S-Nitrosoglutathione reductase (GSNOR) mediates the biosynthesis of jasmonic acid and ethylene induced by feeding of the insect herbivore Manduca sexta and is important for jasmonate-elicited responses in Nicotiana attenuata

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Abstract

S-nitrosoglutathione reductase (GSNOR) reduces the nitric oxide (NO) adduct S-nitrosoglutathione (GSNO), an essential reservoir for NO bioactivity. In plants, GSNOR has been found to be important in resistance to bacterial and fungal pathogens, but whether it is also involved in plant–herbivore interactions was not known. Using a virus-induced gene silencing (VIGS) system, the activity of GSNOR in a wild tobacco species, Nicotiana attenuata, was knocked down and the function of GSNOR in defence against the insect herbivore Manduca sexta was examined. Silencing GSNOR decreased the herbivory-induced accumulation of jasmonic acid (JA) and ethylene, two important phytohormones regulating plant defence levels, without compromising the activity of two mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK). Decreased activity of trypsin proteinase inhibitors (TPIs) were detected in GSNOR-silenced plants after simulated M. sexta feeding and bioassays indicated that GSNOR-silenced plants have elevated susceptibility to M. sexta attack. Furthermore, GSNOR is required for methyl jasmonate (MeJA)-induced accumulation of defence-related secondary metabolites (TPI, caffeoylputrescine, and diterpene glycosides) but is not needed for the transcriptional regulation of JAZ3 (jasmonate ZIM-domain 3) and TD (threonine deaminase), indicating that GSNOR mediates certain but not all jasmonate-inducible responses. This work highlights the important role of GSNOR in plant resistance to herbivory and jasmonate signalling and suggests the potential involvement of NO in plant–herbivore interactions. Our data also suggest that GSNOR could be a target of genetic modification for improving crop resistance to herbivores.

Key words: Defence, ethylene, insect herbivore, jasmonic acid, jasmonate signalling, Manduca sexta, Nicotiana attenuata, S-nitrosoglutathione reductase (GSNOR), secondary metabolites, trypsin proteinase inhibitor.

Introduction

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

Herbivores, especially insects, pose a great challenge for plant survival. Accordingly, plants have developed herbivory-specific defence systems to perceive herbivore attacks and deploy defence responses to optimize their fitness (Heil and Baldwin, 2002; Howe and Jander, 2008; Wu and Baldwin, 2010). Herbivory-induced defence
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 signalling cascades that involve the activation of the mitogen-activated protein kinases (MAPKs), salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), and bursts of jasmonic acid (JA), JA-isoleucine conjugate (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 signalling for herbivory-induced defences (McConn et al., 1997; Halitschke and Baldwin, 2003; Li et al., 2004, 2005; Paschold et al., 2007). Importantly, JA-Ile, but not JA, activates most of the JA-induced responses (Staswick and Tiryaki, 2004). JAZs (jasmonate ZIM-domain proteins) form complexes with MYC2, the major activator of JA-induced transcriptional responses, and thus inhibit the activity of MYC2. Binding of JA-Ile to the COI1 (coronatine insensitive1) receptor facilitates the degradation of the JAZs by the SCF(CO11) ubiquitin ligase-mediated pathway and, in turn, releases MYC2 which activates downstream responses (Chini et al., 2007; Thines et al., 2007). In *N. attenuata*, several compounds have been identified to be important for direct defence against herbivores. These include trypsin proteinase inhibitors (NaTPIs) (Zavala and Baldwin, 2004; Zavala et al., 2004), nicotine (Steppuhn et al., 2004), diterpene glycosides (DTGs) (Jassbi et al., 2008; Heiling et al., 2010), 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 greenhouse 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 JA, the gaseous hormone ethylene seems to play a minor role (Wu and Baldwin, 2010); ethylene potentiates JA-inducible proteinase inhibitors in tomato (O’Donnell et al., 1996) and reduces *M. sexta* herbivory-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) are 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 defences. Wounding induces NO production in marine macroalgae (Ross et al., 2006) and in *Arabidopsis* epidermal cells (Huang et al., 2004). NO negatively regulates proteinase inhibitor transcript levels after wounding, systemic, oligosaccharides, and JA treatment (Orozco-Cardenas and Ryan, 2002). NO is highly diffusible and reactive and it readily nitrosylates cysteine (S-nitrosylation) and tyrosine (tyrosine nitration) residues in various proteins (Lindermayr et al., 2005; Besson-Bard et al., 2008). Importantly, S-nitrosylation has been considered to be an important prototypic, redox-based, post-translational protein modification (Stamler et al., 2001; Wang et al., 2006). However, how NO regulates plant resistance to biotic stresses is still unknown, and very likely protein S-nitrosylation by NO plays a critical role (Feechan et al., 2005; Lindermayr et al., 2005; Grennan, 2007).

Although a bona fide NO synthase has yet to be identified in higher plants, at least three genes are associated with NO levels: NOA1 (nitric oxide associated1), 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 (Yamasaki and Sakihama, 2000; Guo et al., 2003), GSNOR is located in a NO removal pathway: NO rapidly reacts with glutathione and forms S-nitrosylated glutathione (GSNO), and GSNO is further metabolized into the oxidized glutathione disulphide (GSSG) and NH3 by GSNO reductase (Wilson et al., 2008). Consistent with the biochemical property of GSNO, the *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). By contrast, compared with the 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 defence against herbivores was unknown. A reverse genetic approach was used here to investigate the function of GSNOR in *N. attenuata*’s inducible defence against the specialist herbivore *M. sexta*. Virus-induced gene silencing (VIGS) was used to knock down the transcripts of *NaGSNOR*, and traits important in herbivore resistance were examined. It was found that silencing *NaGSNOR* attenuates wounding- and simulated herbivory-induced levels of phytohormones that regulate plant resistance levels and, accordingly, decreased accumulation of the defensive compound NaTPI was detected in *NaGSNOR*-silenced plants. Moreover, 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 defence against herbivores.
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 1.0 l pots 20 d after germination on Petri dishes, and were grown in a climate chamber at 22 °C and under 65% humidity. Light (16 h d\(^{-1}\)) was 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 comparison. For treatment with methyl jasmonate (MJ), MJ was dissolved in heat-liquefied lanolin (5 mg m\(^{-2}\)) and 20 μl of MJ-lanolin paste was applied to the basal part of a leaf; leaves treated with 20 μl of pure lanolin served as controls. All samples were immediately frozen in liquid nitrogen after harvesting and stored at −80 °C until analyses. Neonate *M. sexta* larvae from laboratory colonies were placed on plants (one larva per plant, 30 replicated plants), and the larval masses of a leaf; leaves treated with 20 μl of MJ-lanolin paste were applied to the basal part of a leaf; leaves treated with 20 μl of pure lanolin served as controls. All samples were immediately frozen in liquid nitrogen after harvesting and stored at −80 °C until analyses. Neonate *M. sexta* larvae from laboratory colonies were placed on plants (one larva per plant, 30 replicated plants), and the larval masses were measured on days 4, 9, and 14.

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

No *GSNR* sequences from *Nicotiana* spp. were deposited in the GenBank; therefore an *Arabidopsis* AIGSNOR (At5g43940) sequence was used to blast against the TIGR Plant Transcript Assemblies (http://plantta.jcvi.org/). A 1.48 kb tobacco *NIGSNOR* sequence was found (Plant TA Accession: TAI13797.4097). The partial sequence of *NaGSNR* was amplified from *N. attenuata* cDNA by PCR with primer pair NaGSNR-1 (5′-GAAACCCAA-CAACCTCTGGT-3′) and NaGSNR-2 (5′-CATCCCACCTT-GATTTCCTT-3′), which were designed according to the sequence of *NIGSNOR*. The amplified fragment was cloned into the pJET1.2 vector (Fermentas, St Leon-Rot, Germany) and sequenced.

A 326 bp fragment of *NaGSNR* was cloned into the pTV00 vector to generate the pTV–NaGSNR construct, which was then transformed into *Agrobacterium tumefaciens* (Ratcliffe et al., 2001). Virus-induced gene silencing was done according to Saedler and Baldwin (2004). The initiation of silencing was visually monitored using *phytoene desaturase* (NaPDS)-silenced plants, which showed a photo-bleaching phenotype about 2 weeks after inoculation with *A. tumefaciens* carrying pTV-NaPDS (Saedler and Baldwin, 2004).

The restriction enzymes *EcoRI*, *HindIII*, *EcoRV*, and *XhoI* were used to digest DNA of *N. attenuata*. Five micrograms of digested DNA were separated on a 1% agarose gel and then were further blotted on to a nylon membrane. Hybridization was performed according to Wu et al. (2006) using a probe prepared by PCR amplification of a partial *NaGSNR* sequence with the primer pair NaGSNR-F1 (5′-CTCTCTGGTGATCGAGGATG-3′) and NaGSNR-R1 (5′-TCTCTGGTGATCGAGGATG-3′).

Quantitative real-time PCR (qRT-PCR)

TRIZol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract RNA. cDNA samples were synthesized from 500 ng of total RNA using the 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). An *N. attenuata actin* gene *NaActin* was used to normalize the variation of cDNA concentrations. All qRT-PCR experiments were performed using five biological replicates. The sequences of primer pairs are listed in Supplementary Table S1 at JXB online.

GSNR activity assay

GSNR activity was measured spectrophotometrically at 340 nm using a modified method as described in Sakamoto et al. (2002). In brief, approximately 30 mg of ground leaf tissue were extracted with 300 μl of 50 mM HEPES buffer (pH 8) containing 20% glycerol, 10 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, and 1 mM e-aminocaproic acid. The samples were centrifuged at 4 °C, 16 000 g for 15 min and the supernatants were further desalted using protein desalting spin columns (Thermo Fisher 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 disappearance was observed for 75 s. 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 GSNR activity was expressed as nmol NADH degraded min\(^{-1}\) mg\(^{-1}\) protein.

In-gel kinase activity assay

Each protein sample was extracted from pooled leaves from five replicated plants. About 100 mg of leaf tissue were resuspended in 300 μl of extraction buffer [100 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na\(_2\)VO\(_4\), 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulphonyl fluoride, 10% glycerol, one proteinase inhibitor cocktail tablet per 10 ml extraction buffer (Roche, Mannheim, Germany)]. Samples were centrifuged at 4 °C, 13 000 g for 20 min and the supernatants were transferred to fresh tubes. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) with BSA (Sigma-Aldrich, Hamburg, Germany) as a standard. Ten micrograms of total protein from each sample were used for in-gel kinase activity assay according to a procedure described by Zhang and Klessig (1997). The image of in-gel kinase activity assays were obtained on a phosphorimager (FLA-3000 phosphor imager system, Fuji Photo Film, Stamford, CT, USA). The same amount of each sample was run on a duplicated gel without the kinase substrate myelin basic protein and the gel was stained with the GelCode Blue Safe Stain reagent (Thermo Fisher Scientific).

Quantification of JA, JA-Ile, SA, ethylene, and direct defence 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 briefly crushed leaf tissue were added to 2 ml Eppendorf tubes containing 1 g of ceramic beads (MP Biomedicals, Illkirch, France). After adding 1 ml of ethyl acetate which contained 200 ng of JA[D\(_3\)], 40 ng of JA-[\(^{13}C\)]Ile, and 40 ng of SA[D\(_3\)] as internal standards, the tissue was homogenized on a Geno/Grinder 2000 at 1700 strokes min\(^{-1}\) 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. An HPLC-MS/MS (Varian, Palo Alto, CA, USA) was used to analyse the concentration of JA and JA-Ile in the supernatants. For ethylene quantification, five leaves were untreated as controls or were treated with W+OS and, after recording their fresh mass, they were immediately sealed in a 250 ml three-neck round bottom flask for 4 h under light. The ethylene contents in the flasks were measured on a photoacoustic laser spectrometer (INVIVO, Sankt Augustin, Germany) by comparing sample ethylene peak areas with peak areas generated by an ethylene standard (Von Dahl et al., 2007). Five replicates were done for ethylene measurements.
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). Contents of nicotine, diterpene glycosides, and caffeoylputrescine were analysed on an HPLC as described in Keinanen et al. (2001).

Results

Herbivory but not wounding transiently reduces the activity of NaGSNOR

A fragment of NaGSNOR [GenBank: ADB43258] and Arabidopsis AtGSNOR1 [GenBank: NP_199207] (Martínez et al., 1996), respectively (see Supplementary Fig. S1 at JXB online). 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 (see Supplementary Fig. S2 at JXB online).

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 AtGSNOR in 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, mechanical wounding was done by applying 20 μl of water to wounds (W+W). Initially, NaGSNOR transcripts were slightly reduced 30 min after both treatments (W+W, W+OS), but regained the levels seen in non-treated plants by 3 h (Fig. 1A). However, 6 h after W+W and W+OS treatment, NaGSNOR transcript levels increased 2.2-fold and 4.3-fold compared with those in non-treated plants. It was next examined whether the activity of NaGSNOR is regulated by 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 and showed no changes even after 6 h (Fig. 1B), suggesting that herbivory (probably the OS of M. sexta) but not wounding, specifically and transiently reduces the activity.
of NaGSNOR. In addition, transcript levels of NaGSNOR after wounding and herbivory do not correlate with the activity levels of NaGSNOR.

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 the 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 used to determine the role of NaGSNOR in the response of N. attenuata to wounding and M. sexta feeding. A pTV-NaGSNOR construct was prepared by inserting a partial NaGSNOR coding sequence into the pTV00 vector (Ratcliff et al., 2001; Saedler and Baldwin, 2004). N. attenuata plants inoculated with Agrobacterium carrying pTV-NaGSNOR and empty vector (pTV00) formed NaGSNOR-VIGS and EV plants respectively. VIGS efficiently reduced the transcript levels of NaGSNOR in NaGSNOR-VIGS to about 3%
of those in EV (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 (see Supplementary Fig. S3 at JXB online) and in the elongated stage, NaGSNOR-VIGS plants exhibited stunted stalks, a reduced number of flower buds, and epinastic leaves. All experiments were done at the rosette stage.

Given the central roles of phytohormones in regulating plant resistance to herbivores, it was determined whether NaGSNOR modulates 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 analysed in samples collected 30, 60, and 90 min after treatments. In EV plants, compared with W+W, W+OS elicited 2-fold higher levels of JA by 1 h, indicating that N. attenuata recognized herbivore elicitors, FACs, in M. sexta OS and accumulated high contents of JA; by contrast, JA contents in NaGSNOR-VIGS plants were about half those found in EV plants (Fig. 3A). Similarly, NaGSNOR-VIGS plants challenged with W+W also showed a reduced JA accumulation (Fig. 3A). The JA-Ile levels also showed a tendency to be 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) signalling pathway, it is possible that the suppressed JA levels in NaGSNOR-VIGS resulted from high SA contents in these plants (Pieterse et al., 2009). When untreated, statistically no significantly different levels of SA were detected between EV and NaGSNOR-VIGS (P=0.16), although NaGSNOR-silenced plants tended to
have 35% more SA levels than did EV (Fig. 3C). After W+W and W+OS treatment, compared with EV, NaGSNOR-VIGS also exhibited a tendency of having maximally 50% and 30% higher SA levels ($P > 0.09$ and 0.16, respectively) (Fig. 3C). Wounding does not increase ethylene emission from *N. attenuata* (Von Dahl *et al.*, 2007), hence ethylene emissions were measured in control and W+OS-treated plants. After W+OS, NaGSNOR-VIGS exhibited about 43% reduced ethylene emission compared to EV (Fig. 3D).

Thus, it was inferred that NaGSNOR is required for wounding- and herbivory-induced accumulation of JA and herbivory-elicited biosynthesis of ethylene in *N. attenuata*.

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

In *N. attenuata*, SIPK and WIPK are required 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 0, 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 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 at all times.

**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 d. Stars indicate significantly different larval masses between those fed on EV and on NaGSNOR-VIGS plants (Student’s t test; *, $P = 0.05$; ***, $P < 0.001$; $n = 30$).

**Fig. 7.** Herbivore defence-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$^{-1}$ methyl jasmonate (MJ) or pastes of pure lanolin (Lan) (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 d after treatments. Stars indicate significantly different levels between EV and NaGSNOR-VIGS plants (Student’s t test; *, $P = 0.05$; $n = 5$).
Therefore, the decreased JA and ethylene levels in wounding- and herbivory-induced NaGSNOR-VIGS were not due to impaired MAPK activation.

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

TPIs are important anti-herbivore compounds in solanaceous plants, including *N. attenuata* (Ryan, 1989; Haq et al., 2004; Zavala et al., 2004). To determine the function of NaGSNOR in regulating the response to wounding and herbivory, defence metabolites were determined in EV and NaGSNOR-VIGS plants 3 d 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). This might be the reason why the concentrations of other known JA-inducible secondary metabolites (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, bioassays were performed. Neonate *M. sexta* larvae were grown for 14 d on EV and NaGSNOR-VIGS plants and their masses were recorded on days 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), indicating that NaGSNOR is required for *N. attenuata*’s defence against *M. sexta*.

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). Therefore, it was 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⁻¹) was applied to plants, and plants treated with 20 μl of pure lanolin were used as controls. Defence metabolites (NaTPI, CP, and DTG) were measured 3 d 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). Similarly, MJ application highly increased the levels of CP and DTG contents 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 was inferred that NaGSNOR is required for certain, but not all, JA-induced responses in N. attenuata.

**Discussion**

GSNORs have structural features that are highly conserved in bacteria, animals, and plants (Martinez et al., 1996; Fliegmann and Sandermann, 1997; Liu et al., 2001). In mice, silencing GSNOR leads to increased damage in the lymphatic and liver tissue after being challenged with bacterial endotoxin (Liu et al., 2004). Arabidopsis AtGSNOR1 is a positive regulator of plant immunity against phytopathogens (Feechan et al., 2005). It is shown here that in N. attenuata, NaGSNOR plays an essential role in wounding responses and plant defence against the specialist insect herbivore, M. sexta.

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) haemoglobin levels in red blood cells, which is probably associated with increased NO levels (Liu et al., 2004). Similarly, the 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 (Lindermayr et al., 2005; Grennan, 2007; Besson-Bard et al., 2008). 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 defence-related secondary metabolites, suggesting its role in transducing certain aspects of JA signalling.

In plants, JA plays a central role in defence against herbivore stress (Kessler et al., 2004; Howe and Jander, 2008; 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 biosynthesis is regulated. Our data indicated that NaGSNOR is positively associated with the 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 (Lindermayr et al., 2005; Grennan, 2007; Besson-Bard et al., 2008). 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 defence-related secondary metabolites, suggesting its role in transducing certain aspects of JA signalling.

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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 JA from the inactive MJ (JA is further converted to JA-Ile and therefore activate jasmonate-induced responses) (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 signalling (Pieterse et al., 2009). Whether NaGSNOR-deficient plants have enhanced NPR1 activity and therefore have elevated inhibition of certain JA-induced responses by SA 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., 2011). 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, the abundance of proteins and secondary metabolites, but also the status of protein posttranslational modification (e.g. nitrosylation and phosphorylation) in plant cells (Foyer and Noctor, 2005; Moreau et al., 2010). 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 the optimum induction of herbivory-specific defence reactions, which involves a reconfiguration of the protein nitrosylation status. Given the positive association between GSNOR activity and plant defence levels, it is proposed that GSNOR could, potentially, be a target of genetic modification for improving insect resistance in crops.

Supplementary data

Supplementary data can be found at JXB online.

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

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

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

Supplementary Table S1. Primer pairs used for qRT-PCR.

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