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Research Article

Narboh D, a Respiratory Burst Oxidase Homolog in *Nicotiana attenuata*, is Required for Late Defense Responses After Herbivore Attack

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Abstract

The superoxide (O_2^-)-generating NADPH oxidases are crucial for the defense of plants against attack from pathogens; however, it remains unknown whether they also mediate responses against chewing insect herbivores. The transcripts of the respiratory burst NADPH oxidase homolog *Narboh D* in *Nicotiana attenuate* are rapidly and transiently elicited by wounding, and are amplified when *Manduca sexta* oral secretions (OS) are added to the wounds. The fatty-acid-amino-acid-conjugates (FACs), demonstrably the major elicitors in *M. sexta* OS, are responsible for the increase in *Narboh D* transcripts. Silencing *Narboh D* significantly reduced reactive oxygen species (ROS) levels after OS elicitation, but neither OS-elicited jasmonic acid (JA) or JA-isoleucine (JA-Ile) bursts, pivotal hormones that regulates plant resistance to herbivores, nor early transcripts of herbivore defense-related genes (*NaJAR4* and *NaPAL1*), were influenced. However, late OS-elicited increases in trypsin proteinase inhibitors (TPIs), as well as the transcript levels of defense genes such as *polyphenol oxidase, TPI* and *Thionin* were significantly reduced. In addition, *Narboh D*-silenced plants were more vulnerable to insect herbivores, especially the larvae of the generalist *Spodoptera littoralis*. We thus conclude that *Narboh D*-based defenses play an important role in late herbivore-elicited responses.

Keywords: *Nicotiana attenuata*; Narboh D; *Manduca sexta*; *Spodoptera littoralis*.

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Introduction

The herbivore-induced responses of *Nicotiana attenuate*, a post-fire annual native of the Great Basin Desert of California, Nevada, Idaho, and Utah, have been extensively studied using ecological, chemical, and molecular approaches (Wu and Baldwin 2010). Herbivore feeding by the larvae of *Manduca sexta,* a specialist of *N. attenuata*, or elicitation by the application of larval oral secretions (OS) to puncture wounds, activates a rapid burst of jasmonic acid (JA) and JA-isoleucine (JA-Ile), which in turn induces the accumulation of defense metabolites such as nicotine (Kang et al. 2006; Wang et al. 2007; Wang et al. 2008), 17-hydroxygeranyllinalool diterpenoid glycosides (HGL-DTGs) (Jassbi et al. 2008; Heiling et al. 2010), and the anti-digestive proteins trypsin proteinase inhibitors (TPIs) (van Dam et al. 2001; Zavala et al. 2004). Reducing nicotine, HG-DTGs, and TPI production in transformed plants has demonstrated the importance of these compounds as herbivore defense mechanisms (Steppuhn et al. 2004; Zavala et al. 2004; Jassbi et al. 2008). JA signaling clearly plays a central role in these responses: blocking JA perception by silencing *NaCOI1* or JA biosynthesis by silencing

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NaLOX3 in *N. attenuata* abolishes most of the herbivore-elicited responses (Halitschke and Baldwin 2003; Paschold et al. 2007).

The rapid production of reactive oxygen species (ROS) is a hallmark of defense responses to attack from pathogens, especially in incompatible interactions between resistant plants and avirulent pathogens (Lamb and Dixon 1997). Several studies have reported that insect attack also increases ROS levels at the attack site in lima bean (*Phaseolus lunatus*) (Maffei et al. 2006) and *Medicago truncatula* (Leitner et al. 2005) leaves. ROS can be produced by chloroplasts, mitochondria, and peroxisomes, or by apoplastic cell wall peroxidases, germin-like oxalate oxidases, and amino oxidases (Apel and Hirt 2004; Mittler et al. 2004). However, pharmacological and molecular data indicate that the enzymes responsible for most of the ROS generated during biotic interactions and in response to abiotic stresses are similar to the superoxide (O_2^-)-generating respiratory burst NADPH oxidase homologues (Rboh), originally characterized in mammalian phagocytes (Simon-Plas et al. 2002; Torres et al. 2002; Yoshioka et al. 2003; Sagi et al. 2004; Torres et al. 2005; Lherminier et al. 2009). Interestingly, NADPH oxidase-dependent H_2O_2 is also proposed to function as a second messenger, mediating the systemic expression of various defense-related genes in tomato (*Lycopersicon esculentum*) plants (Orozco-Cardenas and Ryan 1999; Orozco-Cardenas et al. 2001; Sagi et al. 2004). In addition, *rbohD* mutant *Arabidopsis* plants, which lack a functional NADPH oxidase, supported an approximately four-fold higher population growth rate of the aphid *Myzus persicae* than did wild-type plants (Miller et al. 2009). These results suggest that ROS produced by NADPH oxidases play a role in mediating responses against chewing insect herbivores. This inference raises several important questions. Which NADPH oxidase is responsible? Can it be elicited by herbivory-specific elicitors, such as herbivore oral secretions and regurgitants (OS)? Does the ROS derived from herbivore-elicited NADPH oxidase in turn elicit jasmonate (JA) signaling and thereby the outcome of plant-herbivore interactions? In this study, we explore these questions in the model ecological plant, *N. attenuata*.

Here, we report that Narboh D (Nicotiana attenuata respiratory burst NADPH oxidase homologue D), whose transcripts are rapidly and transiently wound- and OS-elicited, is required for the elicitation of late defense responses to attacks from insect herbivores.

Results

OS elicitation increases levels of *Narboh D* transcripts

Because NADPH oxidase-dependent ROS is suggested to function as a second messenger mediating the systemic expression of various defense-related genes in tomato (Orozco-Cardenas and Ryan 1999; Orozco-Cardenas et al. 2001; Sagi et al. 2004) and *Arabidopsis* plants (Miller et al. 2009), and *M. sexta* OS elicit herbivore-specific responses in *N. attenuata*, we searched for NADPH oxidase candidate genes which were amplified specifically by OS treatment. Source-sink transition leaves of *N. attenuata* were wounded with a fabric pattern wheel and 20 μL of *M. sexta* OS were immediately applied to wounds (W + OS treatment); applying water (20 μL) to wounds served as comparisons (W + W treatment). The transcripts of one of the NADPH oxidase genes we cloned were rapidly and transiently elicited by wounding plus water treatments (**Figure 1A**), and they rapidly returned to near basal levels as quickly as in 1 h. Transcript accumulations were more strongly induced by wounding plus OS treatments, as the 1 h-induction peak in **Figure 1A** shows. Because this NADPH oxidase gene displays a high amino acid sequence similarity to *Ntrboh D*, the respiratory burst oxidase homolog D in *N. tabacum* (97% identity; Figure S1), and occurs as a single-copy gene in *N. attenuata* (Figure S3), we refer to it as *Narboh D* (accession number EU104741).

Microarray and biochemical analyses indicated that the fatty-acid-amino-acid-conjugates (FACs) in *M. sexta* OS are responsible for eliciting OS-specific mitogen-activated protein kinases (MAPKs), JA, volatile organic compounds (VOCs), and transcriptional responses in *N. attenuata* (Halitschke et al. 2001; Halitschke et al. 2003; Wu et al. 2007a). To determine if FACs are the specific elicitors of *Narboh D* transcripts, synthetic FACs were applied to wounded *N. attenuata* leaves (W + FAC). The levels of *Narboh D* transcripts in leaves elicited by W + FAC were the same as those in leaves elicited by W + OS, but were significantly higher than in leaves treated with W + W (**Figure 1B**). Furthermore, the removal of FACs by ion-exchange chromatography rendered OS no more capable than water in its ability to induce *Narboh D* transcript levels (**Figure 1B**).

Silencing *Narboh D* reduces ROS production after herbivory

To further investigate the role of *Narboh D* in plant-herbivore interactions, we generated stable *Narboh D*-silenced *N. attenuata* plants by *Agrobacterium*-mediated transformation (Krügel et al. 2002) using a pRESC5 transformation vector containing a 353-bp *Narboh D* fragment in an invert-repeat orientation. Two independently transformed T_2 lines, each harboring a single insertion as confirmed by Southern blot analysis (Figure S3B), were selected for all further experiments (ir*rboh D* lines 1 and 2). The silencing of *Narboh D* gene expression was confirmed by qRT-PCR; the levels of *Narboh D* transcripts in WT plants were increased to 5 fold 1 h after OS-elicitation, but remained unchanged in both ir*rboh D* lines after elicitation (**Figure 2**). DAB (3,3-diaminobenzidine) polymerizes and turns deep brown in the presence of ROS. However, DAB staining

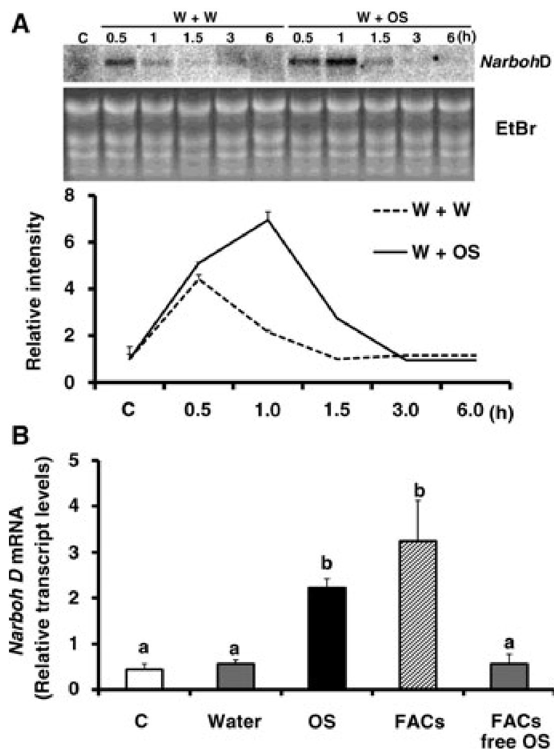

Figure 1. The elicitation of *Narboh D* transcripts.

(A) *Narboh D* transcripts were measured by northern analysis in individual source-sink transition leaves treated by wounding and applying water (W + W) or *Manduca sexta* oral secretions (OS) (W + OS) for 0.5, 1.0, 1.5, 3.0, and 6.0 h (upper panel). Around 10 μg total leaf RNA was loaded in every lane. Control (C) plants remained untreated. An ethidium bromide (EtBr) stained gel served as a loading control. Relative intensities of northern blot signals were calculated from the optical density of the hybridization bands and normalized for the amount of RNA loaded per lane by dividing by the optical density of total RNA (lower panel). The hybridization signals of control samples were arbitrarily set as 1.
(B) Mean (± *SE*) *Narboh D* transcripts were measured by real-time PCR in 4 replicate source-sink transition leaves after 1 h different treatments: wounding plus water (Water), *M. sexta* oral secretions (OS), fatty-acid-amino-acid-conjugates (FACs), or FAC-free OS. Control (C) plants remained untreated. The transcriptional levels of *Narboh D* were normalized by the housekeeping gene *NaActin2*. Different letters indicate statistically significant differences among treatments (Fisher's PLSD test; $P < 0.05$).

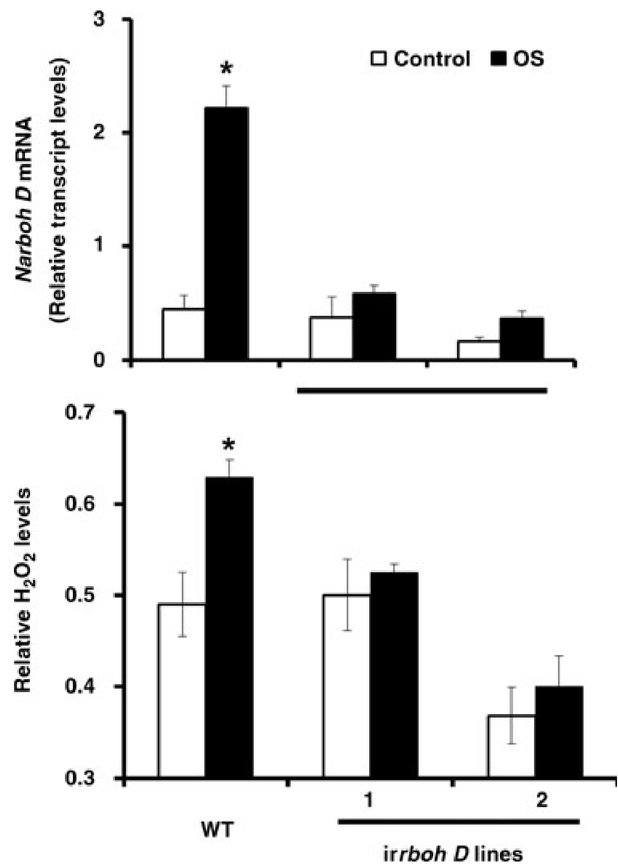

Figure 2. Reduced *Manduca sexta* oral secretions (OS)-elicited ROS levels in *Narboh D*-silenced plants.

Narboh D was stably silenced in *Nicotiana attenuata* by *Agrobacterium*-mediated transformation. Upper panel: Mean (± *SE*) *Narboh D* transcripts were measured by real-time PCR in leaf disks excised from 4 replicate leaves of WT or ir*rbohD* lines after wounding plus 1 h OS treatments. Control plants remained untreated.
Lower panel: Mean (± *SE*) relative H_2O_2 levels accumulated during OS 1 h to 4 h were measured in leaf discs excised from 4 replicate leaves of WT or ir*rbohD* lines treated with wounding plus OS. The asterisks indicate the level of significant difference between WT and ir*rboh D* plants (unpaired *t*-test: *, $P<0.05$).

did not reveal consistent differences between the WT and ir*rboh D* line 1 h after OS-elicitation (Figure S3A). Therefore, we performed the more sensitive and quantitative amplex red hydrogen peroxide assay to evaluate if silencing *Narboh* D decreases OS-elicited ROS accumulation. The results indicate that the relative levels of H_2O_2 increased significantly between 1 to 4 h after OS-elicitation of WT leaves, but not in OS-elicited leaves of either of the ir*rboh D* lines (**Figure 2**), demonstrating that silencing *Narboh D* reduced the accumulation of ROS after OS treatments.

Narboh D and herbivore-elicited early responses

After herbivory, *N. attenuata* produces rapid and transient JA and JA-Ile bursts, which in turn increase the levels of nicotine and TPIs and resistance to the larvae of *M. sexta* (Wang et al. 2008). Whether OS-elicited ROS influence the JA and JA-Ile

bursts was unknown. Silencing *Narboh D* had little influence on these OS-elicited bursts, which attain maximum values at 1 h (Figure S4). Similarly, the OS-elicited early transcripts, *NaJAR4* (the enzyme which conjugates JA with Ile to form JA-Ile) and phenylalanine ammonia lyase (*NaPAL1*), increased to the same levels in both WT and ir*rboh D* plants (Figure S4). These results demonstrate that the *Narboh D*-based ROS signal is not involved in early herbivore-induced responses, especially the JA signaling pathway.

Narboh D and herbivore-elicited late responses

However, 6 h after OS elicitation, *TPI* transcripts were significantly reduced when diphenylene iodonium (DPI), an NADPH oxidase inhibitor widely used to study the function of NADPH oxidases in plants (Orozco-Cardenas and Ryan 1999; Orozco-Cardenas et al. 2001), was added to wounds before OS application, while wound-elicited *TPI* transcripts were unchanged after DPI treatments (**Figure 3A**). These results are consistent with the hypothesis that ROS signals derived from *Narboh D* are involved in OS-elicited, but not wounding-induced, late responses. Indeed, the TPI activity of the OS-elicited ir*rboh D* line 1 was only 46.5% of that of the WT after 48 h treatments. Similar trends were also observed in ir*rboh D* line 2 plants (**Figure 3B**). Wound-elicited TPI activity levels did not differ between leaves of the WT and ir*rboh D* lines (**Figure 3B**).

To re-evaluate the conclusion that OS-induced levels of *TPI* transcripts decreased when treated with DPI, and to further investigate the role of *Narboh D*-based signaling in OS-elicited late responses, we used microarrays enriched in herbivore-elicited genes (a total of 1,404 genes) to compare transcriptional changes in WT and ir*rboh D* plants 10 h after OS elicitation. Among the 115 up-regulated genes in WT plants after OS elicitation, 16% of these genes (Group 1; 18 genes), which are mostly involved in direct defense and include *SnPIN2a*, *NaTPI* and *SnPPO*, were also up-regulated in ir*rboh D* plants, but at much lower levels than those of WT plants (**Figure 4A** and Table S1). However, 73% (Group 3; 84 genes) were not significantly up-regulated in ir*rboh D* plants, which included, for example, *NaThionin* (secondary metabolism) and *NaACO1* (ethylene biosynthesis). Only 11% of the up-regulated genes (Group 2; 13 genes) were induced to the same levels in ir*rboh D* plants as in WT plants. These results revealed that *Narboh D*-based defenses are essential for inducing most OS-elicited late response genes, especially *NaTPI* and *NaThionin*.

Narboh D-based defense is also required for the induction of most of the OS-elicited down-regulated genes (**Figure 4B** and Table S1). Among the 116 down-regulated genes in WT plants after OS treatment, 68% (Group 6; 79 genes) were not significantly down-regulated in ir*rboh D* plants, for example, *Calmodulin* and *SnCAT1*; meanwhile, only 22% (Group 7; 26 genes) were elicited at the same levels in ir*rboh D* plants as in WT plants, such as *NaRCA*. Another 7% (Group 9; 8 genes) were also significantly down-regulated in ir*rboh D* plants, but at greatly reduced levels compared to those of WT plants.

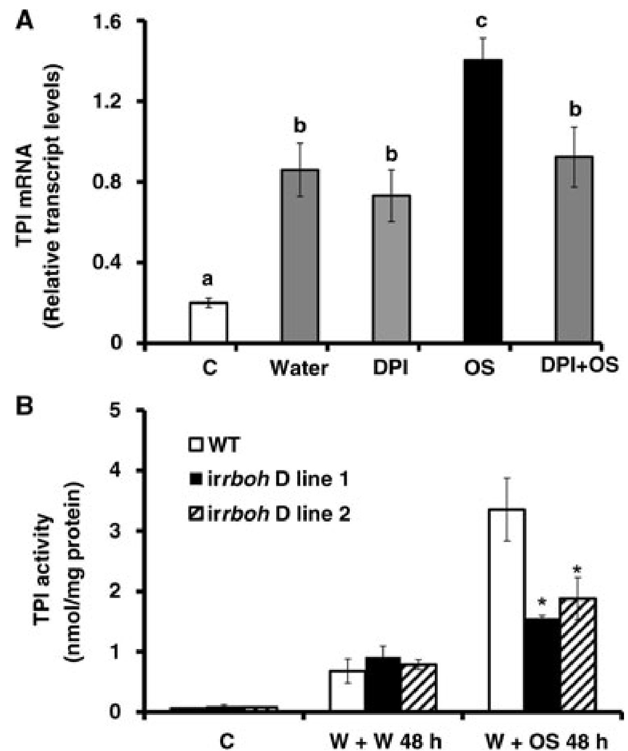

Figure 3. Silencing of *Narboh D* decreases *Manduca sexta* oral secretions (OS)-elicited but not wounding-elicited TPI transcripts and activity.

(A) Mean ($\pm$ *SE*) *NaTPI* transcripts were measured by real-time PCR in leaf disks excised from 4 replicate source-sink transition leaves after 6 h different treatments: wounding plus water (Water), DPI, OS, or DPI and OS. Control (C) plants remained untreated. Different letters indicate statistically significant differences among treatments (Fisher's PLSD test; $P < 0.05$).

(B) Mean ($\pm$ *SE*) TPI activity of 5 replicate WT, ir*rboh D* line 1 and ir*rboh D* line 2 plants 48 h after wounding plus water (W + W) or OS (W + OS) treatments. The treated leaves were harvested. The asterisks indicate the level of significant difference between WT and ir*rboh D* plants (unpaired *t*-test: *, $P < 0.05$). Control (C) plants remained untreated.

Since *Narboh D* is required for the induction of most OS-elicited late transcripts and TPI levels, we hypothesized that insect larvae would perform better on ir*rboh D* plants. After 10 d, larvae of the specialist herbivore *M. sexta* feeding on ir*rboh D* plants gained slightly but not significantly more mass than those on WT plants (**Figure 5A**; unpaired *t*-test, $P = 0.066$). However,

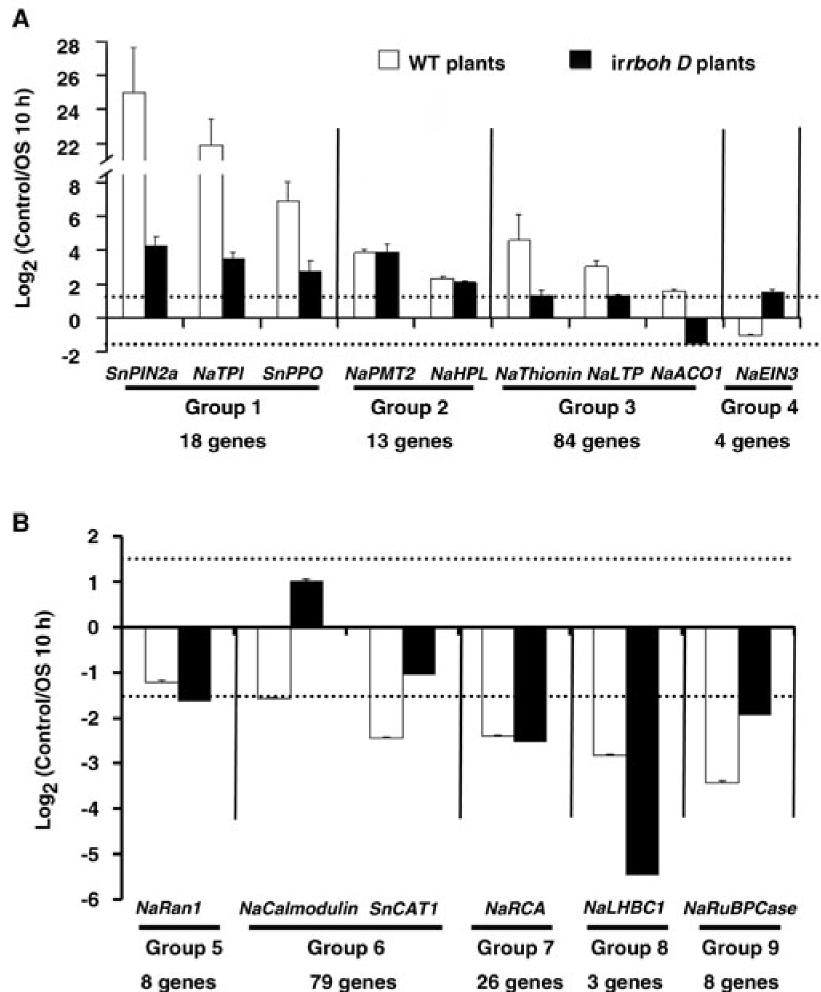

Figure 4. Silencing *Narboh D* greatly influences *Manduca sexta* oral secretions (OS)-elicited late transcript levels.

cDNA samples from untreated source-sink transition leaves were labeled with fluorescent dye Cy5, and samples 10 h after OS-elicitation were labeled with Cy3. For each treatment, 3 pools of WT samples and 3 pools of ir*rboh D* samples were made, each consisting of 3 biological replicates. Each microarray was hybridized with one pool of untreated and OS-elicited samples of labeled cDNA from WT or ir*rboh D* plants. Three microarrays per treatment were hybridized and statistically analyzed. Genes are considered significantly ($P < 0.05$) up-regulated when $\log_2$ (Cy3/Cy5) > 1.5 and down-regulated when $\log_2$ (Cy3/Cy5) < -1.5. The details of regulated gene names and accession numbers are shown in the Supplemental Table.

(A) Mean ($\pm$ *SE*) value of $\log_2$Cy3/Cy5 of representative genes up-regulated in WT or ir*rboh D* plants 10 h after OS-elicitation. All genes are divided into four groups: up-regulated in both WT and ir*rboh D* plants, but the level is lower in ir*rboh D* plants (Group 1, 18 genes); up-regulated to the same levels in both WT and ir*rboh D* plants (Group 2, 13 genes); up-regulated only in WT plants (Group 3, 84 genes); and up-regulated only in ir*rboh D* plants (Group 4, 4 genes). Sn, *Solanum nigrum*; Na, *Nicotiana attenuata*; PIN2a (AF209709), proteinase inhibitor IIa; PPO (A27686), polyphenol oxidase; PMT2 (AF280403), putrescine N-methyltransferase 2; HPL (AJ414400), hydroperoxide lyase; Thionin (AY456268); LTP (AY456267), lipid-transfer protein; ACO1 (AY426756), ACC oxidase 1; EIN3 (AB015855), ethylene insensitive 3.

(B) Mean ($\pm$ *SE*) value of $\log_2$Cy3/Cy5 of representative genes down-regulated in WT or ir*rboh D* plants 10 h after OS-elicitation: down-regulated only in ir*rboh D* plants (Group 5, 8 genes); down-regulated only in WT plants (Group 6, 79 genes); down-regulated to the same level in both WT and ir*rboh D* plants (Group 7, 26 genes); down-regulated in both WT and ir*rboh D* plants, but the level is lower in ir*rboh D* plants (Group 8, 3 genes); down-regulated in both WT and ir*rboh D* plants, but the level is lower in WT plants (Group 9, 8 genes). Ran1 (CA591814), GTP-binding protein (Ran-A1); Calmodulin (AY456265); CAT1 (M93719), catalase; RCA (BU494545), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) activase; LHBC1 (AW191805), lhbC1 gene for LHCII type III; RuBPCase (BU494544), small subunit of RuBPCase.

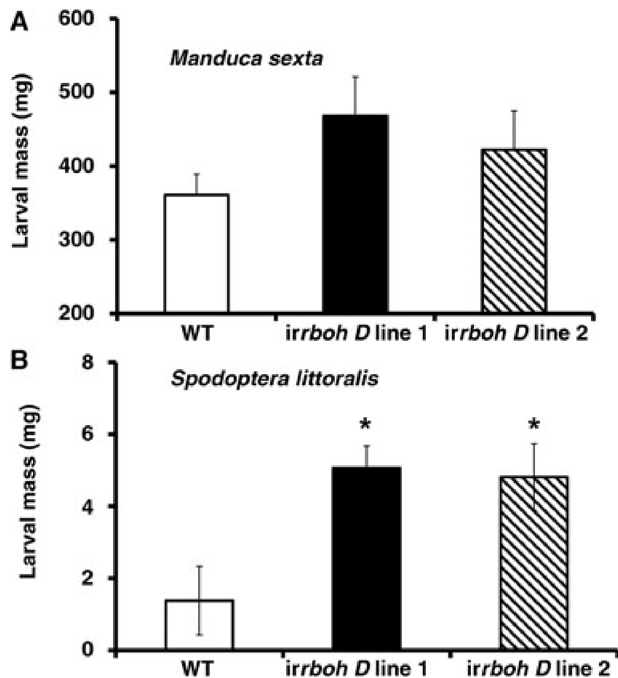

Figure 5. Silencing *Narboh D* decreases herbivore resistance.

(A) Mean (± *SE*) mass of *Manduca sexta* larvae 10 d after feeding on 20 replicates each of WT and ir*rboh D* plants.

(B) Mean (± *SE*) mass of *Spodoptera littoralis* larvae 5 d after feeding on 20 replicate leaves of WT and ir*rboh D* plants within clip cages. The asterisks indicate the level of significant difference between WT and ir*rboh D* plants (unpaired *t*-test: *, $P < 0.05$; **, $P < 0.001$).

larvae of the generalist herbivore *Spodoptera littoralis* (which are not specifically adapted to *N. attenuate*) feeding on leaves from ir*rboh D* plants gained significantly more mass after 5 d than those feeding on leaves from WT plants (**Figure 5B**; unpaired *t*-test, $P = 0.004$).

Discussion

The superoxide (O_2^-)-generating plasma membrane NADPH-dependent oxidases, similar to the mammalian calcium-regulated NADPH oxidase, are thought to play a key role in ROS signaling (Simon-Plas et al. 2002; Torres et al. 2002; Yoshioka et al. 2003; Sagi et al. 2004; Torres et al. 2005; Lherminier et al. 2009). However, Terres et al. (2002) failed to detect O_2^- by nitroblue tetrazolium staining or cytochrome c reduction after pathogen infection. H_2O_2, and not the superoxide, is the ROS most detected in plant-pathogen interactions (Torres et al. 2002; Yoshioka et al. 2003; Torres et al. 2005; Lherminier et al. 2009). These data suggest that rapid dismutation converts superoxide into H_2O_2. In *Narboh D*-silenced plants (ir*rboh D* lines), we also observed a reduction in H_2O_2 accumulation after OS-elicitation compared to similarly-treated WT plants, indicating that *Narboh D* is responsible for ROS production after OS treatments. However, we cannot exclude the role of other ROS sources in mediating the signaling activated by herbivore attack.

Like *Atrboh D* mutants in *Arabidopsis* (Torres et al. 2002), *Narboh D*-silenced plants are morphologically normal; they grow as well as WT plants do in their seedling stage (Figure S2A), but are significantly smaller than WT plants at the rosette stage before bolting when grown in soil (Figure S2B). In contrast, silencing the *Rboh* homolog in tomato by antisense technology leads to dramatic changes in plant morphology: enhanced branching, curling leaflets, indeterminate inflorescences, and fasciated reproductive organs (Sagi et al. 2004), suggesting that the *Rboh* in tomato may function differently from *Narboh D* in *N. attenuata* and *Atrboh D* in *Arabidopsis*.

We did not observe a reduction in wounding-elicited *TPI* transcripts in either DPI-treated WT leaves of *N. attenuata* plants (**Figure 3A**), or in ir*rboh D* plants (Figure S5), although NAPDH oxidase-dependent H_2O_2 has been proposed to function as a second messenger mediating the wound-induced systemic expression of various defense-related genes in tomato plants (Orozco-Cardenas and Ryan 1999; Orozco-Cardenas et al. 2001; Sagi et al. 2004). Furthermore, the levels of TPI activity elicited by wounding were the same in WT and ir*rboh D* plants (**Figure 3B**). These results indicate that *Narboh D*-based signals are not involved in wound-elicited TPI responses in *N. attenuata*, unlike what has been observed in tomato.

However, silencing *Narboh D* does have a large effect on OS-elicited late responses. Most defense genes, including *SnPIN2a*, *NaTPI*, *SnPPO*, *NaThionin*, *NaACO1*, and *NaLTP*, were elicited to much lower levels in ir*rboh D* plants than in WT plants (**Figure 4**). The decreased OS-elicited *NaTPI* transcripts in ir*rboh D* plants are consistent with the results that *NaTPI* transcripts were significantly reduced when DPI was added to wounds before OS application. In addition, the level of TPI activity 48 h after OS elicitation was also significantly reduced in ir*rboh D* plants leaves (**Figure 3B**). These changes in ir*rboh D* plants are likely responsible for the increased larvae performance (**Figure 5**), especially that of *S. littoralis*, which is not particularly adapted to *N. attenuata* plants.

JA signaling clearly plays a central role in herbivore-induced responses of *N. attenuata*, as blocking JA perception by silencing *COI1* in *N. attenuata* (ir*COI1* plants) abolishes most of the herbivore-elicited responses (Paschold et al. 2007). Herbivory-induced accumulation of JA is regulated by many factors; for example, plant ontogeny, and nitric oxide-associated protein 1 (NOA1) and DICER-like (DCL) proteins (Diezel et al. 2011;

Wunsche et al. 2011; Bozorov et al. 2012). Here, we show that OS-elicited levels of JA/JA-Ile are not altered in *Narboh D*-silenced plants, indicating that Narboh D is not involved in the regulation of JA accumulation. However, OS-elicited late responses are greatly influenced in *Narboh D*-silenced plants. Thus, Narboh D-based ROS signaling may function independently of JA signaling. FACs are known to be responsible for eliciting OS-specific MAPK, JA, VOCs, and transcriptional responses in *N. attenuata* (Halitschke et al. 2001; Halitschke et al. 2003; Wu et al. 2007a). Levels of *Narboh D* transcripts were more highly elicited by FACs than by wounding alone, and removing FACs from OS by ion-exchange chromatography removes the elicitation activity of OS, suggesting that FACs are the elicitor of *Narboh D* transcripts (**Figure 1**). It is still not clear how NADPH oxidase is regulated by FACs. Recently, it was proposed that the activity of NADPH oxidase is modulated by Ca^{2+}, CDPK, Rac GTPase (Morel et al. 2004; Kobayashi et al. 2007; Wong et al. 2007; Takahashi et al. 2012), 14-3-3 protein (Elmayan et al. 2007), and MAPK in *Nicotiana benthamiana* (Asai et al. 2008), which provide clues for how NADPH oxidase may be regulated by FACs.

In summary, our results demonstrate that *Narboh D* is required for OS-elicited late responses in *N. attenuata* (**Figure 4**). After herbivory, when plants perceive the FACs in the OS, both the Narboh D-based signaling and the JA-signaling pathways are activated and collaborate. This triggers late transcriptional changes and the accumulation of TPIs, (**Figure 6**) which lower the performance of the generalist herbivore, *Spodoptera littoralis*.

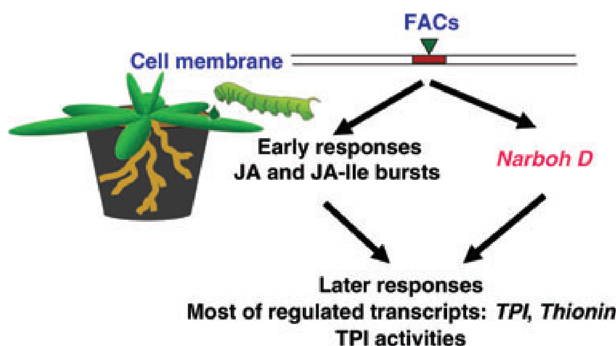

Figure 6. Model of the role of *Narboh D*-based signals in plant-herbivore interaction.

After insect herbivore attack, herbivore elicitors (fatty-acid-amino-acid-conjugates, FACs) activate plant early (in 1 to 3 h) and late defense responses (later than 6 h) by binding to putative receptors in plasma membranes. *Narboh D*-based signaling (reactive oxygen species, ROS) do not influence the early jasmonic acid (JA) and JA-isoleucine (JA-ile) bursts, but contribute to later responses such as the accumulation of *NaTPI*, *NaThionin* transcripts and TPI activities.

Materials and Methods

Plant growth

We used seeds of the 21st generation of an inbred line of *Nicotiana attenuata* Torr. Ex Watts (Solanaceae) for transformation and in all experiments. Seed germination and plant growth were conducted as described by Krügel et al. (2002).

Isolation of *Narboh D*

We cloned *Narboh D* using an RT-PCR with primers designed from sequences of *Ntrboh D* (accession number AJ309006) and *Nbrobh B* (accession number AB079499) (forward primer *Narboh D* 155 and reverse primer *Narboh D* 3235; primer information is listed below). The 3 kb cDNA product (accession number EU104741), which contains the entire open reading frame, was cloned into a pGEM-T EASY vector (Promega, Madison, WI, USA) and sequenced.

Leaf treatments

All treatments were performed on source-sink transition leaves of rosette-stage plants. For wounding plus water or oral secretions (OS) treatments, leaves were wounded with a fabric pattern wheel, and 20 μL of water (or one-fifth diluted *Manduca sexta* OS) was rubbed into the puncture wounds. Fatty-acid-amino-acid-conjugate (FAC) A, FAC B, FAC C, and FAC D were synthesized in-house (Halitschke et al. 2001). Each FAC was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mM, and then diluted in water to 0.2 mM. FAC-free OS were prepared by passing OS four times through spin columns filled with Amberlite IRA-400 resin (Sigma-Aldrich, St. Louis, MO, USA) (Halitschke et al. 2001). Twenty μL of FACs and FACs-free OS solution were applied to wounded leaves. Diphenylene iodonium chloride (DPI, Sigma-Aldrich), an inhibitor of NADPH oxidase, was applied to the wounds at a final concentration of 50 μM, also in quantities of 20 μL.

Generation of *Narboh D* stably silenced plants

We generated stably silenced plants through *Agrobacterium*-mediated transformation as described by Krügel et al. (2002). In brief, the *Narboh D* fragment amplified with forward primer: 5′-ATTGGTGGGTCTTGGAAT -3′ and reverse primer: 5′-AACGAGCATCACCTTCTTCA -3′, was inserted into the pRESC5 transformation vector in an inverted-repeat orientation. Hypocotyls from the seedling stage were cut into 3 mm pieces with a scalpel which had been dipped into the *Agrobacterium* suspension before use. After callus induction and selection, light green shoots started to develop. Subsequently, the callus with shoots was sub-cultured every 3 w

until plantlets were formed. Plantlets were sub-cultured onto rooting media every 3 w until roots appeared, after which plants were carefully removed from the gel and planted into soil. When seeds were collected, they were screened with hygromycin. Two single-insertion lines (ir*rboh D* lines 1 and 2) were identified, bred to homozygosity, and used for experimentation.

Analysis of TPI activity, JA, and JA-Ile

Trypsin proteinase inhibitor (TPI) activity was analyzed by radial diffusion as described by van Dam et al. (2001).

JA and JA-Ile were extracted and quantified by LC-MS as described by Wu et al. (2008). One mL of ethyl acetate spiked with 200 ng of $^{13}C_2$-JA, D_4-SA, and para-coumaric acid, used as internal standards for JA, SA, and JA-Ile quantification, respectively, was added to each briefly-crushed frozen leaf sample (approximately 300 mg). Five replicated leaf samples were used for each treatment. Samples were then ground using a FastPrep homogenizer (Thermo Electron, Waltham, USA). After being centrifuged at 13,000 *g* for 10 min at 4 °C, the supernatant were transferred to fresh tubes and evaporated to dryness on a vacuum concentrator (Eppendorf, Hamburg, Germany). Each residue was resuspended in 0.5 mL of 70% methanol (v/v) and centrifuged (15 min, 13,000 *g*, 4 °C) to remove particles. The supernatant was analyzed on an HPLC-tandem mass spectrometry device (1200L LC-MS system, Varian), and each phytohormone was quantified by comparing its peak area with that of an internal standard.

Microarray analysis

To analyze the late transcriptional responses to OS elicitation in ir*rboh D* and WT plants, control and OS-elicited leaves were harvested 10 h after treatments and pooled from three plants per treatment before RNA extraction. Thus, for each treatment we harvested three pools, each consisting of three biological replicates. mRNA was isolated, reverse transcribed, and labeled as previously described by Wang et al. (2008). Samples from untreated control plants were Cy5-labeled and hybridized against OS-elicited samples which were labeled with Cy3. For all hybridizations, three replicate microarrays were performed and statistically analyzed.

We used a custom-made 1.4 k microarray consisting of 50-mer oligonucleotides. Sequences were selected from herbivore-induced genes identified in differential experiments and from public databases. All 1,405 clones were spotted fourfold on each array. Spot intensities (SIs) for Cy3 and Cy5 were extracted from image files using AIDA software (Raytest). Raw signal intensities were local-background (LBg) subtracted and LOWESS normalized using MIDAS (Saeed et al. 2003). Spots below 1.5 times signal-to-noise ratio (=1.5x SI/ LBg) were set to zero. For statistical analysis, all Bg-corrected-SIs were 2Log transformed. Single slides were evaluated on the basis of an average treatment/control ratio >1.5 or <-1.5, and a *P*-value (*t*-test after exclusion of zero-values) <0.05 as the criteria for significant regulation. To analyze the three replicate microarrays from experiments, a nested-ANOVA was performed on a total of 12 normalized Cy3 and Cy5 values for each oligo. The obtained *P*-values of the factor treatment for all oligos were adjusted for multiple testing using the procedure for controlling the false discovery rate described by Benjamini et al. (1995).

The details of regulated gene names and accession numbers are provided in the Supplemental Table.

DAB staining and H_2O_2 measurements

An accurate quantification assay of H_2O_2 in leaf extracts proved to be very difficult and problematic (Queval et al. 2008). Indeed, a very high background H_2O_2 concentration was detected in *N. attenuata* leaves using the ferrous xylenol orange assay described by Queval et al. (2008). Therefore, we tried using DAB (3,3-diaminobenzidine) staining and Amplex red staining methods to see the relative accumulation of ROS after OS treatments over a certain time.

DAB staining was performed as previously described (Orozco-Cardenas and Ryan 1999) and photographed with a stereomicroscope. Briefly, leaves were excised at the base of the petioles with a razor blade and treated with a 1 mg/mL solution of DAB for 5 h. DAB-treated leaves were wounded with a fabric pattern wheel and treated with *M. sexta* OS. After treatment, the leaves were continually supplied with DAB for another 4 h. Experiments were terminated by immersing the leaves in ethanol (96%) overnight with agitation. DAB polymerizes and turns deep brown in the presence of ROS, and the intensity of the brown color around wounding sites can be qualitatively assessed and photographed.

H_2O_2 was measured in leaf disks using an Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Carlsbad, CA, USA). In brief, source-sink transition leaves of 5 replicate plants were wounded with a fabric pattern wheel, and the puncture wounds were immediately treated with *M. sexta* OS. After 1 h, leaf disks from the treated leaf parts were excised with a No. 4 cork borer and incubated with 400 μL reaction buffer (50 mM sodium phosphate buffer, pH 7.4) for 10 min with gentle agitation. Next, the reaction buffer was quickly replaced with freshly-made reaction solution (50 μM Amplex red reagent and 0.1 U/mL HRP in 50 mM sodium phosphate buffer, pH 7.4). After 3 h, OD_{560} of the reaction solutions were recorded as an estimate of OS-elicited H_2O_2 accumulation between 1- to 4-h

treatments. The value of control leaf discs were arbitrarily set to 1.

Nucleic acid analysis

Southern and northern blot analyses were performed as previously described (Wu et al. 2007b). DNA (5 μg) was digested with *Bam*H I and *Bcl* I (for WT plants), and *Eco*R I (for ir*rboh D* lines), and then blotted onto a nylon membrane. A probe was prepared by labeling the *Narboh D* fragment (amplified with forward primer NarbohD155 and reverse primer NarbohD1894) with ^{32}P using the RediPrime II random prime labeling kit (Amersham, UK). A fragment of *hpt* II was used as a probe for Southern hybridization to confirm the single insertion of the transgenic lines.

To analyze the accumulation of *Narboh D* transcripts in WT plants in response to wounding plus water or *M. sexta* oral secretions (OS), leaves on 3 replicated plants were wounded with a fabric pattern wheel, the puncture wounds were immediately treated with water or *M. sexta* OS, and leaves were harvested 0, 0.5, 1.0, 3.0, 6.0 h after elicitation. Leaf samples harvested were immediately frozen in liquid nitrogen. A fragment of *Narboh D* (forward primer *Narboh D* 155 and reverse primer *Narboh D* 1894) was amplified by PCR and used as a specific probe for hybridization.

Real-time PCR assay

cDNA was prepared from 500 ng total RNA with multiScribe reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA). The PCR products were detected by gene-specific double fluorescent dye-labeled TaqMan® probes. Real-time PCR was performed on an ABI PRISM 7700 Sequence Detection System (qPCR Core Kit, Eurogentec, Seraing, Liège, Belgium) according to the manufacturer's instructions under the following cycle conditions: 10 min 95 °C; 40 cycles: 30 s 95 °C, 30 s 60 °C. For each analysis, a linear standard curve, threshold cycle number versus log (designated transcript level), was constructed using a series dilution of a specific cDNA standard; the levels of the transcript in all unknown samples were determined according to the standard curve. An *N. attenuata* actin2 gene, which is a housekeeping gene that had been shown to have constant transcript levels by microarray analysis, RNA gel blotting, and qRT-PCR after W + W and W + OS treatments (Bubner et al., unpubl. data), was used as an internal standard for normalizing cDNA concentration variations. Relative transcript levels of genes were obtained by dividing the extrapolated transcript levels of the target genes by the levels of actin2 from the same sample.

The transcripts of *Narboh D* were determined by primers NarbohD71 and NarbohD172 with *NaActin2* for normalization under the following cycle conditions: 10 min 95 °C; 40 cycles: 30 s 95 °C, 30 s 54 °C.

Gene-specific primers used for cloning *Narboh D* gene and real-time PCR

Narboh D 155 forward primer	5′-TTCAACCTATCTTCTTTCATTTTCA -3′
Narboh D 3235 reverse primer	5′-ATACTAACATCACAACCACAACTA -3′
Narboh D 1894 reverse primer	5′-TTGGCAAACTAACTCTATTCCTTCT-3′
Narboh D 71 forward primer	5′-ACAGAGCGTACAGTGGT -3′
Narboh D 172 reverse primer	5′-TGGTTCCGATGTCGGTAGATT -3′
NaJAR4 forward primer	5′-ATGCCAGTCGGTCTAACTGAA -3′
NaJAR4 reverse primer	5′-TGCCATTGTGGAATCCTTTTAT -3′
NaPAL1 forward primer	5′-TTTGCATACGCTGATGACGC -3′
NaPAL1 reverse primer	5′-TGGAAGATAGAGCTGTTCGCG -3′
NaTPI forward primer	5′-TCAGGAGATAGTAAATATGGCTGTTCA -3′
NaTPI reverse primer	5′-ATCTGCATGTTCCACATTGCTTA -3′
NaActin forward primer	5′-GGTCGTACCACCGGTATTGTG-3′
NaActin reverse primer	5′-GTCAAGACGGAGAATGGCATG-3′

Gene-specific double fluorescent dye-labeled TaqMan® probes for real-time PCR

NaJAR4: 5′-CAGGTCTGTATCGCTATAGGCTCGGTGATGT -3′

NaPAL1: 5′-CAGAAACTGAGGCAAGTACTCGTCGACCAC -3′

NaTPI: 5′-TCCTTGCTCTCCTCCTCTTATTTGGAATGTCT -3′

NaActin : 5′-TCAGCCACACCGTCCCAATTTATGAGG -3′

Caterpillar performance

Eggs of *M. sexta* were acquired from North Carolina State University and kept in a growth chamber (Snijders Scientific, Tilburg, The Netherlands) at 26 °C for 16 h light, and 24 °C for 8 h dark, until the larvae hatched. Freshly-hatched neonates were placed directly on the source-sink transition leaves of 20 WT or ir*rboh D* plants. In each treatment, 20 larvae were weighed after 10 d of feeding.

For the performance assays with *Spodoptera littoralis*, a lepidopteran herbivore not specifically adapted to *N. attenuata*, larvae were obtained from Dr. Wilhelm Boland at the Department of Bioorganic Chemistry in the Max-Planck Institute for Chemical Ecology (http://www.ice.mpg.de). Three d after

hatching, 30 randomly-selected larvae were placed on the transition leaf of rosette WT, and ir*rboh D* line 1 and line 2 plants, with each larva enclosed individually in a clip cage confining them to one leaf. After 5 d, we measured the mass of each surviving caterpillar.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The amino acid sequence of *Narboh D* (EU104741) compared with tobacco Ntrboh D (AJ309006; Simon-plas et al. 2002); identical sequences are shaded with black.

Figure S2. *Narboh D*-silenced plants are morphologically normal and grow as well as WT plants do in their seedling stages, but are significantly smaller than WT plants at the rosette stage before bolting in soil.
(A) The growth of WT and ir*rboh D* line 1 seedlings 11 d after germination.
(B) 27-d-old WT and ir*rboh D* plants in soil.

Figure S3. 3,3-diaminobenzidine (DAB) staining and Southern blot.

(A) DAB staining of WT and ir*rboh D* plant leaves 4 h after treatments of wounding plus *Manduca sexta* oral secretions (OS). DAB polymerizes and turns deep brown around the wounds.

(B) Southern blot analysis showed that only one copy of *Narboh D* is present in the *Nicotiana attenuata* genome (left). Two stably transformed lines with single-insertions were confirmed with Southern blot analysis with *Eco*R I and probed with the *hpt II* gene (right).

Figure S4. Silencing of *NaRboh D* does not influence *Manduca sexta* oral secretions (OS)-elicited early responses, including jasmonic acid (JA), JA-Ile bursts, and *NaJAR4* and *NaPAL1* transcripts.

(A) Mean (± *SE*) JA and JA-Ile levels were measured in 5 replicate source-sink transition leaves of WT and ir*rboh D* plants after being treated with wounding plus OS 1 h. Control plants remained untreated.

(B) Mean (± *SE*) *NaJAR4* and *NaPAL1* transcripts were measured by real-time PCR in 4 replicate source-sink transition leaves of WT and ir*rboh D* plants 1 h after wounding plus OS elicitation. Control plants remained untreated.

Figure S5. Mean (± *SE*) *NaTPI* transcripts were measured by real-time PCR in 4 replicate source-sink transition leaves of WT and ir*rboh D* plants 6 h after wounding plus water (W+W) treatments. Control plants remained untreated.

Table S1. Up-regulated or down-regulated genes 10 h after *Manduca sexta* oral secretions (OS)-elicitation in WT and ir*rboh D* plants.