DELLA proteins modulate *Arabidopsis* defences induced in response to caterpillar herbivory

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Abstract

Upon insect herbivory, many plant species change the direction of metabolic flux from growth into defence. Two key pathways modulating these processes are the gibberellin (GA)/DELLA pathway and the jasmonate pathway. In this study, the effect of caterpillar herbivory on plant-induced responses was compared between wild-type *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 defence 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 wild-type 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 independently 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. The present results show a link between DELLA proteins and early, induced plant defences in response to insect herbivory; in particular, these proteins are necessary for caterpillar LS-associated attenuation of defence hormones.

Key words: *Arabidopsis thaliana*, caterpillar labial saliva, DELLA proteins, gibberellins, induced plant defences, *Spodoptera exigua*.

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 which shape the plant’s response. In general, jasmonic acid (JA) and related compounds are implicated in plant defence responses against chewing insect herbivores, whereas gibberellins (GAs) promote plant growth and development (Ballare, 2011; Erb et al., 2012). In addition, caterpillar salivary effectors modulate plant defences, often suppressing...
JA-induced plant responses (Musser et al., 2002; Bede et al., 2006; Weech et al., 2008; Dziezel et al., 2009; Tian et al., 2012).

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<sup>COI</sup> 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; Thines et al., 2007; Katsir et al., 2008; Yan et al., 2009; Sheard et al., 2010; Fernandez-Calvo et al., 2011; Erb et al., 2012). Lipoygenase 2 (AtLOX2), Plant Defensin 1.2 (AtPDF1.2), and Vegetative Storage Protein 2 (AtVSP2) are well characterized markers of MYC-regulated gene expression (Bell and Mullet, 1993; Lorenzo et al., 2004; Dombrecht et al., 2007; Pre et al., 2008; Robert-Seilaniantz et al., 2011; Kazan and Manners, 2013), 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 defence responses. In Arabidopsis, key defensive strategies include the production of antinutritive proteins, such as trypsin inhibitors (TIs) and laccase-like multicopper oxidase (LMCO), and secondary metabolites, such as glucosinolates (GSs) (Duffey and Stout, 1996; 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 (Broadway et al., 1986; van Dam et al., 2001; Cipollini et al., 2004; Clauss and Mitchell-Olde, 2004; Weech et al., 2008; Tian et al., 2012). 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 (Zavala et al., 2004; McCraig et al., 2005; 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, 200 GSs have been identified, which are broadly categorized into aliphatic, indole, and aromatic GSs (Clarke, 2010). Over 35 GSs have been identified in Arabidopsis, with representative GSs of the aliphatic and indolyl pathways, such as 3-hydroxypropyl glucosinolate and glucobrassicin, respectively, being prominent in Landsberg (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).

Caterpillar labial salivary (LS) effectors modulate the jasmonate pathway and subsequent induced defence 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 (Weech et al., 2008; Dziezel et al., 2009; Tian et al., 2012). Presently, evidence suggests that caterpillar LS-mediated suppression of induced plant defences involves the activation of the salicylic acid (SA)/nonexpressor of pathogenesis-related protein 1 (NPR1) pathway (Mur et al., 2006; Weech et al., 2008). Spodoptera exigua growth (biomass) was higher when caterpillars were fed on coi1 mutant plants compared with etr1 and npr1 genotypes (Mewis et al., 2005); this suggests that JA pathway COI1 is needed for defence responses but insects use the SA/NPR1 and ethylene pathways to circumvent plant defences, such as GSs. Noctuid caterpillar LS is rich in oxidoreductase enzymes, such as glucose oxidase (GOX), that is believed to be a key effector in the modulation of host plant responses (Eichenseer et al., 1999; Musser et al., 2002; Weech et al., 2008; Afshar et al., 2010; Eichenseer et al., 2010). 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 defences by the SA/NPR1 pathway occurs downstream of SCF<sup>COI</sup>-mediated protein degradation instead through the ORA59 transcription factor. However, other plant hormone pathways, such as GAs, must also contribute to this cross-talk to optimize and fine-tune the plant’s response to changing environmental conditions.

Diterpenoid GA phytohormones promote growth-related physiological processes in flowering plants (Sun, 2011; Hauvermale et al., 2012; Davière and Achard, 2013). Binding of GA to its receptor, Giberellin Insensitive Dwarf 1 (GID1), leads to the degradation of the negative growth regulator DELLA proteins by the 26S-proteasome pathway (Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004; Hartweck and Olsewski, 2006; Murase et al., 2008; 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).

Cross-talk between the GA and JA pathway most probably occurs via DELLA proteins (Hou et al., 2010; Wild et al., 2012; Yang et al., 2012). In vegetative tissues, JA signalling 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 defence responses by repressing the activity of negative regulator JAZ proteins. Also, by interfering with GA degradation of DELLA proteins, JA prioritizes defensive over growth-related pathways.
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 defences 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 (Musser et al., 2002; Bede et al., 2006; Achard et al., 2008; Weech et al., 2008; Paudel et al., 2013). 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 fourth instar S. exigua caterpillars were compared in wild-type 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 h after the onset of herbivory to evaluate the role of JA versus GA trade-offs in this plant–insect interaction. Systemic changes in five plant hormones were recorded, including JA, its biologically active conjugate JA-Ile, and its precursor 12-oxo-phytodienoic acid (OPDA), which is also an important signalling molecule in plant–insect interactions (Farmer et al., 2003; Taki et al., 2005; Fonseca et al., 2009). Additionally, changes in SA and abscisic acid (ABA) were analysed. Increases in ABA levels are often observed in response to mechanical wounding, possibly as a response to water losses due to the damage (Erb et al., 2012). In addition, representative genes of the JA/ethylene 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 (Penninckx et al., 1998; Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; 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 (Zhang et al., 1999; Glazebrook, 2005; Mur et al., 2006; Kusnierczyk et al., 2007; Walling, 2008). Given the competition between DELLA proteins and MYC2 for the JAZ proteins, a decrease in positively regulated MYC2-dependent markers was expected in the quad-della mutant following insect herbivory (Hou et al., 2010; Wild et al., 2012; Wild and Achard, 2013). Also, since caterpillar LS effector(s) may exert the suppression of JA-induced responses through the generation of ROS, DELLA proteins scavenge these compounds, and DELLA proteins suppress the NPR1 pathway, a stronger caterpillar LS-dependent suppression of JA-mediated responses was expected in the quad-della mutants (Musser et al., 2002; Bede et al., 2006; Achard et al., 2008; Weech et al., 2008; Alonso-Ramírez et al., 2009; Paudel et al., 2013). In addition to measuring hormone levels and gene expression, other inducible plant defences, namely TI, LMCOs, and GS, that, alone or in combination, may negatively affect the herbivore, were also assessed.

### Materials and methods

#### Chemicals

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

#### Plant cultivation

Wild-type A. thaliana cv Ler and the quad-della mutant (goi-1, rga-t2, rg1-1, rg2-1) seeds were grown in pasteurized (80 °C for 2 h) Agro Mix. After stratification at 4 °C for 2 d, the seeds germinated in a phytotron (8:16h light:dark, 250 µE m⁻² s⁻¹, 23 °C). As GAs regulate multiple aspects of plant development, wild-type 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 bottom watered as needed with dilute 0.15 g l⁻¹ N-P-K fertilizer. At ~2 weeks, plants were removed to leave three 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 h light:dark, 28–40% humidity, 22 °C). Eggs collected from mated adults were used to maintain the colony for >30 generations.

#### Herbivory experiment

Plants ~5 weeks old [growth stages 1.11–1.14 (Boyes et al., 2001)] were either kept as controls control (no insects) or subject to herbivory by fourth instar S. exigua caterpillars with intact (cat) or impaired (caut) LS secretions. To prevent LS secretions, caterpillar spinerettes were cauterized (caut insects) (Musser et al., 2002). As caterpillar LS contains high levels of the enzyme GOX, success of cautization was tested by allowing caterpillars to feed on glass discs pre-soaked 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 h before the beginning of the herbivory experiment to allow them to adjust to a plant diet.

To either the wild type (Ler) or the quad-della mutant, three fourth 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 h after the plant’s transition to dark. To minimize the effect of plant volatile signalling in the growth cabinets, pexiglass plates separated the different treatments (control, cat, caut).

After 10 h, caterpillars were removed and plants were flash-frozen in liquid nitrogen. The three 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 used.
were collected to focus on local responses. Samples were stored in an –80 °C freezer until analysis. This experiment was repeated eight independent times. For hormone analysis, gene expression, and GS analysis, four biological replicates were analysed. For defensive protein and biomass loss experiments, eight biological replicates were used.

To calculate biomass loss, aerial tissues were dried for 3 d at 70 °C. From 20% to 29% of plant tissue was consumed by caterpillars, regardless of plant genotype. Cauterization of the caterpillar spinn-neret did not affect feeding.

**Hormone analysis**

Lyophilized plant samples were ground using a TissueLyser (Qiagen) and tissues were sent to the Danforth Plant Science Centre for hormone analysis by liquid chromatography–mass spectroscopy/ tandem mass spectrometry (LC-MS/MS). Samples were spiked with deuterium-labelled internal standards of SA (D5-SA), ABA (D6-ABA), and JA (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 s⁻¹ then centrifuged at 16 000 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 analysed 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 2 min 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⁻¹. 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 –450 V, heater gas 50 a.u., curtain gas 25 a.u., collision activation dissociation, high, temperature 550 °C. Each horizon- lowed by 40 cycles of 95 °C for 15 s, annealing temperature for 30 s. Each well contained Blue qPCR SYBR low Rox (Fisher), and 450 nM forward and reverse primers, and cDNA (1/10 dilution) in a TurboIonSpray (TIS) electrospray ion source in negative mode. Parameters were set to: capillary voltage –450 V, heater gas 50 a.u., curtain gas 25 a.u., collision activation dissociation, high, temperature 550 °C. Each horizon- lowed by 40 cycles of 95 °C for 15 s, annealing temperature for 30 s. Each well contained Blue qPCR SYBR low Rox (Fisher), and 450 nM forward and reverse primers, and cDNA (1/10 dilution). The following PCR program was used: 95 °C for 15 min fol-owed by 40 cycles of 95 °C for 15 s, annealing temperature for 30 s. Dissociation curves confirmed amplicon purity. Two technical replicates were performed.

From the standard curve, relative gene expression was measured. Expression of two reference genes [AtAct2/7 and AtUnk (At4g26410)] was not affected by treatment [Ler: AtAct2/7 F₄<sub>2.9</sub> = 0.73, P = 0.51; AtUnk F₄<sub>2.9</sub> < 0.19, P = 0.83; quad-della mutant: AtAct2/7 F₄<sub>2.9</sub> = 2.43, P = 0.143; AtUnk F₄<sub>2.9</sub> = 0.42, P = 0.67]. The geo-metric mean of AtAct2/7 and AtUnk was used to normalize expres-sion of genes of interest (Vandesompele et al., 2002; Brunner et al., 2004; Pfaffl et al., 2004).

**Defence 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 analysed for LCMO activity, a broad-spectrum proteinase inhibitor solution (1×) was added to prevent protein degradation. Samples were vigorously vortexed and centrifuged at 15 700 g for 10 min. Supernatants were used for protein assays.

**Trypsin inhibitor (TI) assay**

Leaf TI 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 an Infinite M200 Pro micro-plate reader (Tecan). The trypsin substrate, N-benzoyl-dl-arginyl-β-naphthylamide (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 colorimetric 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 TI (concentration range, 0–1.0 µg l⁻¹).

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

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

**Modified Bradford assay**

Soluble protein concentration in leaf extracts was measured by a modified Bradford assay using a bovine serum albumin (BSA) standard curve (5–100 µg ml⁻¹) (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 OD₅₉₀/OD₄₅₀ ratio was used to calculate the 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 of dry material was weighed in a 2 ml Eppendorf tube and extracted twice with 1 ml of 70% methanol solution, followed by 15 min ultrasoni-fication. During the first extraction, the tube was placed in a 90 °C water bath for 10 min after the addition of the methanol to inhibit any myrosinase activity immediately. After sonification, tubes were centrifuged at 2975 g for 10 min. Pooled supernatants were cleaned up by ion exchange chromatography using a diethylaminoethoxy Sephadex A-25 column pre-conditioned with sterile MilliQ water. After washing with 70% methanol (2 × 1 ml), MilliQ water (2 × 1 ml), and 20 mM sodium acetate buffer, pH 5.5 (1 × 1 ml), GSs were des-ulfated by the addition of 10 U of arylsulphatase and incubated at room temperature overnight. Desulfated GSs were eluted with sterile MilliQ water (2 × 0.75 ml). The combined eluate 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 C₁₈ column (Alltima C₁₈, 150 × 4.6 mm, 3 µm, Alltech) using an ACN–water gradient (2–35% ACN from 0 to 30 min; flow rate 0.75 ml min⁻¹). Compounds were detected by a photodiode array detector (PDA). Peaks were integrated at 229 nm (EC, 1990).
GSs were identified based on retention time, UV spectrum, MS analysis of selected Arabidopsis thaliana reference samples, and the following reference standards (Phytoplan, Germany): glucobrassicin (3-methylosphenylpropylGSL), glucoraphanin (4-methylothiobutylGSL), progoitrin (2-hydroxy-3-butenylGSL), sinigrin (2-propenylGSL), gluconapin (3-butenylGSL), glucoerucin (4-pentenylGSL), glucobrassicanapin (4-pentenylGSL), glucoraphanin (indol-3-ylmethylGSL), sinigrin (4-hydroxybenzyGSL), glucotropaeolin (benzyGSL), and 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 (Buchner, 1987; EC, 1990; Brown et al., 2003).

**Statistical analysis**

GA3s are involved in multiple aspects of plant development (Davière and Achard, 2013). Therefore, to avoid potentially confounding phenological differences between wild-type 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. The results from statistical analyses are shown in Supplementary Table S2 at JXB online. The results from statistical analyses are shown in Supplementary Table S2. Levels of the other indole GSs did not change upon caterpillar feeding. An LS-specific induction of GS levels was not observed (Fig 3A, B; Table 1; Supplementary Table S2). Levels of the other indole GSs did not change upon caterpillar feeding.

Caterpillar herbivory results in an increase in the indole glucosinolate 4-methoxyglucobrassicin (4-MGB)

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

Levels of aliphatic GSs were not affected by caterpillar herbivory (Table 1; Supplementary Table S2 at JXB online). In contrast, 4-MGB was induced ~25–40% in response to caterpillar herbivory in Ler but not in the quad-della mutants (Fig. 3A, B; Table 1; Supplementary Table S2). Levels of the other indole GSs did not change upon caterpillar feeding.

**Caterpillar herbivory results in an increase in the indole glucosinolate 4-methoxyglucobrassicin (4-MGB)**

Caterpillar herbivory does not affect early defensive protein activity: TI and LMCO

Constitutive TI levels did not increase in the early response to caterpillar herbivory or LS in either wild-type Ler or the quad-della mutant plants (Fig. 4A; Supplementary Table S2 at JXB online). In wild-type Ler plants, constitutive LMCO activity did not increase in response to herbivory (Fig. 4B; Supplementary Table S2). 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 defence. Two key hormone systems that regulate these physiological processes are GA/DELLA proteins for growth and JAS/JAZ proteins involved in plant defence against chewing herbivores, such as caterpillars (Ballare, 2011; Robert-Seilaniantz et al., 2011). The cross-talk
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). In addition, 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 cross-talk between the GA/DELLA and the JA pathway in the early plant responses to caterpillar herbivory (10h). In addition, the role of caterpillar LS effector(s) in these interactions was determined.

Caterpillar infestation of both wild-type and the quad-della mutant plants results in a strong systemic jasmonate burst, as
Fig. 2. Defence gene expression in Arabidopsis rosette leaves in response to caterpillar herbivory. Arabidopsis plants (Ler, Ler+GA, and quad-della mutant) were subject to herbivory by Spodoptera exigua caterpillars with intact (cat) or impaired (caut) labial salivary secretions for 10h. Expression levels of marker genes of the jasmonate pathway (A) AtPDF1.2 (JA and ethylene dependent), (B) AtVSP2 (JA and MYC2 dependent), (C) AtLOX2 (JA and MYC2 dependent), and (D) PR1 (SA/NPR1 dependent) were measured by quantitative real-time PCR and normalized by the expression of two reference genes (AtAct2/7 and AtUnk). Bars represent the means of 3–4 independent biological replications ±SE. Letters indicate significant differences in response to caterpillar herbivory (P<0.05) (Supplementary Table S2 at JXB online). An asterisk indicates a 5-fold increase in expression levels compared to control plants.

Table 1. Glucosinolate (GS) levels in Arabidopsis rosette leaves subject to caterpillar herbivory

Five-week-old Arabidopsis thaliana subject to herbivory by fourth instar Spodoptera exigua caterpillars for 10h (n=4). Caterpillars had either intact (cat.) or impaired (caut.) labial salivary secretions.

| GS (nmol g⁻¹ DW)        | Ler                   | quad-della mutant     |
|-------------------------|-----------------------|-----------------------|
|                         | Control               | Cat.                  | Caut.                  | Control               | Cat.                  | Caut.                  |
| 3-Hydroxypropyl 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 ± 9.8            | 29.4 ± 3.4             |
| 4-Methoxyglucobrassicin | 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         |

A significant increase in the GS 4-methoxyglucobrassicin was observed in response to herbivory in wild-type Arabidopsis [Ler: F₂,₉=6.17, P=0.02]. Letters indicate significant differences due to herbivory within each genotype (Ler or quad-della mutant).
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 was further analysed. *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 wild-type 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* (Figs 1D, 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 probably reflects temporal differences in the experimental design where in this study and that of Tian et al. (2012) gene expression was evaluated at ≤10 h after the initiation of herbivory compared with that of Paudel et al. (2013) where *AtPR1* transcript levels were measured 36 h after herbivory.

GSs are the principal defensive compound in *Arabidopsis* (Halikier and Gershenzon, 2006; Hopkins et al., 2009).
Levels of aliphatic GSs are not affected by caterpillar herbivory (Table 1), in contrast to previous studies where, in the Col background, Mewis et al. (2005) noticed an increase in short-chain aliphatic methylsulphinyl 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). Principal 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 wild-type 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 wild-type 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 (Thipyapong et al., 1997; Cai et al., 2006; Constabel and Barbehenn, 2008). 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 defence responses take priority over GA-dependent growth processes (Hou et al., 2010; Heinrich et al., 2012; Wild et al., 2012; Yang et al., 2012). The present data suggest that DELLA proteins may be involved in the regulation of GSs and also suppress LMCO activity, which may be related to their role in plant cell wall fortification (Thipyapong et al., 1997; Cai et al., 2006; Constabel and Barbehenn, 2008).

Caterpillar labial saliva-specific responses

Since caterpillar LS has been implicated as a stratagem to modify plant-induced defences (Musser et al., 2002; Weech et al., 2008; Tian et al., 2012), plant-induced responses to caterpillars with intact versus impaired LS secretions were compared. Arabidopsis plants subject to herbivory by caterpillars with impaired LS secretions have significantly higher jasmonate levels (OPDA, JA, and JA-Ile) compared with normal S. exigua, indicating that the LS contains effector(s) that suppress this jasmonate burst in response to herbivory (Fig. 1A–C). Weech et al. (2008) observed a similar distinction in JA 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 defence hormones.

Even though an 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 is strongly induced in response to herbivory; however, caterpillar LS differences in transcript expression are not observed. Similar observations for AtLOX2 have been made previously (Weech et al., 2008; Tian et al., 2012; Paudel et al., 2013). However, AtPDF1.2 suppression by caterpillar LS effectors is well recognized (Weech et al., 2008; Paudel et al., 2013). This probably 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 defences is believed to involve the SA/NPR1/TGA pathways possibly by a mechanism as elucidated by Van der Does et al. (2013) (Weech et al., 2008; Paudel et al., 2013).

In the quad-della mutant, expression of AtLOX2 and AtVSP2 parallels that of wild-type 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).

A caterpillar LS-specific difference in SA levels was not observed, and this is reflected in the expression of the marker gene *AtPR1* in the wild-type plant (Figs 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 analysed in this study were not affected by caterpillar LS (Fig. 3 and 4).

**Conclusion**

The present results show a link between DELLA proteins and the regulation of plant defences, such as GSs, in response to insect stress (Fig. 4B) and in the caterpillar LS-mediated suppression of plant defence hormone biosynthesis (Fig. 1A–C).

Previous models propose that caterpillar LS effector(s) manipulate plant defences through the generation of ROS, such as hydrogen peroxide, that activate the NPR1/TGA pathway to modulate induced plant defences (Eichenseer et al., 1999; Musser et al., 2002; Weech et al., 2008; Tian et al., 2012; Paudel et al., 2013). DELLA proteins are known to scavenge hydrogen peroxide (Achard et al., 2008). In addition, treatment of *Arabidopsis* with GAs results in the activation of the NPR1 pathway (Alonso-Ramírez et al., 2009). Therefore, in the quad-DELLA mutant, a stronger LS-dependent response was expected, but was not observed. 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 appear to be multiple points of cross-talk between the JA defence pathway and the GA/DELLA pathway to ensure prioritization of plant responses to changing environmental conditions (Fig. 5). Future studies will continue to further elucidate the underlying mechanism.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Table S1.** Primers to check for genomic contamination and quantitative real-time PCR.

**Table S2.** Statistical results of plant–insect experiments.

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