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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 26*S* 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-hydroxylpropyl glucosinolate and glucobrassicin, respectively, being prominent in Landsberg (L*er*) 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 by *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 salicyclic 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 *coi1* 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.

Diterpenoid GA phytohormones promote growth-related physiological processes in flowering plants (Davière and Achard, 2013; Hauvermale *et al.*, 2012; Sun, 2011). Binding of GA to its receptor, Gibberellin Insensitive Dwarf1 (GID1) leads to the degradation of the negative growth regulator DELLA proteins by the 26*S*-proteasome pathway (Dill *et al.*, 2004; Fu *et al.*, 2004; Hartweck and Olsewski, 2006; Murase *et al.*, 2008; Sasaki *et al.*, 2003; Shimada *et al.*, 2008). The five DELLA proteins in *Arabidopsis* exhibit temporal and spatial differences but are functionally redundant (Gallego-Bartolomé *et al.*, 2011; Hauvermale *et al.*, 2012). *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* (L*er*) 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* (L*er*) 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 phytorium (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 (Boyes *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 (L*er*) 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 C_{18} 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 (N_2) 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 (L*er*: *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 by15 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 eluated 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 (Alltima 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-methylsulfenylpropylGSL), glucoerucin (4-methylthiobutylGSL), progoitrin (2-hydroxy-3-butenylGSL), sinigrin (2-propenylGSL), gluconapin (3-butenylGSL) , glucobrassicanapin (4-pentenylGSL), glucobrassicin (indol-3-ylmethylGSL), sinalbin (4-hydoxybenzylGSL), 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 L*er* and quad-*della* mutant plants,** statistical differences ($p \leq 0.05$) 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 L*er* 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 was measured through the analysis of secondary metabolites and defense-related proteins. Both indole and aliphatic GS were identified in L*er* 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 L*er* 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 L*er* or the quad-*della* mutant plants (Fig. 4A, Supplemental Table 2). In wildtype L*er* 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 L*er* 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 *AtPR1* (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 *AtPR1* 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 *AtPR1* 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 alsosuppress 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 epression 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 4th 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 (L*er* or quad-*della* mutant).

	L*er*			**quad-*della* mutant**		
GS (nmol/g DW)	Control	Cat.	Caut.	Control	Cat.	Caut.
3-Hydroxy propyl GS	4020.7 ± 516.4	4415.8 ± 604.4	4792.3 ± 897.1	4966.4 ± 712.4	3820.9 ± 674.8	4850.7 ± 499.8
Glucoiberin	196.0 ± 84.5	258.9 ± 113.4	182.3 ± 5.9	331.0 ± 109.4	296.9 ± 83.1	381.1 ± 118.2
Glucoraphanin	93.1 ± 11.43	233.0 ± 138.3	112.5 ± 21.5	142.4 ± 24.0	85.8 ± 9.4	126.5 ± 16.4
Glucobrassicin	2203.4 ± 293.9	2392.3 ± 297.8	2738.6 ± 300.7	2251.7 ± 256.6	2335.3 ± 230.5	2534.2 ± 89.2
Neo-glucobrassicin	29.9 ± 6.0	39.7 ± 8.3	47.5 ± 8.0	25.5 ± 5.1	35.3 ± 3.8	29.4 ± 3.4
4-Methyoxy-glucobrassin	190.3 ± 24.3[a]	269.4 ± 20.1[b]	275.1 ± 9.8[b]	207.0 ± 36.1[a]	258.4 ± 13.1[a]	252.1 ± 18.4[a]

Figure Legends

Fig. 1. Phytohormone levels in *Arabidopsis* rosette leaves subject to caterpillar herbivory. *Arabidopsis* plants ((L*er*, L*er* + 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 ((L*er*, L*er* + 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 (L*er*, L*er* + 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 ((L*er*, L*er* + 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

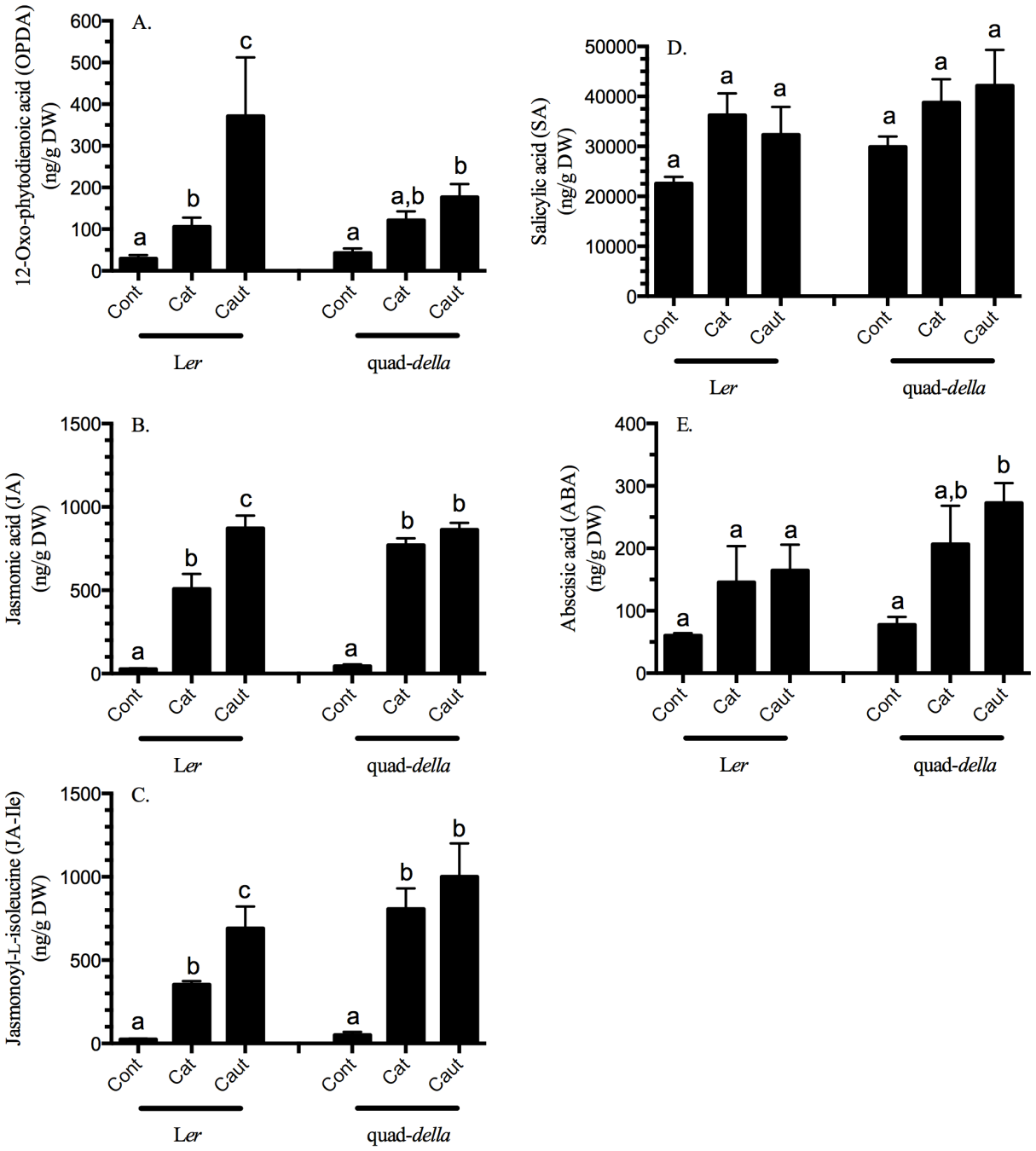


Fig. 2

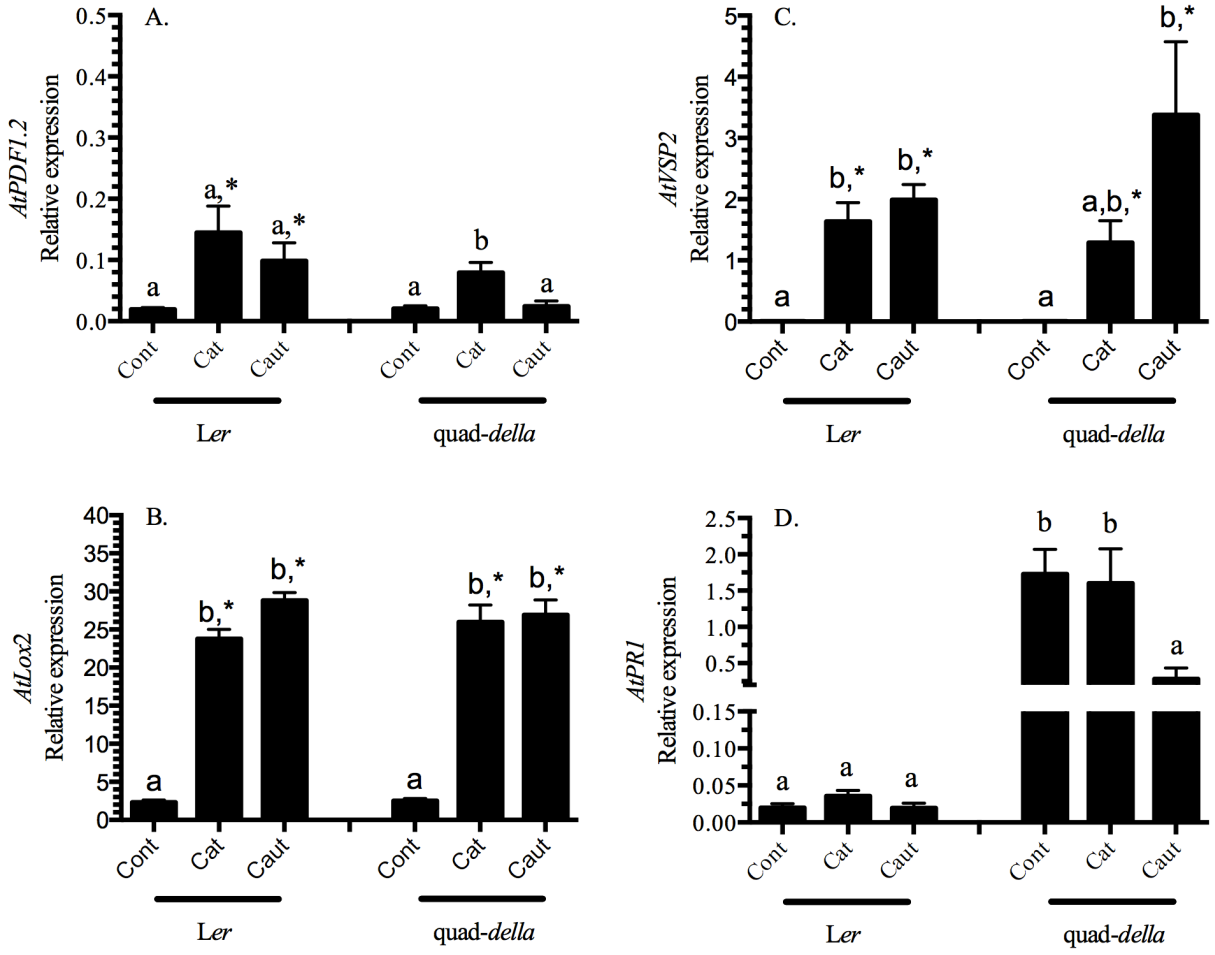

A.
AtPDF1.2
Relative expression
0.5
0.4
0.3
0.2
0.1
0.0
a
a,*
a,*
a
b
a
Cont
Cat
Caut
Cont
Cat
Caut
Ler
quad-della
C.
AtVSP2
Relative expression
5
4
3
2
1
0
a
b,*
b,*
a
a,b,*
b,*
Cont
Cat
Caut
Cont
Cat
Caut
Ler
quad-della
B.
AtLox2
Relative expression
40
35
30
25
20
15
10
5
0
a
b,*
b,*
a
b,*
b,*
Cont
Cat
Caut
Cont
Cat
Caut
Ler
quad-della
D.
AtPR1
Relative expression
2.5
2.0
1.5
1.0
0.5
0.15
0.10
0.05
0.00
a
a
a
b
b
a
Cont
Cat
Caut
Cont
Cat
Caut
Ler
quad-della

Fig. 3

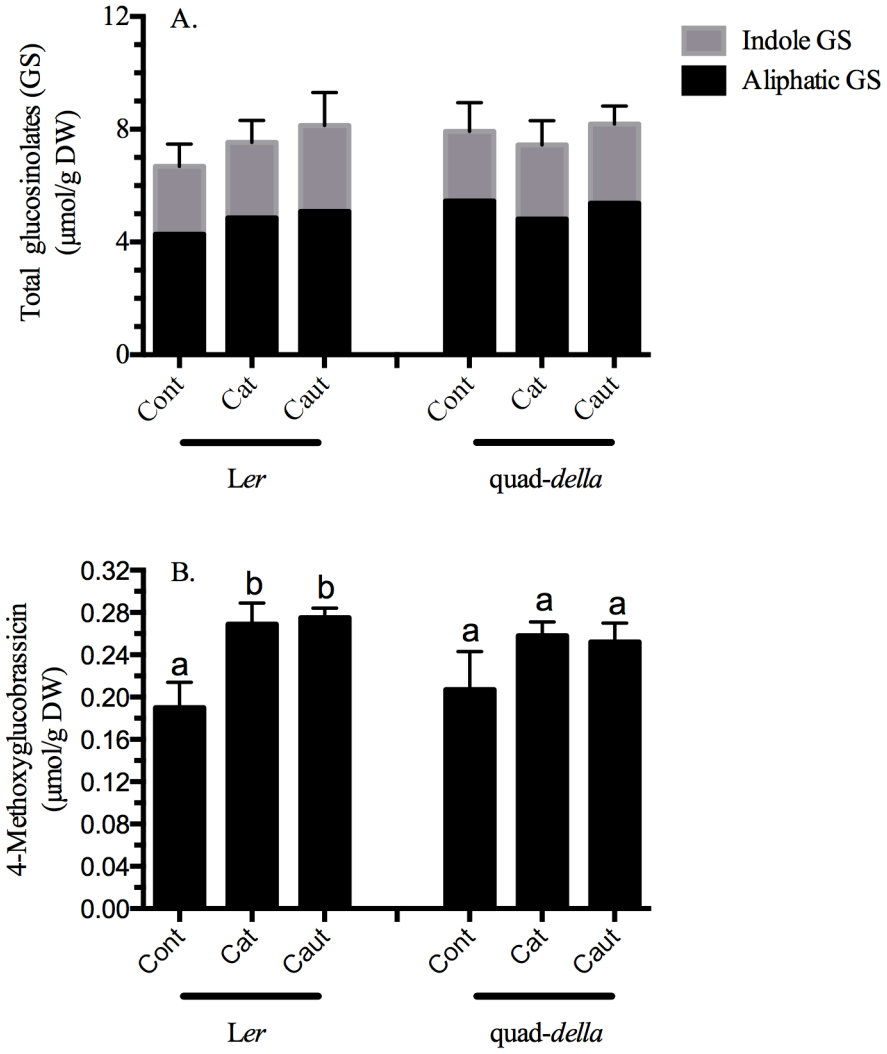
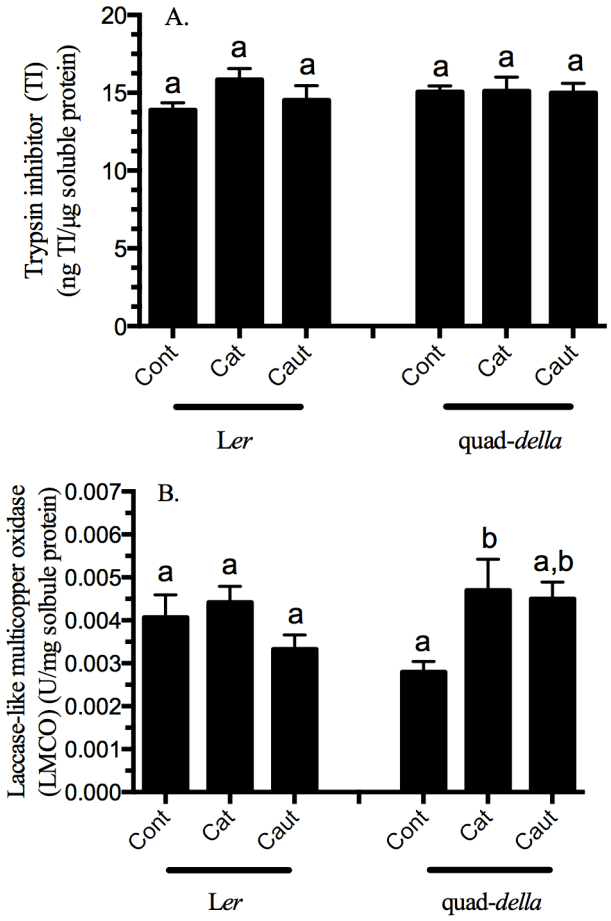

A.
Total glucosinolates (GS) (µmol/g DW)
12
8
4
0
Indole GS
Aliphatic GS
Cont
Cat
Caut
Cont
Cat
Caut
Ler
quad-della
B.
4-Methoxyglucobrassicin (µmol/g DW)
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
a
b
b
a
a
a
Cont
Cat
Caut
Cont
Cat
Caut
Ler
quad-della

Fig. 4


A.
Trypsin inhibitor (TI)
(ng TI/µg soluble protein)
20
15
10
5
0
a
a
a
a
a
a
Cont
Cat
Caut
Cont
Cat
Caut
Ler
quad-della
B.
Laccase-like multicopper oxidase
(LMCO) (U/mg solbule protein)
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
a
a
a
a
b
a,b
Cont
Cat
Caut
Cont
Cat
Caut
Ler
quad-della