Journal of Experimental Botany, Vol. 62, No. 13, pp. 4605–4616, 2011
doi:10.1093/jxb/err171 Advance Access publication 27 May, 2011
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

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*

Hendrik Wünsche, Ian T. Baldwin and Jianqiang Wu*

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Hans-Knoell-Str. 8, D-07745 Jena, Germany

* To whom correspondence should be addressed. E-mail: jwu@ice.mpg.de

Received 13 January 2011; Revised 20 April 2011; Accepted 4 May 2011

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

© 2011 The Author(s).
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 $SCF^{(COI1)}$ 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, systemin, 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 been 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 NH_3 by GSNOR (Wilson *et al.*, 2008). Consistent with the biochemical property of GSNOR, 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^{-1}) 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 larvae per plant, 30 replicated plants), and the larval masses were measured on days 4, 9, and 14.

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

No *GSNOR* sequences from *Nicotiana* spp. were deposited in the GenBank; therefore an *Arabidopsis AtGSNOR* (At5g43940) sequence was used to blast against the TIGR Plant Transcript Assemblies (http://plantta.jcvi.org/). A 1.48 kb tobacco *NtGSNOR* sequence was found (Plant TA Accession: TA13797_4097). The partial sequence of *NaGSNOR* was amplified from *N. attenuata* cDNA by PCR with primer pair NaGNSOR-1 (5′-GAACCCAA-CAAGCCTCTGGT-3′) and NaGSNOR-2 (5′-CATCCACCTT-GATTTCCTTCT-3′), which were designed according to the sequence of *NtGSNOR*. 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 (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 *Eco*RI, *Hin*dIII, *Eco*RV, and *Xba*I 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 *NaGSNOR* sequence 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. 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.

GSNOR activity assay

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 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 ε-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 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 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 GSNOR 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_3VO_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_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^{-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.

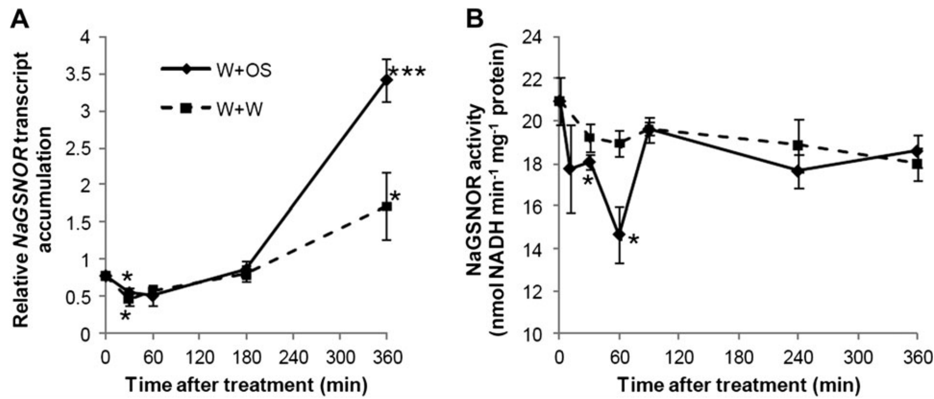

Fig. 1. *NaGSNOR* transcript accumulation and enzyme activity after wounding and simulated herbivory. Transition leaves of *N. attenuata* rosette plants were wounded with a pattern wheel, and were subsequently treated with 20 μl of water (W+W) or 20 μl of *M. sexta* oral secretions (W+OS). Samples were harvested after the indicated times. (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* ⩽0.05; ****P* ⩽0.001; *n*=5).

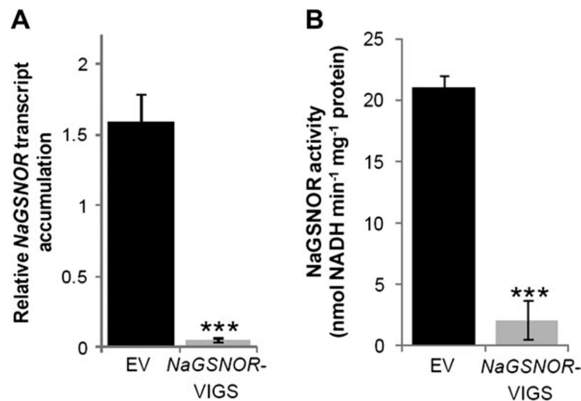

Fig. 2. *NaGSNOR*-VIGS plants have highly diminished transcript levels of *NaGSNOR* and strongly reduced GSNOR activity. *N. attenuata* plants were infiltrated with *Agrobacterium* carrying pTV00 or a pTV-NaGSNOR to generate EV and *NaGSNOR*-VIGS plants, respectively. (A) Transcript levels (mean ±SE) 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* ⩽0.001; *n*=5).

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: HQ830156] with 967 bp was cloned from the *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 (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

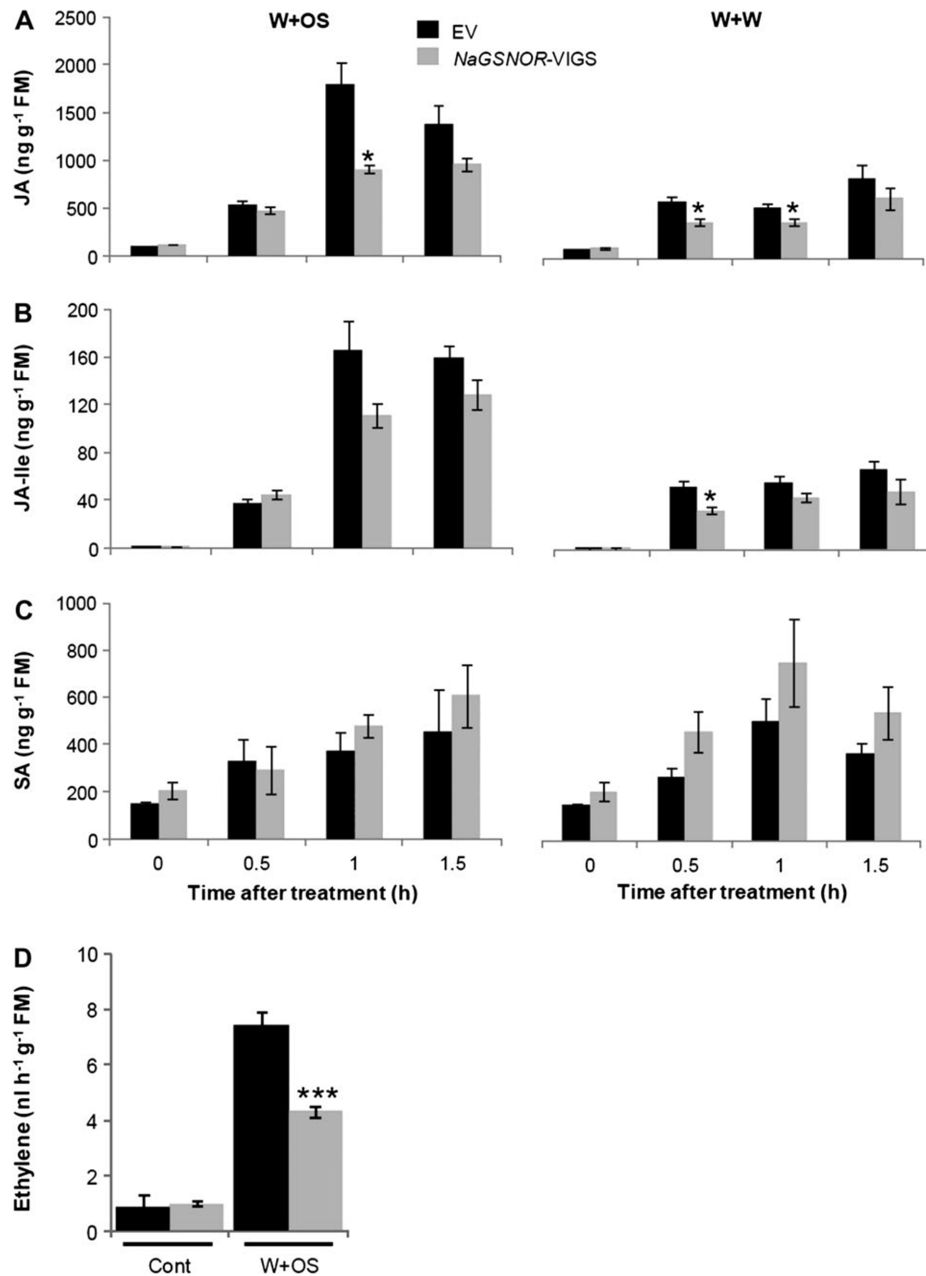

Fig. 3. Wounding- and simulated herbivory-induced levels of phytohormones in EV and *NaGSNOR*-VIGS plants. EV and *NaGSNOR*-VIGS plants were wounded with a pattern wheel and were subsequently treated with 20 µl of water (W+W) or 20 µl of *M. sexta* oral secretions (OS) (W+OS). (A) JA, (B) JA-Ile, and (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; $^{*}P \leqslant 0.05$; $^{***}P \leqslant 0.001$; $n=5$).

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 T_1 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

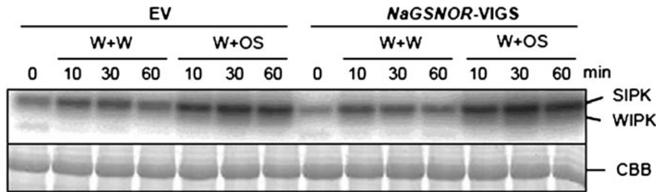

Fig. 4. Silencing *NaGSNOR* does not impair wounding- and simulated herbivory-induced MAPK activity in *N. attenuata*. EV and *NaGSNOR*-VIGS plants were wounded with a pattern wheel and were subsequently applied with 20 μl of water (W+W) or 20 μl of *M. sexta* oral secretions (OS) (W+OS). Samples were harvested after the 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).

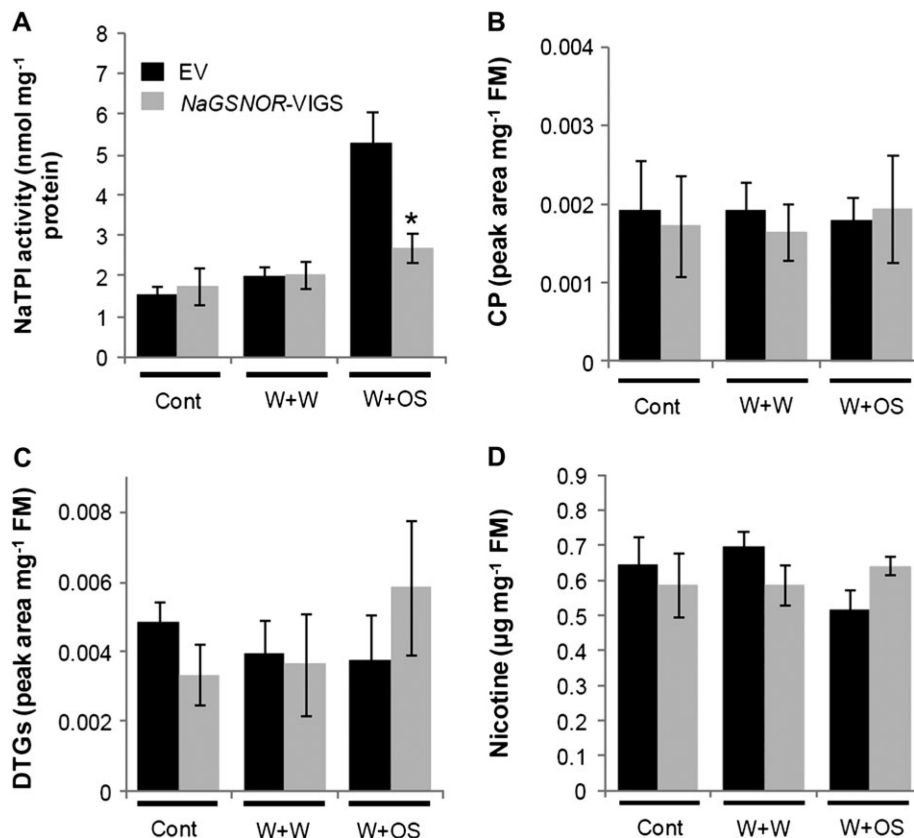

Fig. 5. Accumulation of herbivore defense-related secondary metabolites in EV and *NaGSNOR*-VIGS plants. Leaves of EV and *NaGSNOR*-VIGS plants were wounded with a pattern wheel, and were thereafter applied with 20 μl of water (W+W) or 20 μl of *M. sexta* oral secretions (W+OS). The activity of NaTPI (A), contents of caffeoylputrescine (CP) (B), diterpene glycosides (DTGs) (C), and nicotine (D) (mean ±SE) were determined in EV and *NaGSNOR*-VIGS plants 3 d after treatments; non-treated plants served as controls (Cont). Star indicates significantly different levels between EV and *NaGSNOR*-VIGS plants (Student's t test; $^{*}P \leqslant 0.05$; n=5).

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).

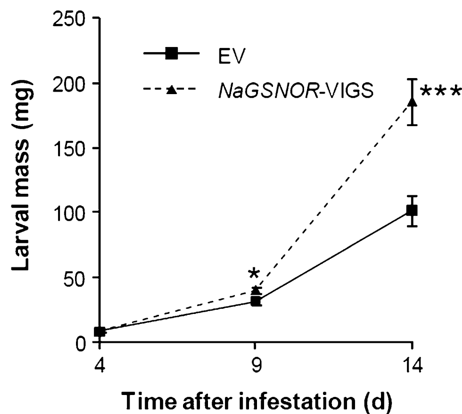

Fig. 6. Silencing *NaGSNOR* in *N. attenuata* compromises plant resistance to insect herbivore, *M. sexta.* Neonate *M. sexta* larvae were placed on rosette-staged EV and *NaGSNOR*-VIGS plants and larval masses (mean ±SE) were measured after 4, 9, and 14 d. Stars indicate significantly different larval masses between those fed on EV and on *NaGSNOR*-VIGS plants (Student's t test; *, $P \leqslant 0.05$; ***$P \leqslant 0.001$; n=30).

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

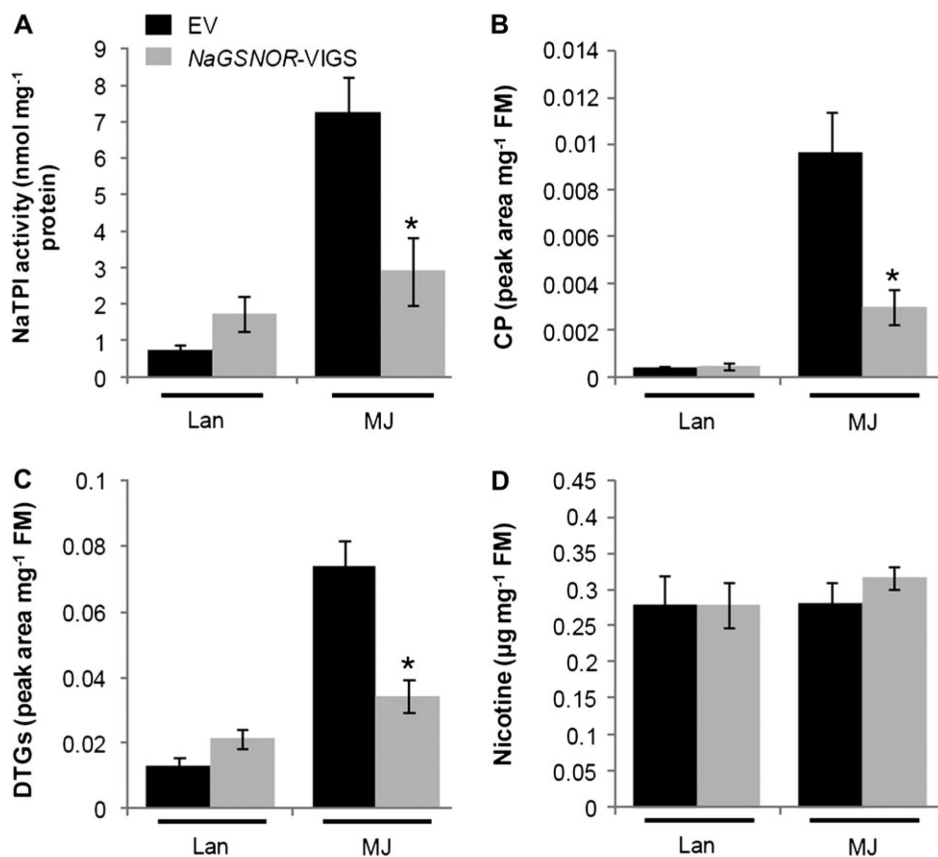

Fig. 7. Herbivore defence-related secondary metabolites in EV and *NaGSNOR*-VIGS plants after methyl jasmonate treatment. EV and *NaGSNOR*-VIGS plants were applied with lanolin pastes (20 µl) containing 5 mg ml^{-1} methyl jasmonate (MJ) or pastes of pure lanolin (Lan) (20 µl) for comparisons. The activity 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; *$P \leqslant 0.05$; n=5).

(Fig. 4). 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 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).

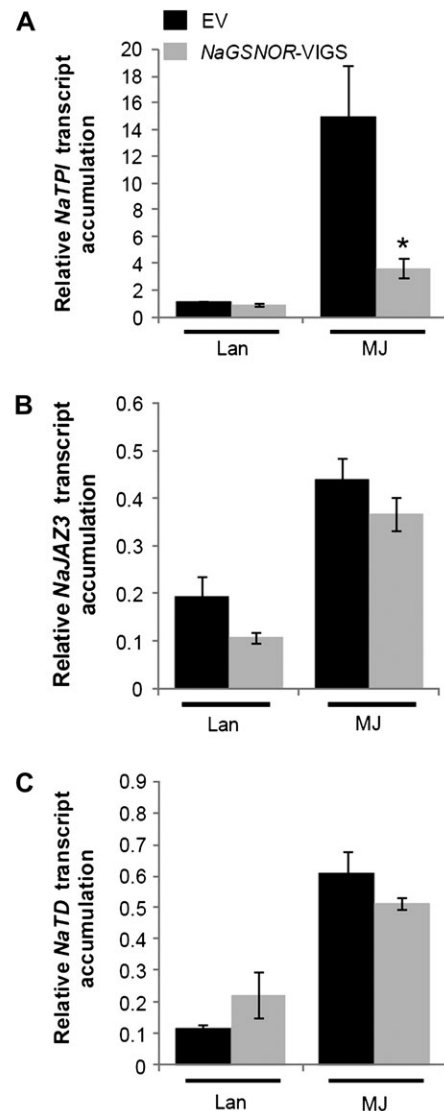

Fig. 8. Transcript levels of *NaTPI*, *NaJAZ3*, and *NaTD* in methyl jasmonate-treated EV and *NaGSNOR*-VIGS plants. EV and *NaGSNOR*-VIGS plants were applied with lanolin pastes (20 μl) containing 5 mg ml^{-1} methyl jasmonate (MJ), or pastes of pure lanolin (Lan) (20 μl) for comparison. The transcript 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; $^{*}P \leq 0.05$; $n=5$).

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 (Martínez *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 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 (Spoel *et al.*, 2003; Diezel *et al.*, 2009; Pieterse *et al.*, 2009), and that NPR1 (non-expresser of PR genes1) is important for the suppression effect of SA on JA accumulation and signalling (Spoel *et al.*, 2003). Importantly, NPR1 is also nitrosylated *in planta* and 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 the nucleus, which is required for the activation of NPR1-induced responses, requires NO. Therefore, in addition to the tendency of increased SA levels in *NaGSNOR*-silenced plants, which may have some effect on the suppression of JA production (Pieterse *et al.*, 2009), there was speculation that the likely elevated levels of GSNO may increase nitrosylation of NPR1 and thereby enhance NPR1 activity, which, in turn, promotes 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 catalyse the oxidation of ACC to form ethylene (Wang *et al.*, 2002). Among these key enzymes, SAMSs (also methionine adenosyltransferases, MATs) are 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 a MAPK-independent manner or that 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 signalling 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 the transcriptional regulation of *NaJAZ3* and *NaTD*. It is very unlikely that silencing *NaGSNOR* compromises the activity of the JA-Ile receptor, COI1, or the activity of the $SCF^{(COI1)}$ 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 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.

Acknowledgements

We thank Dr Klaus Gase, Thomas Hahn, Susan Kutschbach, and Antje Wissgott for sequencing and preparation of the VIGS construct, Dr Tamara Krügel, Andreas Weber, and Andreas Schünzel for their help with plant cultivation, Dahai Yang, Christian Hettenhausen, Stefan Meldau, Maria Heinrich, and Christina Bartnitzek for technical assistance and discussions, and the Max Planck Society for funding.

References

Baldwin IT. 2001. An ecologically motivated analysis of plant–herbivore interactions in native tobacco. *Plant Physiology* **127,** 1449–1458.

Besson-Bard A, Pugin A, Wendehenne D. 2008. New insights into nitric oxide signaling in plants. *Annual Review of Plant Biology* **59,** 21–39.

Chaki M, Valderrama R, Fernández-Ocaña AM, *et al*. 2011. Mechanical wounding induces a nitrosative stress by down-regulation of GSNO reductase and an increase in *S*-nitrosothiols in sunflower (*Helianthus annuus*) seedlings. *Journal of Experimental Botany* **62,** 1803–1813.

Chen M-S. 2008. Inducible direct plant defense against insect herbivores: a review. *Insect Science* **15,** 101–114.

Chini A, Fonseca S, Fernandez G, *et al*. 2007. The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448,** 666–671.

Diaz M, Achkor H, Titarenko E, Martlnez MC. 2003. The gene encoding glutathione-dependent formaldehyde dehydrogenase/gsno reductase is responsive to wounding, jasmonic acid and salicylic acid. *FEBS Letters* **543,** 136–139.

Diezel C, von Dahl CC, Gaquerel E, Baldwin IT. 2009. Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. *Plant Physiology* **150,** 1576–1586.

Dodds PN, Rathjen JP. 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics* **11,** 539–548.

Feechan A, Kwon E, Yun B-W, Wang Y, Pallas JA, Loake GJ. 2005. A central role for *S*-nitrosothiols in plant disease resistance. *Proceedings of the National Academy of Sciences, USA* **102,** 8054–8059.

Fliegmann J, Sandermann H. 1997. Maize glutathione-dependent formaldehyde dehydrogenase cdna: a novel plant gene of detoxification. *Plant Molecular Biology* **34,** 843–854.

Foyer CH, Noctor G. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell* **17,** 1866–1875.

Grennan AK. 2007. Protein *S*-nitrosylation: potential targets and roles in signal transduction. *Plant Physiology* **144,** 1237–1239.

Guo F-Q, Okamoto M, Crawford NM. 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **302,** 100–103.

Halitschke R, Baldwin IT. 2003. Antisense lox expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata*. *The Plant Journal* **36,** 794–807.

Halitschke R, Schittko U, Pohnert G, Boland W, Baldwin IT. 2001. Molecular interactions between the specialist herbivore

Manduca sexta (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiology* **125,** 711–717.

Haq SK, Atif SM, Khan RH. 2004. Protein proteinase inhibitor genes in combat against insects, pests, and pathogens: natural and engineered phytoprotection. *Archives of Biochemistry and Biophysics* **431,** 145–159.

Heil M, Baldwin IT. 2002. Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends in Plant Science* **7,** 61–67.

Heiling S, Schuman MC, Schoettner M, Mukerjee P, Berger B, Schneider B, Jassbi AR, Baldwin IT. 2010. Jasmonate and pphsystemin regulate key malonylation steps in the biosynthesis of 17-hydroxygeranyllinalool diterpene glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. *The Plant Cell* **22,** 273–292.

Howe GA, Jander G. 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology* **59,** 41–66.

Huang X, Stettmaier K, Michel C, Hutzler P, Mueller M, Durner J. 2004. Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. *Planta* **218,** 938–946.

Jassbi AR, Gase K, Hettenhausen C, Schmidt A, Baldwin IT. 2008. Silencing geranylgeranyl diphosphate synthase in *Nicotiana attenuata* dramatically impairs resistance to tobacco hornworm. *Plant Physiology* **146,** 974–986.

Jongsma MA, Bakker PL, Visser B, Stiekema WJ. 1994. Trypsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta* **195,** 29–35.

Kahl J, Siemens DH, Aerts RJ, Gabler R, Kuhnemann F, Preston CA, Baldwin IT. 2000. Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. *Planta* **210,** 336–342.

Kang J-H, Wang L, Giri A, Baldwin IT. 2006. Silencing threonine deaminase and JAR4 in *Nicotiana attenuata* impairs jasmonic acid-isoleucine-mediated defenses against *Manduca sexta*. *The Plant Cell* **18,** 3303–3320.

Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL. 2004. Exploring the temperature-stress metabolome of *Arabidopsis*. *Plant Physiology* **136,** 4159–4168.

Kaur H, Heinzel N, Schoettner M, Baldwin IT, Galis I. 2010. R2R3-NaMYB8 regulates the accumulation of phenylpropanoid–polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. *Plant Physiology* **152,** 1731–1747.

Keinanen M, Oldham NJ, Baldwin IT. 2001. Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in Nicotiana attenuata. *Journal of Agricultural and Food Chemistry* **49,** 3553–3558.

Kessler A, Baldwin IT. 2002. Plant responses to insect herbivory: the emerging molecular analysis. *Annual Review of Plant Biology* **53,** 299–328.

Kessler A, Halitschke R, Baldwin IT. 2004. Silencing the jasmonate cascade: induced plant defenses and insect populations. *Science* **305,** 665–668.

Krügel T, Lim M, Gase K, Halitschke R, Baldwin IT. 2002. *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. *Chemoecology* **12,** 177–183.

Lee U, Wie C, Fernandez BO, Feelisch M, Vierling E. 2008. Modulation of nitrosative stress by *S*-nitrosoglutathione reductase is critical for thermotolerance and plant growth in *Arabidopsis*. *The Plant Cell* **20,** 786–802.

Li C, Schilmiller AL, Liu G, *et al*. 2005. Role of {beta}-oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. *The Plant Cell* **17,** 971–986.

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA. 2004. The tomato homolog of coronatine-insensitive1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. *The Plant Cell* **16,** 126–143.

Lindermayr C, Saalbach G, Bahnweg G, Durner J. 2006. Differential inhibition of *Arabidopsis* methionine adenosyltransferases by protein *S*-nitrosylation. *Journal of Biological Chemistry* **281,** 4285–4291.

Lindermayr C, Saalbach G, Durner J. 2005. Proteomic identification of *S*-nitrosylated proteins in *Arabidopsis*. *Plant Physiology* **137,** 921–930.

Lindermayr C, Sell S, Muller B, Leister D, Durner J. 2010. Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. *The Plant Cell* **22,** 2894–2907.

Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. 2001. A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. *Nature* **410,** 490–494.

Liu L, Yan Y, Zeng M, *et al*. 2004. Essential roles of *S*-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell* **116,** 617–628.

Liu Y, Zhang S. 2004. Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in *Arabidopsis*. *The Plant Cell* **16,** 3386–3399.

Martínez MC, Achkor H, Persson B, Fernández MR, Shafqat J, Farrés J, Jörnvall H, Parés X. 1996. *Arabidopsis* formaldehyde dehydrogenase. Molecular properties of plant class III alcohol dehydrogenase provide further insights into the origins, structure and function of plant class P and liver class I alcohol dehydrogenases. *European Journal of Biochemistry* **241,** 849–857.

McConn M, Creelman RA, Bell E, Mullet JE, Browse J. 1997. Jasmonate is essential for insect defense in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **94,** 5473–5477.

Mittler R. 2006. Abiotic stress, the field environment and stress combination. *Trends in Plant Science* **11,** 15–19.

Moreau M, Lindermayr C, Durner J, Klessig DF. 2010. No synthesis and signaling in plants: where do we stand? *Physiologia Plantarum* **138,** 372–383.

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ. 1996. Ethylene as a signal mediating the wound response of tomato plants. *Science* **274,** 1914–1917.

Orozco-Cardenas ML, Narvaez-Vasquez J, Ryan CA. 2001. Hydrogen peroxide acts as a second messenger for the induction of

defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *The Plant Cell* **13,** 179–191.

Orozco-Cardenas ML, Ryan CA. 2002. Nitric oxide negatively modulates wound signaling in tomato plants. *Plant Physiology* **130,** 487–493.

Paschold A, Halitschke R, Baldwin IT. 2007. Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. *The Plant Journal* **51,** 79–91.

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM. 2009. Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* **5,** 308–316.

Ratcliff F, Martin-Hernandez AM, Baulcombe DC. 2001. Technical advance: tobacco rattle virus as a vector for analysis of gene function by silencing. *The Plant Journal* **25,** 237–245.

Romero-Puertas MC, Campostrini N, Mattè A, Righetti PG, Perazzolli M, Zolla L, Roepstorff P, Delledonne M. 2008. Proteomic analysis of *S*-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. *Protomics* **8,** 1459–1469.

Ross C, Küpper FC, Jacobs RS. 2006. Involvement of reactive oxygen species and reactive nitrogen species in the wound response of *Dasycladus vermicularis*. *Chemistry and Biology* **13,** 353–364.

Rusterucci C, Espunya MC, Diaz M, Chabannes M, Martinez MC. 2007. *S*-nitrosoglutathione reductase affords protection against pathogens in arabidopsis, both locally and systemically. *Plant Physiology* **143,** 1282–1292.

Ryan CA. 1989. Proteinase inhibitor gene families: strategies for transformation to improve plant defenses against herbivores. *BioEssays* **10,** 20–24.

Saedler R, Baldwin IT. 2004. Virus-induced gene silencing of jasmonate-induced direct defences, nicotine and trypsin proteinase-inhibitors in *Nicotiana attenuata*. *Journal of Experimental Botany* **55,** 151–157.

Sagi M, Davydov O, Orazova S, Yesbergenova Z, Ophir R, Stratmann JW, Fluhr R. 2004. Plant respiratory burst oxidase homologs impinge on wound responsiveness and development in *Lycopersicon esculentum*. *The Plant Cell* **16,** 616–628.

Sakamoto A, Ueda M, Morikawa H. 2002. *Arabidopsis* glutathione-dependent formaldehyde dehydrogenase is an *S*-nitrosoglutathione reductase. *FEBS Letters* **515,** 20–24.

Shohael A, Ali M, Yu K-W, Hahn E-J, Paek K- Y. 2006. Effect of temperature on secondary metabolites production and antioxidant enzyme activities in *Eleutherococcus senticosus* somatic embryos. *Plant Cell, Tissue and Organ Culture* **85,** 219–228.

Spoel SH, Koornneef A, Claessens SMC, *et al*. 2003. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *The Plant Cell* **15,** 760–770.

Stamler JS, Lamas S, Fang FC. 2001. Nitrosylation: the prototypic redox-based signaling mechanism. *Cell* **106,** 675–683.

Staswick PE, Tiryaki I. 2004. The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. *The Plant Cell* **16,** 2117–2127.

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT. 2004. Nicotine's defensive function in nature. *PLoS Biology* **2,** e217.

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X. 2008. Plant immunity requires conformational charges of NPR1 via *S*-nitrosylation and thioredoxins. *Science* **321,** 952–956.

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J. 2007. JAZ repressor proteins are targets of the scfcoi1 complex during jasmonate signalling. *Nature* **448,** 661–665.

Von Dahl CC, Winz RA, Halitschke R, Kühnemann F, Gase K, Baldwin IT. 2007. Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*. *The Plant Journal* **51,** 293–307.

Wang KL-C, Li H, Ecker JR. 2002. Ethylene biosynthesis and signaling networks. *The Plant Cell* **14,** S131–S151.

Wang Y, Yun BW, Kwon E, Hong JK, Yoon J, Loake GJ. 2006. *S*-nitrosylation: an emerging redox-based post-translational modification in plants. *Journal of Experimental Botany* **57,** 1777–1784.

Wasternack C. 2007. Jasmonates: aAn update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of Botany* **100,** 681–697.

Wilson ID, Neill SJ, Hancock JT. 2008. Nitric oxide synthesis and signalling in plants. *Plant, Cell and Environment* **31,** 622–631.

Wu J, Baldwin IT. 2009. Herbivory-induced signalling in plants: perception and action. *Plant, Cell and Environment* **32,** 1161–1174.

Wu J, Baldwin IT. 2010. New insights into plant responses to the attack from insect herbivores. *Annual Review of Genetics* **44,** 1–24.

Wu J, Hettenhausen C, Baldwin I. 2006. Evolution of proteinase inhibitor defenses in North American allopolyploid species of *Nicotiana*. *Planta* **224,** 750–760.

Wu J, Hettenhausen C, Meldau S, Baldwin IT. 2007. Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. *The Plant Cell* **19,** 1096–1122.

Wu J, Wang L, Baldwin I. 2008. Methyl jasmonate-elicited herbivore resistance: does MeJA function as a signal without being hydrolyzed to JA? *Planta* **227,** 1161–1168.

Yamasaki H, Sakihama Y. 2000. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: *in vitro* evidence for the NR-dependent formation of active nitrogen species. *FEBS Letters* **468,** 89–92.

Zavala J, Baldwin IT. 2004. Fitness benefits of trypsin proteinase inhibitor expression in *Nicotiana attenuata* are greater than their costs when plants are attacked. *BMC Ecology* **4,** .

Zavala JA, Patankar AG, Gase K, Hui D, Baldwin IT. 2004. Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. *Plant Physiology* **134,** 1181–1190.

Zhang S, Klessig DF. 1997. Salicylic acid activates a 48-kD MAP kinase in tobacco. *The Plant Cell* **9,** 809–824.