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DELLA proteins modulate Arabidopsis defenses induced in response to caterpillar herbivory

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Abbreviations:
GS: glucosinolate, LMCO: laccase-like multicopper oxidase, LS: labial saliva, TI: trypsin inhibitor, ROS: reactive oxygen species

Abstract

Upon insect herbivory, many plant species change the direction of metabolic flux from growth into defense. Two key pathways modulating these processes are the gibberellin (GA)/DELLA pathway and the jasmonate pathway, respectively. In this study, the effect of caterpillar herbivory on plant induced responses was compared between wildtype Arabidopsis thaliana (L.) Heynh. and quad-della mutants that have constitutively elevated GA responses. The labial saliva (LS) of caterpillars of the beet armyworm, Spodoptera exigua, is known to influence induced plant defense responses. To determine the role of this herbivore cue in determining metabolic shifts, plants were subject to herbivory by caterpillars with intact or impaired LS secretions. In both wildtype and quad-della plants, a jasmonate burst is an early response to caterpillar herbivory. Negative growth regulator DELLA proteins are required for the LS-mediated suppression of hormone levels. Jasmonate-dependent marker genes are induced in response to herbivory independent of LS, with the exception of AtPDF1.2 that showed LS-dependent expression in the quad-della mutant. Early expression of the salicylic acid (SA)-marker gene, AtPR1, was not affected by herbivory which also reflected SA hormone levels; however, this gene showed LS-dependent expression in the quad-della mutant. DELLA proteins may positively regulate glucosinolate levels and suppress laccase-like multicopper oxidase activity in response to herbivory. Our results show a link between DELLA proteins and early induced plant defenses in response to insect herbivory; in particular, these proteins are necessary for caterpillar LS-associated attenuation of defense hormones.

Introduction

Confronted with caterpillar attack, plants often redirect metabolic flux away from growth and into defensive compounds (Schwachtje and Baldwin, 2008). These physiological processes are regulated through distinct hormone-mediated pathways shape the plant’s response. In general, jasmonic acid (JA) and related compounds are implicated in plant defense responses against chewing insect herbivores while gibberellins (GAs) promote plant growth and development.
(Ballare, 2011; Erb et al., 2012). In addition, caterpillar salivary effectors modulate plant defenses, often suppressing JA-induced plant responses (Bede et al., 2006; Diezel et al., 2009; Musser et al., 2002; Tian et al., 2012; Weech et al., 2008).

When Arabidopsis thaliana (L.) Heynh is wounded by caterpillar herbivory, a rapid, transient increase in jasmonate biosynthesis results in the accumulation of the bioactive form of JA, 7-jasmonoyl-L-isoleucine (JA-Ile)(Fonseca et al., 2009). By bridging jasmonate ZIM-domain (JAZ) proteins with the E3 ubiquitin ligase SCF^{COI1} complex, JA-Ile promotes the targeted degradation of the JAZ protein by the 26S proteasome, releasing MYC2/3/4 transcription factors leading to induced plant responses (Chini et al., 2007; Erb et al., 2012; Fernandez-Calvo et al., 2011; Katsir et al., 2008; Sheard et al., 2010; Thines et al., 2007; Yan et al., 2009). Lipoxygenase2 (AtLOX2), Plant Defensin 1.2 (AtPDF1.2) and Vegetative Storage Protein2 (AtVSP2) are well characterized markers of MYC-regulated gene expression (Bell and Mullet, 1993; Dombrecht et al., 2007; Kazan and Manners, 2013; Lorenzo et al., 2004; Pre et al., 2008; Robert-Seilaniantz et al., 2011); although late expression of PDF1.2 is also positively regulated through TGA transcription factors (Zander et al., 2010).

Activation of the jasmonate pathway results in the induction of the plant defense responses. In Arabidopsis, key defensive strategies include the production of antinutritive proteins, such as trypsin inhibitors (TI) and laccase-like multicopper oxidase (LMCO) and secondary metabolites, such as glucosinolates (GSs) (Van Poecke, 2007). In many plant systems, TIs are induced in response to caterpillar herbivory and bind to gut serine proteinases impeding protein digestion and, hence, insect growth (Tian et al., 2012; Weech et al., 2008). LMCOs have diverse plant physiological functions, including interfering with protein digestion by oxidizing plant-derived polyphenolics in the insect gut generating quinones that react with protein amino acid residues preventing their absorption (Constabel and Barbehenn, 2008). Arabidopsis and other members of the Brassicaceae also contain signature GSs (Brown et al., 2003; Halkier and Gershenzon, 2006; Hopkins et al., 2009). To date, about 200 GSs have been identified, which are broadly categorized into aliphatic, indole and aromatic GSs (Clarke, 2010). Over 35 GS have been identified in Arabidopsis with representative GS of the aliphatic and indoyl pathways, such as 3-hydroxypropyl glucosinolate and glucobrassicin, respectively, being prominent in Landsberg (Ler) leaves (Brown et al., 2003; Kliebenstein et al., 2001). Wounding by chewing insect herbivores disrupt cellular compartments allowing contact between the enzyme myrosinase and vacuolar-localized GSs generating a diversity of toxic and noxious compounds, such as (iso)thiocyanates and nitriles (Halkier and Gershenzon, 2006). The product that is formed and its toxicity to insect herbivores greatly depends on the GS side chain. Generalist caterpillars of the beet armyworm, Spodoptera exigua (Hübner), are adversely affected by the aliphatic class of GSs whereas aphids are mainly affected by indole GSs (Kusnierczyk et al., 2007; Mewis et al., 2005; Mosleh Arany et al., 2008).
Caterpillar labial salivary (LS) effectors modulate the jasmonate pathway and subsequent induced defense responses. Usually, feeding damage as well as mechanical wounding increase the biosynthesis of jasmonate signalling hormones (Ballere, 2011). However, when responses are compared between plants fed upon *S. exigua* caterpillars with intact or impaired LS secretions or when caterpillar LS is added to wounded plant tissues, these responses may be suppressed and/or delayed (Diezel *et al.*, 2009; Tian *et al.*, 2012; Weech *et al.*, 2008). Presently, evidence suggests that caterpillar LS-mediated suppression of induced plant defenses involves the activation of the salicylic acid (SA)/nonexpressor of pathogenesis-related protein1 (NPR1) pathway (Mur *et al.*, 2006; Weech *et al.*, 2008). *S. exigua* growth (biomass) was higher when caterpillars were fed on *coil* mutant plants compared to *etr1* and *npr1* genotypes (Mewis *et al.*, 2005); this suggests that JA pathway COI1 is needed for defense responses but insects use the SA/NPR1 and ethylene pathways to circumvent plant defenses, such as GSs. Noctuid caterpillar LS is rich in oxidoreductase enzymes, such as glucose oxidase (GOX), that is believed to be key effector in the modulation of host plant responses (Afshar *et al.*, 2010; Eichenseer *et al.*, 1999; Eichenseer *et al.*, 2010; Musser *et al.*, 2002; Weech *et al.*, 2008)). The hydrogen peroxide generated by GOX may act as an upstream signal activating the SA/NPR1 pathway (Shapiro and Zhang, 2001). Recently, Van der Does *et al.* (2013) showed that negative regulation of the JA-induced defenses by SA/NPR1 pathway occurs downstream of SCF^{COI1}-mediated protein degradation instead through the ORA59 transcription factor. However, other plant hormone pathways, such as GAs, must also contribute to this crosstalk to optimize and fine tune the plant’s response to changing environmental conditions.


*Arabidopsis* quadruple-della (quad-della) mutant plants have knockouts in four of these five DELLA proteins, *gai-t6, rga-t2, rgl1-1* and *rgl2-1*, resulting in constitutively elevated GA responses (Achard *et al.*, 2008).

Crosstalk between the GA and JA pathway most likely occurs via DELLA proteins (Hou *et al.*, 2010; Wild *et al.*, 2012; Yang *et al.*, 2012). In vegetative tissues, JA signaling induces expression of the gene encoding the DELLA protein RGL3 which competes with MYC2 for binding to JAZ proteins (Hou *et al.*, 2010; Wild *et al.*, 2012). Thereby, DELLA proteins act to enhance JA-induced defense responses by repressing the activity of the negative regulator JAZ proteins. Also, by interfering with GA-degradation of DELLA proteins, JA prioritizes defensive
over growth-related pathways (Heinrich et al., 2012; Yang et al., 2012). In floral tissues, DELLA proteins interact directly with MYC2 to repress JA-dependent expression of genes encoding sesquiterpene synthases (Hong et al., 2012). Since caterpillar LS-mediated suppression of induced plant defenses is believed to involve effectors that generate reactive oxygen species (ROS), such as hydrogen peroxide, and DELLA proteins act to scavenge and reduce ROS levels, DELLA proteins may also play a role in plant-insect interactions by weakening caterpillar LS-dependent induced responses (Achard et al., 2008; Bede et al., 2006; Musser et al., 2002; Paudel et al., 2013; Weech et al., 2008). Expression of NPR1 is induced by treatment of Arabidopsis with GAs (Alonso-Ramírez et al., 2009). This implies that DELLA proteins may act to suppress the NPR1 pathway that would, again, weaken caterpillar LS-mediated attenuation of induced responses.

In this study, Arabidopsis responses to herbivory by 4th instar S. exigua caterpillars were compared in wildtype Landsberg erecta (Ler) and quad-della mutant plants. The role of LS in these interactions was determined by using caterpillars manipulated to generate two populations; one with intact LS secretions and the other with impaired LS secretions. The focus of this study was early changes at the hormonal, gene expression and defensive protein and metabolite levels within the first 10 hrs after the onset of herbivory to evaluate the role of JA vs GA trade-offs in this plant-insect interaction. We recorded systemic changes in five plant hormones, including jasmonic acid (JA), its biologically active conjugate jasmonoyl-L-isoleucine (JA-Ile), and its precursor OPDA, which is also an important signaling molecule in plant-insect interactions (Farmer et al., 2003; Fonseca et al., 2009; Taki et al., 2005). Additionally, we analyzed changes in SA and abscisic acid (ABA). Increases in ABA levels are often observed in response to mechanical wounding, possibly as a response to water loses due to the damage (Erb et al., 2012).

In addition, representative genes of the JA/ET pathway (AtPDF1.2), the JA/MYC2 pathway (AtLOX2 and VSP2) and the SA pathway (AtPR1) were analysed. Expression of AtPDF1.2b (At2g26020), is negatively regulated by MYC2 (Boter et al., 2004; Dombrecht et al., 2007; Lorenzo et al., 2004; Penninckx et al., 1998; Pre et al., 2008). In addition, late expression of this gene is further activated by the NPR1/TGA pathway (Zander et al., 2010). LOX2 is the rate-limiting enzyme in JA biosynthesis and rapidly induced in response to jasmonate, wounding or caterpillar herbivory (Bell and Mullet, 1993). AtVSP2 expression is another marker for the MYC2-branch of the JA pathway (Dombrecht et al., 2007). Pathogenesis-related 1 (AtPR1, At2g14610) expression, a marker of the SA/NPR1 pathway, is induced in response to infection by biotrophic pathogens and aphids (Glazebrook, 2005; Kusnierczyk et al., 2007; Mur et al., 2006; Walling, 2008; Zhang et al., 1999). Given the competition between DELLA proteins and MYC2 for the JAZ proteins, we expected a decrease in positively regulated MYC2-dependent markers in the quad-della mutant following insect herbivory (Hou et al., 2010; Wild and Achard, 2013; Wild et al., 2012). Also, since caterpillar LS effector(s) may exert the suppression of JA-
induced responses through the generation of ROS and DELLA proteins scavenge these compounds and DELLA proteins suppress the NPR1 pathway, we expected a stronger caterpillar LS-dependent suppression of JA-mediated responses in the quad-della mutants (Achard et al., 2008; Alonso-Ramírez et al., 2009; Bede et al., 2006; Musser et al., 2002; Paudel et al., 2013; Weech et al., 2008). In addition to measuring hormone levels and gene expression, we also assessed other inducible plant defences, i.e. TI, LMCOs and GS, that, alone or in combination, may negatively affect the herbivore..

**Materials and Methods**

**Chemicals**

Chemicals used in this study were obtained from Sigma Chemical Company, unless otherwise specified.

**Plant cultivation**

Wild type *Arabidopsis thaliana* cv Landsberg erecta (Ler) and the quadruple-della mutant (quad-della: gai-t6, rga-t2, rgl1-1, rgl2-1) seeds were grown in pasteurized (80°C for 2 hrs) Agro Mix. After stratification at 4°C for 2 days, the seeds germinated in a phytotrim (8:16 light:dark, 250 µE m⁻² s⁻¹, 23°C). As GAs regulate multiple aspects of plant development, wildtype and quad-della mutants were grown under short day conditions to synchronize vegetative growth and prevent the onset of bolting and flowering (Cheng et al., 2004, Davière and Achard 2013). Plants were bottomed watered as needed with dilute 0.15 g/L N-P-K fertilizer. At approx. 2 weeks, plants were removed to leave 3 evenly-spaced *Arabidopsis* plants per pot.

**Insect maintenance**

*Spodoptera exigua* caterpillars were maintained on a meridic wheat germ-based artificial diet (Bio-Serv) (16:8 light:dark, 28-40% humidity, 22°C). Eggs collected from mated adults were used to maintain the colony for >30 generations.

**Herbivory experiment**
Approx. 5 week old plants (growth stages 1.11-1.14 (Boy es et al., 2001)) were either control (no insects) or subject to herbivory by 4th instar S. exigua caterpillars with intact (cat.) or impaired (caut.) LS secretions. To prevent LS secretions, caterpillar spinnerets were cauterized (caut.) insects (Musser et al., 2002). As caterpillar LS contains high levels of the enzyme glucose oxidase (GOX), success of cauterization was tested by allowing caterpillars to feed on glass discs presoaked in glucose/sucrose solution (5 mg each sugar) and observing GOX activity through the peroxidase/3,3’-diaminobenzidine assay (Weech et al., 2008). Both subsets of caterpillars (cat. and caut.) were allowed to feed on wild type Arabidopsis for 12 hours before the beginning of the herbivory experiment to allow them to adjust to a plant diet.

To either wildtype (Ler) or the quad-della mutant, three 4th instar caterpillars were placed in each pot that was then enclosed by netting to prevent caterpillar escape. As S. exigua caterpillars feed more actively at night, the experiments were initiated in the dark. Insects were placed on the plants 4 hr after the plant’s transition to dark. To minimize the effect of plant volatile signaling in the growth cabinets, pexiglass plates separated the different treatments (control, cat., caut.).

After 10 hrs, caterpillars were removed and plants were flash-frozen in liquid nitrogen. The 3 plants in each pot were pooled to prepare one sample. For hormone analysis, the entire above ground portions of the plant were taken. For gene expression and defensive compound and protein analyses, only caterpillar-damaged leaves were collected to focus on local responses. Samples were stored in a -80°C freezer until analysis. This experiment was repeated 8 independent times. For hormone analysis, gene expression and GS analysis, 4 biological replicates were analyzed. For defensive protein and biomass loss experiments, 8 biological replicates were used.

To calculate biomass loss, aerial tissues were dried for 3 d at 70°C. Twenty to 29% of plant tissue was consumed by caterpillars, regardless of plant genotype. Cauterization of the caterpillar spinneret did not affect feeding.

**Hormone analysis**

Lyophilized plant samples were ground using a TissueLyser (Qiagen) and tissues sent to the Danforth Plant Science Centre for hormone analysis by liquid chromatography-mass spectroscopy/tandem mass spectroscopy (LC-MS/MS). Samples were spiked with deuterium-labeled internal standards of salicylic acid (D5-SA), abscisic acid (D6-ABA) and jasmonic acid (D2-JA). Samples were extracted in ice-cold methanol:acetonitrile (MeOH:ACN, 1:1, v/v) using a TissueLyser for 2 min at a frequency of 15 Hz/sec, then centrifuged at 16,000 x g for 5 min at 4°C. Supernatants were transferred to new tubes and the pellets re-extracted. After the
supernatants were pooled, samples were evaporated using a Labconco Speedvac. Pellets were redissolved in 200 µL of 30% MeOH and analyzed by LC-MS/MS.

LC separation was conducted on a Shimadzu system by reverse-phase chromatography on a monolithic C18 column (Onyx, 4.6 mm x 100 mm, Phenomenex). A gradient of 40% solvent A (0.1% acetic acid in HPLC-grade water (v/v)) held for two minutes to 100% solvent B (90% ACN with 0.1% acetic acid (v/v)) for 5 min was used with a flow rate of 1 mL/min. The LC system was interfaced with an AB Sciex QTRAP mass spectrometer equipped with a TurboIonSpray (TIS) electrospray ion source in negative mode. Parameters were set to: capillary voltage -4500, nebulizer gas (N2) 50 arbitrary units (a.u.), heater gas 50 a.u., curtain gas 25 a.u., collision activation dissociation, high, temperature 550°C. Each hormone was detected using MRM transitions that were previously optimized using each standard and deuterium-labeled standard. Concentrations were determined using a standard curve prepared from a series of standard samples containing different hormone concentrations.

Gene expression

Total RNA was extracted from Arabidopsis leaves finely ground in liquid nitrogen using a sterile mortar and pestle using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. After assessing RNA quality spectrophotometrically, genomic contamination was enzymatically degraded and verified by using a primer pair that spanned an intronic region (AtLMCO4, Supplemental Table I).

Transcript levels were measured in duplicate by quantitative real time-polymerase chain reaction (qRT-PCR) using Absolute Blue qPCR SYBR low ROX mix (Fisher Scientific) according to the manufacturer’s instructions. Each well contained Blue qPCR SYBR low Rox (Fisher), 1 or 3 nM forward and reverse primers and cDNA (1/10 dilution). The following PCR program was used: 95°C for 15 min followed by 40 cycles of 95°C for 15 sec, annealing temperature for 30 sec (Supplemental Table 1), 72°C for 30 sec. Dissociation curves confirmed amplicon purity. Two technical plates were performed.

From the standard curve, relative gene expression was measured. Expression of two reference genes (AtAct2/7 and AtUnk (At4g26410)) were not affected by treatment (Ler: AtAct2/7 F(2,9) = 0.73, p = 0.51; AtUnk F(2,9) < 0.19, p = 0.83; Quad-della mutant: AtAct2/7 F(2,9) = 2.43, p = 0.143; AtUnk F(2,9) = 0.42, p = 0.67) (Supplement Table X). The geometric mean of AtAct2/7 and AtUnk was used to normalize expression of genes-of-interest (Brunner et al., 2004; Pfaffl et al., 2004; Vandesompele et al., 2002).

Defense protein analysis
**Protein extraction**

Samples were ground to a fine powder in liquid nitrogen. Proteins were extracted in ice-cold extraction buffer 0.1 M sodium phosphate buffer, pH 7.0 containing 0.1% Triton X-100 and 7% polyvinylpyrrolidone. For the extraction of proteins to be analyzed for LCMO activity, a broad-spectrum proteinase inhibitor solution (1 x) was added to prevent protein degradation. Samples were vigorously vortexed and centrifuged at 13,000 rpm for 10 min. Supernatants were used for protein assays.

**Trypsin inhibitor (TI) assay**

Leaf trypsin inhibitor activity was measured according to Lara *et al.* (2000). In a 96-well plate format, trypsin (0.5 µg) was added to samples prepared in triplicate and incubated for 20 min at 37°C with gentle shaking in a Infinite M200 Pro microplate reader (Tecan). The trypsin substrate, N-benzoyl-DL-arginyl-β-naphthylamine (final concentration: 3 mM), was added. After an 80 min. incubation, the reaction was inhibited by the addition of 4% HCl. After addition of the colourmetric reagent, p-dimethyl-amino-cinnamaldehyde (final concentration: 0.24%), the product absorbance was read at 540 nm. All plates contained negative controls and a standard curve of soybean trypsin inhibitor (concentration range, 0-5 µg).

**Laccase-like multicopper oxidase (LMCO) activity**

LMCO, also known as polyphenol oxidase (PPO), activity was measured according to Espín *et al.* (1997) with minor modifications. To samples in triplicate, N,N-dimethyl formamide (final concentration: 2%), 3-methyl-2-benzothiazolinone hydrozone hydrochloride monohydrate (MBTH, final concentration: 2 mM) and dopamine hydrochloride (final concentration: 35 mM) are sequentially added. Controls included tyrosinase and enzyme-free and boiled controls. Activity was monitored by measuring absorbance at 476 nm at 30 sec intervals for 5 min at 35°C and LMCO activity was calculated using the molar extinction co-efficient of the MBTH-quinone adduct (20,700 M⁻¹ cm⁻¹).

**Modified Bradford assay**

Soluble protein concentration in leaf extracts were measured by a modified Bradford assay using a bovine serum albumin (BSA) standard curve (5-100 µg/mL) (Bradford, 1976; Zor and Selinger, 1996). Samples and BSA standard curve were incubated with Bradford reagent for 10
min followed by measurement of absorbance at 590 nm and 450 nm. The ratio of OD$_{590}$/OD$_{450}$ was used to calculate soluble protein concentration.

**Glucosinolate analysis**

GS analysis was performed as previously described (Hogge et al., 1988; Kliebenstein et al., 2001). Lyophilized samples were finely ground using a pre-cooled TissueLyser (Qiagen) and 50.0 mg dry material was weighed in a 2 mL Eppendorf tube, extracted twice with 1 mL of 70% methanol solution followed by 15 minutes ultra-sonification. During the first extraction, the tube was placed in a 90°C water bath for 10 minutes after the addition of the methanol to immediately inhibit any myrosinase activity. After sonification, tubes were centrifuged at 4500 rpm (2975 rcf) for 10 minutes. Pooled supernatants were cleaned-up by ion exchange chromatography using a diethylaminoethyl Sephadex A-25 column preconditioned with sterile MilliQ water. After washing with 70% methanol (2 x 1 mL), MilliQ water (2 x 1 mL) and 20 mM sodium acetate buffer, pH 5.5 (1 x 1 mL), GSs were desulfated by the addition of 10 U arylsulfatase and incubated at room temperature overnight. Desulphated GSs were eluted with sterile milliQ water (2 x 0.75 mL). The combined eluted was freeze-dried and redissolved in MilliQ water (1 mL).

Desulphoglucosinolates were separated by high performance liquid chromatography (DIONEX summit HPLC) on a reversed phase C$_{18}$ column (Alltech C$_{18}$, 150 x 4.6 mm, 3µm, Alltech) using an acetonitrile-water gradient (2-35% acetonitrile from 0-30 min; flow rate 0.75 mL/min). Compounds were detected by a photodiode array detector (PDA). Peaks were integrated at 229 nm (EC, 1990).

GS were identified based on retention time, UV spectrum, MS analysis of selected *A. thaliana* reference samples and the following reference standards (Phytoplan, Germany); glucoiberin (3-methylsulfonylpropylGSL), glucoerucin (4-methylthiobutylGSL), progoitrin (2-hydroxy-3-butenylGSL), sinigrin (2-propenylGSL), gluconapin (3-butenylGSL), glucobrassicinapin (4-pentenyGSL), glucobrassicin (indol-3-ylmethylGSL), sinalbin (4-hydroxybenzylGSL), glucotropaeolin (benzylGSL), gluconasturtiin (2-phenylethylGSL). Sinigrin (63, 188, 375, 500 and 625 µM; Sigma-Aldrich) was used as an external standard. Correction factors were used to calculate GS concentrations based on the sinigrin reference curve (Brown et al., 2003; Buchner, 1987; EC, 1990).

**Statistical analysis**

GAs are involved in multiple aspects of plant development (Davière and Achard, 2013). Therefore, to avoid potentially confounding phenological differences between wildtype Ler and quad-della mutant plants, statistical differences (p ≤ 0.05) were determined within each
genotype by one-factor analysis of variance (ANOVA) using SPSS version 20 (SPSS Inc.)
followed by a Tukey HSD post hoc test. Results from statistical analyses are shown in
Supplemental Table 2. Gene expression can be highly variable; therefore, either a statistically
significant difference or >5-fold increase over constitutive control levels was considered an
increase in transcript levels.

**Results**

**Caterpillar herbivory results in a foliar labial saliva-dependent jasmonate burst**

A rapid jasmonate (OPDA, JA, JA-Ile) burst was observed systemically in response to caterpillar
herbivory (Fig. 1 A-C, Supplemental Table 2). It is important to note that significantly higher
jasmonate levels were observed in plants attacked by caterpillars with impaired LS secretions
compared to normal caterpillars (Fig. 1A-C, Supplemental Table 2). This LS-dependent
suppression of JA-related hormone levels was alleviated in the quad-della mutant indicating that
DELLA proteins are required for the caterpillar LS-mediated interference with plant defense
responses.

Caterpillar or LS-dependent changes in SA hormone levels was not observed in wildtype
or quad-della mutant (Fig. 1D, Supplemental Table 2). ABA levels were highly variable and
though a trend might be seen, further studies are needed to understand the role of ABA in these
interactions (Fig. 1E, Supplemental Table 2).

**Early gene expression in response to caterpillar herbivory**

Early transcript expression of defense-related genes was analyzed in caterpillar-wounded tissues.
Expression of the JA-dependent marker gene *AtPDF1.2* expression increased over 5-fold in
response to caterpillar herbivory (Fig. 2A, Supplemental Table 2); a LS-dependent difference
was not observed in wildtype plants. In comparison, in the quad-della mutant, an increase in
*AtPDF1.2* expression was dependent upon caterpillar secretion of LS. Both *AtLOX2* and *AtVSP2*
exhibited the same expression pattern and were strongly induced in response to herbivory in the
wildtype Ler and quad-della mutant plants (Fig. 2B and C, Supplemental Table 2); a caterpillar
LS effect was not observed.

Caterpillar herbivory did not affect *AtPR1* expression in wildtype plants (Fig. 2D,
Supplemental Table 2). In comparison, high constitutive *AtPR1* levels in the quad-della mutant
plants were suppressed in response to herbivory by caterpillars with impaired LS secretions.
Caterpillar herbivory results in an increase in the indole glucosinolate 4-methoxyglucobrassicin (4-MGB)

Local defense responses of the plant were measured through the analysis of secondary metabolites and defense-related proteins. Both indole and aliphatic GS were identified in Ler leaves (Table 1, Fig. 3A) Though indole GS levels were comparable to previous reports, lower levels of aliphatic compounds were identified in this study which may reflect the differences in growth conditions (Brown et al., 2003; Kliebenstein et al., 2001); an approximate 50% decrease in levels of the main aliphatic GS, 2-hydroxypropyl GS, accounts for much of this discrepancy.

Levels of aliphatic GS were not affected by caterpillar herbivory (Table 1, Supplemental Table 2). In contrast, 4-methoxyglucobrassicin (4-MGB) was induced ~25-40% in response to caterpillar herbivory in Ler but not in the quad-della mutants (Fig. 3A and B, Table 1, Supplemental Table 2). Levels of the other indole GS did not change upon caterpillar feeding.

A LS-specific induction of GS levels was not observed (Fig 3A and B, Table 1, Supplemental Table 2). However, the increase in 4-MGB observed in response to caterpillar herbivory was alleviated in the quad-della mutant suggesting that DELLA proteins are important in the JA-dependent regulation of GS biosynthesis.

Caterpillar herbivory does not affect early defensive protein activity: trypsin inhibitor (TI) and laccase-like multicopper oxidases (LMCO)

Constitutive TI levels did not increase in the early response to caterpillar herbivory or LS in either wildtype Ler or the quad-della mutant plants (Fig. 4A, Supplemental Table 2). In wildtype Ler plants, constitutive LMCO activity did not increase in response to herbivory (Fig. 4B, Supplemental Table 2). In comparison, a significant increase in LMCO activity was observed in the quad-della mutant when plants were infested by caterpillars with intact salivary secretions.

DISCUSSION

Responses to caterpillar herbivory

As a plant faces multiple challenges in the environment, there are trade-offs between growth and defense. Two key hormone systems that regulate these physiological processes are gibberellin/DELLA proteins for growth and JAs/JAZ proteins involved in plant defense against chewing herbivores, such as caterpillars (Ballare, 2011; Robert-Seilaniantz et al., 2011). The crosstalk between these two pathways integrates environmental information with plant development to shape the physiological response of the plant. JA interferes with the GA-
mediated degradation of the negative growth regulator DELLa proteins (Heinrich et al., 2012; Yang et al., 2012). As well, DELLa proteins enhance JA-dependent responses by competing with the transcriptional activator MYC2 for the negative regulator JAZ proteins (Hou et al., 2010; Wild et al., 2012). This study investigated the potential crosstalk between the GA/DELLa and the JA pathway in the early plant responses to caterpillar herbivory (10 hr). In addition, the role of caterpillar labial salivary effector(s) in these interactions was determined.

Caterpillar infestation of both wildtype and the quad-della mutant plants results in a strong systemic jasmonate burst as has been witnessed in many other plant-caterpillar models, including wild tobacco-Manduca sexta and tomato-Helicoverpa zea (Fig. 1A-C)(Diezel et al., 2009; Tian et al., 2012). In contrast, caterpillar-specific changes in SA hormone levels are not observed in these two genotypes as was also noted by Weech et al. (2008) and Tian et al. (2012) (Fig. 1D).

Transcript expression of marker genes of the JA- and SA-pathways were further analyzed. AtVSP2 and AtLOX2 are well characterized markers of the MYC2 branch of the JA pathway (Bell and Mullet, 1993; Dombrecht et al., 2007; Kazan and Manners, 2013). AtPDF1.2 is induced synergistically in response to JA and ethylene, negatively regulated by MYC2 and late expression requires the NPR1/TGA pathway (Penninckx et al., 1998; Zander et al., 2010). Given the strong jasmonate burst, it is not surprising that in Ler wildtype and quad-della mutant plants, AtVSP2, AtLOX2 and AtPDF1.2 are strongly induced in response to caterpillar herbivory (Fig. 2A-C).

In contrast, caterpillar herbivory did not affect SA hormone levels or expression of the SA-dependent gene AtPRI (Fig. 1D, Fig. 2D). Tian et al. (2012) also found that SA-dependent early gene expression was not affected by caterpillar herbivory. In stark contrast, Paudel et al. (2013) observed a strong 5-fold induction of AtPRI expression in response to caterpillar herbivory. This likely reflects temporal differences in the experimental design where in this study and Tian et al. (2012) evaluated gene expression at 10 hr or less after the initiation of herbivory compared to Paudel et al. (2013) where AtPRI transcript levels were measured 36 hr after herbivory.

Glucosinolates are the principal defensive compound in Arabidopsis (Halikier and Gershenzon, 2006; Hopkins et al., 2009). Levels of aliphatic GS are not affected by caterpillar herbivory (Table 1); contrary to previous studies where in the Col background, Mewis et al. (2005) noticed an increase in short-chain aliphatic methylsulfinyl GS in response to S. exigua herbivory. However, the levels and types of GS and, presumably, the regulation differ between Arabidopsis genotypes (Kliebenstein et al., 2001; Kusnierczyk et al., 2007). In response to caterpillar feeding, local levels of the indole GS 4-MGB significantly increase (Fig. 3A). Principle component analysis of Arabidopsis ecotypes identified this GS as an important compound negatively effecting S. exigua larval growth (Mosleh Arany et al., 2008). However,
this increase in 4-MGB was only observed in wildtype but not in the quad-della mutant plants, suggesting that DELLA proteins may be involved in the regulation of some branches of GS biosynthesis.

TI or LMCO activity do not increase in the early responses of wildtype Arabidopsis plants to caterpillar herbivory (Fig. 4A,B). In comparison, LMCO increases in quad-della mutant plants infested by caterpillars with intact salivary secretions. This result was unexpected. However, LMCO enzymes are involved in many physiological functions in the plant, including the lignification of cell walls (Cai et al., 2006; Constabel and Barbehenn, 2008; Thipyapong et al., 1997). Therefore, DELLA proteins may negatively regulate LMCO activity in response to caterpillar herbivory.

Together, these data support previous research which shows that in response to stress, JA-mediated defense responses take priority over GA-dependent growth processes (Heinrich et al., 2012; Hou et al. 2010; Wild et al., 2012; Yang et al., 2012). Our data suggests that DELLA proteins may be involved in the regulation of GS and also suppress LMCO activity, which may be related to their role in plant cell wall fortification (Cai et al., 2006; Constabel and Barbehenn, 2008; Thipyapong et al., 1997).

Caterpillar labial saliva-specific responses

Since caterpillar LS has been implicated as a stratagem to modify plant induced defenses (Musser et al., 2002; Tian et al., 2012; Weech et al., 2008), we compared plant induced responses to caterpillars with intact vs. impaired LS secretions. Arabidopsis plants subject to herbivory by caterpillars with impaired LS secretions have a significantly higher jasmonate levels (OPDA, JA, JA-Ile) compared to normal S. exigua, indicating that the labial saliva contains effector(s) that suppress this jasmonate burst in response to herbivory (Fig. 1A-C). Weech et al. (2008) observed a similar distinction in jasmonic acid levels between plants infested by caterpillars with intact and impaired salivary secretions. In contrast, in the quad-della mutants, the LS-dependent difference in jasmonate levels is not observed (Fig. 1A-C). Therefore, DELLA proteins are required for caterpillar LS-dependent suppression of plant defense hormones.

Even though a LS-specific difference in jasmonate levels is observed, the expression of JA-dependent genes shows a slightly different pattern (Fig. 2A-C). Expression of AtPDF1.2, AtLOX2 and AtVSP2 are strongly induced in response to herbivory; however, caterpillar LS-differences in transcript expression are not observed. Similar observations for AtLOX2 have been previously made (Paudel et al., 2013; Tian et al., 2012; Weech et al., 2008). However, AtPDF1.2 suppression by caterpillar LS effectors is well recognized (Paudel et al., 2013; Weech et al., 2008). This likely reflects the temporal regulation of this gene. Zander et al. (2010) have shown that the SA/NPR1-dependent TGA transcription factors regulate late but not early AtPDF1.2
gene expression and caterpillar LS-mediated suppression of plant induced defenses is believed to involve the SA/NPR1/TGA pathway possibly by a mechanism as elucidated by Van der Does et al. (2013) (Paudel et al., 2013; Weech et al., 2008).

In the quad-della mutant, expression of AtLOX2 and AtVSP2 parallel the wildtype plants (Fig. 2B,C). In contrast, expression of AtPDF1.2 was only induced in response to herbivory by caterpillars with intact salivary secretions in the quad-della mutant suggesting a complex relationship with DELLA proteins in the regulation of this gene (Fig. 2A).

Caterpillar LS-specific differences in SA levels was not observed and this is reflected in the expression of the marker gene AtPR1 in the wildtype plant (Fig. 1D, 2D). In contrast, high constitutive AtPR1 levels of the quad-della mutant were suppressed in response to herbivory by caterpillars with impaired LS secretions (Fig. 2D). A possible explanation is that herbivory by caterpillars with impaired LS secretions leads to a strong activation of JA-responses which is known to interfere with the SA/NPR1 pathway and, thus, a suppression of AtPR1 expression is observed (Laurie-Berry et al., 2006; Zarate et al., 2007).

Plant defensive compounds and protein activity analyzed in this study were not affected by caterpillar LS (Fig. 3 and 4).

CONCLUSION

Our results show a link between DELLA proteins and the regulation plant defenses, such as GS, in response to insect stress (Fig. 4B) and in the caterpillar LS-mediated suppression of plant defense hormone biosynthesis (Fig. 1A-C). Previous models propose that caterpillar LS effector(s) manipulate plant defenses through the generation of ROS, such as hydrogen peroxide, that activate the NPR1/TGA pathway to modulate induced plant defenses (Eichenseer et al., 1999; Musser et al., 2002; Paudel et al., 2013; Tian et al., 2012; Weech et al., 2008). DELLA proteins are known to scavenge hydrogen peroxide (Achard et al., 2008). As well, treatment of Arabidopsis with GAs results in the activation of the NPR1 pathway (Alonso-Ramírez et al., 2009). Therefore, in the quad-della mutant, we expected a stronger LS-dependent response which was not observed. Therefore, the mechanism underlying the involvement of GA/DELLA in these plant-insect interactions is as yet unknown but may involve competition between DELLA proteins and MYC transcription factors for negative regulator JAZ proteins (Hou et al., 2010; Wild et al., 2012; Yang et al., 2012). Therefore, there appears to be multiple points of crosstalk between the JA defense pathway and the GA/DELLA pathway to ensure prioritization of plant responses to changing environmental conditions. Future studies will continue to further elucidate the underlying mechanism.

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Table 1. Glucosinolate (GS) levels in *Arabidopsis* rosette leaves subject to caterpillar herbivore. 5 week-old *Arabidopsis thaliana* subject to 4\textsuperscript{th} instar *Spodoptera exigua* caterpillar herbivory for 10 hr (n = 4). Caterpillars either had intact (cat.) or impaired (caut.) labial salivary secretions. A significant increase in GS 4-methoxyglucobrassicin was observed in response to herbivory in wildtype *Arabidopsis* (Ler: F\(_{2,9}\) = 6.17, p = 0.02). Alphabetic letters indicate significant differences due to herbivory within each genotype (Ler or quad-	extit{della} mutant).

<table>
<thead>
<tr>
<th>GS (nmol/g DW)</th>
<th>Ler</th>
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<th>Ler</th>
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<tr>
<td></td>
<td>Control</td>
<td>Cat.</td>
<td>Caut.</td>
<td>Control</td>
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<tr>
<td>3-Hydroxy propyl GS</td>
<td>4020.7 ± 516.4</td>
<td>4415.8 ± 604.4</td>
<td>4792.3 ± 897.1</td>
<td>4966.4 ± 712.4</td>
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<tr>
<td>Glucoiberin</td>
<td>196.0 ± 84.5</td>
<td>258.9 ± 113.4</td>
<td>182.3 ± 5.9</td>
<td>331.0 ± 109.4</td>
</tr>
<tr>
<td>Glucoraphanin</td>
<td>93.1 ± 11.43</td>
<td>233.0 ± 138.3</td>
<td>112.5 ± 21.5</td>
<td>142.4 ± 24.0</td>
</tr>
<tr>
<td>Glucobrassicin</td>
<td>2203.4 ± 293.9</td>
<td>2392.3 ± 297.8</td>
<td>2738.6 ± 300.7</td>
<td>2251.7 ± 256.6</td>
</tr>
<tr>
<td>Neo-glucobrassicin</td>
<td>29.9 ± 6.0</td>
<td>39.7 ± 8.3</td>
<td>47.5 ± 8.0</td>
<td>25.5 ± 5.1</td>
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<tr>
<td>4-Methoxyglucobrassin</td>
<td>190.3 ± 24.3</td>
<td>269.4 ± 20.1</td>
<td>275.1 ± 9.8</td>
<td>207.0 ± 36.1</td>
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Figure Legends

**Fig. 1. Phytohormone levels in Arabidopsis rosette leaves subject to caterpillar herbivory.** Arabidopsis plants ((Ler, Ler + GA, quad-della mutant) were subject to herbivory by Spodoptera exigua caterpillars with intact (cat) or impaired (caut) labial salivary secretions for 10 hr. Plant hormones A) 12-oxo-phytodienoic acid (OPDA), B) jasmonic acid (JA), C) jasmonoyl-isoleucine (JA-Ile), D) salicylic acid (SA) and E) abscisic acid (ABA) were measured by LC-MS/MS. Bars represent the means of three to four independent biological replications ± standard error. Alphabetical letters indicate significant differences in response to caterpillar herbivory (p < 0.05) (Supplemental Table 2). An asterix indicates ≥5-fold increase in expression levels compared to control plants.

**Fig. 2. Defense gene expression in Arabidopsis rosette leaves in response to caterpillar herbivory.** Arabidopsis plants ((Ler, Ler + GA, quad-della mutant) were subject to herbivory by Spodoptera exigua caterpillars with intact (cat) or impaired (caut) labial salivary secretions for 10 hr. Expression levels of marker genes of the jasmonate-pathway A) AtPDF1.2 (JA- and Et-dependent) B) AtVSP2 (JA-, MYC2-dependent), C) AtLOX2 (JA-, MYC2-dependent) and D) PR1 (SA-/NPR1-dependent) were measured by quantitative real time-polymerase chain reaction and normalized by the expression of two reference genes (AtAct2/7 and AtUnk). Bars represent the means of three to four independent biological replications ± standard error. Alphabetical letters indicate significant differences in response to caterpillar herbivory (p < 0.05) (Supplemental Table 2).

**Fig. 3. Glucosinolate (GS) profile in Arabidopsis rosette leaves subject to caterpillar herbivory.** Arabidopsis plants (Ler, Ler + GA, quad-della mutant) were subject to herbivory by Spodoptera exigua caterpillars with intact (cat) or impaired (caut) labial salivary secretions for 10 hr. Compounds extracted from lyophilized samples were desulfated and subject to HPLC analysis. A) represents the total profile of GS in Arabidopsis plants; a significant change in total or individual GS levels were not observed under these treatments with the exception of 4-methoxyglucobrassicin (4-MGB) which is highlighted in B). Bars represent the means of three to four independent biological replications ± standard error. Alphabetical letters indicate significant differences in response to caterpillar herbivory (p < 0.05) (Supplemental Table 2).

**Fig. 4. Levels and activities of defensive proteins in Arabidopsis rosette leaves subject to caterpillar herbivory.** 5 week old Arabidopsis plants ((Ler, Ler + GA, quad-della mutant) were subject to herbivory by 4th instar Spodoptera exigua caterpillars with intact (cat) or impaired (caut) labial salivary secretions for 10 hr. Defensive proteins, A) trypsin inhibitor levels and B)
laccase-like multicopper oxidase (LMCO) activity, were measured. Bars represent the means of three to four independent biological replications ± standard error. Alphabetical letters indicate significant differences in response to caterpillar herbivory (p < 0.05) (Supplemental Table 2).
Figures

Fig. 1
Fig. 2

A. 

B. 

C. 

D.
Fig. 3

A. Total glucosinolates (GS) (μmol/g DW)

- Indole GS
- Aliphatic GS

B. 4-Methoxyglucobrassicin (μmol/g DW)

- Ler
- quad-della
Fig. 4

A. Tryptsin inhibitor (TI) (ng TI/μg soluble protein)

B. Lactase-like multicopper oxidase (LMCO) (U/mg soluble protein)