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Oviposition by *Spodoptera exigua* on *Nicotiana attenuata* primes induced plant defence against larval herbivory

Michele Bandoly[1], Monika Hilker[2] and Anke Steppuhn[1,*]

[1]*Molecular Ecology, Dahlem Centre of Plant Sciences (DCPS), Institute of Biology, Freie Universität (FU) Berlin, Haderslebener Str. 9, 12163 Berlin, Germany, and*

[2]*DCPS, Applied Zoology/Animal Ecology, Institute of Biology, FU Berlin, Haderslebener Str. 9, 12163 Berlin, Germany*

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*For correspondence (e-mail a.steppuhn@fu-berlin.de).

SUMMARY

Plants exhibit multifarious defence traits against herbivory that are constitutively expressed or induced upon attack. Insect egg deposition often precedes impending larval attack, and several plants can increase their resistance against larvae after experiencing the oviposition by an herbivore. The nature of such oviposition-mediated resistance remains unknown, and here we aim to determine plant traits that explain it. We test whether oviposition on a host plant can induce plant defence responses or enhance (prime) the induction of defence traits in response to larval herbivory. We exposed *Nicotiana attenuata* plants to oviposition by moths of a generalist herbivore, *Spodoptera exigua.* Its larvae suffered higher mortality, retarded development and inflicted less feeding damage on oviposition-experienced than on oviposition-unexperienced plants. While oviposition alone did not induce any of the examined defence traits, oviposited plants exhibited a stronger inducibility of known defence traits, i.e. caffeoylputrescine (CP) and trypsin protease inhibitors (TPIs). We found no effects of oviposition on phytohormone levels, but on the feeding-inducible accumulation of the transcription factor NaMyb8 that is governing biosynthesis of phenylpropanoid–polyamine conjugates, including CP. Comparison of larval performance on wild-type plants, CP-deficient plants (silenced *NaMyb8* gene), and TPI-deficient plants (silenced *NaPI* gene) revealed that priming of plant resistance to larvae by prior oviposition required *NaMyb8*-mediated defence traits. Our results show that plants can use insect egg deposition as a warning signal to prime their feeding-induced defence.

Keywords: *Nicotiana attenuata*, *Spodoptera exigua*, plant defence, priming, induction, herbivore oviposition, secondary metabolites, Myb transcription factor.

INTRODUCTION

When plants mount their anti-herbivore defence on demand, they save the costs of defence production in the absence of herbivory and can express herbivore-specific responses (Schaller, 2008). However, the lag time until effective levels of plant defence traits are produced is the drawback of inducible defences, which may be curtailed when responding to stimuli that reliably predict herbivory. Exposure of plants to environmental stimuli that indicate an increased probability of impending stress may prime plants for more efficient stress responses (Conrath *et al.*, 2006; Frost *et al.*, 2008; Conrath, 2011). Thus priming is a strategy that increases phenotypic plasticity beyond the inducibility of resistance traits and may enable plants to increase their fitness. For example, priming of pathogen defences in *Arabidopsis thaliana* provided the same level of resistance to phytopathogens as pre-inducing pathogen defences, but opposite to the latter, priming had no negative impact on the plant's fitness when no pathogen attack occurred (van Hulten *et al.*, 2006). While priming of plant defence against pathogens by previous interactions with microorganisms is a well described phenomenon (Conrath *et al.*, 2001, 2002; Ton *et al.*, 2005; Ahn *et al.*, 2007), fewer studies considered priming of anti-herbivore defence by exposure of plants to volatiles released from feeding-damaged neighbouring plants (Engelberth *et al.*, 2004; Heil and Kost, 2006; Frost *et al.*, 2007). For example, *Zea mays* exposed to green leaf volatiles that are released upon feeding damage respond to herbivory with an increased production of defence compounds and jasmonic acid (JA), a phytohormone that is mediating many plant defence responses against herbivores (Engelberth *et al.*, 2004). In addition, it has been shown that herbivore attack can even increase plant resistance in the subsequent generations

likely due to the priming of defence induction (Rasmann *et al.*, 2012). Here we investigate whether plants may use the egg deposition of an herbivorous insect to prime its feeding-induced defences against the larvae.

While volatiles of damaged plant neighbours and previous herbivory can indicate a risk of future herbivory, oviposition by herbivorous insects on their host plants is arguably the most reliable predictor of future attack (Hilker and Meiners, 2006; Beyaert *et al.*, 2012). Many plants can respond to oviposition with defences that reduce egg survival (reviewed in (Hilker and Fatouros, 2015; Reymond, 2013), for example, by attraction of egg parasitoids (Fatouros *et al.*, 2008; Blenn *et al.*, 2012). In addition to oviposition-induced plant defences targeting the eggs *per se*, recent studies found that insect oviposition on plants may change the plant quality in such a way that the performance of larvae hatching from the eggs is altered. In some plant species, larval performance was reduced on previously oviposited plants (Beyaert *et al.*, 2012; Geiselhardt *et al.*, 2013; Pashalidou *et al.*, 2013). However, herbivores may also benefit from plant responses to insect eggs. In *A. thaliana*, treatments of leaves with extracts of eggs from specialist (*Pieris brassicae*) or generalist (*Spodoptera littoralis*) lepidopteran herbivores can result in suppression of feeding-induced defence genes, which correlates with increased larval performance of the generalist on these leaves (Bruessow *et al.*, 2010). The suppression of the JA-mediated plant response to herbivores is likely mediated by the antagonistic interaction with pathogen-associated signalling pathways that are activated in *A. thaliana* leaves in response to egg deposition and egg extract treatments (Little *et al.*, 2007). The egg-mediated activation of pathogen resistance in *A. thaliana* may prevent the detrimental effect of bacterial pathogens on feeding larvae (Hilfiker *et al.*, 2014). However, on *A. thaliana* and other brassicaceous plants, larval performance of *P. brassicae* can also be negatively affected on previously oviposited leaves, but it remained open by which plant traits these effects were mediated (Geiselhardt *et al.*, 2013; Pashalidou *et al.*, 2013, 2015). To date no study determined how oviposition can increase plant resistance to herbivorous larvae. It is yet unknown whether oviposition-induced changes in the plant quality or whether feeding-induced traits that are primed by previous oviposition are responsible for increased anti-herbivore resistance in oviposited plants.

Here we aim to determine which plant traits can explain the enhanced anti-herbivore resistance of plants warned by insect egg deposition and reveal whether oviposition may serve as a priming signal to enhance the feeding-induced plant defence response or simply induces plant resistance traits by itself. Therefore, we investigate whether oviposition by *Spodoptera exigua* on *Nicotiana attenuata*: (i) affects plant resistance against *S. exigua* larvae; (ii) directly induces plant defence against feeding larvae or primes the feeding-induced plant defence against larvae; and (iii) whether oviposition-primed defence traits are mediating the increased resistance of oviposition-experienced plants against larval herbivory.

The wild tobacco, *N. attenuata*, is known to mount several feeding-inducible secondary metabolites and proteins such as nicotine, phenylpropanoid-polyamine conjugates (PPCs) including caffeoylputrescine (CP), flavonoids such as rutin, diterpene glycosides (DTGs), and trypsin protease inhibitors (TPIs). The induction of the biosynthesis of these traits is known to depend on the wound hormone JA (Halitschke and Baldwin, 2004). The anti-herbivore defence functions of many of these traits have been shown by silencing their biosynthetic genes (Steppuhn *et al.*, 2004; Steppuhn and Baldwin, 2007; Jassbi *et al.*, 2008; Kaur *et al.*, 2010). The generalist lepidopteran herbivore *S. exigua* feeds on *N. attenuata* in native populations (Steppuhn *et al.*, 2004) and is strongly affected by feeding-induced plant defences such as nicotine and TPIs (Steppuhn and Baldwin, 2007).

To examine the ecological effect of *S. exigua* oviposition on the plant's resistance to the larvae, we analysed different performance parameters of larvae feeding on plants with and without prior oviposition experience in several experimental repetitions. The larvae on previously oviposited plants performed worse and to elucidate the plant traits that may explain this effect, we screened an array of plant defence traits in full-factorial experiments. We investigated undamaged and herbivory-damaged plants with and without prior oviposition-experience, and in addition we analysed JA- and transcript accumulation of the NaMyb8 transcription factor. To functionally link the primed responses with the decreased performance of *S. exigua* on oviposition-experienced plants, we compared the effect of prior oviposition on the mortality of larvae feeding on wild-type (WT) *N. attenuata* with that on plants that are stably transformed to silence expression of genes required for the production of candidate anti-herbivore defence compounds.

RESULTS

Prior oviposition increases plant resistance to larvae

On oviposition-experienced *N. attenuata* plants *S. exigua* larvae performed worse than on unexperienced plants. Their mortality was always higher, their mass at day 4 was reduced, and they developed more slowly (Figure 1). The feeding damage (assessed in two experiments) mirrored the strongly negative effects of prior oviposition on larval performance: Oviposition-experienced plants suffered significantly less feeding damage than unexperienced plants.

Figure 1 depicts average effect sizes of mortality and body mass data of three experimental repetitions. Although larval mortality had different levels in the experi-

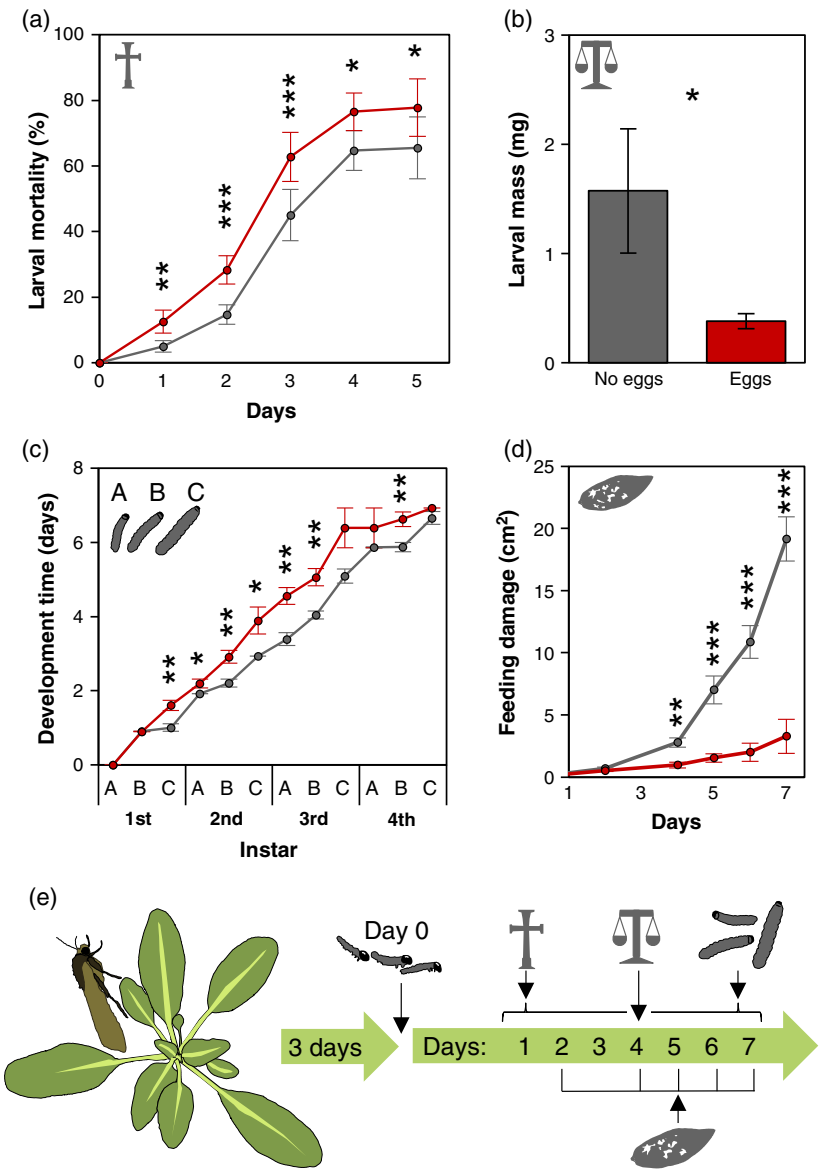

Figure 1. *Spodoptera exigua* larvae perform worse on oviposition-experienced *Nicotiana attenuata*.
(a, b) Mean ± standard error of the mean of: (a) mortality (N_{Plant} = 18/28 at day 4); and (b) average larval mass per plant (N_{Plant} = 19) on oviposition-experienced (red) and oviposition-unexperienced (grey) plants pooled from three experiments with 6, 12, and 10 plant replicates respectively.
(c, d) (c) Developmental time of larvae (initially 27 per oviposition treatment) derived from daily observations of larval instars and sub-instars (A – freshly moulted, B – vigorously feeding, C – preparing to moult) and (d) feeding-damaged leaf area in one of the experiments (N_{Plant} = 6).
(e) Experimental schedule: 3 days after moths oviposited on the plant, we removed eggs and applied neonates. Arrows indicate when the parameters were determined. Asterisks (*/**/***) indicate significant differences between oviposition-experienced and oviposition–unexperienced plants according to Fisher's exact test (a) and paired (b, d) or Welch's (c) *t*-test at $P < 0.05/0.01/0.001$.

mental repetitions, it was significantly increased on oviposition-experienced plants in each of the three experiments (Table S1). In the first experiment, larval mortality on unexperienced plants was moderate but increased by 40% on oviposited plants, where larvae developed more slowly and had an 80% lower mass at day 4. Larvae of our *S. exigua* culture performed worse on plants after some generations, so that all larvae died within a week when feeding on *N. attenuata* in the second experiment. Even these susceptible larvae died significantly faster on oviposited plants and caused significantly less feeding damage. However, 90% of the larvae vanished before day 4 and we detected no differences in the mass of surviving larvae. The enormous mortality rates of *S. exigua* larvae on *N. attenuata* moderately decreased after introducing individuals from another origin into our rearing. In consequence about 70% of the larvae survived until day 4 in the third experiment in which the effect of prior oviposition on larval mass was again indicated by trend. Thus, the effect size of oviposition-mediated resistance depended on the general susceptibility of the larvae and was most pronounced when a proportion of them could develop on unexperienced *N. attenuata* plants. Under natural conditions *S. exigua* is frequently observed to attack *N. attenuata* and can develop on it for at least 2 weeks (Steppuhn *et al.*, 2004) suggesting that the first experiment more realistically reflects this interaction.

We determined levels of known plant defence traits in the experiment with the most striking differences in larval performance but found no differences between

oviposition-experienced and unexperienced plants in the levels of nicotine, phenylpropanoids, and DTGs, and TPI activity was even reduced on oviposition-experienced plants (Table S2). However, at the examined time points (4 and 6 days after larvae had started feeding) unexperienced plants already suffered 2.8-fold and 5.4-fold more feeding damage than oviposition-experienced plants (Figure 1d). Levels of most metabolites correlated positively with feeding damage but for levels of CP and TPI activity this correlation exhibited greater slopes in oviposition-experienced than in unexperienced plants (Figure S1).

Because oviposited *N. attenuata* plants were less damaged than unexperienced plants but still expressed similar levels of feeding-inducible metabolites, we hypothesized that oviposition had increased the plant's responsiveness to feeding damage.

Oviposition primes the induction of defence traits in response to feeding

To test the above-mentioned hypothesis, we exposed oviposition-experienced and unexperienced plants to the same extent of damage in a full-factorial design and measured secondary metabolite levels and activities of defence proteins (TPI and polyphenol oxidase, PPO). We mimicked herbivory by adding *S. exigua* oral secretions (OS) to puncture wounds on leaves of oviposited and unexperienced plants or not. As unwounded but oviposition-experienced plants were not altered in any of the defence traits, the oviposition *per se* did not induce the defence traits measured. In response to wounding and application of OS, plants increased their levels of nicotine, CP, CA, total DTGs, and activities of TPIs and PPOs in the leaves (Figure 2 (left) and Tables S3 and S4). This induction was significantly higher in oviposition-experienced than in unexperienced plants for levels of CP and TPI activity (by 53% and 43% at day 4) but not for nicotine and the other defence traits.

In a second approach, we kept feeding damage on oviposited and unexperienced plants equal by replacing larvae if required to ensure the same number and size of larvae on both types of plants. Similar to the experiment with mimicked herbivory, the feeding-induced production of CP and TPI activity was also increased (Figure 2 (right) and Table S3). Higher CP levels in oviposition-experienced plants were already detected 1 day after larvae had started feeding (Table S3).

Oviposition does not alter feeding-induced accumulation of JA but that of *NaMyb8* transcripts

Because feeding-induced PPC biosynthesis and TPI production are under control of the JA signalling pathway, we determined levels of JA and its bioactive conjugate to isoleucine (JA-Ile) in response to *S. exigua* oviposition and herbivory in a full-factorial experiment. We found that neither constitutive nor feeding-induced levels of JA or JA-Ile were altered in oviposition-experienced compared with unexperienced *N. attenuata* plants (Figure 3a,b and Table S5). This result is consistent with the finding that no other JA-inducible defences such as nicotine and PPO activity were altered in oviposition-experienced, feeding-damaged plants when compared with egg-free, feeding-damaged plants.

To investigate signalling components further downstream of the JA-pathway we examined transcript accumulation of the NaMyb8 transcription factor in the same experiment. This transcription factor has been proposed to rather specifically regulate the production of PPCs (Galis *et al.*, 2006; Kaur *et al.*, 2010; Onkokesung *et al.*, 2012). We found that prior *S. exigua* oviposition significantly affected the feeding-induced transcript accumulation of *NaMyb8.* Interestingly, plants without feeding damage exhibited about 70% lower transcript levels of *NaMyb8* when oviposition-experienced. However, upon *S. exigua* feeding, *NaMyb8* transcript levels of oviposition-experienced plants increased 136-fold, whereas unexperienced plants were induced 34-fold (Figure 3c and Table S5).

Oviposition-priming of *N. attenuata*'s resistance to larvae depends on *NaMyb8*

Larvae of *S. exigua* are known to perform better on ir-PI plants deficient for TPI production (Steppuhn and Baldwin, 2007). To examine whether NaMyb8-regulated defences can also affect *S. exigua* larvae, we compared larval mortality on ir-Myb8 and WT plants. Larval mortality was about 20% lower in two independently transformed ir-Myb8 lines in comparison with WT plants (Figure 4a). We then examined the effect of *S. exigua* oviposition on larvae feeding on ir-PI, ir-Myb8, and WT plants. Because early mortality of larvae was consistently increased on previously oviposited plants throughout all experiments, we used this parameter to evaluate the effect of oviposition on *S. exigua* larval performance in the later assays with transgenic *N. attenuata* plants. Larval mortality on oviposition-experienced WT plants was again significantly higher than on unexperienced WT plants (Figure 4b). While this effect was also determined for the first time point examined when larvae fed on ir-PI plants, it was not present when larvae were feeding on the ir-Myb8 genotype. Thus, the negative effect of previously oviposited plants on *S. exigua* larval performance depends on *NaMyb8*-mediated defences.

DISCUSSION

Plants are known to increase their phenotypic plasticity by priming strategies. Here we provide evidence for another environmental cue that plants can use as a priming signal: the egg deposition of an herbivorous insect on a plant, which is reliably predicting forthcoming herbivory.

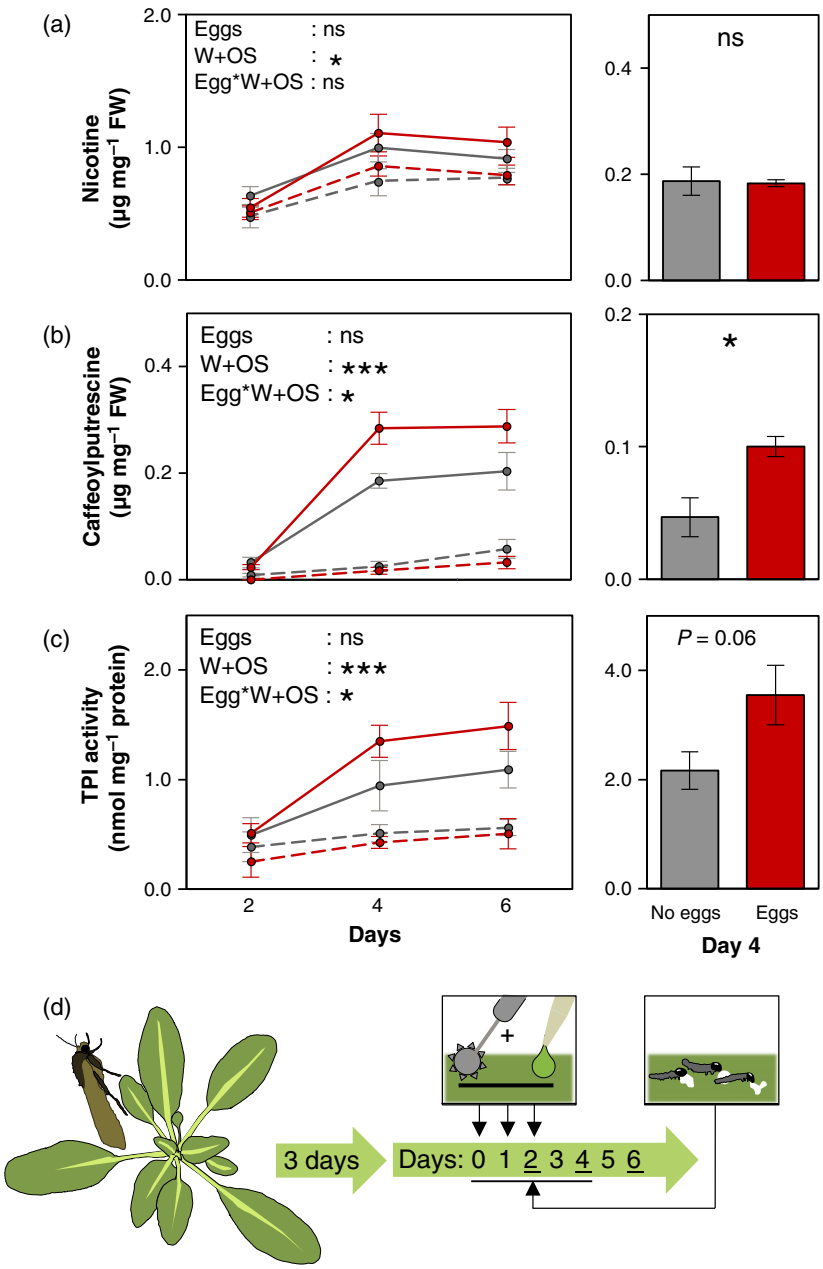

Figure 2. Anti-herbivore defences are more strongly induced in oviposition-experienced *Nicotiana attenuata*.
(a–c) Mean levels (±standard error of the mean of (a) nicotine; (b) caffeoylputrescine; and (c) trypsin protease inhibitor (TPI) activity in leaves of oviposition-experienced (red) or unexperienced (grey) *N. attenuata* plants.
(d) Experimental schedule: 3 days after oviposition, eggs were removed and plants were either: line charts (left): kept untreated (dashed lines) or repeatedly wounded adding *Spodoptera exigua* oral secretions (OS; solid lines) (N_{Plant} = 7); or bar charts (right): exposed to *S. exigua* herbivory of equal extent on oviposition-experienced and unexperienced plants for 4 days (N_{Plant} = 6). Asterisks (*/***) indicate significant differences according to GLM (left) or Welch's *t*-test (right) $P < 0.05/0.001$.

Previous studies provided evidence that insect egg deposition may alter anti-herbivore resistance of different plants, but could not reveal which plant traits were affecting larval performance (Beyaert *et al.*, 2012; Geiselhardt *et al.*, 2013; Pashalidou *et al.*, 2013). We showed that wild tobacco plants use oviposition by the generalist herbivore *S. exigua* as a signal to increase their resistance against the forthcoming larval herbivory and examined which plant traits that are defensive against herbivorous larvae were altered in oviposition-experienced plants. We found that *S. exigua* oviposition on *N. attenuata* did not induce, but primed a distinct subset of its feeding-inducible defences. Finally, we functionally linked the oviposition-mediated increase in plant resistance to *S. exigua* to the production of oviposition-primed defences that are dependent on the transcription factor NaMyb8.

The effect of previously oviposited *N. attenuata* plants on the larval performance were assessed in leaves systemic to the oviposition and ranged from a moderate increase in mortality of about 10% up to striking effects of 40% increased mortality and 80% reduced larval mass gain accompanied by a prolonged developmental time (Table S1). Consequently, oviposition-experienced plants suffered less feeding damage (Figure 1d and Table S1).

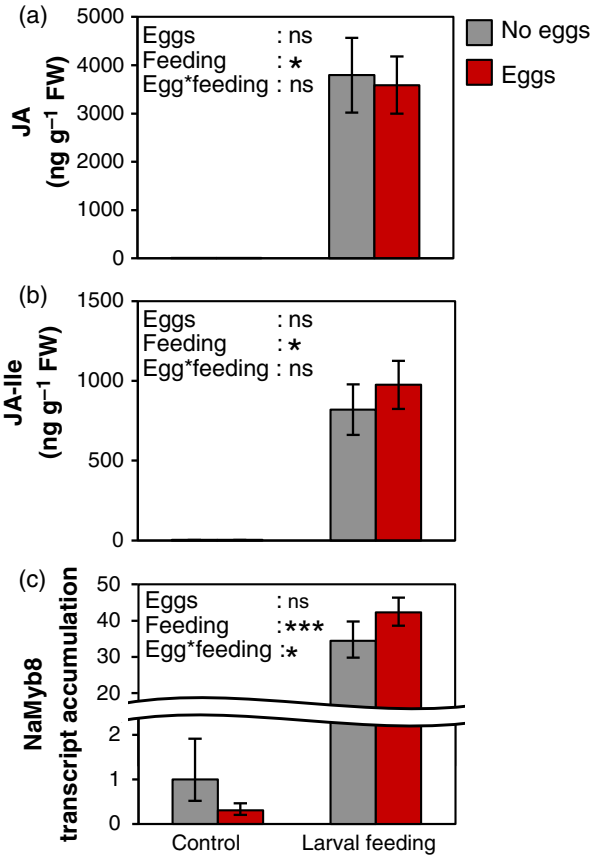

Figure 3. Feeding-induced accumulations of *NaMyb8* transcripts and JA/JA-Ile in oviposition-experienced and in unexperienced plants.
(a–c) Accumulations (mean ± standard error of the mean) of (a) jasmonic acid (JA), (b) its bioactive conjugate to isoleucine (JA-Ile), and (c) *NaMyb8* transcripts in *Nicotiana attenuata* with and without prior *Spodoptera exigua* oviposition. Three days after oviposition, eggs were removed and plants were either exposed to 30 *S. exigua* larvae for 24 h (N_{Plant} = 9/11 unexperienced/oviposition-experienced plants) or remained untreated as control plants (N_{Plant} = 5/11 unexperienced/oviposition-experienced plants). Relative quantity of *NaMyb8* transcripts normalized to β-*actin* as reference gene is displayed relative to untreated plants without eggs. Significant differences according to a generalised linear model (performed on the log_2-normalized data): */*** ($P < 0.05/0.001$).

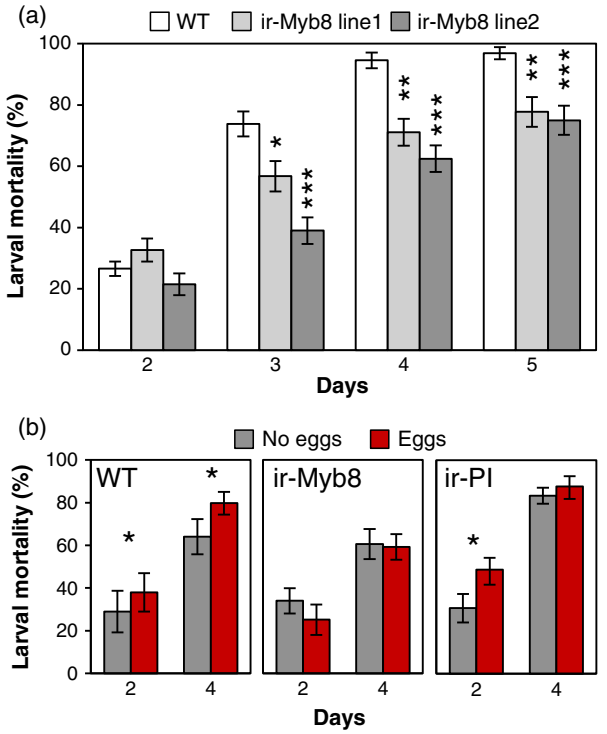

Figure 4. Silencing of *Nicotiana attenuata Myb8* but not *PI* abolishes oviposition-mediated increase of *Spodoptera exigua* larval mortality.
(a, b) Mean ± standard error of the mean of *S. exigua* larval mortality: (a) on wild-type *N. attenuata* (WT: N_{Plant} = 16) plants and on two independently transformed lines to silence *NaMyb8* gene expression (ir-Myb8 line 1/2: N_{Plant} = 6/10) over 5 days; and (b) on oviposition-experienced and oviposition-unexperienced plants after 2 and 4 days of feeding on WT (N_{Plant} = 10) or plants stably transformed to silence *NaPI* (ir-PI) or *NaMyb8* (line 2) gene expression (each: N_{Plant} = 8). Asterisks (*/**/***) indicate significant differences between (a) WT and ir-Myb8 lines or (b) oviposition-experienced and unexperienced plants of each genotype according to Fisher's exact test at $P < 0.05/0.01/0.001$.

While such negative effects of previously oviposited plants on larval performance have been shown in some other studies (Beyaert *et al.*, 2012; Geiselhardt *et al.*, 2013; Pashalidou *et al.*, 2013), the plant traits that cause these effects were unknown.

Insect egg deposition may directly induce plant defence as, for example, oviposition by predacious bugs on tomato enhances plant resistance to thrips feeding likely by activating JA signalling, which was evident in the enhanced gene expression of *PI-I* and *AOS*, a JA-biosynthesis gene, in oviposited plants (De Puysseleyr *et al.*, 2011). However, in this case, oviposition involves wounding of plant tissue, and wounding is known to directly induce these responses (Ryan, 2000). While similar mechanisms may occur in plant–herbivore interactions that involve ovipositional wounding such as oviposition by the pine sawfly on *Pinus sylvestris* (Beyaert *et al.*, 2012), the oviposition by lepidopteran species does not inflict plant damage suggesting that other mechanisms mediate the increased resistance of oviposited plants in these systems. Furthermore, *N. attenuata* shows no signs of an oviposition-induced response paralleling the plant's response to pathogens (e.g. chlorotic spots, ROS induction or formation of neoplasms), which can even interfere with herbivory-induced traits as shown in *A. thaliana* (Little *et al.*, 2007; Bruessow *et al.*, 2010; Hilfiker *et al.*, 2014).

We assessed the induction kinetics of defence metabolites in the leaves of *N. attenuata* and found that none of the defences measured were induced by the oviposition *per se.* However, plants with prior insect egg deposition responded to a standardised induction by wounding and application of *S. exigua* OS with an enhanced production of TPIs and CP (Figure 2). We verified this finding in an

experiment, in which *S. exigua* herbivory was standardised for the inflicted feeding damage on previously oviposited and unexperienced plants. Similarly, oviposition by a lepidopteran herbivore primed the wound-induced accumulations of *PI* transcripts in tomato, which was accompanied by enhanced JA levels; however, whether this affected production of defence traits and plant resistance was not determined (Kim *et al.*, 2012). We tested whether *S. exigua* oviposition may also prime herbivory-induced JA levels, which may mediate priming of inducible TPI activity and CP levels. Herbivory-induced production of JA and JA-Ile were not altered by prior oviposition (Figure 3a, b), which may explain why the priming effect of *S. exigua* oviposition on feeding-inducible defences of *N. attenuata* was limited to a distinct subset of numerous JA-mediated defence traits. Damage-induced volatiles of neighbouring sagebrush plants are also known to prime a particular subset of JA-mediated feeding-induced defence traits of *N. attenuata*, including TPIs and DTGs (Kessler *et al.*, 2006), which is associated with increased resistance to lepidopteran larvae. In both cases – the priming of *N. attenuata*'s defence by sagebrush volatiles or by *S. exigua* oviposition – the underlying mechanisms must operate downstream of the feeding-induced JA-burst. The JA signalling pathway triggers different defence responses *via* the activation of an E3 ubiquitin ligase complex by JA-Ile binding to an F-box protein (COI1) that is part of this complex, which results in the degradation of JAZ repressor proteins that are blocking different transcription factors (Pauwels *et al.*, 2010).

The NaMyb8 transcription factor belongs to the most abundant class of Myb transcription factors in plants (R2R3-Myb) that have been recognized as intermediate targets in different plant regulatory networks (Dubos *et al.*, 2010). Transgenic ir-Myb8 *N. attenuata* plants are deficient in the production of PPCs including CP, but are not altered in other JA-inducible traits examined suggesting a rather specific role of *NaMyb8* in regulating production of phenylpropanoids (Kaur *et al.*, 2010). We found that the herbivory-induced increase in *NaMyb8* transcript accumulation was significantly affected by prior *S. exigua* oviposition in the same experiment in which the induction of JA and JA-Ile were not altered (Figure 3c). We examined these signalling components in plants that have been continuously fed by 30 *S. exigua* larvae for 24 h so that the defence signalling was strongly activated. We cannot exclude that JA levels were affected at the onset of this response, but since the oviposition-priming of CP and TPI production was a long lasting effect, this result suggests that the priming mechanism for the increased feeding-mediated induction of CP in oviposited plants acts downstream of the JA-pathway and upstream of *NaMyb8* transcript accumulation. Several priming mechanisms of feeding-induced plant responses have been discussed; they range from the accumulation of signalling molecules to epigenetic modifications or regulations by small RNA (Bruce *et al.*, 2007; Galis *et al.*, 2009). The priming by insect oviposition must involve a systemic signal, as the feeding-induced defence production is altered in egg-free leaves of oviposition-experienced plants.

Finally, we addressed the question whether the defences primed by the *S. exigua* oviposition could indeed explain the reduced performance of *S. exigua* larvae on oviposition-experienced plants. It was known from previous studies that *S. exigua* larvae suffer from *N. attenuata*'s TPI production (Steppuhn and Baldwin, 2007). We confirmed here that larval performance of *S. exigua* is also reduced by the *NaMyb8*-mediated defences. Similar to the effects described for two other lepidopteran species, *Manduca sexta* and *S. littoralis* (Kaur *et al.*, 2010), *S. exigua* larvae survived much better on CP-deficient ir-Myb8 than on WT plants (Figure 4a). Thus, *NaMyb8* mediated defences can obviously increase mortality of *S. exigua* larvae feeding on *N. attenuata.*

To functionally link the effect of previously oviposited plants on *S. exigua* larval mortality to the primed defence traits, we compared larval mortality on oviposition-experienced plants and unexperienced plants in the WT, ir-PI, and ir-Myb8 genotypes and found that oviposition onto the latter genotype did not affect *S. exigua* mortality (Figure 4b). Thus, the oviposition-mediated reduction in larval performance requires *NaMyb8*-mediated plant defences, such as CP. We cannot yet disentangle the contribution of CP from that of other *NaMyb8* mediated defence traits such as other PPCs. However, in a previous study the reduced resistance of ir-Myb8 plants to lepidopteran larvae was partially restored by spraying leaves with CP in concentrations comparable to that of feeding-induced leaves (Kaur *et al.*, 2010) suggesting an important role of CP for plant defence against lepidopteran herbivores. TPIs alone cannot explain the effect of oviposition-mediated increase in plant resistance to *S. exigua*, because early larval mortality was still increased when larvae fed upon the previously oviposited ir-PI plants.

Altogether, our data show that *S. exigua* oviposition on *N. attenuata* primes *NaMyb8*-mediated feeding-inducible defences and renders the plant more resistant against larval feeding (Figure 5). The perception of oviposition by plants probably evolved because of the high reliability of eggs as first sign of future herbivory. However, when oviposition itself is not damaging the plant and when the eggs are killed by predators or parasitoids before the herbivorous larvae can hatch, the plant remains unattacked. Hence, it may be advantageous for the plant to use oviposition as a signal to prime – but not yet to induce – defence to herbivory, because the costs of producing defences are avoided before the actual attack occurs. Here we show that a plant can indeed use insect egg deposition as alarm sig-

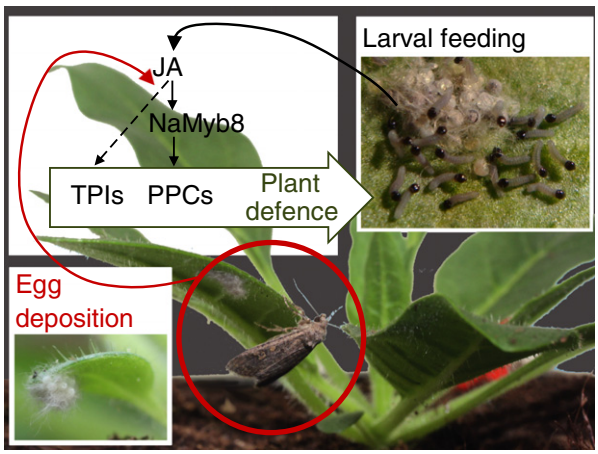

Figure 5. Scheme of defence priming by oviposition.
Eggs are deposited by an *S. exigua* moth on *N. attenuata* plants; upon feeding by the hatching larvae, the oviposited plants show an elevated (red arrow) induction of jasmonic acid (JA)-mediated defences (black arrows): trypsin protease inhibitor activity (TPI) and phenylpropanoid–polyamine conjugates (PPCs) including caffeoylputrescine. PPCs are under control of the transcription factor *NaMyb8*, which is required for the increased resistance of oviposition-experienced plants due to a priming mechanism acting downstream of JA.

nal to adjust specific setscrews in their signalling network to improve their anti-herbivore resistance.

EXPERIMENTAL PROCEDURES

Plants and insects

We performed experiments with WT type *Nicotiana attenuata* Torr. ex Watson (Solanaceae) plants of an inbred line (15th generation) derived from field collected seeds (Great Basin Desert, Utah) (Baldwin, 1998). Additionally we used stably transformed plants carrying inverted repeat constructs (ir) to post-transcriptionally silence gene expression of either *NaMyb8* or *NaPI* (GenBank: GU451752, DQ158200.1) genes. We used T4 plants of line A04-186 carrying the pSOL3PI construct and two independently transformed lines carrying the pSOL8Myb8 construct (line 1: A07-810, line 2: A07-818) thoroughly characterized in previous studies (Steppuhn and Baldwin, 2007; Kaur *et al.*, 2010). Vector construction, the pSOL3PI and pSOL8Myb8 plasmids and *Agrobacterium*-mediated transformation procedure are described elsewhere (Krügel *et al.*, 2002; Bubner *et al.*, 2006). Plants grew in a greenhouse (24°C (±10):15°C; 16:8 h L:D). After germination on agar plates (Gamborg's B5 medium; Duchefa, Haarlem, The Netherlands, https://www.duchefa-biochemie.com) for 7–10 days, we transferred seedlings to individual cells (height: 6 cm; ø: 4 cm) of propagation trays with potting soil (Einheitserde® Classic type T, Uetersen, Germany, http://www.einheitserde.de). Three week-old plants were planted in 1.5 L pots with potting soil.

A culture of *Spodoptera exigua* Hübner (Noctuidae) was established from eggs obtained from Bayer CropScience (Monheim, Germany, http://www.cropscience.bayer.com) and Wageningen University (Wageningen, The Netherlands, http://www.wageningenur.nl) in a climate chamber (24°C; 16:8 h L:D). We fed larvae on a bean flour-based artificial diet (Table S6) in vented plastic boxes and kept the moths in flight cages supplied with 20% honey solution and paper tissue as substrate for oviposition.

Experiments

Overall, we conducted eight experiments (exps. I–VIII, overview in Table S7) and generally exposed 4–5-week-old *N. attenuata* rosettes to mated *S. exigua* moths in a flight cage (40 × 71 × 40 cm) for 12 h. Control plants were exposed in cages containing only male *S. exigua* moths. We received varying number of oviposited plants with 88 ± 10 (mean ± standard error of the mean) eggs per plant at 1–2 old leaves. We matched oviposition-experienced and unexperienced plants of each replicate for their ontogeny (by size and elongation state). After 3 days, which is in the range of the natural egg incubation time, we removed the eggs gently with a soft brush without damaging the leaf surface. The removal of eggs allowed to standardise the onset of larval feeding that started within 12 h after egg removal by transfer of neonate larvae to the plants.

Spodoptera exigua performance and feeding damage on oviposition-experienced and oviposition-unexperienced *N. attenuata* (exps. I–III)

In greenhouse experiments, we recorded mortality, mass, and developmental time of *S. exigua* larvae feeding on oviposited or unexperienced *N. attenuata* plants (exp. I/exp. II/exp. III: N = 6/12/10 plants per treatment). We applied different numbers of neonates dependent on their availability but oviposition-experienced and unexperienced plants of the same replicate always received equal numbers of *S. exigua* neonates (exp. I/exp. II/exp. III: 3–5/20/5–15). Larvae were kept on a standardised leaf position (on the second youngest source leaf in relation to the sink-source transition leaf that we determined from its morphological characteristics) systemic to the oviposited leaf using vented clip cages (ø 6.5 × 2.5 cm) because allocation of defensive compounds depends on leaf ontogeny (van Dam *et al.*, 2001; Kaur *et al.*, 2010). In all experiments, we determined mean larval mass and larval mortality after 4 days of feeding. In two experiments, we recorded mortality daily for 1 week and additionally determined accumulated feeding damage at day 2, days 4–7 or after 5 days of feeding (in exp. I and exp. II respectively) from photographs of the leaves on white paper (including a 1 cm^2 reference area). We processed photographs with Photoshop© CS5 (Adobe Systems Incorporated, USA, http://www.adobe.com) to obtain binary images which we analysed using ImageJ 1.47v (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij, 1997–2014) for determination of the consumed leaf area. In exp. I, we also examined the developmental times of larvae according to daily recordings of their instar and sub-instar (A: freshly moulted; B: vigorously feeding; C: larvae that prepare for moulting; Figure S2).

In experiment I, we additionally sampled the leaves that larvae had fed upon for analysis of secondary metabolites. Larvae were daily positioned one leaf position higher from day 2 on, and we harvested the leaf that larvae had fed upon from days 3–4 and 5–6.

Oviposition-experienced and -unexperienced *N. attenuata* with equal damage to examine defence induction (exp. IV and exp. V)

We either used standardised induction of plant defence that largely mimics that of herbivory (Halitschke *et al.*, 2001; Diezel *et al.*, 2009) or induction by equalized larval herbivory. In experiment IV, we elicited the herbivory-induced defence response by wounding and the application of the *S. exigua* OS that is known to contain fatty acid amino acid conjugates and glucose oxidase as elicitors (Diezel *et al.*, 2009). We collected the OS from third-instar larvae

that had fed on WT *N. attenuata* leaf material with a Teflon tube in 2 ml glass vials that were connected to a vacuum pump. We centrifuged the collected OS to remove solid particles and diluted it 1:1 with water. We stored the OS supernatant for 1 day at −80°C. Every day of the first 3 days after egg removal, we treated one leaf per plant by adding 10 μl of *S. exigua* OS to a row of puncture wounds inflicted with a pattern wheel on each leaf side. The oviposited leaf or that at the corresponding position of plants without oviposition, consecutively the second youngest and then the youngest fully expanded leaf were damage-induced three times per day in 3-h-intervals. In the order of their treatment, we harvested these leaves and the same leaf positions of unwounded plants with and without oviposition-experience 2, 4 and 6 days after the first wounding treatment. In experiment V, we adjusted the number and size of larvae feeding on plants with and without prior oviposition to equally induce plants of both treatment groups by the same larval feeding activity. After egg removal, we applied 10 sec instar larvae and recorded their performance daily. To ensure an equal number and size of feeding larvae on plants with and without prior oviposition of each replicate, we replaced larvae from a separate batch of larvae kept on *N. attenuata* leaves if required. We harvested the local leaf that had been fed upon after 1 and 4 days and also analysed the inflicted feeding damage from pictures of the leaves to control the adjustment.

Short-term induction of oviposition-experienced and -unexperienced *N. attenuata* to examine signalling components (exp. VI)

We exposed oviposition-experienced and -unexperienced plants to feeding damage by 30 neonate *S. exigua* larvae at the second fully expanded leaf position but left control plants untreated. The experimental procedure was the same as described above but after potting we kept plants outside in mosquito tents (195 × 155 × 160 cm; at FU campus during June 2014: 22–32/12–18°C day/night). After 24 h, fed leaves and the corresponding leaves of control plants were harvested, flash frozen in liquid nitrogen and stored at −80°C.

Mortality of *S. exigua* larvae on ir-Myb8 compared with WT *N. attenuata* (exp. VII)

In order to investigate whether Myb8-mediated traits can affect *S. exigua* performance, we exposed ir-Myb8 and WT plants to *S. exigua* larvae. After 2 and 4 days of feeding on their host plants, we compared mortality of *S. exigua* larvae that had been placed as neonates onto the second fully expanded source leaf in clip cages. We verified deficiency of CP in leaf material of ir-Myb8 plants 4 days after larvae started feeding (Table S8).

Effect of oviposition on mortality of *S. exigua* larvae on WT, ir-Myb8, and ir-PI plants (exp. VIII)

In comparison to WT plants, we exposed ir-Myb8 (line 818) and ir-PI plants (line 186) to *S. exigua* oviposition. We compared mortality of larvae that had been placed as neonates onto the second fully expanded source leaf of oviposition-experienced and unexperienced plants after 2 and 4 days. Deficiency of CP and of TPI production was verified in leaf samples 4 days after larvae started feeding ir-Myb8 and ir-PI plants, respectively (Table S8).

Analysis of leaf secondary metabolites by HPLC

We quantified levels of defence metabolites in the harvested leaf material of exps. I, and IV–VIII. Nicotine, CP, CA, rutin, DTGs were analysed by high performance liquid chromatography and photo diode array detection (HPLC-DAD) as previously described (Keinänen *et al.*, 2001) with minor modifications. The in liquid nitrogen flash-frozen leaf samples were powdered using mortar and pestle in liquid nitrogen and 100 mg fresh mass were extracted in 1 ml extraction buffer (40% MeOH, 0.5% acidic acid) by vortexing. After centrifuging the extracts twice (16 000 ***g*** for 10 min), we analysed 20 μl of the resulting supernatant by HPLC-DAD (Shimadzu SPD-M20A, Tokyo, Japan) for secondary metabolites. Compounds were separated on a C18-column (Inertsil ODS 3.3 μm, 4.6 × 150 mm; pre-column: Inertsil 3.3 μm, 4.6 × 15 mm) with water (0.25% H_3PO_4) and acetonitrile as eluents in gradient mode (0 min: 0% acetonitrile; 6 min: 12% acetonitrile; 10 min: 25% acetonitrile; 30 min: 80% acetonitrile; flow rate: 0.5 ml min^{-1}). We identified the compounds according to retention times and ultraviolet (UV) light spectra. We quantified the compounds according to peak areas at wavelengths 254 nm (nicotine), 320 nm (CP, CA) and 360 nm (rutin) on basis of external standard curves of nicotine, CA and rutin, whereas DTGs detected at 210 nm were expressed as summed peak areas per fresh leaf mass.

Analysis of defensive leaf proteins

Activities of TPI and polyphenol oxidase (PPO) were determined from another 100 mg of the powdered leaf material by microwell plate assays as previously described (Bode *et al.*, 2013) except for leaf TPI activity in plants of experiment I (Figure S1 and Table S2) that were analysed by a radial diffusion assay according to van Dam *et al.* (2001) described to more detail in the Supporting Information (Appendix S1). Leaf samples of all other experiments (Figure 2 and Tables S3 and S8) were extracted in 0.6 ml of 5% (w/v) PVPP in 0.1 M KPO4 (pH 7.3) and 50 μl of 10% Triton X-100 by vortexing and centrifugation (for 20 min at 12 000 ***g*** and 4°C). For analysis of TPI activity we added 20 μl of the supernatant into each well of a 96-well plate containing 20 μl 0.1 M Tris–CL (pH 8.0) and 10 μl trypsin solution (1 mg mL^{-1} 0.1 M Tris). After 5 min incubation at 37°C, we added 20 μl substrate solution (3.1 g L^{-1} *N*-benzoyl-DL-arginine-β-naphthylamide in dimethyl sulfoxide). After a second incubation of 20 min, absorbance at 550 nm was read with a microwell plate reader before we stopped the reaction by adding 100 μl 2% HCL in ethanol. The absorbance at 550 nm was read again, 20 min after adding 100 μl dye reagent (0.06% *p*-dimethylaminocinnamaldehyde in ethanol). We calculated the TPI activity relative to a standard curve of a STI dilution series that we run on each plate. To determine activity of PPO, we filled 15 μl of the same protein extract (see paragraph above) in each well of a microwell plate. Then we added 200 μl of substrate buffer (2.92 mmol caffeic acid in 0.1 M KPO4, pH = 8.0) and measured the absorbance at 435 nm every 15 sec for 5 min and calculated PPO activities as change in absorbance at 435 nm per min and g fresh mass extracted.

We performed all microwell plate reads in triplicates with a Multiskan™ GO (Thermo Scientific, Waltham, Massachusetts, USA, http://www.thermoscientific.com). All chemicals were supplied by Sigma-Aldrich (St. Louis, Missouri, USA, https://www.sigmaaldrich.com) or Carl Roth (Karlsruhe, Germany).

Analysis of phytohormones

To determine levels of phytohormones and in leaf samples of exp. VII, we used a LC-MS/MS-based phytohormone analysis method previously described (Wang *et al.*, 2007) with minor modifications. We added 1 ml ethyl acetate spiked with internal standards (30.2 ng μl^{-1} D6-JA, 10 ng μl^{-1} D6-JA-Ile, purchased from High Purity Compounds Standards GmbH, Cunnersdorf, Germany,

http://www.hp-compounds.de) to approximately 150 mg leaf material in 2-ml screw-cap tubes containing 1.25 g homogenization matrix (Zirconox®, 2.8–3.3 mm; Mühlmeier Mahltechnik, Bärnau, Germany, http://www.muehlmeier.de). The pre-ground tissue was homogenized for 40 sec in a FastPrep®-24 instrument (MP Biomedicals , Solon, USA, http://www.mpbio.com) at 5 m sec^{-1}. After centrifugation (10 min at 16 200 ***g*** at 4°C), we transferred the supernatant into 2-ml reaction tubes and re-extracted the pellet in the same way. We dried the combined supernatants in a vacuum concentrator (concentrator 5301; Eppendorf, Hamburg, Germany, https://www.eppendorf.com). The extract was re-eluted in 400 μl 70% methanol with 0.1% formic acid (v/v) and vortexed for 10 min. After centrifugation (10 min at 16 200 ***g***) the supernatant was transferred into HPLC vials, and 7 μl were subjected to analysis by UPLC-ESI-MS/MS (Synapt G2-S HDMS; Waters®, Milford, Massachusetts, USA, http://www.waters.com). Chromatographic separation was carried out on a C18 column (Acquinity UPLC BEH-C18, ø 2.1 × 50 mm, particle size 1.7 μm) with constant flow of 250 μl min^{-1} at 30°C with water and methanol (both containing 0.1% formic acid) as eluents A and B in gradient mode (eluent B: 0 min: 30%; 1 min: 30%; 4.5 min: 90%; 8 min: 90%; 9 min: 30%; 3 min equilibration time between the runs). We used negative ionization mode and tandem mass spectrometry with parent ion/daughter ion selections of 209/59 for JA, 322/130 for JA-Ile, 215/59 for D6-JA and 328/130 D6-JA-Ile. Phytohormones were quantified using MassLynx™ Software (version 4.1; Waters) according to peak area of the respective fragment ions relative to the internal standards.

Analysis of NaMyb8 transcript accumulation

We extracted total RNA of 100 mg powdered leaf material harvested from exp. VII with TRIzol® Reagent and removed contaminating DNA with TURBO DNA-free™ (both Ambion® Life Technologies: http://tools.lifetechnologies.com/content/sfs/manuals) according to the manufacturer's instructions. Total RNA, adjusted to 200 ng μl^{-1} according to spectrophotometrical concentration measures (Multiskan™ GO Microwell plate Spectrophotometer), was inspected by agarose gel electrophoresis for equal concentrations, integrity and purity. We used 1 μl of the total RNA to synthesize cDNA with the Reverse Transcriptase Core kit (Eurogentec, Seraing, Belgium, http://www.eurogentec.com) according to the manufacturer's instructions. SYBR®Green I-based real-time PCR was performed in triplicates using a qPCR kit without ROX (Eurogentec, Seraing, Belgium) on a Stratagene™ Mx3005P® instrument (Agilent Technologies, Santa Clara, California, USA, http://www.agilent.com) in 10 μl-reactions containing 1 μl cDNA and 100 nM of specific primers (NaMyb8 F: CACCCTAATTGGCGTGCACTTCCC, NaMyb8 R: GTTC GTCCCGGTAACCTTGCTGC; NaActin F: CCGGTATTGTGTTGGACT CTGGTG, NaActin R: CAGCTGAGGTGGTGAACATGTAACC). Melting curves were inspected, and application plots were analysed by LinRegPCR (http://LinRegPCR.HFRC.nl) for plate-specific amplification efficiency, which was used to calculate transcript accumulation (RQ) relative to the reference gene (β-actin). The $\log_2$ normalized RQ was used for statistical evaluation (Table S5), and the transcript accumulation was displayed as expression relative to the untreated control levels (Figure 3c).

Statistical analysis

We analysed data using Excel 13 (Microsoft, USA, http://www.microsoftstore.com) and SPSS Statistics 21 (IBM, USA, http://www.ibm.com), screened data graphically for normal distribution (Q-Q-plots), tested for variance homogeneity (Levene's or *F*-test), and generally used two-sided tests.

We used Fisher's exact test (http://www.langsrud.com/stat/Fishertest.htm) to determine mortality difference between *S. exigua* larvae feeding on oviposition-experienced and unexperienced plants of either WT, ir-Myb8 or ir-PI *N. attenuata* (exps. I–III and VIII). Similarly, we compared larval mortality on WT and ir-Myb8 lines (exp. VII). As we conducted all experiments with matched pairs, we used paired *t*-tests to compare oviposition-experienced and unexperienced plants within pairs with larvae surviving to day 4 over all three experiments. We compared larval mass within each experiment and developmental time (exp. I) between larvae on oviposition-experienced and unexperienced plants with Welch's *t*-tests to avoid loss of replicates due to larval mortality. We used paired *t*-tests to compare feeding damage on oviposition-experienced and unexperienced plants (exp. I and exp. II).

Similarly, we compared levels of defence traits (nicotine, CP, CA, rutin, DTGs, TPI and PPO activity) in oviposition-experienced and unexperienced plants with larval herbivory with either paired (exp. I) or Welch's (exp. V, exp. VII and exp. VIII) *t*-tests. We analysed defence traits of the two-factorial data set (exp. IV) using full-factorial linear mixed models with egg deposition (oviposition-experienced/-unexperienced) and damage (W+OS/none) as the main factors and the plant replicates as subjects for the repeated measures over different days (covariance type AR(1)).

Equally, two-factorial data on accumulations of *NaMyb8* transcripts, JA, and JA-Ile (exp. VI) were analysed using full-factorial generalised linear models (with maximum likelihood estimates). The $\log_2$ normalized RQ was used for statistical evaluation of the transcript accumulation.

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AUTHOR CONTRIBUTIONS

AS, MB and MH designed the study. MB and AS performed the experiments, chemical and data analysis. MB and AS wrote the first draft of the manuscript and all authors revised it, and agreed upon the final version.

SUPPORTING INFORMATION

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

Figure S1. Concentrations of caffeoylputrescine and TPI activity correlate with feeding damage (exp. I).

Figure S2. *Spodoptera exigua* larval sub-stages within each instar and egg deposition on the plant.

Table S1. Performance parameters (exps. I–III).

Table S2. Defence parameters in *Nicotiana attenuata* with larval feeding (exp. I).

Table S3. Defence parameters of equally damaged *Nicotiana attenuata* (exps. IV and V).

Table S4. GLM of defence parameters of equally damaged *Nicotiana attenuata* (exp. IV).

Table S5. GLM of *NaMyb8* transcript and phytohormone accumulation in *Nicotiana attenuata* (exp. VI).

Table S6. Composition of *Spodoptera exigua* artificial diet.

Table S7. Summary of parameters in conducted experiments.

Table S8. Deficiency of defence parameters in ir-Myb8 and ir-PI compared to WT plants.

Appendix S1. Analysis of TPI activity by radial diffusion assay (exp. I).

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