensive metabolites and have compromised resistance to *M. sexta* larvae. Transcript analyses after methyl jasmonate (MeJA) treatment revealed that *NaSGT1* is important for the normal regulation of MeJA-induced transcriptional responses.
- This work demonstrates the importance of SGT1 in the regulatory network that deploys defense responses against herbivores, and highlights the significance of SGT1 in plants' responses to JA.

Introduction

Plants have evolved sophisticated strategies to adapt their physiology to changing environmental conditions. The interplay among complex signaling networks, including various pathways regulated by phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene and ABA, dramatically influences plants' stress responses (Chow & McCourt, 2006; Pieterse *et al.*, 2009; Santner & Estelle, 2009). By tightly controlling the accumulation and perception of phytohormones, plants counteract environmental stresses. In recent years, new genetic and biochemical tools have greatly advanced our understanding of the biosynthesis, signal transduction, and physiological functions of phytohormones in various plant species. Among these, JA plays a crucial role in plant development and adaptation to environmental stresses.

Jasmonic acid is important for plants' defense against insects and microbial pathogens, resistance to drought, ultraviolet radiation, ozone and other abiotic stresses (Conconi *et al.*, 1996; Glazebrook, 2005; Howe & Jander, 2008; Browse, 2009). It is also involved in plant senescence and reproductive development (Feys *et al.*, 1994; Li *et al.*, 2004; Buchanan-Wollaston *et al.*, 2005). JA biosynthesis and signaling are embedded in a complex network that includes crosstalk with other phytohormones such as SA and auxin (Harms *et al.*, 1998; Spoel *et al.*, 2003; Diezel *et al.*, 2009; Grunewald *et al.*, 2009), light signaling (Moreno *et al.*, 2009) and mitogen-activated protein kinase (MAPK) pathways (Brodersen *et al.*, 2006, Wu *et al.*,

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2007). Recently, significant progress has been made in understanding the molecular basis of JA signaling. The F-box protein COI1 (CORONATINE-INSENSITIVE 1) is part of a Skp/Cullin/F-box complex [SCF$^{(COI1)}$] that acts as an ubiquitin ligase and is required for physiological responses mediated by JA (Xu *et al.*, 2002; Chini *et al.*, 2007; Paschold *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008; Yan *et al.*, 2009). JARs (JASMONATE RESISTANT) conjugate JA with isoleucine to form JA-Ile (Staswick & Tiryaki, 2004). The binding of JA-Ile to SCF$^{(COI1)}$ facilitates the ubiquitination of JAZs (JASMONATE ZIM DOMAIN proteins), which are negative regulators of JA-induced transcriptional changes, and thereafter the degradation of JAZs through a 26S proteasome-mediated proteolytic pathway, and thus finally activates JA-induced responses (Chini *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008).

Both JA/JA-Ile and COI1 are required for herbivory-induced defense reactions in plants. In some plant species, induced resistance to insect herbivores is activated when tissues are wounded and certain molecules in herbivore oral secretions (OS) are recognized (reviewed in Howe & Jander, 2008; Wu & Baldwin, 2009). Recognition of herbivory triggers a set of diverse physiological responses that enhance plants' defense levels against the attacking herbivores (Howe & Jander, 2008; Wu & Baldwin, 2009). The interaction between *Nicotiana attenuata*, a wild tobacco plant that grows in western North America, and its lepidopteran herbivore *Manduca sexta* has been intensively studied. After *M. sexta* attack, fatty acid-amino acid conjugates (FACs) present in the OS of *M. sexta* are rapidly recognized by *N. attenuata*; FACs amplify and modify wound-induced responses in *N. attenuata*, including the activation of MAPKs and the initiation of the biosynthesis of JA and JA-Ile (Wu *et al.*, 2007; Kallenbach *et al.*, 2010). In *N. attenuata,* JA/JA-Ile and COI1 control the accumulation of various anti-herbivore secondary metabolites (Halitschke & Baldwin, 2003; Paschold *et al.*, 2007; Wang *et al.*, 2007), including trypsin proteinase inhibitors (TPIs), which inhibit the digestion of proteins in insect midguts, phenolic compounds, and diterpeneglycosides (DTGs) (Zavala *et al.*, 2004; Paschold *et al.*, 2007; Wang *et al.*, 2007; Jassbi *et al.*, 2008; Kaur *et al.*, 2010; Heiling *et al.*, 2010). Clearly, the accumulation and signaling of JA/JA-Ile play a pivotal role in plant resistance to herbivores.

SGT1 (SUPPRESSOR OF G-TWO ALLELE OF SKP1) is conserved in all eukaryotes (Shirasu, 2009). It is required for immune responses to pathogens in humans and plants (Muskett & Parker, 2003; Mayor *et al.*, 2007). It is thought to confer at least partial resistance to pathogens through its interaction with HSP90 (reviewed in Shirasu, 2009). In plants, SGT1 and HSP90 mediate the stability of NB-LRR type R proteins (Lu *et al.*, 2003; Boter *et al.*, 2007). RAR1 physically binds to SGT1 and HSP90 and thereby stabilizes the interaction between them (Boter *et al.*, 2007). RAR1 is required for resistance conferred by some, but not all *R* genes, whose functions are dependent on SGT1and HSP90 (Liu *et al.*, 2004; Boter *et al.*, 2007). Furthermore, SGT1 also interacts with components of SCF–ubiquitin ligase complexes in yeast and plants, suggesting that it functions in the proteasome-mediated protein degradation pathway (Kitagawa *et al.*, 1999; Azevedo *et al.*, 2002; Liu *et al.*, 2002). Auxin is perceived by its receptor TIR1, an F-box protein; the binding of auxin to SCF$^{(TIR1)}$ triggers degradation of Aux/IAA proteins which are negative regulators of auxin-induced transcriptional responses (Tan *et al.*, 2007). In Arabidopsis, it was found that SGT1 is required for SCF$^{(TIR1)}$-mediated auxin responses; moreover, *sgt1b* mutants are slightly insensitive to jasmonate-induced inhibition on root growth; given that COI1 is also a part of SCF–ubiquitin ligase complex, a role for SGT1 in SCF$^{(COI1)}$-mediated JA responses has been suggested (Gray *et al.*, 2003; Lorenzo & Solano, 2005).

The roles of SGT1 in plant resistance to pathogens have been intensively studied. However, whether SGT1 is also involved in induced resistance to leaf-chewing herbivores has not yet been examined. In this work, we investigated whether SGT1 is important for herbivory-induced defenses in *N. attenuata*. We used virus-induced gene silencing (VIGS) to knock down the transcript levels of *NaSGT1* and analysed the role of *NaSGT1* in modulating herbivory-induced responses in *N. attenuata*. Silencing *NaSGT1* diminishes wounding- and herbivory-induced accumulation of JA and JA-Ile. The reduced herbivory-elicited JA concentrations in *NaSGT1*-silenced plants do not result from the antagonistic effect of SA, and these plants have normal levels of MAPK activity. Accordingly, *NaSGT1*-silenced plants have decreased amounts of defensive metabolites after herbivory, and *M. sexta* larvae gain more weight on these plants than on empty vector plants. Furthermore, *NaSGT1* is required for the normal regulation of methyl jasmonate (MeJA)-induced transcriptional responses in *N. attenuata*. This study highlights the important role of SGT1 in plant resistance to leaf-chewing herbivores.

Materials and Methods

Plant growth, virus-induced gene silencing, and sample treatments

Nicotiana attenuata 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 rearing are described in Krügel *et al.* (2002). Plants were grown at 20–22°C under 16 h of light. To analyse secondary metabolites and trypsin protease inhibitor activity, plants were transferred to a glasshouse set at 26°C during the time-course of the experiment. A VIGS system was used to silence the accumulation of *NaSGT1* transcripts

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following a VIGS procedure optimized for *N. attenuata* (Ratcliff *et al.*, 2001; Saedler & Baldwin, 2004).

Leaves were wounded with a pattern wheel; immediately thereafter either 10 µl of water or 10 µl of *M. sexta* oral secretions (OS, 1 : 5 diluted) was applied to the wounds (W + W and W + OS treatment). For MeJA treatments, MeJA was dissolved in heat-liquefied lanolin at specified concentrations; 10 µl of the solid paste was applied to leaves, and pure lanolin was applied as controls. Samples were harvested at indicated times, frozen in liquid nitrogen and stored at −80°C until use.

Cloning of *NaSGT1* cDNA sequence and sequence alignments

The open reading frame of *NaSGT1* was amplified from total cDNA prepared from 1 h W + OS-treated *N. attenuata* leaf tissue by PCR, using Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) and primers SGT1-1 (5′-ATG GCG TCC GAT CTG GAG ATT A-3′) and SGT1-2 (5′-GAT TTC CCA TTT CTT CAG CTC-3′). The PCR product was cloned into a pJET1.2/blunt Cloning Vector (Fermentas, Vilnius, Lithuania) and sequenced. The *NaSGT1* sequence was further confirmed by blasting a *N. attenuata* transcriptome database obtained by 454 sequencing.

The *SGT1* sequences in *N. benthamiana*, tomato (*Solanum lycopersicum*) and Arabidopsis were retrieved from GenBank. Alignments of nucleotide and amino acid sequences were done using the Clustal W algorithm (DNASTAR, Lasergene 8, Madison, WI, USA).

Analysis of JA, JA-Ile and SA

One milliliter of ethyl acetate spiked with 200 ng of D_2-JA, 40 ng of D_4-SA and 40 ng of $^{13}C_6$-JA-Ile (the internal standards for JA, SA and JA-Ile/JA-Leu, respectively) was added to each sample (*c.* 150 mg). Samples were homogenized on a FastPrep homogenizer (Thermo Electron, Waltham, MA, USA). After being centrifuged at maximum speed for 10 min at 4°C, supernatants were transferred to 2-ml Eppendorf tubes and evaporated to dryness on a vacuum concentrator (Eppendorf, Hamburg, Germany). The residue was resuspended in 0.5 ml of 70% methanol (v : v) and centrifuged to clarify phases. The supernatants were analysed on an high-pressure liquid chromatography (HPLC)-mass spectrometry (MS)/MS (1200L LC-MS system; Varian, Foster City, CA, USA).

Secondary metabolite analysis

Extraction and analysis of secondary metabolites was modified from Keinanen *et al.* (2001). A 200 mg aliquot of tissue in FastPrep tubes containing 0.9 g of FastPrep matrix (Sili GmbH, Warmensteinach, Germany) was homogenized in 1 ml of 40% methanol containing 0.5% acetic acid on a FastPrep homogenizer (Thermo Electron) for 45 s and then samples were centrifuged at 4°C for 12 min at maximum speed. The supernatants were transferred into 1.5-ml Eppendorf tubes, centrifuged and finally transferred to glass vials. Analysis was done on an HPLC (HPLC 1100 series; Agilent, Foster City, CA, USA), installed with an ODS Inertsil C-18 column (3 µm, 150 × 4.6 mm i.d.; GL Sciences, Tokyo, Japan). 0.25% H_3PO_4 in water and acetonitrile were used as the mobile phase. Solutions of nicotine, caffeoylputrescine, chlorogenic acid were used as external standards for quantification.

Trypsin proteinase inhibitor activity analysis

The TPI activity was quantified using a radial diffusion assay protocol described by Jongsma *et al.* (1994).

Protein extraction and in-gel kinase activity assay

Leaf tissue pooled from four replicate leaves was ground in liquid nitrogen and 250 µl of extraction buffer (100 mM Hepes pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, complete proteinase inhibitor cocktail tablets (Roche)) was added to 100 mg tissue. Leaf tissue was then completely suspended by vortexing. After being centrifuged at 4°C for 20 min, supernatants of samples were transferred to fresh tubes. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent with BSA (Sigma) as a standard. Kinase activity assay was performed as described in Zhang & Klessig (1997).

Manduca sexta bioassay

Freshly hatched *M. sexta* larvae obtained from in-house colonies were placed on rosette leaves of plants (1 larva/plant, 37 replicated plants) 3 wk after agroinfiltration for VIGS. Mass gain was measured after the number of days indicated.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Half a microgram of total RNA was reverse-transcribed using oligo(dT)$_{18}$ and SuperScript reverse transcriptase II (Invitrogen). Quantitative real-time PCR (qPCR) was performed on an Mx3005P Multiplex qPCR system (Stratagene, Santa Clara, CA, USA) using the qPCR Core kit for SYBR Green I (Eurogentec, Seraing, Belgium). An *NaActin* gene was used an internal standard to correct the variation of cDNA concentrations. All qPCR primers used are listed in the Supporting Information, Table S2.

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Southern blotting analysis

Both DNA extraction and Southern blotting were done following the procedure described in Wu *et al.* (2006).

Two-dimensional gel proteomics, mass spectrometry and peptide analysis

Protein extraction, purification and two-dimensional gel electrophoresis were done according to Giri *et al.* (2006). Each protein sample was extracted from pooled leaves of six replicated plants. Three replicated gels loaded with different protein samples were run and evaluated for each plant type (VIGS-EV and VIGS-*NaSGT1*) and treatment. Gel image scanning and measurement of intensity values of protein spots, peptide analysis and database search were done as described in Giri *et al.* (2006).

Analysis of free linolenic acid, 13-hydroperoxy-linolenic acid and OPDA

Extraction and analysis was done according to Kallenbach *et al.* (2010).

Statistical analysis

Statistical analyses were done using STATVIEW (SAS Institute Inc., Cary, NC, USA) and R (http://www.r-project.org).

Sequence data from this article can be found in the GenBank/EMBL database under accession number GU265726 (*NaSGT1*).

Results

Silencing *NaSGT1* in *N. attenuata*

Two copies of *SGT1*, *AtSGT1a* and *AtSGT1b,* exist in the Arabidopsis genome. Mutations in *AtSGT1b* but not in *AtSGT1a* decrease plant resistance to pathogens, and *sgt1a sgt1b* double mutants are lethal (Austin *et al.*, 2002; Azevedo *et al.*, 2006). Two isoforms of *SGT1* were also found in tomato, and silencing these *SGT1* homologues with VIGS leads to different degrees of plant growth defects and reduces *Mi-1*-mediated resistance to nematodes and aphids (Bhattarai *et al.*, 2007). Similarly, two *SGT1* homologues exist in *N. benthamiana*; silencing *NbSGT1-1* results in reduced growth and compromised pathogen defense mediated by various *R* genes (Peart *et al.*, 2002).

An *N. attenuata NaSGT1* cDNA sequence was obtained by cloning, whose coding sequence showed 99%, 97% and *c.* 70% similarity to *SGT1* in *N. benthamiana*, tomato and Arabidopsis (70% to *AtSGT1a*, 69% to *AtSGT1b*), respectively (Fig. S1a). Similar levels of homology were seen for their deduced amino acid sequences (Fig. S1b). Although cloning and scrutinizing a *N. attenuata* transcriptome database prepared by 454 sequencing of transcripts from all plant parts and developmental stages did not reveal any additional sequences of *SGT1* (data not shown), Southern blotting analysis indicated that there is likely another *SGT1* isoform in the *N. attenuata* genome (Fig. S2). To investigate the function of *NaSGT1* in *N. attenuata*'s defense responses to its specialist herbivore *M. sexta*, we used a VIGS system optimized for *N. attenuata* to knock down the transcript levels of *NaSGT1* (Ratcliff *et al.*, 2001; Saedler & Baldwin, 2004). *N. attenuata* inoculated with *Agrobacterium tumefaciens* transformed with pTV00 empty vector and pTV-NaSGT1 containing a 316 bp fragment of *NaSGT1* (Fig. S3) formed VIGS-EV and VIGS-*NaSGT1* plants, respectively (Fig. 1a). To determine whether the pTV-NaSGT1 construct effectively silenced *NaSGT1* transcript levels, VIGS-EV and VIGS-*NaSGT1* plants were wounded with a pattern wheel and 20 µl of *M. sexta* OS was applied immediately to the wounds (W + OS); 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). In VIGS-EV plants, 3 h after W + W and W + OS treatment, *NaSGT1* transcript levels elevated more than twofold, and W + W tended to induce slightly higher levels of *NaSGT1* than did W + OS treatment (*t*-test; $P = 0.07$). Compared with VIGS-EV plants, *NaSGT1* transcript levels reduced by 61% to 73% in VIGS-*NaSGT1* plants (Fig. 1b), confirming the effectiveness of the VIGS. Furthermore, given the high similarity between the two *N. benthamiana SGT1* genes, we assume that our silencing approach knocked down the expression of both genes in *N. attenuata*, if in fact the *SGT1* homologue exists and is expressed. Silencing *NaSGT1* altered plant growth, leading to decreased rosette diameter (Fig. 1c). In later stages, *NaSGT1*-silenced plants also lost apical dominance and had stunted growth (data not shown), suggesting that the role *NaSGT1* plays in plant development in *N. attenuata* is similar to its role in other solanaceous plants. We performed all analyses of *NaSGT1*-dependent defense responses on the leaves of rosette-stage plants (Fig. 1a).

Silencing *NaSGT1* reduces the accumulation of wounding- and herbivory-induced JA and JA-Ile

The role of SGT1 in plant defense against pathogens has been intensively studied (Muskett & Parker, 2003). However, whether SGT1 is important for plants' induced resistance to phytophagous insects has not been explored. Mechanical wounding and herbivory quickly activate JA and JA-Ile biosynthesis in *N. attenuata* and JA/JA-Ile accumulation is essential for inducing most anti-herbivory defense compounds (Halitschke & Baldwin, 2003; Wang *et al.*, 2007; Heiling *et al.*, 2010). To determine if *NaSGT1*

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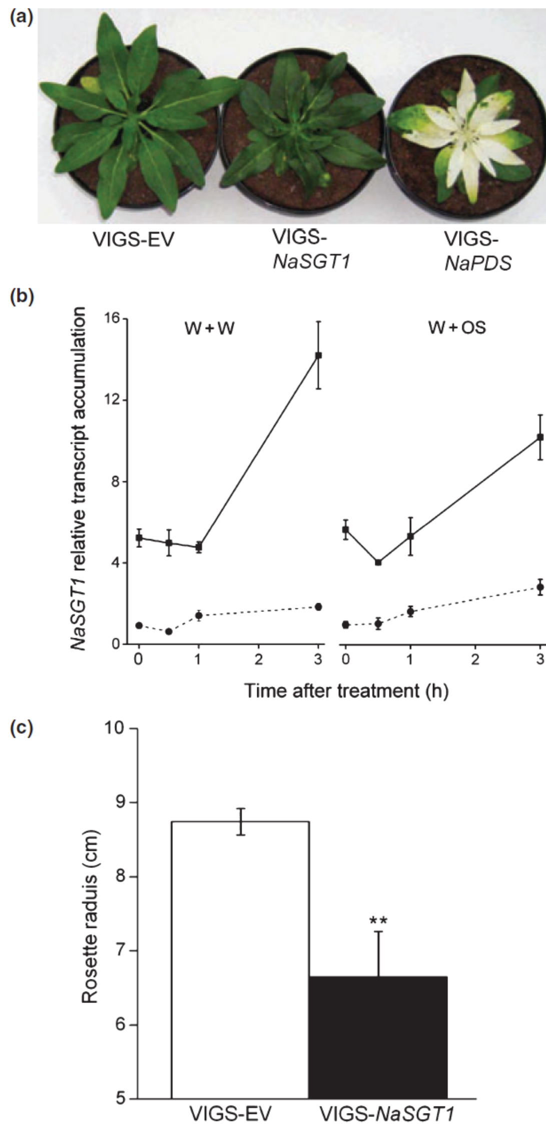

Fig. 1 Silencing *NaSGT1* with virus-induced gene silencing (VIGS). (a) Plants at rosette stage. *Nicotiana attenuata* plants were inoculated with *Agrobacterium* carrying VIGS construct, pTV00, pTV-NaSGT1, or pTV-NaPDS to form VIGS-EV, VIGS-*NaSGT1* and VIGS-*NaPDS* (*phytoene desaturase*) plants, respectively. VIGS-*NaPDS* plants were used to visually determine the degree of gene silencing, because they have a photo-bleaching phenotype. (b) Mean (± SE) *NaSGT1* transcript levels in VIGS-EV and VIGS-*NaSGT1* plants. Leaves from five replicated plants at the rosette stage were wounded with a pattern wheel, treated with 10 µl of water (W + W) or with 10 µl of *Manduca sexta* oral secretions (W + OS), and harvested at the times indicated. Transcript levels of *NaSGT1* in these plants were analysed with qPCR. (c) Mean (± SE) rosette radiuses were measured 20 d after plants were inoculated with *Agrobacterium*-carrying VIGS constructs. Asterisks represent significant differences between VIGS-EV and VIGS-*NaSGT1* plants (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $N = 5$).

is important for the elicitation of JA and JA-Ile, we analysed their concentrations in VIGS-EV and VIGS-*NaSGT1* plants after W + W and W + OS treatment. One hour after W + W treatment, JA and JA-Ile concentrations in *NaSGT1*-silenced plants were reduced by 65% and 66%, respectively, compared with those in VIGS-EV plants (Fig. 2a). Similarly, 1 h after W + OS treatment, JA and JA-Ile concentrations in VIGS-*NaSGT1* plants were only 32% and 36% as high as their concentrations in VIGS-EV plants (Fig. 2a). These data indicate that *N. attenuata*'s highly elevated concentrations of JA/JA-Ile in response to wounding and herbivory are *NaSGT1*-dependent.

As the physical interaction of SGT1 with HSP90 and RAR1 is required for plant resistance to pathogens, we also analysed herbivory-induced concentrations of JA and JA-Ile in *NaHSP90-1*- and *NaRAR1*-silenced plants (Fig. S4). The concentrations of W + OS-induced JA and JA-Ile were reduced only in VIGS-*NaHSP90-1* plants. Thus, herbivory-induced JA accumulation in *N. attenuata* requires *NaSGT1* and *NaHSP90-1* but not *NaRAR1*, although it is still not known whether NaSGT1 binds to NaHSP90-1 and NaRAR1 in *N. attenuata* and whether these physical interactions are required for the normal regulation of herbivory-induced accumulation of JA and JA-Ile. As *NaHSP90-1*-silenced plants displayed strong developmental defects such as dwarfism and necrosis of apical meristems after they reached the early elongation stage, their defense responses against insects were not further investigated.

In order to understand the mechanism underlying the impaired herbivory-induced accumulation of JA in VIGS-*NaSGT1* plants, we analysed the concentrations of the JA precursors, free linolenic acid (LA), 13-hydroperoxy-linolenic acid (18-3 OOH) and 12-oxo-phytodienoic acid (OPDA) within 30 min after W + OS treatment (Fig. 2b). The LA concentrations were reduced by 40% before and after W + OS elicitation in VIGS-*NaSGT1* plants compared with those in VIGS-EV plants. The 18-3 OOH concentrations were also reduced, although to a lesser extent than those of LA (Fig. 2b). In VIGS-EV plants, 15 min after W + OS treatment, OPDA concentrations increased 10-fold; remarkably, in VIGS-*NaSGT1* plants, OPDA concentrations remained the same after W + OS treatment. Thus, *NaSGT1* is clearly required for the normal accumulation of herbivory-induced OPDA. The same extraction method also confirmed the attenuated amounts of JA 30 min after W + OS treatment in VIGS-*NaSGT1* plants (Fig. 2b). Therefore, *NaSGT1* is important for the normal accumulation of various herbivory-induced JA precursors and JA.

Impaired JA accumulation in VIGS-*NaSGT1* plants is independent of MAPK activity and high SA levels

Two MAPKs, NaSIPK and NaWIPK, are rapidly activated by *M. sexta* herbivory, and silencing *NaSIPK* and *NaWIPK*

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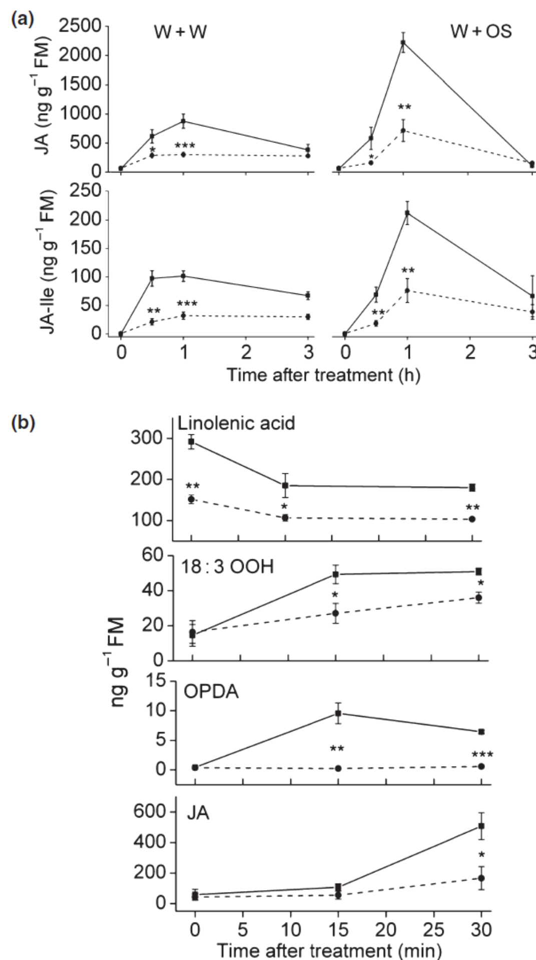

Fig. 2 Silencing *NaSGT1* diminishes wounding- and herbivory-induced jasmonic acid (JA) accumulation. (a) Mean (± SE) levels of JA and JA-Ile. Leaves were wounded with a pattern wheel and treated with 10 µl of water (W + W) or 10 µl of *Manduca sexta* oral secretions (W + OS). Samples were harvested at indicated times. JA and JA-Ile content were analysed on an high-pressure liquid chromatography (HPLC)-mass spectrometry (MS)/MS (n = 5). (b) Concentrations (± SE) of several precursors of JA. Leaves were treated with W + OS and samples were collected at times indicated (n = 6 to 10). Asterisks represent significant differences between VIGS-EV and VIGS-*NaSGT1* plants (Student's t-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

compromises wounding- and herbivory-induced JA/JA-Ile accumulation in *N. attenuata* (Wu *et al.*, 2007; Meldau *et al.*, 2009). To test if silencing *NaSGT1* impairs MAPK activity, leading to reduced JA and JA-Ile accumulation, we treated VIGS-EV and VIGS-*NaSGT1* plants with W + OS and examined the levels of NaSIPK and NaWIPK activity with an in-gel kinase assay. Levels of NaSIPK and NaWIPK activity were not altered in *NaSGT1*-silenced plants compared with those in VIGS-EV plants (Fig. 3a). *NaSIPK*- and *NaWIPK*-silenced plants also show highly diminished transcript levels of genes involved in JA and JA-Ile biosynthesis (Wu *et al.*, 2007). However, in VIGS-*NaSGT1* plants, the transcript levels of genes involved in JA and JA-Ile biosynthesis did not differ markedly from the transcript levels of the same genes in VIGS-EV plants (Fig. S5). These data suggest that NaSGT1 mediates the accumulation of herbivory-induced JA and JA-Ile in a pathway that is either downstream or parallel to the MAPK cascade; moreover, the reduced amounts of JA and JA-Ile do not result from a mis-regulation of the transcript levels of their biosynthetic genes in *NaSGT1*-silenced plants.

The suppression of JA biosynthesis by high SA levels has been reported in different plant species (Spoel *et al.*, 2003; Mur *et al.*, 2006; Diezel *et al.*, 2009). We analysed basal, W + W- and W + OS-induced SA concentrations in VIGS-EV and VIGS-*NaSGT1* plants. Basal SA concentrations in VIGS-*NaSGT1* plants were twice as high as those in VIGS-EV plants (Fig. 3b). One hour after W + W and W + OS treatment, concentrations of SA were four times higher in VIGS-*NaSGT1* plants than in VIGS-EV plants, suggesting that *NaSGT1* is important for SA homeostasis in *N. attenuata* (Fig. 3b). To test if the high concentrations of SA account for the suppressed JA accumulation after herbivory, we silenced *NaSGT1* in plants that were transformed with bacterial *salicylate hydroxylase* under the 35S promoter (35S:*NahG*). Analysis of SA concentrations indicated that NahG effectively abolished the accumulation of SA in all plants (Fig. 3c). Reducing SA concentrations in VIGS-EV plants resulted in a *c.* 40% increase in JA accumulation 1 h after W + OS treatment (Fig. 3d). However, reducing SA levels by > 90% in VIGS-*NaSGT1* plants did not significantly increase JA levels 1 h after W + OS treatment, demonstrating that the high SA levels do not account for the drastically diminished herbivory-elicited JA accumulation in *NaSGT1*-silenced plants.

NaSGT1-silenced plants have decreased amounts of herbivory-induced defensive secondary metabolites and have reduced resistance to the specialist herbivore, *M. sexta*

Transgenic *N. attenuata* plants with reduced JA or JA-Ile concentrations have compromised resistance to herbivores because of their impaired ability to induce anti-herbivore defensive compounds (Halitschke & Baldwin, 2003; Wang *et al.*, 2007). As silencing *NaSGT1* impaired the accumulation of wounding- and herbivory-induced JA and JA-Ile, we examined whether this was correlated with altered concentrations of defensive metabolites. Diterpeneglycosides are highly abundant defensive metabolites in *N. attenuata* whose concentrations increase after *M. sexta* herbivory in a jasmonate-dependent manner (Jassbi *et al.*, 2008; Heiling *et al.*,

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2010). Caffeoylputrescine (CP) and TPIs are both anti-herbivory compounds that are induced by JA and JA-Ile and *M. sexta* attack (Zavala *et al.*, 2004; Kaur *et al.*, 2010). We analysed the concentrations of CP and DTGs and the activity of TPIs in *NaSGT1*-silenced plants 3 d after W + W and W + OS treatment. Basal levels of CP were slightly elevated in *NaSGT1*-silenced plants (Fig. 4a, Students *t*-test, $P = 0.08$) but CP levels were not further induced by W + W and W + OS treatment; by contrast, VIGS-EV plants showed increased levels of CP after these treatments. The DTG concentrations were not altered by W + W treatment and VIGS-*NaSGT1* and VIGS-EV plants did not differ in DTG concentrations in control and W + W-treated leaves, whereas the W + OS treatment increased DTG levels in VIGS-EV plants but not in VIGS-*NaSGT1* plants: DTG levels were 50% of those in VIGS-EV plants (Fig. 4b). Similarly, basal TPI activity was higher in *NaSGT1*-silenced plants than in VIGS-EV plants, but no difference was found after W + W treatment (Fig. 4c). After W + OS treatment, TPI activity in VIGS-*NaSGT1* plants was only 50% of that in VIGS-EV plants (Fig. 4c). Thus, in line with the low JA levels in W + W- and W + OS-treated VIGS-*NaSGT1* plants, these treatments did not induce accumulation of defensive secondary metabolites, while CP, DTG and TPI concentrations were increased after these treatments in VIGS-EV plants.

We next examined if silencing *NaSGT1* compromises the resistance of *N. attenuata* to its specialist herbivore *M. sexta*. *M. sexta* neonates were placed on rosette-staged VIGS-EV and VIGS-*NaSGT1* plants and larval mass gain was measured. After 9 d, the mass of *M. sexta* larvae was *c.* 1.5 times greater in those that fed on *NaSGT1*-silenced plants than in those that fed on VIGS-EV plants (Fig. 4d). On day 11, mass was 40% higher in larvae that fed on VIGS-*NaSGT1* plants than on VIGS-EV plants. We conclude that silencing *NaSGT1* reduces *N. attenuata*'s resistance to its specialist herbivore, *M. sexta*; this is most likely because of diminished herbivory-induced JA and JA-Ile concentrations, which result in insufficiency of induced anti-herbivore defensive metabolites.

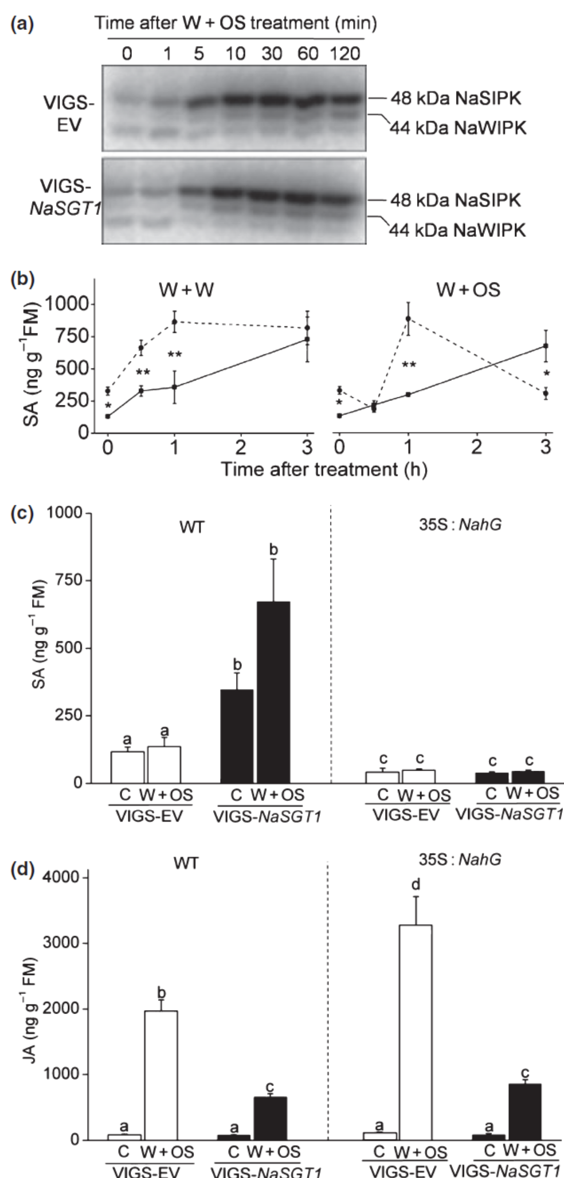

Fig. 3 Mitogen-activated protein kinase (MAPK) activity and elevated salicylic acid (SA) levels in VIGS-*NaSGT1* plants do not account for plants' attenuated jasmonic acid (JA) levels. (a) Silencing *NaSGT1* does not compromise MAPK activation. Plants were wounded with a pattern wheel and 10 µl of *Manduca sexta* oral secretions (OS) was applied to the wounds (W + OS) and leaves of five replicate plants were harvested at indicated times. Kinase activity was measured with an in-gel activity assay using myelin basic protein as the substrate. (b) Mean (± SE) SA levels in VIGS-EV and VIGS-*NaSGT1* plants. Leaves were wounded and treated with 10 µl of water (W + W) or treated with W + OS, and were harvested at indicated times. Asterisks represent significant differences between VIGS-EV and VIGS-*NaSGT1* plants (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $N = 5$). (c) and (d) Mean concentrations (± SE) of SA and JA. Wild-type (WT) plants and plants expressing 35S:*NahG* were used to generate VIGS-EV and VIGS-*NaSGT1* plants. One hour after W + OS treatment, samples were collected and their SA and JA content were analysed; untreated samples served as controls (C). Different small letters represent statistically significant differences based on the minimum adequate model (Fig. 3c: ANOVA, $F_{3,26} = 167.84$, $P < 0.0001$; Fig. 3d: ANOVA, $F_{3,25} = 147.91$, $P < 0.0001$).

Silencing *NaSGT1* alters *N. attenuata*'s transcriptional response to methyl jasmonate

We next examined the possibility that silencing *NaSGT1* compromises the activity/stability of SCF^{COI1} complex and

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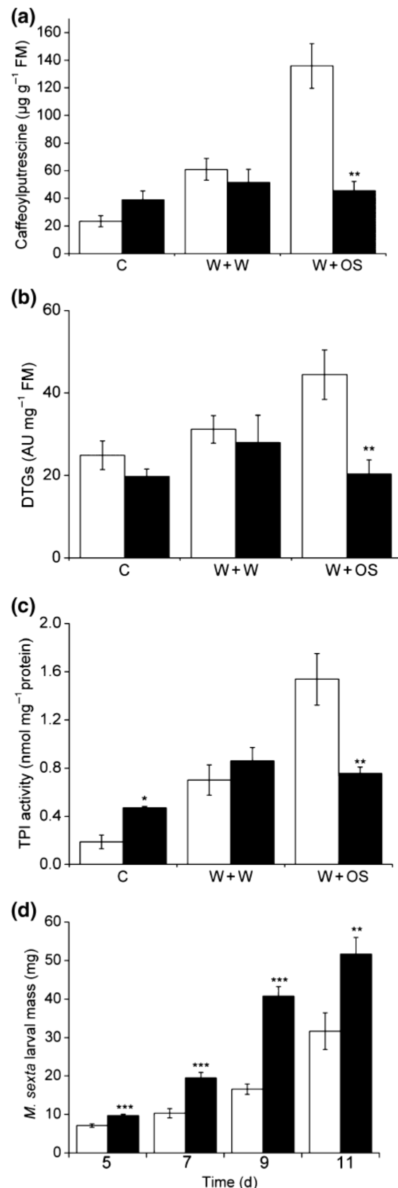

Fig. 4 Silencing *NaSGT1* compromises resistance of *Nicotiana attenuata* to *Manduca sexta*. (a–c) Levels of defensive metabolite caffeoylputrescine, diterpeneglycoside (DTG) and trypsin proteinase inhibitors (TPI) activity in VIGS-EV and VIGS-*NaSGT1* plants. Plants were wounded with a pattern wheel and treated with 10 µl of water (W + W) or 10 µl of *M. sexta* oral secretions (W + OS). Three days after treatment, samples were harvested and analyzed; non-treated samples served as controls (C) ($N = 5$). (d) *M. sexta* larval performance on VIGS-EV and VIGS-*NaSGT1* plants. *Manduca sexta* neonates were placed on rosette leaves 3 wk after *Agro*-inoculation of VIGS constructs, and their mass was recorded at the times indicated. Asterisks represent significant differences between VIGS-EV and VIGS-*NaSGT1* plants (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $N = 37$).

thus decreases JA-induced responses in *N. attenuata*. As our attempts to create transformed plants that were stably silenced in *NaSGT1* were unsuccessful, assays of MeJA-induced root growth inhibition for *N. attenuata* were not carried out. Apart from inhibition of plant root growth, jasmonate application elicits various transcriptional changes. We therefore analysed changes in gene transcript accumulation after MeJA treatment in VIGS-EV and VIGS-*NaSGT1* plants. We treated the leaves of VIGS-EV and VIGS-*NaSGT1* plants with lanolin pastes (10 µl) alone or lanolin pastes containing 5, 50 and 150 µg of MeJA; leaves were harvested at different time-points after treatment, and transcript accumulations of several genes that are known to be highly induced by MeJA in *N. attenuata* were analysed using quantitative real time-PCR. As most of the physiological changes induced by JA require JA perception through COI1, we also created VIGS-*NaSGT1* and VIGS-EV plants in *COI1*-silenced *N. attenuata* background (*irNaCOI1* plants) (Paschold *et al.*, 2007) and induced these plants with 150 µg MeJA in 10 µl lanolin, so that we could examine whether NaSGT1 mediates the transcript accumulation of JA-inducible genes in a COI1-independent pathway. Compared with no treatment, lanolin treatment alone did not alter transcript levels of the genes examined (data not shown). The induction of all genes that we measured required JA perception through COI1 (Fig. 5). Compared with those in VIGS-EV plants, silencing *NaSGT1* reduced the transcript levels of several genes that are upregulated by MeJA treatment, including *threonine deaminase* (*NaTD*) involved in converting Thr to Ile for JA-Ile biosynthesis and *NaLOX2*, which is important for fatty acid peroxidation (Fig. 5). Conversely, the relative transcript levels of *NaTPI*, which is frequently used as a JA-inducible marker in solanaceous plants, were higher after MeJA treatment in VIGS-*NaSGT1* plants than in VIGS-EV plants. Elevated transcript levels were seen for an *α-dioxygenase* (*Na α-DOX*) gene in VIGS-*NaSGT1* plants. The transcript levels of *NaHPL* (*hydroperoxide lyase*), which is involved in C_6 metabolism, did not differ in VIGS-*NaSGT1* and VIG-EV plants. These data demonstrate that *NaSGT1* is required for COI1-dependent transcriptional regulation of MeJA-induced genes in *N. attenuata*.

Silencing *NaSGT1* leads to altered levels of pathogenesis-related proteins and chaperone proteins

Given the remarkably altered defense against herbivores in *NaSGT1*-silenced plants, NaSGT1 might modulate the abundance of many proteins related to defenses. We employed a quantitative proteomic approach to examine the difference of protein abundance in VIGS-EV and VIGS-*NaSGT1* plants. VIGS-EV and VIGS-*NaSGT1* plants were treated with W + OS three times in 2-h intervals, leaf samples were harvested 6 h after the initial

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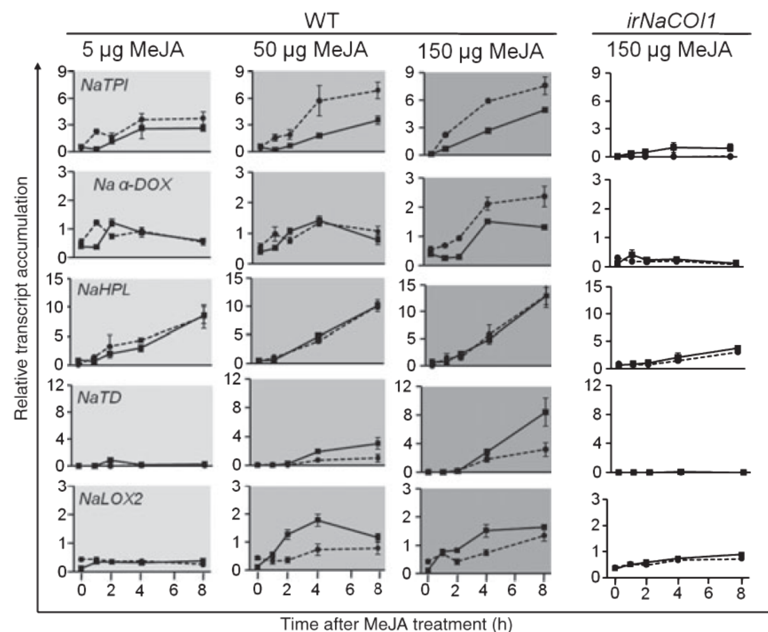

Fig. 5 Silencing *NaSGT1* alters *Nicotiana attenuata* plants' transcriptional responses to methyl jasmonate (MeJA). Relative transcript levels of jasmonic acid (JA)-inducible genes in VIGS-EV and VIGS-*NaSGT1* plants after the application of MeJA. Leaves of VIGS-EV and VIGS-*NaSGT1* plants were treated with 10 µl of lanolin paste containing different amount of MeJA. Transcript levels of *NaTPI*, *Na α-DOX*, *NaHPL*, *NaTD* and *NaLOX2* were examined by quantitative real-time PCR using cDNA prepared from RNA isolated from five biological replicated plants.

treatment and leaves from non-treated plants were harvested as controls. We extracted total proteins from these samples and separated them using two-dimensional electrophoresis (2-DE). Protein spots that differentially accumulated in all three biological replicates were sequenced and blasted in protein databases. In untreated samples, among the roughly 1200 protein spots detected on these gels, only a few differences were found between gels running samples from VIGS-EV and from VIGS-*NaSGT1* plants. Very similar results were obtained from W + OS-treated samples (data not shown). Four proteins accumulated in higher amounts in VIGS-*NaSGT1* plants than in VIGS-EV plants (Tables 1, S1; Fig. S6), all of which belonged to the pathogenesis-related (PR) protein family. Three of these proteins, namely α-galactosidase, osmotin and a hevein-like protein, were detected in VIGS-*NaSGT1* plants but not in VIGS-EV plants (Tables 1, S1; Fig. S6). A protein spot identified as an endochitinase was found to have > 10-fold higher intensity in gels loaded with samples from VIGS-NaSGT1 plants than in gels loaded with samples from VIGS-EV plants (Table 1; Fig. S6). To further understand the molecular basis for the accumulation of these stress-related proteins, their transcript levels were also analysed. Relative transcript accumulation of *NaEndochitinase* showed several hundred-fold increase and *NaOsmotin* and *NaHevein* were *c.* 50 and 40 times increased in VIGS-*NaSGT1* plants compared with VIGS-EV plants; however, the transcript levels of the gene encoding α-galactosidase showed no differences between untreated control VIGS-EV and VIGS-*NaSGT1* plants; higher levels of transcripts were seen in W + OS-induced plants (Fig. S7). Given that similarly higher amounts of α-galactosidase were found VIGS-*NaSGT1* plants regardless of treatments, possibly the abundance of α-galactosidase was regulated on a posttranscriptional level.

Only two proteins had consistently reduced levels in all replicated samples fromVIGS-*NaSGT1* plants (Tables 1, S1; Fig. S6). Blasting peptide sequences of these protein spots revealed their homology to chaperone proteins (Tables 1, S1). One of them showed similarity to a chloroplast trigger factor-like chaperone which was identified in a proteomic analysis of Arabidopsis chloroplasts (Peltier *et al.*, 2006); this type of chaperone is highly conserved and facilitates the folding of nascent proteins derived from ribosomes in prokaryotes and eukaryotes (Hartl & Hayer-Hartl, 2002). The other chaperone, NaCPN, was identified recently in a proteomic study of *N. attenuata* as a protein that is induced by *M. sexta* attack (Giri *et al.*, 2006). Silencing *NaCPN* in *N. attenuata* using VIGS decreases the accumulation of defensive metabolites after herbivory and results in increased *M. sexta* larval mass gain (Mitra *et al.*, 2008). In untreated plants, no difference of relative transcript levels of both chaperones were found between VIGS-EV and VIGS-*NaSGT1* plants, although slight

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Table 1 Differentially accumulated proteins in VIGS-EV and VIGS-*NaSGT1* plants

Protein name, accession number, and species	Peptide(s)	Mean (± SE) of normalized spot intensities		
		VIGS-EV	VIGS-*NaSGT1*	*P*
Endochitinase P08252 *Nicotiana tabacum*	(P)SCHDVLLG(R) (Y)APGFGTSGDTTA(R) (Y)SNNGTGVSPGDNLDCGNQ(R) (T)STYNLLGVSPGDNLDTDNE(R) (G)PLQLSHNYGGYGPANS(T)	594 ± 104	7663 ± 1058	0.001
Hevein-like protein AAO63574 *Hevea brasiliensis*	(V)TNTGTGAKVSD(R)	n.d.*	250 ± 68.6	0.01
Osmotin Q01591 *Solanum lycopersicum*	(Q)GTWVLNAP(R) (N)NCPYTVWAASTLPGN(R)	n.d.*	1476 ± 571	0.03
α-Galactosidase AAP04002 *Carica papaya*	(A)PLLLGCDL(R)	n.d.*	131.33 ± 5.36	≤ 0.001
Trigger factor-like chloroplast chaperone D1HZU8 *Vitis vinifera*	(N)VPEDLLVGYVG(K) (L)SEQQLASLSSP(R) (A)AVESLLK(R)	354 ± 39	177 ± 7.5	0.01
NaCPN (herbivory-induced chaperone) A1BQV8 *Nicotiana attenuata*	(E)VELEDPVENLGASLV(R) (M)VAEYENC(K) (Y)GYNAATG(K) (V)VAAGANPLQKN(K) (G)YLSPYFVTDNE(K)	678 ± 38	320 ± 38	0.001

*n.d. = not detected.

decreases were detected after W + OS treatment (Fig. S7). Therefore, the reduced protein levels of these two chaperones in VIGS-*NaSGT1* plants were possibly modulated on a posttranscriptional level.

These data suggest that NaSGT1 is involved in modulating the abundance of certain defense-related proteins and the accumulation of these proteins may be regulated at transcriptional and posttranscriptional levels. Alternatively, higher SA concentrations in *NaSGT1*-silenced plants could cause the accumulation of these defense-related proteins.

Discussion

SGT1 is a highly conserved protein among all eukaryotes. In plants and humans, SGT1 is important for immune responses triggered by pathogen elicitors (Mayor *et al.*, 2007; Shirasu, 2009). Here we investigated the role of SGT1 in herbivory-induced defense reactions in *N. attenuata* using a reverse genetic approach. In *N. attenuata,* NaSGT1 is important for the wounding- and herbivory-induced accumulation of JA and JA-Ile, the major hormonal regulators of plant defense against phytophagous insects. Accordingly, silencing *NaSGT1* results in decreased levels of defensive metabolites and compromises *N. attenuata*'s resistance to *M. sexta* larvae. Moreover, *NaSGT1* is also involved in mediating transcriptional responses to MeJA, indicating the importance of SGT1 in JA signaling.

Treating Arabidopsis with cell wall protein (CWP) fraction purified from non-pathogenic biocontrol agent *Pythium oligandrum* upregulates several JA-inducible genes and increases plant resistance to pathogen *Pseudomonas syringae* pv. *tomato* DC3000; these CWP-induced responses in Arabidopsis are SGT1-dependent (Kawamura *et al.*, 2009). Wang *et al.* (2010) showed that SGT1 is important for the process of cell death during both compatible and incompatible plant-pathogen interactions in *N. benthamiana*. El Oirdi & Bouarab (2007) demonstrated that silencing *SGT1* in *N. benthamiana* compromises the hypersensitive response induced by *Botrytis cinera*, a necrotrophic pathogen. The authors hypothesize that *B. cinera* promotes *NbSGT1* expression to exploit the antagonistic effects between SA and JA (El Oirdi & Bouarab, 2007). Our phytohormone analysis indicated that SGT1 is an important regulator of basal and herbivory-induced SA and JA concentrations in plants. Whether SGT1 also mediates the homeostasis of pathogen-elicited SA and JA levels – thus knocking out (or down) *SGT1* alters plant resistance to pathogens – deserves further study. At least three scenarios may account for the increased basal levels of SA in VIGS-*NaSGT1* plants. Silencing *NaSGT1* alters SA biosynthesis in the chloroplasts in a manner that is independent of JA-mediated suppression. Higher basal concentrations of SA were also found in *NaCOI1*-silenced *N. attenuata*, suggesting that impaired JA signaling increases SA concentrations

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(Kallenbach *et al.*, 2010); thus higher SA contents in *NaSGT1*-silenced plants may result from reduced COI1 activity in the pathway of JA–SA interactions. *Agrobacterium* and tobacco rattle virus used in VIGS may also contribute to increased SA concentrations in VIGS-*NaSGT1* plants.

Chemical analyses of precursors of JA revealed that NaSGT1 is associated with the concentrations of herbivory-induced free LA. More importantly, herbivory-induced OPDA accumulation is greatly compromised when *NaSGT1* is silenced, although the concentrations of 18-3 OOH, the precursor of OPDA, were only slightly reduced. Therefore, it is likely that the reduced activity of enzymes that convert 18-3 OOH to OPDA accounts for most of the decreases in OPDA accumulation. Transcript levels of *NaAOS* (allene oxide synthase) and *NaAOC* (allene oxide cyclase), the genes involved in these conversion steps, do not markedly differ in VIGS-*NaSGT1* and VIGS-EV plants (Fig. S5). This suggests that NaSGT1 regulates the herbivory-elicited accumulation of OPDA in a post-transcriptional manner.

Mitogen-activated protein kinases, namely SIPK and WIPK, and SA are known to regulate JA accumulation in plants (Harms *et al.*, 1998; Spoel *et al.*, 2003; Schweighofer *et al.*, 2007; Seo *et al.*, 2007; Wu *et al.*, 2007; Diezel *et al.*, 2009). However, activity levels of SIPK and WIPK are not altered in VIGS-*NaSGT1* plants. Therefore, SGT1 is not required for the herbivory-induced activation of SIPK and WIPK and likely mediates the accumulation of JA in a pathway independent of MAPK signaling. The possibility that high SA concentrations resulted from silencing *NaSGT1* accounted for the decreased JA levels in these plants can be excluded, as silencing *NaSGT1* in plants expressing 35S:*NahG*, which had highly decreased concentrations of SA, still resulted in greatly diminished levels of herbivory-induced JA. The enzymes that convert LA to OPDA are located in chloroplasts. How NaSGT1 is involved in the regulation of JA biosynthesis in plastids requires further investigation.

In all eukaryotes studied to date, SGT1 interacts with SCF-ubiquitin ligase complexes (Kitagawa *et al.*, 1999; Azevedo *et al.*, 2002; Liu *et al.*, 2002). In plants, auxin and JA-Ile are recognized by SCF^{TIR1} and SCF^{COI1} ubiquitin ligase, respectively, resulting in proteasomal degradation of negative transcriptional regulators (Chini *et al.*, 2007; Tan *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2009). Mutation in Arabidopsis *AtSGT1b* partially reduces plants' sensitivity to auxin and MeJA (Gray *et al.*, 2003; Lorenzo & Solano, 2005) and decreases the transcript levels of JA-inducible genes after being treated with cell wall protein fraction purified from *P. oligandrum* (Kawamura *et al.*, 2009). Uppalapati *et al.* (2010) demonstrated that SGT1 is required for the induction of chlorosis and cell death elicited by the JA-Ile analogue, coronatine (produced by *P. syringae* pv. *tomato* DC3000), and for coronatine-induced Arabidopsis root growth inhibition and the expression of *AtLOX2* and *AtCORI1*, two coronatine-inducible genes. These data suggest that SGT1 may be important for the function of multiple SCF-regulated pathways, including SCF^{COI1}. However, our transcriptional analyses demonstrated that silencing *NaSGT1* does not generally decrease plant transcriptional responsiveness to MeJA in *N. attenuata*; instead, NaSGT1 appears to be involved in fine-tuning JA-induced transcript accumulation, although the mechanism is unclear. Silencing *NaCOI1* in both VIGS-EV and VIGS-*NaSGT1* plants greatly decreases transcript levels of all the JA-inducible genes that we examined after MeJA treatment. This implies that SCF^{COI1} retains its activity in the absence of NaSGT1, and probably NaSGT1 is involved in modulating the stability of transcription factors that target JA-inducible genes. Furthermore, in *N. attenuata*, abolishing JA signaling by silencing *COI1* leads to decreased levels of W + OS-induced JA (Paschold *et al.*, 2008). The likely unaltered COI1 function in *NaSGT1*-silenced plants also suggests that the decreased W + W- and W + OS-induced JA concentrations do not result from feedback mechanisms within JA signaling circuits. Elegant studies have revealed that SCF^{COI1} directly binds to JAZ protein and facilitates its degradation by ubiquitination (Chini *et al.*, 2007; Thines *et al.*, 2007). Directly examining the activity of SCF^{COI1} by determining the stability of JAZ–GUS (glucuronidase) or JAZ–GFP (green fluorescent protein) fusion proteins in WT (or empty vector) and SGT1-deficient plants will shed light on this question. Whether SGT1 is generally positively associated with other JA-induced responses (i.e. transcriptional changes) in Arabidopsis and other plant species, how NaSGT1 is involved in modifying MeJA-induced transcriptional changes in *N. attenuata* and whether NaSGT1 is also required for auxin-induced responses merit future study.

In addition to SCF ubiquitin ligases, SGT1 physically interacts with chaperone proteins (e.g. HSP90 and HSP70) (Kitagawa *et al.*, 1999; Azevedo *et al.*, 2002; Shirasu, 2009). All these proteins are involved in regulating the activity of their targets by influencing target proteins' stability and proper folding. Using a proteomic approach, we detected surprisingly few proteins whose abundance is altered in *NaSGT1*-silenced plants. Therefore, we speculate that SGT1 is mainly involved in regulating the abundance and activity of regulatory proteins, as these have relatively low concentrations. The proteomic analysis revealed that VIGS-*NaSGT1* plants accumulate large amounts of PR proteins whose transcript levels are also greatly elevated. It is possible that the over-accumulated SA in VIGS-*NaSGT1* plants accounts for the high abundance of these PR proteins. Interestingly, the flowers of Arabidopsis *sgt1b* mutants have enhanced resistance to the flower pathogen *Fusarium culmorum* (Cuzick *et al.*, 2008). As many PR proteins, such

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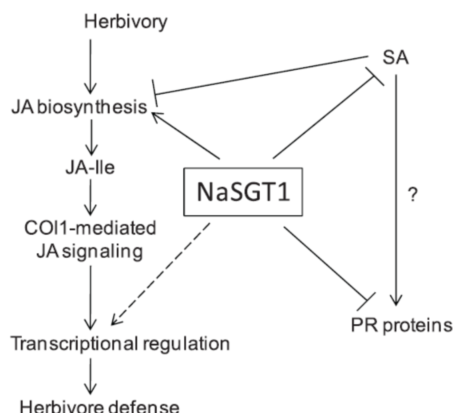

Fig. 6 A model summarizing the function NaSGT1 in *Nicotiana attenuata*'s resistance to *Manduca sexta*. Herbivory by *M. sexta* elicits biosynthesis of jasmonic acid (JA), which is further converted to JA-isoleucine conjugate (JA-Ile). Binding of JA-Ile to the COI1 receptor further induces herbivore defense responses. NaSGT1 is required for herbivory-induced JA accumulation and modulates some but not all of jasmonate-induced COI1-dependent defense responses (indicated by the dashed arrow). Furthermore, NaSGT1 negatively regulates the concentration of salicylic acid (SA) and pathogenesis-related (PR) proteins.

as endochitinases, possess antifungal properties, it is tempting to speculate that the increased resistance might result from elevated PR protein content in flowers of Arabidopsis *sgt1b* mutants. Furthermore, our transcriptional analyses revealed that the abundance of the differentially accumulated abundant proteins between VIGS-EV and VIGS-*NaSGT1* plants are regulated on transcriptional and post-transcriptional levels, demonstrating the complex mode of regulation of protein concentrations by NaSGT1.

In summary, we show that SGT1 is a key element in the regulatory network of plants in response to herbivory. SGT1 is required for herbivory-induced SA and JA homeostasis and normal MeJA-induced transcriptional responses, and is needed for the biosynthesis of secondary metabolites that defend plants against phytophagous insects (Fig. 6). Given the critical role of SGT1 in plant and human resistance to pathogens, and its well-conserved distribution in all eukaryotes, we propose that SGT1 plays a fundamental role in eukaryotes' resistance to biotic stresses. In plants, whether SGT1 is also implicated in abiotic stress resistance and how SGT1 is involved in plants' defense against herbivores are interesting questions to explore.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Nucleotide and amino acid sequence alignment of *Nicotiana attenuata* SGT1 with SGT1 homologues in *Nicotiana benthamiana* and Arabidopsis.

Fig. S2 Southern blotting analysis of *NaSGT1* in *Nicotiana attenuate*.

Fig. S3 Partial sequence of *NaSGT1* used for preparing pTV-NaSGT1.

Fig. S4 Silencing *NaHSP90-1* but not *NaRAR1* diminishes herbivory-induced jasmonic acid (JA) accumulation.

Fig. S5 Transcript levels of genes involved in jasmonic acid (JA) and JA-Ile biosynthesis.

Fig. S6 Differentially accumulated proteins in leaves of VIGS-EV and VIGS-*NaSGT1* plants**.**

Fig. S7 Transcript levels of differentially accumulated proteins in VIGS-EV and VIGS-*NaSGT1* plants.

Table S1 Alignment details of sequenced peptides (query) with the identified proteins

Table S2 Sequences of primers used in quantitative real-time PCR (qPCR)

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(b)

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MASDLEI RAKEAFI DDHFELAVDLYTQAI AMTPKNAELFADRAQANI KLNYFT-EAVVDA  N. attenuata SGT1
MASDLEI RAKEAFI DDHFELAVDLYTQAI AMTPKNAELFADRAQANI KLNYFT-EAVVDA  N. benthamiana SGT1
MASDLETRAKEAFI DDHFELAVDLYTQAI AMTPKNAELFADRAQANI KLNYFT-EAVVDA  Tomato SGT1
MAKELADKAKEAFVDDDFDVAVDLYSKAI DLDPNCAEFFADRAQAYI KLESFTAEAVADA  Arabidopsis SGT1a
MAKELAEKAKEAFLDDDFDVAVDLYSKAI DLDPNCAAFFADRAQANI KI DNFT-EAVVDA  Arabidopsis SGT1b

NKAI ELDPSMSKAYLRKGLACMKLEEYQTAKAALETGASLAPAESRFTKLI KECDERI AE  N. attenuata SGT1
NKAI ELDPSMSKAYLRKGLACMKLEEYQTAKAALETGASLAPAESRFTKLI KECDERI AE  N. benthamiana SGT1
NKAI ELDPSMSKAYLRKGLACMKLEEYQTAKAALQTGASLAPAESRFTKLI KECDERI AE  Tomato SGT1
NKAI ELDPSLTKAYLRKGTACMKLEEYRTAKTALEKGASI TPSESKFKKLI DECNFLI TE  Arabidopsis SGT1a
NKAI ELEPTLAKAYLRKGTACMKLEEYSTAKAALEKGASVAPNEPKFKKMI DECDLRI AE  Arabidopsis SGT1b

EAGELPNQSVDKTSGNVVTPPASESLDNVAVAPKDAQPTVNLSYQGSAARPKYRHEFYQK  N. attenuata SGT1
EAGELPNQSVDKTSGNVVAPPASESLGNVAVAPKDAQPTVNLSYQGSAARPKYRHEFYQK  N. benthamiana SGT1
EAGELPNQSVDKTSGNVVTAPP-ESLDNVAVAPKDAQPSVNLSYQGSAARPKYRHEFYQK  Tomato SGT1
EEKDLV-QPVPSTLPSSVTAPP---------VSELDVTPT----------AK--YRHEYYQK  Arabidopsis SGT1a
EEKDLV-QPMPPSLPSSSTTPL---------ATEADAPPVP---I PAAPAKPMFRHEFYQK  Arabidopsis SGT1b

PEEVVVTI FAKGI PAKNVVVDFGEQI LSVSI DVPGDETYSFQPRLFGKI TPAKCRYEVMS  N. attenuata SGT1
PEEVVVTI FAKGI PAKNVIVDFGEQI LSVSI DVPGDETYSFQPRLFGKI TPAKCRYEVMS  N. benthamiana SGT1
PEEVVVTI FAKGI PAKNVVVDFGEQI LSVSI DVPGEEAYSFQPRLFGKI TPAKCRYEVMS  Tomato SGT1
PEEVVVTVFAKGI PKQNVNI DFGEQI LSVVI EVPGEDAYYLQPRLFGKI I PDKCKYEVLS  Arabidopsis SGT1a
PEEAVVTI FAKKVPKENVTVEFGEQI LSVVI DVAGEEAYHLQPRLFGKI I PEKCRFEVLS  Arabidopsis SGT1b

TKI EI RLAKAEPLHWTSLEYTREPAVVQRPNVSSDAP-RPSYPSSKLRHVDWDKLEAEVK  N. attenuata SGT1
TKI EI RLAKAEPLHWTSLEYTRESAVVQRPNVSSDAP-RPSYPSSKLRHTDWDKLEAEVK  N. benthamiana SGT1
TKI EI RLAKAEPLHWTSLEYTREPAVVQRPNVSSDAP-RPSYPSSKLRHVDWDKLEAEVK  Tomato SGT1
TKI EI CLAKADI I TWASLEHGKGPAVLPKPNVSSEVSQRPAYPSSKK-VKDWDKLEAEVK  Arabidopsis SGT1a
TKVEI RLAKAEI I TWASLEYGKGQSVLPKPNVSSALSQRPVYPSSKP-AKDWDKLEAEVK  Arabidopsis SGT1b

KEEKDEKLDGDAALNKFFRDI YKDADEDTRRAMMKSFVESNGTVLSTNWKEVGAKKVEGS  N. attenuata SGT1
KEEKDEKLDGDAALNKFFRDI YKDADEDTRRAMMKSFVESNGTVLSTNWKEVGAKKVEGS  N. benthamiana SGT1
KEEKDEKLDGDAALNKFFRDI YKDADEDTRRAMMKSFVESNGTVLSTNWKEVGAKKVEGS  Tomato SGT1
KQEKDEKLEGDAALNKFFREI YQNADEDMRRAMSKSFVESNGTVLSTNWQEVGTKTI EST  Arabidopsis SGT1a
KQEKDEKLDGDAAMNKFFSDI YSSADEDMRRAMNKSFAESNGTVLSTNWKEVGTKKVEST  Arabidopsis SGT1b

PPDGMELKKWEI .  N. attenuata SGT1
PPDGMELKKWEI .  N. benthamiana SGT1
PPDGMELKKWEI .  Tomato SGT1
PPDGMELKKWEI .  Arabidopsis SGT1a
PPDGMELKKWEY.  Arabidopsis SGT1b
```

Fig. S1 Nucleotide and amino acid sequence alignment of *N. attenuata* SGT1 with SGT1 homologues in *N. benthamiana* and Arabidopsis

(a) Nucleotide sequence (open reading frame) alignment of *N. attenuata SGT1* with *SGT1* homologues in *N. benthamiana* (accession number: AY899199), tomato (*Solanum lycopersicum*; accession number: EF011105), and Arabidopsis [SGT1a (locus tag: AT4G23570) and SGT1b (locus tag: AT4G11260). (b) Decoded amino acid sequence alignment of *N. attenuata* SGT1 with SGT1 homologues in *N. benthamiana*, tomato, and Arabidopsis. Nucleotide and amino acid residues that are highlighted with black background indicate difference from the consensus sequence. Alignments were made using Clustal W algorithm.

Fig. S2

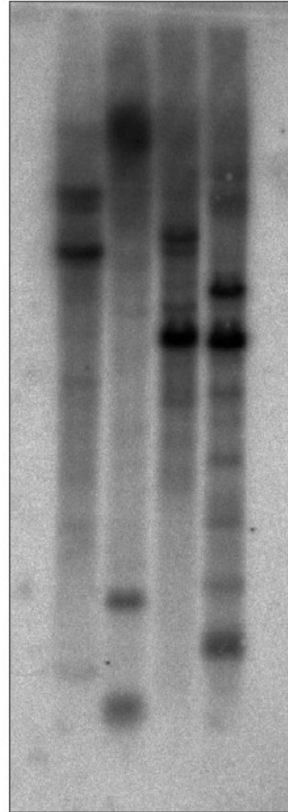

Fig. S2 Southern blotting analysis of *NaSGT1* in *N. attenuata*

Genomic DNA extracted from wild-type *N. attenuata* was digested with various endonucleases. Digested DNA samples were run on a 1% agarose gel (left to right: *Eco*R I, *Hind* III, *Eco*R V, and *Xba* I) and then blotted on a nylon membrane. The membrane was hybridized with radio-labeled probe prepared from partial *N. attenuata NaSGT1* cDNA.

Fig. S3

5′-
TGATGAACGCATTGCAGAGGAAGCTGGAGAACTACCTAAT
CAGTCGGTGGATAAAACCTCGGGAAATGTCGTAACTCCCC
CTGCATCTGAGTCTTTGGACAATGTTGCTGTTGCGCCTAAA
GATGCTCAACCAACTGTCAACCTGTCCTATCAAGGATCTGC
TGCCAGACCAAAATACAGGCATGAATTTTACCAGAAGCCA
GAGGAGGTGGTGGTGACTATATTTGCCAAGGGAATACCAG
CCAAGAATGTTGTTGTTGACTTTGGTGAACAAATACTTAGT
GTTAGCATTGATGTGCCGGGTGACGAAACTTATTCTTTCCA
GCCTAGGTTGTTTGGGAAGATAACACCTGCAAAATGCAGA
TACGAAGTGATTTCC-3′

Fig. S3 Partial sequence of *NaSGT1* used for preparing pTV-NaSGT1

Fig. S4

(a) 5′-
CATTGACAGTGGTATTGGCATGACAAAGGCTGATCTGGTG
AACAACCTGGGTACAATTGCGAGGTCAGGGACCAAGGAAT
TCATGGAAGCTCTTGCAGCTGGTGCTGATGTTAGCATGATT
GGGCAATTTGGTGTTGGTTTCTACTCTGCTTACTTGGTAGC
TGAGAAGGTTATCGTGACCACAAAGCACAATGATGATGAG
CAATATGTCTGGGAATCTCAAGCTGGTGGTTCTTTCACTGT
TACCAGGGATACATCCGGTGAGAACCTTGGTAGGGGTACA
AAAATTACCCTCTTTCTCAAGGAGGATCAACTTGAATACCT
TGAAGAACGTAGGCTC-3′

(b)
5′-
ACCAGTGATAGCAAAGCCAGCTGCCAACCGGAATAGAGC
AATTCCCGCACCAACTTCTATGGCCAATGTATCACCGAAG
GATGCTTGTCCTAGATGCCGCCAGGGATTCTTTTGTTCTGA
TCACGGTTCACAACCTAGAGAAGCAATTCCAAAAGCATCA
AATACAACAACATCTGTACCTTCTGAGAGCAATACAGGAC
AGCAAAGCCATCCTGCTCCCGTGAAGAAGAAAGTTGATAT
AAACGAGCCCCAAATTTGTAAAAACAAGGGCTGTGGTAAG
ACCTTCACAGAAAAGGAAAATCATGACACTGCTTGCAGTT
ACCATCCTGGCCCCGCTATCTTCCATGACCGAA-3′

(c)

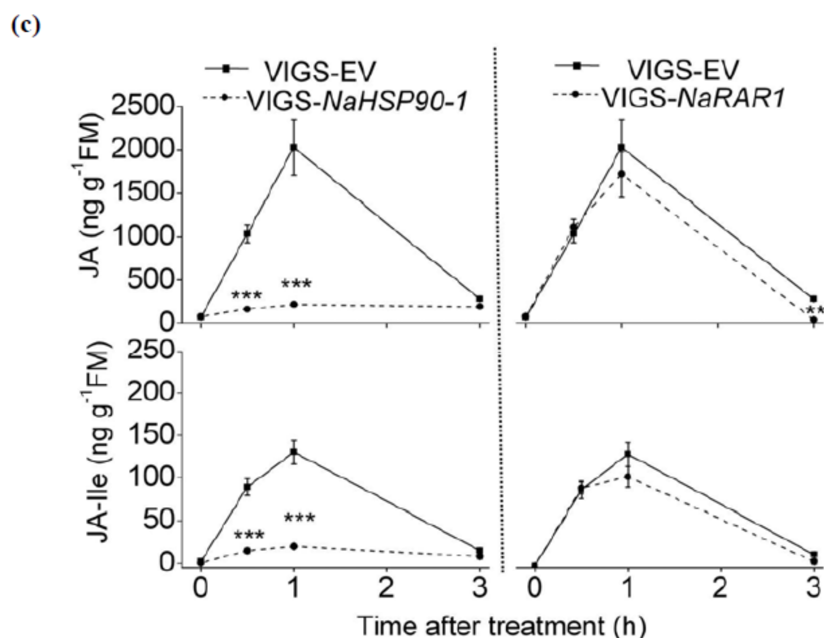

Fig. S4 Silencing *NaHSP90-1* but not *NaRAR1* diminishes herbivory-induced JA accumulation

Partial sequence of *NaHSP90-1* (a) and *NaRAR1* (b) used for preparing VIGS constructs. (c), Mean (± SE) JA and JA-Ile levels in VIGS-EV, VIGS-*NaHSP90-1*, and VIGS-*NaRAR1* plants. Plants were wounded with a pattern wheel and treated with 10 µl of *M. sexta* oral secretions (W+OS). JA and JA-Ile were analyzed in samples harvested at indicated times. Stars represent significant differences between VIGS-EV and VIGS-*NaHSP90-1* or VIGS-*NaRAR1* plants (student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N = 5).

Fig. S5

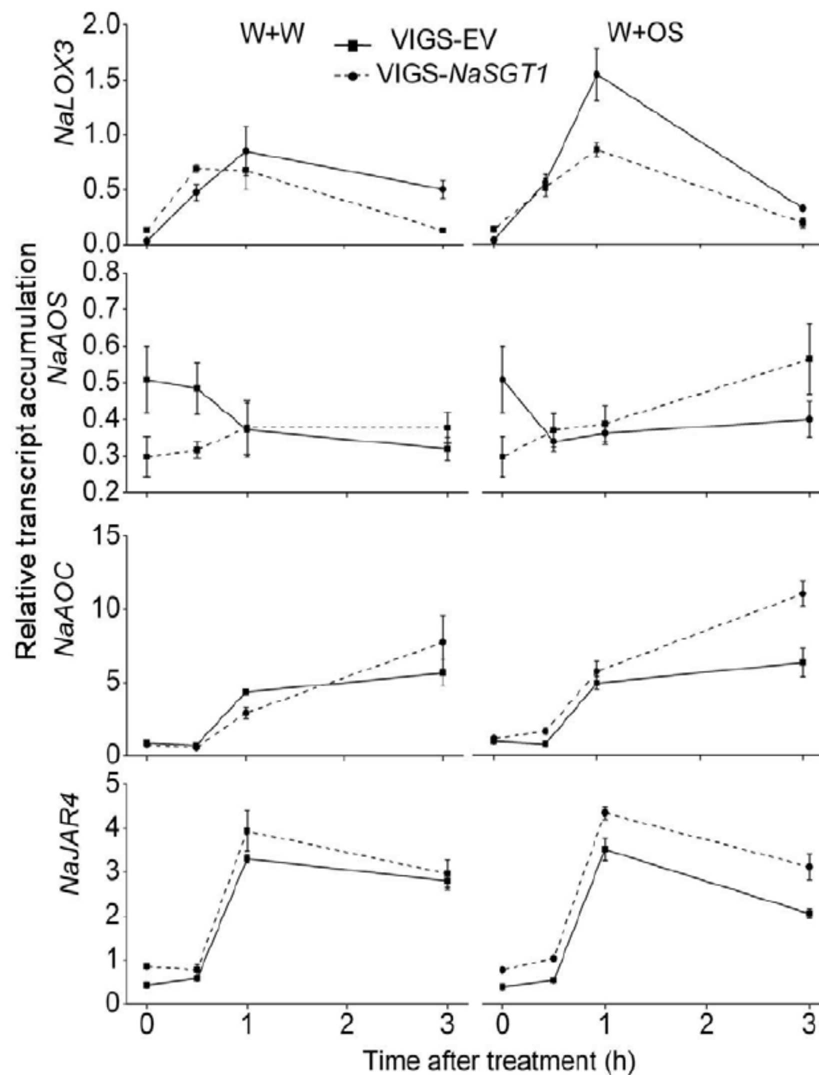

Fig. S5 Transcript levels of genes involved in JA and JA-Ile biosynthesis

Mean (± SE) relative transcript accumulation of *NaLOX3*, *NaAOS*, *NaAOC*, and *NaJAR4*. VIGS-EV and VIGS-*NaSGT1* plants were wounded with a pattern wheel and treated with 10 µl of water (W+W) or with 10 µl of *M. sexta* oral secretions (W+OS). Transcript levels of these genes were measured in samples collected at indicated times (N = 5).

Fig. S6

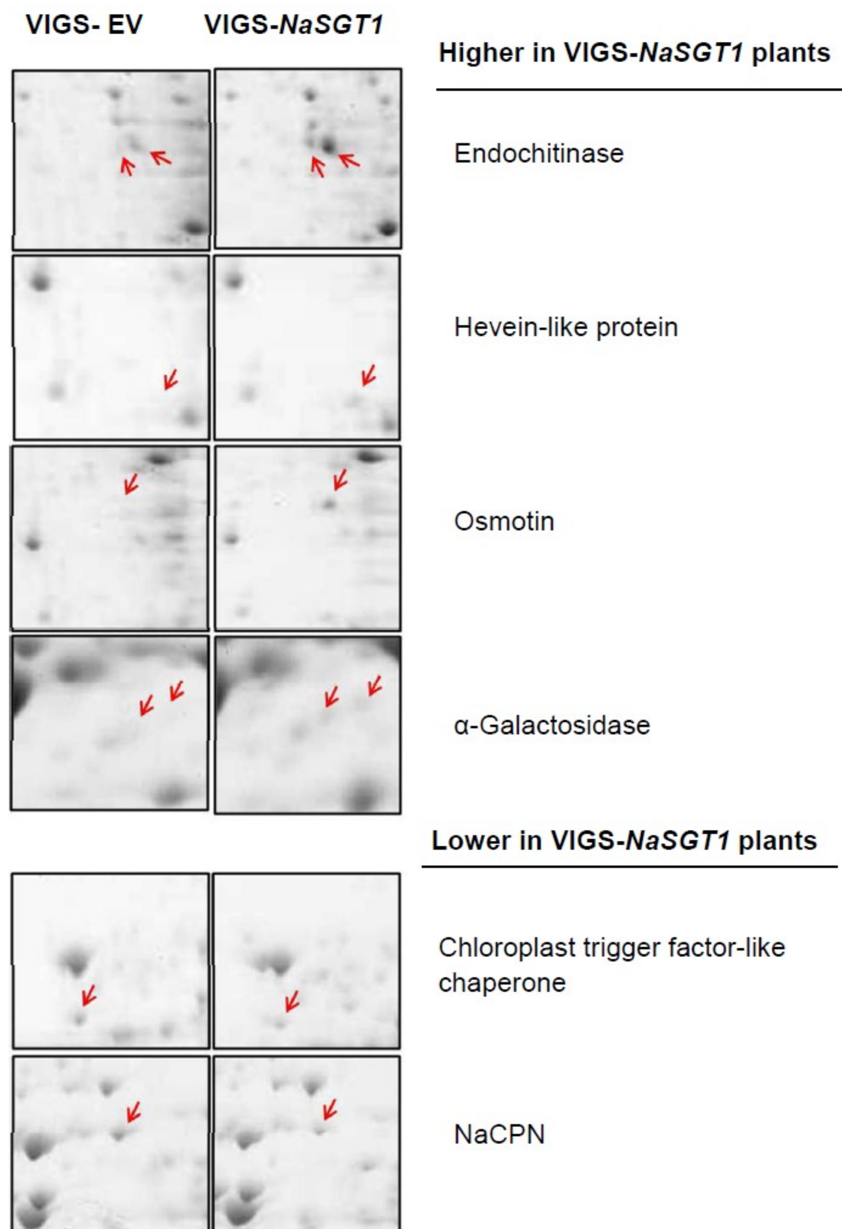

Fig. S6 Differentially accumulated proteins in leaves of VIGS-EV and VIGS-*NaSGT1* plants

Images of selected areas of Coomassie-stained 2-DE gels. Proteins were extracted from leaves of VIGS-EV and VIGS-*NaSGT1* plants and separated on 2-DE gels. Red arrows indicate differentially accumulated proteins in VIGS-EV and VIGS-*NaSGT1* plants in all 3 independently replicated gels.

Fig. S7

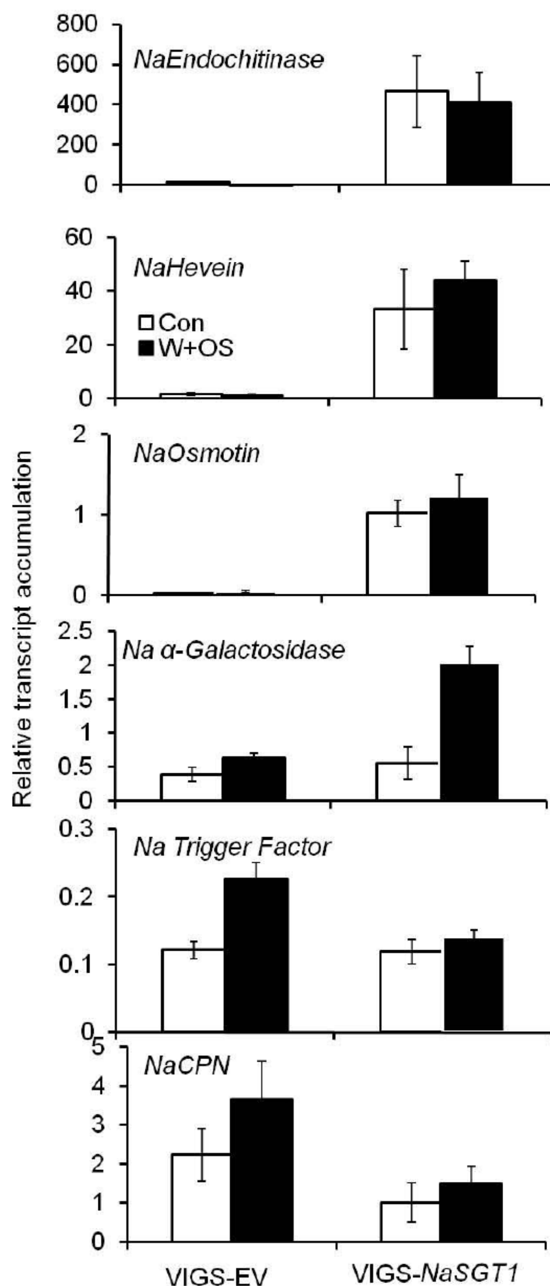

Fig. S7 Transcript levels of differentially accumulated proteins in VIGS-EV and VIGS-*NaSGT1* plants

VIGS-EV and VIGS-*NaSGT1* plants were untreated (Con) or wounded with a pattern wheel and treated with 10 µl of *M. sexta* oral secretions (W+OS). The transcript levels of indicated genes were measured with qPCR.

Table S1 Alignment details of sequenced peptides (query) with the identified proteins

Protein Name	Peptide alignment details
Endochitinase	Score = 28.6 bits (60) Identities = 8/10 (80%), Positives = 10/10 (100%) Query: 1 PSCHDVLLGR 10 PSCHDV++GR Sbjct: 243 PSCHDVIIGR 252
	Score = 38.8 bits (84) Identities = 12/12 (100%), Positives = 12/12 (100%) Query: 3 PGFGTSGDTTAR 14 PGFGTSGDTTAR Sbjct: 119 PGFGTSGDTTAR 130
	Score = 47.3 bits (104) Identities = 14/14 (100%), Positives = 14/14 (100%) Query: 7 GVSPGDNLDCGNQR 20 GVSPGDNLDCGNQR Sbjct: 304 GVSPGDNLDCGNQR 317
	Score = 35.8 bits (77) Identities = 12/15 (80%), Positives = 13/15 (86%) Query: 7 LGVSPGDNLDTDNER 21 LGVSPGDNLD N+R Sbjct: 303 LGVSPGDNLDCGNQR 317
	Score = 30.3 bits (64) Identities = 10/14 (71%), Positives = 12/14 (85%) Query: 1 GPLQLSHNYGGYGP 14 GP+Q+SHNY YGP Sbjct: 192 GPIQISHNY-NYGP 204
Hevein-like protein	Score = 32.0 bits (68) Identities = 10/10 (100%), Positives = 10/10 (100%) Query: 1 VTNTGTGAKV 10 VTNTGTGAKV Sbjct: 52 VTNTGTGAKV 61
Osmotin	Score = 26.9 bits (56) Identities = 7/8 (87%), Positives = 8/8 (100%) Query: 3 TWVLNAPR 10 TWV+NAPR Sbjct: 52 TWVINAPR 59
	Score = 43.9 bits (96) Identities = 12/12 (100%), Positives = 12/12 (100%) Query: 1 NNCPYTVWAAST 12 NNCPYTVWAAST Sbjct: 28 NNCPYTVWAAST 39
α-Galactosidase	Score = 34.6 bits (74) Identities = 10/10 (100%), Positives = 10/10 (100%) Query: 1 APLLLGCDLR 10 APLLLGCDLR Sbjct: 287 APLLLGCDLR 296
Chloroplast trigger factor-like chaperone	Score = 23.5 bits (48) Identities = 6/7 (85%), Positives = 7/7 (100%) Query: 1 NVPEDLL 7 N+PEDLL Sbjct: 34 NIPEDLL 40
	Score = 35.8 bits (77) Identities = 11/12 (91%), Positives = 12/12 (100%) Query: 1 LSEQQLASLSSP 12 L+EQQLASLSSP Sbjct: 434 LNEQQLASLSSP 445
	Score = 22.3 bits (45), Identities = 7/8 (87%), Positives = 8/8 (100%) Query: 2 AVESLLKR 9 AVESLL+R Sbjct: 313 AVESLLER 320
NaCPN	Score = 52.4 bits (116) Identities = 16/17 (94%), Positives = 17/17 (100%) Query: 1 EVELEDPVENLGASLVR 17 EVELEDPVEN+GASLVR Sbjct: 10 EVELEDPVENIGASLVR 26
	Score = 26.1 bits (54) Identities = 8/8 (100%), Positives = 8/8 (100%) Query: 1 VVAAGANP 8 VVAAGANP Sbjct: 57 VVAAGANP 64

Table S2 Sequences of primers used in quantitative real-time PCR (qPCR)

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
NaSGT1	CCACAAAGGATTTCATCATGGCT	GAAATTGAGACATGTGGATTGGG
NaActin	GGTCGTACCACCGGTATTGTG	GTCAAGACGGAGAATGGCATG
NaLOX3	GAATTTGAGAACAAGGAGAGTACTG	CTTAACAATTGTTTCCTTCAGATCTTCC
NaLOX2	TTAGTAGAAAATGAGCACCACAAGG	TTGCACTTGGTGTTTGAGATGGTA
NaAOC	CTATATACCGGAGACCTAAAGAAGA	AGTATCCTCGTAAGTCAAGTACGAT
NaAOS	GACGGCAAGAGTTTTCCCAC	TAACCGCCGGTGAGTTCAGT
NaHPL	CACTTAGACTTAGTCCACCTGTGC	AACACAAACTTTTCAGGATCATCA
NaTPI	TCAGGAGATAGTAAATATGGCTGTTCA	ATCTGCATGTTCCACATTGCTTA
NaHevein	ACTGCATTTTGTGGCCCTGTTG	TCCATCTGTATCGAGCTGCCG
NaJAR4	ATGCCAGTCGGTCTAACTGAA	TGCCATTGTGGAATCCTTTTAT
Na αDOX	GTGTTGCTAGGTACAATGAATTTC	CAACCATCAGATCCAATTCTTCT
NaEndochitinase	GTGGCCACAGATGCAGTCATC	ACACGATTGGCTGCTCGGTCA
NaOsmotin	TGTCCAACAACATGGGCAACTTG	CGCCTATGGGTGTCGACGC
Na α-Galactosidae	TGGAACGATCCAGACATGTTGGA	CATGGGTAGTTTGGTCAATCGAAC
NaCPN	TGGGTAGGAAAGGTGTAGTGACT	CTTGTCTACCAGAAGCAGCTTG
NaTriggerFactor	TTCATGCTCAATTCACAGTTGATTGT	CTTTGTAGCAATGCTTGCTTTACC
NaOsmotin	TACATGCCAAACCGGTGACTGT	GCGAAAGTCATCGGTATATTGAATC

MINI-REVIEW

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For security and stability
SGT1 in plant defense and development

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Key words: SGT1, HSP90, RAR1, immunity, development, jasmonate, coronatine, pathogen, herbivore

Abbreviations: SGT1, suppressor of G-two allele of SKP1; HSP, heat shock protein; RAR1, required for MLA12 resistance; JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine; Ub, ubiquitin; SCF, SKP1-Cdc53-F box; COR, coronatine; NLR, nucleotide-binding domain and leucine-rich repeat-containing

SGT1 (suppressor of G-two allele of SKP1) is highly conserved among all eukaryotes. In plants, SGT1 interacts with various proteins, including molecular chaperones (HSP70 and HSP90) and certain SCF ubiquitin ligases, and hence SGT1 likely functions in protein folding and stability. Since these protein complexes are involved in many aspects of plant biology, plants with a defective SGT1 display a plethora of phenotypic alterations. In this mini-review we highlight the interaction between SGT1 with other protein complexes and summarize the function of SGT1 in plant defense responses and development, including the recent advancements in the understanding of the role of SGT1 in jasmonic acid (JA) biosynthesis and signaling.

Interaction of SGT1 with Other Proteins

SGT1 proteins are highly conserved among eukaryotes. In plants, humans and yeast, SGT1 proteins all consist of five domains: the SGT1-specific motif (SGS), a tetratricopeptide repeat domain (TPR), two variable regions (VR1 and VR2), and the CS motif.[1] The TPR and CS domain have similarities to regions of proteins that interact with heat shock protein 90 (HSP90), a molecular chaperone that is important for protein folding and stability (reviewed in ref. 2). Indeed, recent studies indicated that SGT1 binds HSP90 in humans, yeast and plants.[3-5] In plants, the interaction between SGT1 and HSP90 is mediated via its CS domain.[3,6] Additionally, SGT1 can interact with HSP70 through the SGS domain.[7] Its conserved interactions with heat shock proteins suggest that SGT1 plays a role in chaperone-mediated assembly or conformational regulation of diverse protein complexes.[2]

The HSP90 chaperone complex is often closely connected with the ubiquitin (Ub)-dependent protein degradation pathway via the 26S proteasome.[8] The Ub/26S proteasome pathway is responsible for regulating the stability of most short-lived regulatory proteins either constitutively or during stress responses.[9-11]

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SCF (SKP1, Cullin, F box) E3 ligases are part of the Ub-mediated machinery that targets proteins for proteasome-mediated degradation.[12,13] Conjugation of Ub to specific proteins requires three enzymes, E1 (ubiquitin activating enzymes), E2 (ubiquitin conjugating enzymes) and E3 (ubiquitin ligase enzymes). SCFs are a class of E3s that function as scaffolds between the activated Ub-E2 complex and the target protein.[12] Within SCF complexes, SKP1 links the ubiquitin ligase catalytic core with various F-box proteins that recognize different substrates through specific protein-protein interaction motifs.[12] More than a decade ago, SGT1 was shown to interact with SKP1 through its TPR domain in yeast.[14] Similar associations between SGT1 and SKP1 homologues have also been reported in plants.[1] Complementation of yeast *sgt1* mutations using the Arabidopsis *SGT1* gene is consistent with the notion that SGT1 has a conserved role in interacting with the SCF E3 ligase complexes.[1] However, SGT1 seems not to be a core SCF subunit as the *sgt1* mutant does not exhibit the full spectrum of plant SCF mutant phenotypes.[15] Additionally, it associates with SCF complexes at substoichiometric levels and is dispensable for yeast SCF ubiquitin-ligase activity in vitro, suggesting that SGT1 might be a regulatory component of SCF complexes.[14] SGT1 also associates with the COP9 signalosome (CSN),[1,16] which is a possible regulator of SCF and other Cullin-based ubiquitin ligases.[17-19]

These findings indicate a role of SGT1 in linking HSP90 chaperone-mediated protein stability and targeted protein degradation through the interaction with ubiquitin ligase complexes. Therefore, it is conceivable that SGT1 is involved in various aspects of plant biology, including plant defense responses and development.

Role of SGT1 in Plant Resistance to Pathogens and Herbivores

SGT1 was first found to confer resistance to *Peronospora parasitica* in Arabidopsis in a mutant screen.[20] SGT1 interacts with RAR1 (Required for MLA12 Resistance),[1,3] which is a 25-kD cytoplasmic protein with two zinc-binding CHORDs (cysteine- and histidine-rich domains) and is essential for resistance conferred by multiple *R* genes recognizing distinct avirulent *Phytophthora parasitica* or *Pseudomonas syringae* pv. *tomato* isolates.[21] Although the

mechanism is still not well understood, SGT1 regulates defense responses triggered by various pathogens. A typical plant response to pathogen attack is the recognition of pathogen-derived ligands by plant disease-resistance (*R*) gene products,[22,23] which are usually nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins. It has been found that SGT1 is involved in resistance mediated by diverse NLR proteins, including Bs2, Bs4, LR2, MLA, Mi, N, Rx, RPS4, Prf, I2 and R3a (reviewed in ref. 2). Interfering with HSP90 function, on the other hand, compromises resistance mediated by Rx, RPM1, Mla, N, Prf, Mi, I2, R3a, Lr21 and RPS2, demonstrating variability in the dependence of *R* gene-mediated resistance on SGT1 and HSP90. Additionally, SGT1 is also required for non-NLR-type immunity triggered by Cf4, Cf9 or RPW8 (reviewed in ref. 2).

At least two scenarios account for the fundamental role of SGT1 in pathogen resistance. Several studies indicated that the steady state levels of *R* genes depend on SGT1 and HSP90.[3,24,25] Secondly, SGT1 and HSP90 may function in stabilizing the three dimensional conformation of NLR complexes,[2] since coimmunoprecipitation studies suggested that SGT1 can bind to HSP90 and an NLR protein simultaneously, therefore SGT1 may assist correct folding or intermolecular associations of specific R protein complexes.[26-28] Ubiquitination-based protein degradation pathways play critical roles in plant resistance to pathogens.[29,30] SGT1 is known to physically interact with SKP1, a part of the SCF E3 ubiquitin ligase, and thus is probably involved in protein ubiquitination. However, evidence demonstrating a functional link between specific E3 ligase complexes and SGT1 in biotic stress resistance remains rudimentary.

Jasmonic acid (JA) is a phytohormone that is important for plant resistance to certain necrotrophic fungi and phytophagous insect herbivores.[31] Among the numerous SCF E3 ligase complexes, SCF^{COI1} (COI1: coronatine insensitive 1) is required for the physiological responses induced by JA.[31,32] JA is converted to its isoleucine (Ile) conjugate, JA-Ile, by JARs (jasmonate resistance) and binding of JA-Ile to SCF^{COI1} activates the ubiquitination of JAZ (Jasmonate ZIM-Domain) proteins, which repress JA responses.[33,34] Several studies suggested the involvement of SGT1 in certain jasmonate-mediated responses. The Arabidopsis *sgt1b* mutant is moderately insensitive to JA-induced inhibition of root growth.[35] Kawamura et al. found that a cell wall protein (CWP) fraction purified from the oomycete *Pythium oligandrum* elevates the transcript levels of certain JA-inducible genes in Arabidopsis; however, CWP-induced transcriptional responses were completely compromised in *sgt1a-1* and *sgt1b*,[36] suggesting that SGT1 is probably involved in JA signal transduction. One of the best-studied roles of JA in plant defense responses is the regulation of resistance to herbivores.[37] Silencing *SGT1* in *Nicotiana attenuata* leads to highly diminished wounding- and herbivore feeding-induced JA levels and compromises defense against the insect herbivore, *Manduca sexta*, demonstrating that SGT1 is also involved in JA biosynthesis, although the exact mechanism is still unclear.[38] Quantification of the precursors of JA, linolenic acid, 13-hydroperoxy-linolenic acid and 12-oxo-phytodienoic acid (OPDA), indicated that silencing *SGT1* affects early steps of JA biosynthesis.[55] Examining the transcript levels of several JA-inducible genes indicated that compared with those in empty vector control plants, these genes in *SGT1*-silenced *N. attenuata* do not generally show decreased transcriptional levels after induction of JA, instead, SGT1 appears to be involved in fine-tuning JA-induced transcript accumulations. Additional evidence for a role of SGT1 in JA signaling was provided from the work with coronatine (COR). COR is a phytotoxin produced by *P. syringae*,[39-41] that has structural and functional similarity to JA-Ile [42,43] and its activity in plants is COI1-dependent.[41,43,44] Uppalapati et al. demonstrated that SGT1 is required for the induction of chlorosis and cell death elicited by COR, for COR-induced Arabidopsis root growth inhibition and the expression of *LOX2* (*Lipoxygenase 2*) and *CORI1* (*Coronatine-Induced Protein 1*).[45]

Although these lines of evidence have pointed to the role of SGT1 in SCF^{COI1}-mediated JA signaling, more studies are needed to examine whether SGT1 physically interacts with SCF^{COI1} and thus modulates JA (or COR)-induced responses, instead of regulating these responses indirectly in a SCF^{COI1}-independent pathway.

SGT1 and Plant Development

The Arabidopsis genome consists of two copies of SGT1, SGT1a and SGT1b, and double knock-out mutants are lethal.[20,24] In tomato knocking down the expression of the two *SGT1* homologues with virus-induced gene silencing (VIGS), a transient gene silencing approach, leads to different degrees of plant growth defects.[46] Consistently, VIGS *SGT1* in *N. benthamiana* and *N. attenuata* results in highly reduced growth[38,47] and silencing *SGT1* in *N. attenuata* leads to necrotic lesions in apical stem meristems but not in other tissues, suggesting an important role for SGT1 in rapidly dividing tissues (unpublished data). These data demonstrate a critical and conserved role of SGT1 in plant growth and development. One explanation for the growth defects in SGT1-deficient plants could be the altered accumulation or signaling of hormones that are important for plant development. Gray et al. found that Arabidopsis *eta3* mutant, whose SGT1b is truncated due to mutation, has altered auxin sensitivity.[35] Auxin is perceived by TIR1, an F-box protein. Auxin perception triggers 26S proteasome-mediated degradation of Auxin/Indole-3-Acetic Acid (Aux/IAA) proteins, which are negative transcriptional regulators of auxin-induced gene expression.[48] Although not yet confirmed by experiments, it is possible that in Arabidopsis SGT1b at least associates with TIR1 and assists the ubiquitination process of Aux/IAAs and thereby regulates plant developmental processes.

The 26S proteasome-mediated pathways play critical roles in controlling plant proteomes.[49] It is possible that SGT1 modulates SCF E3 ligases and thus SGT1 plays an essential role in plant development. A subunit of 26S proteasome, Rpn3, was found to have high expression levels in rapidly dividing tissues,[50-52] suggesting that 26S proteasome may be particularly active and abundant in these tissues. Regulatory proteins that are important for meristem activity might be the targets of the SGT1-E3 ubiquitin ligases complexes and this scenario may partly account

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for the stunted growth of SGT1-deficient plants. Analyzing the role of SGT1 in plant responses to other growth-related phytohormones, such as brassinosteroids or gibberellins, will provide further insight into the mechanisms by which SGT1 modulates plant growth and development.

Conclusions and Perspective

SGT1-deficient plants display complex phenotypic alterations, including diminished resistance to biotic stresses and growth defects. The physical association between SGT1 and HSP70 and HSP90 chaperones indicates that SGT1 is an important regulator of protein folding. Some evidence suggests that in plants SGT1 also interacts with certain SCF E3 ligases and thereby controls protein stability.[2]

To further understand the role of SGT1 in plant physiology, more research is needed to identify proteins, whose conformation and/or stability are SGT1-dependent. Although it was shown that in barley, SGT1 coimmunoprecipates with SKP1 and CUL1, biochemical work is needed to confirm whether SGT1 also coimmunoprecipiates with known E3 ligases that function in hormone signaling pathways, such as SCF^{TIR1} and SCF^{COI1}, which have been suggested to be regulated by SGT1. Further studies on whether and how SGT1 interacts with these ligases and identification of other SGT1-associated SCF E3 ligases will enhance our understanding of the function of this well-conserved protein in plants.

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Lipase Activity in Insect Oral Secretions Mediates Defense Responses in Arabidopsis[1][C][W][OA]

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How plants perceive herbivory is not yet well understood. We investigated early responses of the model plant Arabidopsis (*Arabidopsis thaliana*) to attack from the generalist grasshopper herbivore, *Schistocerca gregaria* (Caelifera). When compared with wounding alone, *S. gregaria* attack and the application of grasshopper oral secretions (GS) to puncture wounds elicited a rapid accumulation of various oxylipins, including 13-hydroperoxy octadecatrienoic acid, 12-oxo-phytodienoic acid (OPDA), jasmonic acid, and jasmonic acid-isoleucine. Additionally, GS increased cytosolic calcium levels, mitogen-activated protein kinase (MPK3 and MPK6) activity, and ethylene emission but not the accumulation of hydrogen peroxide. Although GS contain caeliferin A16:0, a putative elicitor of caeliferan herbivores, treatment with pure, synthetic caeliferin A16:0 did not induce any of the observed responses. With mutant plants, we demonstrate that the observed changes in oxylipin levels are independent of MPK3 and MPK6 activity but that MPK6 is important for the GS-induced ethylene release. Biochemical and pharmacological analyses revealed that the lipase activity of GS plays a central role in the GS-induced accumulation of oxylipins, especially OPDA, which could be fully mimicked by treating puncture wounds only with a lipase from *Rhizopus arrhizus*. GS elicitation increased the levels of OPDA-responsive transcripts. Because the oral secretions of most insects used to study herbivory-induced responses in Arabidopsis rapidly elicit similar accumulations of OPDA, we suggest that lipids containing OPDA (arabidopsides) play an important role in the activation of herbivory-induced responses.

Insect herbivores represent one of the major factors limiting plant growth and fitness. Plants, therefore, have evolved sophisticated strategies to detect and resist insect herbivory. Plants respond to specific compounds that indicate the presence of herbivores; these are classified as herbivory-associated molecular patterns (HAMPs; Felton and Tumlinson, 2008; Mithöfer and Boland, 2008). Well-known HAMPS are fatty acid derivatives present in herbivore saliva, such as fatty acid-amino acid conjugates, bruchins, and caeliferins, or benzyl cyanides present in oviposition fluids (Hilker and Meiners, 2006). Plants differ in their sensitivity to HAMPs, which is consistent with expectations of specific HAMP recognition systems (Schmelz et al., 2009). The amphiphilic nature of some elicitors can also destabilize plant membranes, leading to the activation of downstream responses (Maffei et al., 2004; Maischak et al., 2007). Plants can also sense the herbivory-mediated degradation or digestion products of plant material such as pectin, oligogalacturonide fragments, oligosaccharides, or fragments of the chloroplastic ATP synthase (Doares et al., 1995; Creelman and Mullet, 1997; Bergey et al., 1999; Schmelz et al., 2006).

Herbivore attack is transduced into specific defense responses by a sophisticated signaling system (for review, see Wu and Baldwin, 2010). This complex system, which consists of evolutionarily conserved defense pathways, is activated in various plant species by the perception of herbivory and HAMPS. One of these conserved responses is the rapid activation of the jasmonate (JA) biosynthetic pathway by wounding (for review, see Howe, 2004) or HAMP detection (Schmelz et al., 2009; for review, see Wu and Baldwin, 2010). JA biosynthesis starts in the chloroplast with the release of α-linolenic acid, which is converted to 13-hydroperoxy octadecatrienoic acid (13-HPOT) by 13-lipoxygenases and subsequently into cyclopentenone 12-oxo-phytodienoic acid (OPDA) by the activity of allene oxide synthase and allene oxide cyclase. After transfer to the peroxisome and the reduction of OPDA by OPDA reductase 3 (OPR3), followed by three cycles of decarboxylation, JA is formed (for review, see Wasternack, 2007). JA is subsequently conjugated with Ile to form jasmonoyl-Ile (JA-Ile) by JA:amino acid synthetase. JA-Ile is bound by the CORONATINE-INSENSITIVE (COI1)-JA-zim domain protein complex, leading to the degradation of negative transcriptional regulators and thus the activation of signal transduction (Thines et al., 2007; Chini et al., 2009; Sheard et al., 2010).

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Until now, it was not completely elucidated in Arabidopsis (*Arabidopsis thaliana*) which lipases are involved in the supply of the precursors for JA biosynthesis. In addition to DONGLE and DEFECTIVE IN ANTHER DEHISCENCE1, several other phospholipase A family lipases are known to participate in the formation of JA. However, no mutants defective in one or several of these lipases are completely compromised in JA formation under unelicited conditions or after wounding (Ishiguro et al., 2001; Hyun et al., 2008; Ellinger et al., 2010). It is also still unclear which role these lipases play in the formation of JA after herbivory.

In Arabidopsis, OPDA levels increase after wounding and herbivore attack (Reymond et al., 2000, 2004). OPDA is recognized not only as a precursor of JA biosynthesis but also as a signal in its own right. OPDA induces COI1-dependent and -independent transcriptional regulation (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008), changes in intracellular calcium levels (Walter et al., 2007), and alterations of the cellular redox status (Böttcher and Pollmann, 2009). Additionally, OPDA was shown to affect the growth of insect herbivores (Dabrowska et al., 2009). In Arabidopsis, OPDA is esterified in galactolipids, called arabidopsides (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2006; Böttcher and Weiler, 2007; Kourtchenko et al., 2007).

Another important phytohormone that is regulated by wounding and herbivory is ethylene (ET). ET interacts with JA responses (for review, see Adie et al., 2007) and tunes herbivory-related local and systemic responses (Rojo et al., 1999; Kahl et al., 2000). Aside from their inducibility, little is known about the regulation of herbivory-induced JA and ET biosynthesis. In tomato (*Solanum lycopersicum*) and in two tobacco species, *Nicotiana tabacum* and *Nicotiana attenuata*, mitogen-activated protein kinases (MAPKs) are known to be important regulators of wound- and herbivory-induced JA and ET levels (Kandoth et al., 2007; Seo et al., 2007; Wu et al., 2007). However, how MAPKs function in herbivory-induced JA and ET biosynthesis in the model plant Arabidopsis is not well understood, although indirect evidence points to the involvement of a MAPK phosphatase (Schweighofer et al., 2007). In addition to MAPK activation, Ca^{2+} signaling and reactive oxygen species (ROS) are induced by wounding and herbivory (Orozco-Cárdenas and Ryan, 1999; Maffei et al., 2004, 2006; Sagi et al., 2004; Leitner et al., 2005) and mediate herbivory-induced gene expression (Kanchiswamy et al., 2010).

It was recently claimed that the application of caeliferin A16:0 to wounds elicits JA and ET biosynthesis in Arabidopsis (Schmelz et al., 2009), suggesting that this HAMP may play a central role in grasshopper oral secretion (GS)-elicited responses. Here, we use the well-established genetic and molecular tools available for Arabidopsis to examine the regulatory machinery of GS-induced defense signaling. We show that, unlike wounding alone, applying GS to wounded Arabidopsis leaves highly promotes OPDA, JA, JA-Ile, and ET levels, MAPK activity (MPK3 and MPK6), and cytosolic calcium levels ($[Ca^{2+}]_{cyt}$). We show that the observed responses are independent of caeliferin A16:0 and that lipase activity in herbivore-derived oral secretions (OS) mediates the accumulation of oxylipin levels in Arabidopsis leaves. Our results suggest a new function for arabidopsides in the elicitation of herbivore-induced defense responses.

RESULTS

Grasshopper-Induced Defense Responses in Arabidopsis

The signal transduction machinery in Arabidopsis that is induced by wounding or herbivore feeding is well known (Reymond et al., 2000, 2004; Stintzi et al., 2001; Taki et al., 2005; Ehlting et al., 2008), but little is known about the influence of OS on Arabidopsis wound-induced defense responses. Recent studies indicate that herbivore-derived signals influence wound-induced transcript and phytohormone levels (Reymond et al., 2000, 2004; Schmelz et al., 2009).

We found that feeding of the locust *Schistocerca gregaria* induced JA accumulation and ET release in Arabidopsis (Supplemental Fig. S1). To distinguish wound- and *S. gregaria*-induced responses and to standardize herbivory elicitation, we mechanically wounded leaves and immediately treated the wounds with either water (W+W) or GS (W+GS) to investigate GS-specific defense responses (Baldwin, 1990). In addition to testing the OS of *S. gregaria*, we also tested OS from a population of native grasshoppers (*Chorthippus* spp.) collected near the Max Planck Institute for Chemical Ecology in Jena, Germany, for the ability of their secretions to alter wound-induced oxylipin levels in Arabidopsis leaves. We found that the OS of these closely related species induced similar responses in Arabidopsis leaves, suggesting that common mechanisms may underlie their elicitation. Because native grasshoppers were only temporarily available and we had a continuous supply of *S. gregaria* nymphs, we continued our analyses using the latter (see "Materials and Methods").

The various oxylipins we profiled after W+W and W+GS treatments are known to be part of the JA biosynthesis pathway. For 13-HPOT, significant increases were observed from 5 to 15 min and 2 h after leaves were treated with W+GS compared with W+W treatments (Fig. 1A). OPDA levels in leaves after W+GS treatment were highly elevated in comparison with levels after W+W treatment (Fig. 1A; Supplemental Fig. S2). From 5 min to 2 h after W+GS treatment, OPDA levels increased 8- to 10-fold compared with W+W treatments. JA and JA-Ile levels were slightly elevated within the first 15 min after W+GS treatment, while 2 h after W+GS induction, JA levels had more than doubled; compared with JA levels in plants treated by W+W, JA-Ile levels increased more than 1.5-fold (Fig.

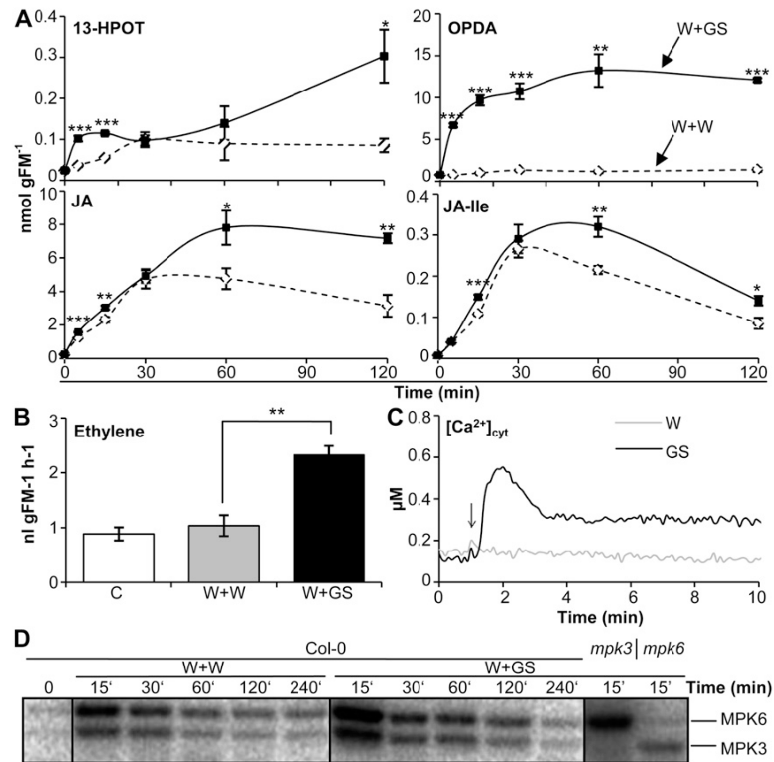

Figure 1. Responses induced by *S. gregaria* GS in Arabidopsis (Col-0). A, Mean levels ± SE ($n \geq 3$) of 13-HPOT, free OPDA, JA, and JA-Ile. Leaves were wounded and either water (W+W) or GS (W+GS) was applied, or leaves remained untreated (time point 0). Samples were harvested at the indicated time points. B, ET emissions ± SE ($n \geq 3$). Leaves were W+W or W+GS treated or untreated (C). C, $[Ca^{2+}]_{cyt}$. Leaves were treated with water (W) or GS. The application time point is indicated by the arrow. The graph shows representative curves. D, MPK3 and MPK6 activities. Leaves of Col-0 as well as *mpk3* and *mpk6* mutant plants were W+W or W+GS treated. Treated samples as well as untreated control samples were harvested at the indicated time points. Asterisks indicate significant differences between W+W and W+GS treatments at the same time point (independent-samples *t* test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). FM, Fresh mass.

1A). These data show that the application of GS to wounded leaves amplifies the levels of various oxylipins more than does wounding alone.

Another herbivory-induced phytohormone is ET (Kahl et al., 2000). Comparisons showed that W+GS-treated plants released more than 2-fold the amount of ET in the first 5 h after treatment than did W+W-treated plants (Fig. 1B). Similar changes in oxylipin and ET levels were observed in Arabidopsis accessions Columbia (Col-0), Landsberg *erecta*, and Wassilewskija (Supplemental Fig. S3).

Changes in $[Ca^{2+}]_{cyt}$ after herbivore feeding were reported recently in lima bean (*Phaseolus lunatus*; Maffei et al., 2004). Therefore, these were measured after the application of GS (Fig. 1C). GS induced an increase in $[Ca^{2+}]_{cyt}$ within a few seconds. The $[Ca^{2+}]_{cyt}$ rose from around 0.15 μM before treatment to around 0.6 μM within less than 1 min after GS application and then decreased again. After 1 to 2 min, $[Ca^{2+}]_{cyt}$ stabilized; the baseline was higher than before treatment (Fig. 1C).

It is known that MAPKs can be activated by HAMP recognition (Wu et al., 2007). Our data show that two MAPKs, MPK3 and MPK6, were more active after treatment by W+GS than by W+W (Fig. 1D). The most pronounced difference was observed 15 min after treatment. Taken together, these data clearly show that GS are potent elicitors of well-described defense responses in Arabidopsis.

GS-Induced Changes Are Not Dependent on Caeliferin A16:0

Caeliferins were reported to be active elicitors in *Schistocerca americana* OS in maize (*Zea mays*; Alborn et al., 2007). Additionally, caeliferin A16:0 was shown to elicit JA and ET levels in Arabidopsis (Schmelz et al., 2009). We also detected caeliferin A16:0 in the GS of *S. gregaria* (Supplemental Fig. S4, C and D). To test whether caeliferin A16:0 was responsible for the observed effects of GS on wounded leaves, authentic synthetic caeliferin A16:0 was tested for its ability to induce OPDA, JA, JA-Ile (Fig. 2A), and ET (Fig. 2B) as well as changes in $[Ca^{2+}]_{cyt}$ (Fig. 2C). Surprisingly, our attempts to induce defense responses by applying caeliferin A16:0 to Arabidopsis leaves were unsuccessful (Fig. 2). We increased the caeliferin concentration up to 2 mM without being able to induce any of the described responses. In contrast, applying GS significantly induced OPDA levels (Fig. 2A; *t* test, $P < 0.001$, GS versus buffer [B_1]) and JA accumulation (Fig. 2A; *t* test, $P < 0.05$, GS versus B_1) as well as the release of ET (Fig. 2B; *t* test, $P < 0.05$, GS versus B_1). The differences in JA and JA-Ile accumulation between treatments with B_1 or GS were less pronounced when compared with water as a control, most likely due to the slight activity of the phosphate buffer itself. The changes in $[Ca^{2+}]_{cyt}$ after GS application were also not mimicked by the application of caeliferin A16:0, which did not change $[Ca^{2+}]_{cyt}$ (Fig. 2C).

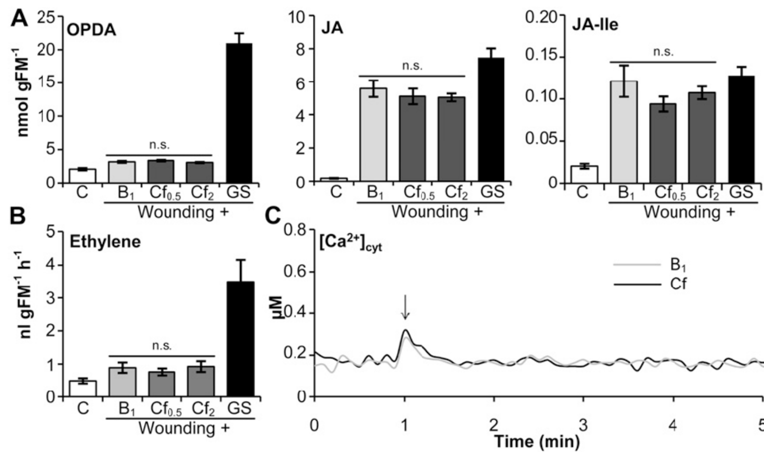

Figure 2. Caeliferin A16:0 does not mediate responses induced by *S. gregaria* GS in Arabidopsis (Col-0). A, Mean levels ± SE ($n \geq 4$) of free OPDA, JA, and JA-Ile. Leaves were wounded and 50 mM sodium phosphate buffer, pH 8 (B_1), 0.5 mM caeliferin A16:0 ($Cf_{0.5}$), 2 mM caeliferin A16:0 (Cf_2), or GS was applied, or leaves remained untreated (C). Samples were harvested after 2 h. B, ET emissions ± SE ($n = 4$). Either leaves were wounded and B_1, $Cf_{0.5}$, Cf_2, or GS was applied, or leaves were kept untreated (C). C, $[Ca^{2+}]_{cyt}$. Leaves were treated with B_1 or caeliferin A16:0 (Cf). The application time point is indicated by the arrow. The graph shows representative curves. For statistical analysis, see text (independent-samples *t* test: n.s. = no significant difference [$P > 0.05$]) FM, Fresh mass.

MPK3/6 Function in GS-Induced Responses in Arabidopsis

The homologs of Arabidopsis MPK6 and MPK3 in tomato and *N. attenuata* are known to regulate herbivory-induced oxylipin and ET accumulation (Kandoth et al., 2007; Wu et al., 2007; Kallenbach et al., 2010). We used *mpk3* and *mpk6* mutant plants to analyze their influence on these GS-induced phytohormones. Increased levels of 13-HPOT, OPDA, JA, and JA-Ile were observed in leaves 120 min after W+W and W+GS treatment (Fig. 3A). In contrast to their homologs in *N. tabacum* and *N. attenuata*, *mpk3* and *mpk6* mutant plants did not exhibit significant reductions in their levels of 13-HPOT, OPDA, JA, and JA-Ile levels after elicitation when compared with wild-type plants. Interestingly, MPK6-impaired plants showed even slightly elevated JA and OPDA levels (Fig. 3A; *t* test, $P < 0.05$, wild-type versus *mpk6* plants after W+GS treatment).

SIPK is known to participate in HAMP-induced ET release in *N. attenuata*, and MPK3 and MPK6 are important for pathogen-induced ET release in Arabidopsis (Wu et al., 2007; Han et al., 2010). We tested whether MPK6, the homolog of SIPK, is necessary for W+GS-induced ET release in Arabidopsis. After W+GS treatment, ET emissions in *mpk6* plants were reduced about 30% compared with emissions in wild-type plants (Col-0; Fig. 3B; *t* test, $P < 0.01$, wild-type versus *mpk6* plants after W+GS treatment). In contrast, the *mpk3* mutant plants showed a slight but not significant tendency to release more ET after W+GS treatment (Fig. 3B). W+W-induced ET levels in these mutants were not impaired. These data demonstrate that GS-induced oxylipin accumulation is not mediated by MPK3 and MPK6 alone in Arabidopsis; however, GS-induced ET levels are partially MPK6 dependent.

ROS Are Not Induced by GS in Arabidopsis

The production of ROS in response to herbivory is reported for *Medicago truncatula*, lima bean, and *N. attenuata* (Leitner et al., 2005; Maffei et al., 2006; Diezel et al., 2009). To assess whether hydrogen peroxide (H_2O_2) is induced by GS, 3,3-diaminobenzidine (DAB) staining was performed (Fig. 4A). The intensity of the dark brown color correlates with the abundance of a DAB-derived polymer produced in the presence of H_2O_2. For this reason, the color can be correlated with the amount of H_2O_2. H_2O_2 accumulation was examined at 1, 2, 4, 6, and 8 h after W+W and W+GS treatments. DAB staining was detected directly at the wound sites and increased with time (Fig. 4A). In leaves treated with W+W or with W+GS, there was no difference in DAB staining intensities.

Herbivory-derived H_2O_2 was shown to reduce JA accumulation and ET emissions in *N. attenuata* (Diezel et al., 2009). The *respiratory burst oxidase homolog* (*rboh*) mutants are deficient in NADPH oxidases, which are proposed to be responsible for wound- and herbivory-induced H_2O_2 production (Orozco-Cárdenas and Ryan, 1999; Orozco-Cárdenas et al., 2001; Sagi et al., 2004). For that reason, the responses of *rbohd* mutant plants and wild-type plants, both types treated with W+GS, were compared. Mutant plants were examined with respect to their accumulation of H_2O_2 (Fig. 4B), JA (Fig. 4C), and ET (Fig. 4D). The H_2O_2 accumulation after W+GS was strongly reduced in the *rbohd* mutant. The *rbohd* mutant plants tended to accumulate more JA after W+GS treatment. Interestingly, ET emissions after W+GS were more than 50% higher in the mutants than in the wild-type plants (Fig. 4D; ANOVA, $P < 0.01$, W+GS-treated wild type versus the mutant line). These results demonstrate that although H_2O_2 is not particularly induced by GS, wound-induced H_2O_2 levels negatively affect GS-induced responses.

GS-Induced Oxylipin Responses Are Mediated by Insect Lipases

To narrow the spectrum of possible effectors, fractionated GS was used for induction experiments. Less than 10 kD is depleted of large compounds like proteins

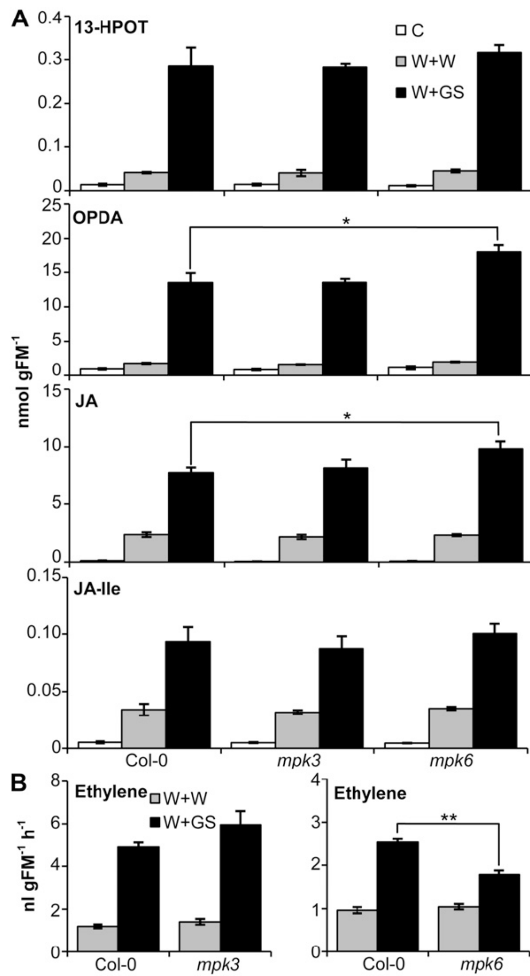

Figure 3. Role of MPK3 and MPK6 in responses induced by *S. gregaria* GS in Arabidopsis. A, Mean levels ± SE ($n = 5$) of 13-HPOT, free OPDA, JA, and JA-Ile. Leaves of Col-0 plants as well as *mpk3* and *mpk6* mutants were wounded and water (W+W) or GS (W+GS) was applied, or leaves remained untreated (C). Samples were harvested 2 h after treatment. B, ET emissions ± SE ($n \geq 3$). Leaves of Col-0 plants as well as *mpk3* and *mpk6* mutant plants were W+W or W+GS treated. Asterisks indicate significant differences between wild-type and mutant plants for the same treatment (independent-samples *t* test: * $P < 0.05$, ** $P < 0.01$). FM, Fresh mass.

(data not shown). The application of the less than-10-kD fraction to wounded leaves did not significantly increase the accumulation of OPDA and JA (Fig. 5A). In contrast, the greater than-10-kD fraction induced a strong increase in these phytohormones, similar to GS (Fig. 5A; *t* test, $P < 0.05$, greater than-10-kD GS fraction or GS application versus water application). This indicates that the OPDA and JA accumulation is likely mediated by large molecules. The application of both the greater than-10-kD GS fraction and GS to wounded leaves significantly induced JA-Ile accumulation (Fig. 5A; *t* test, $P < 0.05$, greater than-10-kD GS fraction or GS application versus water application). Interestingly, the JA-Ile levels are somewhat induced by the less than-10-kD GS fraction as well (Fig. 5A). Additionally, GS was boiled or treated with isopropanol in combination with heat to inhibit enzyme activities. The treated GS induced no increase in OPDA levels, in contrast to untreated GS (Fig. 5B; *t* test, $P < 0.01$, W+GS versus W+W), indicating that the elicitor of OPDA accumulation is heat sensitive. We next tested if the high release of OPDA observed in GS-treated leaves depends on enzymatic activity in the leaves. Importantly, GS but not boiled or heat- and isopropanol-treated GS were able to increase levels of free OPDA from heat- and isopropanol-treated leaf tissue (Fig. 5C; *t* test, $P < 0.01$, GS application versus water application). For boiled leaf tissue, similar results were obtained (data not shown). These data imply that enzymatic activity in GS but not leaf tissue is mediating the GS-induced accumulation of OPDA in Arabidopsis.

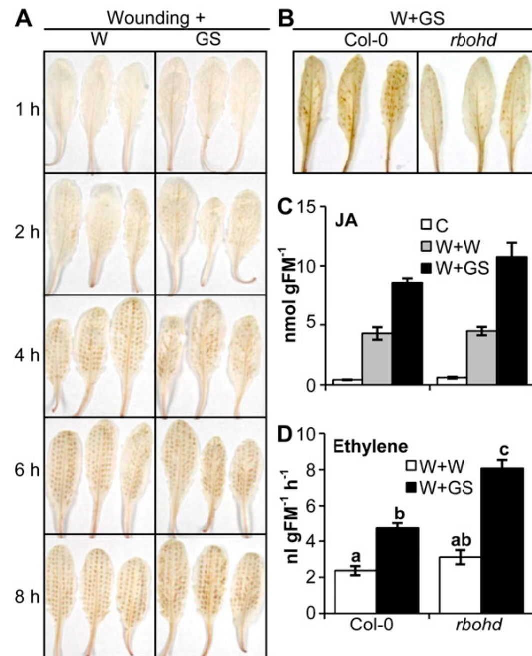

Figure 4. Role of wound-induced H_2O_2 for JA and ET levels in Arabidopsis induced by *S. gregaria* GS. A, Representative H_2O_2 accumulation. Leaves of Col-0 plants preincubated with DAB were wounded and water (W+W) or GS (W+GS) was applied. At the indicated time points, the reaction was stopped. B, Representative H_2O_2 accumulation. Leaves of Col-0 plants as well as *rbohd* mutant plants preincubated with DAB were W+GS treated. After 4 h, the reaction was stopped. C, Mean levels ± SE ($n \geq 3$) of JA. Leaves of Col-0 plants as well as *rbohd* mutant plants were either W+W or W+GS treated, or leaves remained untreated (C). Samples were harvested after 2 h. D, ET emissions ± SE ($n \geq 3$). Leaves of Col-0 plants as well as *rbohd* mutant plants were treated with W+W or W+GS. Different letters indicate significant differences among treatments (ANOVA: $P < 0.05$, Tukey's honestly significant difference test). FM, Fresh mass. [See online article for color version of this figure.]

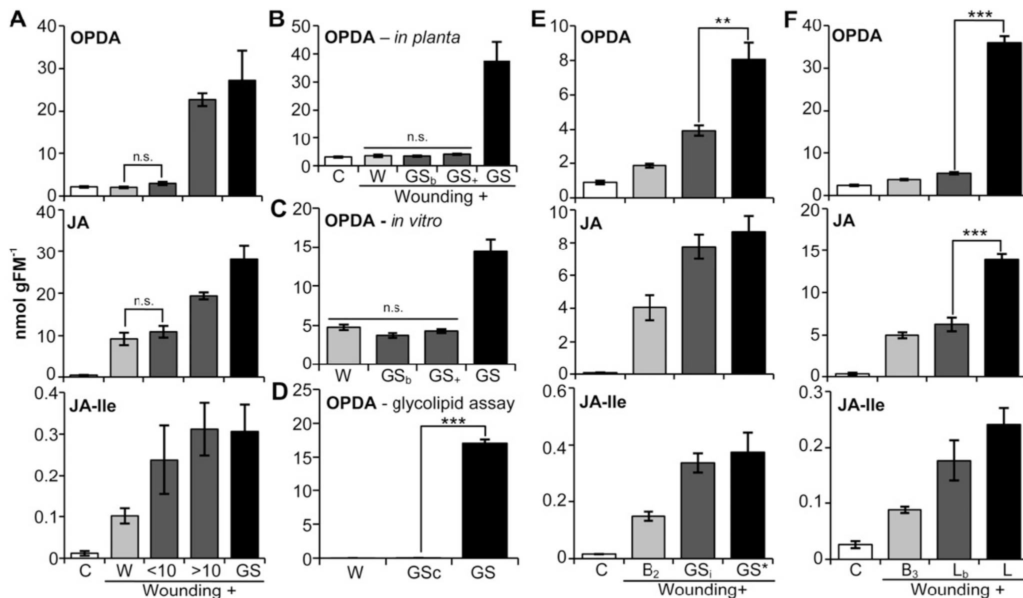

Figure 5. Lipase-dependent oxylipin accumulation in Arabidopsis (Col-0). A, Mean levels ± SE ($n = 4$) of free OPDA, JA, and JA-Ile. Leaves were wounded and water (W), the less than-10-kD *S. gregaria* GS fraction (<10), the greater than-10-kD GS fraction (>10), or GS was applied, or leaves remained untreated (C). Samples were harvested 2 h after treatment. B, In planta OPDA accumulation ± SE ($n = 4$). Wounded leaves were treated with W, boiled GS (GS_b), isopropanol- and heat-treated GS (GS_+), or GS. Samples were harvested after 2 h. C, In vitro OPDA release ± SE ($n \geq 3$). Isopropanol- and heat-treated plant material was incubated with W, GS_b, GS_+, or GS for 10 min. D, OPDA release from glycolipids ± SE ($n \geq 3$). Glycolipid extract from Arabidopsis (Col-0) was incubated with W or GS or GS was added without incubation time (GSc). E, Mean levels ± SE ($n = 5$) of free OPDA, JA, and JA-Ile. Leaves were wounded and either 2.5% (v/v) ethanol (B_2) with 1 mM orlistat-treated GS (GS_i) or with 2.5% (v/v) ethanol-incubated GS (GS*) was applied, or leaves remained untreated (C). Samples were harvested 2 h after treatment. F, Mean levels ± SE ($n \geq 3$) of free OPDA, JA, and JA-Ile. The wounded leaves were treated with 0.1 M Tris-HCl, pH 7.5 (B_3), boiled fungal lipase (L_b), or fungal lipase (L). Samples were harvested after 2 h. Asterisks indicate significant differences between the indicated treatments (independent-samples *t* test: n.s. = no significant difference [$P > 0.05$], ** $P < 0.01$, *** $P < 0.001$). FM, Fresh mass.

Compared with other plant species, Arabidopsis possesses high levels of esterified OPDA bound to lipids; these are referred to as arabidopsides (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2006; Böttcher and Weiler, 2007; Kourtchenko et al., 2007). Our data indicate that enzymatic activity in GS leads to the high accumulation of OPDA after contact with wounded leaf tissues. To test if lipase activity of GS can release OPDA out of arabidopsides, we performed lipase activity assays using glycolipid extracts of Arabidopsis (Col-0) leaves. Figure 5D shows that glycolipids without GS or glycolipids and GS together but without incubation time yielded only marginal amounts of free OPDA, whereas glycolipids and GS incubated together yielded high levels of free OPDA (Fig. 5D; *t* test, $P < 0.001$, GS without incubation time versus GS). These data demonstrate that lipase activity in GS can hydrolyze OPDA out of glycolipids in Arabidopsis.

To analyze if the GS proteome contains lipase-like proteins, we used two-dimensional PAGE (2-D PAGE) and de novo sequencing of protein spots. As expected for a digestive fluid, two of the protein spots we identified had high homology to insect lipases (lipase, Q177T4, *Aedes aegypti*; similar to lipase, XM_001948948.1, *Acyrthosiphon pisum*; Supplemental Table S1). To determine if lipase activity in GS accounts for OPDA accumulation in wounded Arabidopsis leaves, we incubated GS with the lipase inhibitor orlistat prior to treatment (Fig. 5E). Compared with untreated GS, the orlistat-treated GS induced 50% less OPDA in wounded Arabidopsis leaves (Fig. 5E; *t* test, $P < 0.01$, W+GS_i versus W+GS* [see Fig. 5 legend for definitions]), whereas orlistat added to GS directly before application induced no changes (Supplemental Fig. S5; *t* test, $P = 0.740$, $GS_{(i)}$ versus GS*). Because results from orlistat addition to the GS with and without incubation time differed significantly (Supplemental Fig. S5; *t* test, $P < 0.05$, $GS_{(i)}$ versus GS_i), it can be assumed that orlistat-induced changes are mediated by altered GS properties and not by the interaction between orlistat and leaf tissue. However, other than reduced OPDA levels, only slight decreases in JA and JA-Ile levels were observed after lipase inhibitor treatment of the GS.

To test whether an externally added lipase not only contributes to but could also be sufficient to induce the accumulation of JAs on wounded leaves, plants were treated with a lipase solution (Fig. 5F; Supplemental Fig. S6). The sn1-specific lipase from the fungus *Rhizopus arrhizus* was shown to release OPDA from arabidopsides (Stelmach et al., 2001). The application of *R. arrhizus* lipase solution (L) to wounded leaves (W+L) highly increased the levels of 13-HPOT, OPDA, JA, and JA-Ile in comparison with the application of the corresponding buffer ($W+B_3$) 2 h after treatment (Fig. 5F; Supplemental Fig. S6; *t* test, $P < 0.01$, W+L versus $W+B_3$). Levels of OPDA and JA were significantly reduced after application of the heat-treated lipase solution (L_b) than after application of untreated lipase solution (Fig. 5F; *t* test, $P < 0.001$, $W+L_b$ versus W+L). The heat-treated lipase solution probably maintained some residual activity leading to slightly, but nevertheless significantly elevated, levels of OPDA (Fig. 5F; *t* test, $P < 0.01$, $W+L_b$ versus $W+B_3$); JA-Ile levels tended to be higher in leaves treated with $W+L_b$ than in leaves treated with $W+B_3$ (Fig. 5F; *t* test, $P \leq 0.07$, $W+L_b$ versus $W+B_3$). These data provide evidence for an important role of lipase activity in GS-mediated oxylipin accumulation in Arabidopsis leaves. To test whether GS and L are only able to induce responses on the wounding side, GS and L were applied to the surface of unwounded leaves. Supplemental Figure S7 shows that GS was not able to induce JA and JA-Ile levels when only applied to the leaf surface (*t* test, $P > 0.9$, GS versus W), whereas OPDA levels were marginally but significantly increased (*t* test, $P < 0.01$, GS versus W). The L, which is supposed to possess higher lipase activity than GS, also slightly induced OPDA levels as well as JA and JA-Ile levels (Supplemental Fig. S7; *t* test, $P < 0.05$, L versus B_3). GS- andL-mediated changes in the OPDA levels were nearly nine times higher in combination with wounding when compared with application to unwounded leaf surfaces. Also, L-mediated changes in JA and JA-Ile levels were approximately five times higher when applied to wounded leaves compared with unwounded leaves. It is possible that the accumulation of oxylipins in response to GS and L application to the unwounded leaf surface is a result of small wounding events produced by the treatment or harvesting procedure. These data demonstrate that GS-mediated accumulation of oxylipins requires contact of GS with wounded leaf tissue.

Lipase-Independent Defense Elicitations

We tested if lipase-independent elicitors in GS mediate defense responses in Arabidopsis. Application of the less than-10-kD fraction of GS, which was shown to not induce lipase-mediated responses (Fig. 5A), induced similar $[Ca^{2+}]_{cyt}$ when compared with the greater than-10-kD fraction (Fig. 6A). Additionally, GS-induced $[Ca^{2+}]_{cyt}$ changes could not be abolished by treating the GS with heat before use (Fig. 6A). However, $[Ca^{2+}]_{cyt}$ measurements after the application of *R. arrhizus* lipase revealed that this lipase is also sufficient to induce changes in $[Ca^{2+}]_{cyt}$ (Fig. 6A). Regardless, the signature of the $[Ca^{2+}]_{cyt}$ after fungal lipase treatment showed a different pattern than after GS treatment. The first peak, which is normally present after GS application, was missing, suggesting that lipase activity is not the main elicitor of $[Ca^{2+}]_{cyt}$. There are some hints that the amphiphilic nature of some elicitors can change $[Ca^{2+}]_{cyt}$ (Maffei et al., 2004; Maischak et al., 2007). However, the $[Ca^{2+}]_{cyt}$ signature after Triton X-100 treatments showed a steady increase of $[Ca^{2+}]_{cyt}$ over

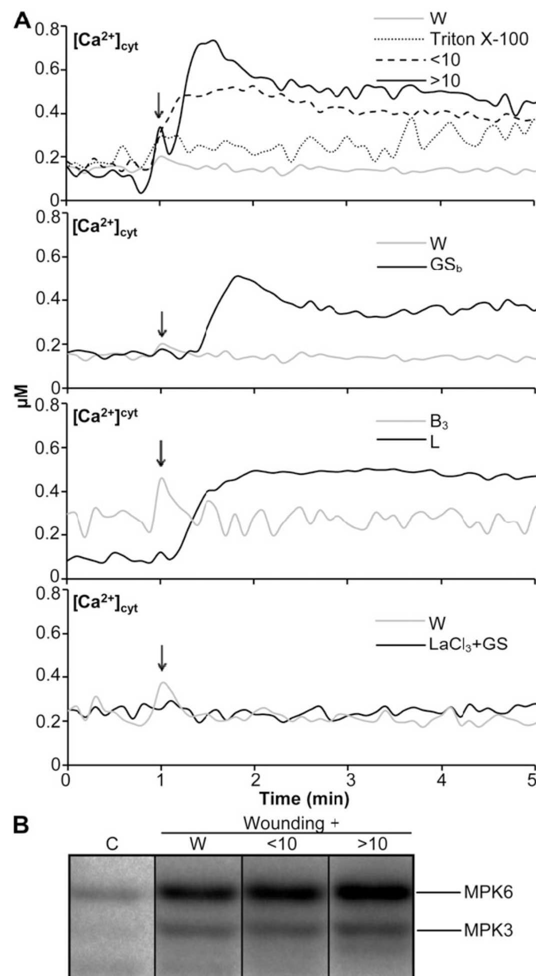

Figure 6. Lipase-independent defense responses in Arabidopsis. A, $[Ca^{2+}]_{cyt}$. Leaves were treated with water (W), Triton X-100, the less than-10-kD GS fraction (<10), the greater than-10-kD GS fraction (>10), boiled GS (GS_b), *R. arrhizus* lipase (L), or the corresponding buffer (B_3). Additionally, leaves were preincubated in 1 mM $LaCl_3$ for 1 h and then treated with GS ($LaCl_3$+GS). The test substance application is indicated by the arrows. The graphs show representative curves. B, MPK3 and MPK6 activities. Leaves of Col-0 plants were wounded and water, the less than-10-kD GS fraction, or the greater than-10-kD GS fraction was applied.

time and lacked the early peak, demonstrating that detergent effects alone likely do not elicit the immediate GS-induced $[Ca^{2+}]_{cyt}$ increase. Furthermore, the GS-induced $[Ca^{2+}]_{cyt}$ changes could be abolished by pretreatment with the Ca^{2+} channel inhibitor $LaCl_3$, suggesting that an increase in $[Ca^{2+}]_{cyt}$ is mediated through Ca^{2+} channel-driven Ca^{2+} import (Fig. 6A).

Wounding and application of the less than-10-kD GS fraction induced higher MAPK activity when compared with W+W treatments, although the relative increase is less pronounced when compared with application of the greater than-10-kD fraction (Fig. 6B). These data demonstrate that, in parallel to GS inherent lipase activity, other unknown elicitors of GS also induced defense responses in Arabidopsis leaves. Possible elicitors could be fragments of plant cells, other defense-inducing enzymes, or herbivore-derived elicitors (for review, see Heil, 2009). Future research will focus on the analyses of additional elicitors in GS.

GS-Induced Changes in OPDA-Regulated Transcripts

OPDA was shown to specifically change the transcript abundance of various genes in Arabidopsis (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008). After finding high levels of OPDA induced by GS, we tested if these correlate with the activation of OPDA-related gene expression. We analyzed the expression of two marker genes, glutathione *S*-transferase 1 (*GST1*) and *OPR1* (Fig. 7), which were shown to be regulated by OPDA (Stintzi et al., 2001). *GST1* showed a 2.5- to 3-fold increase in transcript abundance 4 h after treatment with W+GS compared with W+W. After 7 h, transcript levels in W+GS-treated leaves were more than 4.5-fold higher than those in W+W-treated leaves. *OPR1* showed a 1.5- to 2.5-fold increase in transcript abundance after GS application at 4 and 7 h after treatments, respectively. Interestingly, we observed that only the OPDA-inducing greater than-10-kD GS fraction was able to increase the *GST1* and *OPR1* transcript abundance, whereas the less than-10-kD fraction, which was not able to induce OPDA levels, did not mediate similar inductions (Fig. 7). These data show that the induction of OPDA-mediated gene expression is part of the Arabidopsis response to GS.

High OPDA Accumulation upon Herbivory Is a Common Response in Arabidopsis

Most studies analyzing herbivory-induced responses in Arabidopsis elicit plants with lepidopteran insects (Reymond et al., 2000, 2004; Stotz et al., 2000; De Vos et al., 2005; Mewis et al., 2006; Ehlting et al., 2008). To test if lepidopteran insect species show similar oxylipin patterns, we applied the OS of *Heliothis virescence*, *Spodoptera littoralis*, *Spodoptera frugiperda*, *Manduca sexta*, *Pieris rapae*, and *Heliothis subflexa* to wounded Arabidopsis leaves (Fig. 8). The data clearly show that all OS are able to quickly induce elevated levels of OPDA in

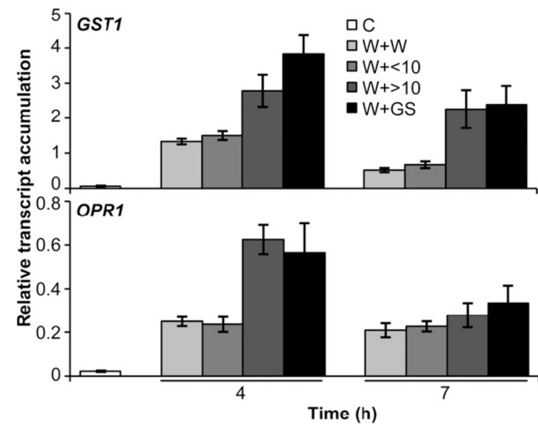

Figure 7. *S. gregaria* GS induce OPDA-mediated gene expression. Relative abundance ± SE (n = 5) of *GST1* and *OPR1* transcripts is shown. Leaves of Arabidopsis plants (Wassilewskija) were wounded and water (W+W), the less than-10-kD GS fraction (W+<10), the greater than-10-kD GS fraction (W+>10), or GS (W+GS) was applied, or leaves remained untreated (C). Samples were harvested at the indicated time points. After RNA preparation and cDNA synthesis, the relative transcript abundance in correlation with the eIF4A1 transcript levels was determined by qPCR.

the wounded leaves of Arabidopsis. Additionally, JA and JA-Ile levels were elevated after treatment with wounding and OS application compared with W+W. Unlike JA levels, JA-Ile levels varied according to the application of the different OS. Additionally, the data show that the food provided to the insects had only a minor influence on the oxylipin-eliciting properties of the tested OS. These results demonstrate that the high OPDA release upon herbivory is induced by unrelated insect herbivore species; in other words, OPDA accumulation appears to be a general response of Arabidopsis plants to herbivory.

DISCUSSION

Plants have evolved sophisticated mechanisms to distinguish herbivory from mechanical wounding. Insect herbivore attack differentially regulates phytohormone levels, transcriptional responses, and defense metabolites in Arabidopsis plants, and its effect differs from that of mechanical wounding alone (Reymond et al., 2000, 2004; Stintzi et al., 2001; Mewis et al., 2006; Ehlting et al., 2008; Schmelz et al., 2009). However, when compared with what we know about other plant species, our knowledge about the molecular events leading to the recognition of herbivory and transduction to specific responses in the model plant Arabidopsis is limited. Here, we show that grasshopper elicitation differentially affects early wound-induced molecular events, such as oxylipin release, ET accumulation, MAPK activity, and intracellular Ca^{2+} levels, in Arabidopsis (Fig. 1). Our data show, to our knowledge for the first time, that lipase

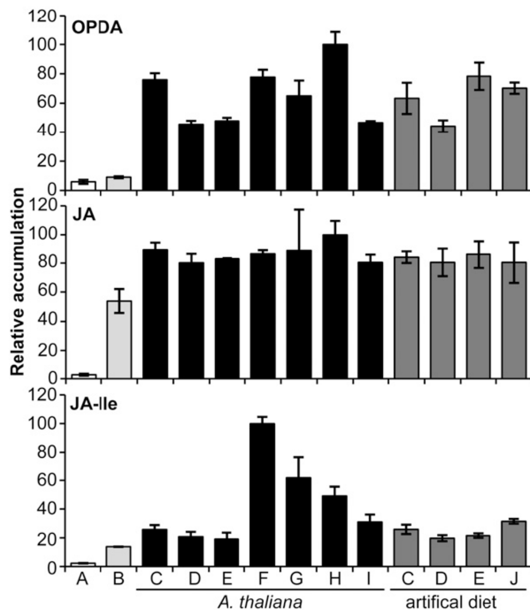

Figure 8. Oral secretions of various insects induce OPDA, JA, and JA-Ile accumulation in Arabidopsis (Col-0). Relative abundance ± SE (n = 4) of OPDA, JA, and JA-Ile is shown. Leaves were wounded and treated with water (B) or regurgitate from *S. gregaria* (C), *H. virescence* (D), *S. littoralis* (E), *S. frugiperda* (F), *M. sexta* (G), a population of native grasshoppers (*Chorthippus* spp.; H), *P. rapae* (I), or *H. subflexa* (J), or leaves remained untreated (A). Insects were fed either Arabidopsis or artificial diet as indicated. Samples were harvested after 2 h.

activity in insect OS can directly elicit changes in oxylipins, especially OPDA. Thus, we provide a new mechanism for how insect feeding can alter the levels of herbivory-induced defense metabolites.

Oxylipin Accumulation Induced by GS-Inherent Lipase Activity

Arabidopsis contains OPDA esterified to membrane lipids, classified as arabidopsides (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2006; Böttcher and Weiler, 2007; Kourtchenko et al., 2007). Arabidopsides were described to possess senescence-promoting (Hisamatsu et al., 2006), growth-inhibiting (Nakajyo et al., 2006), antimicrobial (Andersson et al., 2006), and antifungal (Stelmach et al., 2001; Kourtchenko et al., 2007) properties. It was also proposed that arabidopsides function as storage molecules for OPDA and serve as substrates for JA biosynthesis (Kourtchenko et al., 2007). It was already shown that arabidopside levels are highly induced by wounding (Stelmach et al., 2001; Buseman et al., 2006; Böttcher and Weiler, 2007). Dabrowska et al. (2009) proposed that OPDA has a direct negative influence on herbivores; accordingly, arabidopsides may act as direct defense compounds against herbivores. Previous work showed that damage from feeding *P. rapae* in contrast to wounding elicits higher levels of free OPDA than of JA in Arabidopsis (Reymond et al., 2000, 2004; Stintzi et al., 2001). The same was observed after damage from grasshopper feeding (Supplemental Fig. S1A). Our data show that damage from grasshopper feeding and treatment with GS on wounded leaves quickly elicits levels of free OPDA in Arabidopsis (Fig. 1A; Supplemental Fig. S1A) and that this increase depends on lipase activity in GS but not in the plant (Fig. 5). High OPDA levels are found mainly at the site of wounding (Supplemental Fig. S8), and the identification of lipase-like proteins in GS further supports our conclusion that insect lipases are among the main elicitors of OPDA released in the wounded tissues of Arabidopsis. Since we found that the OS of all insects we tested elicit high levels of free OPDA in Arabidopsis leaves (Fig. 8), we hypothesize that OPDA release mediated by insect lipases might be a general response to herbivory in Arabidopsis. Accordingly, OPDA accumulation induced by the OS from the lepidopteran *M. sexta* could also be significantly reduced by pretreatment with the lipase inhibitor orlistat (Supplemental Fig. S9). Elicitations with the purified insect lipases that mediate these reactions will help to further clarify the role of these lipases in herbivory-induced OPDA release. Our data add an additional mechanism to those already known from the literature on how plants recognize the herbivory-mediated degradation of their metabolites (for review, see Heil, 2009).

OPDA was shown to elicit the transcript levels of many JA-responsive and non-JA-responsive genes in Arabidopsis in a COI1-dependent or COI1-independent manner (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008). We demonstrate that two of these genes (*OPR1* and *GST1*) were significantly induced by the OPDA-releasing fraction of GS (Fig. 7), which indicates that in Arabidopsis, OPDA-related signaling is induced by grasshopper feeding. Although the function and mechanism of OPDA signaling in defense responses to herbivores are not fully understood, we hypothesize that OPDA-mediated responses in Arabidopsis might help distinguish mechanical wounding from herbivore attack. In our proposed model, lipases from insect OS release OPDA from arabidopsides in damaged leaf tissue, leading to the activation of OPDA-related signaling (Fig. 9). It is tempting to speculate that high levels of the signaling molecule OPDA in arabidopsides might have evolved as a way of rapidly detecting herbivory in Arabidopsis and related species. Stumpe et al. (2010) showed that OPDA, but not JA, is present in the moss *Physcomitrella patens*, indicating the presence of an evolutionarily conserved function of OPDA in plants. Future studies will investigate whether OPDA orchestrates herbivory-induced defense responses in Arabidopsis.

Our data show that W+GS treatment induced higher levels of JA and JA-Ile than did W+W treatment. OPDA, released by insect feeding, might enter surrounding intact cells to serve as a precursor for JA

Figure 9. Summary of *S. gregaria* GS-induced responses in Arabidopsis. The model shows herbivore-damaged cells (gray) and intact cells (green). GS lipase activity releases esterified OPDA from lipids (eOPDA) and possibly also 13-HPOT (eHPOT). OPDA may directly elicit signaling events or supply substrates for JA biosynthesis in adjacent cells. GS activates MPK3 and MPK6 through an unknown mechanism. MPK6 activity positively affects ET release. GS triggers a Ca^{2+} influx in the cytosol mediated by Ca^{2+} channels. The released OPDA might contribute to the observed Ca^{2+} response. Wound-induced H_2O_2 levels slightly suppress JA and ET accumulation. Signaling events are indicated by dotted arrows, and fluxes and metabolic processes are indicated by solid arrows. Hypothetical events are shaded gray instead of black.

biosynthesis (Miersch and Wasternack, 2000). It could also be possible that extracellular OPDA serves only as a signal to induce responses that include changes in $[Ca^{2+}]$ (Walter et al., 2007) or gene expression (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008); these then may elevate levels of JA and JA-Ile biosynthesis. The JA levels were not as much affected by pretreatment of GS with orlistat than the OPDA levels, perhaps because the released OPDA is still sufficient for JA biosynthesis (Fig. 5D). Despite higher levels of JAs, slightly higher levels of both esterified and free OPDA were observed after W+GS treatment in comparison with W+W treatment (Supplemental Fig. S2), suggesting elevated de novo biosynthesis of OPDA. It is tempting to speculate that wound-induced accumulation of esterified OPDA is involved in priming plant defense responses against herbivores. Interestingly, treatment with either GS or fungal lipase was sufficient to elevate levels of the potential OPDA precursor 13-HPOT (Fig. 1A; Supplemental Fig. S6). The presence of membrane-bound 13-HPOT in Arabidopsis was proposed already (Stenzel et al., 2003; Andersson et al., 2006). It remains unclear whether the measured increase in 13-HPOT is derived from de novo synthesis by radical- or lipoxygenase-dependent mechanisms or is released from lipid-bound forms. Since 13-HPOT participates in various biochemical pathways, such as the biosynthesis of JAs and of phytoprostanes, and was found to have signaling activity such as inducing changes in gene expression (Stenzel et al., 2003; Mueller, 2004; Mueller and Berger, 2009), we propose that its accumulation after GS induction is important for herbivory-induced signaling in Arabidopsis.

Lipase Activity-Independent Defense Responses

In addition to lipase activity, our results indicate the activity of other important effectors in GS. Even if the nature of the elicitor remains unknown, we show that the less than-10-kD GS fraction possesses the ability to induce changes in the $[Ca^{2+}]_{cyt}$ (Fig. 6A). Boiling GS was not able to abolish the $[Ca^{2+}]_{cyt}$ changes after GS application (Fig. 6A), and the application of fungal lipase solution induced $[Ca^{2+}]_{cyt}$ signatures other than the GS (Fig. 6A). Nevertheless, as OPDA, JA, and JA-Ile were shown to induce $[Ca^{2+}]$ changes in tobacco cells (Walter et al., 2007) and JA and JA-Ile in Arabidopsis (Sun et al., 2010), it seems likely that the relative increases in oxylipins could influence intracellular Ca^{2+} levels after W+GS treatment. Recently, the detergent effects of insect OS have been suggested to influence plant responses to herbivory (Maischak et al., 2007). We show that the observed $[Ca^{2+}]_{cyt}$ changes have a signature that differs from that of a detergent (Fig. 6A), one that can be completely blocked by the Ca^{2+} channel inhibitor $LaCl_3$ (Bush, 1995; Fig. 6A); this indicates that the observed changes are mediated by Ca^{2+} channels and likely not by detergent-like compounds in the GS. $[Ca^{2+}]_{cyt}$ changes are among the earliest herbivory-induced signals (Maffei et al., 2007) and therefore may influence later herbivory-induced responses. Ca^{2+}-regulated protein kinases have long been hypothesized to play a role in ET emission and recently were shown to influence herbivory-induced transcriptional responses in Arabidopsis (Tatsuki and Mori, 2001; Kanchiswamy et al., 2010), demonstrating that Ca^{2+} plays an important role in herbivory-induced

responses in Arabidopsis. Further experiments with Arabidopsis plants altered in Ca^{2+}-dependent signaling mediators are needed to elucidate the role of this signaling molecule in herbivory-induced responses.

The less than-10-kD fraction of GS still induced JA-Ile levels (Fig. 5A). W+W- and W+GS-induced JA-Ile is present in several times lower quantities than wound-induced JA, indicating that the JA levels are not limiting for JA-Ile biosynthesis. It could be possible that the less than-10-kD fraction of GS is still inducing the biosynthetic steps involved in the conversion of JA to JA-Ile. Therefore, lipase-independent pathways might be involved in regulating JA-Ile accumulation after W+GS treatment. It might be possible that Ca^{2+}-dependent signaling is involved in JA-Ile biosynthesis, since $[Ca^{2+}]_{cyt}$ is still induced by the less than-10-kD fraction of GS.

A response that is partially independent of the lipase activity-containing fraction is the activation of MPK3 and MPK6, which was observed after wounding and the application of the less than-10-kD GS fraction (Fig. 6B). Future research will focus on the GS-derived elicitors of $[Ca^{2+}]_{cyt}$ increase and MAPK activation and their roles in herbivory-induced downstream responses.

MAPKs Partially Contribute to GS-Induced Phytohormone Levels

From tomato and *N. attenuata*, it is known that the herbivory-induced accumulation of JA and its precursors is mediated by MAPKs (MPK1, MPK2, and MPK3 for tomato, SIPK and WIPK for *N. attenuata*; Kandoth et al., 2007; Kallenbach et al., 2010). We tested whether their homologs in Arabidopsis, MPK3 and MPK6, regulate GS-induced changes in oxylipin contents, as both MAPKs were activated rapidly after GS treatment (Fig. 1D). To our surprise, neither *mpk3* nor *mpk6* mutants showed reduced W+GS-induced oxylipin levels (Fig. 3A). Therefore, the function of these two MAPKs in herbivory-induced oxylipin elicitation might not be evolutionarily conserved. However, we found that MPK6 mediates ET accumulation after GS treatment (Fig. 3B), which is in line with the role of the MPK6 homolog SIPK in herbivory-induced ET emissions in *N. attenuata* (Wu et al., 2007). The tendency to higher ET emissions in the *mpk3* plants (Fig. 3B) could be due to higher MPK6 activity after W+GS treatment, since both MPK3 and MPK6 can be activated partially by the same MAPK kinases (Andreasson and Ellis, 2010). In the absence of MPK3, higher MPK6 activity and ET release could be due to enhanced MPK6 phosphorylation by upstream MAPK kinases. Even in the *mpk6* mutants, W+GS treatment increased the emission of ET to higher levels than did W+W treatment, indicating that other signaling pathways are involved in the regulation of the GS-dependent ET release. MPK3 was shown to be necessary for the ET release in *mpk6* mutant seedlings treated with the necrotrophic fungus *Botrytis cinerea* (Han et al., 2010). Using plants silenced in both MPK3 and MPK6 will help to further identify the possibly overlapping roles of both MAPKs in the regulation of ET after herbivory in Arabidopsis (Han et al., 2010). Interestingly, the OPDA and JA levels were 33% and 26% increased in *mpk6* plants (Fig. 3A; *t* test, $P < 0.05$, wild-type versus *mpk6* plants after W+GS treatment). If *mpk6* plants have altered steady-state levels of OPDA-containing lipids or if structural or developmental properties of *mpk6* plants lead to higher oxylipin levels after W+GS treatments are interesting questions for future research. Also, the cross talk with the phytohormone ET (for review, see Adie et al., 2007), which is emitted in lower amounts after W+GS treatment in *mpk6* mutant plants compared with wild-type plants (Fig. 3B), might influence oxylipin levels.

GS-Induced Defense Responses Are Altered by Wound-Induced H_2O_2

H_2O_2 production after wounding and herbivory was reported to alter wound- and herbivory-induced plant responses (Orozco-Cárdenas and Ryan, 1999; Sagi et al., 2004; Maffei et al., 2006; Diezel et al., 2009). Additionally, it was reported that H_2O_2 production is regulated by MAPK and Ca^{2+} signaling; both are induced by GS treatments (Keller et al., 1998; Sagi and Fluhr, 2001; Asai et al., 2008). We, too, found H_2O_2 to be induced by wounding; however, DAB staining revealed no effect of GS treatment on wounded leaves. Nevertheless, DAB staining is not sensitive enough to record subtle changes in H_2O_2 production, which might still be differentially induced when W+W is compared with W+GS treatments. By using *rbohd* mutant plants with highly reduced wound-induced H_2O_2 production, we demonstrate that wound-induced H_2O_2 is a negative regulator of W+GS-induced JA and ET levels. These data are consistent with a previous report from Diezel et al. (2009) showing that herbivory-induced H_2O_2 negatively influences JA and ET in *N. attenuata*. Future work is needed to understand the role of wound-induced H_2O_2 in mediating herbivory-induced responses in Arabidopsis.

Caeliferin A16:0 Is Not Sufficient to Induce GS-Mediated Defense Responses

Recently, herbivore-derived elicitor screening in various plant species revealed that caeliferin A16:0 induced JA accumulation and ET release in Arabidopsis (Schmelz et al., 2009). However, we were unable to induce any of the GS-induced responses using synthetic caeliferin A16:0, although we were able to detect this compound in the OS of *S. gregaria*. We cannot rule out the possibility that different chemical compositions of test substances, plant treatments, or plant growth conditions may have affected the plant's sensitivity to caeliferin A16:0.

CONCLUSION

Our data highlight a previously unknown mechanism for how herbivory leads to increased levels of

oxylipins in plants. We demonstrate that lipase activity in the OS of grasshoppers leads to increased levels of OPDA and OPDA-related signaling in Arabidopsis. The finding that similar increases in OPDA levels are induced by various insects in Arabidopsis suggests a common modus operandi and indicates the role arabidopsides may play in herbivore perception. Lipase-independent grasshopper-induced activation of $[Ca^{2+}]_{cyt}$ levels and MAPK activation demonstrate parallel induction of complex signaling cascades by herbivores (Fig. 9).

MATERIALS AND METHODS

Plant Material

Experiments were carried out with Arabidopsis (*Arabidopsis thaliana*) ecotypes Col-0, Landsberg *erecta*, and Wassilewskija as well as the mutants *mpk3* (provided by Brian Ellis, Michael Smith Laboratories, Vancouver, Canada), *mpk6* (provided by Justin Lee, Leibniz-Institut für Pflanzenbiochemie, Halle, Germany), and *rbohd* (provided by Miguel Angel Torres, Universidad Politécnica de Madrid, Spain).

Plant Growth

Arabidopsis plants were grown on substrate consisting of 80% Fruhstorfer Nullerde, 10% vermiculite, and 10% sand, fertilized with Triabon (1 g L^{-1}) and Osmocote Exact Mini (1 g L^{-1}), and treated with *Steinernema feltiae*. The controlled-environment chamber provided 10 h of light per day, a temperature of 21°C, and 60% humidity. The light intensity was 190 to 220 μmol m^{-2} s^{-1}.

Insects

Manduca sexta larvae were obtained from in-house colonies. Larval regurgitate was collected from third-instar larvae. *Spodoptera frugiperda*, *Spodoptera littoralis*, *Heliothis subflexa*, and *Heliothis virescence* were cultivated on an agar-based diet (Bergomaz and Boppre, 1986) or Arabidopsis. *Schistocerca gregaria* were obtained from Bugs International and b.t.b.e. Insektenzucht GmbH and were fed Arabidopsis. *Pieris rapae* were provided by Marcel Dicke (Wageningen University, The Netherlands) and were fed Arabidopsis. Additionally, the grasshopper population of the lawn at the Beutenberg Campus in Jena, next to the Max Planck Institute for Chemical Ecology, served as a source for grasshoppers (*Chorthippus* spp.) termed "native grasshoppers."

Elicitors and Test Substances

Synthetic caeliferin A16:0 was purchased from ChemPep and dissolved in 50 mM Na_2HPO_4 buffer (pH 8) stock solution as described previously (Schmelz et al., 2009). Structure and exact mass were verified by NMR (Supplemental Fig. S10) and high-resolution liquid chromatography-mass spectrometry (Supplemental Fig. S4, A and B). *Rhizopus arrhizus* lipase (Sigma-Aldrich) was dissolved in 100 mM Tris-HCl buffer (pH 7.5) to a concentration of 0.5 units μL^{-1}. OS (including GS) were collected according to Turlings et al. (1993) with the modifications of Alborn et al. (2003). For fractionation, GS were filtered using a 10-kD cutoff-column (Viraspin 500; GE Healthcare). The flow-through fractions were termed as less than 10 kD, and the supernatants were termed as greater than 10 kD. After fractionation, flow through and supernatant were adjusted to the original volume with aqua bidest. To inhibit enzyme activity, the GS and leaf tissue were incubated with 5 volumes of isopropanol at 80°C for 10 min (Heller and Steinberg, 1972; Yadav et al., 1998; Sana et al., 2004). Before use, the GS isopropanol solution was completely evaporated under N_2 and the pellet was dissolved in the original volume of water. For heat inhibition, GS and fungal lipase solution were boiled for 10 min. It is possible that other substances than enzymes also are affected by the two methods mentioned before. For in vitro treatment, 100 mg of enzyme-inhibited plant tissue was incubated with 5 μL of test substance for 10 min at room temperature. For lipase inhibition tests, GS and *M. sexta* OS were incubated with 1 mM tetrahydrolipstatin (orlistat; Cayman Chemicals; dissolved in ethanol) for 1 h at 37°C. The water, GS, and *M. sexta* OS controls were treated with the same amount of ethanol and incubated for 1 h at 37°C. As an additional control, 1 mM orlistat was added to the accordingly treated GS directly before application. GS and *M. sexta* OS for lipase inhibitor treatments were diluted 1:10 before treatment. GS, GS fractions, and heat-treated GS were used in a 1:5 dilution.

Leaf Treatments

To mimic herbivory-induced responses, four fully expanded leaves from 4- to 5-week-old rosette-stage plants were mechanically wounded by rolling a fabric pattern wheel three times on each side of the leaf, followed by the immediate application of 5 μL of the test substance. The test substance was distributed over the entire wounded leaf. At the indicated time intervals, the entire treated leaves without petioles were collected, immediately frozen in liquid nitrogen, and stored at −80°C.

For the natural feeding experiments, four starved *S. gregaria* nymphs were placed on each Arabidopsis plant in separate boxes (20 cm × 28 cm × 20 cm), and damaged leaves were harvested at the indicated time points.

Protein Extraction, Separation by 2-D Gel, and Analysis of Protein Spots

Proteins from GS were extracted with the phenolic extraction procedure described by Giri et al. (2006). A 2-D Quant kit (Amersham Bioscience) was used for protein quantification. 2-D gel electrophoresis, digestion, and analysis were done according to Giri et al. (2006). ProteinLynx Global Server Browser version 2.3 software (Waters) was used for baseline subtraction and smoothing, de novo peptide sequence identification, and database searches. The peptide fragment spectra were searched against the UniProt_TREMBL or UniProt_Swissprot database (parameters were as follows: peptide mass tolerance, 20 ppm; estimated calibration error, 0.005 D; carbamidomethylation of Cys and possible oxidation of Met). The BLAST search was performed internally using the MS-BLAST algorithm (Lit17) with a minimum of one peptide matching at an expect score of 100, with no-gaphspmax100-sort_by_totalscore -span1 advanced options and PAM30MS search matrix.

Phytohormone and Hydroperoxy Fatty Acid Analysis

Oxylipins (13-HPOT, OPDA, JA, and JA-Ile) were extracted and analyzed as described by Kallenbach et al. (2010). It is particularly important to do the extraction on ice to avoid enzymatic cleavage of OPDA-containing lipids during the extraction procedure. To quantify JA and JA-Ile, 200 ng of [9,10-^{2}H]dihydro-JA and 40 ng of [$^{13}C_6$]JA-Ile were added as internal standards to each sample. 13-HPOT was quantified using 15-hydroperoxyeicosadienoic acid (10 ng per sample; Cayman Chemicals) as an internal standard, and OPDA was quantified based on the JA standard and an empirically determined conversion ($m_{OPDA} = 1.19 \times m_{JA}$). For the extraction and analysis of free and esterified OPDA, the published phytohormone extraction method was slightly modified. After ethyl acetate extraction and evaporation, the sample was redissolved in 1 mL of methanol, and 1 mL of 15% (m/v) KOH was added, incubated for 1 h at 60°C, and neutralized with 2 mL of 1 M citric acid. After reextraction with ethyl acetate, the ethyl acetate phase was evaporated and the pellet was resuspended in 70% methanol for analysis. The extraction and analysis of free OPDA by the normal phytohormone extraction method in parallel samples enabled the calculation of the esterified OPDA.

[9,10-^{2}H]Dihydro-JA was synthesized by saponification and deuteration of methyl JA (Sigma-Aldrich), [$^{13}C_6$]JA-Ile was synthesized out of [$^{13}C_6$]L-Ile (Cambridge Isotope Laboratories), and JA (Sigma-Aldrich) and methanol for mass spectrometry analysis were purchased by VWR International.

ET Release

To analyze herbivory-induced ET release, all fully expanded leaves were mechanically wounded by rolling a fabric pattern wheel twice on each side of the midrib, followed by the immediate application of 3 μL of the test substance to the wounding site, or plants were induced by damage from feeding *S. gregaria*. For feeding experiments, four starved *S. gregaria* nymphs were placed on the plants, each plant in a separate box (20 cm × 28 cm × 20 cm), for the indicated times. ET released was measured from detached plants that were allowed to accumulate ET in a 50-mL flask for 5 h with a photoacoustic spectrometer (INVIVO; https://www.invivo-gmbh.de) as described by von Dahl et al. (2007).

Glycolipid Assay

The extraction of lipids was done by the isopropanol-hexane method (Hara and Radin, 1978) with the modifications described by Bonaventure et al. (2003). The lipid extracts from 2 g of Arabidopsis plant tissue were evaporated under N_2 and dissolved in 2 mL of Cl_3CH:acetic acid (100:1 by volume) and loaded on a 750-mg silica gel 60 column preconditioned with Cl_3CH:acetic acid (100:1 by volume). After two washing steps with 6 mL of Cl_3CH:acetic acid (100:1 by volume) and 5 mL of Cl_3CH:acetone (80:20 by volume) to remove neutral lipids, the glycolipids were eluted by 5 mL of acetone and 5 mL of acetone:acetic acid (100:1 by volume). The glycolipid fractions were combined and equally divided to 15 aliquots. The presence of lipid-bound OPDA was tested by KOH ester hydrolysis as described above. The extracted glycolipid fraction contained more than 35 nmol lipid-bound OPDA g^{-1} fresh weight leaf material used for lipid extraction, whereas no free OPDA could be determined. The solvents were evaporated completely under N_2, and the aliquots were immediately used for lipase activity assays.

For the glycolipid assays, 35 μL of water or 1:5 diluted GS was added to each aliquot and incubated for 30 min at room temperature. After the addition of 50 μL of ethyl acetate, the samples were incubated for 10 min at room temperature. Samples were mixed several times during incubation. GS treatment without incubation was performed by using water-incubated samples and application of 35 μL of 1:5 diluted GS directly before extraction. Afterward, OPDA was extracted with the ethyl acetate-phytohormone extraction method described above.

Transcript Analysis

After treatments, the RNA from five biological replicates was extracted. For cDNA synthesis, RNA was reverse transcribed using oligo(dT) primer and ReverAid reverse transcriptase (Invitrogen). Quantitative (q)PCR was performed on a Stratagene Mx3005P qPCR machine using a SYBR Green-containing reaction mix (Eurogentec; qPCR Core kit for SYBR Green I No ROX). The Arabidopsis eIF4A1 elongation factor was used as a standard for normalizing the cDNA concentrations. The primer sequences are summarized in Supplemental Table S2.

MAPK Assay

Protein extraction was performed according to Wu et al. (2007), and the determination of kinase activity was performed as described by Zhang and Klessig (1997) using myelin basic protein as a substrate. After reaction and the washing steps, the gels were dried on a gel dryer (Bio-Rad), and the images were obtained with a FLA-3000 phosphor imager system. For each sample, three biological replicates were pooled.

$[Ca^{2+}]_{cyt}$ Measurements

For the experiments, transgenic Arabidopsis (Col-0) plants expressing apoaequorin in the cytosol (Knight et al., 1997) were grown on Hoagland medium with 1% agar. After 21 d, leaves were dissected and placed onto 96-well plates. The leaves were incubated in 150 μL of 5 μM coelenterazine in the dark overnight at 21°C. After the addition of 40 μL of test substance, the luminescence was quantified with a microplate luminometer (Luminoscan Ascent, version 2.4; Thermo Fisher Scientific). Prior to the treatment with the test substance, the background luminescence was determined for 1 min, and after the measurement, the samples were discharged by adding a 0.1 M $CaCl_2$ and 10% ethanol solution. The measurements were calibrated using the equation of Rentel and Knight (2004). $LaCl_3$ (1 mM) treatments were done 1 h before treatment.

Additional wounding proved not to be necessary, as the leaves were excised from the plant and therefore were already prewounded. Experiments with and without additional wounding by a needle showed similar results (data not shown). Since thigmomorphogenic stress, such as touching, can induce Ca^{2+} responses, we used no additional wounding prior to the application of test substances in the main experiments.

H_2O_2 Measurement

DAB staining was modified after Thordal-Christensen et al. (1997). For DAB staining, fully expanded leaves from rosette-stage plants were cut from the plant and petioles were exposed to a 1 mg mL^{-1} DAB solution at pH 3.8. After 4 h of incubation, the plants were subjected to the treatment. After the indicated incubation times, the leaves were subjected to DAB destaining solution (1:1:3 glycerol:acetic acid:ethanol) at 60°C.

Statistical Analysis

Data were analyzed with SPSS Statistics 17.0. Depending on the hypothesis that was being tested, either one-way ANOVA followed by Tukey's honestly significant difference test or independent-sample *t* test was used as indicated. Each experiment was repeated at least twice.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers X65052 (eIF4A1), Y11727 (GST1), BT000007 (MPK3), AY120737 (MPK6), BT020365 (OPR1), and BT002651 (RbohD).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Feeding damage from *S. gregaria* induces changes in phytohormone levels of Arabidopsis.

Supplemental Figure S2. *S. gregaria* GS induce changes in free and esterified OPDA levels.

Supplemental Figure S3. *S. gregaria* GS induce phytohormone changes in different Arabidopsis accessions.

Supplemental Figure S4. Mass spectrometry analysis of caeliferin A16:0.

Supplemental Figure S5. Lipase-dependent oxylipin accumulation in Arabidopsis (Col-0).

Supplemental Figure S6. Lipase activity induces the accumulation of 13-HPOT levels in Arabidopsis (Col-0) leaves.

Supplemental Figure S7. *S. gregaria* GS and fungal lipase solution (L)-induced oxylipin levels in Arabidopsis (Col-0).

Supplemental Figure S8. OPDA accumulates in close proximity to the wounded site.

Supplemental Figure S9. *M. sexta* OS induce lipase-dependent OPDA accumulation in Arabidopsis (Col-0).

Supplemental Figure S10. NMR analysis of synthetic caeliferin A16:0.

Supplemental Table S1. Sequences of lipase-like proteins in *S. gregaria* GS.

Supplemental Table S2. Primers used for qPCR.

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Grasshopper oral secretions increase salicylic acid and abscisic acid levels in wounded leaves of *Arabidopsis thaliana*

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Key words: abscisic acid, Arabidopsis, herbivory, salicylic acid, *Schistocerca gregaria*

Abbreviations: ABA, abscisic acid; JA, jasmonate; ET, ethylene; OS, oral secretions; SA, salicylic acid

Recent investigations showed that the model plant *Arabidopsis thaliana* specifically responds to herbivory-associated molecular patterns by activating a sophisticated signaling network. The lipase activity of insect oral secretions was shown to elevate oxylipin levels when applied to puncture wounds in leaves. The results also demonstrated that the oral secretions of the generalist *Schistocerca gregaria* contained other, probably non-proteinous, elicitors of plant defense responses which induced mitogen-activated protein kinases, calcium signaling and ethylene levels.[1] This addendum presents data on the levels of additional phytohormones that are elevated after application of *S. gregaria* oral secretion to wounded leaves. Abscisic acid and salicylic acid levels are significantly elevated after elicitation with *S. gregaria* oral secretions, adding another layer of complexity to the herbivory-induced response of *A. thaliana*.

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Grasshopper-Induced Defense Responses

Most studies in *Arabidopsis thaliana* have focused on the wound response or the response to feeding by herbivorous insects.[2-6] Although there are some hints for herbivory associated effectors,[3,4] it remains largely unknown to which extend the herbivore responses of *A. thaliana* are induced by the wounding process or if effectors from insect oral secretions alter the wound response. Using standardized wounding with and without application of oral secretions (OS) allows researchers to distinguish simple wound responses from herbivory-induced processes.[7] Experiments with OS of *Schistocerca gregaria* and different lepidopterans showed that lipase activity in OS in addition with other unknown effectors are able to induce levels of 13-hydroperoxy octadecatrienoic acid (13-HPOT), 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA) and jasmonyl isoleucine (JA-Ile), increase the emission of ethylene (ET), enhance mitogen-activated protein kinase (MAPK) activity and alter the cytosolic calcium levels ($[Ca^{2+}]_{cyt}$).[1]

SA

Results from pathogen research in *A. thaliana* showed that different phytohormones such as the JA/ET group as well as the salicylic acid (SA) group are part of a sophisticated network that interacts with synergistic as well as compensatory effects.[8] The JA/ET group is known to play a major role in the defense responses to herbivory by biting and chewing insects[9] and, insect herbivory-induced SA levels were known to negatively influence JA/ET levels and signaling.[10-14] The application of *S. gregaria* OS to wounded leaves doubled the levels of SA when compared with wounding alone (**Fig. 1**). It might be possible that the increased SA accumulation is a direct response to yet uncharacterized herbivory-associated effectors or is indirectly triggered by other OS-induced signaling events. Nevertheless it might be also possible that OS contain certain microbial elicitors, which are responsible for the observed increase in SA levels. Elevated SA levels in *S. gregaria* OS elicited leaves might play a role in fine-tuning the herbivore defense response and/or be important for the defense of wounded leaves against pathogens, which might use wound sites as entry points for infection. The latter possibility is particularly interesting as it is already known that some herbivores are vectors for plant pathogens.[15] Despite a beneficial role in herbivore or pathogen defense it might be also possible that the oral secretion of *S. gregaria* manipulates *A. thaliana* defense responses by inducing SA accumulation, leading to the suppression of jasmonate mediated anti-herbivore defense responses, as has been reported for other model systems.[14]

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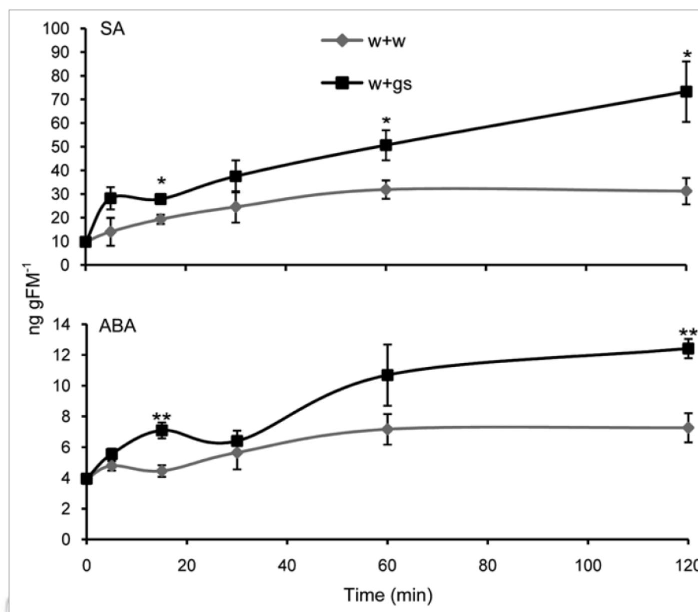

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Figure 1. Salicylic acid (SA) and abscisic acid (ABA) levels induced by *Schistocerca gregaria* oral secretions (GS) in *Arabidopsis thaliana* (Col-0). Mean levels (±SE; n ≥ 3) of SA and ABA. Leaves were wounded and either water (w+w) or GS (w+gs) was applied or leaves were untreated (time point 0).[1] Samples were harvested at the indicated time points. Compounds were extracted with the chloroform-methanol method[17] and analyzed as previously discribed for underivatised compounds.[18] Asterisks indicate significant differences between w+w and w+gs treated plants for the same time point (independent sample t-test; *p < 0.05; **p < 0.01). FM, Fresh mass.

Additional support for a role of SA in modulating herbivory-induced transcript levels in *A. thaliana* was reported recently, by showing altered transcript levels in herbivore infested wild type plants when compared with herbivory-elicited SA signaling and SA biosynthesis mutants.[16] Using such mutants to study the role of grasshopper OS-mediated SA accumulation will be an interesting aspect for future research.

ABA

Abscisic acid (ABA) is mainly known to play a role in the response to water stress but in *A. thaliana* wounding induces similar responses to those elicited by dehydration.[4] Feeding by biting or chewing insects produces wounds and therefore also water stress. Interestingly, infestation by *Pieris rapae* larvae induced less severe water stress-induced gene expression than mechanical wounding, suggesting that the feeding strategy of *P. rapae* might minimize the water stress in *A. thaliana*.[4] We found that the application of *S. gregaria* OS to wounded leaves increased wound-induced ABA levels by 70% when compared with wounding and water treatments (**Fig. 1**). It remains unknown which features of *S. gregaria* OS are responsible for the observed increase in ABA levels. In addition to the presence of specific elicitors, it might be possible that physicochemical properties such as viscosity or the osmotic potential of the OS might affect the water stress response at the wounding site and thereby influence the ABA levels. It was shown that *A. thaliana* mutants impaired in ABA biosynthesis significantly affected herbivore performance and herbivory-induced transcript rates.[16] Our data are consistent with the hypothesis that ABA might be involved in the regulation of defense and tolerance responses of *A. thaliana* after herbivore attack. Using plants impaired in ABA signaling will further shed light on the role of *S. gregaria* OS-induced ABA levels.

Conclusion

Our data indicate that insect oral secretions activate multiple signaling pathways in wounded *A. thaliana* leaves. It will be an interesting task for future investigations to understand the extent to which herbivory-induced signaling pathways are directly or indirectly interconnected or act in parallel to regulate *A. thaliana's* physiological adaptations to insect attack. The analysis of the signaling network after natural and artificial herbivore treatments will be necessary to unravel the complex functions of specific phytohormones in plant responses to herbivore attack.

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Manuscript VI

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The role of phytohormones in attacker-specific plant reactions

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Abstract

The capacity to perceive and respond is integral to biological immune systems. But to what extent can plants specifically recognize and respond to insects? Current findings suggest that plants possess potent surveillance systems to detect general patterns of cellular damage as well as highly specific herbivore-associated cues. The jasmonate (JA) pathway emerges as the major signaling cassette that integrates information perceived at the plant-insect interface into broad-spectrum defense responses. Specificity can be achieved via i) JA-independent processes and ii) spatio-temporal changes of JA-modulating hormones, including ethylene, salicylic acid, abscisic acid, auxin, cytokinins, brassinosteroids and gibberellins. The identification of receptors and ligands and an integrative view of hormone-mediated response systems are crucial to understand specificity in plant immunity to insect herbivores.

Know your enemy- a golden rule of plant defense?

“If you know your enemies and know yourself, you can win a hundred battles without a single loss”, states Sun Tzu in his ancient military treatise “The Art of War”. Plants, as primary producers of organic matter in terrestrial ecosystems, must continuously resist a multitude of attackers and, and, unlike the armies of Sun Tzu, do not have the option of retreating to safe ground. But have plants evolved the capacity to “know” the attacking enemies and adjust their defenses accordingly? In this review we use the current paradigm of molecular specificity in plant-pathogen interactions as a framework to discuss potential mechanisms by which plants specifically recognize insect herbivores. We then merge this concept with current knowledge of hormone signaling pathways to evaluate the potential role of hormones in shaping defense responses to specific types of insect herbivores. Important knowledge gaps about specificity underlying plant-enemy interactions are discussed in the context of framing future mechanistic questions in this exciting field of research.

Plants recognize herbivores via mechanical and chemical cues

An appropriate defense response to a biotic threat requires initial recognition. Pathogens are recognized when common microbial compounds called pathogen-associated molecular patterns (PAMPs) are detected by pattern recognition receptors (PRRs) on the surface of the host plant cell, leading to PAMP-triggered immunity (PTI; Figure 1). Damage-associated molecular patterns (DAMPs), which are endogenous molecules that are produced by the plant after infection, are also recognized by PRRs to trigger defensive reactions [1]. Pathogens can evade this innate immune response through the action of effector molecules that, upon delivery into the host cell, suppress PTI. Some plant genotypes again contain disease resistance (R) proteins that specifically recognize pathogen effectors, resulting in effector-triggered immunity (ETI) [2]. The molecular identification of ligands and receptors involved in PTI and ETI has enabled well-founded conclusions about the specificity of recognition in plant-pathogen interactions: In general, PTI is based on non-specific recognition of common microbial molecules, whereas ETI is triggered by highly pathogen-specific compounds [3].

To what extent can the PTI/ETI model inform research aimed at elucidating the specificity of recognition in plant-herbivore interactions? In comparison to pathogens, insects are highly complex multicellular organisms with intricate lifestyles and behavioral patterns. The various cues emanating from these patterns may be used by the plant to recognize the threat of herbivory and to mount appropriate defensive responses [4] (Figure 1). The first contact often occurs between the leaf surface and the tarsi of an arriving insect. Landing and walking on a plant will exert pressure, break trichomes, and deposit chemicals from tarsal pads on the leaf [4]. Plants have evolved mechanisms to sense pressure. The Venus fly trap, for example, closes immediately after stimulation of its sensory hairs by insects [5]. Non-carnivorous plants are highly sensitive to touch as well [6]. In at least some cases, mechanostimulation by repeated touching is sufficient to induce the accumulation of jasmonic acid (JA) [7], the precursor of the defense hormone jasmonoyl-L-isoleucine (JA-Ile). Breaking of tomato leaf trichomes by adult moths or caterpillars induces hydrogen peroxide (H_2O_2) formation and expression of defensive proteinase inhibitors [8]. There is currently no indication that this type of “early warning”

response is specific for particular insect species, and the observed effects may mostly be related to DAMP-like effects (see below).

Oviposition represents another opportunity for plants to detect insect herbivores. The formation of necrotic zones following egg deposition was observed in *Brassica nigra* and certain potato clones [9, 10]. In pea plants, long-chain alpha,omega-diols deposited during oviposition of pea weevils trigger the formation of undifferentiated cells beneath the eggs, which increase plant resistance [11]. Oviposition can be accompanied by wounding, and in the interaction between the elm leaf-beetle and the field elm, for example, oviduct secretions induce defenses only when they are released into oviposition wound sites [12]. Overall, some oviposition-associated cues seem to act as PAMP-like molecular patterns that can be used by plants to recognize and predict herbivore attack. Consistent with the PTI/ETI framework, oviposition effectors may be produced by herbivores to suppress the plant immune response (Box 1). Taken together, these findings suggest that oviposition events trigger plant defense reactions in an insect- and potentially even species-specific manner.

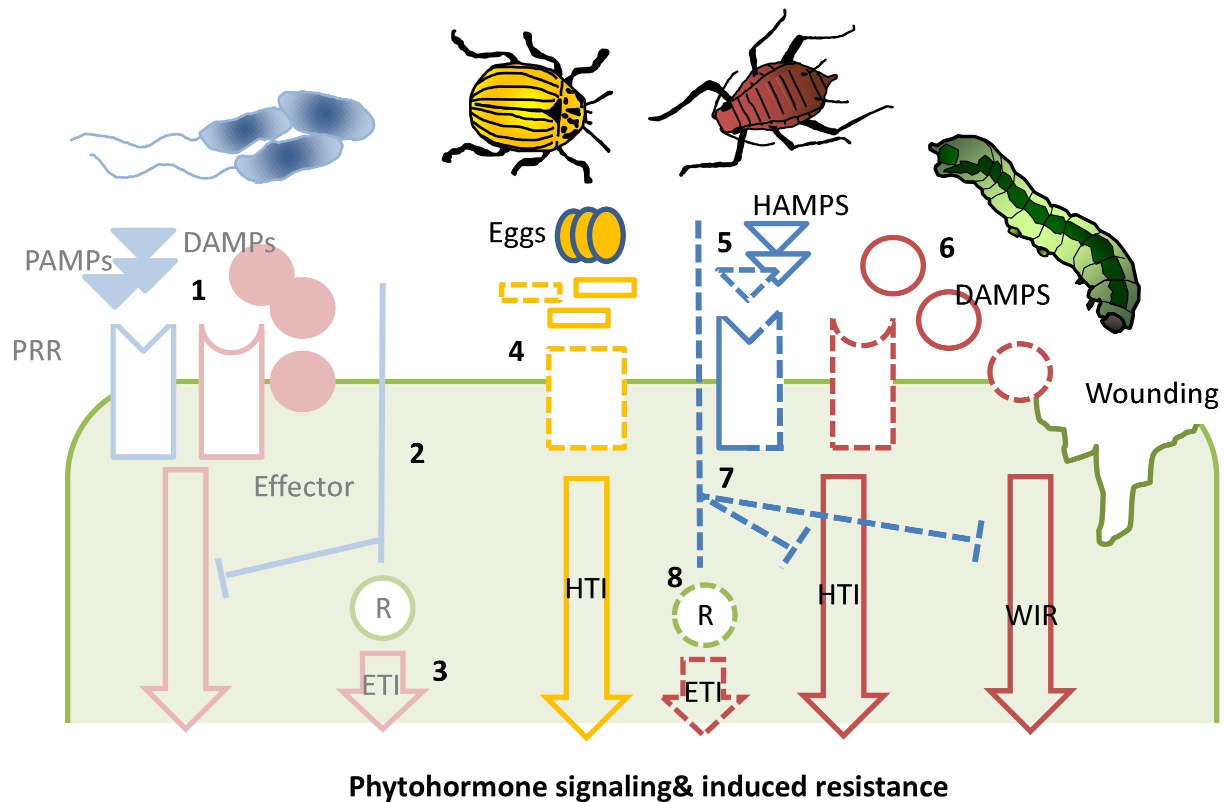

Figure 1: Molecular recognition of pathogens and herbivores by plants. 1. Pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) are recognized by pattern recognition receptors (PRRs) and lead to PAMP-triggered immunity (PTI). 2. Pathogen effectors suppress PTI. 3. Resistance gene products recognize effectors and lead to effector-triggered immunity (ETI). 4. Oviposition-associated compounds are recognized by unknown receptors and trigger defensive responses. 5. Putative herbivore-associated molecular patterns (HAMPs) are recognized by receptors and lead to herbivore-triggered immunity (HTI). 6. Wounding leads to the release of DAMPs and to wound-induced resistance (WIR). 7. Effector-like molecules from insects can suppress HTI and WIR. Uncharacterized elements are indicated by dashed lines.

Actual herbivory disrupts the integrity of plant tissue, an event that can hardly be ignored by plants. Many plant defense responses can be triggered by mechanical wounding alone [13-15], leading to wound-induced resistance (WIR). Extensive studies

of the wound response in model plants such as tomato and Arabidopsis have identified plant-derived compounds that trigger anti-insect defense responses. Such compounds are potentially recognized by PRRs and thus can be defined conceptually as DAMPs [16]. Among the DAMPs shown to activate anti-insect defenses in tomato are cell wall-derived oligosaccharides and the peptide signal systemin [17]. These studies lend support to the idea that many plant defense responses against herbivores are mediated by recognition of the plant's "damaged self" [16, 18]. Analogous to danger signal models in the vertebrate immune system, wound trauma inflicted to plant tissue is likely to disrupt intracellular compartmentalization in ways that lead to the production of molecules that trigger general plant immune responses.

It is becoming clear that a second layer of perception in addition to WIR can provide the plants with the capacity to detect herbivores more specifically: Plants seem to recognize compounds that are released by herbivores during feeding. Extensive genetic analysis of the Hessian fly-wheat interaction [19, 20] and the cloning of receptor-like resistance (*R)* genes provide solid evidence for a high degree of specificity of perception in this case [21-23]. The recognition systems for hemipteran and dipteran parasites, together with the identification of possible effectors (Box 1), appear to conform to the general PTI/ETI theory [24]. Less is known about the mechanisms by which plants perceive chewing insects such as beetles and caterpillars, which constitute the vast majority of plant herbivore species. Numerous studies have shown that insect oral secretions, when applied to artificial wound sites, amplify the plant's wound response [25-28]. Identified elicitors include fatty acid-amino acid conjugates (FACs), sulfur-containing fatty acids (califerins), peptides from digested plant proteins, and lipases [29-33]. The eliciting activity of this diverse group of compounds suggests that they can be conceptually classified as herbivore-associated molecular patterns (HAMPs), which presumably are recognized by PRRs at the cell surface [34, 35] to trigger HAMP-induced immunity (HTI). Despite the fact that HAMP receptors have not been identified, several trends have emerged concerning the specificity of elicitor-mediated recognition of chewing herbivores by plants. First, herbivore-derived elicitors boost the amplitude of wound-induced defense responses [29, 30, 32, 33, 36]. Second, different herbivore species produce qualitatively and quantitatively different elicitor combinations [37, 38].

Third, the activity of the different known elicitors varies between plant species [36]. Taken together, these observations suggest the potential for elicitor-mediated, species-specific recognition of chewing insects by plants.

Plant hormone regulatory networks integrate different herbivore recognition cues

Following the recognition of an attacker, plants employ different signaling cascades to reprogram their phenotype. Extended PTI/ETI models in plant-pathogen interactions suggest that although the recognition of pathogens can be very specific, plants have a "common downstream signaling machinery" [39] that is activated upon recognition of many different attackers. To what extent is this paradigm valid for plant-insect interactions? The wound hormone JA-Ile is widely considered to be a master regulator of plant resistance to arthropod herbivores as well as various pathogens [14, 16, 40-44], and may therefore represent the core signaling pathway for activating resistance to insects. Disruption of plant tissue integrity during insect feeding triggers the production of JA-Ile and activation of a well-defined signal transduction chain leading to transcriptional activation of defense responses. Thus, it is the defining feature of most, if not all herbivores, namely the need to obtain nutrition from plant tissues, which betrays the presence of the attacker to the host. A major unresolved question is the extent to which herbivore-induced production of JA-Ile is promoted by plant-derived "self" (DAMPs) *versus* herbivore-derived "non-self" (HAMPs) signals [18]. The fact that mechanical wounding is sufficient to trigger robust local and systemic increases in JA-Ile levels within minutes of leaf injury indicates that herbivore-associated cues are not strictly required to activate the response [16, 45, 46]. However, the severity of crushing-type wounds typically used in these studies may bypass a requirement for HAMPs in the elicitation of herbivore-induced responses. Research aimed at identifying herbivore-derived elicitors has therefore relied on the application of insect oral secretions to wound sites created by mild wounding regimens that do not elicit a strong defense response in the absence of oral secretion [34, 35, 47-49].

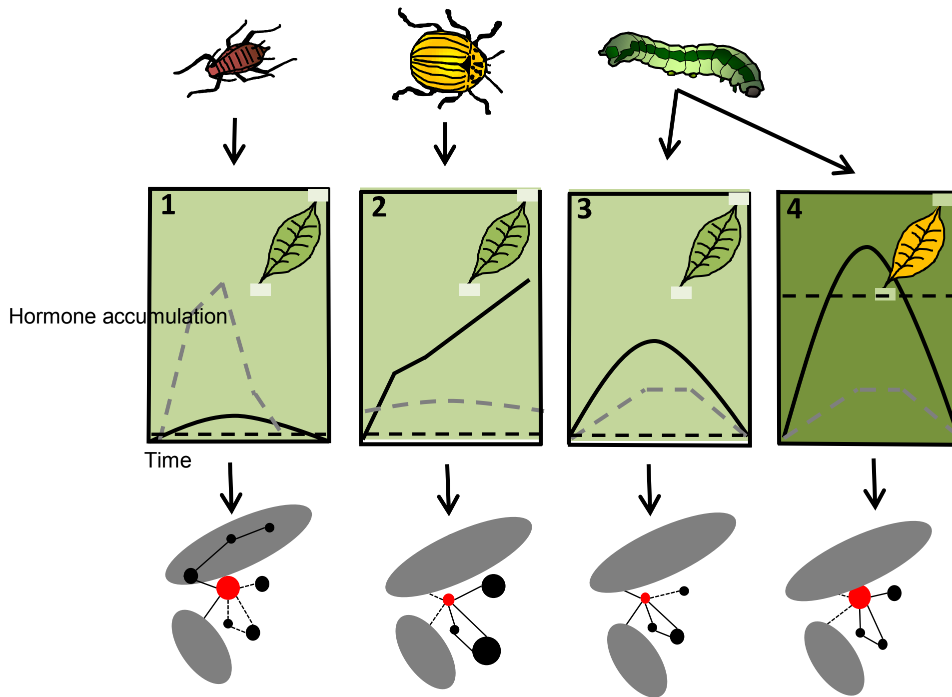

Figure 2: Herbivore perception triggers tissue-specific hormone responses. A conceptual model of how the perception of different herbivore species by the same plant can induce differential changes in the levels of hormones (solid and dashed, black and grey lines, 1,2,3) is shown. Hypothetical hormone responses during perception of the same herbivore (3 and 4) are altered by tissue-specific differences (indicated by green and yellow leaves) in the basal levels of other hormones (dotted black lines in 3 and 4). The integration of spatiotemporal changes of hormone signaling into the downstream transcriptional network can lead to herbivore- and tissue- specific responses.

We argue here that the JA pathway represents a conserved core-signaling machinery that is activated by both non-specific and specific recognition patterns following herbivore attack. But how do plants fine tune their defense machinery to appropriately mount herbivore-specific responses beyond jasmonates? We propose two potential answers to this question. First, plants may use JA-independent, parallel pathways to create distinct response patterns. Second, specificity may be mediated through the activation of spatio-temporal modulators of the JA-response (Figure 2). Evidence for the first concept comes from studies on the recognition and response system of tomato to the aphid *Macrosiphum euphorbiae. Mi-1*, a putative receptor,

triggers salicylic acid (SA)-mediated signaling [50] and resistance independently of the JA pathway [51]. Plant recognition of and response to many other hemipterans seems to follow a similar pattern [52], thereby providing evidence that plants use JA-independent hormone response pathways to achieve specific resistance against phloem feeders.

Most herbivores, however, inflict much greater cell damage than phloem feeders, and will activate JA signaling and resistance. In this case, specificity may be achieved via cross-talk with other hormones (Figure 3). Indeed, JA-induced changes in gene expression typically depend on the context in which the hormone is perceived [53,54]. The best studied hormones that alter JA-mediated defense responses and herbivore resistance are salicylic acid (SA) and ethylene (ET). In general, SA antagonizes JA-induced resistance, whereas ET can have both positive and negative effects. A number of current studies show that SA and ET are specifically modulated by different herbivore elicitors [28, 55], and may thereby provide a degree of hormone-mediated specificity. SA-JA-ET crosstalk has been reviewed in detail elsewhere (see also this special issue). On the other hand, abscisic acid (ABA), auxins, gibberellins (GB), cytokinins (CK) and brassinosteroids (BR) have received less attention as potential factors that regulate herbivore resistance. The following discussion highlights examples from the recent literature indicating an important role of these hormones in mediating specificity in herbivory-induced defense responses.

Absisic acid

Abscisic acid (ABA) levels in maize are increase during attack by the specialist root herbivore *Diabrotica virgifera virgifera*, but not by mechanical wounding alone [26, 56] and in Arabidopsis after induction with wounding and oral secretions of the generalist herbivore *Schistocerca gregaria* [33]. ABA levels also increased in a goldenrod species (*Solidago altissima*) after induction by *Heliothis virescens*, but not by the gall-inducing insect *Gnorimoschema gallaesolidaginis* [57]. ABA synthesis and signaling affect herbivore-induced transcript levels and JA biosynthesis in Arabidopsis [58, 59]. Furthermore, ABA regulates JA-inducible defense responses in maize [26] and ABA-deficient plants show decreased resistance to herbivores in tomato [60]. ABA, together with JA, synergistically induces MYC2-dependent gene expression during wound

responses, thereby modulating the primary JA response [61-63]. JA also regulates the expression of the PYR/PYL/RCAR family ABA receptor protein *NtPYL4* in tobacco, thereby affecting ABA-induced levels of root alkaloids [64]. The same study demonstrated that *AtPYL4* and *AtPYL5* mutants in Arabidopsis are more sensitive to JA-induced growth inhibition and less sensitive to JA-induced anthocyanin accumulation. Although the molecular mechanisms behind ABA-JA crosstalk are still elusive, recent findings suggest that both pathways share similar regulatory proteins. The co-repressor TOPLESS (TPL) interacts with EAR-motif (ethylene-responsive element binding factor-associated amphiphilic repression) proteins to repress transcription [65]. The EAR-motif protein NINJA (Novel Interactor of JAZ) connects TPL to the JAZ complex, thereby mediating repression of genes demarcated by JAZ-bound transcription factors such as MYC2. TPL also interacts with NINJA-related proteins that are part of a complex that mediates ABA-induced degradation of negative transcriptional regulators [66]. Taken together, these findings indicate that ABA and JA are tightly interconnected and that regulation of ABA levels in response to herbivory can modulate JA-driven defense responses (Figure 3). However, because ABA is also an important signal for responses to desiccation, an effect which accompanies herbivore attack in many cases, it remains to be determined to what extent this stress hormone is involved in recognition-mediated responses to insect feeding. The fact that the application of *Spodoptera littoralis* regurgitant to Arabidopsis can reduce wound-induced stomatal closure and water loss [67], whereas *S. gregaria* oral secretions induce ABA levels [33], indeed suggests specific elicitor-mediated regulation of this hormone.

Auxin

Levels of the auxin indole-3-acetic acid (IAA) are elevated in plants attacked by gall-feeding insects [57, 68]. In contrast, IAA levels in *N. attenuata* leaves are reduced within three days after simulated herbivory [69]. It is known that plant resistance to pathogens can be modulated through changes in auxin sensitivity. For example, the perception of the bacterial elicitor flagellin decreases auxin sensitivity, thereby elevating resistance to *P. syringae* [70]. Concomitantly, *P. syringae* suppresses host defense by promoting auxin production via delivery of effectors into the plant cell [71, 72]. Also, treatments with synthetic auxin directly suppress SA-induced defense responses [73],

which can be linked to SA-mediated resistance to phloem-feeding insects [50]. Whether insects prone to SA-mediated defenses can alter auxin homeostasis or signaling to suppress host defense is not known. In addition to regulation of SA signaling, recent studies suggest an intimate molecular interplay between auxin and JA signaling: Auxin formation in Arabidopsis roots is enhanced by JA-mediated induction of genes involved in auxin biosynthesis and transport [63, 74]. In *N. attenuata* leaves, JA negatively regulates wound-induced decreases in auxin content [69], demonstrating that the effects of JA on auxin biosynthesis are tissue and possibly also species specific [53]. Importantly, these data suggest that herbivore-induced JA levels might affect auxin homeostasis. Conversely, there is evidence to indicate that auxin enhances JA biosynthesis and signaling [75-77]. JAZ1 and MYC2 are co-regulated by auxin and JA, demonstrating the potential for crosstalk between both hormones [76, 77]. Analogous to ABA signaling, EAR-motif containing AUX/IAA proteins, which are negative regulators of auxin-induced responses, also interact with TPL [66], suggesting that TPL acts as integrator of multiple hormone pathways. Another protein that links auxin and JA responses is SGT1 (suppressor of G-two allele of SKP1), which connects chaperone-mediated protein assembly and ubiquitin-mediated protein degradation. *SGT1* mutants of Arabidopsis are compromised in their sensitivity to both auxin and JA [78], and silencing *SGT1* in *N. attenuata* attenuates JA levels, defense metabolite accumulation, and resistance to *M. sexta* [79]. Auxin can also regulate plant defense responses independently of SA and JA [80, 81]. These findings demonstrate that auxin is a potent modifier of herbivore-relevant defense responses and indicate that plants may modulate auxin levels to mediate attacker specificity.

Gibberellins

Recent studies with plants altered in gibberellin (GB) signaling suggest a role for GB in herbivore-induced defense responses. DELLA proteins are negative transcriptional regulators of gibberellic acid (GA)-induced gene expression and are considered to play key roles in integrating plant responses to diverse developmental and environmental stimuli [82]. Remarkably, GAs affect JA signaling through competitive binding of DELLAs to the JAZ proteins, therefore preventing JAZ-MYC2 interaction and promoting MYC2-induced transcriptional responses [83]. GA perception leads to

degradation of DELLAs, which ultimately leads to inhibition of MYC2 and diminished JA responses. Accordingly, alteration of DELLA levels affects JA biosynthesis and signaling [84, 85].

Cytokinins

In *N. attenuata,* cytokinin (CK)-related transcripts are among the genes that are most strongly regulated by FAC elicitors [86, 87], suggesting a role of CK in the hormonal regulatory network. In addition, gall-forming insects and possibly some leaf miners regulate plant CK levels, presumably to maintain the sink status of the infected tissues [57, 68, 88]. Isopentenyltransferases (IPT) represent the rate-limiting step in CK biosynthesis, and IPT overexpression increases resistance of common tobacco to the lepidopteran herbivore *Manduca sexta* [89]. Several lines of evidence also support an important role for CK in activation of JA biosynthesis. Transgenic tobacco plants that overexpress a small GTP-binding protein accumulate high levels of CK, resulting in increased rates of JA production after wounding, a response that can be mimicked by long term CK treatments [90]. Additionally, CK treatments of hybrid poplar leaves increase the wound-induced JA burst and the expression of genes involved in JA biosynthesis [91]. The same study shows that wounding and CK treatments of sink, but not source leaves, impairs Gypsy moth larval performance, suggesting that CK-mediated resistance to insects depends on leaf ontogeny. CK levels in leaves are thought to be regulated by ontogenic constraints because the hormone accumulates to high levels in younger leaves, whereas reduced CK levels promote leaf senescence [92, 93]. Because CKs regulate herbivore-induced defenses, the CK status of a given tissue might determine the intensity of the defense response of that particular tissue after perception of herbivory, and thus contribute to tissue-specific responses in herbivory-induced signaling [94] (Figure 2).

Brassinosteroids

Recent findings also suggest important roles for brassinosteroids (BR) in herbivore resistance. BRs antagonize JA-mediated trichome density and defense metabolite accumulation in tomato [95]. BRs are also known to repress JA-governed inhibition of root growth [96]. BR are perceived by BR insensitive 1 (BRI1), a leucine-rich

repeat receptor-like kinase [97, 98]. BRI1-associated Kinase 1 (BAK1) interacts with BRI1 and plays an essential role in BR signaling [99]. Apart from BR signaling, BAK1 also interacts with the flagellin receptor FLS2 and is required for multiple PAMP-elicited responses [100]. Silencing BAK1 in *N. attenuata* reduces wound- and herbivory-induced JA and JA-Ile levels and JA-induced trypsin proteinase inhibitor (TPI) activitiy [101]. Whether or not BAK regulates HAMP or DAMP perception to modulate JA levels, or whether the effects in BAK1-silenced plants are due to changes in BR perception requires further analysis [101].

Taken together, these examples illustrate the many possibilities plants have to modulate the JA-pathway in order to achieve specific responses. However, apart from JA/SA crosstalk (see other review in this special issue), clear examples of specific induction of JA-modulators following herbivore recognition remain scarce. The identification of herbivore receptors followed by in-depth analysis of their downstream targets will help to fill this gap of knowledge.

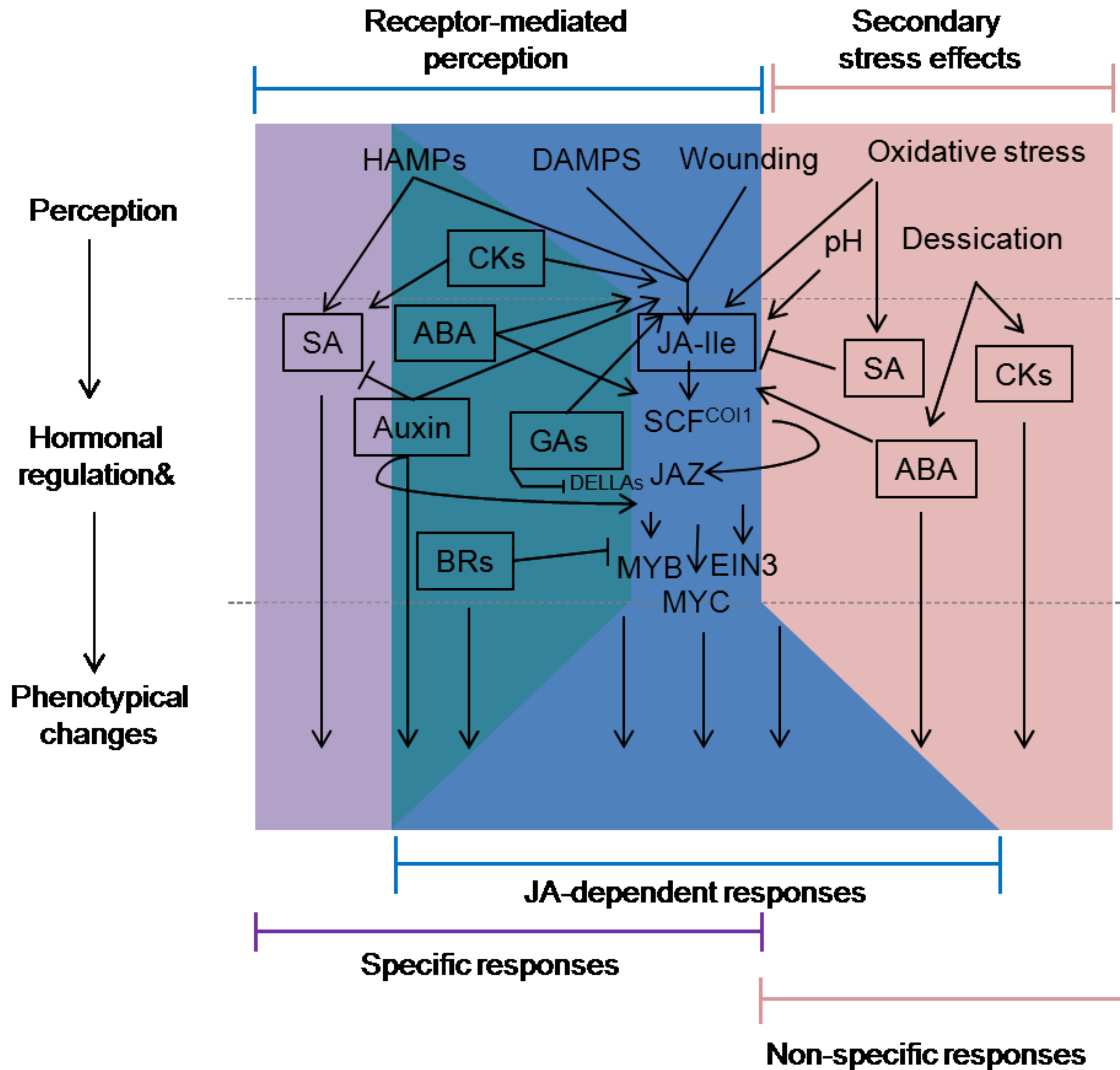

Figure 3: The jasmonate core pathway and its modulating factors. A conceptual, non-exhaustive overview is presented. General and specific herbivore-associated patterns, including HAMPs, DAMPs and wounding activate the jasmonate pathway (blue area). Increased accumulation of JA-Ile promotes interaction of JAZ proteins with the SCF ubiquitin ligase SCF^{COI1}. Ubiquitin-dependent degradation of JAZs by the 26S proteasome releases transcription factors from their JAZ-bound repressed state, thereby activating the expression of transcriptional regulons that promote defense and inhibit vegetative growth. JA-independent hormonal pathways are also induced (purple area), and a number of hormones, including salicylic acid (SA), ethylene (ET), auxin, gibberellins (GA), cytokinins (CK) and brassinosteroids (BR) modulate JA metabolism and signaling (light blue are). Herbivory also leads to oxidative stress, changes in intracellular pH and dessication, which modulate the JA pathway either directly or indirectly through other hormones. Together, this leads to complex phenotypic changes that comprise both specific and general responses, the majority of which can be linked back to the jasmonate pathway.

Do recognition-induced plant hormone networks trigger specific and appropriate defense responses?

The recognition systems used by plants to perceive herbivore attack are integrated with hormone response pathways that reprogram the plant. But what is the evidence that the resulting responses are a) specific and b) appropriate (see Glossary) for defense against the attacking herbivore?

Again, for hymenopterans, compelling examples of gene-for-gene resistance link specific recognition to both specific and appropriate responses [52]. In the case of chewing herbivores, many studies demonstrate differential responses of plants to different insect species as well [102-105], but clear evidence that these responses provide specific resistance are rare. Given the complexity of host transcriptional responses to herbivory, some of these differences may be attributed to a variety of experimental factors that influence the response. Plant growth conditions, plant and insect developmental stage, herbivore density and treatment duration are among the parameters that are expected to have major effects on transcript profiles. Also, in some cases, different insects from the same feeding guild elicit similar or converging responses via the general JA-signaling cassette [106-109]. The question thus arises whether from an adaptive point of view, plants benefit from tailoring their response to different chewing herbivores, or whether a generalized response following recognition is the most pertinent strategy?

Induced resistance to a broad spectrum of insects may be analogous to the scorched earth military strategy aimed at denying resources to potential enemies - regardless of who the enemy is. There is good evidence to indicate, however, that among the multitude of defenses induced by one herbivore species, specific responses target specific types of herbivores, even within the same feeding guild. One example comes from work on the JA-regulated defensive enzyme threonine deaminase (TD2), which degrades the essential amino acid Thr in the lepidopteran gut [110]. Although TD2 expression in tomato leaves is induced in response to attack by both caterpillars and beetles, the defensive activity of the enzyme is activated in the gut of lepidopteran but

not coleopteran herbivores [111]. Levels of the JA-regulated non-protein amino acid N-acetylornithine in Arabidopsis increases in response to feeding by caterpillars and aphids, but this defense appears to target only the latter herbivore [44]. Overall, generalist and specialist herbivores may be susceptible to different types of defenses (see other articles in this special issue), and plants may benefit from detecting highly adapted herbivores and adjusting their regulation of quantitative and qualitative direct and indirect defenses. Clearly, further research is required to disentangle whether differential responses of plants to chewing herbivores are truly specific, and whether plants have evolved to tailor their response to different chewing attackers.

Conclusions and future directions: Piecing together the recognition-response puzzle

A recurring theme in all spheres of plant-herbivore biology is the ability of each partner to perceive and respond to chemical cues generated by the other partner; this exchange of information provides an excellent focal point for elucidating basic chemical and molecular principles of plant-herbivore interactions. Understanding the mechanisms behind perception and response may also inform studies about their evolutionary history. In contrast to well-established models describing the evolution of plant-pathogen interactions [112], our understanding of how molecular recognition and response systems shape plant-herbivore relationships is still in its infancy, and many important questions remain unanswered (see “outstanding questions”). Nevertheless, the current literature supports several general conclusions about specificity in plant-herbivore interactions. First, plants perceive different arthropods by integrating various environmental cues, ranging from mechanostimulation by walking insects to the contact with salivary components during feeding. Second, the perception of herbivores triggers regulatory responses that include different phytohormones, with the JA pathway playing a dominant role in host resistance. Third, although the core JA signaling module is highly conserved, it is becoming increasingly clear that multiple hormone response pathways interact to translate initial perception events into appropriate responses that increase plant fitness in the presence of hostile aggressors.

Outstanding questions:

1. Which receptors are involved in plant perception of herbivores?
2. How prevalent are herbivore effectors, and how do they act to suppress the plant immune system?
3. Are herbivore effectors recognized by plant *R* genes in accordance with the ETI/PTI model in plant-pathogen interactions?
4. How do plants integrate information derived from multiple herbivore- and plant-derived cues?
5. Which JA-independent processes mediate specific plant responses to herbivore attack?
6. What is the precise role of growth hormones (gibberellins, cytokinins, auxin and brassinosteroids) in modulating plant immunity to herbivores?
7. Is the recognition of a specific chewing herbivore translated into a distinct and appropriate defense response?

Box 1: Herbivore effectors

Just as plants recognize a variety of herbivore-derived cues, there is evidence that herbivores may use effector molecules to suppress plant defenses:

- Oviposition fluids trigger the SA pathway in Arabidopsis, which increases the growth of *S. littoralis* larvae [113, 114]. Also, oviposition can suppress herbivore-induced plant volatiles in maize [115].
- During feeding, insects secrete effector-like compounds to suppress plant immunity. The best known example is glucose oxidase produced by the salivary glands of lepidopteran insects [25]. Aphids also produce effector-like compounds [116], and other examples of herbivore-mediated suppression of plant defenses via yet unknown mechanisms have been documented [35, 67].
- Herbivores may produce plant hormones or hormone mimics to manipulate the host defense responses [117].
- Insect herbivores are hosts to microorganisms (e.g., endosymbionts) and surface dwelling parasites that produce compounds that potentially interfere or otherwise affect plant immunity [35]. For example, a recent study suggests that *Wolbachia* endosymbionts suppress the induction of maize genes involved in defense against the root feeder *Diabrotica virgifera* [118]. Bacterial symbionts are also involved in the production of cytokinins that are secreted by leaf-miners to inhibit leaf senescence [119].

The PTI/ETI paradigm indicates that plants have evolved various ways to recognize and respond defensively to pathogen effectors. Have plants acquired the capacity to recognize insect effectors as well? Although emerging evidence indicates that this may indeed the case for hymenopterans [120] ligands and receptors that constitute this form of recognition have yet to be identified and characterized [116]. Future research focusing on the identification of insect effectors and their mechanism of action will likely mark a new phase in plant-interaction research.

Glossary Box

Specificity of recognition: The extent to which a plant can discriminate the presence and/or attack by different herbivores. Specific recognition of arthropod herbivores can occur at different levels, ranging from phyla (i.e. distinct detection of arthropods compared to vertebrates) to species (i.e. distinct detection of two different herbivore species). Currently, little known about the molecular mechanisms underlying plant recognition of herbivores, but generalized examples based on the PTI/ETI paradigm are informative. For example, a high degree of specificity in recognition could be achieved by R gene products that evolved to recognize effector molecules in adapted insects. Low-level specificity may involve the action of mechanosensors that detect insect movement on the leaf surface. Receptor-mediated recognition of HAMPs and/or DAMPs produced at the site of insect feeding is expected to provide an intermediate level of specificity because these signals are common in plant interactions with multiple insect species.

Specificity of response: The extent to which plant physiological and/or metabolic changes elicited by specific perception of a given herbivore are distinct from changes elicited by the perception of other attackers. Unlike the adaptive immune system in animals that creates an immunological memory of a specific invading pathogen, the recognition of many insects (e.g. chewing herbivores) are channeled into a general defense response. Many measured differences in responses are not based on specific perception, but are likely to be artifacts of secondary stress factors. These responses are referred to as “distinct” or “different”, but not “specific”. Examples of specific responses are the different phenotypical changes triggered by different putative aphid receptors [121].

Appropriate response: A phenotypical change following herbivory that provides a benefit to the plant. This benefit may be realized either by increasing resistance and fending off the attacker, or by changing the primary metabolism to enable a more effective regrowth after attack. Appropriate responses are not necessarily based on specific recognition and specific metabolic changes, as plants may use general mechanisms to defend themselves against a variety of attackers. Different chewing herbivores for example are likely to be susceptible to the same defensive mechanisms.

On the other hand, phloem feeders may require different measures of protection, as they feed on specialized cells only. From an adaptive point of view, truly specific responses can be expected to be appropriate, as this is a prerequisite for their evolution.

Hormone crosstalk: A phenomenon in which a signal transmitted through one hormone pathway stimulates or represses signal output (e.g., a physiological or defense-related response) from another signaling pathway. Interactions between the hormone signals may be direct or indirect (see below).

Direct crosstalk: A phenomenon in which two or more hormone pathways either share a common signaling component (e.g., the use of TPL by both the JA and auxin pathways), or contain components that physically interact to modify signal output from the pathways involved (e.g., JAZ-DELLA interaction). The biological significance of direct crosstalk in shaping the outcome of plant-insect interactions remains to be demonstrated.

Indirect crosstalk: A common phenomenon in which two or more hormone pathways are integrated at the hormone response gene-network level rather than at the upstream level of signal transduction. One example is the JA-induced expression of *NtPYL4*, which affects the ability of ABA to regulate alkaloid production in tobacco.

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3. Discussion

When I started my PhD, only little was known about mechanisms behind herbivore perception and signaling, leading to downstream activation of hormonal pathways and defense responses. The research on herbivory-induced signaling was mainly inspired by studies focusing on plant-pathogen interactions. One of these examples where signaling mediators from plant-pathogen studies were also found to be important in herbivore-induced signaling were MAPKs. Plant perception of pathogens often involves the activation of MAPKs (reviewed in Rodriguez *et al.*, 2010). SIPK, WIPK and their homologues in other plant species, e.g. MPK6 and MPK3 in *A. thaliana*, are the most thoroughly studied MAPKs in plants. They are activated by pathogen-derived elicitors like flagellin (Asai *et al.*, 2002; Bardwell *et al.*, 2004), by tobacco mosaic virus (TMV) in *N. tabacum* plants that express the N-resistance gene (Zhang et al, 1998), by the fungal Avr9 effector in tobacco cells that expresses the Cf-9 resistance gene (Romeis *et al.*, 1999), and play roles in the interaction of Arabidopsis with growth-promoting fungi (Vadassery *et al.*, 2009). Additionally, they function in various plant hormone signaling pathways (Yoo *et al.*, 2008; Bethke *et al.*, 2009). SA and JA were shown to directly activate SIPK and WIPK and their homologues, and AtMPK6 has been proposed to play a role in jasmonate signaling (Zhang *et al.*, 1997; Takahashi *et al.*, 2007). Recently, it was demonstrated that SIPK and WIPK regulate wound- and herbivory-induced levels of JA and ethylene (Wu *et al.*, 2007).

However, evidence that SIPK or WIPK affect plants' vulnerability to biotic stresses in their natural environments was missing. We showed that silencing SIPK and WIPK greatly diminished transcriptional activation of major defensive pathways and the accumulation of important anti-herbivore metabolites, as was suggested by the impaired ability of SIPK- and WIPK-deficient plants to induce JA biosynthesis after herbivory (Wu *et al*. 2007). It was therefore surprising that silencing these kinases did not result in increased susceptibility of *N. attenuata* to herbivores. The number of plant metabolites known to affect oviposition or growth of phytophagous insects is steadily increasing. An important role for VOCs for insect attraction and performance is emerging (Dicke and Baldwin, 2010). Both kinase-silenced plant lines have greatly reduced expression of genes involved in VOC and GLV biosynthesis; consistently they emitted less VOCs,

especially GLVs, during continuous herbivore feeding. Supplementing GLVs increased consumption and weight gain of *M. sexta* on SIPK- and WIPK-deficient plants, suggesting that reduced GLV emission could "mask" the defenseless status of these plants. We tested this hypothesis by crossing asLOX3 (reduced defense) with irHPL (reduced GLVs). Although this cross showed reduced accumulation of defensive metabolites, it was significantly more resistant to *M. sexta* than asLOX3 plants. These data demonstrate that manipulating plant volatile emissions could be used to increase resistance of vulnerable plants to herbivorous insects. Our study suggests that applying this strategy to crop plants, which have lost defensive metabolites during domestication, could help to increase their resistance to herbivorous insects. However, this work also raises the question of why plants emit high levels of GLVs, if this increases their apparency to herbivores? One possible answer to this question is that GLVs can simultaneously increase plants' resistance to herbivores in nature by attracting natural enemies and therefore serve as indirect defenses. Insect predators like *Cotesia glomerata* use GLVs as detection cues to find plants infested by herbivores; *A. thaliana* plants that emit higher levels of GLVs, due to sense expression At*HPL*, were more attractive to *C. glomerata* than to WT plants (Shiojiri *et al.*, 2006). In *N. attenuata* the generalist predator *G. pallens* is attracted to GLVs emitted from herbivore-attacked *N. attenuata* plants and GLV deficient plants were less attractive to *G. pallens* (Halitschke *et al.*, 2007). Recently, it was also shown that GLVs, especially *cis*-3-hexenol, can be rapidly isomerized by *M.* sexta oral secretions. The isomerized forms attract *G. pallens*, which leads to increased predation rates of *M. sexta* in nature (Allmann and Baldwin, 2010). Additionally, some GLVs are repellant for ovipositing *M. sexta* moths (Kessler and Baldwin, 2001). Thus, by attracting natural enemies and repelling ovipositing moths through herbivory-induced GLV release, plants can efficiently decrease their herbivore load. GLVs are also implicated in plant resistance to pathogens; for example, *trans*-2-hexenal, a GLV that is commonly emitted after herbivore wounding (De Moraes *et al.*, 2001), was shown to have bactericidal effects (Croft *et al.*, 1993). Additionally, GLVs are implicated as signaling molecules. *Cis*-3-hexenyl acetate was also shown to prime defense responses in poplar (Frost *et al*., 2008). These data may explain why GLV emissions are still retained, even if they increase consumption rates by herbivores. It is tempting to speculate that phytophagous insects respond to high GLV levels with

increased food intake in order to more quickly outgrow their vulnerable phase and thus compensate for a higher risk of detection by predators. If this scenario holds true, why do then insect larvae like *M. sexta* do not consume more plant material, even in the absence of higher GLV levels? Obviously growing faster might have its trade-offs. Analyzing fitness of these “fast-feed” caterpillars would help to answer these questions.

From data in Manuscript I it becomes clear that SIPK and WIPK are important regulators of VOC emissions, most likely by transcriptional regulation of VOC biosynthetic genes. It would be interesting to test whether SIPK or WIPK regulate VOC biosynthesis in a post-transcriptional fashion, as has been reported for the biosynthesis of ethylene, where a homologue of SIPK in Arabidopsis directly phosphorylates the enzyme that regulates the rate limiting step in ethylene biosynthesis and therefore inhibits its turnover, leading to increased ethylene emissions (Joo *et al.*, 2008). Further studies are required to fully understand the function of NaSIPK and NaWIPK in regulating metabolic cascades and resistance during stress conditions like herbivory and pathogen attack.

Beside MAPKs, many other regulators of plant defense responses have been identified first in studies analyzing resistance to pathogens. Among them, SGT1, a conserved eukaryotic protein, was found to regulate immune responses in plants and humans (Mayor *et al.*, 2007; Shirasu, 2009). In plants, SGT1 interacts with HSP90 and RAR1 to mediate stability of NB-LRR-type R proteins, therefore ensuring plants’ ability to detect and resist pathogens (Lu *et al.*, 2003; Boter *et al.*, 2007). Plant resistance to herbivores is often increased when insect-derived elicitors are perceived by the plant (reviewed in Wu and Baldwin, 2009). However, the perception mechanism of phytophagous herbivores by plants is not well resolved. We tested whether SGT1 is important for regulation of defense responses in *N. attenuata* that are induced by perception of FACs derived from the insects’ oral secretions of *M. sexta*. Silencing SGT1 in *N. attenuata* greatly diminished JA levels after induction with wounding and application of *M. sexta* OS. These results show that SGT1 is important for early responses triggered by wounding and *M. sexta* OS treatment. Within minutes after treatment with wounding and FACs, SIPK and WIPK are activated in *N. attenuata* and regulate JA biosynthesis after wounding and herbivory (Wu *et al.*, 2007). However, we

found that silencing SGT1 did not alter the activation of SIPK and WIPK, suggesting that FAC perception *per se* is not affected by SGT1. This raises the question of how SGT1 modulates JA biosynthesis. Early precursors in the JA biosynthetic pathway, especially OPDA, were greatly reduced in SGT1-deficient plants. However, these plants did not show reduced levels of transcript accumulation of genes important for JA biosynthesis, indicating that post-transcriptional mechanisms are involved. Since SGT1 assembles with protein chaperones, it might be possible that protein stability of JA biosynthetic enzymes is impaired in plants lacking SGT1. Studies of the stability of proteins required for JA biosynthesis are needed to elucidate the function of SGT1 in this process.

Turnover of proteins involved in plant immunity via ubiquitinylation plays an important role in regulating plant defense responses (Trujillo and Shirasu, 2010). The ubiquitinylation machinery consists of activating (E1), conjugating (E2), and ligating (E3) enzymes (Vierstra, 2009). Attention has centered on ubiquitin ligases (E3s) because they specify the target protein (substrate) that is selected for degradation. Jasmonate perception requires the F-Box protein COI1 that is part of the SCF ubiquitin ligase complex targeting JAZ proteins, negative transcriptional regulators of jasmonate-responsive gene expression (Thines *et al.,* 2007; Chini *et al.*, 2007). In all eukaryotes studied so far, SGT1 interacts with SCF-ubiquitin ligase complexes (Kitagawa *et al.*, 1999; Azevedo *et al.*, 2002; Liu *et al.*, 2002). Consistently, *sgt1b* mutants of Arabidopsis are altered in their response to auxin, which is perceived by another E3 ligase complex containing TIR1; *sgt1b* mutants were also partially suppressed in their response to JA in a root growth inhibition assay (Gray *et al.,* 2003). While our manuscript was in preparation, another report demonstrated that SGT1 is important for the defense response triggered by *Pseudomonas syringae* pv. *tomato* in Arabidopsis and tomato (Uppalapati *et al.,* 2010). *P. syringae* pv. *tomato* produces the JA-Ile homologue coronatine which binds to COI1 and triggers necrotic lesions surrounded by chlorosis. Arabidopsis *sgt1b* mutants and tomato plants silenced in SGT1 show reduction of disease-associated symptoms (cell death and chlorosis), suggesting a molecular connection between COR/COI1 and SGT.

We tested whether silencing SGT1 impairs jasmonate perception in *N. attenuata* by analyzing the transcriptional responses of leaves treated with different concentrations

of methyl jasmonate (MeJA). In line with the results of Gray *et al.* (2003) and Uppalapati *et al.* (2010), we found that SGT1-deficient plants were impaired in their accumulation of some jasmonate-responsive gene transcripts (NaTD, NaLOX2). However, other transcripts did not show differential accumulation (NaHPL), while transcript levels of some genes (NaTPI, NaDOX) were induced to higher transcript levels in SGT1 silenced plants when compared to empty vector controls. Since induction of all these genes is regulated by COI1, we propose that silencing SGT1 modulates COI1 activity. These data show that silencing SGT1 alters the jasmonate response in *N. attenuata*. Whether SGT1 directly interacts with the COI1 complex, and therefore modulates the degradation of specific JAZ proteins that may regulate different JA responsive genes, is an interesting question for future research.

As mentioned previously, SGT1 regulates the stability of proteins involved in plant immunity. We tested whether we could find signatures of such regulation by displaying protein patterns using 2D gelelectrophoresis from leaves of SGT1-silencend plants compared to EV plants. However, we found only very few changes, including accumulation of PR proteins and reduced levels of certain chaperones. Likely because proteins involved in signaling and stress perception may occur at low levels, we were not able to find them in our 2D approach. Removing RubisCO (Ribulose-1,5-bisphosphat-carboxylase/-oxygenase), the most abundant protein in plant protein extracts, might increase the relative amounts of proteins involved in plant signaling and increase the likelihood of finding proteins regulated by SGT1. Further studies are also required to show whether the accumulation of PR proteins in SGT1-silenced plants is a result of higher basal SA levels, e.g. using the NahG background which efficiently reduces free SA levels. The importance of the reduced levels of the two chaperones is unclear, since the differential accumulation cannot be entirely explained by their transcriptional levels. Interestingly, one of these chaperones was recently identified in a proteomic study as highly regulated by *M. sexta* feeding and silencing this chaperone decreased *N. attenuata's* resistance to *M. sexta* (Mitra *et al.,* 2008). It is likely that this chaperone influences the stability of proteins that play roles in resistance against herbivores. Future studies designed to analyze their involvement in the defense response of *N. attenuata* after herbivore attack are needed to anwser these questions. In summary, the data in

Manuscript II show that SGT1 is an important player in plant defense responses against herbivores, and our results provide inspiration for future experiments to dissect the functions of SGT1.

Although Arabidosis is the gold standard for studying perception of pathogens, early signaling events involved in herbivore perception in *A. thaliana* are poorly understood. The reason for this is likely the lack of herbivore derived elicitors that activate defense responses in Arabidpsis. In 2009, Schmelz and colleagues demonstrated that a previously identified elicitor from American grasshopper species, named Caeliferin A16-0, is a potent elicitor of JA and Ethylene emissions when applied to wounded Arabidopsis leaves (Ahlborn *et al*., 2007; Schmelz *et al*., 2009). We also identified Caeliferin A16-0 in OS of European and African grasshopper species and when applied to wounded Arabidopsis leaves, these OSs induced a whealth of physiological responses (manuscript IV). However we were unable to induce any of these responses using synthetic Caeliferin A16-0. Due to its stereochemical properties, this elicitor can occur in different conformations, and it is not clear yet which isoform represents the active elicitor. Similar cases have been reported for JA-Ile, where only the (3R,7S) JA-Ile is the active form that binds to the COI1-JAZ complex (Fonseca *et al*., 2009). We used synthetic Caeliferin A16-0, which, although it should consist of racemeric mixture, might have a conformation that was not perceived by the plant when it was applied to wounded leaves. Experiments using purified stereoisomers might help to clarify the discrepancies between our data and the results presented by Schmelz and colleagues. Instead of Caeliferin A16-0, we identified that lipase activity derived from OS of grasshoppers releases membrane-bound OPDA and activates OPDA-induced transcripts. All OSs from insects that we tested, released high amounts of OPDA when applied to wounded leaves. These results are not surprising, since we apply a digestive fluid to the leaves, possibly comprising of dozens of enzymes involved in cleavage of macromolecules. OPDA accumulations were also observed when insect feeding, instead of standardized treatments, were used to elicit Arabidopsis leaves (Mansucript IV, Reymond *et al*., 2000; Stintzi *et al*., 2001; Reymond *et al*., 2004), demonstrating that OPDA release is a common response to herbivory in this plant. OPDA was demonstrated to possess signaling activity and we detected OPDA related transcripts to

be induced by OS treatments (manuscript IV). Taken together, these data suggest that we described a new general mechanism of how plants can perceive herbivory, namely by the detection of membrane hydrolysis products that are not released by mere wounding but by enzymatic activity derived from insect OS. In general, digestive lipases are relatively non-specific concerning their substrate specificity. Beside OPDA-containing lipids (Arabidopsides), more than 260 different types of lipids were detected recently in Arabidopsis (Giavalisco *et al*., 2011). It is quite likely that most of these lipids can be reached by OS during herbivore feeding on Arabidopsis leaves. Digestion products may influence physiological processes at the feeding site and systemic responses, e.g. priming (Galis *et al*. 2009). Characterization and functional analysis of the role of insect-induced accumulation of hydrolysis products in Arabidopsis and other plant species will further our understanding of the mechanisms behind herbivore recognition in the plant-insect interface.

Besides identifying lipase activity as possible mediator of plant-insect recognition, we were also able to show that small molecules (<10 kDa) are present in OS that elicit MAPK activity and Ca^{2+} release. Further analysis of this fraction may reveal novel elicitors of insect-induced responses in Arabidopsis. We also detected the induction of ABA, ET and SA by GS treatments of wounded leaves, but not by wounding alone (manuscripts IV and V). However, we did not test if the <10 kDa fractions also activated these hormonal responses. In future experiments measuring ABA, SA and ET levels could serve as a screening tool to further characterize active chemical constituents in different OS fractions, as has been done for grasshopper OSs in maize (Alborn *et al*., 2007). It would be intriguing if different elicitors activate specific phytohormone branches.

The work on *N. attenuata* and Arabidosis revealed that multiple elicitors activate defense signaling pathways which in turn modulate various hormonal sectors. In Arabidopsis, we found that hydroperoxides accumulate within seconds after single W+OS elicitations, followed by a steady increase in OPDA, JA, JA-Ile, SA and ABA (manuscripts IV and V). In addition, we detected high induction of Ethylene release during the first 4 hrs after treatment. How do plants integrate these hormonal responses? SA, for example, is well known for antagonizing the JA-pathway and some

insects activate SA leading to impaired JA-mediated plant defense responses; whereas ABA can promote the JA biosynthesis and signaling, leading to increased resistance to herbivores. In manuscript VI, we postulate that, after recognition of herbivory, spatiotemporal activation of different hormonal pathways are integrated by a given tissue and result in specific physiological changes. While the JA pathway is central for activation of herbivory-related defenses, modulation of this pathway by other hormone signals, including ABA, SA, Ethylene, Auxin, Cytokinins, Gibberellins and Brassinosteroids, are possibly involved in mediating herbivore-specific defense responses. Using transgenic plants and mutants altered in 1) levels of these hormones and in 2) hormone signaling components are needed to tackle these hypotheses. To analyze the significance of fine-tuned hormonal responses after herbivore attack we urgently need to reduce the physiological noise that is introduced by constitutive activation of some genes, or by constitutive gene silencing technology. Inducible promotor systems might provide useful means to circumvent many pleiotropic effects.

In conclusion, this thesis provides new insights into the mechanisms that underlie plant perception of herbivory and raises novel hypotheses on how plants integrate the complex signals that they perceive during herbivore attack.

5. Summary

How plants detect herbivory and how specific perception events activate defense pathways are only poorly understood. Herbivore detection can be divided into 1) perception of molecular events inflicted by tissue damage, classified as damage-associated molecular patterns (DAMPS) and 2) detection of herbivore-derived elicitors, classified as herbivore-associated molecular patters (HAMPS). In this thesis I analyzed herbivore-perception and herbivory-induced signaling in two plants species, the solanaceous plant *N. attenuata* and the brassicacean plant *A. thaliana*. Two protein kinases in *N. attenuata*, SIPK and WIPK, were previously shown to be quickly activated by DAMPS and HAMPS. Silencing both kinases highly diminished the accumulation of phytohormones and defense metabolites in *N. attenuata*, however, these kinase-silenced plants were not particularly susceptible to native herbivores when transplanted into the plants natural environment. This surprising effect could be explained by reduced emissions of green leaf volatiles (GLVs) from attacked SIPK and WIPK silenced plants, when compared to WT plants. GLVs were shown to attract insect herbivores and stimulate tissue consumption and complementing SIPK and WIPK silenced plants with GLVs increased insect performance on these plants. We propose that altering GLV levels in crop plants could increase resistance to insect herbivores.

Another new finding presented in this thesis is the importance of SGT1in plant perception of herbivory in *N. attenuata*. SGT1 is a highly conserved eukaryotic protein that was described to play important roles in plant and human immune responses to pathogens. Silencing SGT1 high ly diminished HAMP and DAMP-induced jasmonate (JA) biosynthesis in *N.attenuata* and resistance to the specialist herbivore *Manduca sexta*. We demonstrated that SGT1 modulates perception of JA in a Coi1 dependent manner. These data, together with the current knowledge about the role of SGT1 in defense and development, are summarized in a mini-review that is included in this thesis.

In comparison to *N. attenuata*, herbivore perception and the regulation of phytohormones in response to DAMPS and HAMPS is much less understood in other plant species. To gain insight into the perception of herbivory, *A. thaliana* was used as

model system, because the wealth of genetic and molecular tools available for this plant allows the intimate analysis of early herbivore perception events. The study presented here revealed that *A. thaliana* responds differently to herbivore elicitation when compared to *N. atteunata*. We found that lipase activity in insect's oral secretions releases the JA precursor OPDA from membrane lipids, leading to activation of gene expression and likely also JA biosynthesis. Thus, we identified lipase activity in insect´s salivae as new HAMP. Using mutant plants, we also demonstrated that the homologues of *N. attenuata`s* SIPK and WIPK, MPK6 and MPK3 respectively are not involved in activation of DAMP and HAMP-induced jasmonate biosynthesis in *A. thaliana*, however, the role of MPK6/SIPK in regulating Ethylene emission is conserved between both plants. Using a standardized methodology, we analyzed kinetics of various early herbivore perception events in *A. thaliana* including MAPK activity, hydrogen peroxide accumulation, intracellular Calcium levels, ABA and SA accumulations and production of fatty acid hydroperoxides. Based on these data we present a new model about how herbivore perception in plants can lead to the activation of hormonal pathways. Why do plants activate so many different hormone pathways in response to herbivore attack? An attempt to answer this question is provided in the last manuscript of this thesis, where we hypothesize that spatiotemporal regulation of various hormonal pathways are integrated via interaction of complex hormone response networks leading to herbivore-specific defense or tolerance responses. Surgical manipulations of these pathways using inducible genetic tools together with standardized treatments will help to understand the role of different hormone pathways in this complex network.

6. Zusammenfassung

Pflanzen haben während der Koevolution mit Insekten Resistenzen entwickelt. Sie können Insekten anhand von Bestandteilen ihres Oralsekretes erkennen und daraufhin verteidigungsrelavante Prozesse einleiten. Die Wahrnehmung des Insektes aktiviert unter anderem pflanzliche Verteidigungshormone, Genexpression und metabolische Prozesse. Über die Wahrnehmung von Insekten, sowie die Regulation der Verteidigung nach Herbivorenfraß ist aber erst wenig bekannt.

In dieser Arbeit demonstrieren wir an zwei Modellsystemen wie Pflanzen Herbivorie wahrnehmen und welche regulatorischen Elemente für die Aktivierung der Verteidigung verantwortlich sind. Wir konnten nachweisen, dass zwei Proteinkinasen in *Nicotiana attenuata*, SIPK und WIPK, für die Regulation der Insekten-induzierten Verteidigung wichtig sind. Genetisch veränderte Pflanzen mit reduzierter SIPK und WIPK Expression zeigten, dass diese Pflanzen deutlich niedrigere Konzentrationen an wichtigen Sekundärmetaboliten haben, die bei der Resistenz gegen Insekten eine Rolle spielen. Freilandversuche und Gewächshausexperimente haben jedoch demonstriert, dass diese transgenen Pflanzen interessanterweise keine erhöhte Anfälligkeit gegenüber Insekten haben. Unsere Analysen ergaben, dass SIPK und WIPK die Herbivor-induzierten Emissonen von verschiedenen Duftstoffen, insbesondere bestimmte kurzkettige Aldehyde (GLVs), regulieren. Eine Applikation von GLVs zu den transgenen SIPK und WIPK Pflanzen führte dazu, dass Raupen deutlich mehr Blattmaterial konsumierten und schneller wuchsen als Wildtyp-Pflanzen. Zusätzliche Experimente mit transgenen Linien, die besonders anfällig gegenüber Insekten sind, haben bewiesen, dass deren Resistenz wiedererlangt werden kann, wenn man die GLV Biosynthese genetisch reduziert. Diese Daten implizieren, dass die genetische Reduktion bestimmter Duftstoffe die Resistenz von Pflanzen erhöht, auch wenn diese Pflanzen ansonsten sehr anfällig für Insektenfraß sind. Dieses Wissen könnte z.B. landwirtschaftlich genutzt werden um die Resistenz gegen Insekten zu erhöhen.

Wir haben zusätzlich die Funktion von SGT1 (suppressor of G-two allele of SKP1) in der induzierten Abwehr von *N. attenuata* nach Insektenfrass untersucht. SGT1 ist ein, für die Immunantwort gegen Pathogene in Pflanzen und Menschen essentielles

Protein, dessen Sequenz evolutorisch hochkonserviert ist. Unsere Versuche bestätigten, dass SGT bedeutend ist, für die Verwundungs- und Herbivoren-induzierte Biosynthese von Jasmonsäure und deren Vorstufen. Virus-induced gene silencing (VIGS)-SGT1 Pflanzen verfügen über einen reduzierten Gehalt von Sekundärmetaboliten und eine geringere Resistenz gegen Insekten. Unsere Daten zeigten auch, dass SGT1 eine bedeutende Rolle für die Wahrnehmung von Jasmonsäure spielt. Zukünftige Experimente werden untersuchen, wie SGT1 die Stabilität und den Abbau von Proteinen reguliert, welche bei der pflanzlichen Immunantwort von Bedeutung sind.

Neben Studien mit *N. attenuata* haben wir die Wahrnehmung von Insektenfrass in der Modellpflanze *Arabidopsis thaliana* untersucht. Wir konnten erstmals nachweisen, dass eine Lipaseaktivität im Oralsekret von Insekten Verteidigungsreaktionen in der Pflanze auslöst. Zusätzlich zeigten wir, dass dieser Effekt durch alle, von uns getesteten, Insekten hervorgerufen wird. Durch die detaillierte Analyse verschiedener Parameter pflanzlicher Stressantworten, erstellten wir ein neues Modell, welches die Reaktion von Arabidopsis (und möglicherweise anderer Pflanzen) auf Insektenfraß thematisiert. In diesem Modell spielt die Regulation von verschiedenen Phytohormonenen eine große Rolle. Wie integrieren Pflanzen unterschiedliche Phytohormonsignale nach einer Herbivorenattacke? Diese Frage wird abschließend in einem Übersichtsartikel diskutiert und ist Gegenstand derzeitiger Forschung.

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8. Curriculum vitae

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2006-2011	Ph.D. student in Molecular Ecology, MPI for Chemical Ecology, Jena, Germany
2004	ERASMUS Fellowship, University of Wales, Bangor, UK
2000-2006	Diploma in Biology, Friedrich-Schiller-University, Jena

Professional Positions

2006-2011	Research Assistant (PhD), MPI for Chemical Ecology, Jena, Germany
2004-2006	Research Assistant (HiWi), MPI for Chemical Ecology, Jena, Germany
2004-2006	Research Assistant (Tutor), Instutite for Microbiology, FSU Jena, Germany

Invited Seminars

2006	Workshop on proteomic insights into plant herbivore interactions, Pune, India

Selected Presentations at Conferences

2011	Botanikertagung 2011, German Botanical Society, Humboldt University, Institute for Biology, Berlin, DE, Sep 2011
	ICE Symposium, MPI for Chemical Ecology, Jena, Germany
2010	9th IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany
2009	5th Plant Science Student Conference, Halle (Saale), Germany

Student Supervision& Commitees

2011	PhD Committee: Martin Schäfer, FSU Jena
2011	B.Sc. Thesis, Maria Knyrim, FSU Jena
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2011	M.Sc Thesis, Christoph Brütting
2011	M.Sc Thesis, Julia Kästner
2010	M.Sc Thesis, M.Sc. Martin Schäfer
2010	M. Sc Thesis, M. Sc. Christine Fischer
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Teaching

2004-2006 Microbial Physiology at Institute for Applied and Environmental Microbiology, FSU Jena (Prof. Gabriele Diekert)

Languages:

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Memberships

Member of the *Deutsche Botanische Gesllschaft*
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Radio Organizer of a monthly science radioshow at local radiostation

Blog Writing PhD Blog for Federal Ministry of Education and Research (BMBF)

Publications

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Erb M, Meldau S, Howe GA (2011). The role of phytohormones in attacker-specific plant reactions. Submitted as invited review to Trends in Plant Science

Oral presentations

Meldau S. New stories from old hormones: roles of cytokinins in plant-herbivore interactions. Botanikertagung 2011, German Botanical Society, Humboldt University, Institute for Biology, Berlin, DE, Sep 2011

Meldau S. New stories from old hormones: Cytokinins mediate plant responses to herbivores. ICE Symposium, MPI for Chemical Ecology, Jena, DE, Sep 2011

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Meldau S. SGT1 regulates JA biosynthesis and perception in Nicotiana attenuata. 5th Plant Science Student Conference, Halle (Saale), DE, Jun 2009

Meldau S. SGT1 regulates JA-biosynthesis and signaling in Nicotiana attenuata and Arabidopsis thaliana. ICE Symposium, MPI for Chemical Ecology, Jena, DE, Jun 2009

Meldau S. Can being unapparent to herbivores diminish for reduced defenses. Plant Interactions with the Environment, Universität Neuchâtel, Neuchâtel, CH, Sep 2008

Meldau S. Apparency and Defense: Two MAPK and their role in plant susceptibility to herbivores. 7th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2008

Meldau S. Function of MAPK signaling in plant herbivore interaction. Workshop on proteomic insights into plant-insect interaction, National Chemical Laboratory, Pune, IN, Dec 2006

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Poster Presentations

Weinhold A., Baldwin I.T., Groten K., Meldau DG., Meldau S., Wissgott A. Plant Microbe Interactions - From Koch's postulates to Microbial Communities. SAB Meeting 2010, MPI for Chemical Ecology, Jena, DE, Oct 2010

Wu J., Hettenhausen C., Meldau S., Baldwin I.T. MAPKs Regulate Plants’ Responses to Herbivory in Nicotiana attenuata. Gordon Research Conference - Evolutionary and Ecological Functional Genomics, Tilton, US, Jul 2009

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9. Eigenständigkeitserklärung

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

Personen die an der Durchführung und Auswertung des Materials und bei der Herstellung der Manuskripte beteiligt waren sind am Beginn der Arbeit ("Manuscript Overview", Seiten 10-15) und jedes Manuskriptes angegeben. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Die vorgelegte Arbeit wurde weder an der Friedrich-Schiller-Universität Jena, noch an einer anderen Hochschule als Dissertation eingereicht.

Stefan Meldau

10. Eigenanteil

Hiermit bestätige Ich dass die Angaben über den Arbeitsanteil aller beteiligten Autoren der Manuskripte (Seiten 11-16) richtig ausgewiesen wurden.

Prof. Ian T. Baldwin