Involvement of two nitric oxide-associated genes, *NOA1* and *GSNOR,* in *Nicotiana attenuata*'s resistance to the specialist insect herbivore *Manduca sexta*

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1. Introduction

A wild tobacco, *Nicotiana attenuata* Torr. ex Watson, which is also named coyote tobacco, is phylogenetically assigned to the Nightshade family, Solanaceae. Many species in this plant family are very common vegetables or species contain very toxic compounds, which are fatal in minute amount. There are 67 species known that belong to the *Nicotiana* genus and most *Nicotiana* species are native to America (48 species) and Australia (18 species), but one species is also occurring in Namibia (Goodspeed, 1954; Hunziker, 2001).

N. attenuata is a 0.5 to 1.5 m high plant with prolate-lanceolate to elliptic leaf blades of 2-10 cm length and 2-4 cm width and its glandular complexion is caused by trichomes spread over the surface of leaves and stem (Goodspeed, 1954). The natural habitat of *N. attenuata* ranges from northwest Mexico, east to the Great Basin and north to southern Canada (Baldwin, 2001). This environment typically resembles cold semi-deserts at 1000 to 2600 m altitude (Goodspeed, 1954). Recently burned areas promote the largest populations 1 - 3 years after fires, but this tobacco plant grows also at ruderal sides (e.g. along roadsides, dry washes, rocky or sandy grounds with outcrops) (Preston and Baldwin, 1999; Wells, 1959). For that reason, *N. attenuata* demonstrates a notable affinity to burned environments and a specific germination trait, the so called ‘fire chasing’ behavior, is responsible for this habitat selection. In most *N. attenuata* seeds, smoke cues terminate dormancy and initiate germination into favorable post fire conditions (Preston and Baldwin, 1999).

The natural history of *N. attenuata* is one of successful coexistence with herbivores and exemplifies this ‘narrow’ ecological situation on an ecological and molecular biological level (Baldwin, 2001). In nature, a number of different specialist or generalist herbivores have been identified to challenge the survival and reproduction of the *N. attenuata*. The specialized herbivore species are the three beetles *Trichobaris mucorea*, *Epitrix hirtipennis*, and *Epitrix subcrinita* and the larvae of the two lepidopteran species *Manduca sexta* and *Manduca quinquemaculata*. The *Manduca spp.* larvae are capable of devouring the entire green tissue part of *N. attenuata*; they consume about three plants in their life time. Generalist herbivores that may occur on this tobacco are the hare *Lepus californicus*, the suckfly *Tupiocoris notatus*,

grasshoppers e.g. *Trimerotropis spp.*, and the larvae of two other lepidopteran species *Heliothis virescens* and *Spodoptera exigua*.

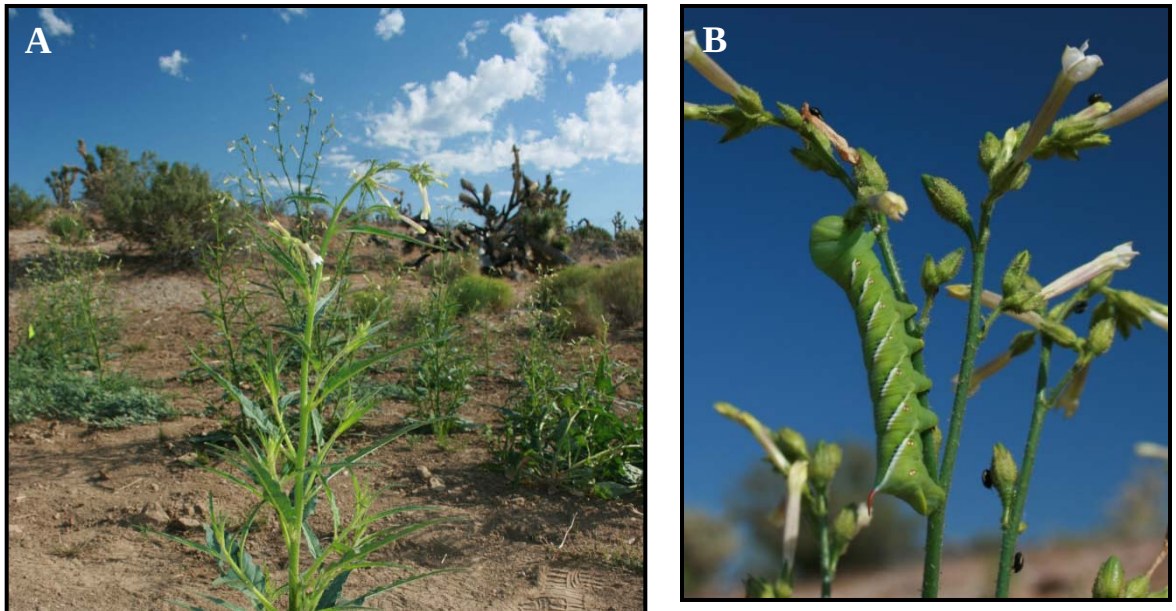

Fig. 1. *N. attenuata* in its natural environment with herbivores (courtesy: D. Kessler).

(A) A flowering *N.* attenuata plant in Utah, USA. (B) Fourth instar *M. sexta* larvae and seed feeding negro bugs (*Corimelaena extensa*) on flowers and seed capsules.

An array of elaborate defense systems, including receptors and sensors, complex regulatory networks, secondary metabolite compounds and proteins, evolved in plants to manage these adverse conditions and protect plants in a direct or indirect way (Chen, 2008; Dodds and Rathjen, 2010; Mittler, 2006; Wu and Baldwin, 2010). Of importance for this *N. attenuata – M. sexta* interaction are oral secretions and regurgitant of *M. sexta* larvae that contain fatty acid-amino acid conjugates, which present the biochemical signal for induction of plant responses (Halitschke *et al.*, 2001; Roda *et al.*, 2004). These compounds are present in caterpillars after their first meal throughout the entire alimentary channel and in the frass, but they cannot be detected in salivary glands, mandibular glands, in eggs, or neonates (Roda *et al.*, 2004).

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The four fatty acid-amino acid conjugates in the larval regurgitant that elicit the most prominent inducible direct and indirect plant defense mechanisms are N-linolenoyl-L-glutamine, N-linolenoyl-L-glutamate, N-linoleoyl-L-glutamine and N-linoleoyl-L-glutamate. These fatty acid-amino acid conjugates account for the biggest plant responses, which are characterized by activation of MAPK signaling, generation of a jasmonic acid burst, increased trypsin proteinase inhibitor concentration (direct defense) (Wu *et al.*, 2007) and increased emission of cis-α-bergamotene (indirect defense) (Halitschke *et al.*, 2001). These plant reactions were elicited by application of oral secretions or synthetic fatty acid-amino acid conjugates on wounded leaf tissue, and caterpillar feeding.

Large transcriptional responses following herbivore-specific cues are part of the necessary adjustments that enable attacked plants to defend themselves. A *N. attenuata* plant that faces a serious threat to its existence (e.g. by *Manduca sp*. caterpillars) is forced to rearrange its metabolism in order to permit appropriate direct and indirect defenses and to ensure optimal fitness under these conditions. Differential display technique and microarray analysis were used to identify changes in the mRNA expression pattern, which are specifically triggered by *M. sexta* caterpillar attack and treatment of wounded *N. attenuata* leaves with fatty acid-amino acid conjugates (Halitschke *et al.*, 2003; Hermsmeier *et al.*, 2001). Highly up-regulated transcripts of genes range from processes related to stress, wounding, pathogen, carbon, and nitrogen shifting. Several transcripts whose products are important for photosynthesis were strongly down-regulated. Among these are also up-regulated transcripts that are encoded by genes of the oxylipin-signaling cascade such as lipoxygenase3 (*NaLOX3*, supplying fatty acid hydroperoxide substrates), allene oxide synthase (*NaAOS*, using fatty acid hydroperoxides for jasmonic acid biosynthesis), and hydroperoxide lyase (*NaHPL*, using fatty acid hydroperoxides for green leaf volatile biosynthesis) (Blee, 1998; Feussner and Wasternack, 2002; Halitschke *et al.*, 2004). The enzymes LOX3 and AOS take part in the biosynthesis of signaling compound jasmonic acid (JA) (Schaller and Stintzi, 2009). The perception of herbivore attack and the dominant role of JA to set up defense responses in plants against herbivores has been intensively studied (Creelman and Mullet, 1997; Halitschke and Baldwin, 2003; Howe and Jander, 2008; Reymond and Farmer, 1998; Wu and Baldwin, 2010).

The role of JA biosynthesis and its signaling activity for plant defenses has been highlighted in Arabidopsis, tomato, and *N. attenuata* (Halitschke and Baldwin, 2003; Li *et al.*,

2005; Li *et al.*, 2004; McConn *et al.*, 1997; Paschold *et al.*, 2007). In *N. attenuata*, herbivory of *M. sexta* or simulated herbivory via application of larval oral secretions (OS) on wounded leaves elicit mitogen-activated protein kinases (MAPK) activity that is necessary to trigger the following JA burst and inducible anti-herbivore defenses (Kang *et al.*, 2006; Kessler *et al.*, 2004; Wu *et al.*, 2007). Accordingly, preventing the synthesis of JA precursors or JA signaling in mutant plants makes them vulnerable to the herbivore community (Halitschke and Baldwin, 2003; Li *et al.*, 2005; McConn *et al.*, 1997; Paschold *et al.*, 2007). Moreover, wounding and herbivory induce Jasmonate Resistance (JAR) enzymes which synthesize the conjugates between JA and amino acids (Kang *et al.*, 2006; Staswick and Tiryaki, 2004). In *N. attenuata*, JAR4 and JAR6 are required for induction of NaTPI activity (Wang *et al.*, 2007). It has been demonstrated that the conjugation product of JA and isoleucine, JA-Ile, is inducing most of the JA inducible defenses (Kang *et al.*, 2006; Staswick and Tiryaki, 2004). The perception of JA-Ile by the F-box protein Coronatine insensitive 1 (COI1) initiates the proteasomal degradation of JAZ (jasmonate ZIM-domain) proteins via $SCF^{(COI1)}$ E3-ubiquitin ligase complex and thereby disabling the JAZ suppression on MYC2 transcription factor that controls transcriptional activation of JA responsive genes (Chini *et al.*, 2009; Chini *et al.*, 2007; Sheard *et al.*, 2010; Thines *et al.*, 2007).

Interestingly, evidence has been accumulated for JA-SA antagonism that governs the outcome of plant defense responses during herbivory (Baldwin *et al.*, 1997; Doares *et al.*, 1995; Pena-Cortés *et al.*, 1993). High SA levels suppress JA synthesis in Arabidopsis and tobacco (Diezel *et al.*, 2009; Mur *et al.*, 2006). In Arabidopsis, SA represses JA-dependent gene expression via NPR1 and this effect can be influenced by ethylene (Leon-Reyes *et al.*, 2009). NPR1 has been recognized as a key regulator of the antagonizing effects of SA on JA signaling (Koornneef and Pieterse, 2008). In contrast, the NaNPR1 in *N. attenuata* is preventing SA accumulation in order to enable JA signaling and JA-dependent defense elicitation (Rayapuram and Baldwin, 2007). However, the function of SA in plant defense to chewing insects is mostly unknown (Wu and Baldwin, 2010); ethylene and JA suppress SA accumulation or SA suppresses JA burst in different plant-herbivore interactions (Diezel *et al.*, 2009). Ethylene is not the principal signal but in combination with JA facilitates induction of proteinase inhibitors that impair digestion of proteins in insect herbivores (O'Donnell *et al.*, 1996). Furthermore, the ethylene burst is required for optimal *M. sexta* OS-induced nicotine accumulation in *N. attenuata* (Kahl *et al.*, 2000; Von Dahl *et al.*, 2007).

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An important measure against herbivores in *N. attenuata*'s defense arsenal is trypsin protease inhibitor (NaTPI) (Van Dam *et al.*, 2001; Zavala *et al.*, 2004). NaTPI enhances the fitness of plants by inhibiting the midgut proteases of *M. sexta* larvae, which are therefore experiencing reduced growth and increased mortality to the benefit of the attacked and NaTPI deploying plant (Glawe *et al.*, 2003). In addition, other significant JA-dependent anti-herbivore defense metabolites in *N. attenuata* that act as toxins or feeding deterrent are the earlier mentioned nicotine (Steppuhn *et al.*, 2004) and diterpene glycosides (DTGs) (Heiling *et al.*, 2010; Jassbi *et al.*, 2008). The two foremost phenylpropanoid-polyamine conjugates present in *N. attenuata* are caffeoylputrescine (CP) and dicaffeoylspermidine (DCS), which have been shown to be distributed in the plant in a manner to protect plant fitness enhancing tissues against herbivory (Kaur *et al.*, 2010).

Besides JA, SA and ethylene, other low molecular mass compounds have been suggested to be in involved in signaling processes during plant-insect interaction (Wu and Baldwin, 2009, 2010). For instance, hydrogen peroxide appears to mediate a signaling process in tomato during response to wounding or methyl jasmonate which is increasing accumulation of the transcripts cathepsin D inhibitor, metallocarboxypeptidase inhibitor, protease inhibitors I and II, and polyphenol oxidase (Orozco-Cardenas *et al.*, 2001; Sagi *et al.*, 2004). Another small molecule is nitric oxide and its function in signaling processes in animals has been thoroughly highlighted (Schmidt and Walter, 1994).

In plants, seed germination, de-etiolation, hypocotyl elongation, stomatal closure and pathogen defense are processes in which NO has been identified to have a regulatory role (Beligni and Lamattina, 2000; Delledonne *et al.*, 1998; Guo *et al.*, 2003; Neill *et al.*, 2008; Shimazaki *et al.*, 2007). NO is increasingly suspected to play a role during plant-insect interaction as well. By utilizing the NO-specific fluorescent dye DAF-2 DA, an increase of NO levels has been detected in epidermal cells of wounded Arabidopsis leaves and in the macroalga *Dasycladus vermicularis* (Huang *et al.*, 2004; Ross *et al.*, 2006). These reports provide evidence that NO plays a role in plant systems after herbivore associated elicitation cues. That NO is involved in regulation of defense traits such as cathepsin D inhibitor, metallo-carboxypeptidase inhibitor, protease inhibitors I and II in tomato downstream of JA adds further evidence for a functional role of NO during plant-insect interaction (Orozco-Cardenas and Ryan, 2002).

Introduction

The NO production in plant cells remains unclear. Even though, a nitric oxide synthase (NOS) has only recently been discovered in the green alga *Ostreococcus tauri* with some resemblance to human NOS (Foresi *et al.*, 2010), the identification of a genuine NOS in higher plants has not been accomplished to date. It has been well established that at least three genes control NO abundance in plants (Besson-Bard *et al.*, 2008; Wilson *et al.*, 2008): NOA (nitric oxide associated), NR (nitrate reductase), both contribute to higher NO levels (Guo *et al.*, 2003; Yamasaki and Sakihama, 2000), and GSNOR (S-nitrosoglutathione reductase) that is associated with NO removing processes (Wilson *et al.*, 2008).

A previously discovered putative AtNOS1 in Arabidopsis (Guo *et al.*, 2003) has been renamed as AtNOA1 because it did not possess arginine-dependent NOS activity, although *noa1* mutant plants have reduced NO levels (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006). The plastid targeted NOA protein has binding domains for nucleic acids and peptides and it is presumably participating in protein synthesis and redox control (Flores-Perez *et al.*, 2008; Guo and Crawford, 2005; Moreau *et al.*, 2008; Sudhamsu *et al.*, 2008). However, mutant *noa1* plants have been used to study the role of NO during stomata closure, salt stress, oxidative stress and floral transition (Guo *et al.*, 2003; He *et al.*, 2004; Zhao *et al.*, 2007a; Zhao *et al.*, 2007b). In addition, NOA1 is also involved in plant resistance to biotic stresses. Silencing *AtNOA1* in Arabidopsis compromises the plants' ability to defend against *Pseudomonas syringae* pv *tomato* DC3000 and silencing *NbNOA1* in *N. benthamiana* is necessary to thwart infections by a necrotrophic pathogen (Asai and Yoshioka, 2009; Zeidler *et al.*, 2004). Any involvement of NOA in plant defenses against herbivory has not been reported so far.

Glutathione is a potent scavenger of NO and is converted to S-nitrosylated glutathione (GSNO) during this process (Clancy *et al.*, 1994). Furthermore GSNO is thought to function as a NO donor, where GSNO is a potential NO pool that regulates protein function by cysteine nitrosylation (Besson-Bard *et al.*, 2008; Gupta, 2011; Jaffrey *et al.*, 2001; Stamler *et al.*, 1992). GSNOR, a conserved protein from bacteria to animals, controls GSNO abundance by decomposing it into the oxidized glutathione disulfide (GSSG) and NH_3 (Liu *et al.*, 2001; Sakamoto *et al.*, 2002). Accordingly, increased levels of NO and SNO were detected in Arabidopsis plants with silenced *AtGSNOR1* (Feechan *et al.*, 2005; Lee *et al.*, 2008). Enhanced SNO levels were associated with compromised resistance to the plant pathogens *Pseudomonas syringae* pv. *tomato* DC3000, *Blumeria graminis* (powdery mildew) and *Hyaloperonospora*

parasitica (downy mildew) (Feechan *et al.*, 2005). In contrast, another oomycete, *Peronospora parasitica* Noco2, performed better in antisense *gsnor* Arabidopsis plants than in WT plants (Rusterucci *et al.*, 2007). Interestingly, the herbivory associated wounding stress is decreasing GSNOR transcript and protein amount in Arabidopsis leaves (Diaz *et al.*, 2003) and GSNOR is reduced at transcriptional, protein and activity levels after stem wounding in *Helianthus anuus* seedlings, consequently GSNO content in stem is increasing (Chaki *et al.*, 2010).

This aim of this thesis is to study the roles of NaNOA1 and NaGSNOR in *N. attenuata*'s resistance against the larvae of *M. sexta* by analyzing the related anti-herbivory defense signaling and defense metabolite accumulation.

In the first chapter of this thesis, we studied how NOA1 is involved in anti-herbivory defense responses. To do so, we used *NOA1*-silenced *N. attenuata* plants and compared inducible herbivore defense traits with those in WT plants. We found that NaNOA1 is important for the normal generation of an herbivory induced JA and JA-Ile burst. In addition, the inducible accumulation of some but not all of the carbon-based defense metabolites were severely reduced or absent in *NOA1*-silenced plants. In contrast, the nitrogen-rich defense compounds NaTPI and nicotine accumulated to levels similar to those measured in WT plants.

In chapter two, we describe that after silencing *NaGSNOR* by virus-induced gene silencing (VIGS) in *N. attenuata* plants, herbivory-induced defense traits such as the JA/JA-Ile burst, ethylene emission and NaTPI were compromised. In addition, NaGSNOR is also involved in JA signaling, which mediates certain JA-inducible responses. Thereby, NaGSNOR is required to maintain plant resistance against *M. sexta*.

2. Chapter I

Silencing *NOA1* elevates herbivory-induced JA accumulation and compromises most of carbon-based defense metabolites in *Nicotiana attenuata*

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Abstract

NOA1 (nitric oxide-associated protein 1) is involved in various abiotic stress responses and is required for plant resistance to pathogen infections. However, the role of NOA1 in plant-herbivore interactions has not been explored. We created *NOA1*-silenced *Nicotiana attenuata* plants (irNaNOA1). Compared with wild-type (WT) plants, irNaNOA1 plants had highly decreased photosynthesis rates. We further examined various traits important for plant defense against its specialist herbivore *Manduca sexta* by treating WT and irNaNOA1 plants with mechanical wounding and *M. sexta* oral secretions. *NOA1*-silenced plants showed elevated levels of herbivory-induced JA (jasmonic acid), but decreased JA-isoleucine conjugate (JA-Ile). The decreased JA-Ile levels did not result from compromised JAR activity in irNOA1 plants. Moreover, nitrogen-rich defensive compounds, nicotine and trypsin proteinase inhibitors, did not differ between WT and irNaNOA1 plants. By contrast, concentrations of most carbon-based defensive compounds were lower in these plants than in WT plants, although the levels of chlorogenic acid were not changed. Therefore, silencing *NOA1* alters the allocation of carbon resources within the phenylpropanoid pathway. These data suggest the involvement of NOA1 in *N. attenuata*'s defense against *M. sexta* attack, and highlight its role in photosynthesis, and biosynthesis of jasmonates and secondary metabolites.

Chapter I NOA1 in plant defense against herbivores

Introduction

The function of nitric oxide (NO) as a signaling molecule in animals has been intensively studied (Schmidt and Walter 1994). Increasing lines of evidence have also revealed its roles in plants (Delledonne *et al.* 1998). NO is involved in seed germination, de-etiolation, hypocotyl elongation, and stomatal closure (Beligni and Lamattina 2000; Guo *et al.* 2003; Shimazaki *et al.* 2007; Neill *et al.* 2008). Unlike the intensively studied NO synthase (NOS) in mammals, NOS has not been isolated in plants. However several pathways have been implicated in modulating plant NO levels (Wilson *et al.* 2008).

AtNOA1 (NO-associated 1), previously known as AtNOS1 (NO synthase 1), was first identified as an NO synthase in Arabidopsis (Guo *et al.* 2003). However, it was renamed as AtNOA1, as further studies indicated that it is not a NOS, even though knocking out *AtNOA1* decreases plants' NO levels (Crawford *et al.* 2006; Zemojtel *et al.* 2006). Recent studies suggested that NOA is located in plastids and functions at least as a cGTPase homologous to the YqeH of *Bacillus subtilis,* with recognition domains for nucleic acids and peptides that are probably required for ribosome assembly and stability (Moreau *et al.* 2008; Sudhamsu *et al.* 2008). Therefore, NOA was proposed to be also involved in protein synthesis and redox control in plastids (Guo and Crawford 2005; Flores-Perez *et al.* 2008).

Although its exact biochemical function remains elusive, plants impaired in NOA1 have frequently been used to study the functions of NOA1 and NO in plants (Guo *et al.* 2003; He *et al.* 2004; Zeidler *et al.* 2004; Zhao *et al.* 2007a; Asai and Yoshioka 2009). Arabidopsis *noa1* mutants are impaired in abscisic acid-mediated stomatal closure due to deficient NO generation (Guo *et al.* 2003). These mutants also have compromised resistance to salt and oxidative stress (Zhao *et al.* 2007a; Zhao *et al.* 2007b). Another study on *noa1* plants highlights the regulatory effect of nitric oxide on the expression of genes which controls the timing of the floral transition (He *et al.* 2004). Apart from its role in plant development and abiotic stress resistance, NOA1 plays a role in resistance to bacterial pathogens: *noa1* mutants are highly susceptible to *Pseudomonas syringae* pv *tomato* DC3000 (Zeidler *et al.* 2004). Moreover, disease lesions caused by infection of a necrotrophic pathogen in *Nicotiana benthamiana* that have silenced *NbNOA1* levels progress more rapidly than in wild-type (WT) plants (Asai and Yoshioka 2009). Whether NOA1 plays a role in plant defense against herbivores has not been examined.

Chapter I NOA1 in plant defense against herbivores

Substantial progress has been made in understanding how plants perceive herbivore attack and deploy defense responses (Howe and Jander 2008; Wu and Baldwin 2010). *Nicotiana attenuata* is a native annual plant of the semi arid deserts of the North American continent (Baldwin 2001; Kessler and Baldwin 2002). Feeding activity of *N. attenuata*'s specialized herbivore, *Manduca sexta* larvae, or the application of larval oral secretions (OS) on wounded leaves triggers the activation of mitogen-activated protein kinases (MAPKs) and subsequently a jasmonic acid (JA) burst that induces defense responses (Kessler *et al.* 2004; Kang *et al.* 2006; Wu *et al.* 2007). The pivotal role of JA in regulating herbivory-induced defenses has been demonstrated in plants with compromised JA biosynthesis (McConn *et al.* 1997; Halitschke and Baldwin 2003; Li *et al.* 2005) or JA signaling (Li *et al.* 2004; Paschold *et al.* 2007). Several secondary metabolites in *N. attenuata* have been found to play important roles in the direct defenses of plants against herbivores. Trypsin protease inhibitors (TPI) (Van Dam *et al.* 2001; Zavala and Baldwin 2004), nicotine (Steppuhn *et al.* 2004), diterpene glycosides (DTGs) (Jassbi *et al.* 2008; Heiling *et al.* 2010), and the phenylpropanoid-polyamine conjugates, caffeoylputrescine (CP) and dicaffeoylspermidine (DCS) (Kaur *et al.* 2010) are all anti-herbivore compounds whose levels are mediated by JA signaling.

To study the function of NOA1 in plants' inducible defense against herbivores, we examined herbivore resistance traits in transgenic *N. attenuata* plants that are silenced in *NaNOA1*. We show that NaNOA1 is required for normal herbivory-induced accumulations of JA and its amino acid conjugate, JA-isoleucine conjugate (JA-Ile). Furthermore, silencing *NaNOA1* abolishes most, but not all, of the carbon-based defensive metabolites, likely due to these plants' compromised photosynthesis rates; however, compared with WT plants NaNOA1-silenced plants have normal levels of nitrogen-rich defensive compounds, TPI and nicotine.

Results

Molecular cloning and silencing of *NaNOA1* in *N. attenuata*

Partial *NaNOA1* sequence [GenBank: HM755675] was amplified from cDNA prepared from *N. attenuata* leaf tissue using primers designed from the sequence of *NbNOA1* [GenBank: AB303300] from *N. benthamiana*. Alignment of the deduced NaNOA1 protein sequence (Supporting Figure S1) indicated 96% and 67% sequence homology to *N. benthamiana* NbNOA1 and Arabidopsis AtNOA1, respectively (Guo *et al.* 2003; Asai and Yoshioka 2009). Furthermore,

like *AtNOA1* in Arabidopsis, Southern blotting analysis confirmed that *NaNOA1* is also a single copy gene in *N. attenuata* genome (Supporting Figure S2A).

To analyze if transcript levels of *NaNOA1* are induced by herbivory and mechanical wounding, we wounded *N. attenuata* leaves with a pattern wheel and applied 20 µL of *M. sexta* OS (W+OS), which mimics *N. attenuata*'s responses to *M. sexta* herbivory (Halitschke *et al.* 2001), and application of 20 µL of water (W+W) was used to induce wound-related responses. Samples were collected at different times and the transcript levels of *NaNOA1* were analyzed by qRT-PCR. The *NaNOA1* transcript levels decreased slightly 0.5 h after either treatment (Figure 1A). Thereafter, W+W treatment enhanced *NaNOA1* transcript levels by 1 h and returned to basal levels by 3 h; in contrast, *NaNOA1* levels returned to those of untreated samples 1 h after W+OS treatment without any further changes (Figure 1A). From these results, we conclude that transcript levels of *NaNOA1* in *N. attenuata* are not highly induced by *M. sexta* herbivory.

Agrobacterium tumefaciens was transformed with pSOL8 vector containing a partial sequence of *NaNOA1* in an inverted repeat orientation, and was subsequently used for *N. attenuata* transformation to obtain irNaNOA1 plants (Krügel *et al.* 2002). Using Southern hybridization we identified two independently transformed lines (N247 and N267) harboring a single transgene insertion (Supporting Figure S2B) which were chosen for further experiments. *NaNOA1* transcript levels in both transgenic lines were reduced by 74% compared to those in WT plants (Figure 1B).

The irNaNOA1 plants exhibited a yellowish leaf phenotype (Supporting Figure S3) resembling that of *noa1* mutants in Arabidopsis (Guo *et al.* 2003; Guo and Crawford 2005) The vigor and tension of these leaves were similar to WT leaves. irNaNOA1 plants had about 40% of the chlorophyll levels of WT plants (Figure 2A). Consistently, the photosynthetic rates of N247 and N267 were about 20% of those in WT plants at ambient CO_2 concentration (400 μmol mol^{-1}, light intensity 1,200 µmol m^{-2} s^{-1}) (Figure 2B).

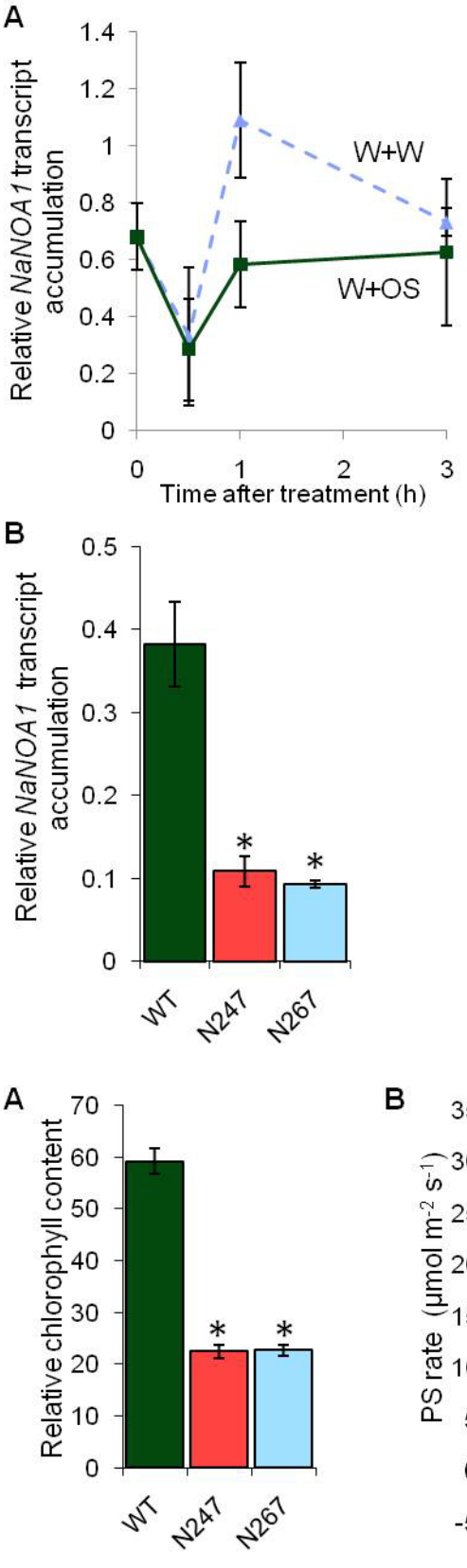

Figure 1. Quantitative real-time PCR (qPCR) analysis of *NaNOA1* transcripts.

(A) Transcriptional changes of *NaNOA1* after wounding and herbivory. *N. attenuata* plants were wounded with a pattern wheel and immediately 20 µL of *M. sexta* oral secretions or water were applied to wounds (W+OS and W+W, respectively). Samples were harvested at indicated time. (B) Relative transcript levels of *NaNOA1* in wild-type (WT) and irNaNOA1 plants (N247 and N267). Average relative transcript levels (± SE) were measured with qPCR. Stars indicate significantly differently levels between irNaNOA1 and WT plants ($p \leq 0.05$; Student's *t*-test).

Figure 2. Relative chlorophyll contents and photosynthesis rates of wild-type and irNaNOA1 plants.

Source-sink transition leaves of rosette-staged wild-type (WT) and irNaNOA1 plants (N247 and N267) were used to measure (A) relative chlorophyll content (N = 7) and (B) photosynthetic rates (N = 6) at different CO_2 concentrations (0, 200, 400, 600, 800 and 1000 µmol mol^{-1}). Data points are mean values ± SE. Stars indicate significantly different levels between WT and irNaNOA1 plants ($p \leq 0.05$; Student's *t*-test).

Herbivory induces elevated levels of JA but decreased levels of JA-Ile in irNaNOA1 plants

AtNOA1 in Arabidopsis and NbNOA1 in *N. benthamiana* are known to be required for defense against pathogens (Zeidler *et al.* 2004; Kato *et al.* 2008; Asai and Yoshioka 2009). We sought to determine whether silencing *NaNOA1* altered traits that are important for *N. attenuata*'s defense against herbivores.

JA plays a pivotal role in plant-herbivore interactions. The precursor of JA, 12-oxo-phytodienic acid (OPDA), is synthesized in chloroplasts and is further transported to peroxisomes to form JA (Schaller and Stintzi 2009). Recent studies have revealed that the conjugate between JA and Ile, JA-Ile but not JA, binds to the receptor COI1 to activate most of JA-elicited responses (Thines *et al.* 2007; Staswick 2008; Chini *et al.* 2009). Thus, we measured the levels of OPDA, JA, and JA-Ile in W+OS-treated rosette leaves in WT, N247, and N267 plants over 90 minutes (Figure 3). After W+OS treatment, both WT and irNaNOA1 plants showed enhanced levels of OPDA; however, OPDA contents in irNaNOA1 plants were about 1 fold higher than in WT plants (Figure 3A). Similarly, JA contents in irNaNOA1 plants were around twice as much as those in WT plants 30 min after W+OS treatment, which subsequently decreased to WT levels (Figure 3B). Surprisingly, the larger and more rapid JA burst in irNaNOA1 plants didn't result in higher JA-Ile contents; instead considerably reduced levels of JA-Ile were detected in these plants: 1 h after W+OS treatment, JA-Ile contents of N247 and N267 plants were only 38% and 60% of those in WT plants (Figure 3C). It is known that W+W treatment of *N. attenuata* leaves induces JA accumulation as well; though, the JA level in WT plants is much less than after W+OS treatment (Halitschke *et al.* 2001). We also assessed the ability of wounding to induce OPDA, JA and JA-Ile in irNaNOA1 plants and found the pattern of jasmonate accumulation in W+W-treated leaves was similar to the jasmonate accumulation in W+OS elicited leaves

(Supporting Figure S4). However, the amount of OPDA, JA and JA-Ile in WT and irNaNOA plants was about 50%, 40% and 70% of that in W+OS elicited leaves, respectively.

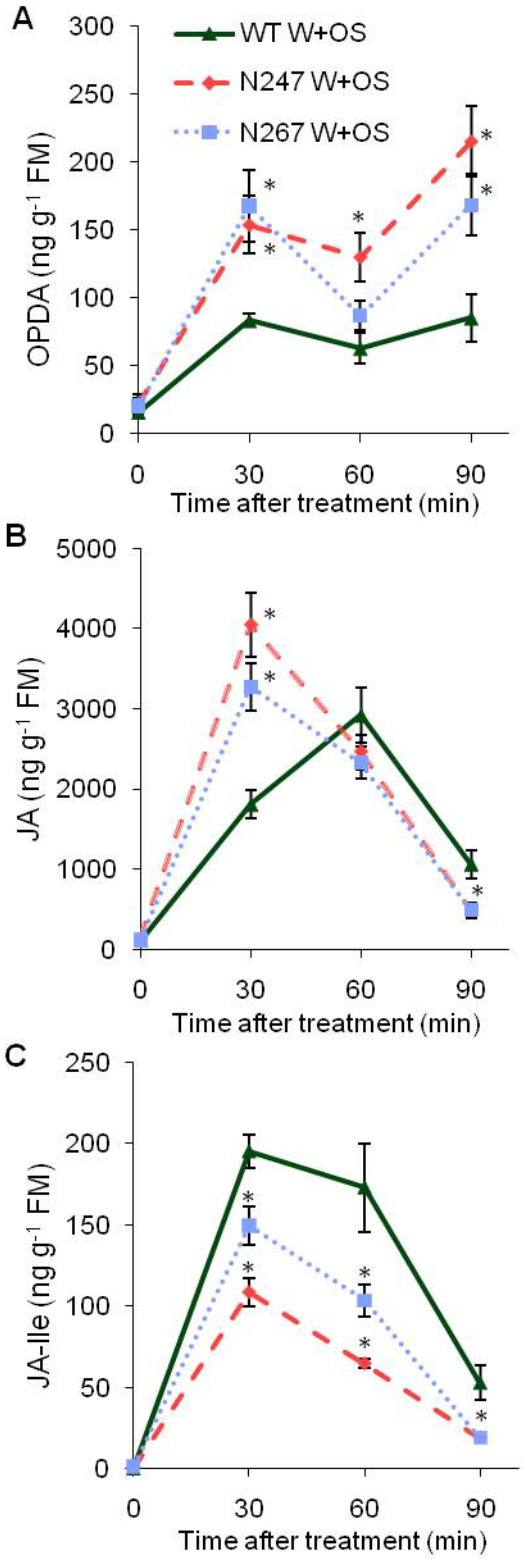

Figure 3. Silencing *NaNOA1* increases herbivory-induced OPDA and JA accumulation, but decreases JA-Ile accumulation.

Wild-type (WT) and irNaNOA1 plants (N247, N267) were wounded with a pattern wheel and subsequently applied with 20 µL of *M. sexta* oral secretions (OS) (W+OS). Samples were collected at indicated times, and the contents (± SE) of (A) OPDA, (B) JA, and (C) JA-Ile were measured on a HPLC-MS/MS. Stars indicate significantly different levels between irNaNOA1 lines and WT plants ($p \leq 0.05$; Student's *t*-test; N = 4).

The early JA burst in irNaNOA plants was absent and JA accumulation was not significantly different when compared with WT plants. Thus silencing *NaNOA1* leads to enhanced levels of herbivory-induced JA but decreased levels of JA-Ile in *N. attenuata*.

Since salicylate-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) signaling are located upstream of herbivory-elicited JA synthesis (Wu *et al.* 2007; Wu and Baldwin 2010), we analyzed SIPK and WIPK activity in WT and irNaNOA1 plants. After W+OS treatment, the kinase activity in irNaNOA1 plants was comparable to that of WT plants (Supporting Figure S5). Thus the elevated JA contents in herbivory-induced irNaNOA1 plants were not correlated with elevated MAPK activity. We next measured the transcript levels of *glycerolipase A1* (*GLA1*), *lipoxygenase 3* (*LOX3*), *allene oxide synthase* (*AOS*), *allene oxide cyclase* (*AOC*), and *OPDA reductase 3* (*OPR3*) in both WT and irNaNOA plants, which encode enzymes important for JA biosynthesis (Schaller and Stintzi 2009; Kallenbach *et al.* 2010). None of these genes showed significantly higher transcript levels in irNaNOA1 plants than in WT plants (Supporting Figure S6). Therefore, it is likely that NaNOA1 negatively influences herbivory-induced JA accumulation at a posttranscriptional level.

To examine the possibility that irNaNOA1 plants have increased turnover rates of JA-Ile which results in decreased levels of herbivory-induced JA-Ile, we treated plants with W+OS and 30 min later, 500 ng of JA-[$^{13}C_6$]Ile were applied to the wounded area on both WT and irNaNOA1 plants (3 cm² leaf area). The leaf material was collected immediately or 90 min after the JA-[$^{13}C_6$]Ile application to estimate the turnover rate of JA-Ile. Compared with the initial amount of JA-[$^{13}C_6$]Ile applied to the leaves, 76% was recovered from WT, 68% and 70% were recovered from N247 and N267 leaves (Figure 4A). From these results we conclude that irNaNOA1 plants do not have JA-Ile turnover rates higher than those of WT plants.

To test if the supply of Ile in the wound tissues limited the production of JA-Ile, we supplemented Ile to *M. sexta* OS and applied 20 µL of this Ile-containing OS to freshly wounded leaves of WT and irNaNOA1 plants. Ile supplementation fully restored JA-Ile levels in irNaNOA1 plants (Figure 4B). In addition, supplementing OS with both JA and Ile strongly increased JA-Ile contents in all plants (Figure 4B inset), indicating that silencing *NaNOA1* didn't

attenuate the activity of JARs, the enzymes that conjugate JA and Ile to form JA-Ile (Wang *et al.* 2007; Staswick 2008). From these results, we inferred that irNaNOA1 plants may have limited

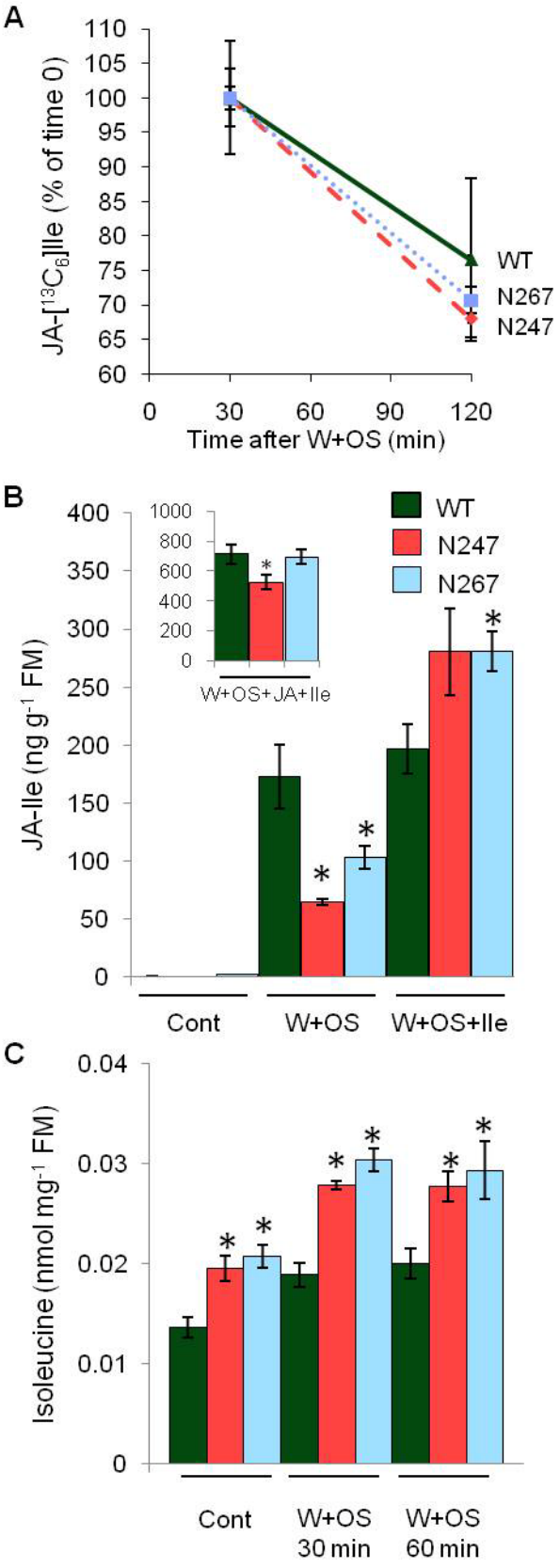

Figure 4. Levels of JAR activity, free Ile, and the turnover rates of JA-Ile in wild-type and irNaNOA1 plants.

(A) 30 min after leaves were wounded with a pattern wheel and applied with 20 µl of *M. sexta* oral secretions (OS) (W+OS), 500ng of JA-[$^{13}C_6$]Ile were applied on a 3 cm² leaf area. Samples harvested immediately served as 100% reference; samples harvested after 90 min (120 min after W+OS treatment) were used to determine amount of JA-[$^{13}C_6$]Ile turnover. JA-[$^{13}C_6$]Ile (± SE) were quantified on a HPLC-MS/MS. (B) Leaves of wild-type (WT) and irNaNOA1 plants (N247, N267) were treated with W+OS or an excess amount (0.625 µmol) of Ile or JA and Ile [W+OS+Ile and W+OS+JA+Ile (inset), respectively]. Mean JA-Ile levels (± SE) were analyzed in samples harvested 1 h after treatments; non-treated plants served as controls (Cont). (C) Free total Ile content (± SE) were measured in WT and irNaNOA1 plants before (Control) and 30 min and 60 min after W+OS treatments. Data points are mean values ± SE (N = 5). Stars indicate $p \leq 0.05$ (Student's *t*-test) significance levels between NOA-silenced lines and WT.

Ile contents which compromised the production of herbivory-induced JA-Ile. However, quantifying the contents of free Ile in WT and irNaNOA1 plants indicated otherwise: irNaNOA1 plants had higher levels of free Ile contents before and after W+OS induction than did WT plants (Figure 4C). In *N. attenuata*, JAR4 and JAR6 conjugate JA with Ile and Val but not with other amino acids (Wang *et al.* 2007). HPLC-MS/MS analysis indicated that in addition to JA-Ile, only JA-Val was detected in WT and irNaNOA1 plants, which also showed significantly lower levels (71% in N247 and 53% in N267 plants compared with those in WT plants) (Supporting Figure S7).

Defense metabolites and *Manduca sexta* performance in irNaNOA plants

One of *N. attenuata*'s defenses against *M. sexta* is the deployment of TPIs, which is a trait largely controlled by JA/JA-Ile signaling (Zavala and Baldwin 2004; Paschold *et al.* 2007). W+OS treatment highly elevated the TPI activity in WT plants (Figure 5A); in comparison, slightly higher or similar levels of TPI activity were found in irNaNOA1 plants before and after W+OS elicitation (Figure 5A). Several other secondary metabolites in *N. attenuata* have been identified to play important defensive roles in plant-herbivore interactions; these include phenolic compounds, chlorogenic acid (CGA) and rutin (Isman and Duffey 1982; Bi *et al.* 1997), an alkaloid, nicotine (Baldwin 1996), diterpene glycosides (DTGs) (Jassbi *et al.* 2008; Heiling *et al.* 2010), and the phenylpropanoid-polyamine conjugates, dicaffeoylspermidine (DCS) and caffeoylputrescine (CP) (Kaur *et al.* 2010). All these metabolites were analyzed in rosette leaves harvested 3 days after W+OS treatment. CGA and nicotine did not show large differences between non-treated and W+OS-treated WT and irNaNOA1 plants (Figure 5B and C). In contrast, when irNaNOA1 plants were untreated, levels of rutin, DTGs, DCS, and CP were at most 42%, 25%, 50%, and 0% (not detectable) of those in WT plants; after W+OS induction, contents of rutin, DTGs, DCS, and CP in WT plants were still at least 2.2, 4.4, 1.8, and 14.5 fold higher than those in irNaNOA1 plants (Figure 5D-G).

We further examined the growth of the specialist herbivore, *M. sexta*, on WT and irNaNOA1 plants to determine the resistance levels of these plants. Neonate larvae were placed on rosette plants for 12 days and their mass gain was recorded. By day 12, larvae feeding on WT

plants were around 100% heavier than those that fed on irNaNOA1 plants (Figure 6). We measured the total protein contents of WT and irNaNOA1 plants and found that WT plants contained 50% more protein than did irNaNOA1 plants (Supporting Figure S8), suggesting that despite compromised accumulation of defensive compounds the slower growth of *M. sexta* larvae on these plants was likely caused by the low protein contents of irNaNOA1 plants.

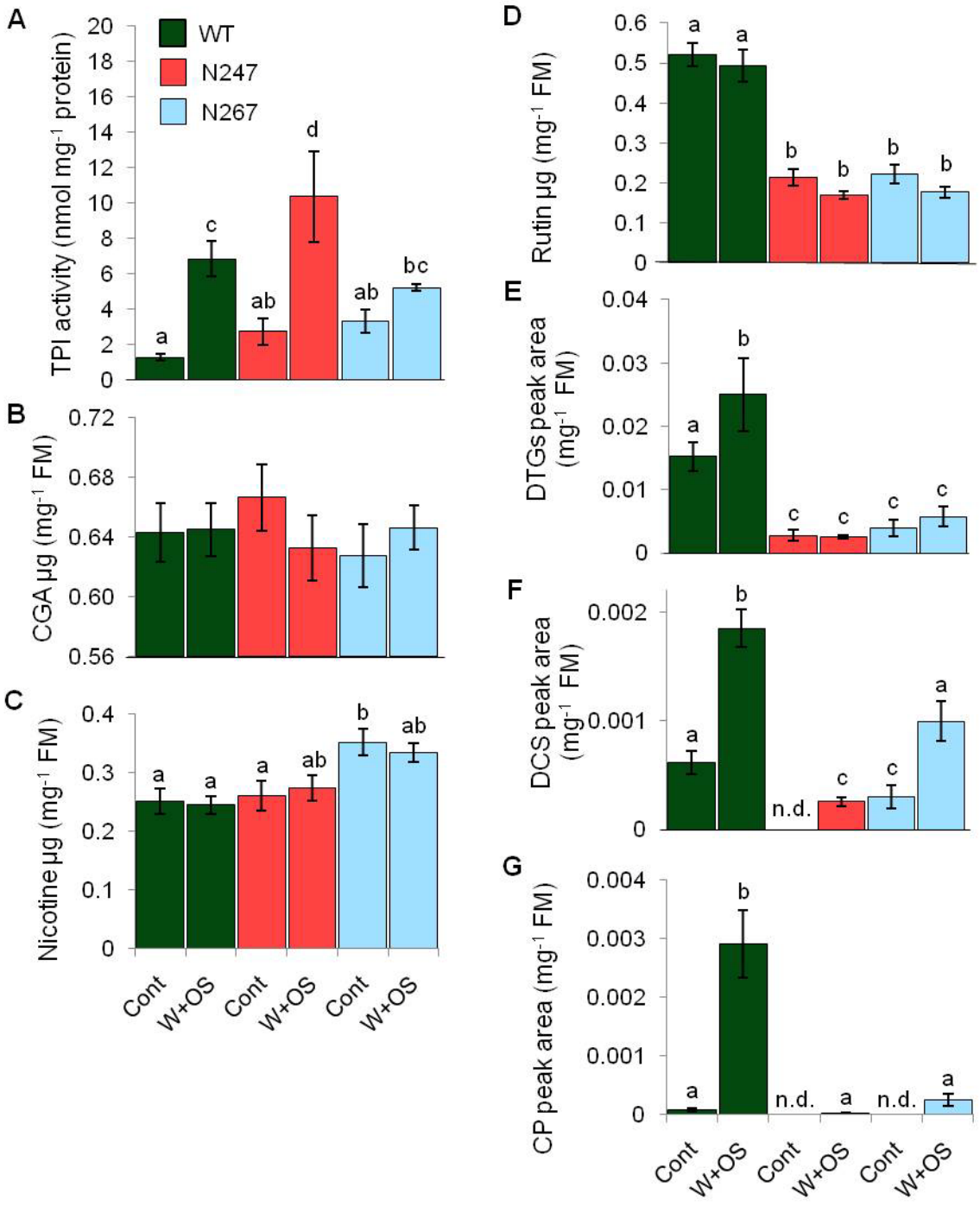

Figure 5. Accumulation of defense-related secondary metabolites in wild-type and irNaNOA1 plants.

Wild-type (WT) and irNaNOA1 plants (N247, N267) were wounded with a pattern wheel and subsequently applied with 20 µL of *M. sexta* oral secretions (OS) (W+OS); untreated plants served as controls (Cont). TPI activity (± SE) (A) and the contents (± SE) of chlorogenic acid (B), nicotine (C), rutin (D), diterpene glycosides (E), dicaffeoylspermidine (F) and caffeoylputrescine (G) were analyzed in rosette leaves of WT and irNaNOA1 plants 3 days after W+OS treatment. Different small letters represent statistically significant differences (ANOVA, N = 5). n.d. = not detected.

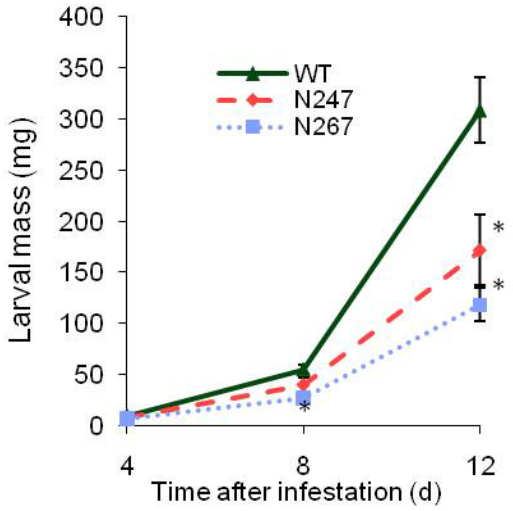

Figure 6. Silencing *NaNOA1* increases *N. attenuata*'s defense against *M. sexta* larvae.

M. sexta larvae were placed on rosette-staged wild-type (WT) and irNaNOA1 plants (N247 and N267) and larval mass was measured after 4, 8, and 12 days. Data points are mean values ± SE (at least N = 21). Stars indicate significantly different larval mass of those fed on WT and irNaNOA1 plants ($p \leq 0.05$; Student's *t*-test).

Discussion

In this study, we show that NaNOA1 is involved in plant defense against herbivores. Silencing *NaNOA1* alters various traits that are important for plant resistance to herbivores in *N. attenuata*.

JA biosynthesis and signaling play critical roles in plant resistance against herbivores (Howe and Jander 2008; Schaller and Stintzi 2009; Wu and Baldwin 2010). In *NaNOA1*-silenced plants, we found that herbivory induces a more rapid and larger JA burst, which was correlated with enhanced OPDA levels. OPDA is the last precursor in the JA biosynthesis pathway which is synthesized in chloroplasts before being transported to peroxisomes (Schaller and Stintzi 2009). Both the rapid nature of JA biosynthesis after wounding or herbivory and several lines of genetic evidence obtained from plants overexpressing JA biosynthesis genes indicated that the constitutive levels of JA biosynthetic enzyme activity determine the levels of JA accumulation

(Delker *et al.* 2006). Thus, we examined the basal transcript levels of genes involved in JA biosynthesis in irNaNOA1 plants. No large differences were found between WT and irNaNOA1 plants and we infer that NaNOA1 influences the levels of JA biosynthetic enzyme activity at a posttranscriptional level.

GLA1, LOX3, AOS, and AOC, which are required for OPDA biosynthesis, are all located in chloroplasts (Ryu 2004; Farmaki *et al.* 2007; Kallenbach *et al.* 2010). The Arabidopsis mutant *noa1* contains smaller chloroplasts with overall markedly reduced thylakoid membrane structures (Flores-Perez *et al.* 2008). Thus, we hypothesize that silencing *NaNOA1* alters the chloroplast structure which results in physical relocations of enzymes involved in OPDA biosynthesis and therefore enhances substrate-enzyme vicinity and consequently augments OPDA biosynthesis. Moreover, it has been shown that Arabidopsis *noa1* mutants are more prone to suffer from oxidative stress, and lipid peroxidation is consequently more extensive than in WT Arabidopsis plants (Zhao *et al.* 2007b). The altered lipid metabolism may elevate the supply of lipid substrates for various lipid metabolizing pathways including OPDA/JA biosynthesis.

Catalyzed by JARs, the conjugation product between JA and Ile, JA-Ile (but not JA itself) binds to $SCF^{(COI1)}$ E3 ubiquitin ligase and facilitates the degradation of JAZs (JASMONATE ZIM-DOMAIN proteins), which are suppressors of JA-induced responses (Thines *et al.* 2007; Staswick 2008; Chini *et al.* 2009). In contrast to the elevated JA levels in herbivory-induced irNaNOA1 plants, the JA-Ile levels are markedly reduced in irNaNOA1 plants (Figure 3C). Several possible mechanisms may account for this: irNaNOA1 plants may have decreased levels of free Ile, compromised JAR activity, or altered JAR substrate specificity. Our analyses revealed that irNaNOA1 plants had increased levels of free Ile and uncompromised JAR activity, and we found no evidence for alterations in JAR's substrate specificity. The increased levels of JA and Ile may be associated with altered chloroplast structures in irNaNOA1 plants. Chloroplasts are the source of Ile and JA biosynthesis, while JA adenylation and conjugation to amino acids, as mediated by JAR, occur in the cytosol. Hence at least two scenarios may account for the compromised JA-Ile levels in irNaNOA1 plants: 1) amino acid transporters in the outer membranes of the chloroplast (Pohlmeyer *et al.* 1997) may have decreased activity to export Ile into the cytosol and thereby limit JA-Ile production in the cytosol; 2) JA is highly accumulated in peroxisomes, where JA is finally formed, and irNaNOA1 plants may be compromised in their ability to export JA from peroxisomes to cytosol and thus resulting in attenuated levels of JA-Ile.

In Arabidopsis *noa1* mutants, altered chloroplast structures have been demonstrated (Flores-Perez *et al.* 2008). Consistently, we show that *NaNOA1*-silenced plants have greatly decreased photosynthesis rates. It is conceivable that low rates of photosynthesis could result in reductions in attenuated carbon-rich secondary metabolites. However not all C-rich compounds were found to have decreased levels. The concentrations of DCS, CP, rutin, and DTGs are suppressed in irNaNOA1 plants, while CGA contents are similar in WT and *NaNOA1*-silenced plants. Although most of the C-based defensive metabolites are lower in irNaNOA1 plants, the contents of N-rich compound, nicotine and TPI, were not altered. The synthesis of nicotine takes place in roots (Tso and Jeffrey 1957) and is converted from the precursors, putrescine and nicotinic acid (Cane *et al.* 2005). Although nicotine and CP share the same precursor (putrescine), compared with WT plants, herbivory-induced irNaNOA1 plants contain very limited amount of CP, but normal (or even slightly higher) levels of nicotine. It is likely that this is a result of the spatial separation of CP and nicotine biosynthesis (root and shoot, respectively). In line with this, silencing *putrescine methyltransferase* (*PMT*) in *N. attenuata* inhibits nicotine production but does not increase the production of CP (Steppuhn *et al.* 2004; Steppuhn *et al.* 2008). Putrescine is synthesized either by ornithine decarboxylase (ODC) or arginine decarboxylase (ADC) pathways (Cane *et al.* 2005). In *N. tabacum*, the ODC but not the ADC pathway plays a major role in supplying the putrescine that is subsequently used for nicotine synthesis (Chintapakorn and Hamill 2007). Therefore, the decreased CP and DCS accumulation in irNaNOA1 plants probably results from attenuated activity of the ADC pathway located in chloroplasts (Borrell *et al.* 1995) and the likely unaltered activity of the ODC pathway in root accounts for the lack of changes in nicotine contents in irNaNOA1 plants. The compromised levels of CP and DCS might also be caused by a decreased supply of the caffeoyl moiety, which is derived from caffeoyl-CoA. Alternatively, enzymes that conjugate polyamine, i.e. putrescine, with caffeoyl-CoA may have lower activity in irNaNOA1 plants than in WT plants, and thus result in lower CP and DCS contents. The greatly diminished levels of most of the C-based defensive metabolites in irNaNOA1 plants demonstrate the dependency of their biosynthesis on photosynthesis; however, unaltered CGA contents in these plants also suggests that NaNOA1 may also have functions in specifically influencing the activity of enzymes involved in the biosynthesis of certain C-based defensive compounds. CGA biosynthesis requires quinic acid (Niggeweg *et al.* 2004), derived from the shikimate pathway, and caffeoyl-CoA, synthesized in the phenylpropanoid pathway. The unaltered CGA levels suggests that the supply of quinic acid

and caffeoyl-CoA is sufficient for CGA synthesis in irNaNOA1 plants, and the enzyme activity of hydroxycinnamoyl-CoA quinate: hydroxycinnamoyl transferase (HQT) (Niggeweg *et al.* 2004) remains unaltered in irNaNOA1 plants. CGA and quercetin (the flavonol moiety of rutin) are both synthesized via phenylpropanoid pathway and share common precursors, for instance cinnamic acid (Niggeweg *et al.* 2004; Vogt 2010). However, low photosynthetic rates in irNaNOA1 plants may result in reduced levels of glucose and/or rhamnose for the rutinose (the disaccharide moiety of rutin) biosynthesis, which in turn could compromise rutin accumulation in irNaNOA1 plants. DTGs are composed of two moieties, a diterpene and a sugar moiety, and plastid-located methylerythritol phosphate (MEP) pathway is important for the biosynthesis of diterpenes. Arabidopsis *rif1*/*noa1* mutants have increased levels of DXS and DXR proteins (Flores-Perez *et al.* 2008). Thus the decreased levels of DTGs in irNaNOA1 plants are not likely a result of decreased activity of the methylerythritol phosphate (MEP) pathway but rather from attenuated levels of substrates required by the MEP pathway and compromised sugar biosynthesis due to low photosynthesis rates of irNaNOA1 plants.

Although the exact mechanism remains unclear, NOA1 is required for normal chloroplast function (Flores-Perez *et al.* 2008). In agreement with this, silencing *NaNOA1* highly compromises plant photosynthesis capacity. Comparing these plants with plants silenced in *ribulose-1,5-bisphosphate carboxylase/oxygenase activase* (*RCA*), a gene important for modulating the activity of RuBPCase, is particularly intriguing. Like irNaNOA1 plants, *N. attenuata* silenced in *RCA* (irRCA plants) show decreased levels of photosynthesis rates, and have reduced herbivory-induced JA-Ile (Mitra and Baldwin 2008). In irRCA plants, it was suspected that reduced photosynthetic rates decrease ATP supply for adenylation of JA during the conjugation reaction between JA and Ile (Mitra and Baldwin 2008). However, treating wounds with OS supplemented with Ile fully restores JA-Ile levels in irNaNOA1 plants to levels even slightly higher than those in WT plants treated with supplemented OS, indicating that ATP levels in irNaNOA1 plants are more than sufficient for the JA-Ile conjugation reaction. irRCA and irNaNOA1 plants have additional differences in their defensive compounds. Silencing *NaNOA1* doesn't affect the levels of TPI activity; however, the levels of TPI activity in irRCA plants are reduced (Mitra and Baldwin 2008). Furthermore, studies in *N. tabacum* and *N. attenuata* showed that decreasing plant photosynthetic rates impairs the accumulation of C-based secondary metabolites, including CGA, rutin, and DTGs (Matt *et al.* 2002; Mitra and Baldwin 2008). In

comparison, silencing *NaNOA1* results in highly diminished contents of rutin, DTGs, DCS and CP, but not of CGA. Therefore, the accumulation of C-based defensive compounds, DTGs, DCS, CP, and CGA is likely dependent on photosynthesis, but among these the accumulation of CGA is also specifically regulated by NaNOA1. NO functions in regulating plant development and stress responses (Beligni and Lamattina 1999; Guo *et al.* 2003; Arasimowicz and Floryszak-Wieczorek 2007; Shimazaki *et al.* 2007; Neill *et al.* 2008), and it is clear that NOA1 has other functions than modulating NO levels (Flores-Perez *et al.* 2008; Moreau *et al.* 2008; Sudhamsu *et al.* 2008). The contribution of the putative low levels of NO in irNaNOA1 plants to these ecologically important traits merits further investigation.

NOA1 is important for ribosome assembly and stability in plastids, which may partially explain the decreased protein contents in irNaNOA1 plants (Flores-Perez *et al.* 2008; Moreau *et al.* 2008; Sudhamsu *et al.* 2008). In addition, decreased photosynthetic capacity probably also accounts for the low protein contents in irNaNOA1 plants. In spite of highly attenuated concentrations of defensive compounds, *M. sexta* larvae perform worse on irNaNOA1 plants compared to WT plants, a result which is likely due to the lower protein contents of these plants. We speculate that irNaNOA1 plants would have decreased levels of resistance, if their nutrient deficiency could be restored. In Arabidopsis and *N. benthamiana*, compromising the transcript accumulation of *NOA1* leads to abolished resistance to pathogens (Zeidler *et al.* 2004; Asai and Yoshioka 2009). It would be interesting to examine whether silencing (or knocking out) *NOA1* also leads to reduced levels of anti-pathogen compounds in plants.

In summary, NaNOA1 is involved in plant chloroplast functions, such as photosynthesis and JA/JA-Ile biosynthesis. Silencing *NaNOA1* doesn't alter herbivory-induced N-based defenses, such as TPI and nicotine, but compromises the accumulation of most but not all C-based defensive compounds that are involved in herbivore resistance.

Methods

Plant growth and treatment and larval performance

For all experiments and transformation, we used a *Nicotiana attenuata* line maintained in our lab, which has been inbred for 22 generations. Plant growth conditions and seed germination were performed according to Krügel *et al.* (2002). Transition leaves of rosette-sized plants were used for experimentation and measurements. For simulating herbivory, we used a fabric pattern

wheel to create standardized wounds on leaf lamina and applied 20 µL of 1/5 diluted *M. sexta* OS (oral secretion) to the wounds (W+OS) or 20 µL of water (W+W). For determination of JAR activity, we supplemented OS with Ile and/or JA to achieve a concentration of 0.625 µmol (Ile and/or JA)/20 µL of OS and applied these amended OS solutions to wounds and quantified the resulting JA burst (Kang *et al.* 2006).

Specialist herbivore *M. sexta* was obtained from a colony maintained in our lab. Each rosette plant was infested with one neonate larvae. Larval mass was assessed on day 4, 8, and 12.

Cloning of *NaNOA1* and Southern blotting analysis

We used the primer pair NaNOA1-1 (5'-CCTTGCTCTATCCTCCTTATC-3') and NaNOA1-2 (5'-GGGTCTAACTTCTGATTCCTC-3') to amplify a partial sequence of *NaNOA1*. The PCR product was cloned and sequenced. A 294 bp region of *NaNOA1* (Supporting Figure S9) was inserted into a pSOL8 vector in an inverted-repeat fashion to form pSOL8-irNaNOA1, and this construct was further used for *Agrobacterium tumefaciens*-mediated transformation to generate irNaNOA1 plants (Krügel *et al.* 2002). Homozygous lines N247 and N267 were isolated by segregation screening of T2 seedlings' hygromycin resistance.

Five µg of DNA, digested with *Eco*R I, *Hind* III, *Eco*R V, or *Xba* I, were separated on a 1% agarose gel. Southern blotting and hybridization was done following Wu *et al.* (2006). For determination of NaNOA1's copy numbers in WT plants, probe was prepared using primer pair NaNOA1-3 (5'-GGAGCCAAGTTGGAAACTTTAAAGA-3') and NaNOA1-4 (5'-ATGGCTATGGAGCTGCCGCT-3') and a plasmid containing *NaNOA1* cDNA serving as the PCR template. The primer pair hptII-1 (5'-CTGACGGACAATGGCCGC-3') and hptII-2 (5'-CCGGATCGGACGATTGCG-3') was used to prepare a probe for the hygromycin resistance gene to confirm a single transgene insertion in the irNaNOA1 plants.

Quantitative real-time PCR (qRT-PCR)

Total RNA samples from at least 3 biological replicates were extracted with the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) from 500 ng of total RNA. qPCR was done on a Stratagene MX3005P (Agilent Technologies, Santa Clara, CA, USA) using qPCR core kit with or without SYBR Green (Eurogentec, Cologne, Germany). Primers and probes used for SYBR and Taqman assays are listed in additional file1 (Supporting Table S1).

Chlorophyll and photosynthesis rate

Photosynthesis rates were measured at 0, 200, 400, 600, 800, and 1000 µmol mol^{-1} CO_2 and constant light levels of 1200 µmol m^{-2} s^{-1} with a Li-COR 6400 Portable Photosynthesis System (Li-COR Biosciences, Lincoln, NE, USA). Six replicates were measured for each CO_2 concentration. With a chlorophyll meter (SPAD-502 :Konica Minolta, Langenhagen, Germany), we estimated the relative chlorophyll contents using 7 replicated plants from each line.

Protein extraction and In-gel MAPK activity assay

Protein extraction, concentration estimation, and MAPK assay were performed according to Wu *et al.* (2007).

Quantification of jasmonates, JA-Ile turnover and direct defense metabolites

For 12-oxo-phytodienoic acid (OPDA), JA, JA-Ile, and JA-Val analysis, leaf tissue was rapidly crushed in liquid nitrogen. Approximately 100 mg aliquots were combined with ~ 1 g ceramic beads (MP Biomedicals, Illkirch, France) in 2 mL reaction tubes. One mL ethyl acetate, spiked with 200 ng of JA[D^2], 40 ng of JA-[$^{13}C_6$]Ile as internal standards, was added to the tissue powder and the tissue was homogenized with a Geno/Grinder 2000 at 1700 strokes/min for 2 minutes (SPEX CertiPrep, Metuchen, New Jersey, USA). Plant tissue fragments were separated from the organic phase by a 10 min centrifugation at 4°C. The organic phase was collected and completely dried on a vacuum dryer (Eppendorf, Hamburg, Germany). The pellets were resuspended in 500 µL of 70% (v/v) methanol and centrifuged to remove insoluble particles. A HPLC-MS/MS (Varian, Palo Alto, CA, USA) was used to analyze the concentration of JA, JA-Ile, and JA-Val. To estimate JA-Ile turnover rates, a leaf area of 3 cm^2 was elicited by W+OS and 30 min later 500 ng of JA-[$^{13}C_6$]Ile were applied to the wounds (Paschold *et al.* 2008); these leaf regions were immediately harvested for quantifying initial JA-[$^{13}C_6$]Ile or were harvested after 90 min for estimating the remaining amount of JA-[$^{13}C_6$]Ile using HPLC-MS/MS as indicated above.

Source-sink transition leaves of WT and irNaNOA1 plants were harvested 3 days after W+OS treatment. About ~ 200 mg of fresh leaf tissue were used for protein extraction and quantification of TPI activity (Jongsma *et al.* 1994; Van Dam *et al.* 2001). Contents of nicotine, chlorogenic acid, rutin, diterpene glycosides, caffeoylputrescine and dicaffeoylspermidine were analyzed on an HPLC as described in Keinanen *et al.* (2001).

Analysis of free isoleucine contents

Leaves were harvested, frozen in liquid nitrogen and ground to a fine powder. Aliquots of ~ 100 mg were mixed with 300 µL of extraction buffer (0.1 M Tris-HCl, pH 7.6, 50 g L^{-1} PVPP, 2 g L^{-1} phenylthiourea, 5 g L^{-1} diethyldithiocarbamate) by vortexing for 2 min. Insoluble particles were separated by centrifugation at 16,000 g for 10 min. 200 µL of the supernatant was mixed with 300 µL of 0.5 M sodium borate (pH 11). After 15 min of incubation at room temperature samples were again centrifuged. A standard curve was created by injecting 1, 2, 5, and 10 µL of an amino acid standard solution (Sigma-Aldrich, Steinheim, Germany). The autosampler was programmed to inject 30 µL of OPA-2ME [potassium borate buffer (pH 11) containing 10 µL mL^{-1} 2-mercaptoethanol and 11.4 mg mL^{-1} *o*-phthaldialdehyde)] into each sample to derivatize amino acids; samples were mixed twice, incubated for 3 min and injected into HPLC. The HPLC running conditions were following de Kraker *et al*. (2007).

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Supplemental Figures and Table

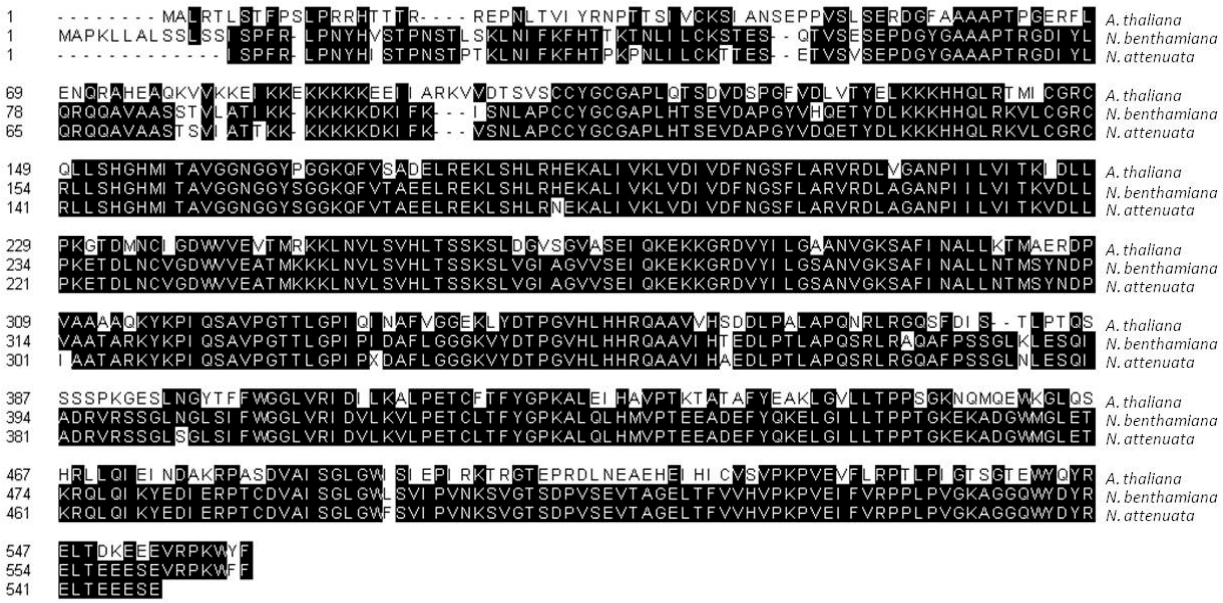

Figure S1. Protein sequence alignment of NaNOA1 and NOA1 in Arabidopsis and *N. benthamiana.*

AtNOA1 (NP_190329) and NbNOA1 (BAF93184) were retrieved from GenBank. The sequences were aligned using ClustalW algorithm. Shaded sequence sections indicate exact matches with the consensus sequence.

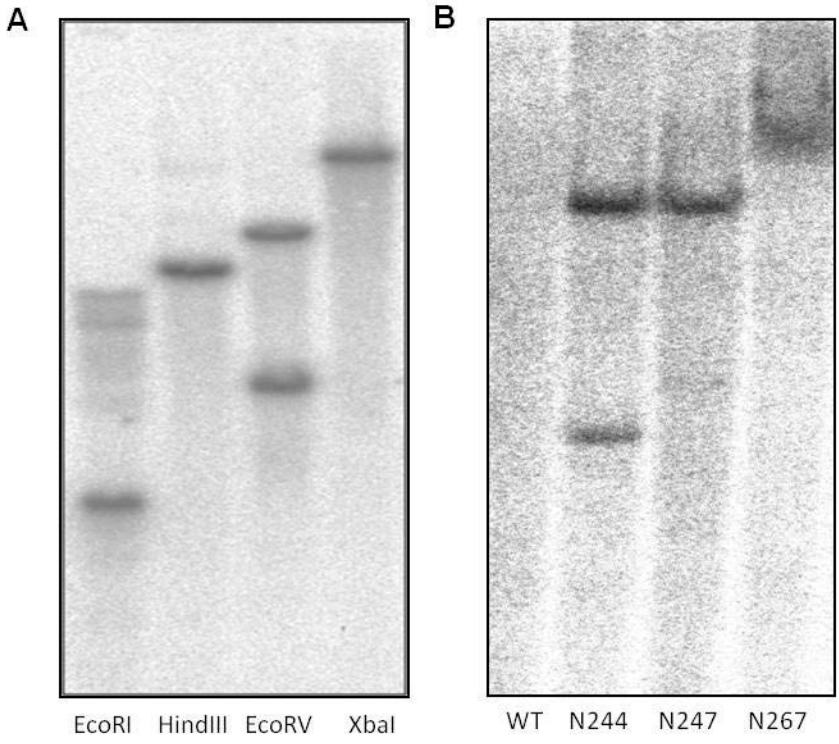

Figure S2. Southern blotting analyses of *NaNOA1* in wild-type *N. attenuata* and transgene numbers in irNaNOA1 plants.

(A) Wild-type (WT) *N. attenuata* genomic DNA was digested with various restriction enzymes. DNA was separated on a 1% agarose gel and blotted to a nylon membrane. The blot was hybridized with a partial *NaNOA1* cDNA probe. (B) Genomic DNA extracted from WT and irNaNOA1 lines was digested with *Eco*R V. DNA was separated on a 1% agarose gel and blotted to a nylon membrane. The blot was hybridized with a *hptII* (hygromycin resistance gene) probe.

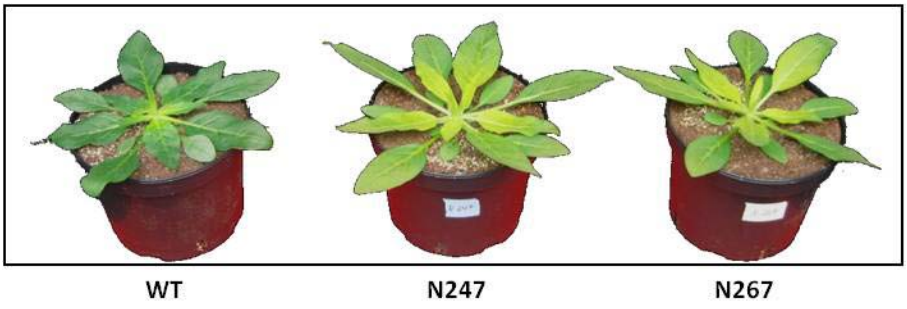

Figure S3. Morphology of wild-type (WT) and irNaNOA1plants.

Comparison of rosette-staged *Nicotiana attenuata* WT and irNaNOA1 plants (N247 and N267).

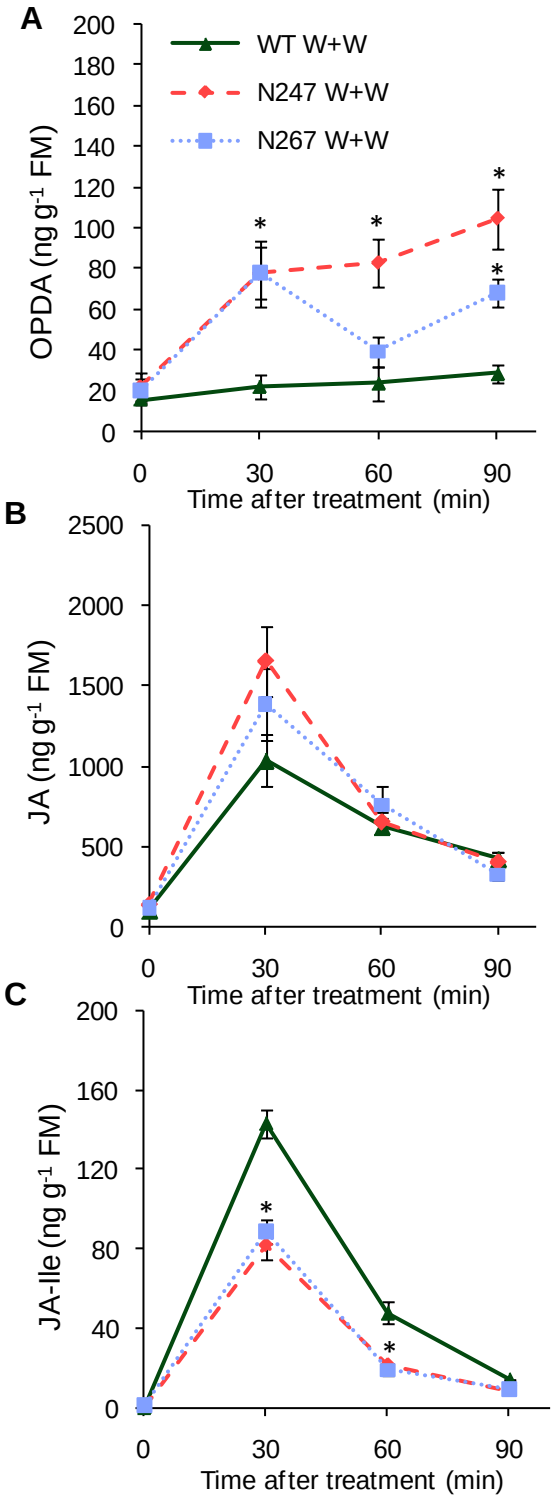

Figure S4. Silencing *NaNOA1* increases herbivory-induced OPDA and JA accumulation, but decreases JA-Ile accumulation after W+W treatment.

Wild-type (WT) and irNaNOA1 plants (N247, N267) were wounded with a pattern wheel and subsequently applied with 20 µL of water (W+W). Samples were collected at indicated times, and the contents (± SE) of (A) OPDA, (B) JA, and (C) JA-Ile were measured on a HPLC-MS/MS. Stars indicate significantly different levels between irNaNOA1 lines and WT plants ($p \leq 0.05$; Student's *t*-test; N = 4).

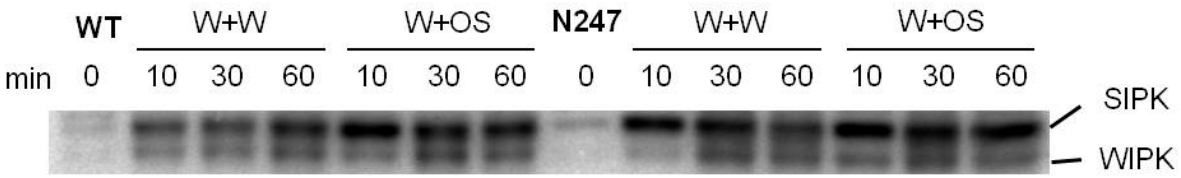

Figure S5. SIPK and WIPK activity in WT and irNaNOA1 plants.

In-gel kinase assay with myelin basic protein as the substrate was used to determine SIPK and WIPK activity. Rosette transition leaves of wild-type (WT) and irNaNOA1 (N247) plants were collected after W+W or W+OS treatment at the indicated time points. Five biological replicates were combined for each time point and treatment.

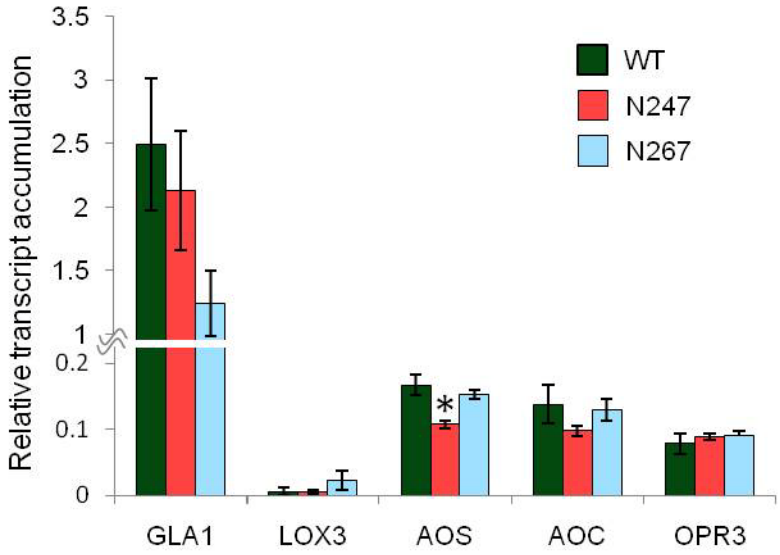

Figure S6. Quantitative real time PCR analysis of JA biosynthetic genes in wild-type and irNaNOA1 plants.

The relative transcript levels (± SE) of *GLA1*, *LOX3*, *AOS*, *AOC*, and *OPR3* were measured in uninduced transition leaves of wild-type (WT) and irNaNOA1 (N247, N267) plants. Star indicates significantly different levels between irNaNOA1 lines and WT plants ($p \leq 0.05$; Student's *t*-test, N = 5).

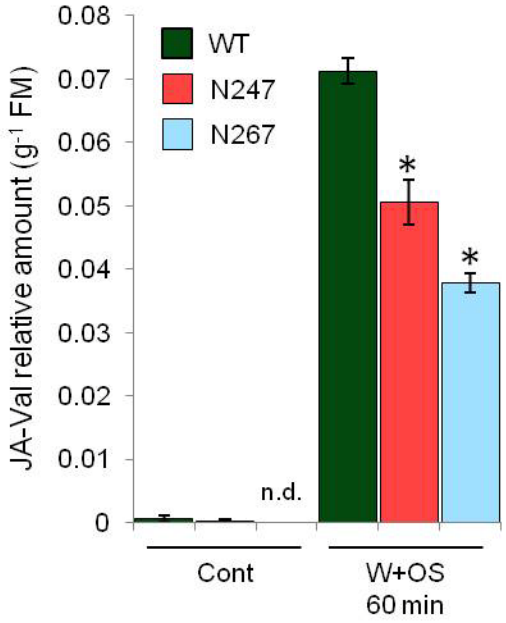

Figure S7. JA-Val accumulation is reduced in irNaNOA1 plants.

Leaves of wild-type (WT) and irNaNOA1 plants (N247, N267) were wounded with a pattern wheel and treated with 20 µl of *M. sexta* oral secretions (OS) (W+OS). JA-Val contents (± SE) were measured in WT, N247 and N267 plants before (Cont) and 60 min after W+OS treatment. Stars indicate significantly different levels between irNaNOA1 and WT plants ($p \leq 0.05$; Student's *t*-test, N = 5). n.d. = not detected.

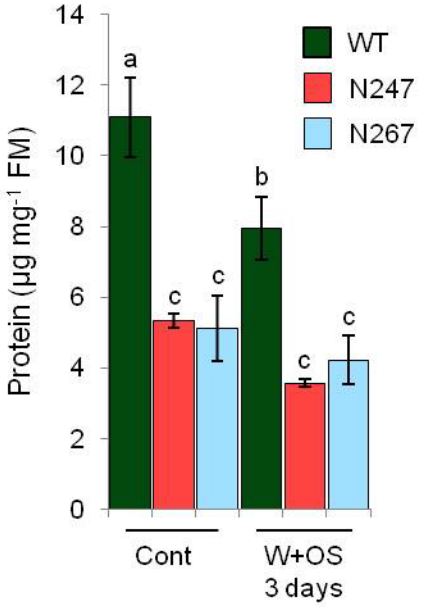

Figure S8. irNaNOA1 plants have reduced total protein contents.

Wild-type (WT) and irNaNOA1 plants were left untreated (Cont) or treated with W+OS. After 3 days samples were harvested for determination of protein contents (± SE). Different letters indicate significant differences (ANOVA, Fisher's PLSD; $F_{5,12} = 14.063$, $p = 0.0001$).

5'-TCCGAACTACCACATTTCAACTCCTAATTCTACACCTACCAAGCTTAACATCTTCAAATTC
CACACTCCCAAACCCAACCTTATCCTCTGCAAAACCACAGAATCAGAGACTGTGTCAGTATC
CGAACCCGATGGCTATGGAGCTGCCGCTCCGACCCGAGGCGACATATACCTTCAACGCCA
ACAGGCCGTGGCTGCATCTACGTCTGTGATAGCCACAACTAAGAAGAAAAAGAAGAAGAAA
GATAAAATCTTTAAAGTTTCCAACTTGGCTCCTTGCTGTTACGGTTGTGG-3'

Figure S9. Partial sequence of *NaNOA1* used for constructing pSOL8-irNaNOA1.

Supporting Information Table S1 Primer pairs and TaqMan probes used for qRT-PCR

Genes	Primer pairs	
NaNOA1	5'-GGAGCCAAGTTGGAAACTTTAAAGA	5'-ATGGCTATGGAGCTGCCGCT
GLA1	5'-AGTAGCAGATGATGTTAGTACATGTA	5'-ACATGTGAATATGCCCATGGCATACT
LOX3	5'- GGCAGTGAAATTCAAAGTAAGAGC	5'-CCCAAAATTTGAATCCACAACA
AOS	5'-GACGGCAAGAGTTTTCCCAC	5'-TAACCGCCGGTGAGTTCAGT
AOC	5'-ATCGTACTTGACTTACGAGGATACT	5'-TCACAAGCTTTAGCTTCAGGTGCTT
OPR3	5'-ATGCCAGATGGAACTCATGCTATTT	5'-TATGAATTTGCAACGGTTGGCTAGT
Actin	5'-GGTCGTACCACCGGTATTGTG	5'-GTCAAGACGGAGAATGGCATG
	Double fluorescent dye-labeled TaqMan probes	
Actin	5'-TCAGCCACACCGTCCCAATTTATGAGG	
LOX3	5'-CAGTGAGGAACAAGAACAAGGAAGATCTGAAG	

3. Chapter II

S-Nitrosoglutathione reductase (GSNOR) mediates resistance of *Nicotiana attenuata* to specialist insect herbivore *Manduca sexta*

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Abstract

The enzyme *S*-nitrosoglutathione reductase (GSNOR) is conserved from bacteria to plants and animals and plays an important role in the metabolism of nitric oxide (NO) by reducing nitrosylated glutathione (GSNO), an essential reservoir for NO bioactivity. Recent studies indicated that GSNOR is important for plant resistance to bacterial and fungal pathogens, but its role in herbivore resistance has not been examined. Using a virus-induced gene-silencing (VIGS) system, we knocked down the activity of NaGSNOR in a wild tobacco species, *Nicotiana attenuata*, and examined several traits important for defense against herbivory of specialist insect herbivore, *Manduca sexta*. Silencing *NaGSNOR* reduced herbivory-induced bursts of jasmonic acid (JA) and ethylene emissions, two important hormones regulating plant defense against herbivores. Consequently, highly reduced levels of trypsin proteinase inhibitor (TPI) activity were detected in *NaGSNOR*-silenced plants after *M. sexta* herbivory and *NaGSNOR*-silenced plants showed increased susceptible to *M. sexta* attack. We found that the attenuated levels of defense-related phytohormones did not result from compromised mitogen-activated protein kinase (MAPK) activity. Furthermore, after methyl jasmonate (MeJA) treatment the increased transcript levels of *TPI* and contents of defense-related secondary metabolites (TPI, caffeoylputrescine, and diterpene glycosides) were NaGSNOR-dependent; however, levels of *JAZ3* (*jasmonate ZIM-domain 3*) and *TD* (*threonine deaminase*) transcripts were not altered in *NaGSNOR*-silenced plants. Thus, NaGSNOR mediates certain but not all transcriptional accumulation of jasmonate-inducible genes. This work highlights the important role of GSNOR in plant resistance to herbivory and suggests potential involvement of NO and redox signaling in plant-herbivore interactions.

Chapter II GSNOR mediates plant resistance to herbivores

Introduction

Plants are constantly challenged by various environmental stresses, such as herbivory, pathogen infection, unfavorable temperatures, drought, and UV-B radiation. Accordingly, plants have evolved to cope with these stresses using sophisticated defense systems, which include receptors and sensors, highly complex regulatory networks, compounds and proteins that directly or indirectly protect plants from these unfavorable conditions (Chen, 2008; Dodds and Rathjen, 2010; Mittler, 2006; Wu and Baldwin, 2010).

Herbivores, especially insects, pose a great challenge for plant survival. Accordingly, plants have equipped with herbivory-specific defense systems to perceive herbivore attacks and deploy defense responses to optimize their fitness (Heil and Baldwin, 2002; Howe and Jander, 2008; Wu and Baldwin, 2010). Herbivory-induced defense responses have been intensively studied in *Nicotiana attenuata*, a native annual plant of the semi arid deserts which ranges from northwest Mexico, east to the Great Basin, and north to southern Canada (Baldwin, 2001; Kessler and Baldwin, 2002). Feeding of *Manduca sexta*, a specialist herbivore for *N. attenuata*, or the application of *M. sexta* larval oral secretions (OS) on wounded leaves activates signaling cascades that involve activation of mitogen-activated protein kinases (MAPKs) and bursts of jasmonic acid (JA), JA-isoleucine (JA-Ile), salicylic acid (SA), and ethylene (Kang *et al.*, 2006; Von Dahl *et al.*, 2007; Wu *et al.*, 2007).

Many studies in Arabidopsis, tomato, and *N. attenuata* have demonstrated the critical roles of JA biosynthesis and signaling for herbivory-induced defenses (Halitschke and Baldwin, 2003; Li *et al.*, 2005; Li *et al.*, 2004; McConn *et al.*, 1997; Paschold *et al.*, 2007). Recent studies revealed that JA-Ile, but not JA, activates most of JA-induced responses (Staswick and Tiryaki, 2004). JA-Ile binds to COI1 receptor and thus facilitates the degradation of JAZ (jasmonate ZIM-domain) proteins by the $SCF^{(COI1)}$ ubiquitin ligase-mediated process; JAZ proteins negatively regulate the activity of MYC2, the major activator of JA-induced transcriptional responses in Arabidopsis (Chini *et al.*, 2007; Thines *et al.*, 2007). In *N. attenuata*, several compounds have been identified to be important for the direct defense against herbivores. These include trypsin proteinase inhibitors (NaTPIs) (Zavala and Baldwin, 2004; Zavala *et al.*, 2004), nicotine (Steppuhn *et al.*, 2004), diterpene glycosides (DTGs) (Heiling *et al.*, 2010; Jassbi *et al.*, 2008), and the phenylpropanoid-polyamine conjugate caffeoylputrescine (CP) (Kaur *et al.*, 2010). Silencing the JA-Ile receptor *COI1* greatly impairs the accumulation of these metabolites and

dramatically attenuates *N. attenuata*'s resistance against *M. sexta* attack in the glasshouse and in nature (Paschold *et al.*, 2007). The function of SA in resistance to chewing insects remains largely elusive (Wu and Baldwin, 2010), although in some plant-herbivore interactions, SA appears to suppress JA accumulation (Diezel *et al.*, 2009). Compared with dramatic effect of JA, the role played by the gaseous hormone, ethylene, is more limited, potentiating JA-inducible proteinase inhibitors in tomato (*Solanum lycopersicum*) (O'Donnell *et al.*, 1996) and reducing *M. sexta* OS-induced nicotine accumulation in *N. attenuata* (Kahl *et al.*, 2000; Von Dahl *et al.*, 2007).

Emerging evidence has revealed other small molecules in the regulatory networks in plant-herbivore interactions (Wu and Baldwin, 2009, 2010). In tomato, reactive oxygen species (ROS) have been shown to be important for the transcript accumulation of several herbivore-resistant genes (Orozco-Cardenas *et al.*, 2001; Sagi *et al.*, 2004). Moreover, nitric oxide (NO), one of the reactive nitrogen species (RNS), seems to be also involved in herbivore defenses. Wounding induces NO production in marine macroalga (Ross *et al.*, 2006) and in Arabidopsis epidermal cells (Huang *et al.*, 2004). NO negatively regulates proteinase inhibitor transcript levels after wounding, systemin, oligosaccharides, and JA treatment (Orozco-Cardenas and Ryan, 2002).

Although a *bona fide* NO synthase has yet to been identified in higher plants, at least three genes are associated with NO levels: NOA (nitric oxide associated), NR (nitrate reductase), and GSNOR (*S*-nitrosoglutathione reductase) (Besson-Bard *et al.*, 2008; Wilson *et al.*, 2008). Unlike NOA and NR, which are positively associated with NO levels in plants (Guo *et al.*, 2003; Yamasaki and Sakihama, 2000), GSNOR is located in a NO removal pathway (Wilson *et al.*, 2008). NO rapidly reacts with glutathione and forms *S*-nitrosylated glutathione (GSNO), and GSNO is further metabolized into the oxidized glutathione disulfide (GSSG) and NH_3 by GSNOR. Consistent with the biochemical property of GSNOR, Arabidopsis *gsnor* mutant exhibits elevated NO levels, stunted growth, impaired flower development, and compromised thermotolerance (Lee *et al.*, 2008). Apart from its role in plant development and interaction with abiotic environmental factors, GSNOR also positively controls plant immunity to *Pseudomonas syringae* pv. *tomato* DC3000, *Blumeria graminis* (powdery mildew) and *Hyaloperonospora parasitica* (downy mildew) (Feechan *et al.*, 2005). In contrast, compared with wild-type,

Arabidopsis antisense *GSNOR* plants are less susceptible to *Peronospora parasitica* Noco2 (oomycete) (Rusterucci *et al.*, 2007).

Although the function of GSNOR in plant-pathogen interactions has been explored, its role in plant defense against herbivores has not. Here, we used a reverse genetic approach to investigate the role of GSNOR in *N. attenuata*'s inducible defense against the specialist herbivore *M. sexta*. Virus-induced gene silencing (VIGS) was employed to knock down the transcripts of *NaGSNOR*, and traits important in herbivore resistance were examined. We found that silencing *NaGSNOR* attenuates wounding- and simulated herbivory-induced levels of JA, JA-Ile, and ethylene, but not of SA. Accordingly, decreased accumulation of defensive compound, NaTPI, was detected in *NaGSNOR*-silenced plants and many, but not all jasmonate-inducible responses are compromised in *NaGSNOR*-silenced plants, indicating the involvement of NaGSNOR in transducing certain jasmonate-induced responses. Taken together, our data highlight the important role of NaGSNOR in plant defense against herbivores.

Results

Herbivory but not wounding transiently reduces the activity of NaGSNOR

A fragment of *NaGSNOR* [GenBank: HQ830156] with 967 bp was cloned from *N. attenuata* cDNA pool. The deduced NaGSNOR partial protein sequence showed 98% and 92% similarity to tomato (*Solanum lycopersicum*) SlGSNOR [GenBank: ADB43258] and Arabidopsis AtGSNOR1 [GenBank: NP_199207] (Martínez *et al.*, 1996), respectively (Fig. S1). In the Arabidopsis genome, *AtGSNOR1* is a single gene (Martínez *et al.*, 1996). Similarly, Southern blotting analysis indicated that *NaGSNOR* has only one copy in *N. attenuata* (Fig. S2).

Wounding and chemical components such as fatty-acid amino-acid conjugates (FACs) in the OS of herbivores, which are introduced into wounds during feeding, induce a myriad of reactions on transcriptomic, proteomic, and metabolomic levels (Howe and Jander, 2008; Wu and Baldwin, 2010). The transcript and protein levels of AtGSNOR1in Arabidopsis are down-regulated after wounding (Diaz *et al.*, 2003). To examine whether *M. sexta* herbivory leads to altered *NaGSNOR* transcript accumulation and NaGSNOR activity in *N. attenuata*, rosette leaves of *N. attenuata* were wounded with a pattern wheel and 20 µL of *M. sexta* larval oral secretions (OS) were immediately applied to wounds (W+OS); this treatment effectively mimics herbivory of *M. sexta* (Halitschke *et al.*, 2001). For comparison with mechanical wounding, 20 µL of water

were applied to wounds (W+W). Initially, *NaGSNOR* transcripts were slightly reduced 30 min after both treatments (W+W, W+OS), but regained the levels in control samples after 3 h (Fig. 1A). However, 6 h after W+W and W+OS treatment, *NaGSNOR* transcript levels increased 2.2- and 4.3-fold compared to those in control samples. We next examined whether the activity of NaGSNOR is regulated in *N. attenuata* after wounding and simulated herbivory. After W+W treatment, no obvious changes of NaGSNOR activity were found (Fig. 1B). W+OS treatment suppressed up to 30% of the NaGSNOR activity by 1 h; however, the activity regained the levels found in non-treated plants by 1.5 h (Fig. 1B), suggesting that OS-elicitation but not wounding, specifically and transiently reduces the activity of NaGSNOR.

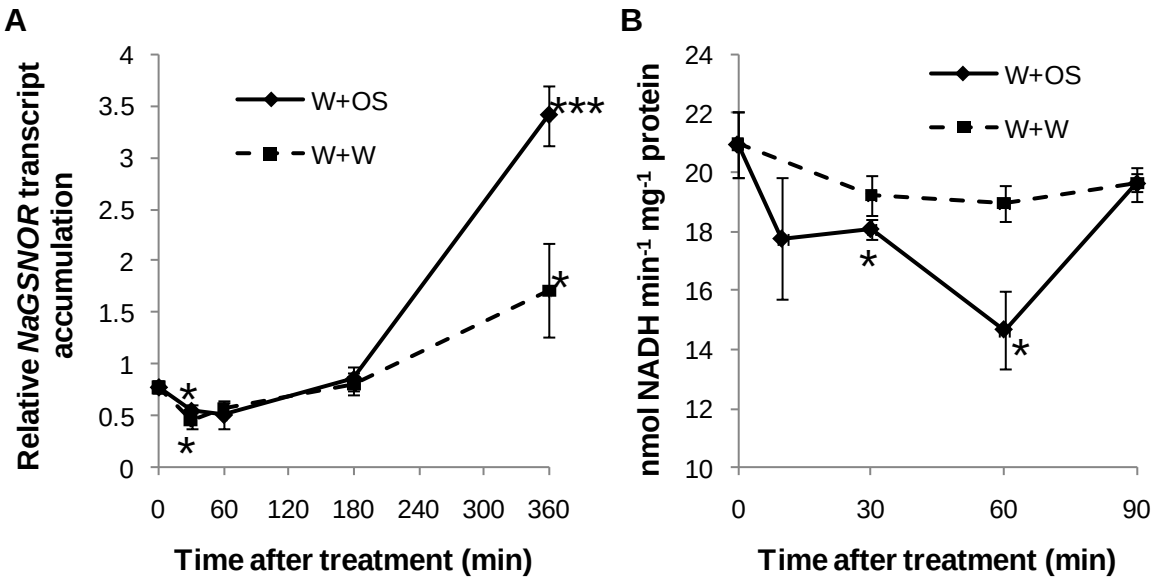

Fig. 1. *NaGSNOR* transcript accumulation and enzyme activity after wounding and simulated herbivory.

Transition leaves of *N. attenuata* rosette plants were wounded with a pattern wheel, and were subsequently treated with 20 µL of water (W+W) or 20 µL of *M. sexta* oral secretions (W+OS). Samples were harvested after indicated time. (A) Transcript levels (mean ± SE) of *NaGSNOR* were measured with qPCR. (B) Activity (mean ± SE) of NaGSNOR. Stars indicate significantly different levels between treated and non-treated samples (Student's *t*-test; *, $p \leq 0.05$; ***, $p \leq 0.001$; N = 5).

Silencing *NaGSNOR* impairs herbivory-induced accumulation of JA and ethylene

RNAi-based gene silencing was first used to generate plants stably silenced in *NaGSNOR*. However, all plants of T1 generation that were well silenced in *NaGSNOR* showed highly stunted growth, reduced apical dominance, epinastic leaves, and finally aborted all flower buds. Thus, a virus-induced gene silencing (VIGS) approach was employed to determine the role of *NaGSNOR*

in the defense of *N. attenuata* against wounding and herbivory of *M. sexta*. A pTV-NaGSNOR construct was prepared by inserting a partial *NaGSNOR* cDNA fragment into the pTV00 vector (Ratcliff *et al.*, 2001; Saedler and Baldwin, 2003). *N. attenuata* plants inoculated with *Agrobacterium* carrying pTV-NaGSNOR and empty vector (pTV00) were called *NaGSNOR*-VIGS and EV plants, respectively. VIGS efficiently reduced the transcript accumulation of *NaGSNOR* in *NaGSNOR*-VIGS plants to about 3% of that in EV plants (Fig. 2A); furthermore, the activity of NaGSNOR was 90% reduced in these plants (Fig. 2B). Consistent with the growth phenotype of Arabidopsis *gsnor* mutant (Lee *et al.*, 2008), the rosette sizes of *NaGSNOR*-VIGS were slightly smaller than those of EV plants (Fig. S3); furthermore, in the elongated stage of growth, *NaGSNOR*-VIGS plants showed stunted stalks with reduced flower buds and epinastic leaves. All experiments were done when plants were in the rosette stage of growth.

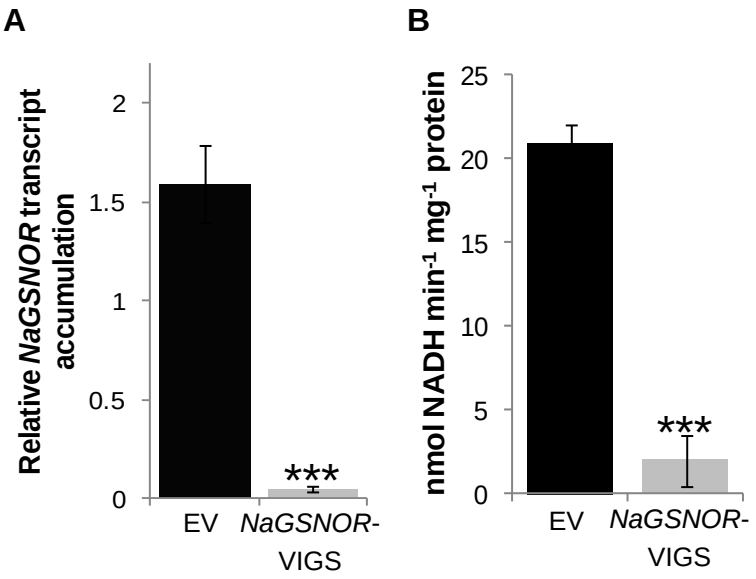

Fig. 2. *NaGSNOR*-VIGS plants have highly diminished transcript levels of *NaGSNOR* and strongly reduced GSNOR activity.

N. attenuata plants were infiltrated with *Agrobacterium* carrying pTV00 or a pTV-NaGSNOR to generate EV and *NaGSNOR*-VIGS plants, respectively. (A) Transcript levels (mean ± SE) of *NaGSNOR* and (B) GSNOR activity (mean ± SE) were determined in EV and *NaGSNOR*-VIGS plants. Stars indicate significantly different levels between EV and *NaGSNOR*-VIGS plants (Student's *t*-test; ***, $p \leq 0.001$; N = 5).

Given the central roles of phytohormones in regulating plant resistance to herbivores, we determined whether the silencing of *NaGSNOR* alters wounding and simulated herbivory-induced

levels of JA/JA-Ile and ethylene. EV and *NaGSNOR*-VIGS plants were treated either with W+W or W+OS and JA contents were analyzed in samples collected 30, 60, and 90 min after treatment. In EV plants, compared with W+W, W+OS elicited 2-fold higher levels of JA 1 h after treatments, indicating that *N. attenuata* recognized herbivore elicitors, FACs, in *M. sexta* OS and accumulated high contents of JA (Fig. 3A). In contrast, 1 h after W+OS treatment, JA contents in *NaGSNOR*-VIGS plants were about half those found in EV plants; *NaGSNOR*-VIGS plants challenged with W+W also showed a reduced JA accumulation (Fig. 3A). Consistently, the JA-Ile levels were also decreased in *NaGSNOR*-VIGS plants after W+W and W+OS treatment (Fig. 3B). Due to the antagonistic nature between the JA and salicylic acid (SA) signaling pathway, it is possible that the suppressed JA levels in *NaGSNOR*-VIGS resulted from high SA contents in these plants (Pieterse *et al.*, 2009). However, when untreated, no significantly different levels of SA were detected between EV and *NaGSNOR*-VIGS; after W+OS and W+W treatments these two types of plants also showed comparable SA levels (Fig. 3C). Wounding does not increase ethylene emission from *N. attenuata* (Von Dahl *et al.*, 2007); hence ethylene emission was measured in control and W+OS-treated plants. After W+OS, *NaGSNOR*-VIGS plants exhibited markedly reduced ethylene emission compared to EV plants (about 43% reduced) (Fig. 3D).

Thus, we infer that *NaGSNOR* is required for the production of herbivory-induced phytohormones, JA and ethylene, in *N. attenuata*.

NaGSNOR-VIGS plants do not have altered activity of SIPK and WIPK

In *N. attenuata*, SIPK and WIPK are important for wounding- and herbivory-induced JA and ethylene biosynthesis (Wu *et al.*, 2007). Using an in-gel kinase activity assay, SIPK and WIPK activity was determined in EV and *NaGSNOR*-VIGS plants 10, 30, and 60 min after W+W and W+OS treatment (Fig. 4). In EV plants, W+W and W+OS rapidly activated SIPK within 10 min, and compared with W+W, W+OS elicited higher levels of SIPK activity. Low WIPK activity was only detected in W+OS-induced samples. Importantly, EV and *NaGSNOR*-VIGS plants showed similar levels of SIPK and WIPK activity (Fig. 4). Therefore, the decreased JA and ethylene levels in NaGSNOR-VIGS were not due to impaired MAPK activation.

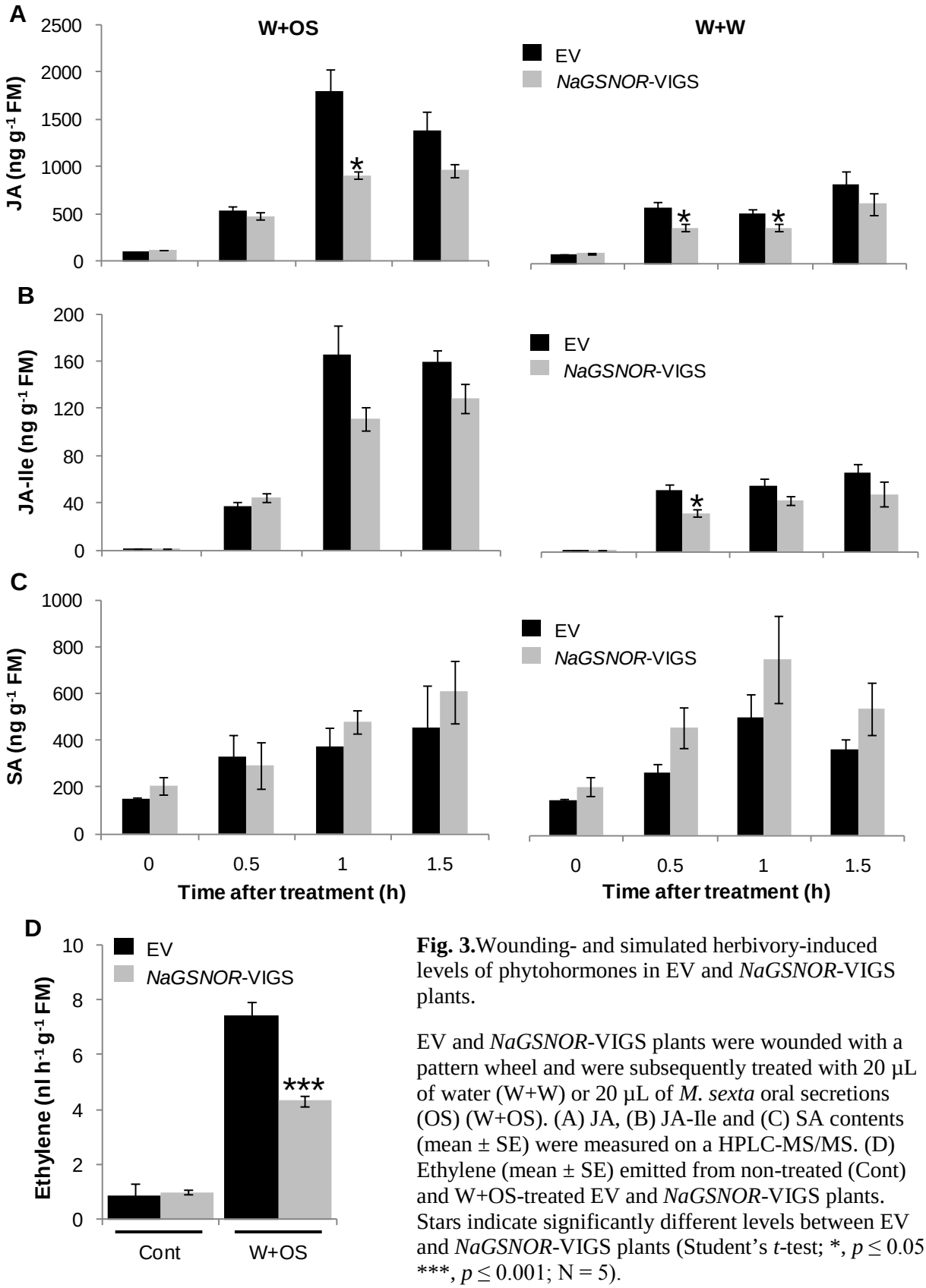

Fig. 3. Wounding- and simulated herbivory-induced levels of phytohormones in EV and *NaGSNOR*-VIGS plants.

EV and *NaGSNOR*-VIGS plants were wounded with a pattern wheel and were subsequently treated with 20 µL of water (W+W) or 20 µL of *M. sexta* oral secretions (OS) (W+OS). (A) JA, (B) JA-Ile and (C) SA contents (mean ± SE) were measured on a HPLC-MS/MS. (D) Ethylene (mean ± SE) emitted from non-treated (Cont) and W+OS-treated EV and *NaGSNOR*-VIGS plants. Stars indicate significantly different levels between EV and *NaGSNOR*-VIGS plants (Student's *t*-test; *, $p \leq 0.05$; ***, $p \leq 0.001$; N = 5).

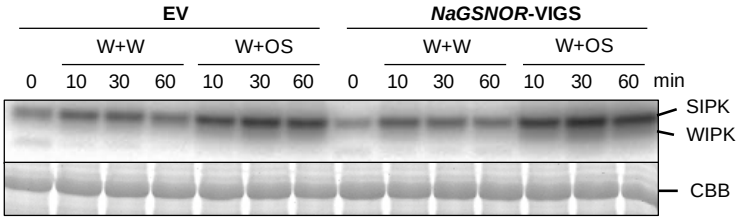

Fig. 4. Silencing *NaGSNOR* does not impair wounding- and simulated herbivory-induced MAPK activity in *N. attenuata*.

EV and *NaGSNOR*-VIGS plants were wounded with a pattern wheel and were subsequently applied with 20 µL of water (W+W) or 20 µL of *M. sexta* oral secretions (OS) (W+OS). Samples were harvested after indicated times. An in-gel kinase activity assay (upper panel) was performed to detect the activity of SIPK and WIPK. Replicated samples were run on a SDS-PAGE gel, and this gel was thereafter stained with Coomassie Brilliant Blue (CBB) for visualization of equal loading (lower panel).

Wounding- and herbivory-induced levels of NaTPI are compromised in *NaGSNOR*-VIGS plants

TPIs are important anti-herbivore compounds in solanaceous plants, including *N. attenuata* (Haq *et al.*, 2004; Ryan, 1989; Zavala *et al.*, 2004). To determine the function of NaGSNOR in regulating defense response to wounding and herbivory, defense metabolites were determined in EV and *NaGSNOR*-VIGS plants 3 days after W+W or W+OS. NaTPI activity was not inducible after W+W and W+OS treatment in *NaGSNOR*-VIGS, whereas W+OS treatment elicited a 3.3 fold increase in EV plants (Fig. 5A). VIGS requires growing plants under reduced temperatures, which significantly influences secondary metabolism and can selectively alter the amount of particular secondary metabolites in plant tissue (Kaplan *et al.*, 2004; Shohael *et al.*, 2006). The concentrations of other known JA-inducible secondary metabolites such as CP, DTGs, and nicotine did not increase after wounding and simulated herbivory treatment, even in EV plants (Fig. 5B, C, D).

To evaluate the resistance levels of *NaGSNOR*-silenced plants against *M. sexta* attack, *M. sexta* growth bioassays were performed. Neonate *M. sexta* larvae were grown 14 days on rosette EV and *NaGSNOR*-VIGS plants and their masses were recorded on day 4, 9, and 14. Average final larval mass on EV plants (102 mg) was only 54% of the mean mass of those reared on *NaGSNOR*-VIGS plants (186 mg) (Fig. 6).

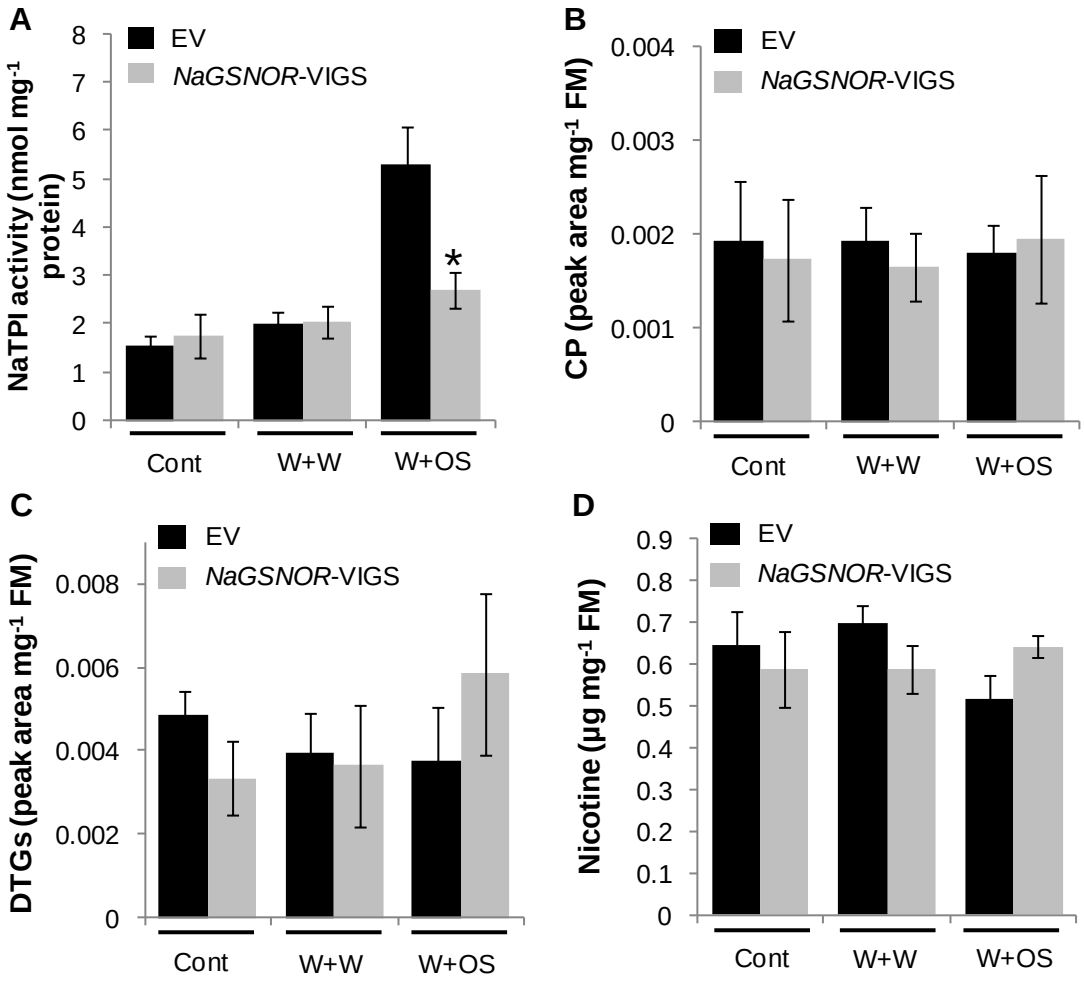

Fig. 5. Accumulation of herbivore defense-related secondary metabolites in EV and *NaGSNOR*-VIGS plants.

Leaves of EV and *NaGSNOR*-VIGS plants were wounded with a pattern wheel, and were thereafter applied with 20 µL of water (W+W) or 20 µL of *M. sexta* oral secretions (W+OS). The activity of NaTPI (A), contents of caffeoylputrescine (CP) (B), diterpene glycosides (DTGs) (C), and nicotine (D) (mean ± SE) were determined in EV and *NaGSNOR*-VIGS plants 3 days after treatments; non-treated plants served as controls (Cont). Star indicates significantly different levels between EV and *NaGSNOR*-VIGS plants (Student's *t*-test; *, $p \leq 0.05$; N = 5).

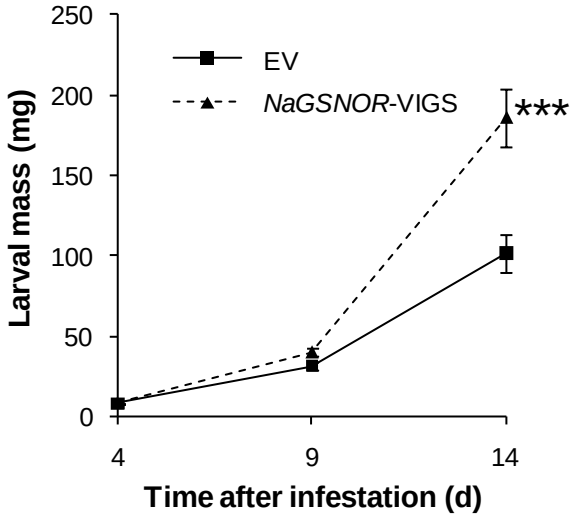

Fig. 6. Silencing *NaGSNOR* in *N. attenuata* compromises plant resistance to insect herbivore, *M. sexta.*

Neonate *M. sexta* larvae were placed on rosette-staged EV and *NaGSNOR*-VIGS plants and larval masses (mean ± SE) were measured after 4, 9, and 14 days. Stars indicate significantly different larval masses between those fed on EV and on *NaGSNOR*-VIGS plants (Student's *t*-test; *, $p \leq 0.05$; ***, $p \leq 0.001$; N = 30).

NaGSNOR-VIGS plants have altered methyl jasmonate-induced responses

Changing NO levels by supplying NO donors to tomato leaves strongly suppresses transcript levels and activity of proteinase inhibitors, whereas levels of several other JA-inducible transcripts are not altered (Orozco-Cardenas and Ryan, 2002). Thus, we determined if silencing *NaGSNOR* also compromises the accumulation of *NaTPI* transcript levels, and other JA-inducible genes and secondary metabolites.

Methyl jasmonate (MJ) in 20 µL of lanolin (5 µg/µL) was applied to plants, and plants treated with 20 µL of pure lanolin were used as controls. Defense metabolites (NaTPI, CP, and DTG) were measured 3 days after these treatments. When treated with lanolin, *NaGSNOR*-VIGS plants exhibited 1 fold higher levels of NaTPI activity than did EV plants (Fig. 7A). After MJ application, NaTPI activity levels increased 9.5 fold in EV plants, while only 1.7-fold in *NaGSNOR*-VIGS (Fig. 7A). Likewise, MJ application highly increased the levels of CP and DTGs in EV, but *NaGSNOR*-VIGS plants had only about 30% and 50% of the CP and DTG contents found in EV plants (Fig. 7B, C). Probably due to the relatively low growing temperatures, neither MJ treatment nor silencing *NaGSNOR* altered the levels of nicotine in any plants (Fig. 7D).

In addition, the transcript levels of several JA-inducible genes were examined. Consistent with the attenuated NaTPI activity in *NaGSNOR*-silenced plants, MJ treatment induced 4 fold

higher *NaTPI* transcript levels in EV plants than in *NaGSNOR*-VIGS plants (Fig. 8A). Although compared with those in EV plants, somewhat lower and higher transcript levels of *NaJAZ3* (*jasmonate ZIM-domain 3*) and *NaTD* (*threonine deaminase*) were found in control plants, after MJ treatment, transcript levels of *NaJAZ3* and *NaTD* were the same in *NaGSNOR*-VIGS and EV plants (Fig. 8B, C).

Therefore, it is inferred that NaGSNOR is required for certain, but not all, JA-induced responses in *N. attenuata*.

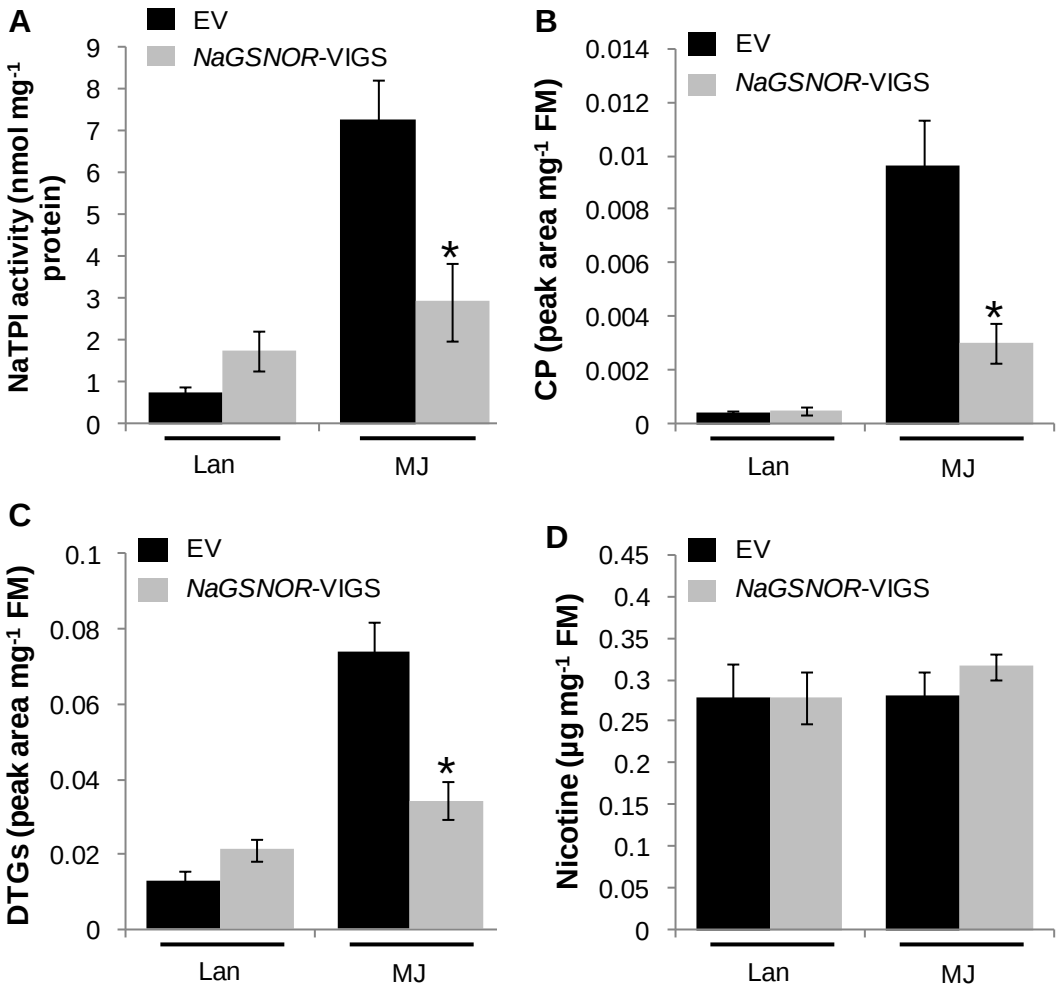

Fig. 7. Herbivore defense-related secondary metabolites in EV and *NaGSNOR*-VIGS plants after methyl jasmonate treatment.

EV and *NaGSNOR*-VIGS plants were applied with lanolin pastes (20 µL) containing 5 mg/mL methyl jasmonate (MJ) or pastes of pure lanolin (20 µL) for comparisons. The activity of NaTPI (A), contents of caffeoylputrescine (CP) (B), diterpene glycosides (DTGs) (C), and nicotine (D) (mean ± SE) were

determined in EV and *NaGSNOR*-VIGS plants 3 days after treatments. Stars indicate significantly different levels between EV and *NaGSNOR*-VIGS plants (Student's *t*-test; *, $p \leq 0.05$; N = 5).

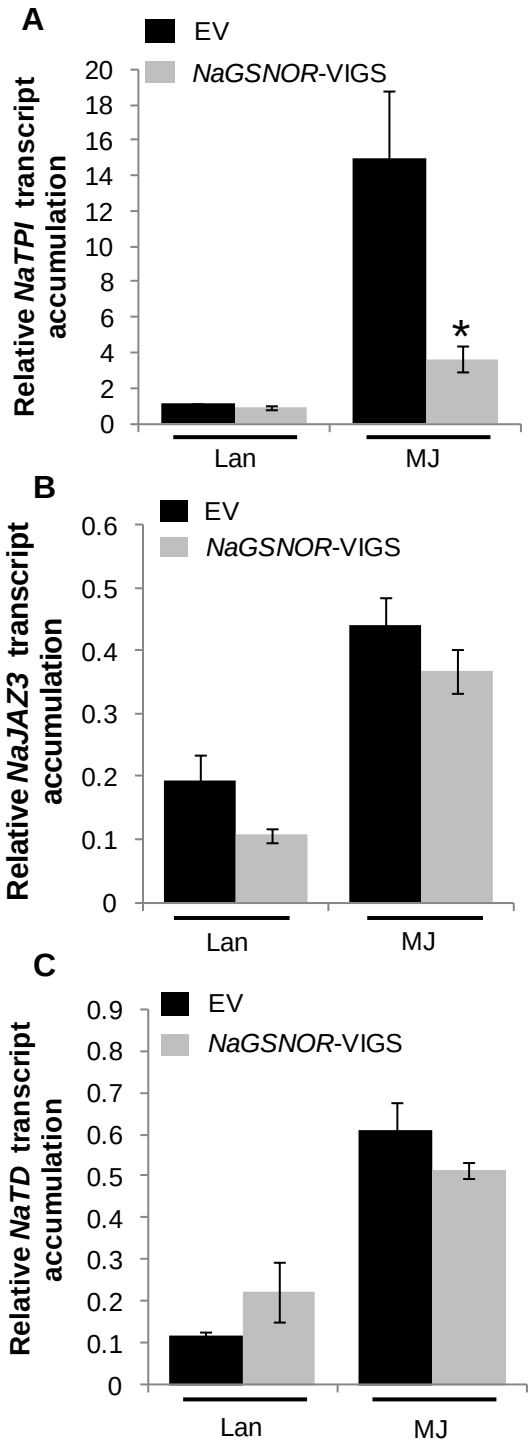

Fig. 8. Transcript levels of *NaTPI*, *NaJAZ3*, and *NaTD* in methyl jasmonate-treated EV and *NaGSNOR*-VIGS plants.

EV and *NaGSNOR*-VIGS plants were applied with lanolin pastes (20 µL) containing 5 mg/mL methyl jasmonate (MJ), or pastes of pure lanolin (20 µL) for comparisons. The transcript levels of *NaTPI* (A), *NaJAZ3* (B), and *NaTD* (C) (mean ± SE) were determined in EV and *NaGSNOR*-VIGS plants 8 h after treatments. Stars indicate significantly different levels between EV and *NaGSNOR*-VIGS plants (Student's *t*-test; *, $p \leq 0.05$; N = 5).

Discussion

GSNOR contains structural features that are highly conserved in bacteria, animals and plants (Fliegmann and Sandermann, 1997; Liu *et al.*, 2001; Martínez *et al.*, 1996). In mice, silencing *GSNOR* leads to increased damage in the lymphatic and liver tissue after being challenged with bacterial endotoxin (Liu *et al.*, 2004). In contrast to the immune suppressing function of GSNOR in mice, AtGSNOR1 is a positive regulator of plant immunity against phytopathogens (Feechan *et al.*, 2005). In *N. attenuata*, NaGSNOR plays an essential role in mediating wounding- and *M. sexta* herbivory-induced responses.

NO rapidly reacts with glutathione and forms GSNO; in addition, it modifies cysteine and tyrosine residues in proteins and therefore forms nitrosylated cysteine and tyrosine. Consistent with the biochemical function of GSNOR, $GSNOR^{-/-}$ mutant mice have high *S*-nitrosothiol (SNO) hemoglobin levels in red blood cells, which is likely associated with increased NO levels (Liu *et al.*, 2004). Similarly, Arabidopsis *gsnor* mutant also exhibits greatly elevated levels of NO, nitrate, SNO, and *N*-nitroso species (Feechan *et al.*, 2005; Lee *et al.*, 2008). Many proteins, especially those involved in signal transduction, are targets of nitrosylation (Besson-Bard *et al.*, 2008; Grennan, 2007; Lindermayr *et al.*, 2005). In agreement with this, NaGSNOR is required for wounding- and simulated herbivory-induced accumulation of phytohormones (JA/JA-Ile and ethylene) and NaGSNOR is also important for certain responses induced by JA, including the accumulation of defense-related secondary metabolites, suggesting its role in transducing certain aspects of JA signaling.

In plants, JA plays a central role in defense against herbivore stress (Howe and Jander, 2008; Kessler *et al.*, 2004; Wu and Baldwin, 2010). Although almost all the enzymes involved in JA biosynthesis have been identified in various plant species (Wasternack, 2007), little is known about how JA accumulation is regulated. Our data indicated that NaGSNOR is positively associated with the levels of wounding- and herbivory-induced JA in *N. attenuata*. However, how NaGSNOR is involved in the regulation of JA homeostasis remains elusive. It is possible that *NaGSNOR*-silenced plants over-accumulate GSNO (a source of NO) which may nitrosylate certain JA biosynthetic enzymes and thus decrease their activity. At least one enzyme in the oxylipin pathway for JA biosynthesis, allene oxide cyclase (AOC), has been identified to be a nitrosylation target (Romero-Puertas *et al.*, 2008). Studies in many plant species demonstrated that SA suppresses the accumulation of JA (Diezel *et al.*, 2009; Pieterse *et al.*, 2009; Spoel *et al.*,

2003), and NPR1 (nonexpresser of PR genes) is important for the suppression effect of SA on JA accumulation and signaling (Spoel *et al.*, 2003). Importantly, NPR1 is also nitrosylated *in planta* and the nitrosylation is important for the homeostasis of NPR1 (Tada *et al.*, 2008). Recently, Lindermayr *et al.* (2010) demonstrated that GSNO nitrosylates both NPR1 and TGA1, an important transcription factor that activates transcription of *PR* (*pathogenesis-related*) genes after binding of NPR1; furthermore, translocation of NPR1 to nucleus, which is required for the activation of NPR1-induced responses, requires NO. Therefore, we speculate that despite similar levels of SA in EV and *NaGSNOR*-VIGS plants, the inferred elevated levels of GSNO may increase nitrosylation of NPR1 and thereby enhance NPR1 activity, promoting the suppression of JA accumulation by SA. This hypothesis needs to be examined further.

Compared with JA biosynthesis, ethylene production requires fewer enzymes. Methionine is converted to *S*-adenosylmethionine (*S*-AdoMet) by *S*-AdoMet synthases (SAMSs), and the conversion of *S*-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) is mediated by ACC synthases (ACSs). ACOs (ACC oxidases) further catalyze the oxidation of ACC to form ethylene (Wang *et al.*, 2002). Among these key enzymes, SAMSs (also methionine adenosyltransferases, MATs) have been found to be targets of nitrosylation (Lindermayr *et al.*, 2005), and an *in vitro* assay suggested that nitrosylation of certain SAMS inhibits its activity (Lindermayr *et al.*, 2006). Consistent with this scenario, in *NaGSNOR*-silenced plants, herbivory-induced ethylene emissions are greatly compromised. Whether silencing *NaGSNOR* alters the activity of other ethylene biosynthetic enzymes (ACSs and ACOs) also requires further study.

In *N. attenuata*, SIPK and WIPK are regulators of wounding- and herbivory-induced biosynthesis of JA (Wu *et al.*, 2007). Moreover, activation of SIPK in *N. attenuata* and its homologue (AtMPK6) in Arabidopsis is required for 50% of the ethylene emitted after herbivory and pathogen elicitor (flagellin) elicitation (Liu and Zhang, 2004; Wu *et al.*, 2007). However, kinase activity assays revealed either that NaGSNOR modulates the levels of JA and ethylene in an MAPK-independent manner or NaGSNOR functions downstream of MAPKs.

Supplying excised tomato leaves with NO donors strongly inhibits JA-induced proteinase inhibitor expression and activity; however, JA-induced transcript levels of several signaling pathway-related genes are not altered (Orozco-Cardenas and Ryan, 2002). Similarly, NaGSNOR appears to be important for some but not all JA-induced responses: after MJ treatment, NaGSNOR activity is required for sufficient up-regulation of the genes that are involved in the

biosynthesis of NaTPI, CP, and DTGs, but is not important for transcriptional regulation of *NaJAZ3* and *NaTD*. It is very unlikely that silencing NaGSNOR compromises the activity of JA-Ile receptor, COI1, or the activity of $SCF^{(COI1)}$ complex, given that at least two JA-inducible genes, *NaJAZ3* and *NaTD*, have similar levels of transcripts in EV and *NaGSNOR*-VIGS plants after MJ induction. This also ruled out the possibility that *NaGSNOR*-VIGS plants have decreased activity of MJ esterase, which releases active JA from inactive MJ (Wu *et al.*, 2008). In addition to its function in suppression of JA accumulation, NPR1 also plays a critical role in mediating the antagonism between SA and JA signaling (Pieterse *et al.*, 2009). Whether NaGSNOR-deficient plants have enhanced NPR1 activity and therefore have elevated inhibition of certain JA-induced responses needs to be examined.

After wounding, Arabidopsis GSNOR exhibits reduced abundance of both transcripts and protein (Diaz *et al.*, 2003), and this is congruent with increased NO levels induced by wounding (Huang *et al.*, 2004). Recently, wounding was also found to attenuate the activity of GSNOR in sunflower seedlings (Chaki *et al.*, 2010). Although wounding does not alter the activity of NaGSNOR in *N. attenuata*, simulated herbivory induces a transient decline. These data suggest that compared with mechanical wounding, herbivory not only specifically modifies transcript levels of various genes, abundance of proteins and secondary metabolites, but also the redox and protein posttranslational modification (e.g. nitrosylation) status of cells (Foyer and Noctor, 2005; Moreau *et al.*, 2010; Stamler *et al.*, 2001). Given that diminishing the activity of NaGSNOR using gene silencing compromises plant resistance to *M. sexta*, the rapid reduction and subsequent regaining of NaGSNOR activity after herbivory implies that a transient decrease of NaGSNOR activity is required for optimum induction of herbivory-specific defense reactions, which involves reconfiguration of redox and nitrosylation status.

Materials and Methods

Plant growth, plant treatment, and herbivore performance assay

Seeds of *N. attenuata* Torr. Ex Watts were from a line that had been inbred for 30 generations. Germination and plant cultivation followed Krügel *et al.* (2002). Plants were transferred into 1 L pots 20 days after germination on Petri dishes, and were grown in a climate chamber at 22°C and under 65% humidity and 16 h light provided by Philips Sun-T Agro 400 sodium lights (Philips, Turnhout, Belgium). Herbivory was simulated by wounding the rosette

sink-source transition leaves of *N. attenuata* with a pattern wheel and immediately applying 20 µL of 1/5 diluted oral secretions (OS) (W+OS) from *M. sexta* to the puncture wounds; plants whose puncture wounds were treated with 20 µL of water (W+W) were used for comparisons. For treatment with methyl jasmonate (MJ), MJ was dissolved in heat-liquefied lanolin (5 mg/mL) and 20 µL of MJ-lanolin paste were applied on the basal part of a leaf; leaves treated with pure lanolin served as controls. All samples were immediately frozen in liquid nitrogen after harvesting and stored in -80 °C until analyses. Neonate *M. sexta* larvae from laboratory colonies were placed on EV and *NaGSNOR*-VIGS plants (1 larvae/plant), and the larval masses were measured on day 4, 9, and 14 (30 replicates were used for this bioassay).

Cloning of NaGSNOR, virus-induced gene-silencing, and Southern blotting analysis

The partial sequence of *NaGSNOR* was amplified from *N. attenuata* cDNA by PCR with primer pair NaGNSOR-1 (5'-GAACCCAACAAGCCTCTGGT-3') and NaGSNOR-2 (5'-CATCCACCTTGATTTCCTTCT-3'). The amplified fragment was cloned into the pJET1.2 vector (Fermentas, St. Leon-Rot, Germany) and sequenced. A 326 bp fragment of *NaGSNOR* was cloned into the pTV00 vector to generate the pTV-NaGSNOR construct, which was then transformed into *Agrobacterium tumefaciens* (Ratcliff *et al.*, 2001). Virus-induced gene silencing was done according to Saedler and Baldwin (2003). The initiation of silencing was visually monitored using *phytoene desaturase* (*NaPDS*)-silenced plants, which showed a photo-bleaching phenotype about 2 weeks after inoculation of *A. tumefaciens* carrying pTV-NaPDS (Saedler and Baldwin, 2003).

The restriction enzymes *Eco*R I, *Hind* III, *Eco*R V, or *Xba* I were used to digest DNA of *N. attenuata*. Five µg of digested DNA were separated on an 1% agarose gel and then were further blotted on a nylon membrane. Hybridization was performed according to Wu *et al.* (2006) using a probe prepared with the primer pair NaGSNOR-F1 (5'-CCTCTGGTGATCGAGGATGT-3') and NaGSNOR-R1 (5'-TCTCCTGGCTGAACCTCAGT-3').

Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract RNA. All qRT-PCR experiments were performed using 5 biological replicates. cDNA was synthesized from 500 ng of total RNA using Superscript II reverse transcriptase (Invitrogen). qRT-PCR analyses were

performed on a Stratagene MX3005P (Agilent Technologies, Santa Clara, CA, USA) using qPCR SYBR Green kits (Eurogentec, Seraing, Belgium). The sequences of primer pairs are listed in Supplementary Table S1.

GSNOR activity assays

GSNOR activity was measured spectrophotometrically at 340 nm using a modified method as described in Sakamoto *et al.* (2002). In brief, approximately 30 mg of frozen tissue powder were extracted with 300 µL of 0.05 M HEPES buffer (pH 8) containing 20% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine and 1 mM ε-aminocaproic acid. The samples were centrifuged at 4 °C, 16,000 g for 15 min and the supernatant were desalted using protein desalting spin columns (Thermo Fischer Scientific, Rockford, IL, USA). Protein concentrations were determined and 30 µL of desalted protein samples containing about 70 - 120 µg of proteins were added to 300 µL of assay mix [20 mM Tris-HCl (pH 8), 0.2 mM NADH, and 0.5 mM EDTA]. The NADH decomposition without GSNO was observed for 75 seconds. The enzymatic reaction was started by adding 10 µL of a GSNO solution into the assay mix to achieve a final GSNO concentration of 400 µM. The resulting GSNOR activity was expressed as nmol NADH degraded per min per mg protein.

Quantification of JA, JA-Ile, SA, ethylene, and direct defense metabolites

Five biological replicates were used for quantification of JA, JA-Ile, and ethylene. For JA and JA-Ile analysis, about 100 mg of frozen and rapidly crushed leaf tissue were added into 2 mL Eppendorf tubes containing 1 g of ceramic beads (MP Biomedicals, Illkirch, France). After adding 1 mL of ethyl acetate that was spiked with 200 ng of JA[D_2], 40 ng of JA-[$^{13}C_6$]Ile and 40 ng of SA[D_4] as internal standards, the tissue was homogenized on a Geno/Grinder 2000 at 1700 strokes/min for 2 min (SPEX CertiPrep, Metuchen, New Jersey, USA). After 10 min centrifugation at 4 °C and 13,000 g, the supernatants were transferred to fresh tubes and completely dried on a vacuum dryer (Eppendorf, Hamburg, Germany). The pellets were extracted with 500 µL of 70% (v/v) methanol, and samples were cleared with another centrifugation step. A HPLC-MS/MS (Varian, Palo Alto, CA, USA) was used to analyze the concentration of JA and JA-Ile in the supernatants. For ethylene quantification, leaves were untreated as controls or treated with W+OS, and after recording their fresh mass they were immediately sealed in 250 mL three-neck round bottom flasks for 4 h under light. The ethylene contents in the flasks were

measured on a photoacoustic laser spectrometer (INVIVO, Bonn, Germany) by comparing sample ethylene peak areas with peak areas generated by an ethylene standard (Von Dahl *et al.*, 2007).

For analyses of TPI activity, leaves were ground in liquid nitrogen and ~ 200 mg of leaf tissue were used for protein extraction and quantification of TPI activity (Jongsma *et al.*, 1994; Van Dam *et al.*, 2001). Contents of nicotine, diterpene glycosides, and caffeoylputrescine were analyzed on an HPLC as described in Keinanen *et al.* (2001).

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Supplemental Figures

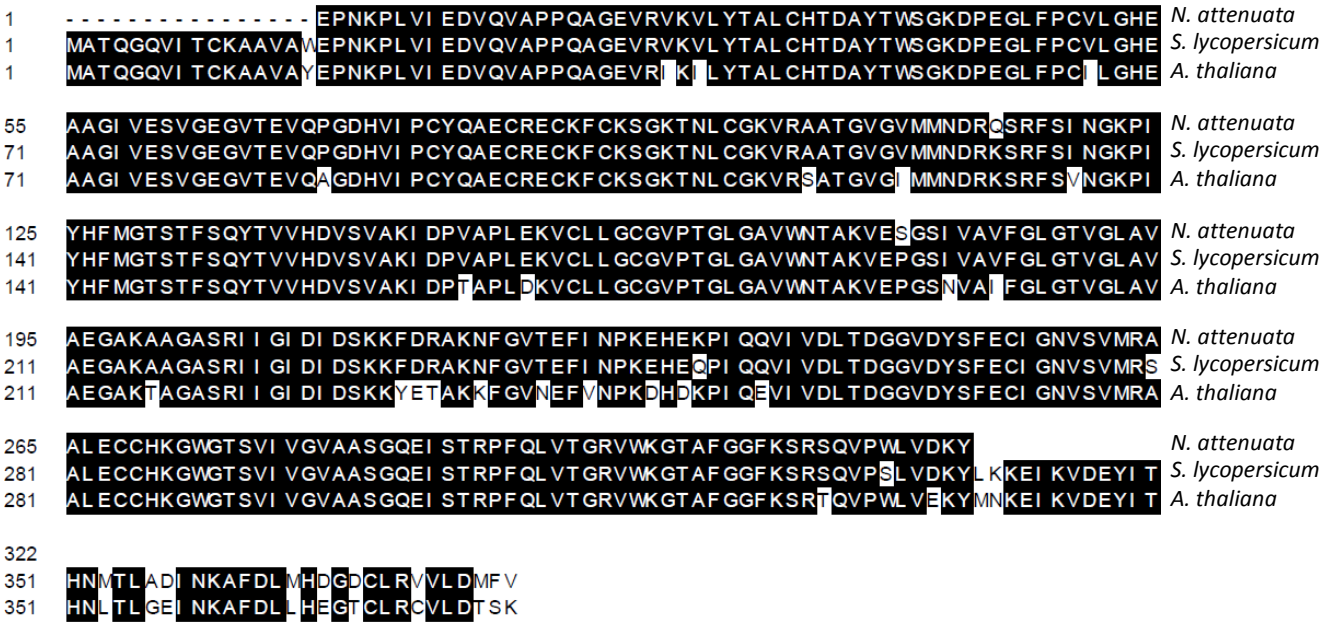

Fig. S1. Alignment of protein sequences of GSNOR in *Nicotiana attenuata*, *Solanum lycopersicum*, and *Arabidopsis thaliana*.

Arabidopsis AtGSNOR1 (NP_199207) and *Solanum lycopersicum* SlGSNOR (ADB43258) were retrieved from GenBank. Protein sequences were aligned using the ClustalW algorithm. Shaded sequences indicate exact matches with the consensus sequence.

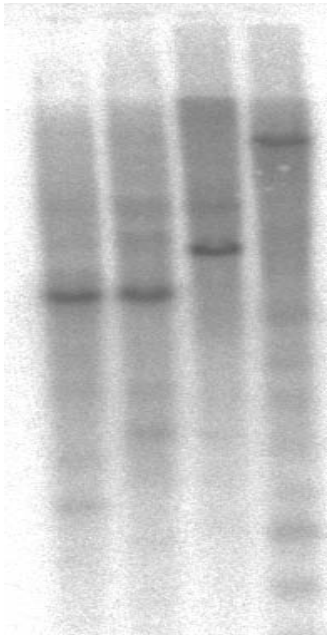

Fig. S2. Southern blotting analysis of *NaGSNOR* in *N. attenuata.*

N. attenuata genomic DNA was digested with various restriction enzymes. Following the separation on a 1% agarose gel, the DNA was blotted onto a nylon membrane and hybridized with a radio-labeled partial *NaGSNOR* cDNA probe.

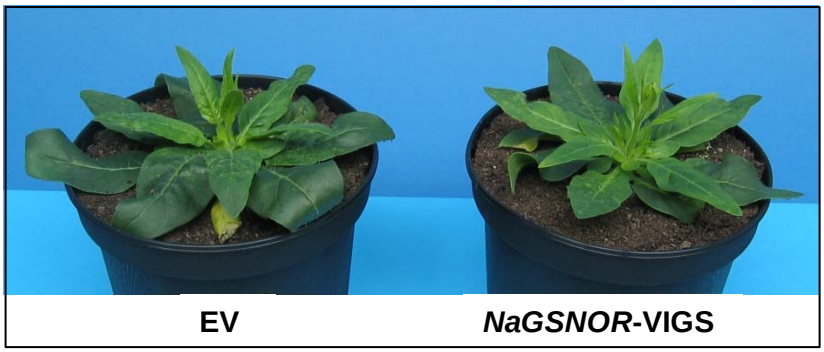

Fig. S3. Morphology of EV and *NaGSNOR*-VIGS plants.

Supplementary Table S1 Primer pairs used for qRT-PCR

Genes	Primer pairs	
NaGSNOR	5’-CCTCTGGTGATCGAGGATGT	5’-TCTCCTGGCTGAACCTCAGT
NaTPI	5'-TCAGGAGATAGTAAATATGGCTGTTCA	5'-ATCTGCATGTTCCACATTGCTTA
NaJAZ3	5'-CAATCGCGAGACGAGCTTCA	5'-CTCAGCTTTCACTGGAAATTGAG
NaTD	5'-TAAGGCATTTGATGGGAGGC	5'-TCTCCCTGTTCACGATAATGGAA
Actin	5’-GGTCGTACCACCGGTATTGTG	5’-GTCAAGACGGAGAATGGCATG

4. Discussion

The plant hormone JA is a major regulator of plant resistance against herbivores (Howe and Jander, 2008; Schaller and Stintzi, 2009; Wu and Baldwin, 2010). A quick JA burst is necessary (Glauser *et al.*, 2008) to induce timely plant defenses against herbivores (Stork *et al.*, 2009). Most biochemical reactions and enzymes in the biosynthetic pathway of JA have been identified, but it is not clear how JA biosynthesis is controlled. JA biosynthesis appears to be mainly dependent on substrate availability, because the transcriptional upregulation of JA biosynthetic enzymes follow the JA burst and AOS mutants with increased constitutive accumulation do not show increased basal JA accumulation (Delker *et al.*, 2006; Wasternack, 2007). In this thesis, NaNOA1, a protein positively associated with NO generation, and NaGSNOR, an enzyme involved in NO removal processes, were found to be involved in the biosynthesis of jasmonates and in *N. attenuata*'s defense against *M. sexta* attack.

Role of NaNOA1 in JA and secondary metabolite accumulation

The translated partial sequence of *NaNOA1* displays homology of 67% to AtNOA1 and 96% to NbNOA (Guo *et al.*, 2003; Kato *et al.*, 2008). Many studies showed that NOA1 influences various phenotypic traits (Ahlfors *et al.*, 2009; Asai and Yoshioka, 2009; Flores-Perez *et al.*, 2008; Guo *et al.*, 2003; He *et al.*, 2004; Zhao *et al.*, 2007a). Hence, it is not surprising that a specific transcriptional regulation after herbivory does not occur in leaves. Silencing *NaNOA1* has strong effects on the various plant herbivore resistance traits in *N. attenuata*. The mechanistic origin of these effects is not clear and merits further discussion.

Silencing *NaNOA1* in *N. attenuata* plants yields an herbivory-induced JA burst that is more rapid and larger than that in WT plants. This is correlated with high OPDA levels in WT plants. Additionally, OPDA, as a precursor of JA, is synthesized in the chloroplast and then being exported to peroxisomes where JA biosynthesis is completed (Schaller and Stintzi, 2009). The two MAPKs, SIPK and WIPK, are involved in the early upstream signaling processes and regulate precursors of JA (Kallenbach *et al.*, 2010; Wu *et al.*, 2007). SIPK regulates the generation of hydroperoxide fatty acids in the early steps of JA biosynthesis and WIPK seems to be involved in the synthesis of OPDA by controlling AOS. However, the increased JA and

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OPDA accumulation in *NaNOA1*-silenced plants is not elicited by elevated SIPK and WIPK activity, because both MAPKs do not change their activity profile after herbivory. In addition, the quick JA biosynthesis after wounding or herbivory and evidence from plants overexpressing JA biosynthesis genes demonstrated that the constitutive levels of JA biosynthetic enzyme activity determine the levels of JA accumulation (Delker *et al.*, 2006). Yet, no increased basal transcript levels of the JA biosynthesis genes GLA1, LOX3, AOS, AOC and OPR3 were detected in irNaNOA1 plants. Thus, the activity of JA biosynthetic enzymes is regulated by NaNOA1 at a posttranslational level.

JA biosynthesis initiates in the chloroplasts and the enzymes GLA1, LOX3, AOS, and AOC, which are located in the chloroplasts, facilitate the biosynthesis of the JA precursor OPDA (Farmaki *et al.*, 2007; Kallenbach *et al.*, 2010; Ryu, 2004). Furthermore, the localization of AOS and two lipases, which are suspected to participate in hormone metabolism, have been found to be on the thylakoid membranes and more specifically in the plastoglobules (Austin *et al.*, 2006; Vidi *et al.*, 2006; Ytterberg *et al.*, 2006). Plastoglobules are monolayer membrane structures budding from the thylakoid membranes that increase in numbers and even form plastoglobule clusters, which remain attached to the thylakoid membranes, during light stress, senescence, and oxidative stress (Austin *et al.*, 2006; Vičánková *et al.*, 2007; Youssef *et al.*, 2010). In addition, plastoglobules serve as a storage compartment for free fatty acids, including the main JA precursor linolenic acid (18:3) (Steinmüller and Tevini, 1985; Youssef *et al.*, 2010). Interestingly, the chloroplasts in the Arabidopsis mutant *noa1* have reduced size and clearly reduced thylakoid membrane structures (Flores-Perez *et al.*, 2008). A JA phenotype similar to that seen in irNaNOA1 plants has been observed in an earlier study (Laudert *et al.*, 2000). Laudert *et al.* (2000) generated AOS over expressing *A. thaliana* and *N. tabacum* lines and analyzed the constitutive JA levels and wounding-induced JA accumulation. The constitutive JA levels were similar in WT and mutant, but the wounding experiment demonstrated a 2 to 3-fold higher and quicker JA accumulation as well. Thus, it is hypothesized that altered plastid membrane structure enables the OPDA biosynthesis enzymes GLA1, LOX3, AOS and AOC to be in close vicinity to provide a better substrate supply for the OPDA biosynthesis enzymes. Moreover, enhanced oxidative stress in Arabidopsis *noa1* mutants causes higher levels of lipid peroxidation than in WT plants (Zhao *et al.*, 2007b). These alterations in the lipid metabolism could provide more lipid substrates for various lipid metabolizing pathways including OPDA/JA biosynthesis.

Discussion

JA-Ile, which is synthesized in cytosol from JA and Ile by JARs, binds to $SCF^{(COI1)}$ E3 ubiquitin ligase that triggers the degradation of JAZs (JASMONATE ZIM-DOMAIN proteins) and in that way is enabling JA-induced responses (Chini *et al.*, 2009; Staswick, 2008; Thines *et al.*, 2007). In contrast to increased JA/OPDA levels, the JA-Ile accumulation is markedly lower after W+OS treatment in irNaNOA1 than in WT plants. An increase of JA-Ile turnover rate apparently does not contribute to reduced JA-Ile accumulation, because the JA-Ile turnover rates in irNaNOA1 are comparable to those in WT plants. Other possible mechanisms may account for this: irNaNOA1 plants may have compromised JAR activity, altered JAR substrate specificity or decreased levels of free Ile. However, irNaNOA1 plants had uncompromised JAR activity and substrate specificity. JA is highly accumulated in peroxisomes, where JA is finally formed, and irNaNOA1 plants may be compromised in their ability to export JA from peroxisomes to cytosol and thus resulting in attenuated levels of JA-Ile. Furthermore, Ile accumulation is higher in *NaNOA1*-silenced plants than in WT under constitutive and herbivory conditions. Hence, isoleucine is not restricting for JA-Ile synthesis via impaired isoleucine production. Hypothetically, increased Ile levels in *NaNOA1*-silenced plants can be caused by elevated threonine deaminase activity in chloroplasts or processes that involve senescence and protein turn over specific ESTs (Guo *et al.*, 2004). Interestingly, free amino acid levels are elevated during senescence (Soudry *et al.*, 2005). In addition, chloroplast outer membrane amino acid transporters (Pohlmeyer *et al.*, 1997) may not fully utilize the export of Ile into the cytosol due to distorted chloroplast structures and thereby limit JA-Ile production in the cytosol.

In agreement with an earlier study where Arabidopsis *noa1* mutants have impaired chloroplast function (Flores-Perez *et al.*, 2008) leaves of *NaNOA1*-silenced plants appear yellowish and have greatly decreased photosynthesis rates. Thus, a reduction of photosynthesis likely results in attenuated levels of carbon-rich secondary metabolites. However not all C-rich compounds were found to have decreased levels. The concentrations of DCS, CP, rutin, and DTGs are suppressed in irNaNOA1 plants, while CGA contents are similar in WT and *NaNOA1*-silenced plants. In contrast, the contents of N-rich compounds nicotine and NaTPI were not altered in irNaNOA1 plants.

The precursors of nicotine, putrescine and nicotinic acid, are converted to nicotine in roots (Cane *et al.*, 2005; Tso and Jeffrey, 1957). Although putrescine is a precursor for nicotine and CP, compared with WT plants, herbivory-induced irNaNOA1 plants contain very limited amount

of CP, but normal (or even slightly higher) levels of nicotine. It is likely that this is a result of the spatial separation of CP and nicotine biosynthesis (root and shoot, respectively). In line with this, silencing *putrescine methyltransferase* (*PMT*) in *N. attenuata* inhibits nicotine production but does not increase the production of CP (Steppuhn *et al.*, 2004; Steppuhn *et al.*, 2008). Putrescine is synthesized either by ornithine decarboxylase (ODC) or arginine decarboxylase (ADC) pathways (Cane *et al.*, 2005). In *N. tabacum*, the ODC but not the ADC pathway plays a major role in supplying the putrescine that is subsequently used for nicotine synthesis (Chintapakorn and Hamill, 2007). Putrescine synthesis via ODC is universal in all eukaryotes, while ADC is localized in chloroplasts (Borrell *et al.*, 1995). Additionally, ODC in potato and tomato is increased in root tips and young growing leaf tissue (Cohen *et al.*, 1982). On the other hand, ADC occurs in all plant tissues of *N. tabacum*, but with lowest expression in roots (Bortolotti *et al.*, 2004). Interestingly, it has been suggested that the ADC pathway originates from the cyanobacterial ancestor of chloroplasts (Illingworth *et al.*, 2003); the ADC and ODC pathways are playing different roles in the biosynthesis of phenylpropanoid-polyamine conjugates. Therefore, the decreased CP and DCS accumulation in irNaNOA1 plants probably result from attenuated activity of the ADC pathway located in chloroplasts (Borrell *et al.*, 1995) and the likely unaltered activity of the ODC pathway in root accounts for the lack of changes in nicotine contents in irNaNOA1 plants. The compromised levels of CP and DCS might also be caused by a decreased supply of the caffeoyl moiety, which is derived from caffeoyl-CoA. Alternatively, enzymes downstream of ADC in the putrescine biosynthesis pathway, e.g. agmatine iminohydrolase and *N*-carbamoylputrescine amidohydrolase (Alcázar *et al.*, 2010) or the enzymes that conjugate caffeoyl-CoA to polyamines like putrescine (Grienenberger *et al.*, 2009; Luo *et al.*, 2009) may have lower activity in irNaNOA1 plants than in WT plants.

The greatly diminished levels of most of the C-based defensive metabolites in irNaNOA1 plants demonstrate the dependency of their biosynthesis on photosynthesis; however, unaltered CGA contents in these plants also suggests that NaNOA1 may also have functions in specifically influencing the activity of enzymes involved in the biosynthesis of certain C-based defensive compounds. CGA biosynthesis requires quinic acid (Niggeweg *et al.*, 2004), derived from the shikimate pathway, and caffeoyl-CoA, synthesized in the phenylpropanoid pathway. The unaltered CGA levels suggest that the supply of quinic acid and caffeoyl-CoA is sufficient for CGA synthesis in irNaNOA1 plants, and the enzyme activity of hydroxycinnamoyl-CoA quinate:

hydroxycinnamoyl transferase (HQT) (Niggeweg *et al.*, 2004) remains unaltered in irNaNOA1 plants. CGA and quercetin (the flavonol moiety of rutin) are both synthesized via phenylpropanoid pathway and share common precursors, for instance cinnamic acid (Niggeweg *et al.*, 2004; Vogt, 2010). However, rutin for example requires sufficient supply of carbohydrates for rutinose, namely glucose and rhamnose, but depressed photosynthesis permits only lower rutin accumulation in irNaNOA1 than in WT plants. Additionally, the chloroplast-localized methylerythritol phosphate pathway (MEP) provide dimethylallyl diphosphate (DMAPP) for the synthesis of geranylgeranyl diphosphate, which is a precursor of not only carotenoids and the chlorophyll's phytol side group but also of DTGs (Flores-Perez *et al.*, 2008; Jassbi *et al.*, 2008). DTGs are composed of a diterpene and a sugar moiety. Arabidopsis *rif1*/*noa1* mutants have increased levels of DXS and DXR proteins (Flores-Perez *et al.*, 2008). Reduced accumulation of DTGs appears to be a consequence of low photosynthesis rates of irNaNOA1 plants with decreased carbohydrate biosynthesis rather than a compromised MEP pathway.

Intriguingly, plants silenced in *ribulose-1,5-bisphosphate carboxylase/oxygenase activase* (*RCA*) (irRCA plants), a gene that modulates the activity of RuBPCase, are also impaired in photosynthesis and have reduced herbivory-induced JA-Ile (Mitra *et al.*, 2008). It is expected that reduced ATP is supplied to the adenylation of JA during the conjugation reaction between JA and Ile due to low photosynthesis rates (Mitra *et al.*, 2008), which could explain the reduced JA-Ile levels in irNaNOA1 plants. Still, this study demonstrated that irNOA1 plants do not suffer from depleted ATP supply, because amending OS with Ile and applying this on wounded leaves induces JA-Ile accumulation not much different than in WT plants. In addition, irRCA and irNaNOA1 plants differ in their profile of anti-herbivory compounds. The NaTPI and CGA accumulation are diminished in irRCA but not in irNOA1 plants (Mitra *et al.*, 2008). Many C-based secondary metabolites depend on photosynthesis (Matt *et al.*, 2002; Mitra *et al.*, 2008), but only rutin, DTGs, DCS and CP were reduced in irNOA1. It appears that DTGs, DCS, CP, and CGA levels may strongly depend on photosynthesis. However, these data suggest that NaNOA1 specifically regulates the biosynthesis of CGA. How this is integrated with the other roles of NOA1 in plant development, stress responses, NO generation and chloroplast function is currently unknown and requires further examination (Arasimowicz and Floryszak-Wieczorek, 2007; Beligni and Lamattina, 1999; Flores-Perez *et al.*, 2008; Guo *et al.*, 2003; Moreau *et al.*, 2008; Neill *et al.*, 2008; Shimazaki *et al.*, 2007; Sudhamsu *et al.*, 2008).

Discussion

Recently, studies on NOA1 offered a more precise suggestion about NOA1's role during chloroplast development, ribosome assembly and stability in the chloroplasts (Flores-Perez *et al.*, 2008; Moreau *et al.*, 2008; Sudhamsu *et al.*, 2008). Impaired protein synthesis and decreased photosynthetic rate are the two major factors that likely contribute to the decreased protein contents in irNaNOA1 leaves. DTGs, CP and DCS have strong negative effects on the growth of *M. sexta* neonate larvae and it is expected that reduction of these compounds benefit insect herbivores. However, attenuated concentrations of defensive compounds do not cause the *M. sexta* larvae to perform better on irNaNOA1 plants. The low protein contents of irNOA1 plants probably account for the poor performance of *M. sexta*. In addition, silencing *AtNOA1* diminishes NO production via a yet unknown process in plants and depending on NO concentration and location in plant tissue, it possibly acts as a scavenger of reactive oxygen species (ROS) or the balanced chemistry between ROS and nitric oxide species is in disarray (Darley-Usmar *et al.*, 1995). Hence, ROS stress cannot be alleviated sufficiently enough by NO with the remaining antioxidant systems to prevent protein oxidation, thylakoid membrane damage and further chlorophyll degradation (Beligni and Lamattina, 1999; Eum *et al.*, 2009; Guo and Crawford, 2005; Shi *et al.*, 2005), which can decrease food quality as well.

NaGSNOR mediates plant defense responses

The structural configuration of GSNOR is highly conserved in bacteria, animals and plants (Fliegmann and Sandermann, 1997; Liu *et al.*, 2001; Martínez *et al.*, 1996). GSNOR is an important regulator of immune function in animal and plant systems (Feechan *et al.*, 2005; Liu *et al.*, 2004). In contrast to NOA1, GSNOR is involved in NO removal processes where it metabolizes the product of NO scavenging, GSNO (Liu *et al.*, 2001; Sakamoto *et al.*, 2002). Controlling NO and GSNO is important to regulate the nitrosylation of cysteine and tyrosine in proteins. Accordingly, the red blood cells of $GSNOR^{-/-}$ mutant mice contain high *S*-nitrosothiol (SNO) hemoglobin levels that are likely associated with increased NO levels (Liu *et al.*, 2004). In addition, silencing GSNOR in Arabidopsis greatly elevates levels of NO, nitrate, SNO, and *N*-nitroso species (Feechan *et al.*, 2005; Lee *et al.*, 2008).

The significance of *S*-nitrosylation of thiol groups, specifically from cysteine, in animals has been established for signal transduction (Akhand *et al.*, 1999), apoptosis (Haendeler *et al.*, 2002), protein-protein interactions (Matsumoto *et al.*, 2003) and protein targeting for proteasomal

degradation by ubiquitylation (Hess *et al.*, 2005). The role of transient protein nitrosylation in plants' physiological processes is just emerging and many proteins, especially those involved in signal transduction, are targets of nitrosylation (Besson-Bard *et al.*, 2008; Grennan, 2007; Lindermayr *et al.*, 2005). Interestingly, several of the Arabidopsis proteins whose homologues have been identified during a study of herbivory-induced proteomic changes in *N. attenuata* are targets of nitrosylation in Arabidopsis as well (Giri *et al.*, 2006; Lindermayr *et al.*, 2005). These proteins include glycerinaldehyde-3-phosphate dehydrogenase, *S*-adenosylmethionine synthase (SAMS), RuBisCo large and small chain, RuBisCo activase, PSII oxygen evolving complex proteins and glutamine synthase. It is apparent that many of these proteins have functions in primary metabolism and that they function directly and indirectly in the interaction between *N. attenuata* and *M. sexta* (Giri *et al.*, 2006; Mitra and Baldwin, 2008; Mitra *et al.*, 2008). This suggests many possibilities for NaGSNOR in modulating different aspects of plant physiology during plant-insect interaction. In this study for example, we found that NaGSNOR is required for W+OS induced accumulation of phytohormones (JA/JA-Ile and ethylene) and for certain responses induced by JA, including the accumulation of defense-related secondary metabolites, suggesting its role in transducing certain aspects of JA signaling.

The individual reactions and most of the enzymes involved in the JA biosynthetic pathway have been indentified, but the regulation of the JA accumulation has not entirely resolved (Wasternack, 2007). Due to silencing *NaGSNOR*, increased abundance of nitric oxide species such as GSNO possibly influences protein function in JA biosynthesis pathway or other processes regulating JA accumulation by enhanced nitrosylation of these proteins. First, an earlier study showed that allene oxide cyclase (AOC) is being postranslationally modified by nitrosylation (Romero-Puertas *et al.*, 2008). Second and more intriguingly, nitrosylation of NPR1 (nonexpresser of PR genes) *in planta* is essential for the homeostasis of NPR1 (Tada *et al.*, 2008). NPR1 is an important mediator of the antagonizing effect of SA on JA accumulation and signaling (Spoel *et al.*, 2003). Moreover, nitrosylation of NPR1 and TGA1, another transcription factor that acts in combination with NPR1, is necessary to activate *PR* (*pathogenesis-related*) genes (Lindermayr *et al.*, 2010). Although, SA levels remain unchanged in *NaGSNOR*-VIGS plants, it is hypothesized that elevated GSNO content in these plants increases NPR1 activity and thereby JA accumulation is more sensitive to SA-mediated antagonism than in EV plants.

Discussion

The emission of the plant hormone ethylene depends on NaGSNOR. Ethylene biosynthesis is initiated by the conversion of methionine to *S*-adenosylmethionine (*S*-AdoMet) by *S*-AdoMet synthases (SAMSs); the conversion of *S*-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) is mediated by ACC synthases (ACSs). ACOs (ACC oxidases) finally facilitate the oxidation of ACC to ethylene (Wang *et al.,* 2002). Some evidence indicates that at least SAMS (also methionine adenosyltransferases, MATs) can be nitrosylated and thereby lose its enzymatic activity (Lindermayr *et al.,* 2006; Lindermayr *et al.,* 2005). How nitrosylation of ACS and ACO by GSNO may contribute to inhibition of their enzymatic activities in e.g. *N. attenuata* is not known. Accordingly, silencing *NaGSNOR* compromises herbivory-induced ethylene emission.

MAPK signaling cascades are important for plants to adapt to diverse environmental stresses (Asai *et al.,* 2002; Romeis *et al.,* 1999; Samuel *et al.,* 2000; Zhang and Klessig, 1998). In *N. attenuata*, W+OS-induced biosynthesis of JA is mediated by SIPK and WIPK signaling (Wu *et al.,* 2007). Also a significant part of herbivory- and pathogen-elicited ethylene emissions depend on SIPK (also AtMPK6 in Arabidopsis) signaling in *N. attenuata* and Arabidopsis (Liu and Zhang, 2004; Wu *et al.,* 2007). From the data we conclude that NaGSNOR is not involved in regulating MAPK activity. Two scenarios are possible: either NaGSNOR controls JA and ethylene in an MAPK-independent way or NaGSNOR functions downstream of MAPKs.

In tomato leaves, NO donors have a two-sided effect on JA-inducible responses. JA-dependent activation of proteinase inhibitor is severely reduced, but transcript accumulation of genes related to signaling is not altered (Orozco-Cardenas and Ryan, 2002). In *N. attenuata*, treatment of leaves with MJ indicated that NaGSNOR is important for the accumulation of the anti-herbivory defense compounds NaTPI, CP and DTGs, whereas the accumulation of *NaJAZ3* and *NaTD* transcripts are independent of NaGSNOR. This suggests that NaGSNOR is important for some but not all JA-induced responses. The function of the JA-Ile receptor, COI1, or the activity of $SCF^{(COI1)}$ complex seems not to be compromised by silencing *NaGSNOR*, because MJ induction still elicits similar *NaJAZ3* and *NaTD* transcript levels in EV and *NaGSNOR*-VIGS plants. Furthermore, clearly MJ esterase activity in *NaGSNOR*-VIGS plants is not inhibited and inactive MJ is cleaved into the signaling compound JA (Wu *et al.,* 2008). It is not clear whether some JA-induced responses are inhibited due to changed regulation of SA-JA cross-talk, likely via the NPR1/TGA1 system.

Discussion

In other plant systems, transcript, protein and activity of GSNOR are reduced after wounding (Chaki *et al.*, 2010; Diaz *et al.*, 2003). Consistently, wounding elicits NO in the epidermal layers of Arabidopsis leaves, detected by a NO-specific fluorescence dye (DAF-2 DA) (Huang *et al.*, 2004). Although wounding alone did not decrease NaGSNOR activity in *N. attenuata* leaves, NaGSNOR activity is temporary reduced by simulated herbivory (larval oral secretion included). In addition to other specific herbivory induced responses, herbivory appears to explicitly alter redox and posttranslational modifications (e.g. nitrosylation) of proteins in plants as well (Foyer and Noctor, 2005; Moreau *et al.*, 2010; Stamler *et al.*, 2001; Wu and Baldwin, 2010). Given that silencing *NaGSNOR* compromises many herbivory-induced responses and thus the resistance of *N. attenuata* to *M. sexta*, NaGSNOR is another regulator of optimal induction of defense responses in this plant-insect interaction.

5. Summary

Plant responses to different abiotic stress factors and to pathogen infection involve NOA1 (nitric oxide-associated protein 1). This protein is targeted to chloroplasts and mitochondria, and is positively associated with nitric oxide (NO) generation. Molecular analysis of NOA1 in Arabidopsis suggests that it is required for ribosome assembly and chloroplast development. Involvement of NOA1 in plant-herbivore interactions has not yet been explored. Another protein involved in NO-metabolism is the enzyme *S*-nitrosoglutathione reductase (GSNOR), which is negatively associated with NO biosynthesis. This protein is conserved between bacteria, plants and animals, and reduces nitrosylated glutathione (GSNO). GSNOR is required for regulating plant resistance to bacterial and fungal pathogens. However, its role in herbivore resistance has not been determined as well.

Agrobacterium tumefaciens-mediated transformation was used to generate *NOA1*-silenced *Nicotiana attenuata* plants (irNaNOA1). In contrast to *N. attenuata* WT plants, these transformed plants grew more slowly and had strongly decreased photosynthetic rates. We examined defense traits important for the plant's resistance to the specialist herbivore *Manduca sexta* via simulated herbivory by mechanical wounding and application of *M. sexta* oral secretions to WT and mutant rosette leaves. The plant defense hormone jasmonic acid (JA) in irNaNOA1 exhibited a faster and higher burst than in WT plants, whereas jasmonic acid-isoleucine (JA-Ile), the conjugation product between JA and isoleucine, was reduced. No decreases of the JA-Ile precursor isoleucine, in the turnover rate of JA-Ile, or in JAR (jasmonic acid resistant) activity were detected to explain this JA-Ile phenotype. In addition, most of the carbon-based defensive compounds such as rutin, diterpene glycosides, caffeoylputrescine, and dicaffeoylspermidine were reduced in irNaNOA1 plants. An exception was chlorogenic acid, which accumulated to similar levels as WT. We infer that silencing *NOA1* alters the allocation of carbon resources within the phenylpropanoid pathway. In contrast to most carbon-based compounds, the nitrogen-rich defensive compounds nicotine and trypsin proteinase inhibitors did not differ between WT and irNaNOA1 plants. Hence, it is proposed that NOA1 plays a role in *N. attenuata*'s defense against *M. sexta* attack, and in biosynthesis of jasmonates and secondary metabolites.

To study the function of NaGSNOR in *N. attenuata* during defense response against *M. sexta*, a virus-induced gene-silencing (VIGS) system was used to decrease NaGSNOR activity. Following herbivory, the accumulation of the two plant defense hormones JA and ethylene was

reduced in *NaGSNOR*-silenced plants. Therefore, *N. attenuata* plants did not induce the major defense compound trypsin proteinase inhibitor (TPI) in response to herbivory and thereby exhibited decreased resistance against *M. sexta*. However, impaired mitogen-activated protein kinase (MAPK) signaling was not responsible for the decreased JA and ethylene burst. In addition, silencing *NaGSNOR* compromised the accumulation of the defense-related secondary metabolites TPI, diterpene glycosides and caffeoylputrescine after methyl jasmonate (MeJA) treatment, whereas MJ-induced transcripts of *JAZ3* (*jasmonate ZIM-domain 3*) and *TD* (*threonine deaminase*) were NaGSNOR-independent. These data suggest that NaGSNOR is important for the plant's resistance to herbivory, and mediates the induction of some but not all jasmonate-inducible defense metabolites.

6. Zusammenfassung

Pflanzen reagieren auf abiotische und biotische Stressfaktoren mit einem vielfältigen Spektrum an physiologischen Antworten. An diesen Reaktionen ist auch NOA1 beteiligt (Stockstoffmonoxid-assoziiertes Protein 1). Dieses Protein kommt in Chloroplasten und Mitochondrien vor. Außerdem beeinflusst NOA1 die Synthese von Stickstoffmonoxid positiv. Molekulare Analysen zeigten das NOA1 in Arabidopsispflanzen für Ribosomenzusammenbau und Chloroplastenentwicklung wichtig sein könnte. Bisher wurde noch nicht untersucht, ob NOA1 während der Interaktion von Pflanzen und Insekten eine Rolle spielt. Ein anderes Protein, das im Stickstoffmonoxidmetabolismus eine Rolle spielt, ist das Enzym *S*-Nitrosoglutathionereduktase (GSNOR). Dieses ist welches negativ mit der Generierung von Stickstoffmonoxid assoziiert. Die GSNOR-Aminosäuresequenz ist in Bakterien, Pflanzen und Tieren hochkonserviert. Es reduziert nitrosyliertes Glutathion und reguliert die Resistenz von Pflanzen gegen phytopathogene Bakterien und Pilze. Eine Funktion von GSNOR in Verteidigungsprozessen gegen pflanzenfressende Insekten wurde bisher noch nicht nachgewiesen.

Durch *Agrobacterium tumefaciens*-vermittelte Transformation wurden *Nicotiana attenuata* Mutanten (irNaNOA1) erzeugt, welche eine reduzierte Expression von *NaNOA1* haben. Im Gegensatz zu Wildtyp *N. attenuata* Pflanzen wuchsen diese transgenen Pflanzen eher langsam und hatten ein stark reduzierte Photosyntheserate. Durch mechanisches Verwunden und Auftragen von *Manduca sexta* Oralsekreten auf Wildtyp und irNaNOA1 Rosettenblätter, wurde ein Herbivorenbefall simuliert, um pflanzliche Marker zu untersuchen, welche wichtig sind für die Resistenz gegen die spezialisierten Larven von *M. sexta*. Ein wichtiges Pflanzenverteidigungshormon, die Jasmonsäure (JA), akkumulierte schneller und die Menge ist höher in irNaNOA1 als in Wildtyppflanzen, während das Produkt aus der Konjugation von JA und Isoleucin, Jasmonsäureisoleucin (JA-Ile), in irNaNOA1 reduziert war. Ein reduziertes Vorkommen von Isoleucin, eine erhöhte Abbaurate von JA-Ile oder eine geringe Aktivität von JAR (jasmonic acid resistant) waren nicht messbar und können somit auch nicht diesen JA-Ile Phänotyp erklären. Zusätzlich waren die Konzentrationen von kohlenstoffbasierten Verteidigungsmetaboliten wie Rutin, Diterpenglykosiden, Caffeoylputrescin und Dicaffeoylspermidin im Blattgewebe von irNaNOA1 Pflanzen stark verringert. Eine Ausnahme war die Chlorogensäure, welche Konzentrationen ähnlich denen in Wildtyppflanzen erreichte.

Zusammenfassung

Wir schlussfolgern daraus, dass das Ausschalten von *NOA1* die Verteilung von kohlenstoffbasierten Ressourcen innerhalb des Phenylpropanoidmetabolismus verändert hat. Im Gegensatz zu den meisten kohlenstoffbasierten Verteidigungsmetaboliten konnten keine Konzentrationsunterschiede zwischen Wildtyp- und irNaNOA1 Pflanzen für die stickstoffreichen Verteidigungsmetabolite Nikotin und Trypsinproteinaseinhibitor (TPI) festgestellt werden. Dies verdeutlicht, das NOA1 eine Rolle spielt bei der Verteidigung von *N. attenuata* gegen *M. sexta*, speziell bei der Biosynthese von Jasmonsäure und Sekundärmetaboliten.

Die Aktivität von NaGSNOR wurde durch Virus-induziertes Gensilencing (VIGS) reduziert, um zu untersuchen, inwiefern NaGSNOR eine Funktion in *N. attenuata*‘s Verteidigungsantwort gegen *M. sexta* hat. Die durch Herbivorie induzierte Anreicherung der zwei Pflanzenverteidigungshormone JA und Ethylen war reduziert in irNaNOA1 Pflanzen. Folglich wurde in *NaGSNOR*-VIGS Pflanzen auch nicht ein wichtiges Verteidigungsmetabolit, TPI, als Antwort auf simulierten Befall mit *M. sexta* induziert und Resistenz gegen *M. sexta* Befall war erheblich verringert. Eine defekte Mitogen-aktivierte Proteinkinase (MAPK) Signaltransduktion war nicht für diese niedrige JA und Ethylenakkumulierung verantwortlich. Zusätzlich kompromittierte eine Reduktion der *NaGSNOR* Expression auch die Methyljasmonat-induzierte Anreicherung von *TPI*-Transkripten, Diterpenglykosiden und Caffeoylputrescin. Die durch Methyljasmonat induzierbare Akkumulation von *JAZ3* (*jasmonate ZIM-domain 3*) and *TD* (*threonine deaminase*) konnte jedoch unabhängig von NaGSNOR stattfinden. Diese Daten deuten darauf hin das NaGSNOR wichtig ist für *N. attenuata*’s Resistenz gegen Fraßfeinde ist und das NaGSNOR einige, aber nicht alle Jasmonsäure-induzierte Verteidigungsmetabolite reguliert.

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Human behavior flows from three main sources: desire, emotion, and knowledge. ~Plato (428/427 BC – 348/347 BC)

9. Curriculum Vitae

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Publications

Mitra, S., Wünsche, H., Giri, A. P., Hivrale, V., Baldwin, I. T. (2008). Silencing 7 herbivory-regulated proteins in *Nicotiana attenuata* to understand their function in plant–herbivore interactions. Functional Ecology, 22, 606-615.

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Oral presentations

Wünsche H. (2008). *Manduca sexta*'s nitrogen assimilation on *Nicotiana attenuata*. 7th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Wünsche H. (2006). Post-Ingestive Proteomics of *Manduca sexta* Feeding on *Nicotiana attenuata*. Workshop on proteomic insights into plant-insect interaction, National Chemical Laboratory, Pune, IN

Wünsche H. (2006). A proteomic analysis of plant-insect interaction between N. attenuate and *M. sexta*: Post-ingestive Proteomics. 5th Biannual IMPRS Symposium, MPI for Chemical Ecology, Jena, DE

Posters

Wünsche H. (2009). Nitric oxide plays a signaling role in *Nicotiana attenuata*'s responses to herbivory. presented at 8th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

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Curriculum Vitae

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10. Selbstständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Hendrik Wünsche