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Increased SA in *NPR1*-silenced plants antagonizes JA and JA-dependent direct and indirect defenses in herbivore-attacked *Nicotiana attenuata* in nature

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Summary

The phytohormone jasmonic acid (JA) is known to mediate herbivore resistance, while salicylic acid (SA) and non-expressor of PR-1 (NPR1) mediate pathogen resistance in many plants. Herbivore attack on *Nicotiana attenuata* elicits increases in JA and JA-mediated defenses, but also increases SA levels and Na-*NPR1* transcripts from the plant's single genomic copy. SA treatment of wild-type plants increases Na-*NPR1* and Na-*PR1* transcripts. Plants silenced in *NPR1* accumulation by RNAi (ir-*npr1*) are highly susceptible to herbivore and pathogen attack when planted in their native habitat in Utah. They are also impaired in their ability to attract *Geocorus pallens* predators, due to their decreased ability to release *cis*-α-bergamotene, a JA-elicited volatile 'alarm call'. In the glasshouse, *Spodoptera exigua* larvae grew better on ir-*npr1* plants, which had low levels of JA, JA–isoleucine/leucine, lipoxygenase-3 (*LOX3*) transcripts and JA-elicited direct defense metabolites (nicotine, caffeoyl putrescine and rutin), but high levels of SA and isochorismate synthase (*ICS*) transcripts, suggesting *de novo* biosynthesis of SA. A microarray analysis revealed downregulation of many JA-elicited genes and upregulation of SA biosynthetic genes. JA treatment restored nicotine levels and resistance to *S. exigua* in ir-*npr1* plants. We conclude that, during herbivore attack, NPR1 negatively regulates SA production, allowing the unfettered elicitation of JA-mediated defenses; when *NPR1* is silenced, the elicited increases in SA production antagonize JA and JA-related defenses, making the plants susceptible to herbivores.

Keywords: ***Nicotiana attenuata,*** **jasmonic acid, salicylic acid,** ***NPR1, Spodoptera exigua.***

Introduction

Plants employ inducible defenses to prevent their tissues from being damaged by herbivores and pathogens. Defenses elicited in response to insect and pathogen attack are known as induced resistance (IR) and systemic acquired resistance (SAR), respectively. IR and SAR are associated with the elicitation of a distinct set of defense genes; these genes are largely mediated by the phytohormones jasmonic acid (JA) and salicylic acid (SA) (Ward *et al.*, 1991; Kessler and Baldwin, 2002; Delaney *et al.*, 1995; Cheong *et al.*, 2002). It has become apparent that herbivore or pathogen attack frequently recruits not one but many signal cascades. For example, bacteria (*Pseudomonas syringae*) can activate both the SA and the JA pathways in *Solanum esculentum* (Stout *et al.*, 1999), while in Arabidopsis, herbivore (*Pieris rapae*) damage elicits both JA- and SA-dependent defenses (De Vos *et al.*, 2006). The specificity of responses in defense gene expression to particular attackers seems to be the result of a network of interconnecting signal cascades that cross-communicate (Feys and Parker, 2000; Glazebrook, 2001; Thomma *et al.*, 2001; Heidel and Baldwin, 2004). Cross-communication among various signaling cascades provides plants with the regulatory potential required to tailor their responses to the diverse herbivore species that attack them (Walling, 2000). Although cross-communication among various signaling cascades in plants infected by pathogens has been extensively studied, not much is known about cross-communication in plants attacked by various types of herbivores. Studies have reported the elicitation of SA and its marker genes (e.g. *NPR1*, *PR-1*) after herbivore attack (Glazebrook, 2001; Heidel and Baldwin, 2004), yet their exact role in IR remains unknown.

The non-expressor of *PR-1* (NPR1) is known to be a major molecular player in SAR. NPR1 functions as a transducer of SA, which is produced after pathogen attack. NPR1 was first

identified in Arabidopsis in genetic screens for SAR-compromised mutants (Cao *et al.*, 1994; Delaney *et al.*, 1995). Pathogen attack results in changes in cytosolic cellular redox as well as increases in the levels of SA; these increases cause the constitutively present NPR1 protein to de-polymerize and form monomers (Mou *et al.*, 2003), which migrate to the nucleus where they associate with transcription factors (TGA family) that induce pathogenesis-related (*PR*) defense genes (Zhang *et al.*, 1999). However, NPR1's function is not restricted to SA-dependent responses; it also interacts with various signaling cascades in response to different attackers. For example, during induced systemic resistance (ISR, a biologically elicited, systemic defense response activated when roots are colonized by particular strains of non-pathogenic rhizobacteria), JA and ethylene responses are mediated via NPR1, independently of SA (Pieterse and Van Loon, 2004). Among other functions, NPR1 can negatively regulate SA biosynthesis during pathogen attack (Shah, 2003). Recently, NPR1 has been shown to mediate the SA-induced suppression of JA-dependent responses (Spoel *et al.*, 2003). These studies have highlighted the diverse roles that NPR1 plays in plants. The *NPR1* gene encodes a protein with a BTB/BOZ domain and an ankyrin-repeat domain; both domains are characteristic of proteins with highly variable functions (Bork, 1993; Cao *et al.*, 1997; Aravind and Koonin, 1999).

Several studies with Arabidopsis have suggested various causal associations between SA and NPR1 expression and herbivore/pathogen performance. For example, *Trichoplusia ni* larvae fed more on constitutive SAR mutants with elevated SA levels than on wild-type (WT) plants, but eliciting SAR with avirulent bacteria, a process that is typically accompanied by elevated SA levels, decreased insect feeding (Cui *et al.*, 2002). In another study, *Spodoptera littoralis* larvae fed less on SAR-compromised *npr1* mutants than on WT plants; SA treatment enhanced feeding only in *npr1* mutants in which SA does not induce SAR (Stotz *et al.*, 2002). These studies suggest that SA and NPR1 have the potential to inhibit JA responses, but the mechanism remains unclear. Moreover, most of these studies have been performed with Arabidopsis grown under laboratory conditions using herbivores or pathogen strains whose ecological relevance is not known. An understanding of NPR1's role in mediating herbivore resistance in plants growing in their natural habitats would be valuable.

IR is well studied in *Nicotiana attenuata*, a native plant of the south-western USA. *N. attenuata* is an annual plant that grows in post-fire environments; as a result, it has to re-establish itself regularly with new plant populations and unpredictable herbivore communities. Because it grows in the post-fire niche, *N. attenuata* has adapted to be able to respond to attack from various herbivores using different blends of secondary metabolites (Kessler and Baldwin, 2002). How *N. attenuata* generally responds to herbivory has been studied using the specialist herbivore *Manduca sexta*. Allowing *M. sexta* to feed on *N. attenuata* or applying larval oral secretions (OS) to puncture wounds in leaves elicits a JA burst, which in turn mediates the accumulation of various direct defense metabolites (Halitschke and Baldwin, 2004). Some of the main anti-herbivory metabolites studied are nicotine (Baldwin, 1999), caffeoyl putrescine, rutin and diterpene glycoside (Keinanen *et al.*, 2001), as well as anti-digestive trypsin protease inhibitors (TPIs) (van Dam *et al.*, 2001; Zavala *et al.*, 2004). The OS-elicited JA burst also influences the accumulation of several indirect defense metabolites. Among the indirect defense compounds that *N. attenuata* produces are volatile organic compounds (VOCs), which attract predators of *M. sexta* eggs and larvae (Halitschke *et al.*, 2000; Kessler and Baldwin, 2001). Plants that have been genetically engineered to accumulate less JA (Halitschke and Baldwin, 2003; Kessler *et al.*, 2004) or are unable to respond to JA (Paschold *et al.*, 2007) have been used to demonstrate that JA acts as a major transducer of signals that are essential to plant defense.

Here we address three questions about NPR1's role in *N. attenuata*'s interactions with its native herbivores. (i) Does NPR1 mediate cross-communication between SA and JA pathways to optimize the function of its direct and indirect defenses? (ii) If so, how does this optimization come about? (iii) How does NPR1 expression influence JA-mediated direct and indirect defenses in plants grown under natural conditions?

We transformed *N. attenuata* plants with an RNAi construct harboring a fragment of Na-*NPR1* in an inverted-repeat orientation (ir-*npr1*) to silence expression of the endogenous Na-*NPR1* gene. We compared the performance of larvae of the second most important native lepidopteran herbivore of *N. attenuata*, *Spodoptera exigua*, on ir-*npr1* and WT lines in experiments conducted in the glasshouse, as well that of native herbivores in the plant's native habitat, the Great Basin Desert. To understand the resistance phenotypes, we measured the production of various phytohormones (total SA, free SA, conjugated SA, JA, JA–amino acid conjugates), direct defense metabolites (nicotine, rutin and caffeoyl putrescine), indirect defense metabolites (VOCs), and gene expression profiles. The results demonstrate that Na-NPR1 and its associated phytohormone, SA, influence JA-dependent IR, and in doing so influence both direct and indirect defenses.

Results

Isolation and characterization of NPR1 *in* N. attenuata *(Na*-NPR1*)*

Using the sequence information of *Nicotiana tabacum NPR1* (Liu *et al.*, 2002) (NCBI accession number AF480488), primers were designed to isolate a 335 bp non-intronic partial

cDNA fragment and a full-length ORF from wounding and *M. sexta* OS-elicited leaves of WT *N. attenuata* (sequence submitted to NCBI database, accession number DQ351939) (Supplementary Figure S1). *N. tabacum* (*NPR1*) and *N. attenuata (NPR1*) are almost identical (>97%) at the nucleotide level. In order to determine the copy number of *NPR1* in *N. attenuata*, a Southern hybridization using the 335 bp partial fragment as the probe was carried out. Analysis revealed that *NPR1* is a single-copy gene in the *N. attenuata* genome (Supplementary Figure S2). Silencing Na-*NPR1* expression by an *Agrobacterium*-mediated transformation procedure (Krügel *et al.*, 2002) using a pRESC5 transformation vector containing an inverted-repeat construct of Na-*NPR1* (Supplementary Figure S3) (see Experimental procedures) yielded three independently transformed lines (Supplementary Figure S4A). Levels of Na-*NPR1* transcripts after SA treatment were significantly decreased (at least 50%) in all three ir-*npr1* lines compared to levels in identically treated WT plants (Supplementary Figure S4B; ANOVA $F_{3,12} = 5.460$, $P < 0.001$). Two ir-*npr1* lines (174 and 213) were selected for all further experiments. In Arabidopsis and tobacco, *NPR1* is known to be SA-responsive, and pathogen attack or SA treatment activates *NPR1*. Activated NPR1 in turn influences the accumulation of transcripts of *PR* genes, the best studied of which is *PR-1* (Ward *et al.*, 1991). In order to determine whether *N. attenuata NPR1* (Na-*NPR1*) responds similarly, we analyzed the transcript accumulation of Na-*NPR1* and Na-*PR1* using quantitative real-time polymerase chain reaction (PCR). After SA treatment, WT *N. attenuata* plants accumulated higher levels (>1-fold) of Na-*NPR1* transcripts (Figure 1; ANOVA $F_{5,12} = 7.04$, $P = 0.002$) as well as of Na-*PR-1* transcripts (>1-fold) (Figure 1; ANOVA $F_{5,12} = 25.36$, $P < 0.0001$) compared to similarly treated ir-*npr1* plants. On the other hand, control plants treated only with water showed basal levels of Na-*NPR1* but not Na-*PR-1* in all genotypes. In summary, silencing Na-*NPR1* reduced Na-*PR1* accumulation, suggesting that Na-*NPR1* regulates Na-*PR-1* expression in a SA-dependent manner. These results confirm that Na-*NPR1* is indeed responsive to SA, and suggest that Na-*NPR1* is also involved in regulating the expression of *PR* genes, which are correlated with disease resistance in cultivated tobacco.

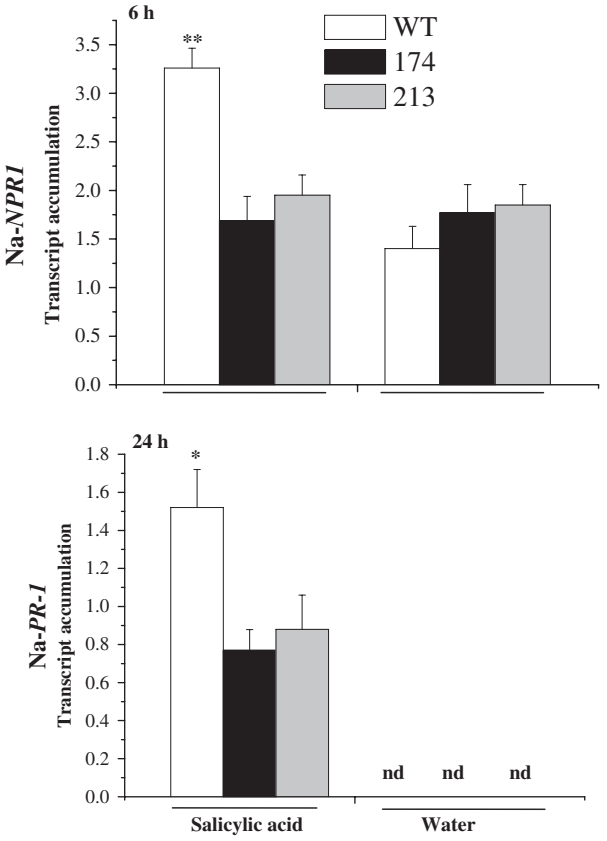

Figure 1. Na-*NPR1* and Na-*PR-1* transcript analysis.
Quantitative real-time polymerase chain reaction analysis of Na-*NPR1* (6 h) and Na-*PR-1* (24 h) transcript accumulation in wild-type (WT) plants and ir-*npr1* lines (174 and 213) of *Nicotiana attenuata* in response to spraying with 1 mM salicylic acid (SA) or water. cDNA (100 ng) from three replicate plants of SA- and water-treated leaves was used in the analysis. A constitutively unregulated sulfite reductase gene (*ECI*) was used for normalization. Asterisks indicate significant differences between WT plants and ir-*npr1* lines (174 and 213) for the respective treatments (*$P < 0.05$, **$P < 0.01$) (nd, not detected).

Characterizing Na-NPR1 *gene expression and protein accumulation in response to herbivore attack*

We measured the kinetics of transcript accumulation in WT and ir-*npr1* plants in response to *S. exigua* attack. The quantitative real-time PCR results showed that Na-*NPR1* transcript accumulation was significantly higher in WT compared to ir-*npr1* plants at 1 h (Figure 2a; ANOVA $F_{5,12} = 5.309$, $P = 0.008$) and 6 h (Figure 2a; ANOVA $F_{5,12} = 25.8$, $P < 0.001$) after the start of *S. exigua* attack. In addition, we also measured Na-NPR1 protein accumulation 24 h after the start of herbivore attack. Western blot analysis revealed that Na-NPR1 was present constitutively. Moreover, *S. exigua* damage marginally increased protein levels in WT plants compared to undamaged WT plants after 24 h. Finally, Na-NPR1 accumulation in *ir-npr1* plants compared to WT plants was substantially reduced (Figure 2b).

Na-NPR1*-silenced plants are susceptible to herbivores and pathogens in nature*

To determine whether Na-NPR1 mediates responses influencing the resistance of *N. attenuata* to herbivores and pathogens under natural conditions, we transplanted size-matched WT and ir-*npr1* (213) pairs into their native habitat in the Great Basin Desert (near Santa Clara, UT, USA), and compared the extent of damage on both genotypes. Total

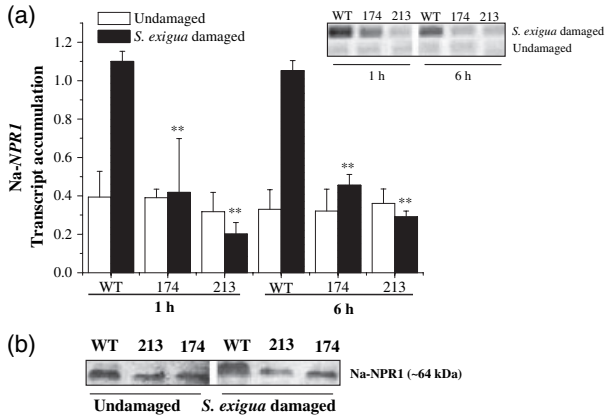

Figure 2. Na-*NPR1* transcript accumulation in response to *Spodoptera exigua* attack.
(a) Na-*NPR1* transcript accumulation in wild-type (WT) plants and ir-*npr1* lines (174 and 213) of *Nicotiana attenuata* in response to *S. exigua* attack. The transcripts from 100 ng cDNA prepared from RNA samples extracted 1 and 6 h after continuous feeding by *S. exigua* larvae were analyzed by quantitative real-time PCR and are expressed as the mean (±SE) of three replicate plants in arbitrary units. A constitutively unregulated sulfite reductase gene (*ECI*) was used for normalization. Asterisks indicate significant differences between *S. exigua*-damaged WT plants and ir-*npr1* lines (174 and 213) at the respective harvest times (**$P < 0.01$). Inset: the real-time PCR products from the same experiments separated on a 1.7% agarose gel.
(b) Western blot analysis of expression of Na-NPR1 protein in WT plants and ir-*npr1* lines (174 and 213). Total leaf protein (20 μg) was separated on an 8.0% sodium dodecylsulphate–polyacraylamide gel and visualized by immunoblotting with Na-NPR1 polyclonal antibody (for loading control, see Supplementary Figure S11).

herbivore damage was significantly higher on ir-*npr1* plants (88% on day 25 after transplanting) than on WT plants (Figure 3a; ANOVA $F_{5,60} = 3.381$, $P = 0.009$). Among the individual herbivores, grasshoppers inflicted significantly more damage (1.3-fold on day 25) on ir-*npr1* (213) than on WT plants (Figure 3b; ANOVA $F_{5,60} = 3.86$, $P = 0.004$). However, flea beetles (*Ephitrix sps*) did not damage ir-*npr1* (213) plants significantly more than WT plants (Figure 3c; ANOVA $F_{5,60} = 0.609$, $P = 0.693$). Interestingly, ir-*npr1* (213) plants also showed significantly more symptoms (2-fold on day 25) that resembled bacterial spots than did the WT plants (inset of Figure 3d; ANOVA $F_{5,60} = 2.48$, $P = 0.01$). The disease symptoms appeared 2 days after a brief rain, which may have vectored soil bacteria to the leaf surfaces via raindrop splash. Because all plants were at the rosette stage, i.e. leaves were in direct contact with the ground, they were lightly covered in soil after the rain. We subsequently identified two *Pseudomonas* species (an unidentified species of *Pseudomonas* strain 4 and *Pseudomonas jessenii*) from the infected field samples (Supplementary Table S1). Both strains of *Pseudomonas* were tested on WT *N. attenuata* under glasshouse conditions, and only *Pseudomonas* sp. strain 4 was found to be pathogenic (Supplementary Figure S5). In summary, we found that Na-*NPR1*-silenced plants were more susceptible than WT plants to herbivores and pathogens in nature.

Na-NPR1 *silencing inhibits JA-mediated indirect defenses in nature*

In nature, *N. attenuata*'s JA-dependent responses are known to elicit the release of VOCs, which in turn attract *Geocorus pallens* (a lepidopteran egg and larval predator) to herbivore-attacked or oral secretion (OS)-elicited plants (Kessler and Baldwin, 2001). To determine whether this indirect defense is altered in an ir-*npr1* line 213, we analyzed the ability of WT and ir-*npr1* (213) plants to attract predators after OS elicitation using an egg predation assay that has been developed to measure a plant's ability to attract

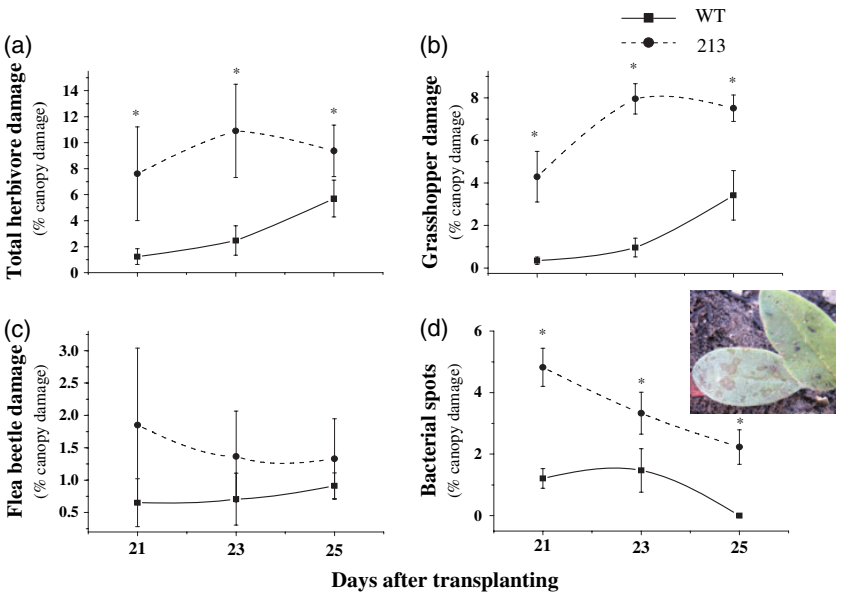

Figure 3. Herbivory on pairs of size-matched ir-*npr1* (213) and wild-type (WT) *Nicotiana attenuata* plants growing in *N. attenuata*'s native habitat in the Great Basin Desert.
Damage caused by (a) all herbivores, (b) grasshoppers, (c) flea beetles (*Epitrix hirtipennis*) and (d) bacteria *Pseudomonas* species. Inset: bacterial damage symptoms. Damage was measured as the percentage of total canopy area damaged 21, 23 and 25 days after plants had been transplanted into the field. Asterisks indicate a significant difference between WT and ir-*npr1* (213) plant pairs at $P < 0.05$ ($n = 15$ pairs).

G. pallens to *M. sexta* eggs (Kessler and Baldwin, 2001). *M. sexta* eggs and larvae are a natural food for this abundant predator, and as gluing eggs to a plant does not elicit a VOC response, this assay allows predation rates to be measured before and after OS-elicited VOC release (Kessler and Baldwin, 2001). *G. pallens* predation on eggs results in a characteristic small feeding hole through which the contents of the egg are emptied. Such emptied eggs are transparent and papery and readily distinguished from non-predated eggs, which appear greenish (inset of Figure 4a). For 42 h after eggs were glued to leaves and before plants were elicited with *M. sexta* OS, few eggs were emptied and there were no significant differences in the number of eggs emptied between WT and ir-*npr1* (213). However, within 17 h of OS elicitation, 41% of the 35 eggs (five eggs each on seven replicate plants) affixed to WT plants had been attacked, compared with only 16% of the 35 eggs glued to ir-*npr1* line 213 (Figure 4a; ANOVA $F_{9,57} = 6.708$, $P < 0.001$). Similar differences were observed after 25 h. Consistent with the predation rate data, after elicitation, WT plants attracted more than three times the number of *G. pallens* compared to ir-*npr1* plants (213) (Figure 4b; ANOVA $F_{9,57} = 3.39$, $P = 0.02$).

Geocorus pallens adults and larvae are known to be attracted to herbivore-attacked plants by OS-elicited VOCs that the plants release into their surroundings. To determine why ir-*npr1* plants were less able to attract predators, we trapped the headspace VOCs of field-grown plants before and after OS elicitation. We analyzed the VOCs and green leaf volatiles (GLVs) from ir-*npr1* (213) and WT plants. The release of terpenoid VOC *cis*-α-bergamotene in particular was significantly lower (51%) from ir-*npr1* than from WT plants (Figure 4c; ANOVA, $F_{1,12} = 8.93$, $P = 0.013$). This finding is consistent with previous field work with *N. attenuata* that highlights the role of *cis*-α-bergamotene in attracting *G. pallens* (Kessler and Baldwin, 2001). Interestingly, other VOCs, such as β-pinene, germacrene, limonene and *cis*-jasmonol, did not differ significantly between WT plants and ir-*npr1* line 213 (Supplementary Table S2), suggesting that these compounds are independent of the Na-NPR1-mediated response. In addition, the level of GLVs such as (*z*)-3-hexanol and (*z*)-3-hexanol acetate, which require a functional Na-HPL (hydroperoxy lyase), released from WT plants and ir-*npr1* line 213 did not differ (Supplementary Table S2).

Na-NPR1 *silencing increases susceptibility to* S. exigua *and decreases direct defenses*

Under field conditions, we observed that ir-*npr1* plants (line 213) were more susceptible than WT plants to generalist grasshoppers. Moreover, in an initial planting early in the growing season, ir-*npr1* plants were heavily attacked by *S. exigua* larvae. Although these larvae were not found in subsequent plantings, these results prompted us to measure

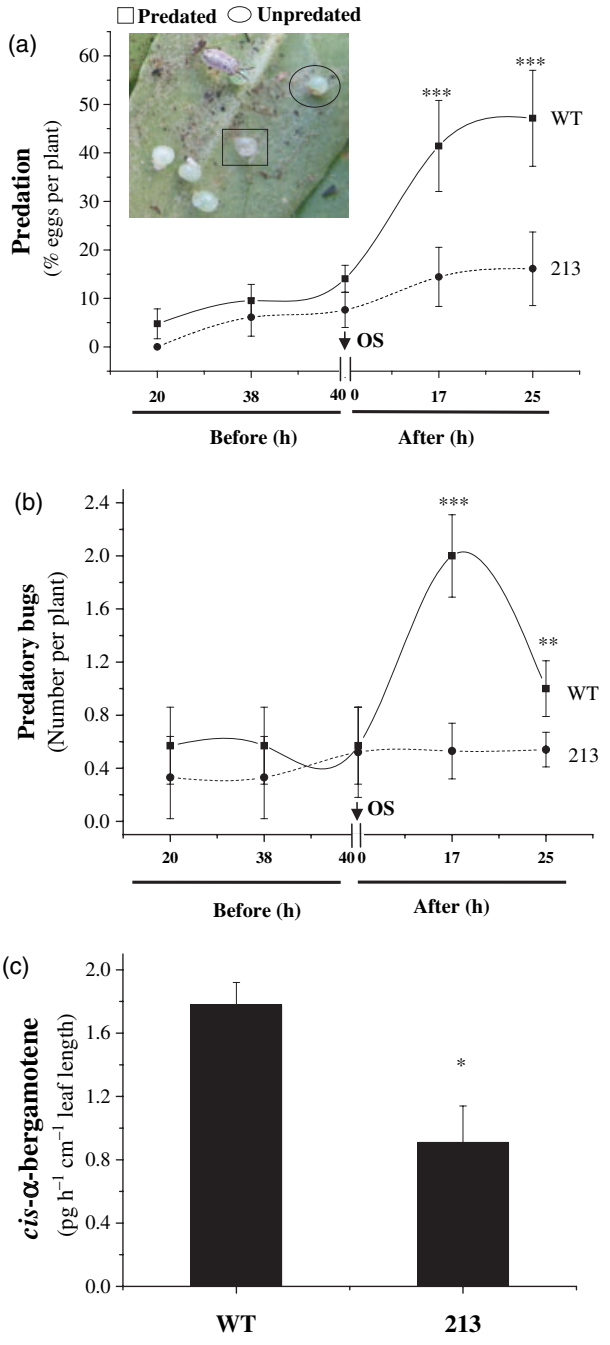

Figure 4. Effect of Na-*NPR1* on indirect defenses in nature.
(a) Mean (±SE) percentage of *Manduca sexta* eggs predated per plant on wild-type (WT) (solid line) and ir-*npr1* (line 213; dashed line) plants before and after elicitation using *M. sexta* oral secretions (OS) ($n = 7$ plant pairs). Five eggs were glued on the second stem leaf of each plant; predation rates by *Geocoris pallens* were monitored for 42 h before OS elicitation and for an additional 25 h after OS elicitation. Inset: predation of *M. sexta* eggs by *G. pallens*.
(b) Mean (±SE) number of *G. pallens* found on WT (solid line) and ir-*npr1* (line 213; dashed line) plants before and after OS (*M. sexta*) elicitation ($n = 7$ plant pairs).
(c) Mean (±SE) emission of *cis*-α-bergamotene from WT and ir-*npr1* plants (line 213) 12 h after a second treatment with OS.
Asterisks indicate significant differences between ir-*npr1* and WT plants (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $n = 7$).

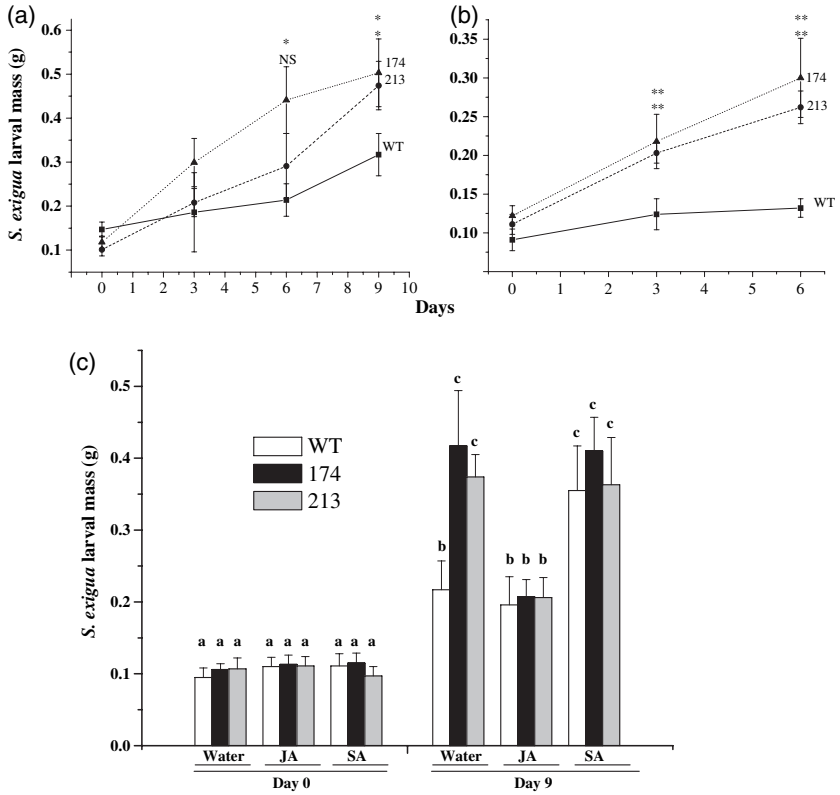

Figure 5. Silencing Na-*NPR1* increases the susceptibility of *Nicotiana attenuata* to attack from *Spodoptera exigua* larvae.
(a) Mean (±SE) mass gain of *S. exigua* larvae on WT plants and ir-*npr1* lines 174 and 213. First-instar larvae were reared on WT *N. attenuata* for 3 days before being placed on wild type (WT) plants and ir-*npr1* lines.
(b) Mean (±SE) mass gain of *S. exigua* larvae on WT plants and ir-*npr1* lines 174 and 213. First-instar larvae were reared on an artificial diet for a week before being placed on the plants.
In both experiments, larvae performed better on ir-*npr1* lines. Asterisks indicate significant differences between larvae feeding on WT plants and those feeding on ir-*npr1* plants (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $n = 15$).
(c) Mean (±SE) mass of *S. exigua* larvae feeding on water, JA- and salicylic acid (SA)-treated WT plants and ir-*npr1* lines (174 and 213). Before the treatments, WT plants and ir-*npr1* lines (174 and 213) were fed upon by *S. exigua* for 3 days to activate Na-NPR1 and its dependent responses. At the beginning of the 4th day, larvae were removed and plants were treated with water, jasmonic acid (JA) and SA. At the end of 4th day, fresh larvae of uniform mass (day 0) were placed on water, JA- and SA-treated WT plants and ir-*npr1* lines (174 and 213), and their final mass was recorded after 9 days. Different letters indicate significant differences between treatments and genotypes ($n = 15$).

the performance of *S. exigua* in the glasshouse. We conducted two experiments. For the first, we used *S. exigua* larvae that had been reared for 3 days on WT *N. attenuata* plants. Larvae that fed on ir-*npr1* lines (174 and 213) gained significantly more mass (49%) by the end of day 9 than those that fed on WT plants (Figure 5a; ANOVA, $F_{2,24} = 2.625$, $P = 0.03$). In the second experiment, we used larvae that had been reared on an artificial diet instead of WT *N. attenuata* plants. In this experiment, the difference in the performance of larvae that fed on ir-*npr1* lines (174 and 213) compared to WT plants was much larger than the difference in performance observed in the first experiment. When weighed on day 6 of the experiment, larvae that fed on ir-*npr1* lines (174 and 213) were >1.0-fold larger than those that fed on WT plants (Figure 5b; ANOVA, $F_{2,24} = 7.525$, $P = 0.001$). The results from these two experiments demonstrate that silencing Na-*NPR1* increases the performance of *S. exigua* larvae, and suggest that prior exposure to a WT *N. attenuata* diet allows the larvae to better cope with *N. attenuata*'s defenses.

To determine whether SA and JA treatments could help explain the reduced resistance to *S. exigua* in the ir-*npr1* lines, JA and SA (each 1 mM), and water were sprayed onto separate plants of WT and ir-*npr1* lines (174 and 213) that had been fed on by *S. exigua* for 3 days beforehand. The reason for using *S. exigua*-damaged plants was to make sure that Na-NPR1 and its dependent responses were activated in all of the plants prior to the treatments. When larval mass was measured on the 9th day, we found firstly that larvae that fed on JA-treated WT plants and on ir-*npr1* lines (174 and 213) increased their body mass up to 85% (day 0 to day 9), while those feeding on SA-treated WT plants and on ir-*npr1* lines (174 and 213) were nearly 2.4-fold larger on day 9 than on day 0 (Figure 5c; ANOVA, $F_{17,145} = 13.66$, $P < 0.001$). Secondly, treatment with JA or SA abolished the differences in larval performance between the lines

(Figure 5c). Larvae that fed on SA-treated WT plants and ir-*npr1* lines (174 and 213) gained similar amounts to those fed on water-treated ir-*npr1* lines (174 and 213), but the mass of all these larvae was significantly higher (2.2-fold) than the mass of those that fed on water-treated WT plants (Figure 5c). In brief, these results demonstrate that SA and JA treatments have opposing effects on larval mass gain, that mass gain is not influenced by the genotype, and that controlled production of SA in WT plants seems vital for resisting *S. exigua*.

Nicotine is known to be an effective defense against generalist herbivores including *S. exigua* larvae (Steppuhn *et al.*, 2004). Interestingly, after 72 h of feeding by *S. exigua*, nicotine levels in ir-*npr1* lines (174 and 213) were at least 62% lower than in WT plants (Figure 6a; ANOVA, genotype: $F_{17,52} = 8.539$, $P < 0.0001$). We also analyzed the concentrations of two other potential defense metabolites: caffeoyl putrescine, which, like nicotine, is elicited by JA signaling, and rutin, which is not (Keinanen *et al.*, 2001). Caffeoyl putrescine, which was observed only in induced tissues, occurred at concentrations >2.0-fold lower in ir-*npr1* lines (174 and 213) than in WT lines (Supplementary Figure S6; ANOVA, genotype: $F_{17,52} = 9.814$, $P < 0.0001$). Similarly, levels of rutin were at least 60% lower in ir-*npr1* lines (174 and 213) than in WT lines (Supplementary Figure S6; ANOVA, genotype: $F_{17,52} = 6.44$, $P < 0.0001$). These results demonstrate that Na-NPR1 expression influences both JA-dependent and JA-independent defense metabolites.

JA treatment restores nicotine levels in NPR1-*silenced* N. attenuata *plants*

Nicotine accumulation in *N. attenuata* is known to be JA-dependent, and treating JA-deficient *LOX3*-silenced plants with JA restores nicotine to levels similar to those found in WT plants (Halitschke and Baldwin, 2003). As *S. exigua*-damaged ir-*npr1* plants accumulate less nicotine than do WT plants, we wished to determine whether JA treatment could complement the nicotine deficits in ir-*npr1* plants. We used ir-*npr1* and WT *N. attenuata* plants that had been attacked for 3 days by *S. exigua* larvae to ensure that the plants were activated for Na-NPR1 and its dependent responses. Twenty-four hours after treatment, a moderate increase in nicotine levels (at least 40%) was observed in WT and ir-*npr1* plants sprayed with JA compared to those sprayed with water (control plants). In both treatments (water and JA), ir-*npr1* plants accumulated significantly less nicotine than did the WT plants (Figure 6b,c; ANOVA, genotype: $F_{1,4} = 16.47$, $P = 0.015$), which is not surprising given that nicotine concentrations reflect a plant's life-time nicotine production (Baldwin and Ohnmeiss, 1994; Ohnmeiss and Baldwin, 1994). However, 48 and 72 h after JA treatment, WT and ir-*npr1* plants accumulated similar and significantly higher (1.9-fold) nicotine levels compared to WT and ir-*npr1* plants sprayed with water (Figure 6b,c; ANOVA, genotype: $F_{17,36} = 53.85$, $P < 0.001$). These results demonstrate that the inability of ir-*npr1* plants to

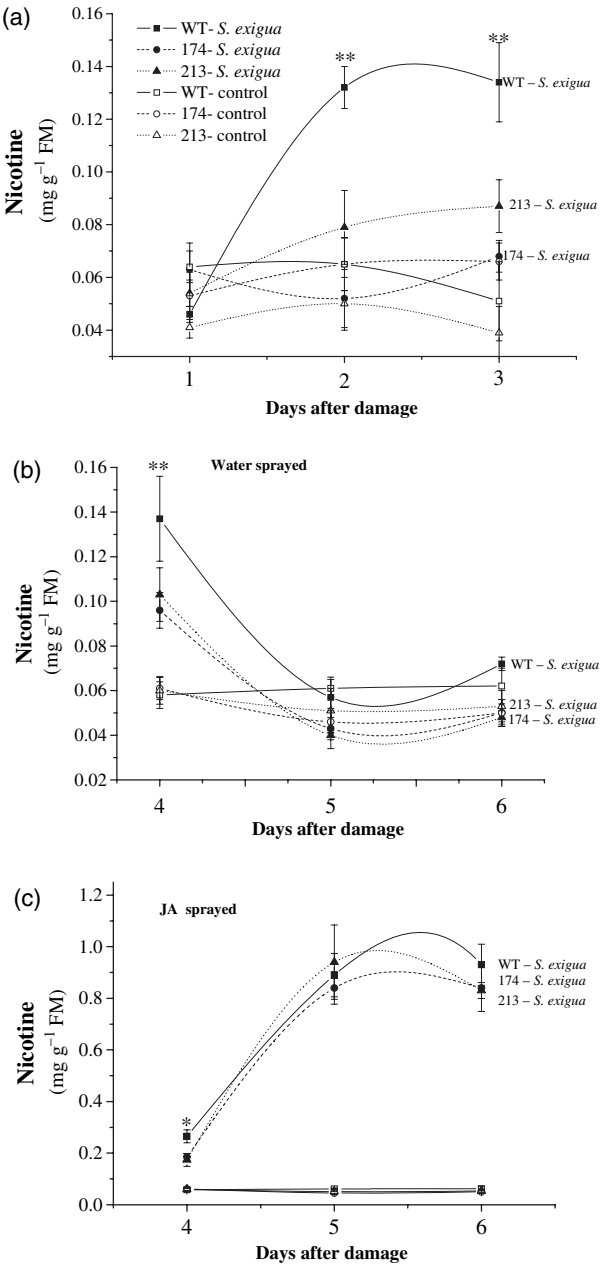

Figure 6. Silencing Na-*NPR1* influences nicotine accumulation in plants attacked by *Spodoptera exigua* larvae or sprayed with exogenous jasmonic acid.
(a) *S. exigua*-damaged leaves harvested 1, 2 and 3 days after the herbivore took its first bite.
(b) Three-day-old *S. exigua*-damaged leaves sprayed with water.
(c) Three-day-old *S. exigua*-damaged leaves sprayed with 1 mM jasmonic acid. In (b) and (c), leaves were harvested 4, 5 and 6 days after the herbivore took its first bite. Values are the means (±SE) of total nicotine accumulated after different treatments. Asterisks indicate that WT *S. exigua*-damaged plants differ significantly from both ir-*npr1* lines (174 and 213) (*$P < 0.05$; **$P < 0.01$; $n = 5$).

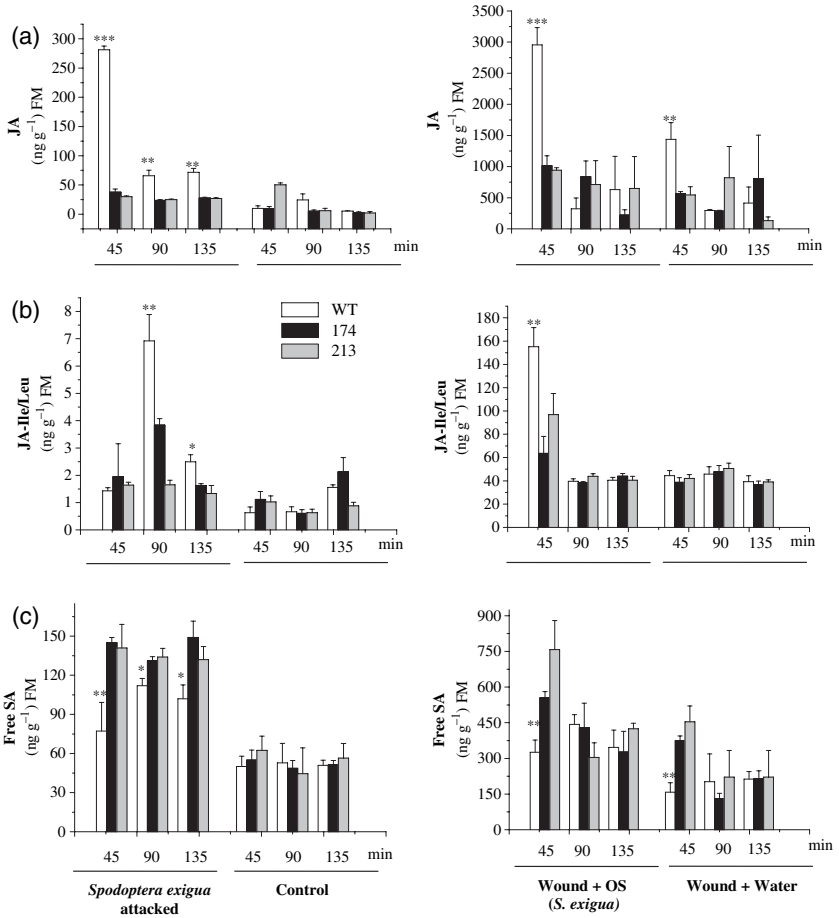

Figure 7. Silencing Na-*NPR1* reduces the levels of jasmonic acid (JA) and JA–Ile/Leu but increases free salicylic acid (SA) in plants attacked by *Spodoptera exigua* larvae (left) or elicited by wounding and OS or wounding and water (right) treatments in glasshouse-grown plants.
(a) Mean (±SE) JA, (b) JA–Ile/Leu, and (c) free SA in leaves of three replicate ir-*npr1* (lines 174 and 213) and WT plants per genotype and treatment. Node +1 leaves were wounded with a fabric pattern wheel and the resulting puncture wounds immediately treated with 20 μl *S. exigua* OS or water. For the *S. exigua* treatments, two larvae were placed in a clip cage on each node +1 leaf. Asterisks indicate significant differences between WT and both ir-*npr1* lines at the designated time (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $n = 7$).

accumulate nicotine following *S. exigua* damage can be restored by JA treatment, and we infer that JA limits nicotine accumulation in ir-*npr1* plants.

Silencing Na-NPR1 *reduces levels of OS-elicited JA and JA–Ile/Leu, but not ethylene bursts*

Manduca sexta larvae attack and OS elicitation are known to elicit transient increases in JA and JA–Ile/Leu levels that are substantially larger than the increases that occur after identical amounts of wounding (Kang *et al.*, 2006). These increases, referred to collectively as the oxylipin burst, are known to be elicited by the fatty-acid amino-acid conjugates (FACs) in OS. Typically the burst attains maximum values within 45 min and subsequently declines rapidly (Halitschke and Baldwin, 2003; Roda *et al.*, 2004). Feeding *S. exigua* larvae elicited significantly higher JA levels (>4-fold) in WT plants compared to ir-*npr1* lines (174 and 213) (Figure 7a; ANOVA, $F_{8,18} = 287.4$, $P < 0.001$). Similar results were observed after treatment with *S. exigua* OS. Wounded and OS-treated WT plants had higher JA levels (3-fold) than did plants of either ir-*npr1* line (174 and 213) 45 min after OS elicitation (Figure 7a; ANOVA, $F_{8,18} = 6.9$, $P < 0.001$). The low JA burst in ir-*npr1* lines was not confined to the OS-elicited response: JA levels in the wound- and water-treated WT plants were also significantly higher (1.4-fold) than those of ir-*npr1* lines (174 and 213) (Figure 7; ANOVA, $F_{8,18} = 3.21$, $P = 0.035$). Trends in the levels of JA–Ile/JA–Leu were similar to those in the levels of JA. In response to feeding *S. exigua*, WT plants accumulated more JA–Ile/JA–Leu (1.0-fold) than did either ir-*npr1* line (Figure 7b; ANOVA, $F_{8,18} = 8.63$, $P < 0.001$). Similar results were found in response to OS elicitation: WT plants accumulated more (1.0-fold) than did either ir-*npr1* line (Figure 7b; ANOVA, $F_{8,18} = 10.3$, $P < 0.0001$). The analysis of leaf samples from field-grown WT plants and ir-*npr1* line 213 also revealed that Na-*NPR1* silencing is correlated with reduced JA accumulation: ir-*npr1* line 213 accumulated less JA (85%) than did WT plants 45 min after OS elicitation (Supplementary Figure S7; ANOVA, $F_{17,36} = 23.44$, $P < 0.001$). That JA and JA–Ile/Leu levels are severely diminished in ir-*npr1* lines (174 and 213) suggests that Na-NPR1 exerts a major influence on the JA pathway, and that JA is the limiting factor for the production of JA–Ile/Leu. In addition, we also measured ethylene 5 h

after *S. exigua* attack and OS elicitation. In WT and ir-*npr1* plants, no significant differences were found between *S. exigua*-damaged (Supplementary Figure S8; ANOVA, $F_{2,6} = 0.211$, $P = 0.815$) and OS-elicited leaves (Supplementary Figure S8; ANOVA, $F_{2,6} = 1.885$, $P = 0.231$). However, ethylene emission was significantly greater from *S. exigua*-attacked WT and ir-*npr1* plants than from undamaged WT and ir-*npr1* plants (Supplementary Figure S8; ANOVA, $F_{5,12} = 8.68$, $P = 0.02$). Similarly, wound- and OS-elicited WT and ir-*npr1* leaves released significantly more ethylene compared to wound- and water-elicited WT and ir-*npr1* leaves (Supplementary Figure S8; ANOVA, $F_{5,12} = 11.69$, $P < 0.001$). These results clearly demonstrate that Na-NPR1 does not influence the herbivore-induced ethylene burst, but strongly influences the oxylipin burst.

Na-NPR1 *silencing increases SA levels in response to* S. exigua *attack and OS elicitation*

NPR1 regulates SA levels in Arabidopsis (Shah, 2003), and our analysis revealed that the same holds for *N. attenuata*. In WT *N. attenuata* plants, free SA increased 45 min after *S. exigua* attack and OS elicitation, and levels remained elevated for 135 min. Interestingly, free SA levels were significantly higher (85%) 45 min after *S. exigua* damage in both ir-*npr1* lines (174 and 213) compared with WT plants (Figure 7c; ANOVA, $F_{8,18} = 4.17$, $P = 0.005$). Similarly, 45 min after OS elicitation, plants from both ir-*npr1* lines (174 and 213) accumulated significantly more free SA (60%) than did WT (Figure 7c; ANOVA, $F_{8,18} = 3.582$, $P = 0.011$). Field-grown ir-*npr1* line 213 also had higher (1.0-fold) free SA levels compared to WT plants 45 min after OS elicitation (Supplementary Figure S7; ANOVA, $F_{17,36} = 7.33$, $P < 0.0001$). Silencing Na-*NPR1* increases free SA, indicating that Na-NPR1 is probably a negative regulator of free SA production. These increases in free SA could result from increases in *de novo* synthesis and/or from increases in the release of free SA from SA conjugates. Because a majority of the SA in *Nicotiana* species occurs as sugar esters (Malamy *et al.*, 1992; Lee and Raskin, 1998), we also measured SA conjugates. Levels of conjugated SA also increased in OS-elicited WT compared to unelicited WT plants after 45 min, and levels in ir-*npr1* lines attacked by *S. exigua* were higher (60%) than in similarly damaged WT plants (Supplementary Figure S9; ANOVA, $F_{8,18} = 3.30$, $P = 0.016$). OS-elicited ir-*npr1* lines also accumulated higher levels of conjugated SA (>4-fold) than did similarly elicited WT plants (Supplementary Figure S9; ANOVA, $F_{8,18} = 2.9$, $P = 0.025$). Given that Na-NPR1 silencing increased both free and conjugated SA levels with similar kinetics, it is likely that *de novo* SA biosynthesis rather than its release from conjugated pools is responsible for the changes observed in SA levels. To better understand the role of SA biosynthesis, we analyzed transcripts of JA and SA biosynthetic genes.

Na-NPR1 modulates LOX3 *and* ICS *transcript accumulation*

We conducted a quantitative real-time PCR analysis of Na-*LOX3*, which encodes an enzyme catalyzing the oxygenation of linolenic acid at the 13-C position in the JA biosynthetic pathway, and of Na-*ICS*, an enzyme catalyzing the conversion of chorismate to isochorismate, which ultimately forms SA. Na-*LOX3* transcript levels in Na-*NPR1*-silenced lines (174 and 213) were significantly reduced (40% and 45%, respectively) compared to their levels in WT plants 1 h after *S. exigua* attack (Figure 8a; ANOVA, $F_{11,24} = 5.86$, $P = 0.0001$). In contrast, Na-*ICS* transcript levels in Na-*NPR1*-silenced lines (174 and 213) increased dramatically (nearly 2.5- and 3-fold, respectively) (Figure 8b; ANOVA, $F_{11,24} = 8.16$, $P < 0.001$). These results demonstrate that Na-*NPR1* negatively regulates SA biosynthesis after herbivore attack. Interestingly, higher levels of Na-*ICS* transcripts were found in unattacked control WT plants (1.2-fold) than in WT plants that were attacked by *S. exigua*, which is consistent with a role for Na-NPR1 as a negative regulator of herbivore-induced SA biosynthesis.

Transcriptional responses of Na-NPR1*-silenced plants to* S. exigua *attack*

To understand how *S. exigua*-induced transcriptional responses are altered in *NPR1*-silenced plants (lines 174 and 213), we performed microarray analysis with a custom microarray enriched in *M. sexta*-induced *N. attenuata* genes (Halitschke and Baldwin, 2003; Voelckel and Baldwin, 2004b). We hybridized arrays using RNA extracted from ir-*npr1* (lines 174 and 213) and WT plants that had been continuously attacked by *S. exigua* larvae for 24 h. For each of the two microarrays hybridized for each genotype, RNA was extracted from three biological replicate plants of WT plants and ir-*npr1* lines (174 and 213). Both *S. exigua*-damaged ir-*npr1* lines (174 and 213) were significantly altered in their expression of 47 and 41 genes, respectively. The summary of genes differentially regulated in both chips is presented in Supplementary Figure S10. The *ICS* gene encoding isochorismate synthase (ICS) was upregulated in both ir-*npr1* lines (174 and 213), a result consistent with the RT-PCR results, as were some genes involved in phenylalanine biosynthesis, suggesting a regulatory role for Na-NPR1 with the phenylpropanoid pathway. JA-responsive genes such as *TPI* (for trypsin protease inhibitor), *TPS* (for terpene synthase, involved in sesquiterpene biosynthesis), and *ODC* (for ornithine decarboxylase, involved in nicotine production) were downregulated in ir-*npr1* lines (174 and 213) but not in WT plants. These results are consistent with the observed changes in the levels of direct and indirect metabolites in ir-*npr1* plants, and clearly demonstrate that Na-NPR1 negatively regulates JA-dependent defense responses. During herbivore damage, WT *N. attenuata* plants

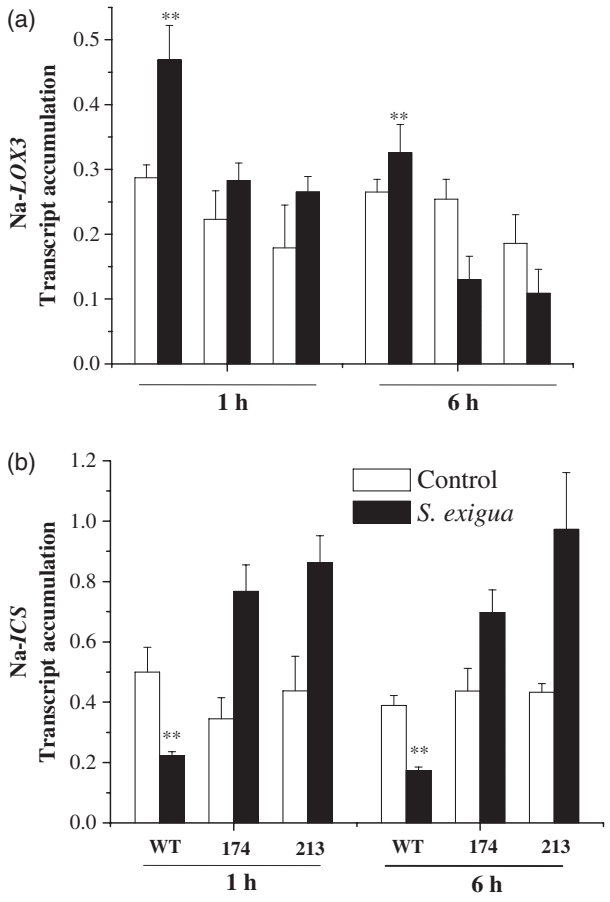

Figure 8. Na-*LOX3* and Na-*ICS* transcript accumulation in response to *Spodoptera exigua* damage in wild-type (WT) and ir-*npr1* lines (174 and 213). A first-instar larva previously reared on wild-type (WT) *Nicotiana attenuata* plants was enclosed in a well-aerated clip cage of diameter 5 cm. These clip cages were attached to the +1 nodal leaves of six replicate WT plants and ir-*npr1* lines (174 and 213). Leaves from three replicate plants of WT and ir-*npr1* lines (174 and 213) were harvested 1 and 6 h after the herbivore took its first bite. As a control, leaf tissues were harvested similarly from WT plants and ir-*npr1* lines (174 and 213) to which the clip cage was attached with no larvae inside. The transcripts from 100 ng cDNA prepared from RNA samples extracted 1 and 6 h after continuous feeding by *S. exigua* larvae were analyzed by quantitative real-time polymerase chain reaction and are expressed as the mean (±SE) of three replicate leaves in arbitrary units. A constitutively unregulated sulfite reductase gene (*ECI*) was used for normalization.
(a) Na-*LOX3* (required for JA production) is upregulated after herbivory in WT but not in ir-*npr1* lines.
(b) Na-*ICS* (required for salicylic acid production) is highly upregulated in ir-*npr1* lines.
Asterisks represent significant differences between WT induced and ir-*npr1* induced lines (174 and 213) (**P* < 0.05; ***P* < 0.01; *n* = 3).

downregulate photosynthetic genes such as *RUBISCO* and *PSII*, perhaps to allocate more resources to the production of defense compounds. Surprisingly, we found that *RUBISCO* and *PSII* were more strongly downregulated in ir-*npr1* lines, suggesting that Na-NPR1-mediated responses might influence growth-related traits. A number of cytochrome P450 genes (CYP 71D2, CYP 81E8) known to be expressed during plant stress were downregulated in ir-*npr1* lines. The transcriptional responses revealed by the microarray analysis suggest that Na-NPR1 may be involved in fine-tuning responses to herbivores by influencing genes that are SA- and JA-dependent as well as growth- and photosynthesis-related.

Discussion

In order to understand NPR1's influence on *N. attenuata's* direct and indirect defenses against herbivores, we compared the performance of herbivores feeding on WT and ir-*npr1* plants and correlated the results with the production of defense metabolites and with changes in the two main phytohormones (SA and JA) that mediate SAR and IR. We conducted this study in *N. attenuata*'s natural habitat as well as under controlled glasshouse conditions. The results demonstrate that, in addition to its well-established role in SAR, *NPR1* influences the genes involved in IR. This was evident when Na-*NPR1*-silenced plants were transplanted into native habitats and found to be more susceptible than WT plants to naturally occurring herbivores as well as to *Pseudomonas* pathogens. The greater susceptibility to herbivores could be attributed to *N. attenuata's* impaired ability to elicit indirect (*cis*-α-bergamotene release) and direct (nicotine) defense responses. As a result, ir-*npr1* plants are less able to attract *G. pallens* predators and less able to increase nicotine levels. Both predator attraction and nicotine accumulation are known to be elicited by JA signaling. Exogenous JA application to nicotine-deficient ir-*npr1* plants restored normal nicotine levels. Field- and glasshouse-grown ir-*npr1* plants were found to accumulate high levels of SA and low levels of JA in response to herbivore attack and OS elicitation; these changes in phytohormone levels were mirrored by changes in the transcript levels of JA and SA biosynthesis genes. We propose that Na-NPR1 suppresses SA accumulation during herbivore attack, which minimizes SA–JA antagonism and allows the unhampered activation of JA-mediated defense responses.

The mechanisms of *N. attenuata's* IR have been well studied, and the importance of LOX3-mediated JA signaling in the production of several secondary metabolites involved in direct and indirect defenses is known (Halitschke and Baldwin, 2003; Steppuhn *et al.*, 2004; Zavala *et al.*, 2004). The increased susceptibility of ir-*npr1* plants to *S. exigua* is correlated with reduced levels of at least three secondary defense metabolites (nicotine, caffeoyl putrescine and rutin). Although how caffeoyl putrescine and rutin function as defenses is unknown, the defensive function of nicotine has been established. Reduced nicotine levels are correlated with increases in the performance of *S. exigua* on ir-*npr1* plants, and previous studies found *S. exigua* larvae to be the most significant lepidopteran herbivore on nicotine-silenced

N. attenuata plants (ir-*pmt*) planted into a native population (Steppuhn *et al.*, 2004). The release of JA-dependent *cis*-α-bergamotene, a VOC known to attract *G. pallens* predators to *M. sexta* eggs and early instar larvae (Kessler and Baldwin, 2001; Halitschke and Baldwin, 2003), is also impaired in ir-*npr1* plants, and as a result ir-*npr1* plants attract fewer predators after OS elicitation. Taken together, these results demonstrate that ir-*npr1* plants have a 'defenseless' phenotype similar to that of JA-deficient plants (Kessler *et al.*, 2004), probably due to increased SA production. Given that JA treatment can restore production of nicotine (a JA-dependent metabolite) in *S. exigua*-damaged ir-*npr1* plants, we propose that this 'defenseless' phenotype results from SA–JA antagonism.

The effects of SA treatment on JA-mediated defenses are commonly interpreted as evidence of SA–JA antagonism. When SA, its methyl ester (MeSA), or SA mimics are applied to wounded or herbivore-attacked plants, the JA burst, JA-mediated gene expression, levels of JA-elicited defensive metabolites and resistance to some herbivores are suppressed (Baldwin *et al.*, 1997; Stout *et al.*, 1999; Stotz *et al.*, 2002; Cipollini *et al.*, 2004; reviewed by Van Poecke, 2007). Comparable examples of such suppression can be found in the eicosanoids of animals, which are derivatives of C20:4 fatty acids. The eicosanoids share biosynthetic and structural similarities with the jasmonates, which are synthesized from 18:3 fatty acids. The cyclo-oxygenase enzymes of animals, like the lipoxygenases of plants, are inhibited by salicylates, the best studied of which is acetylsalicylate (aspirin) (Vane, 1971). Suppressed JA signaling in Arabidopsis plants mutated in *mpk4* can be partially attributed to the plants' high SA levels (Wiermer *et al.*, 2005). In WT Arabidopsis, SA is thought to antagonize JA signaling during pathogen infection, which is corroborated by the diminishment of this antagonism in pathogen-elicited SA-deficient *NahG* plants that have high levels of *LOX2* transcripts and JA (Spoel *et al.*, 2003).

The SA–JA antagonism is not apparent in Arabidopsis *npr1* and *nim1* mutants in the Col-0 and Ws genetic backgrounds, respectively, but these plants do show evidence of altered JA–ethylene signaling when ISR is triggered (Pieterse *et al.*, 1998). ISR, which requires both JA and ethylene signaling, is SA-independent but NPR1-dependent. *npr1 nim1* mutants tend to be more resistant to lepidopteran herbivores (*T. ni*, *S. littoralis* and *S. exigua*), perhaps due to their elevated levels of JA-inducible glucosinolates (Stotz *et al.*, 2002; Cipollini *et al.*, 2004; Mewis *et al.*, 2005), but whether the resistance results from altered SA signaling or JA–ethylene signaling is not clear (Van Poecke, 2007).

After herbivore attack or OS elicitation, ir-*npr1 N. attenuata* plants accumulate higher levels of SA and SA biosynthetic transcripts, but release ethylene in quantities similar to those in WT plants. Moreover, ir-*npr1* plants have diminished herbivory- and OS-elicited JA bursts, as well as low levels of JA biosynthetic transcripts and JA-mediated defenses (Figures 7 and 8, and Supplementary Figures S6–S8). These results are consistent with the view that generalist herbivores, such as *S. littoralis*, may activate the SA pathway concomitantly with the JA pathway, perhaps to weaken JA-mediated resistance by amplifying the SA–JA antagonism (Stotz *et al.*, 2002; Cipollini *et al.*, 2004). In *Nicotiana sylvestris*, MeSA application reduces elicited nicotine accumulation (Baldwin *et al.*, 1996, 1997). In *N. tabacum*, TMV-inoculated plants (which are associated with local and systemic increases in endogenous SA) showed attenuated wound-induced JA and nicotine responses. Moreover, larvae consumed 1.7–2.7 times more leaf tissue from TMV-inoculated plants than from mock-inoculated plants (Preston *et al.*, 1999).

Here we show that, in *N. attenuata*, Na-*NPR1* silencing dramatically increases the levels of free SA and reduces nicotine accumulation following herbivory. How herbivore attack elicits increases in SA levels remains an open question. Perhaps pathogenic factors in the larval OS activate an SA-dependent pathway, just as FACs activate JA signaling. Given that trade-offs between herbivore and pathogen resistance are likely to take place in plants (Bostock, 2005), and generalist herbivores are capable of activating SA-dependent responses, NPR1 may function as a regulatory protein capable of controlling SA production.

NPR1 occurs as a single-copy gene in the *N. attenuata* genome and is clearly responsive to SA (Figure 1). Among the various NPRs studied to date in Arabidopsis, Na-NPR1 is most similar (50%) to *Arabidopsis thaliana* NPR1 (At-NPR1) (Supplementary Figures S12 and S13). As Na-*NPR1*-silenced plants were susceptible to both pathogens and herbivores (Figure 3), the same Na-NPR1 is likely to function in SAR as well as controlling SA production during IR. Apart from inhibiting the JA pathway, high SA levels are also associated with stunted growth (Mauch *et al.*, 2001; Shah, 2003). Although ir-*npr1* plants grew at normal rates in the glasshouse, microarray analysis revealed that their *RUBISCO* and *PSII* transcript levels were lower than those of OS-elicited WT plants. As field-grown ir-*npr1* plants tended to be slightly smaller than WT plants (although statistically not significant) (Supplementary Figure S14), Na-NPR1 may influence growth, but additional experiments are required to understand Na-NPR1's role in growth.

We conclude that the simultaneous activation of multiple signaling pathways involving SA and JA in plants can inhibit the activation of defense responses. To negate these effects, plants have evolved regulatory proteins such as NPR1 that help fine-tune defense responses by controlling SA production and thereby retain the function of the JA pathway.

Experimental procedures

Plant material, treatments and insect rearing

Wild-type *N. attenuata* plants selfed for 14 generations (seeds collected from a native population from the DI Ranch, Santa Clara, UT, USA), and ir-*npr1* lines 174 and 213, in which a 335 bp fragment of Na-*NPR1* is expressed in an inverted repeat orientation in the same WT genotype, were used in the experiment. Germination was carried out according to the procedures described by Krügel *et al.* (2002). For glasshouse studies, experiments were carried out on rosette-stage plants 13 days after they had been transferred to 1-l pots.

For JA and SA treatments in the complementation studies, 1 mM aqueous solutions of JA and SA (both Sigma, http://www.sigmaaldrich.com/) were used. JA was first dissolved in a small amount of ethanol and made up to a concentration of 1 mM with distilled water. SA and JA were sprayed on the entire plant, except for one leaf, till run-off occurred. Each leaf received approximately 400–500 μl of solution. No phytotoxic effects were observed between unsprayed and sprayed leaves.

Neonate larvae of *S. exigua* (Lepidoptera:Noctuidae) hatched from eggs supplied by the Plant Protection Centre of Bayer AG (http://www.bayer.com) were maintained in well-aerated plastic boxes (10 × 10 cm) lined with filter paper to reduce humidity (photoperiod 14–16 h) at 22–24°C. The larvae were fed an artificial diet consisting of 300 g l^{-1} agar, 400 g l^{-1} bean flour, 3 g sodium ascorbate, 3 g ethyl *p*-hydroxybenzoate and 1 g formaldehyde. Small cubes of the diet were placed in plastic rearing boxes on pieces of aluminum foil. For the second experiment, larvae were maintained similarly except that they were fed WT *N. attenuata* leaves. Eggs of *M. sexta* L. (Lepidoptera:Sphingidae), from North Carolina State University (Raleigh, NC, USA), were incubated at 26°C until they hatched, and caterpillars were fed WT *N. attenuata* plants and used for OS collection.

Isolating Na-NPR1

A 335 bp fragment of the genomic *NPR1* gene of *N. attenuata* was amplified using primers derived from conserved cDNA regions of *N. tabacum NPR1* (Liu *et al.*, 2002) using forward primer *NPR1*-5 (3′-CCTGATAAACATGTTAAGAGG-5′) and reverse primer *NPR1*-6 (3′-GCCTAGTGAGCCTCTTGGC-5′) (Supplementary Figure S1). Using *N. tabacum* as the reference sequence (Liu *et al.*, 2002), a full-length ORF was isolated. Two sets of primers were used: (i) forward primer FL3P (3′-ATGGATAATAGTAGGACTGCG -5′) and reverse primer RL5P (3′-TCTTCTTTCTGCTTGCTC-5′), and (ii) forward primer OLF1 (3′-CAGTAAGTCTCCAGAGGAAGGA-5′) and reverse primer OLR1 (3′-CTATTTCCTAAAAGGGAGCTT-5′) (Supplementary Figure S1). Using these two sets of primers, a 1767 bp full-length ORF was PCR-amplified from cDNA prepared from RNA extracted from wounding and *M. sexta* OS-elicited *N. attenuata* leaves. The fragments were excised from the gel, purified using an Amersham gel purification kit (http://www5.amershambiosciences.com/), and cloned in a pGEM-T Easy vector (Promega, http://www.promega.com/) according to the manufacturer's instructions. Positive colonies were identified by blue–white screening and grown overnight, and the plasmid was isolated using a Macheray and Nagel kit (http://www.macheray-nagel.com). The fragments were sequenced and compared with known sequences from the NCBI database, which confirmed the clone to be Na-*NPR1*.

Nucleotide sequences of the ORF of Na-*NPR1* were aligned using the MegAlign program (DNASTAR; http://www.gatcbiotech.com). To determine the phylogenetic relationships among the various *NPR1* sequences from the NCBI database, we used the neighbor-joining method with bootstrap analysis (1000 replicates, Figure S12) (Wu *et al.*, 2006).

Generation and characterization of Na-NPR1-*silenced plants (ir*-npr1*)*

A 335 bp fragment of the cDNA sequence of Na-*NPR1* was inserted into the pRESC5 transformation vector as an inverted-repeat construct (Supplementary Figure S3). This vector was transformed into *N. attenuata* WT plants using an *Agrobacterium*-mediated transformation procedure previously described by Krugel *et al.* (2002). The gene for hygromycin resistance (*hptII*) allowed transformed plants to be identified by selecting hygromycin-resistant individuals (Krugel *et al.*, 2002). Southern hybridization of genomic DNA from independently transformed T_2 generation plants and from WT lines was carried out using a PCR fragment of the *hptII* gene as a probe. Lines harboring a single copy of the transgene resulting from independent transformation events were identified and further screened for homozygosity after germination on GB-5 germination plates supplemented with hygromycin. Two independently transformed single-insert homozygous lines with 100% germination on hygromycin-containing GB5 Petri plates, strongly suppressed in their Na-*NPR1* transcript accumulation (see Supplementary Figure S4) and with WT growth morphologies, were used for all further experiments (lines 174 and 213).

Nucleic acid analysis

DNA was extracted from the leaf tissue of fully developed plants using the cetyl-trimethylammonium bromide method originally developed by Rogers and Bendich (1985) and modified by Paschold *et al.* (2007). For the Southern blot hybridizations, 15 μg of the DNA samples was digested with various restriction enzymes at 37°C overnight, separated on a 0.8% w/v agarose gel, and Southern-blotted onto a nylon membrane (GeneScreen Plus, Perkin Elmer; http://www.perkinelmer.com). The 335 bp fragment of Na-*NPR1* was isolated (see above), and, together with an *hptII* probe (forward primer 5′-CGTCTGTCGAGAAGTTTCTG-3′; reverse primer 3′-CCGGATCGGACGATTGCG-5′) generated by PCR amplification, used as probes for Southern hybridization to confirm the *NPR1* copy number and single-insertion transgenic lines, respectively. Both probes were labeled with α-^{32}P (Rediprime™ II DNA labeling system, Amersham Biosciences).

To analyze Na-*NPR1* transcripts, we extracted total RNA with TRI reagent according to the TIGR protocol (http://www.tigr.org/tdb/potato/images/SGED_SOP_3.1.1.pdf). cDNA was synthesized from 1 μg RNA using SuperScript™ II reverse transcriptase (Invitrogen, http://www.invitrogen.com/). Quantitative real-time PCR (ABI PRISM™ 7000, Applied Biosystems, http://www.appliedbiosystems.com/) was performed using the qPCR™ core reagent kit (Eurogentec; http://www.eurogentec.com), an Na-*NPR1*-specific TaqMan primer pair (forward primer 5′-GTGTCCCTTTTAACCAAAGGA-3′; reverse primer 5′-GCAGATTTTCCTTCCTCT-3′), and a double fluorescent dye-labeled probe (5′-CATCCGATGGCAGAAAAGC ACTTCAAA-3′). Relative gene expression was calculated using a 10-fold dilution series of cDNAs containing Na-*NPR1* transcripts of sulfite reductase (*ECI*), which is not regulated under our experimental conditions (B. Bubner and I.T.B., unpublished data); ECI served as endogenous control genes (Bubner and Baldwin, 2004).

To analyze Na-*NPR1* transcript accumulation in transgenic plants, 1 mM SA was sprayed on leaves until run-off. Tissues were harvested 1 h after elicitation ($n = 3$). A similar procedure was

employed to analyze the kinetics of Na-*NPR1* regulation in response to attack from *S. exigua* larvae, except that the leaf samples were from *S. exigua*-attacked plants that had not been treated with SA. To examine Na-*LOX3* transcript accumulation in *S. exigua*-damaged tissues of WT plants and ir-*npr1* lines, we used an Na-*LOX3*-specific TaqMan primer pair (forward primer 5′-GGCAGTGAAATTCAAAGTAAGAGC-3′; reverse primer 5′-CCCAAAATTTGAATCCACAACA-3′), and a double fluorescent dye-labeled probe (5′-CAGTGAGGAACAAGAACAAGGAAGATCTGAAG-3′). For ICS (Na-*ICS*) analysis, we used a SYBR Green-based RT-PCR approach using Na-*ICS1*-specific primers (forward primer 5′-TTTGCAACCTCCCCAGTC-3′; reverse primer 5′-ACCCCTAGCCCGTGTTC-3′).

Western blot analysis

To isolate polyclonal antibodies against Na-NPR1, a 15-amino-acid peptide was synthesized using the cDNA sequence of Na-*NPR1* (N′-CKG/ARP/SDL/TSD/GRK-C′). This peptide was used to immunize a rabbit, and anti-serum against the synthesized peptide was obtained after 10 weeks (Genemed Synthesis; http://www.genemedsyn.com). Protein samples were separated on an 8.0% sodium dodecylsulphate–polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature in TTBS buffer (12.5 mM Tris–HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% non-fat dried milk, washed three times in TTBS, and incubated for 1 h at room temperature in 1:5000–1:10 000 dilutions of Na-NPR1 anti-serum in TTBS. Blots were washed in TBS-T buffer three times, and incubated for 1 h at room temperature in a 1:10 000 dilution of goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (GeneScreen Plus, Perkin Elmer) in TBS-TMIL buffer. Antibody-bound proteins were visualized by incubating the blots in alkaline phosphatase buffer containing 17 μl NBT and 17 μl BCIP for 30 min.

Direct defense traits

Manduca sexta and *S. exigua* OS were collected from 3rd and 4th instar larvae. OS were diluted 1:2 v/v with de-ionized water before being added to puncture wounds. Leaf tissue (100–150 mg) for the analysis of nicotine, rutin and caffeoyl putrescine was sampled 24, 48 and 72 h after elicitation by treating puncture wounds with water or the OS of *S. exigua*. On separate plants, leaf tissues (100–150 mg) were sampled 24, 48 and 72 h after *S. exigua* began feeding. Secondary metabolites that are strongly correlated with resistance to *S. exigua* in *N. attenuata* were analyzed by HPLC as described by Steppuhn *et al.* (2004). Leaf samples (approximately 100 mg) from *S. exigua*- or wounding and OS-elicited (+1 nodal leaves) glasshouse-grown plants (five replicates for each genotype) were extracted with 2:3 methanol:0.5% acetic acid (v/v), and analyzed by HPLC-DAAD. A standard curve was created using a dilution series of nicotine and rutin, and used to calculate the amounts of nicotine and rutin. In the case of caffeoyl putrescine, for which synthetic standards are not commercially available, the amounts were expressed as relative peak areas. For all metabolites, quantities were normalized to the exact amount of tissue used for the extraction.

Analysis of JA, JA–amino acid conjugates, SA and ethylene

About 200 mg of frozen tissue samples was finely ground and transferred to FastPrep tubes containing 0.9 g of FastPrep matrix, and 1 ml of 90% MeOH spiked with 200 ng ^{13}C-JA and pCA was added to each sample before homogenization on a FastPrep homogenizer (Thermo Electron; http://www.thermo.com) and centrifugation at 6000 ***g*** for 15 min at 4°C. The supernatants were transferred to fresh 2.0 ml Eppendorf tubes, and 1 ml of 100% MeOH was added for re-extraction. The combined supernatants were dried in a vacuum at 45°C, and, after drying, 1 ml of hot water (80°C) was added. The samples were divided into 2500 μl aliquots to measure free and conjugated SA, JA and JA–Ile/Leu; 500 μl of 0.2 M acetate buffer was added to the samples of free SA, JA and JA–Ile/Leu, and 500 μl of 0.2 M acetate buffer containing 0.1 mg ml^{-1} β-glucosidase was added to the sample for conjugated SA analysis. Both samples were incubated at 37°C for 14 h, after which the sample pH was adjusted to 1–1.5. Then, 700 μl of cyclopentane/ethyl acetate/isopropanol (50:50:1) was gently added, and the organic extract was separated and dried under nitrogen. Finally, the dried samples were suspended in 70% MeOH and pipetted to new glass vials before being analyzed by a 1200 L LC/MS-MS-MS system (Varian; http://www.varianinc.com). The instrument was set to a flow rate of 0.1 ml min^{-1}, and 15 μl of each sample was injected onto a Pursuit C8 column (3 μm, 150 × 2 mm) (Varian). A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) was used in gradient mode for separation. Phytohormones were detected in negative ESI mode as described by Wang *et al.* (2007). The levels of JA and its conjugates were estimated based on the peak area of the internal standard; SA was estimated based on a standard curve from the serial dilution of SA. For ethylene, the exact protocol described by Von Dahl and Baldwin (2007) was used. In brief, +1 nodal leaves of three pairs of WT and ir-*npr1* lines (174 and 213) plants were treated with OS (of *S. exigua*) on the mechanically wounded leaves, and immediately sealed in a 250 ml three-necked flask. To analyze ethylene from *S. exigua*-damaged leaves, three pairs of WT and ir-*npr1* plants (lines 174 and 213) were fed on by two *S. exigua* larvae; after they took their first few bites, leaves were sealed in a 250 ml three-necked flask. Ethylene was allowed to accumulate in the flask for 300 min. The headspace was flushed into a photo-acoustic laser spectrometer (Invivo GmbH; http://www.invivo-gmbh.de) with hydrocarbon-free air. Ethylene concentrations were measured by comparing ethylene peak areas with peaks generated by a standard ethylene gas.

Analysis of herbivory

Plants were grown in the glasshouse (16/8 h photoperiod at 200–300 μmol m^{-2} sec^{-1}, 25/21°C and 45–55% relative humidity) in 1-l pots containing soil. To analyze the growth performance of *S. exigua* on glasshouse-grown plants, we placed 3-day-old larvae that had been reared on WT *N. attenuata* leaves on the fully developed leaves of rosette-stage WT and ir-*npr1* plants (n = 15). Each larva was enclosed in a well-aerated clip cage of 5 cm diameter. The larvae were weighed 3, 6 and 9 days after feeding. We then repeated the assay with larvae that had been reared on an artificial diet (see above).

Performance under field conditions

WT and transgenic ir-*npr1* lines were planted into the natural habitat of *N. attenuata* in the south-western USA. Seeds of WT and ir-*npr1* plants were germinated on agar plates. The plates were incubated at 25°C/16 h (200 μm sec^{-1} m^{-2} light) and 20°C/8 h dark. After 10 days, seedlings were transferred to Jiffy 703 pots (1¾ in × 1¾ in, AlwaysGrows; http://www.alwaysgrows.com) that had been soaked in borax solution (0.4 mg 45 ml^{-1} water). The seedlings were fertilized with iron solution (stock solution: 2.78 g

$FeSO4{\cdot}7\ H_2O$ and 3.93 g Titriplex in 1-l H_2O, diluted 100-fold for fertilization) after 7 days. Seedlings were allowed to gradually adapt to the environmental conditions of the Great Basin Desert (high sun exposure and low relative humidity) over 2 weeks in a mesh tent before being transplanted into the field sites. Plants were transplanted in size-matched pairs to an irrigated field plantation at the Lytle Ranch Preserve (Santa Clara, UT, USA) and into native *N. attenuata* populations in a blackbrush and pinyon–juniper forest that had burned in 2005. Seventeen ir-*npr1*–WT pairs of size-matched acclimatized seedlings were planted in transects at the burn site. Seedlings were watered every other day for 2 weeks until roots were established in the native soil. The release of transgenic plants was carried out under APHIS notification 06–003–08n. To comply with 7CFR 340.4, the legal statute that governs the release of transgenic organisms, plants were either harvested and destroyed before the start of flowering (burn site) or flowers were removed before seeds matured (plantation site).

We analyzed the total herbivore damage 21 days after transplanting into the field sites. We estimated the percentage of leaf area removed (in the case of grasshoppers), or the percentage of characteristic damage caused by specific herbivores/pathogens relative to the total leaf area as described by Paschold *et al.* (2007). Damage was expressed as the percentage of canopy damage per plant.

Pathogen identification

We collected leaves infected by naturally occurring pathogens from the field and cultured them on LB medium. Single colonies distinct from other colonies in color and morphology were selected and re-plated. Five isolates were purified from the infected leaves. Plates containing single colonies were sent to AMODIA for identification (http://www.amodia.com). A single colony from five plates was sequenced for 16S ribosomal RNA to identify the pathogen. Three isolates had 16S sequences with more than 98% similarity to those of *Pseudomonas* species, and other two isolates matched the isolates of *Pantoea* species (Suppplementary Table S1).

Indirect defenses

We used a *M. sexta* egg predation assay to measure how well *N. attenuata*'s herbivore-induced VOCs attract the dominant predator of the herbivores, *Geocoris pallens* (Kessler and Baldwin, 2001). Using a natural cellulose glue that is known to have no effect on the predation rate or the VOC emissions, we glued five *M. sexta* eggs to the abaxial side of the second stem leaf of seven matched pairs of ir-*npr1* and WT plants, 30 days after the plants had been planted into the field. We counted the number of predated *M. sexta* eggs (which are transparent and papery) 12, 20, 38 and 42 h after they had been glued to the leaf. We elicited the first stem leaves by wounding and OS 42 h after the eggs had been glued, and measured egg predation again 17, 20 and 25 h after elicitation.

Analysis of VOCs

To trap VOCs released by OS elicitation, we used mechanically punctured leaves of field-grown plants and immediately treated the puncture wounds with *M. sexta* OS. As the OS of both *M. sexta* and *S. exigua* contain common FACs (Voelckel and Baldwin, 2004a), it is possible to mimic each herbivore's response. We enclosed the OS-elicited leaves individually in open-ended polystyrene chambers and trapped VOCs at approximately 350 ml min^{-1} for 8 h using charcoal traps (Orbo M32, Supelco; http://www.sigmaaldrich.com) as described by Kessler and Baldwin (2001). After the experiment, the charcoal traps were stored at −20°C until VOC elution and analysis. Before eluting the VOCs for GC-MS analysis, the traps were spiked with 80 ng tetraline as an internal standard, and eluted with 500 μl dichloromethane as described by Paschold *et al.* (2007).

Oligonucleotide microarray analysis

A customized microarray containing 50-mer oligonucleotides from 1404 herbivore-regulated genes was designed by spotting each oligo four times onto epoxy-coated glass slides (Quantifoil Microtools; http://www.bionity.com) (Voelckel and Baldwin, 2004a,b). First-instar *S. exigua* larvae were placed on the second fully expanded (+1) leaves of five replicate glasshouse-grown WT and ir-*npr1* plants (lines 174 and 213). Leaf tissue was harvested from the damaged leaves and from separate control plants at the same positions. cDNA from damaged leaves of one genotype was Cy3-labeled and cDNA from the corresponding control leaves from the same genotype was Cy5-labeled. A competitive hybridization was performed on the gene-spotted epoxy slides. Each hybridization was replicated twice. An Affymetrix 428™ array scanner (Affymetrix; http://www.affymetrix.com) was used to scan the microarrays sequentially for Cy3- and Cy5-labeled cDNA at a maximum resolution of 10 μm $pixels^{-1}$ with a 16-bit depth. The final data were statistically analyzed using a Lowess normalization procedure within the MIDAS package (TIGR microarray data analysis system, Institute for Genome Research, Washington, DC, USA). For a gene to qualify as up- or downregulated, a minimum of a 1.5-fold change in expression ratio was required, as well as a *t*-test at confidence level (α) of 0.05 for the quadruplicate spots of each gene. A gene was regarded as differentially regulated if it met both criteria in both microarrays from each line. In some cases, a gene was also defined to be significantly regulated when the signal of the gene was present in only one channel and the density was more than 2.5 times the signal-to-noise ratio.

Statistical analysis

Data were analyzed using STATVIEW (Abacus Concepts, Inc.; http://abacus-concepts.com). One-way ANOVA s with Bonferroni-corrected *post hoc* tests were used to analyze the data.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Alignment of the *N. attenuata NPR1* ORF with *NPR1* ORF sequences from various plant species using the CLUSTALW method (http://www.ebi.ac.uk/clustalw).

Figure S2. Southern blot of Na-*NPR1* in WT plants.

Figure S3. Transformation vector used to generate transgenic plants silenced in Na-*NPR1*.

Figure S4. Southern blot of *NPR1*-silenced plants, and Na-*NPR1* transcript accumulation 1 h after SA treatment.

Figure S5. Disease symptoms (8 days post-inoculation) caused by field-isolated *Pseudomonas* sp. (strain 4) on WT *N. attenuata* under glasshouse conditions.

Figure S6. Influence of silencing Na-*NPR1* on rutin and caffeoyl putrescine accumulation in response to *S. exigua* damage.

Figure S7. Silencing Na-*NPR1* reduces JA levels but increases levels of free SA in field-grown OS-elicited plants.

Figure S8. Silencing Na-*NPR1* did not affect the amount of ethylene released after herbivore damage or after wounding and OS elicitation in the leaves of ir-*npr1* (213 and 174) and WT glasshouse-grown plants.

Figure S9. Silencing Na-*NPR1* increases levels of conjugated SA and total SA during continuous herbivory as well as after wounding and OS elicitation in the glasshouse.

Figure S10. Silencing *NPR1* changes herbivory-elicited transcriptional responses.

Figure S11. Loading controls for Western blot analysis shown in (Figure 2b).

Figure S12. Neighbor-joining tree of *NPR1* ORF sequences.

Figure S13. Alignment of *N. attenuata* NPR1 (deduced amino acids) with various NPRs of *Arabidopsis thaliana* using the CLUSTALW method (http://www.ebi.ac.uk/clustalw).

Figure S14. Growth of WT and ir-*npr1* line 213 grown in native populations in the south-western United States.

Table S1. Pathogen identification from infected field samples.

Table S2. Levels of volatile organic compounds (VOCs) emitted by OS-elicited single leaves of field-grown *N. attenuata* WT and ir-*npr1* (213) plants for 8 h.

This material is available as part of the online article from http://www.blackwell-synergy.com

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