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ORIGINAL PAPER

Salicylic acid-mediated reductions in yield in *Nicotiana attenuata* challenged by aphid herbivory

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Abstract Aphid herbivory decreases primary production in natural ecosystems and reduces crop yields. The mechanism for how aphids reduce yield is poorly understood as some studies suggest aphid feeding directly impedes photosynthesis, whereas other studies suggest a change in allocation of resources from growth to defense compounds reduces yield. To determine the mechanisms underlying reduced plant growth by aphids, *Nicotiana attenuata* plants, native tobacco, were infested with *Myzus persicae* ssp. *nicotianae*, tobacco-adapted green peach aphids, at low and high densities, and plant performance including fitness was assessed. To test the direct defense capacity of salicylic acid (SA) on aphid performance, we fed aphids an artificial diet with varying levels of SA and measured their survivorship and fecundity. There was no detectable effect of aphid herbivory on net photosynthesis, yet herbivory reduced plant growth, final biomass (43 % at high aphid density), and seed set (18 % at high aphid density) at both low and high aphid infestation levels. High-density aphid attack during the rosette and flowering stage caused an increase in SA levels, but caused only a transient decrease in jasmonic acid concentration at low aphid density. SA concentrations similar to those found in infested flowering plants decreased aphid fecundity, suggesting that SA was an effective chemical defense response against aphids. These results suggest that as aphid densities increased the proximal cause of reduced growth and yield was not reduced photosynthesis, but instead resources may have been mobilized for defense via the SA pathway, decreasing the availability of resources for building plant biomass.

Keywords Induced defenses · Photosynthesis · Plant–insect interactions · Reactive oxygen species · Trade-off

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Introduction

Aphid herbivory decreases primary production in natural ecosystems (Zvereva et al. 2010) and crop yield in agriculture systems (Kieckhefer 1980). Currently, the mechanism for how aphids reduce yield is poorly understood as some studies suggest aphid feeding directly impedes photosynthesis (e.g., Macedo et al. 2003), whereas other studies suggest a re-allocation of resources from growth to defense compounds reduces yield (e.g., Bezemer and Jones 1998). By understanding the effects of aphid feeding on plant yield, we will improve estimations of net primary productivity in natural ecosystems and help mitigate the effects of herbivore attack in agricultural systems.

Aphids harvest photosynthate and cause cellular damage upon insertion of stylets that puncture cell walls (Tjallingii and Esch 1993) and inject salivary toxins (Kuśnierczyk et al. 2008). Reduced photosynthesis has been observed within a variety of plant species under attack by aphids

(Macedo et al. 2003; Gerloff and Ortman 1971; Wood and Tedders 1986). Aphids can lower chlorophyll content, which reduces the light-capturing ability of the plant and subsequently photosynthetic rates (Cottrell et al. 2009). Aphid feeding also inhibits the electron transport rate in the photosystem II reaction center for photosynthesis (Burd and Elliott 1996; Haile 1999), leading to a reduction in gas exchange. Although there are many other examples of aphids decreasing plant yield, the response is not universal (Welter 1989). Studies have documented increases or no change in photosynthesis under aphid attack (Rabbinge et al. 1981; Ryan et al. 1987; Welter 1989). A lack of photosynthetic response suggests that there may be other factors reducing biomass in some species.

Because insect feeding can induce chemical defense responses in plants, aphid-elicited declines in yield may result from the re-allocation of resources from growth to defense (i.e., the growth–differentiation balance hypothesis; Herms and Mattson 1992). Aphid feeding induces both jasmonic acid (JA)- and salicylic acid (SA)-related defenses depending on the plant–aphid interaction (Heidel and Baldwin 2004; Mohase and van der Westhuizen 2002). However, defining mechanisms for defense elicitation against aphids have been difficult because there is evidence of cross talk between JA and SA, which leads to reciprocal down-regulation (Zarate et al. 2007). Transcriptomic and physiological evidence have revealed a variety of responses, including increases and no changes in JA and SA levels (Mohase and van der Westhuizen 2002; Heidel and Baldwin 2004; Voelckel et al. 2004; De Vos et al. 2005), making the fitness cost of inducible defense for the plant difficult to determine.

The objective of this study was to determine whether the reduction in yield in wild tobacco (*Nicotiana attenuata* Torr ex. S. Watson) under aphid attack was related to reduced photosynthesis. We used *N. attenuata* because it is a model plant system for understanding plant–insect interactions in response to aphid feeding. Because prior evidence suggests aphid feeding reduces transcription of genes regulating photosynthesis and ultimately yield, we hypothesized that this herbivore damage reduces carbon uptake. However, if photosynthesis is not reduced under aphid attack, then the reduction in yield may be caused by the re-allocation of resources from growth to defense.

Materials and methods

Plant and insect material

Nicotiana attenuata plants were germinated in agar plates in an environmental growth chamber (28 °C; 14:10, light:dark) as in Krügel et al. (2002). Plants were transplanted after two weeks to soilless medium (LC1 mix, SunGro Horticulture, Vancouver, BC) in 4-l pots in a greenhouse with supplemental lighting for the duration of the experiment.

The tobacco-adapted form of the green peach aphid, *Myzus persicae* ssp. *nicotianae* (Sulzer) (Eastop and Blackman 2005), was used in all experiments and maintained on *N. attenuata* in an environmental growth chamber (28 ± 2 °C; 14:10, light:dark).

Plant performance experiments

To characterize plant fitness and herbivore-related traits under aphid attack, plant gas exchange, defense-related hormones, and vegetative and reproductive traits were measured at two growth stages (rosette and flowering) and at two levels of aphid density (high and low). Three-week-old plants were blocked by size and then infested by transferring newly hatched (<1 day old) nymphs to each plant at one of three levels of aphid density: high-density plants received 10 aphids, low-density received five aphids, and control plants received no aphids. Aphid population growth was monitored throughout the experiment. Plants were randomly assigned new locations every other day to minimize the effects of environmental variation in the greenhouse.

Measurements of photosynthesis, conductance, and transpiration were made with an automated infrared gas analysis system (LI-6400 Photosynthesis System, LI-COR Biosciences, Lincoln, NE). Aphids were removed prior to enclosing the leaf within the gas exchange cuvette. Measurements were made on the +1 source leaf as determined by Giri et al. (2006) at saturating light intensity (PAR = 1,500 μmol $m^{-2}s^{-1}$), constant air temperature (30 °C), and constant relative humidity (~70 %).

Following senescence, branch number was counted on each plant before harvesting, and 50 seeds from each plant were collected, oven-dried to constant mass at 70 °C, and weighed. Aboveground plant tissues were harvested, dried, and weighed for final biomass.

The status of plant defense signaling was determined by quantifying jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) titers. The same leaf used for gas exchange measurements was harvested, immediately frozen in liquid nitrogen, and stored at −80 °C. Leaf tissues were ground with a mortar and pestle, and approximately 150 mg of each sample was used for JA, SA, and ABA analysis according to Wang et al. (2007). Following grinding, each sample received 1 ml ethyl acetate spiked with isotopically labeled internal standards for JA (200 ng ml^{-1}, ^{5}JA), SA (50 ng ml^{-1}, ^{4}SA), and ABA (50 ng ml^{-1}, ^{6}ABA). Samples were vortexed for 5 min and centrifuged at 13,000 rpm for 20 min at 4 °C, and then the supernatants were transferred to

new tubes. Each sample was re-extracted with 0.5 ml of unlabeled ethyl acetate, shaken, and centrifuged under the same conditions detailed above. The supernatants were combined and evaporated until dryness at 30 °C under a vacuum concentrator. The dried residue was re-suspended in 500 µl 70 % (v/v) methanol, vortexed for at least 5 min, and centrifuged at 13,000 rpm for 10 min, and 400 µl was transferred to vials for analysis by HPLC–MS.

A liquid chromatography–mass spectrometry system (2010 EV, Shimadzu, Columbia, MD, USA) was used to measure hormone content. The mobile phase made up of solvent A (0.05 % formic acid) and solvent B (0.05 % formic acid in methanol) was used in gradient mode for ion separation. The system was operated in negative electrospray ionization mode where 10 uL of sample was injected at a flow rate of 0.2 ml min^{-1} onto a Luna C18 column (250 × 2 mm, 5 um ID, Phenomenex, Torrance, CA, USA) following Wang et al. (2007), with selected reaction monitoring of compound-specific parent ions JA = 209; ^{5}JA = 214; SA = 137; ^{4}SA = 141; ABA = 263; ^{6}ABA = 269 m/z.

Aphid performance experiments

To test how plant defenses influence aphid performance, aphid fecundity and survivorship were assessed on defense-modified genotypes of *N. attenuata*. Performance on wild-type (WT) plants was compared to plants lacking JA signaling and synthesis (antisense lipoxygenase 3; as-lox3, Halitschke and Baldwin 2003), and plants lacking SA signal transduction (inverted repeat non-expresser of pathogen response genes 1; ir-npr1, Rayapuram and Baldwin 2007). Plants with suppressed npr1 function lack the ability to transduce SA signals, but have greater constitutive and inducible SA levels relative to wild-type plants (Rayapuram and Baldwin 2007). All genotypes were germinated and potted under the conditions previously described although plants were potted in 2-l containers. Initially, two to three reproductive adult aphids were placed on 10 plants of each genotype and allowed to produce nymphs for 24 h. Then, all but five <1-day-old nymphs were removed, and the remaining five nymphs were observed daily for survivorship and allowed to reproduce to assess fecundity. Newly produced nymphs were removed daily to prevent density-dependent effects on aphid performance. This experiment was repeated although the second trial was set up with 15 plants of each genotype (combined trials $n = 25$).

Because SA accumulation occurred concurrent with reduced aphid performance in wild-type plants, we tested the capacity for salicylic acid to act as a direct defense against aphid attack. A gradient of concentrations of SA were added to an artificial diet (see Kim and Jander 2007) and assessed as in Ramsey and Jander (2008). Initially, 100 µg of SA was dissolved in 1 ml 100 % ethanol (EtOH) and then serially diluted in 5 ml aliquots of artificial aphid diet to attain five treatment concentrations: 0, 50, 100, 250, and 500 ng ml^{-1} of salicylic acid). Artificial diet (100 µl) was pipetted between two layers of Parafilm stretched over the tops of 30-ml plastic cups with the bottoms replaced with fine mesh to allow airflow. Twenty-five replicates for each treatment were infested with four adult aphids of similar size and color and placed within an environmental growth chamber as described above. The number of adult aphids and nymphs per cup were recorded every 24 h for five consecutive days.

Because salicylic acid modulates the hypersensitive response and, subsequently, redox potentials of locally damages tissues, we also examined the capacity for aphids to locally alter the oxidative environment of leaf tissue. After 6-h feeding on each of the three *N. attenuata* genotypes (WT, as-*lox3*, ir-*npr1*), leaves were harvested, cleared by boiling in a destaining solution containing glycerol, lactic acid, acidic phenol ethanol, and water (1:1:1:2:1) to remove chlorophyll, and stained by infiltrating remaining leaf tissue with 5 mM 3,3′-diaminobenzidine (DAB) at pH 3.8 following (Thordal-Christensen et al. 1997).

Statistical tests

Photosynthesis measurements, plant vegetative and reproductive traits, and hormone levels were analyzed with multifactorial repeated-measures ANOVA where density (high or low) and plant stage were main effects and plant was the repeated subject (Proc Mixed 9.1, SAS, Cary, NC). Aphid survivorship on diets with different levels of SA and on defense-modified genotypes of *N. attenuata* was analyzed by performing a univariate survivorship analysis with log-rank significance test (JMP 4.0, SAS Institute, Cary, NC).

Results

The average rate of net photosynthesis for *N. attenuata* declined as plants transitioned from the rosette stage to the reproductive stage ($F = 25.75$; $df = 1,8$; $P = 0.001$), but aphid-dependent reductions in photosynthesis were not statistically resolved at either density. ($F = 1.78$; $df = 2.24$; $P = 0.19$) (Table 1). However, a low-density aphid infestation caused a small (~7 %) but statistically significant decrease in stomatal conductance of *N. attenuata* rosettes ($t = 3.16$; $df = 16$; $P = 0.006$) but did not alter conductance during flowering ($t = -0.27$; $df = 16$; $P = 0.8$). No effect of low aphid infestation on intercellular CO_2 concentration (C_i) could be resolved for plants at either age (rosette: $t = 1.52$, $df = 16$, $P = 0.14$; flowering: $F = -0.13$, $df = 16$, $P = 0.9$). High aphid density also reduced

Table 1 Mean (±SE) leaf gas exchange (net photosynthesis, stomatal conductance, and intercellular CO_2 concentration, C_i), maximal (F_v'/F_m') and operating (ΦPSII) efficiency of photosystem II, and plant fitness traits (aboveground biomass, seed biomass per 50 seeds and branch number) of *N. attenuata* under aphid attack

	No aphids	Low aphids	High aphids
Rosette			
Aphid number	0a	56 ± 13b	103 ± 14c
Photosynthesis (μmol CO_2 m^{-2} s^{-1})	27.3 ± 0.9	25.2 ± 1.1	25.2 ± 1.0
Conductance (mol H_2O m^{-2} s^{-1})	0.56 ± 0.03a	0.44 ± 0.03b	0.39 ± 0.02b
C_i (μmol CO_2 mol^{-1})	288 ± 4a	276 ± 5a	260 ± 5b
F_v'/F_m'	0.669 ± 0.005	0.669 ± 0.005	0.665 ± 0.005
ΦPSII	0.465 ± 0.008	0.469 ± 0.01	0.466 ± 0.007
Flowering			
Aphid number	0a	444 ± 96b*	1072 ± 160c*
Photosynthesis (μmol CO_2 m^{-2} s^{-1})	21.5 ± 1.3	22.5 ± 1.1	19.5 ± 1.8
Conductance (mol H_2O m^{-2} s^{-1})	0.31 ± 0.03	0.32 ± 0.02	0.35 ± 0.04
C_i	263 ± 7a	264 ± 5a	289 ± 4b
F_v'/F_m'	0.619 ± 0.011	0.614 ± 0.011	0.594 ± 0.014
ΦPSII	0.434 ± 0.002	0.435 ± 0.018	0.389 ± 0.023
Plant biomass (g)	15.4 ± 1.5a	12.3 ± 1.2b	8.8 ± 0.9c
Seed biomass (mg)	6.5 ± 0.2a	5.9 ± 0.2b	5.3 ± 0.2c
Branch number	23 ± 1a	21 ± 0b	19 ± 1b

Aphid number estimated from aphid/leaf count indicated by *. Different letters indicate significant differences between treatments at $P < 0.05$

conductance during the rosette stage ($t = 4.57$; $df = 16$; $P = 0.0003$), but again this effect did not persist in flowering ($t = -1.11$; $df = 16$; $P = 0.28$). This reduction in conductance without a corresponding decrease in photosynthesis likely led to the observed reduction in C_i ($t = 3.6$; $df = 16$; $P = 0.002$); however, C_i increased at flowering ($t = -3.22$; $df = 16$; $P = 0.005$) without a change in conductance.

Plant biomass decreased 20 % under low infestation and 43 % under high infestation ($t = 2.1$; $df = 22$; $P = 0.048$, $t = 4.5$; $df = 22$; $P = 0.0002$, respectively). Seed biomass also declined by 9 and 18 % under low and high aphid infestations, respectively (low: $t = 2.2$, $df = 22$, $P = 0.037$; high: $t = 4.4$, $df = 22$, $P = 0.0002$). Branch number also declined with aphid density ($F = 21.51$, $df = 2.22$, $P < 0.0001$) (Table 1).

The concentration of JA decreased under low-density aphid infestation in the rosette ($t = 2.15$; $df = 19$; $P = 0.044$) but not in the flowering stage ($t = -1.26$; $df = 19$; $P = 0.22$), whereas high-density infestation tended to reduce JA during the rosette stage ($t = 1.74$; $df = 19$; $P = 0.09$) and again did not alter JA during flowering ($t = -0.42$; $df = 19$; $P = 0.7$). In contrast, high-density aphid infestation caused substantial increases in SA concentration at both developmental stages (rosette: $t = -4.03$, $df = 17$, $P = 0.0009$; flowering: $t = -8.01$, $df = 17$, $P = <0.0001$; Fig. 1). Aphid infestation had no detectable effect on ABA concentration ($F = 0.16$; $df = 2.24$; $P = 0.8$) (Fig. 1).

Plant genotype altered aphid survivorship ($\chi^2 = 10.2$; $df = 2$; $P = 0.006$) whereby total aphid survivorship decreased on plants lacking SA signaling (ir-*npr1*; 4.1 ± 0.1 days) relative to plants capable of transducing SA signals (WT:4.5 ± 0.1 days; $\chi^2 = 8.9$; $P = 0.003$, as-*lox3*: 4.5 ± 0.1 days; $\chi^2 = 5.9$; $P = 0.015$). Fecundity also decreased on ir-*npr1* plants (5.1 ± 0.3 nymphs/aphid/day) compared with WT (6.2 ± 0.2 nymphs/aphid/day, $t = -3.25$; $df = 42$; $P = 0.002$) and as-*lox3* (5.9 ± 0.2 nymphs/aphid/day, $t = 2.49$; $df = 42$; $P = 0.017$). Survivorship for aphids feeding on diets with SA concentrations of 250 and 500 ng/ml decreased relative to diets lacking SA (250 ng: $\chi^2 = 24.8$; $P < 0.0001$, 500 ng: $\chi^2 = 11.4$; $P = 0.007$) (Table 2). Aphid feeding increased H_2O_2, leading to noticeable changes in localized oxidative levels on DAB-stained leaves, whereas low background staining in defense-modified plants suggest that having an active defense-signaling network increased constitutive H_2O_2 levels (Fig. 2).

Discussion

Aphid herbivory reduced biomass accumulation and seed production in *N. attenuata* without a measurable reduction in photosynthesis and concomitant with an increase in salicylic acid defenses. These data suggest a trade-off between growth and defense may be modulated by the severity of aphid attack and the direct production of SA, that is, higher infestation levels reduced fitness more than lower infestations and induced greater SA production. Aphids feeding on artificial diets with high SA concentrations experienced decreased survivorship, suggesting that SA itself may be an effective defense against aphids.

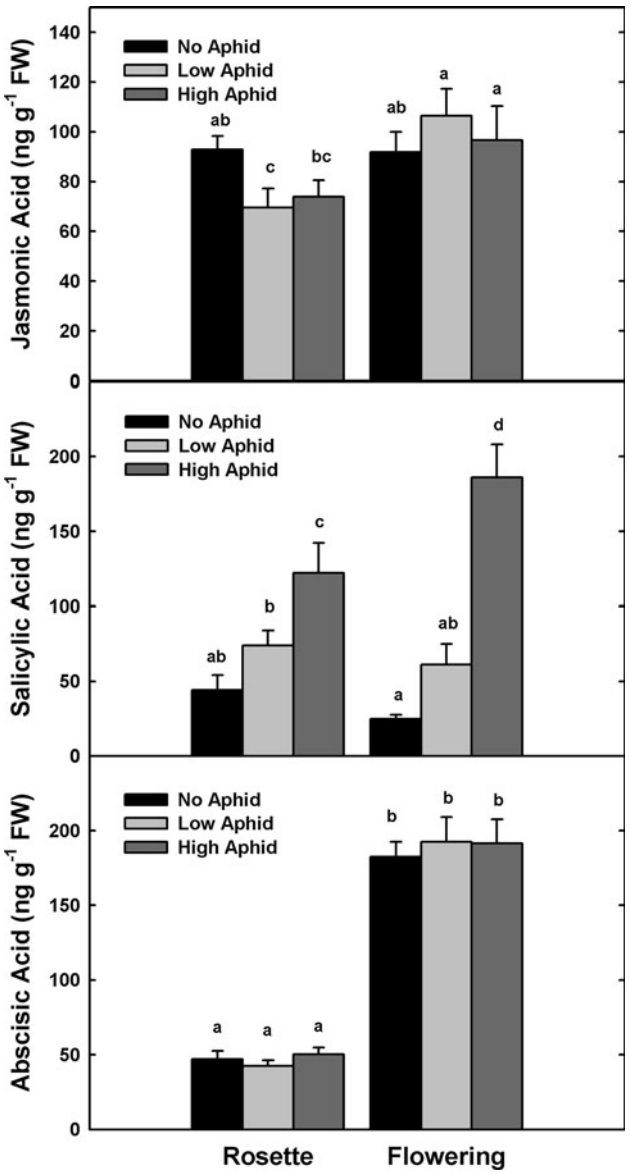

Fig. 1 Mean (±SE) hormone levels in *N. attenuata* elicited by *M. persicae*. *Different letters* indicate significant differences between treatments. The +1 source leaf was harvested on each plant

Table 2 Mean (±SD) survivorship of aphids fed an artificial diet with different levels of SA

Treatment (ng ml^{-1} SA)	Mean survival (days)
Control	4.05 ± 0.11a
50	3.84 ± 0.12ab
100	3.86 ± 0.12ab
250	3.25 ± 0.11c
500	3.44 ± 0.15bc

Different letters indicate significant differences between treatments

No reductions in photosynthetic gas exchange under aphid infestation were resolved during the rosette or flowering stages of *N. attenuata*, similar to other plant and aphid systems (e.g., Ryan et al. 1987; Welter 1989). Despite the lack of photosynthetic response, low and high aphid infestations decreased conductance and C_i during the rosette stage. The reduction in conductance and C_i may have been caused by the aphid elicitation of reactive oxygen species (ROS) that occur near feeding sites (Fig. 2) and have been linked to photosynthetic decline in other aphid–plant interactions (Kerchev et al. 2012). Subtle changes in conductance or biochemical efficiency (C_i) can lead to slight but unresolvable reductions in assimilation and result in long-term reductions in growth and fitness. Because our data do not indicate a strong effect on photosynthesis, other herbivore-induced factors may have contributed to the reduction in CO_2 for carbon fixation and may ultimately have reduced relative growth rate because of a lack of resources. Our DAB staining indicated that localized H_2O_2 occurred near feeding sites, especially in defense-modified mutants (Fig. 2). The lack of response and the higher constitutive H_2O_2 after 6-h feeding in WT plants suggest that modifying either the SA- or JA-defense-signaling network interacts with ROS signaling, and it adds support to the emerging model that ROS signaling forms the basis of plant response to insect herbivory (Kerchev et al. 2012).

High-density aphid attack increased SA levels in both the rosette and flowering stages and ultimately reduced yield in *N. attenuata*. For many plant–aphid interactions, attack elevates SA and initiates the SA signaling cascade (Thaler et al. 2010; e.g., Blande et al. 2010). This cascade increases the systemic acquired resistance (SAR) and pathogenesis-related (PR) gene expression and facilitates the hypersensitive response (HR) in a positive feedback loop where SA increases HR that, in turn, increases SA (Vlot et al. 2009). Yield reductions occur with this increase in SA (Cipollini 2002) and SA-dependent responses (e.g., SAR; Heil et al. 2000) and suggest the reductions in yield we observed were driven by the increase in SA.

The induction of SA defenses increases the titer of SA (and methyl SA) within the phloem as endogenous signaling molecules that initiate SAR and downstream PR defense (Vlot et al. 2009). Aphids feed on phloem and thereby come into direct contact with SA. The literature suggests that *npr1* plants are deficient in SA signal transduction, but still accumulate SA greater than wild-type plants (e.g., Freeman et al. 2005; Ng et al. 2011). Decreased survivorship and fecundity of aphids feeding on *npr1* plants has been observed (Mewis et al. 2005), but no mechanism has been proposed. We observed a direct effect of SA on aphid fecundity during the artificial diet tests, suggesting these mobile phenolics that bear a reactive hydroxyl group also have a direct defense in addition to a role in perceiving attack. During our tests, aphids experienced decreased survivorship on diets with 250 ng/ml SA artificial diets, which is within the range of measured SA

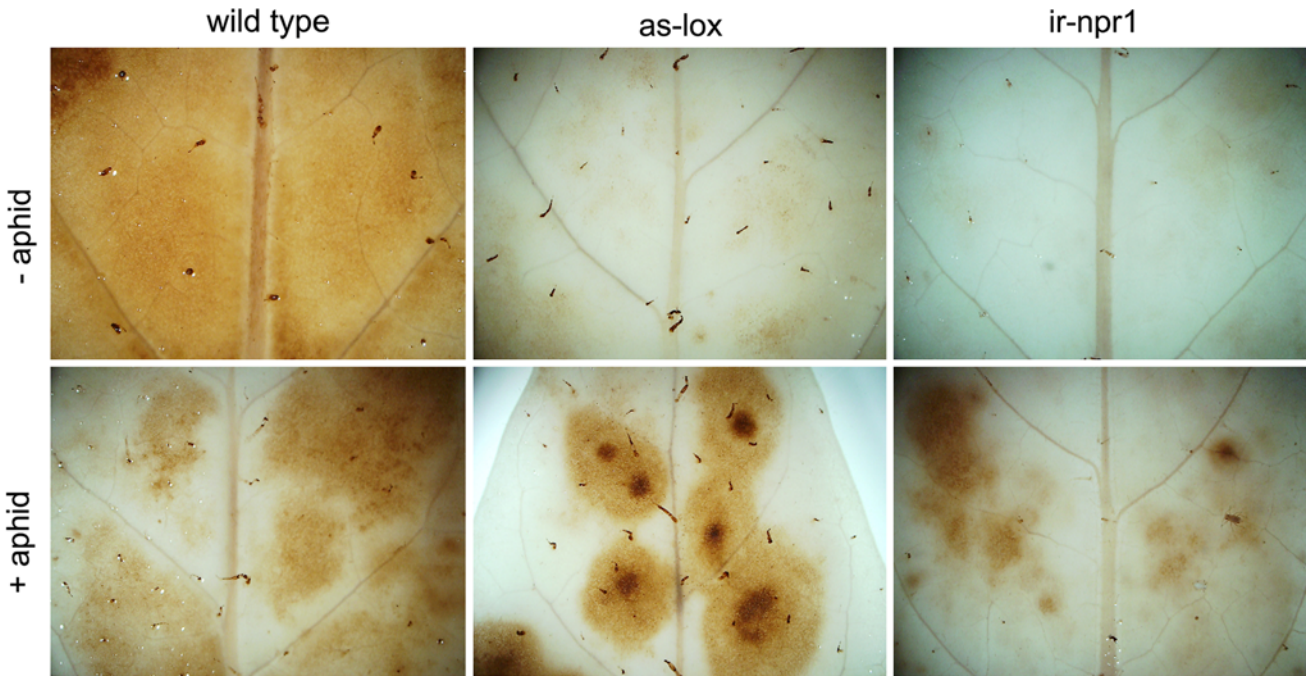

Fig. 2 H_2O_2 accumulation in *N. attenuata* genotypes of various defense capacities was visualized using diaminobenzidine (DAB) staining to characterize constitutive (*top row*) and aphid-elicited (*bottom row*) reactive oxygen formation. Wild-type plants were compared to plants deficient in signal transduction of jasmonic acid (antisense lipoxygenase; as-*lox3*) and salicylic acid (inverted repeat non-expressor of pathogenesis-related genes l; ir-*npr1*). The intensity of *brown coloration* corresponds to the concentration of H_2O_2. *Images* represent 6 h after aphid feeding began

in *N. attenuata* plants (Heidel and Baldwin 2004). Although the mechanism for reduced aphid performance in the presence of SA remains uncharacterized, a test of SA toxicity on the chewing herbivore *Helicoverpa armigera* (Hubner) indicated that larval growth decreased with increasing SA levels in artificial diet and likely resulted from reduced respiratory activity (Akbar et al. 2012).

Aphid feeding decreased JA concentration during the rosette stage, which may be an effect of cross talk between JA and SA and reciprocal down-regulation of JA in response to increased SA production (Zarate et al. 2007). During the flowering stage, JA did not differ between treatments, which may be an effect of plant life stage. Whole plant concentrations of JA increase during senescence (He et al. 2002), so the natural increase in JA may have been more significant than the decrease in JA caused by reciprocal down-regulation.

Another possible contributing factor to reduced yield is the extraction of carbohydrates from the plants by aphids. Aphids extract sucrose from phloem tissue and excrete honeydew, a sugar-rich waste product, onto the leaves of the plant (Baron and Guthrie 1960). Sucrose is the major form of carbohydrate transported through plants, so its reduction may lead to a lack of carbohydrates in sink tissues and a subsequent decrease in growth capability. A single aphid can remove 0.5–3 $\mu l\ h^{-1}$ of phloem fluid (Kennedy and Mittler 1953; Mittler 1958; Weatherley et al. 1959), and an infestation can generate a significant loss of carbon from the plant, contributing to a reduction in yield (Truernit 2001). Burd and Burton (1992) also reported that the loss of turgor in aphid-infested wheat caused a reversible reduction in growth and may have contributed to the small decrease in stomatal conductance observed in this study (Table 1). Measuring honeydew excretion and content will help illustrate the relative importance that photosynthate extraction has on reducing plant yield.

Aphid feeding decreased plant yield and seed production, but did not reduce photosynthesis. Instead, high-density aphid herbivory caused the mobilization of resources for defense via the salicylic acid pathway, which may have decreased the availability of resources for growth and lead to the reduction in yield. SA is a precursor to the production of important lines of defense, including HR, but our results suggest the SA itself may be a form of defense against aphids. Further research is needed on the direct effect of endogenous or induced SA levels to test the efficacy of SA as a direct defense and its role in the reduction in plant yield associated with aphid herbivory.

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