

RESEARCH PAPER

# Caterpillar saliva interferes with induced *Arabidopsis thaliana* defence responses via the systemic acquired resistance pathway

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## Abstract

*Arabidopsis thaliana* (L.) Heynh. genotypes limited in their ability to mount either octadecanoid-dependent induced resistance (IR<sup>-</sup>) or systemic acquired resistance (SAR<sup>-</sup>) were used to characterize the roles of these pathways in plant–herbivore interactions. Molecular and biochemical markers of IR were analysed in plants subject to herbivory by caterpillars of the beet armyworm, *Spodoptera exigua* Hübner, which had either intact or impaired salivary secretions since salivary enzymes, such as glucose oxidase, have been implicated in the ability of caterpillars to circumvent induced plant defences. Transcript expression of genes encoding laccase-like multicopper oxidase [*AtLMCO4* (polyphenol oxidase)] and defensin (*AtPDF1.2*) showed salivary-specific patterns which were disrupted in the SAR<sup>-</sup> mutant plants. The activity of octadecanoid-associated anti-nutritive proteins, such as LMCO and trypsin inhibitor, showed similar patterns. Gene and protein changes parallel plant hormone levels where elevated jasmonic acid was observed in wild-type plants fed upon by caterpillars with impaired salivary secretions compared with plants subject to herbivory by normal caterpillars. This salivary-specific difference in jasmonic acid levels was alleviated in SAR<sup>-</sup> mutants. These results support the model that caterpillar saliva interferes with jasmonate-dependent plant defences by activating the SAR pathway.

**Key words:** Caterpillar, cross-talk, gene expression, induced resistance, plant–insect interactions, salivary elicitors, *Spodoptera exigua*.

## Introduction

Plants defend themselves against the diverse onslaught of pests and pathogens by both general and specific induced defences (Walling, 2000; Kessler and Baldwin, 2002; Bruce and Pickett, 2007; Kessler and Halitschke, 2007). During feeding, wounding of leaf tissue by caterpillars induces the biosynthesis of oxylipin phytohormones, such as jasmonic acid (JA), which results in general octadecanoid-dependent induced resistance (IR) (Farmer *et al.*, 2003; Delessert *et al.*, 2004; Halitschke and Baldwin, 2005). Plants also target their defence responses specifically against the herbivorous insect (Kessler and Halitschke, 2007). In *Nicotiana attenuata* Torr., genes differentially expressed in response to herbivory by three different caterpillar species showed similar expression profiles (Voelckel and Baldwin, 2004); however, the plant's overall transcriptional response to the two generalist noctuid caterpillar species, *Heliothis virescens* Fabricius (tobacco budworm) and *Spodoptera exigua* Hübner (beet armyworm), was more consistent than the response to the sphingid specialist, *Manduca sexta* L. (tobacco hornworm). Similarly, even though the *Arabidopsis* relative, *Boechera divaricarpa* (Nels.) Löve, showed overlapping transcriptional patterns in response to herbivory by caterpillars of the crucifer specialist *Plutella xylostella* L. (diamondback moth) or the generalist *Trichoplusia ni* Hübner (cabbage looper), numerous genes were regulated in distinct patterns that indicated interactions between the octadecanoid/ethylene or octadecanoid/salicylic acid (SA) pathways, respectively, resulting in species-specific responses (Vogel *et al.*, 2007). Presumably, the ability of the plant to modify defence responses to target the herbivore lies in

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the recognition of caterpillar-related elicitors or other signals which are detected by the plant.

After grinding leaf tissue with their mandibles, caterpillars use oral secretions to transport food into their mouthparts. These oral secretions, which are comprised of regurgitant and labial and mandibular saliva, contain a milieu of potential elicitors that are recognized by the plant and shape its defence response. Treatment of wounded corn seedlings or tobacco with volicitin, which is found in the insect regurgitant, induces the synthesis and release of volatiles that lure predators or parasitoids of the herbivorous caterpillar to the plant (Alborn *et al.*, 1997; Turlings *et al.*, 2000; Kessler and Baldwin, 2001, 2002). Labial saliva of some caterpillar species contains oxidoreductases, such as glucose oxidase (GOX), which may enable the insect to circumvent induced plant defences (Musser *et al.*, 2002, 2005).

The basis of GOX action is believed to be through the activation of phytohormone pathways which interact and modify the plant's typical responses (Rojo *et al.*, 2003; Zhu-Salzman *et al.*, 2005; Fujita *et al.*, 2006; Torres *et al.*, 2006; Bodenhausen and Reymond, 2007). In response to wounding or caterpillar herbivory, the rapid biosynthesis of octadecanoids leads to jasmonate-dependent IR (Devoto and Turner, 2003; Howe, 2004; Halitschke and Baldwin, 2005; Wasternack *et al.*, 2006). In systemic tissues, JA and its isoleucine-conjugated form, isoleucine-JA, activates the SCF<sup>Col</sup> ubiquitin-proteasome-mediated degradation of the jasmonate ZIM domain (JAZ) protein, which negatively regulates the master transcription factor MYC2, leading to the induction of JA-dependent plant defences (Devoto *et al.*, 2002; Xu *et al.*, 2002; Chini *et al.*, 2007; Dombrecht *et al.*, 2007; Thines *et al.*, 2007). Another potential target of the SCF<sup>Col</sup> ubiquitin-proteasome is histone deacetylase which adds a layer of epigenetic regulation to JA-responsive gene expression (Devoto *et al.*, 2002). However, production of hydrogen peroxide by caterpillar salivary oxidoreductases is believed to interfere with these responses by acting as an upstream signal to the production of hormones, such as ethylene, or activation of signalling proteins, such as NPR1 (non-expresser of PR genes 1), which attenuate octadecanoid responses (Winz and Baldwin, 2001; Spoel *et al.*, 2003; Pieterse and van Loon, 2004; Fobert and Després, 2005; Foyer and Noctor, 2005; Lou and Baldwin, 2006).

NPR1 is part of the systemic acquired resistance (SAR) pathway which is activated in plant pathogen defence (Dong, 2004; Pieterse and van Loon, 2004). Upstream molecules in this pathway, SA and reactive oxygen species (ROS), positively affect each other's production in a feedforward mechanism that leads to the oxidative burst and, ultimately, the hypersensitive response (Chen *et al.*, 1993; León *et al.*, 1995; Durrant and Dong, 2004). The disulphide bridges of the cytosolic oligomeric NPR1 protein are reduced as cellular redox changes alter the

glutathione redox status and/or biosynthesis (Després *et al.*, 2003; Mou *et al.*, 2003; Ball *et al.*, 2004; Senda and Ogawa, 2004). Monomeric NPR1 enters the nucleus and binds to TGA transcription factors, leading to SA-dependent gene expression (Kinkema *et al.*, 2000; Fan and Dong, 2002; Després *et al.*, 2003; Mou *et al.*, 2003; Blanco *et al.*, 2005; Wang *et al.*, 2006). However, NPR1 also interferes with the IR pathway, either by inhibiting octadecanoid biosynthesis or by preventing degradation of JAZ or histone deacetylase proteins, or by activating transcription of *AtWRKY* transcription factors which repress JA-responsive genes (Devoto *et al.*, 2002; Xu *et al.*, 2002; Spoel *et al.*, 2003; Li *et al.*, 2004; Beckers and Spoel, 2006; Miao and Zentgraf, 2007). Therefore, cross-talk between SAR and IR pathways may be mediated through the production of hydrogen peroxide by caterpillar salivary enzymes, leading to activation of NPR1 and subsequent interference with IR-dependent induced plant defences.

This study used *Arabidopsis thaliana* (L.) Heynh. plants with mutations in the IR or SAR pathways to elucidate the involvement of caterpillar saliva in mediating the cross-talk between these signalling pathways. Chloroplast lipooxygenase2 (LOX2; EC.1.13.11.12) catalyses the second step in octadecanoid biosynthesis. Therefore, *Atlox2* is impaired in its ability to mount IR in response to wounding; however, plants still show constitutive, basal octadecanoid levels (Bell *et al.*, 1995). The SAR<sup>-</sup> double mutant is unable to produce functional proteins of non-race specific disease resistance1 (NDR1-1) and NPR1-2 (Zhang and Shapiro, 2002). The plasma membrane-associated NDR1-1 protein interacts with the negative regulator RPM1-interacting protein (RIN4) to enable downstream SA/ROS-dependent defence responses to proceed (Shapiro and Zhang, 2001; Day *et al.*, 2006). NPR1-2 functions downstream of NDR1-1, and the activated form of this protein interferes with jasmonate-dependent defence responses (Glazebrook *et al.*, 1996; Beckers and Spoel, 2006; Wang *et al.*, 2006). The *Atndr1-1*, *npr1-2* SAR<sup>-</sup> double mutant is unable to accumulate hydrogen peroxide or SA when faced with pathogen challenge (Zhang *et al.*, 2004). Therefore, hydrogen peroxide produced by caterpillar feeding will not enhance SA biosynthesis, and any resultant changes in cellular redox potential should not lead to NPR1-mediated inhibition of the IR pathway in these SAR<sup>-</sup> plants.

Typical markers of the IR pathway in *Arabidopsis*, such as *AtLOX2*, defensin (*AtPDF1.2*), trypsin inhibitor (*AtTI*), and laccase-like multicopper oxidase [LMCO (polyphenol oxidase)] (Bell and Mullet, 1993; Creelman and Mullet, 1997; Koiwa *et al.*, 1997; Penninckx *et al.*, 1998; Cipollini *et al.*, 2004), were monitored in these mutant lines to determine if caterpillar saliva is involved in the cross-talk between the IR and SAR pathways. Wound- and JA-inducible LOX2 is part of a feedforward

cascade that rapidly leads to elevated JA biosynthesis during plant defence signalling (Bell and Mullet, 1993; Sasaki *et al.*, 2001; Wasternack, 2007). The antimicrobial peptide defensin (AtPDF1.2) is another marker of JA-dependent gene expression (Penninckx *et al.*, 1998). TI and LMCO are anti-nutritive defences mounted by the plant (Duffey and Stout, 1996; Felton, 2005). As caterpillars feed on the plant, LMCOs oxidize diphenolics present in the leaf tissue to highly reactive *ortho*-quinones, which covalently modify free amino and sulphhydryl groups on dietary proteins preventing their digestion and absorption by the caterpillar (Duffey and Stout, 1996; Wang and Constabel, 2004; Mayer, 2006; Pourcel *et al.*, 2007). Serine proteinase inhibitors, such as TI, lower the nutritive quality of the diet by inhibiting caterpillar proteinases, such as trypsin, impairing protein digestion (Koiwa *et al.*, 1997; Zavala *et al.*, 2004; Wu *et al.*, 2006). In this study, the effect of mechanical damage or caterpillar herbivory was determined on levels of *AtLOX2*, *AtPDF1.2*, and *AtLMCO4* transcripts, *AtTI* protein levels, and the enzyme activity of *AtLMCO*.

Another tool to determine the influence of caterpillar saliva on plant defence responses involves the cauterization of the spinneret. In the beet armyworm, *S. exigua*, labial saliva is secreted through the caterpillar spinneret during feeding (Musser *et al.*, 2002). Burning this spinneret impairs labial salivary secretions but does not impact herbivory (Musser *et al.*, 2002; Bede *et al.*, 2006). Therefore, *Arabidopsis* plants (parental wild type, and *IR*<sup>−</sup> or *SAR*<sup>−</sup> mutants) were subjected to one of four treatments (control, mechanical damage, and herbivory by caterpillars with intact or impaired salivary secretions), and the resultant effect on *IR* was monitored at the molecular and biochemical levels.

## Materials and methods

### *Arabidopsis* cultivation

*Arabidopsis* seeds [wild type (Col-0; TAIR #CS3749), *IR*<sup>−</sup> mutant (T-DNA line, *Atlox2* (At3g45140); TAIR #CS3748), *SAR*<sup>−</sup> mutant (null mutation in *Atndr1-1* and point mutations in *Atnpr1-2* (At3g50600, At1g642080); TAIR # CS6355] were obtained from the *Arabidopsis* Biological Resource Center (TAIR; Ohio State University), sown in Premiere Promix BX (Premier Horticulture Inc.) mixed with slow-release Nutricote (Plant Products; 14–14–14; 5.2 g kg<sup>−1</sup> Promix), and grown in a phytorium growth cabinet (light intensity 140  $\mu\text{E m}^{-2} \text{s}^{-1}$  set at a 12:12 h light:dark schedule with temperatures of 23/21 °C). Plants were bottom-watered as needed, ~3 times per week. Three 5-week-old plants, one from each genotype and of approximately the same growth stages, were transplanted to larger pots (16 cm diameter). At 6 weeks, pots were encased in a plastic chamber and 7-week-old plants, which were between growth stage 6.2 and 6.5 according to Boyes *et al.* (2001), were used for herbivore experiments.

### Insect rearing

The beet armyworm, *S. exigua* (Hübner) (Lepidoptera: Noctuidae), colony was reared for multiple generations from eggs purchased

from AgriPest Inc. (Zebulon, NC, USA) under defined conditions in a growth cabinet (16:8 h light:dark; 28–40% relative humidity; 28.5 °C). Caterpillars were maintained on a wheat germ-based artificial diet (Bio-Serv, Frenchtown, NJ, USA). Adult moths mated and the eggs were collected to maintain the colony.

### Spinneret cauterization

A subset of fourth instar *S. exigua* caterpillars were subject to spinneret cauterization to impair labial salivary secretions (Musser *et al.*, 2002; Bede *et al.*, 2006). Early fourth instar caterpillars were selected, cooled on ice, and their spinneret ablated using a hot probe. Cauterized caterpillars were allowed to recover and feed on BioServ diet (2–4 h) before testing for salivary GOX activity (Bergmeyer, 1974). Briefly, individual caterpillars were placed in medicine cups containing a filter (Glass microfiber, Whatmann, 24 mm) saturated with a glucose/sucrose solution (50 mg of each sugar ml<sup>−1</sup>; Sigma). Once caterpillars fed on the glass disc, the presence of salivary GOX was tested by incubating the filter paper with 3,3'-diaminobenzidine (1 mg ml<sup>−1</sup>, pH 5.8, Sigma) and the enzyme horseradish peroxidase (2.5 U in 50 mM sodium phosphate buffer, pH 7.0, Sigma). The presence of a dark brown precipitate indicates that the caterpillar secreted GOX during feeding and had normal salivary secretions. Indications of feeding on the filter disc but the absence of the precipitate showed that spinneret cauterization was successful. Both subpopulations of caterpillars, those with normal and impaired salivary secretions, were separately allowed to feed on *Arabidopsis* plants for 1 d prior to the experiment.

### Herbivory experiment

*Arabidopsis* plants were subject to one of three treatments: herbivory by caterpillars with intact or impaired salivary secretions or remaining untouched (controls). For the caterpillar treatments, six fourth instar *S. exigua* caterpillars with either intact or impaired salivary secretions were introduced to their respective plants. As mentioned above, plants were enclosed in plastic containers and netting was firmly secured to the tops to prevent caterpillar escape. After 36 h of treatment, plants were collected and prepared for analysis. For each independent biological replicate, at least two plants of each genotype and treatment were randomly collected for biomass, gene expression, TI and LMCO activity, and hormonal analyses. *Arabidopsis* which were flowering were discarded. Plants for biomass analysis were cleaned, weighed, and pressed for later measurement of dry weight. Aerial tissues of all other plants were collected, quickly frozen in liquid nitrogen and stored at −80 °C until analysis. This experiment was repeated three times independently.

### RNA extraction and cDNA synthesis

For each treatment and replicate, at least one plant of each genotype (27 total) was rapidly ground with a mortar and pestle and total RNA extracted using the RNeasy Mini kit (Qiagen), following the manufacturer's instructions. After DNase treatment [Wipeout, QuantiTect Reverse Transcription kit (Qiagen)], the absence of DNA contamination was confirmed using primers 5'-ATG GGT CGT CAT CAG ATT CAG AGC AGA TAA-3' and 5'-CAT ATA AGA GGT GTG TTA GAG ACA ATA ATA-3' which span an intronic region. A cDNA copy was generated from 1  $\mu\text{g}$  of total RNA following the manufacturer's protocol.

### Quantitative real time-polymerase chain reaction (qRT-PCR)

Gene-specific primers were identified from the literature (Jirage *et al.*, 2001) or designed using AtRTPrimer (Han and Kim, 2006) and expression analysed by qRT-PCR using a Mx3000p thermocycler (Stratagene). Transcript expression was performed in

duplicate using the Brilliant One-Step quantitative RT-PCR kit (Stratagene), according to the manufacturer's protocol. For each gene, purified PCR amplicons, which were sequenced for verification, were used to prepare a standard curve. A non-template control was also included for every gene. Each reaction contained 1× SYBR green I, 0.375 nM ROX, 175 nM or 200 nM each of gene-specific forward and reverse primers (Table 1), serial dilutions of PCR amplicon or 1.25 ng of cDNA, and mastermix, which contained dNTPs, MgSO<sub>4</sub>, and *Taq* polymerase. Thermocycler conditions used were as follows: 95 °C for 10 min; 40 cycles of annealing temperature for 1 min, then 72 °C for 30 s, followed by 95 °C for 1 min; one cycle at annealing temperature for 30 s and 95 °C for 30 s. The annealing temperatures are given in Table 1. Dissociation curves were produced to confirm amplicon purity. Two plate replicates were performed.

From the standard curves, gene copy numbers were estimated using Mx3000p MxPro v3.20 software. Actin (*AtACT2*) expression has been shown to be constitutive when *Arabidopsis* plants were subject to osmotic stress or treated by methyljasmonate (MeJA) and SA, and, therefore, was used in these experiments as the reference gene (Stotz *et al.*, 2000; Dufresne *et al.*, 2008). *AtACT* was stably expressed within a genotype and not affected by treatments (wild type,  $P=0.53$ ; IR<sup>-</sup> mutant,  $P=0.99$ ; SAR<sup>-</sup> mutant,  $P=0.22$ ) (Brunner *et al.*, 2004). Normalized gene expression of *AtPDF1.2*, *AtLOX2*, and *AtLMCO4* was calculated. Within a genotype, transcript expression differences were statistically determined by one-way analysis of variance (ANOVA) using SPSS version 15 (SPSS Inc., Chicago, IL, USA).

#### Protein extraction

*Arabidopsis* plants were ground in liquid nitrogen using a mortar and pestle. To 50 mg of plant material, ice-cold extraction buffer (0.1 M sodium phosphate buffer, pH 7.0 containing 0.1% Triton X-100 and 7% PVP) was added, vortexed, and centrifuged (5 min at 17 310 g), and the supernatant was transferred to a clean 1.5 ml microcentrifuge tube and placed on ice (Wang and Constabel, 2004). Detergent (0.1% Triton X-100) in the extraction buffer is necessary for LMCO activation (Yoruk and Marshall, 2003). For the assay of LMCO activity, broad-spectrum proteinase inhibitor solution (Sigma; final concentration 1×) was added to the extraction buffer (Yoruk and Marshall, 2003). Samples were used immediately to assay TI or LMCO activity.

#### Trypsin inhibitor assay

Analysis of trypsin levels as described by Lara *et al.* (2000) was modified for a 96-well plate format (Corning, flat-bottomed wells). Briefly, bovine trypsin (0.5 µg, treated to reduce chymotrypsin contamination, Sigma) was incubated with either soybean TI (Type 1S, Sigma; concentration range 0.05 ng to 50 µg) or protein extract at 37 °C with gentle rocking. After 30 min, the trypsin substrate *N*-

benzoyl-DL-arginyl-β-naphthylamine (BANA, Sigma, 3.1 mM final concentration) was added and allowed to incubate for an additional hour with gentle shaking. To stop the reaction, 2% HCl was added. The reagent *p*-dimethyl-amino-cinnamaldehyde (Sigma, final concentration 0.24% in ethanol) reacts with β-naphthalene which is released from BANA through trypsin-catalysed hydrolysis. The product was visualized at 540 nm in a microplate reader (BioTek Synergy HT microplate reader). Controls of TI in the absence of trypsin (blank) as well as trypsin in the absence of TI were included. The standard curve, blanks, and controls were performed in duplicate. All samples were analysed in triplicate. After subtraction of the blank, TI levels in plant samples were calculated from the log of the standard curve using Graphpad 4.0 (Prism).

The pH of the caterpillar gut is extremely alkaline (Schultz and Lechowicz, 1986; Dow, 1992). Protein structure and stability studies have also indicated that both trypsin and TIs are extremely stable over pH and temperature ranges (Simon *et al.*, 2001; Garcia *et al.*, 2004). In this study, TI standard curves were initially compared using 1 M borate buffer, pH 9.3 and sodium phosphate buffer, pH 7.0, and a difference was not observed. Therefore, analysis of plant material was conducted in the sodium phosphate-based extraction buffer to allow comparisons with the literature.

#### Laccase-like multicopper oxidase enzyme activity

AtLMCO (EC 1.10.3.2; polyphenol oxidase) (McCraig *et al.*, 2005; Cai *et al.*, 2006) activity was assayed according to Espín *et al.* (1997) adapted to a 96-well format. To triplicate sample extracts or tyrosinase from mushroom (Sigma, 50 U), *N,N*-dimethylformamide (Sigma, final concentration 0.1%) and 3-methyl-2-benzothiazolone hydrazone hydrochloride monohydrate (Sigma, final concentration 0.2 mM prepared in methanol) were added. These chemicals act to stabilize the reactive quinones generated through the LMCO reaction to prevent them from inactivating the enzyme activity. After the addition of dopamine hydrochloride (Sigma, final concentration 35 mM), enzyme activity was monitored at 30 s intervals for 5 min at 476 nm at 35 °C using a multiplate reader. Enzyme-free and boiled controls were included.

Many plant laccases are latent, requiring activation by protease, such as trypsin, in the caterpillar gut or, in the laboratory, using SDS in the extraction buffer (Yoruk and Marshall, 2003). Activation by SDS requires a neutral pH; therefore, these LMCO activity assays were conducted at pH 7.0 (Sellés-Marchant *et al.*, 2007). Trypsin-mediated activation has a broader pH range and, presumably, occurs in the extremely alkaline caterpillar gut (Schultz and Lechowicz, 1986; Dow, 1992; Wang and Constabel, 2004).

#### Protein assay

Levels of soluble, extracted protein were measured by Bradford assay (Pierce) at 595 nm in a 96-well format (Bradford, 1976). A standard curve was generated using duplicate samples of bovine

**Table 1.** Primers used for qRT-PCR expression analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
<i>AtPDF1.2</i> (At2g2620)	CGGCAATGGTGGAAGCA	CATGCATTACTGTTTCCGCAA	59	79	Jirage <i>et al.</i> (2001)
<i>AtLOX2</i> (At3g45140)	GTCCTACTTGCCTTCCCAAAC	ATTGTCAGGGTCACCAACATC	57	160	Designed using AtRTPPrimer
<i>AtLMCO4</i> (At2g38080)	AGGTCCCACAATCTACGCACGA	CCATCCCGTTCTCACTTGTCTCACA	58	115	Designed using AtRTPPrimer
<i>AtACT2</i> (At3g18780)	ACCAGCTCTTCCATCGAGAA	GAACCACCGATCCAGACACT		338	Dufresne <i>et al.</i> (2008)

serum albumin (dilution range: 0.35–6.9 µg). Leaf extracts were analysed in triplicate. Blank controls consisted of Bradford reagent with extraction buffer.

#### Estimation of leaf loss due to caterpillar herbivory

Dry weights of the vegetative tissue from two replicates of each genotype and treatment for each experimental replication were measured to determine the estimated leaf tissue removed by caterpillar herbivory ( $n=6$ ).

#### Hormone analysis

Foliar levels of JA and SA were analysed by high-performance liquid chromatography electrospray tandem mass spectroscopy (HPLC/ES-MS/MS) at the Plant Biotechnology Institute of the National Research Council of Canada. Three biological replications of wild-type or SAR<sup>-</sup> plants subject to herbivory by caterpillars with either intact or impaired salivary secretions were frozen in liquid nitrogen. Plant material was ground to a fine powder in liquid nitrogen. Extraction of ~300 mg samples was performed by adding 3 ml of methanol:water:glacial acetic acid (90:9:1, by vol.) extraction solution and the internal standard [100 ng of 3,4,5,6-d<sub>4</sub>-2-hydroxybenzoic acid and 50 ng of 2,2-d<sub>2</sub>-jasmonic acid dissolved in 15% acetonitrile in water+0.1% formic acid (Galka *et al.*, 2005)]. Following sonication (5 min) and incubation on an orbital shaker (4 °C, 5 min), samples were centrifuged (17 310 g, 10 min) to pellet the debris. The supernatant was transferred to a clean tube and the pellets were re-suspended in 2 ml of the extraction solution. Sonication, extraction on the orbital shaker, and centrifugation were repeated. The supernatant was combined with the initial extracted volume and the pellet was re-suspended in 1 ml of methanol. The extraction step was repeated a third time. After the supernatants were combined, methanol was evaporated under a constant stream of nitrogen. A 2 ml aliquot of 0.1 M NaOH was added to the residual water phase and neutral components were removed by extraction with 3 ml of dichloromethane. After phase separation, the water layer was transferred to a clean tube. The dichloromethane layer was re-extracted with 0.1 M NaOH (2 ml). Both aqueous layers were combined and acidified with 5% aqueous HCl on ice followed by the partitioning with 1 ml of ethyl acetate:cyclohexane (1:1, v/v) solvent mixture. The organic phase was collected and the water phase was extracted a second time with 0.5 ml of ethyl acetate:cyclohexane mixture. The organic fractions were pooled and the solvent was evaporated under a constant stream of nitrogen. Prior to mass spectrometric analysis, the samples were reconstituted in 100 µl of 40% HPLC-grade methanol in 1% aqueous formic acid, which contained 100 ng of 1,2,3,4,5,6-<sup>13</sup>C<sub>6</sub>-2-hydroxybenzoic acid and 50 ng of 12,12,12-d<sub>3</sub>-jasmonic acid to monitor instrument performance.

SA and JA analysis was carried out by HPLC/ES-MS/MS utilizing an HP1100 series binary solvent pump and autosampler (Hewlett-Packard) coupled to a Quattro LC<sup>TM</sup> quadrupole tandem mass spectrometer via a Z-spray<sup>TM</sup> interface (Micromass, Manchester, UK). The analytical HPLC column was a Zorbax Rapid Resolution 2.1×50 mm 1.8 µm column (Agilent Technologies). Mobile phase A comprised 1% formic acid in HPLC-grade water; mobile phase B comprised HPLC-grade methanol. Sample volumes of 5 µl were injected onto the column at a flow rate of 0.20 ml min<sup>-1</sup> under initial conditions of 40% B, which was maintained for 3 min, then increased to 90% B at 3.1 min, and held until 10 min. B was decreased to 40% by 11 min and held until 20 min for column equilibration before the next injection. The analytes were ionized by negative-ion electrospray using the following conditions: capillary potential 2.5 kV; cone voltage 30 V; desolvation gas flow 600 l h<sup>-1</sup>; source and desolvation gas temperatures, 120 °C and 350 °C, respectively. Analytical procedures analogous to those

reported in Ross *et al.* (2004) were employed to determine the quantities of SA and JA in the plant extracts. Briefly, analysis utilizes the Multiple Reaction Monitoring (MRM) function of the MassLynx v3.5 (Micromass) control software wherein the instrument monitors the loss of a neutral CO<sub>2</sub> moiety from the deprotonated parent ion during collision-induced dissociation (CID). The transitions monitored for the standards as well as undeuterated analytes are  $m/z$  (mass-to-charge ratio): 137>93 for SA, 141>97 for d<sub>4</sub>-SA; 143>99 for <sup>13</sup>C<sub>6</sub>-SA; 209>59 for JA; 211>61 for d<sub>2</sub>-JA; and 212>59 for d<sub>3</sub>-JA. The resulting chromatographic traces are quantified off-line by the QuanLynx v4.0 software (Micromass) wherein each trace is integrated and the resulting ratio of signals (undeuterated/internal standard) is compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample).

#### Statistical analysis

The experiment was repeated three times independently. Transcript and hormone levels were analysed in one plant from each experimental replicate ( $n=3$ ). TI levels, LMCO activity and soluble protein levels, as well as the amount of caterpillar damage were analysed in two plants from each experimental replicate ( $n=4-6$ ). Outliers were detected using the Grubbs' test (Prism GraphPad). Within each genotype, data were analysed statistically by ANOVA using SPSS version 14 (SPSS Inc.). Statistical differences were determined using a Tukey HSD *post hoc* test. Significant differences in hormone levels were detected using a two-tailed Student's *t*-test.

## Results and discussion

#### Caterpillar damage

*Arabidopsis* damage resulting from caterpillar herbivory was roughly estimated by measuring aerial tissue dry weight. Between 23% and 31% of plant tissue was removed by caterpillar herbivory, irrespective of *Arabidopsis* genotype or cauterization treatment.

#### Arabidopsis hormone levels

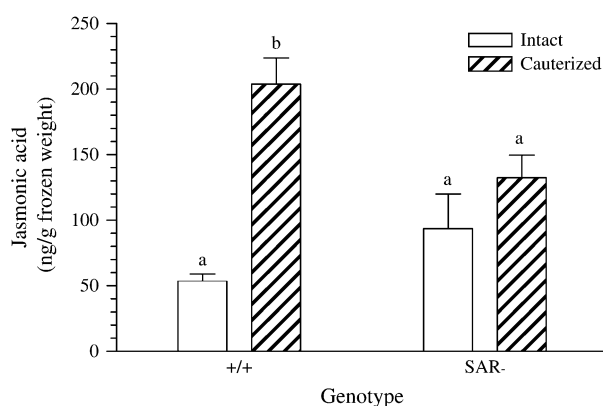
In response to caterpillar feeding, plants mount a rapid induction of biosynthesis of jasmonate and related octadecanoid compounds, such as 12-oxo-phytodienoic acid (OPDA) and dinor OPDA, which together lead to the induction of plant defence responses (Kahl *et al.*, 2000; Reymond *et al.*, 2004). This may manifest itself as a rapid, transient burst which, depending on the plant species, may be followed by a steady increase over a period of at least 24 h (Reymond *et al.*, 2004; Zavala *et al.*, 2004; Rayapuram and Baldwin, 2007). Free JA levels were measured in wild-type and SAR<sup>-</sup> mutant plants which had been subject to herbivory by caterpillars with intact or impaired salivary secretions to determine if salivary elicitors interfere with changes in phytohormone levels. Wild-type plants eaten by cauterized caterpillars compared with plants infested by caterpillars with normal salivary secretions had similar SA levels [ $<5 \mu\text{g g}^{-1}$  frozen weight,  $t_{(4)} = -1.27$ ;  $P=0.27$ ] but  $>4$  times more JA [ $t_{(4)} = -7.27$ ;  $P=0.002$ ] (Fig. 1). Induced levels in this range have been previously observed in *Arabidopsis* plants

subject to herbivory by first instar caterpillars of the crucifer specialist *P. rapae* (Reymond *et al.*, 2004). The salivary-dependent difference in JA levels observed in wild-type plants was not seen in the SAR<sup>-</sup> double mutants [ $t_{(4)} = -1.23$ ;  $P = 0.29$ ].

SAR<sup>-</sup> mutant plants have basal, constitutive levels of SA which do not increase in response to pathogen attack (Zhang *et al.*, 2004). In response to caterpillar herbivory, SA levels in the SAR<sup>-</sup> plants were consistently low ( $<5 \mu\text{g g}^{-1}$  frozen weight) and salivary-specific differences were not observed in either the wild-type or SAR<sup>-</sup> mutant plants [data not shown;  $t_{(4)} = -1.27$ ; wild type,  $P = 0.27$ ; SAR<sup>-</sup> mutant,  $t_{(4)} = 1.40$ ;  $P = 0.22$ ]. These data suggest that a factor(s) in caterpillar saliva acts directly or indirectly as a negative regulator of JA biosynthesis by an SA-independent mechanism.

### Induced plant defences

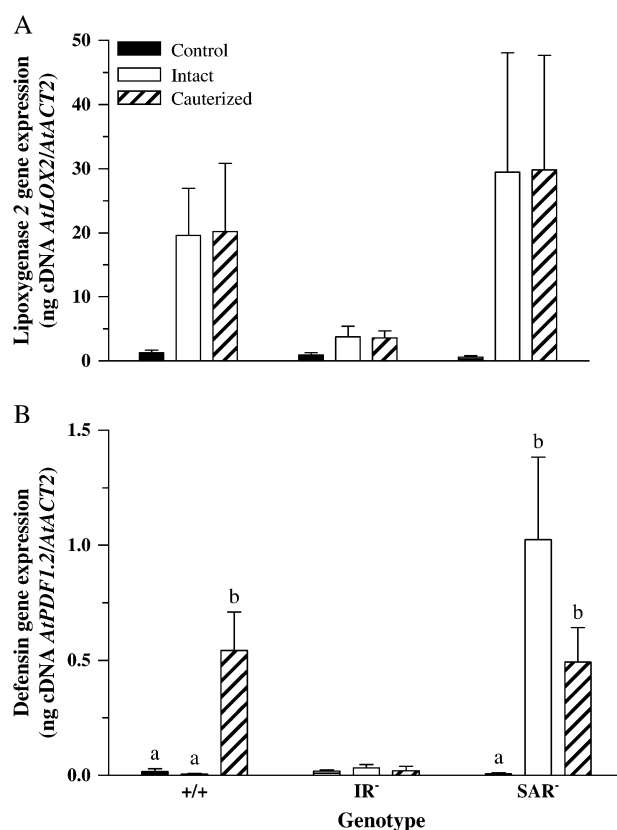
LOX2 catalyses the second step in octadecanoid biosynthesis; the conversion of  $\alpha$ -linolenic acid to 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (Vick and Zimmerman, 1976; Mueller, 1997). In *Arabidopsis* and other plant species, constitutive, basal *AtLOX2* transcript levels are rapidly induced in response to injury (Bell and Mullet, 1993; Heintz *et al.*, 1997; Zheng *et al.*, 2007). Also, octadecanoids may further stimulate *AtLOX2* expression, leading to a transient jasmonate burst (Sasaki *et al.*, 2001; Ziegler *et al.*, 2001). In wild-type and SAR<sup>-</sup> *Arabidopsis* plants, caterpillar herbivory resulted in a  $>4$ -fold increase in *AtLOX2* expression (Fig. 2A); however, due to the large variation in gene expression, significant differences in response to treatment are not observed [wild type,  $F_{(2,6)} = 2.17$ ;  $P = 0.20$ ; SAR mutant,  $F_{(2,6)} = 1.27$ ;  $P = 0.35$ ].



**Fig. 1.** *Arabidopsis* jasmonic acid levels in response to caterpillar herbivory. Levels of free jasmonic acid (JA) were measured in 7-week-old *Arabidopsis* plants [Col-0 parental (+/+) or *Atndr1-1*, *npr1-2* double mutant (SAR<sup>-</sup>)] that were subject to caterpillar herbivory by caterpillars with normal (intact) or impaired (cauterized) salivary secretions for 36 h. Bars represent the means of three independent replicates  $\pm$  SE. Within each genotype, the lower case letters indicate significant differences ( $P \leq 0.05$ ) ( $n = 3$ ).

Transcript *AtLOX2* levels do not show caterpillar salivary-specific regulation, yet JA levels reflect salivary status. In the wild-type, SAR<sup>-</sup> mutant, and, to some extent, even in the IR<sup>-</sup> mutant plants, *AtLOX2* expression levels are elevated in response to caterpillar herbivory regardless of the salivary status of the caterpillars (Fig. 2A). This is reminiscent of a model proposed by Huang *et al.* (2004) where nitric oxide is an upstream signal leading to the activation of *AtLOX2* and the repression of *AtOPDA* via SA. Perhaps, wound-related nitric oxide levels induce *AtLOX2* gene expression, yet the negative regulator associated with caterpillar saliva inhibits JA biosynthesis downstream of this point.

*AtPDF1.2* encodes the ethylene- and JA-dependent defence protein defensin (Penninckx *et al.*, 1998). Wild-type plants damaged by herbivory by cauterized caterpillars show a  $>5$ -fold increase in *AtPDF1.2* expression

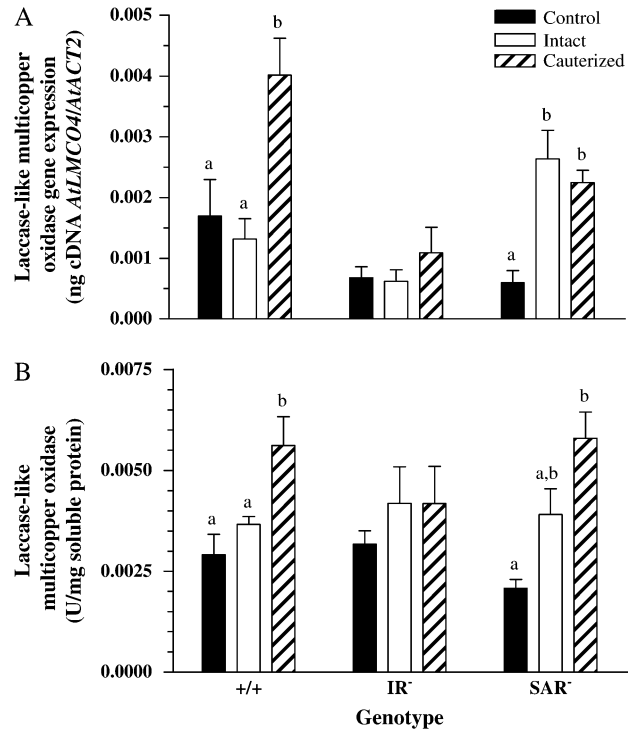


**Fig. 2.** Transcript expression of genes encoding lipoxigenase2 (*AtLOX2*) and defensin (*AtPDF1.2*) in response to caterpillar herbivory. Seven-week-old *Arabidopsis* plants [Col-0 parental (+/+), *Atlox2* mutant (IR<sup>-</sup>), and *Atndr1-1*, *npr1-2* double mutant (SAR<sup>-</sup>)] were subject to caterpillar herbivory by caterpillars with normal (intact) or impaired (cauterized) salivary secretions for 36 h. cDNA was prepared from total RNA extracted from vegetative tissue, and transcript expression was analysed by qRT-PCR using gene-specific primers. *AtLOX2* (A) and *AtPDF1.2* (B) are markers of JA-dependent gene expression (Bell and Mullet, 1993; Penninckx *et al.*, 1998). Bars represent the means of three independent replicates normalized with the reference gene actin (*AtACT2*)  $\pm$  SE. Within each genotype, lower case letters indicate significant differences ( $P \leq 0.05$ ) ( $n = 3$ ).

levels compared with control plants [ $F_{(2,6)}=10.29$ ;  $P=0.012$ ] (Fig. 2B). In comparison, herbivory by caterpillars with normal labial salivary secretions prevents the induction of *AtPDF1.2* transcription. Reymond *et al.* (2004) observed a similar pattern where *AtPDF1.2* gene expression was induced in response to wounding but not herbivory by *S. littoralis* or *P. rapae* caterpillars. In other studies, *P. rapae* caterpillar feeding resulted in strong, localized *AtPDF1.2* transcript expression, but whole leaf levels were too low to be detected (De Vos *et al.*, 2006). A caterpillar-specific increase in *AtPDF1.2* expression was observed in SAR<sup>-</sup> double mutants [ $F_{(2,6)}=5.13$ ;  $P=0.05$ ]; however, in comparison with wild-type plants, a saliva-specific difference was not seen. Herbivory-induced *AtPDF1.2* expression was not observed in the IR<sup>-</sup> mutants [ $F_{(2,6)}=1.54$ ;  $P=0.29$ ].

Caterpillars feeding on plant leaves often face protein limitations, in terms of quality and quantity (Mattson, 1980; Felton, 1996; Bede *et al.*, 2007). Also, plants have jasmonate-dependent anti-nutritive defences that interfere with caterpillar protein digestion, such as proteinase inhibitors, LMCO, or enzymes which deaminate essential amino acids (Duffey and Stout, 1996; Koiwa *et al.*, 1997; Zavala *et al.*, 2004; Chen *et al.*, 2005; Felton, 2005). LMCOs oxidize foliar polyphenolics to reactive quinones which bind to proteins undergoing digestion in the caterpillar gut, preventing their absorption (Duffey and Stout, 1996; Pourcel *et al.*, 2007). Though a recent study has shown that, contrary to expectations, lymantriid caterpillars of *Lymantria dispar* L. (gypsy moth) and *Orgyia leucostigma* Smith (white-marked tussock moth) did not suffer negative consequences by feeding on transgenic poplar trees with elevated LMCO levels (Barbehenn *et al.*, 2007), further studies are needed to ascertain the generality of these observations. *AtLMCO* gene expression and enzyme activity increased in wild-type plants subject to herbivory by caterpillars with impaired labial salivary secretions [LMCO transcript expression,  $F_{(2,7)}=8.38$ ,  $P=0.014$ ; LMCO activity,  $F_{(2,7)}=8.18$ ,  $P=0.015$ ] (Fig. 3A, B). This caterpillar-specific difference is not observed in the SAR<sup>-</sup> double mutant [LMCO transcript expression:  $F_{(2,6)}=811.64$ ,  $P=0.009$ ; LMCO activity,  $F_{(3,9)}=12.18$ ,  $P=0.003$ ]. Since this is a JA-dependent defence, treatment does not affect transcript levels or enzyme activity in the IR<sup>-</sup> mutant [LMCO transcript expression,  $F_{(2,6)}=1.40$ ,  $P=0.30$ ; LMCO activity,  $F_{(2,13)}=0.38$ ,  $P=0.695$ ].

Upon ingestion, many plant LMCOs are latent until activated by trypsin (Yoruk and Marshall, 2003). Therefore, it is interesting that another common jasmonate-dependent plant defence to interfere with caterpillar nutritional intake are serine proteinase inhibitors, such as TIs, which hinder protein digestion by binding to the caterpillar digestive enzyme trypsin in the gut, thereby reducing protein digestion (Koiwa *et al.*, 1997; Cipollini *et al.*, 2004; Clauss and Mitchell-Olds, 2004; Zavala *et al.*,

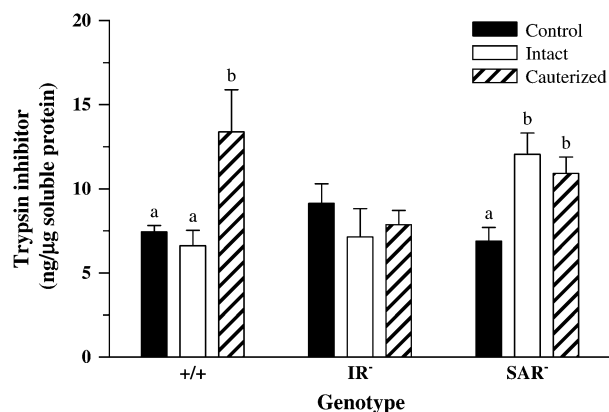


**Fig. 3.** Transcript expression and enzyme activity of laccase-like multicopper oxidase (LMCO) in response to caterpillar herbivory. Seven-week-old *Arabidopsis* plants [Col-0 parental (+/+), *Atlox2* mutant (IR<sup>-</sup>), and *Atndr1-1*, *npr1-2* double mutant (SAR<sup>-</sup>)] were subject to caterpillar herbivory by caterpillars with normal (intact) or impaired (cauterized) salivary secretions for 36 h. (A) *AtLMCO4* gene expression. cDNA was prepared from total RNA extracted from vegetative tissue, and transcript expression was analysed by qRT-PCR using *AtLMCO4*-specific primers. Bars represent the means of three independent replicates normalized with the reference gene *actin* (*AtACT2*)  $\pm$  SE. Within each genotype, lower case letters indicate significant differences ( $P \leq 0.05$ ) ( $n=3$ ). (B) LMCO activity. Bars represent the average LMCO activity per mg of soluble protein of 4–6 plants  $\pm$  SE. Within each genotype, lower case letters indicate significant differences ( $P \leq 0.05$ ) ( $n=4-6$ ).

2004). In *Arabidopsis*, five members of this multigene family were induced in response to herbivory by third instar *P. xylostella* caterpillars (Clauss and Mitchell-Olds, 2004). Caterpillar salivary-specific difference in TI levels observed in wild-type plants are not seen in the SAR<sup>-</sup> double mutant [wild type,  $F_{(2,8)}=7.33$ ,  $P=0.016$ ; SAR<sup>-</sup>,  $F_{(3,6)}=6.90$ ,  $P=0.028$ ] (Fig. 4). As this defence is strongly correlated with JA levels, treatment differences observed in the wild type and SAR<sup>-</sup> mutant plants are not seen in the IR<sup>-</sup> mutant [ $F_{(2,13)}=0.71$ ,  $P=0.51$ ].

#### Caterpillar saliva interferes with JA-dependent plant defences via the SAR pathway

Using well-established *Arabidopsis* mutants subject to herbivory by insects with intact and impaired salivary secretions, this study presents evidence that caterpillar labial saliva interferes with octadecanoid-associated induced plant defences. JA hormone levels, *AtLMCO4* and *AtPDF1.2* transcript levels, and TI and LMCO activity



**Fig. 4.** Trypsin inhibitor levels in response to caterpillar herbivory. Levels of the anti-nutritive protein, trypsin inhibitor, were determined in 7-week-old *Arabidopsis* plants [Col-0 parental (+/+), *Atlox2* mutant (IR<sup>-</sup>), and *Atndr1-1*, *npr1-2* double mutant (SAR<sup>-</sup>)] subject to caterpillar herbivory by caterpillars with normal (intact) or impaired (cauterized) salivary secretions for 36 h. Bars represent means of 3–6 plants  $\pm$  SE. Within each genotype, lower case letters indicate significant differences ( $P \leq 0.05$ ) ( $n=4-6$ ).

show salivary-specific regulation (Figs 1, 2B, 3, 4). Wild-type plants infested by caterpillars with intact salivary secretions did not mount the expected defence responses which are observed when caterpillar salivary secretions are inhibited by cauterization. This salivary-specific difference is alleviated when the SAR<sup>-</sup> pathway is not present.

The *Arabidopsis* SAR<sup>-</sup> mutant plants have a null mutation in the gene encoding the protein NDR1-1 and point mutations in the genes encoding NPR1 and 2 (Zhang and Shapiro, 2002). This double mutation blocks both ROS synthesis and NPR1 function (Zhang *et al.*, 2004). Also, SA does not accumulate in these plants when they are subject to pathogen attack, which was also observed in this study (data not shown).

NPR1 is constitutively present in its cytosolic oligomeric form (Mou *et al.*, 2003). A change in cellular redox potential which, for example, may be associated with increased ROS during plant–pathogen interactions or caterpillar salivary oxidoreductases, reduces NPR1 intermolecular disulphide bonds via activation of glutathione biosynthesis and/or altering glutathione redox status, releasing the monomeric protein (Mou *et al.*, 2003; Ball *et al.*, 2004; Senda and Ogawa, 2004). Activated NPR1 may enter the nucleus and associate with TGA- or WRKY-type transcription factors, leading to SA-dependent gene expression (Després *et al.*, 2003; Mou *et al.*, 2003; Wang *et al.*, 2006). NPR1 is also believed to interfere with JA-dependent gene expression; however, the mechanism by which this occurs is unclear and it may occur by inhibiting octadecanoid biosynthesis or by preventing the JA-dependent, ubiquitin–proteasome-mediated degradation of the JAZ protein or histone deacetylase, or by activating WRKY transcription factors

(Devoto *et al.*, 2002; Xu *et al.*, 2002; Beckers and Spoel, 2006; Miao and Zentgraf, 2007; Thines *et al.*, 2007). Recent evidence also supports the involvement of a glutaredoxin protein downstream of NPR1 which upon interaction with TGA2,5,6 transcription factors binds to the promoter region and represses transcription of JA-dependent genes, such as *AtPDF1.2* (Ndamukong *et al.*, 2007).

In this study, the salivary-related difference in the induction of JA-related defences is not observed in the SAR<sup>-</sup> mutants. The small sample size, between three and six depending on the assay, could lead to the observation of false negatives; however, the consistency in response supports the conclusion that a negative JA-regulatory factor present in the caterpillar saliva mediates the activation of NPR1, perhaps through a change in cellular redox potential, leading to the attenuation of octadecanoid-associated defence responses. In wild tobacco, *N. attenuata*, *NaNPR1*-silenced plants have blocked plant defences and were more susceptible to caterpillar herbivory (Rayapuram and Baldwin, 2007). In the *Nicotiana* system, reduced NPR1 levels are believed to affect SA biosynthesis positively, resulting in antagonism of JA-dependent defences. In the present study, SA levels did not change in response to caterpillar herbivory, possibly demonstrating species-specific differences in the regulation of plant defence pathways.

## Conclusion

Caterpillar elicitors, present in the regurgitant or saliva, modify plant defence responses by affecting interactions between signalling pathways. In response to mechanical injury, plants mount defence and healing responses which are primarily mediated by octadecanoid-related compounds, such as JA (Léon *et al.*, 2001; Howe, 2004). However, other phytohormones, such as ethylene, abscisic acid, nitric oxide, and auxin, and signalling proteins, such as NPR1, can attenuate or accentuate these plant responses by interacting at signalling nodes where it is clear that microlocalization and equilibrium ratios play a critical role in mediating the final cellular response (Orozco-Cárdenas and Ryan, 2002; Gupta and Luan, 2003; Anderson *et al.*, 2004; Huang *et al.*, 2004; Li *et al.*, 2004; Lorenzo *et al.*, 2004; Bostock, 2005; Devoto and Turner, 2005; Lorenzo and Solano, 2005; Wang *et al.*, 2006; Adie *et al.*, 2007; Bodenhausen and Reymond, 2007; Dombrecht *et al.*, 2007; Miao and Zentgraf, 2007; Spoel *et al.*, 2007; von Dahl and Baldwin, 2007). One adaptive strategy of caterpillars to circumvent induced plant defences may be to activate these other signalling pathways which interfere with the plant's normal octadecanoid responses (Musser *et al.*, 2002, 2005). This study raises the possibility that labial salivary enzymes secreted onto leaves as caterpillars are feeding interfere with octadecanoid-responsive plant defences by activating the



SAR pathway (Devoto *et al.*, 2002; Xu *et al.*, 2002; Després *et al.*, 2003; Mou *et al.*, 2003; Spoel *et al.*, 2003; Li *et al.*, 2004; Pieterse and van Loon, 2004; Fobert and Després, 2005; Foyer and Noctor, 2005; Beckers and Spoel, 2006; Miao and Zentgraf, 2007).

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