The role of DELLA proteins in plant-insect interactions

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOS</td>
<td>allene oxide synthase</td>
</tr>
<tr>
<td>BANA</td>
<td>N-benzoyl-DL-arginyl-β-naphthylamine</td>
</tr>
<tr>
<td>COI1</td>
<td>CORONATINE INSENSITIVE1</td>
</tr>
<tr>
<td>COR</td>
<td>coronatine</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethyl formamide</td>
</tr>
<tr>
<td>DOPA</td>
<td>dopamine hydrochloride</td>
</tr>
<tr>
<td>EIN3</td>
<td>ETHYLENE INSENSITIVE3</td>
</tr>
<tr>
<td>EIL1</td>
<td>EIN3-LIKE1</td>
</tr>
<tr>
<td>ERF1</td>
<td>ETHYLENE RESPONSE FACTOR1</td>
</tr>
<tr>
<td>ET</td>
<td>ethylene</td>
</tr>
<tr>
<td>FAC</td>
<td>fatty acid–amino acid conjugate</td>
</tr>
<tr>
<td>GA</td>
<td>gibberellin</td>
</tr>
<tr>
<td>GA20ox</td>
<td>GA20-oxidase</td>
</tr>
<tr>
<td>GA2ox</td>
<td>GA2-oxidase</td>
</tr>
<tr>
<td>GA3ox</td>
<td>GA3-oxidase</td>
</tr>
<tr>
<td>GAI</td>
<td>GA INSENSITIVE</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic deoxyribonucleic acid</td>
</tr>
<tr>
<td>GID1</td>
<td>GIBBERELLIN INSENSITIVE DWARF1</td>
</tr>
<tr>
<td>GOX</td>
<td>glucose oxidase</td>
</tr>
<tr>
<td>HAMP</td>
<td>herbivore-associated molecular pattern</td>
</tr>
<tr>
<td>HrP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>JA</td>
<td>jasmonate</td>
</tr>
<tr>
<td>JAR1</td>
<td>JASMONATE RESISTANT1</td>
</tr>
<tr>
<td>JA-Ile</td>
<td>jasmonoyl-L-isoleucine</td>
</tr>
<tr>
<td>JAZ</td>
<td>JASMONATE ZIM-DOMAIN</td>
</tr>
</tbody>
</table>
LC-MS/MS  liquid chromatography-tandem mass spectrometry
Ler  Landsberg erecta
LMCO  laccase-like multicopper oxidase
LOX2  LIPOXYGENASE2
MBTH  3-methyl-2-benzothiazolinone hydrozone
NPR1  NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1
NINJA  NOVEL INTERACTOR OF JAZ
OPDA  12-oxo-phytodienoic acid
OS  oral secretion
PCR  polymerase chain reaction
PDF1.2  PLANT DEFENSIN1.2
PI  proteinase inhibitor
PIF  phytochrome interacting factor
PIN  PIN-FORMED
PPO  polyphenol oxidase
PR1  PATHOGENESIS-RELATED GENE1
qRT-PCR  quantitative real time-polymerase chain reaction
quad-della  quadruple-della Arabidopsis mutant
RGA  REPRESSOR OF ga1-3
RGL1  RGA-LIKE1
RGL2  RGA-LIKE2
RGL3  RGA-LIKE3
ROS  reactive oxygen species
SA  salicylic acid
SCL3  SCARECROW-LIKE3
SLY1  SLEEPY1
TI  trypsin inhibitor
TPL  TOPELESS
VSP2  VEGETATIVE STORAGE PROTEIN2
Abstract

Jasmonates (JAs) play a major role in plant defense against herbivores while some caterpillar species use effectors in their labial saliva to suppress the induction of JA-mediated defense responses. On the other hand, activation of plant defense is associated with slowed plant growth which is controlled by gibberellins (GA) and growth repressor DELLA proteins. Recent studies have shown that DELLA proteins play an important role in plant stress response and are involved in the crosstalk between JA and GA pathways. However, the role of DELLA proteins in plant-insect interactions remains unclear. In this study, wild-type Arabidopsis, wild-type sprayed with GA and a quadruple-della Arabidopsis mutant (quad-della) were subject to herbivory by beet armyworm Spodoptera exigua caterpillars with either intact or impaired labial salivary secretions. Wild-type Arabidopsis, Arabidopsis + GA and the quad-della mutant showed a JA burst in response to herbivory. This was reflected in increased transcript levels of the JA-dependent gene markers, such as AtPDF1.2, AtLOX2 and AtVSP2. A caterpillar saliva-specific pattern of JA hormone levels (JA, JA-isoleucine, OPDA) were observed in the wild-type background but not in the quad-della mutant, suggesting that DELLAAs are involved in plant response to caterpillar saliva, probably by mediating the crosstalk between JA- and salicylic acid (SA)- dependent pathways. Additionally, high constitutive expression of the SA pathway marker gene AtPRI1 was observed in the quad-della mutant but not in wild-type Arabidopsis, which indicates that DELLAAs play a role in maintaining the homeostasis of SA signalling by repressing its constitutive induction.
Résumé

Les Jasmonates (JAs) font une partie importante dans la défense des plantes contre les herbivores. Certaines espèces de chenilles utilisent des effecteurs dans leur salive labiale pour supprimer l'induction de réponses de défense induites par les JAs. En revanche, l'activation de défense de la plante est associée au ralentissement de la croissance des plantes. La croissance des plantes est contrôlée par les gibbérellines (GA) et les répresseurs de la croissance, les protéines DELLA. Des études récentes ont montré que les protéines DELLA font partie de la réponse des plantes au stress et participent à la diaphonie entre les voies métaboliques du JA et GA. Cependant, le rôle des protéines DELLA reste incertain. Dans cette étude, Arabidopsis type sauvage, Arabidopsis type sauvage traités avec une pulvérisation du GA, et le mutant quadruple-della (quad-della) souffert des attaques par le herbivore Spodoptera exigua. Les chenilles sont normales ou avec les facultés affaiblies dans les glandes salivaires. Les trois groupes des plantes ont montré une explosion du JA en réponse aux herbivores. Cette réponse a été reflétée dans les niveaux de transcription des gènes dépendant de JA, comme AtPDF1.2, AtLOX2 et AtVSP2. Un motif spécifique à la salive des niveaux des hormones JA (JA, JA-isoleucine, OPDA) a été observé dans le type sauvage mais pas dans les mutants. Ce résultat suggère que DELLA sont impliquées dans la réponse de la plante à la salive probablement par la médiation de la diaphonie entre les voies métaboliques du JA et du acide salicylique (SA). Ailleurs, une forte expression du gène AtPRI1, qui est partie de la voie d’SA, a été observé dans le mutant quad-della. Ce résultat suggère que DELLA sont impliquées dans l'homéostasie de la signalisation de SA en réprimant son expression constitutive.
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Contributions of authors

The project presented hereafter was designed by the candidate and Dr. Jacqueline C. Bede, Department of Plant Science, Macdonald Campus of McGill University. The candidate performed herbivory experiment, insect performance experiment and collected all samples. The samples for hormone analysis were analyzed at Donald Danforth Plant Science Center. The candidate performed qRT-PCR, TI assay, LMCO assay and data analysis. JCB supervised the whole project and helped the candidate edit this thesis.

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1. Introduction

1.1. General introduction

When attacked by insect herbivores, plants often redirect their limited resources away from primary metabolic processes, such as growth and development, into induced defenses (Kessler and Baldwin, 2002). This involves the crosstalk between different plant signalling pathways. In general, jasmonates (JAs) play a major role in plant defense against herbivores while gibberellins (GAs) promote plant growth and development (Ballare, 2011).

In response to chewing herbivores, such as caterpillars, increases in jasmonoyl-L-isoleucine (JA-Ile) triggers the degradation of the signalling repressor JASMONATE ZIM-DOMAIN (JAZ) proteins, which leads to the release of the transcriptional regulator MYC2 and the induction of plant defense responses (Pieterse et al., 2012). Some caterpillar species use effector(s) in their labial saliva to suppress the induction of JA-mediated defense responses for their own benefit (Musser et al., 2002; Weech et al., 2008). The mechanism responsible for this suppression is unknown but is believed to involve activation of the salicylic acid (SA)-mediated pathway that has been shown to antagonize JA signalling (Robert-Seilaniantz et al., 2011). GAs positively regulate plant organ expansion and developmental processes by triggering the degradation of the growth repressor DELLA proteins (Sun, 2011). However, under adverse conditions, DELLA protein levels increase, suppressing plant growth and regulating plant stress responses (Sun, 2011). For example, under high salinity conditions, DELLAs prevent cell death and enhance plant salt tolerance by promoting the scavenging of reactive oxygen species (ROS) in plant cells (Achard et al., 2006; Achard et al., 2008). In response to necrotrophic pathogen attack, DELLAs reinforce JA-mediated defense reactions to help the plant protect itself (Navarro et al., 2008).

Increasing evidence shows that the crosstalk between GA and JA pathways is mediated by DELLA and JAZ proteins. DELLAs and MYC2 compete for binding to JAZ (Hou et al., 2010; Wild et al., 2012); therefore, high cellular DELLA levels promote the release of MYC2 from JAZ/MYC2 complex and, thus,
activate the MYC2-dependent defense gene expression. This is supported by a recent study showing that JA prioritize plant defense over growth through the JAZ-DELLA-phytochrome interacting factor (PIF) signalling cascade (Yang et al., 2012).

Although DELLA proteins have been investigated in many plant stress responses, little attention has been paid to the role of DELLAs in plant-insect interactions. As herbivores are a major threat to agriculture, it is important to investigate the role of DELLAs in plant defense against insects.

In this study, we used the model plant Arabidopsis thaliana and caterpillars of the beet armyworm, Spodoptera exigua, to investigate the role of DELLA proteins in plant defense responses. Hormone, gene expression and defensive protein changes were measured in wild-type Arabidopsis Landsberg erecta (Ler), Ler sprayed with GA (Ler + GA) and an Arabidopsis DELLA-deficient mutant (quad-della) subject to herbivory by beet armyworm caterpillars with either intact or impaired salivary secretions. As well, an insect performance experiment was performed on the Ler, Ler + GA and quad-della plants. This research provides information to the role of DELLA proteins in plant-insect interactions and the crosstalk between JA and GA. Ultimately, it gives us a more complete picture of DELLAs’ role in plants subject to adverse conditions.
1.2. Hypotheses

Under chewing insect attack, DELLA proteins promote JA-mediated plant defense and attenuate the antagonistic effect of caterpillar saliva-activated SA signalling on JA signalling. Therefore,

- In the DELLA-deficient *Arabidopsis* mutant, JA-mediated defense responses will be partially impaired. As well, caterpillar labial saliva will be more effective in suppressing JA signalling in the DELLA-deficient mutants.
- Caterpillars will perform better on the DELLA-deficient mutant compared to wild-type plants.

1.3. Objective

To investigate the role of DELLA proteins in plant-insect interactions, herbivory-induced plant defenses will be monitored in wild-type *Arabidopsis* (Ler), GA-treated wild-type *Arabidopsis* (GA triggers the degradation of DELLAs) and the quadruple-della *Arabidopsis* plants (a quadruple DELLA knockout mutant), in terms of:

- Plant hormone levels
- Transcript levels of JA- and SA-dependent gene markers
- Activity of JA-dependent defensive proteins
- Insect pupa weight after feeding on plants
2. Literature Review

2.1. Plant-insect interactions

In ecological scenarios, two important factors that shape the plant kingdom are plant-insect interactions and plant-plant growth competition (Ballare, 2009; Howe and Jander, 2008). In response to herbivory by phytophagous insects, plants have evolved sophisticated and exquisitely-regulated defense systems to protect themselves. Physical barriers, such as leaf toughness, trichomes and cuticles increase plant fitness under herbivory (Howe and Jander, 2008). Defensive secondary metabolites, another major part of plant defense against herbivores, usually function as repellents or toxins (Kessler and Baldwin, 2002). Additionally, volatile organic compounds, such as green leaf volatiles, may be indirect defenses involved in attracting natural enemies of herbivores (Kessler and Baldwin, 2002). Collectively, plant direct and indirect defenses function to protect the plant. Plant defense responses can be constitutive and induced. Constitutive defense are generated by the plant regardless of herbivory (Howe and Jander, 2008). However, plant defense is costly and high level of constitutive defenses may result in growth inhibition and compromised competitive ability (Kempel et al., 2011). Insect-induced plant resistance, on the other hand, is only activated upon the recognition of herbivory (Kessler and Baldwin, 2002). By deploying quick and strong defense responses only when needed, plants optimize their defense reactions and minimize the fitness cost due to resource allocation (Baldwin, 1998, 2001; Kessler and Baldwin, 2002). This trade-off between growth and defense is important to the survival of the plant, and, often referred to as the ‘dilemma of plants’ (Herms and Mattson, 1992). Plant growth-defense balance is orchestrated by the interplay of various phytohormone signalling pathways while the mechanism underlying is largely unknown (Ballare, 2011; Bostock, 2005).

2.2. Jasmonate-mediated defense signalling

Jasmonic acid (JA) and related compounds are oxylipin phytohormones implicated in many physiological processes throughout the life cycle of higher plants (Ballare, 2011). JA plays a central role in plant responses to many abiotic
and biotic stresses, including wounding, herbivory and necrotrophic pathogen infection (Erb et al., 2012; Howe, 2004; Wasternack, 2007). JAs also regulate plant developmental processes, such as root growth, tuber formation, tendril coiling, flower development and senescence (Wasternack, 2007).

Upon insect attack, JA signalling is induced both locally and systemically, which helps the whole plant establish a defensive status (Kessler and Baldwin, 2002). JA plays a pivotal role in regulating global gene reprogramming in response to wounding or insect attack (De Vos et al., 2005; Devoto et al., 2005; Halitschke et al., 2003; Reymond et al., 2004; Reymond et al., 2000). Plant mutants defective in their JA biosynthesis or signalling usually have an attenuated defense response to chewing insects and, as a result, there is better herbivore performance (Howe and Jander, 2008; Kessler and Baldwin, 2002). For example, silencing of the JA biosynthetic enzyme lipoxygenase in *Nicotiana attenuata* makes the plant more vulnerable and attractive to herbivores (Kessler et al., 2004). Similarly, knockouts of the JA signalling component COII or overexpression of JASMONATE ZIM-DOMAIN (JAZ) protein in *Arabidopsis* results in low defense gene expression and defensive compound production in response to herbivory (Chung et al., 2008; Reymond et al., 2004).

Insect attack by chewing herbivores that wound the plant during feeding stimulates rapid JA biosynthesis (Howe and Jander, 2008; Reymond et al., 2004). In addition to wounding signals, the plant recognizes herbivore-associated molecular patterns (HAMPs). Larval footsteps that cause damage to leaf surface tissues stimulate rapid local reactions (Hall et al., 2004). Herbivore-specific elicitors, such as fatty acid–amino acid conjugates (FACs) from insect oral secretions (OS), play important roles in shaping the plant defense response while the receptors that perceive these signals are still not identified (Diezel et al., 2009). Furthermore, insect feeding duration, biting frequency and sites can be sensed by plants and triggers specific plant responses (Mithöfer et al., 2005). The recognition of insect attack leads to signal transduction pathways, including Ca^{2+} flux and reactive oxygen species (ROS) burst, that results in JA biosynthesis (Zhao et al., 2005). Derived from α-linolenic acid, JA is synthesized through the
allene oxide synthase (AOS)-dependent octadecanoid pathway with almost all its biosynthetic enzymes having been identified (Schaller et al., 2004; Wasternack, 2007). Newly synthesized JA is further conjugated with isoleucine to generate its bioactive form, jasmonoyl-L-isoleucine (JA-Ile), catalyzed by JASMONATE RESISTANT1 (JAR1) (Kang et al., 2006; Staswick and Tiryaki, 2004).

Bioactive JA, jasmonoyl-L-isoleucine, is perceived by a protein complex formed of CORONATINE INSENSITIVE1 (COI1) and JASMONATE ZIM-DOMAIN (JAZ) proteins (Fig. 2.1) (Katsir et al., 2008; Sheard et al., 2010; Yan et al., 2009). Specifically, the F-BOX protein COI1 is a subunit of the E3 ubiquitin-ligase Skp1/Cullin/F-box complex (SCF^COI1) and has a binding pocket for JA-Ile (Devoto et al., 2002; Sheard et al., 2010; Xie et al., 1998; Xu et al., 2002; Yan et al., 2009). JAZ repressor proteins interact with JA-dependent transcriptional factors and inhibit their activity (Chini et al., 2007; Thines et al., 2007). By promoting the interaction between COI1 and JAZ, JA-Ile triggers the degradation of JAZ through the COI1-dependent 26S proteasome pathway and, thus, releases the downstream regulators and activates JA signalling responses (Pauwels and Goossens, 2011).

In Arabidopsis, two downstream subsets of JA pathway, MYC and ERF branches, have been elucidated (Fig. 2.1). Controlled by basic helix-loop-helix zipper (bHLHzip)-type transcription factors MYC2/3/4, the MYC branch is responsible for the JA-mediated wounding response and herbivory defense (Abe et al., 2003; Boter et al., 2004; Cheng et al., 2011; Fernández-Calvo et al., 2011; Lorenzo et al., 2004). In MYC2 knockout plants or JAZ overexpressing plants, compromised host resistance to herbivores and diminished transcript level of the MYC branch marker gene VEGETATIVE STORAGE PROTEIN2 (VSP2) was observed (Chung et al., 2008; Dombrecht et al., 2007). JAZ proteins repress the MYC branch signalling by physically interacting with MYC-type transcription factors and recruiting the corepressor TOPLESS (TPL) through the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Cheng et al., 2011; Chung et al., 2008; Fernández-Calvo et al., 2011; Pauwels et al., 2010). In contrast to the MYC branch, the principal regulators of the ERF branch, ETHYLENE
RESPONSE FACTOR1 (ERF1) and ORA59, integrate ethylene (ET) and jasmonate signals and positively regulate the pathogen defense-related genes, such as *PLANT DEFENSIN1.2* (*PDF1.2*) (Lorenzo *et al.*, 2003; Penninckx *et al.*, 1998; Pré *et al.*, 2008). The connection between ERF1 and JAZ was shown by a recent study demonstrating that *ERF1* expression is activated through ET-mediated stabilization of transcriptional regulators ETHYLENE INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1). JA promotes the removal of JAZs which inhibit EIN3 and EIL1, activating the ERF1-mediated responses (Zhu *et al.*, 2011). Therefore, distinct roles of MYC branch and ERF branch confer specific JA defence responses in an attacker-dependent manner, which enables the plant to allocate the limited resource in a best way (Dombrecht *et al.*, 2007; Pieterse *et al.*, 2012; Verhage *et al.*, 2011).

After JA perception, JA-induced defense output can be characterized by the expression of jasmonate-responsive genes, the biosynthesis of secondary metabolites and the production of defensive proteins (Howe and Jander, 2008). As described above, *PDF1.2* and *VSP2* are the most widely used marker genes for JA signalling. Plant defensin, encoded by the *PDF1.2* gene, has antifungal and antibacterial activity (Penninckx *et al.*, 1996; Penninckx *et al.*, 1998). The *PDF1.2* promoter contains a GCC-box which is thought to be partially responsible for JA/ET-mediated induction of the gene (Brown *et al.*, 2003). VSP2 is a storage protein which also has anti-insect function due to its acid phosphatase activity. Addition of AtVSP2 to insect diets results in delayed development and increased mortality of insects (Liu *et al.*, 2005). Regulated by MYC2, *VSP2* expression can be stimulated by JA treatment, wounding and insect feeding (Dombrecht *et al.*, 2007). *LIPOXYGENASE2* (*LOX2*), another commonly used JA marker gene, encodes a lipoxygenase involved in JA biosynthesis which catalyzes the conversion of α-linolenic acid to 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid (Wasternack, 2007). Transcript level of *LOX2* increases rapidly upon treatment of octadecanoids through a feedforward mechanism regulated by MYC2 (Bell and Mullet, 1993).
In addition to defense marker genes, a number of proteins with anti-herbivore functions increase in response to herbivory. Proteinase inhibitors (PIs), which are harmful to the growth and development of insects, have been studied in many plant species (Ryan, 1990). PIs bind to the active domains of their target insect gut-associated proteinases, preventing proteolysis and absorption of amino acids in insect midgut (Zhu-Salzman et al., 2008). Polyphenol oxidase (PPO), which belongs to the laccase-like multicopper oxidase (LMCO) family, is another well studied anti-digestive enzyme, especially in tomato and other Solanaceous plants (Constabel and Ryan, 1998). The induction of PPO by MeJA and herbivory has been reported in herbaceous crops and trees (Constabel and Barbehenn, 2008). PPO catalyzes the oxidation of foliar polyphenolics to reactive quinones that bind with dietary proteins and, thus, interferes with nutrient uptake in caterpillar gut (Constabel and Barbehenn, 2008; Felton et al., 1992). Additionally, amino acid-degrading enzymes, such as arginase and threonine deaminase, are novel anti-nutritive defensive proteins discovered in plants (Chen et al., 2005).

Herbivory-induced secondary metabolites often act as toxins and are diverse among different plant species, such as glucosinolates in Arabidopsis, nicotine in tobacco and saponin in Medicago (Howe and Jander, 2008; Mithöfer and Boland, 2012). Emitting volatile organic compounds to attract predators or prime themselves against future attacks is another strategy used by plants to fend off herbivores (Conrath et al., 2006). Airborne signals sensed by plants help them to establish a primed status, displaying faster and stronger cellular defense responses once induced by following attackers (Engelberth et al., 2004; Frost et al., 2007; Heil and Silva Bueno, 2007). In summary, defensive proteins and secondary metabolites act synergistically to repel insect herbivores by a combination of their anti-digestive, toxic and priming characteristics.

2.3. Crosstalk between JA and other phytohormones

Rather than a linear cascade, the JA pathway interacts with many other hormone pathways, that allows the plant to fine-tune its overall signalling network in a complex environment (Fig. 2.1). The most understood crosstalk in plant
defense is the antagonism between JA and salicylic acid (SA) pathways (Robert-Seilaniantz *et al.*, 2011).

SA treatment, that potentiates plant defense to biotrophic pathogens, comprises resistance to insect herbivores by attenuating JA responses (Cipollini *et al.*, 2004; Stotz *et al.*, 2002). The key protein of SA-mediated systemic acquired resistance, NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), is involved in the inhibition of JA defense response, but the mechanism of how NPR1 represses JA response remains to be elucidated (Dong, 2004; Spoel *et al.*, 2003). NPR1 is regulated in response to cellular redox charge (Tada *et al.*, 2008). Redox-based modifications of regulatory proteins affect their activity, localization, stability and protein interaction profile and, therefore, modulate the molecular players in JA-SA crosstalk (Koornneef *et al.*, 2008; Spoel and Loake, 2011). This is supported by a study showing that SA-mediated increase of glutathione is involved in suppressing MeJA-induced *PDF1.2* expression (Koornneef *et al.*, 2008). Other studies showed that several SA-related transcriptional factors, including TGAs and WRKYs, also act as negative regulators of JA-mediated defense responses (Gao *et al.*, 2011; Li *et al.*, 2004a; Ndamukong *et al.*, 2007).

As discussed earlier, ET and JA act synergistically through ERF1, leading to the induction of *PDF1.2* expression (Lorenzo *et al.*, 2003). ET also plays an important role in modulating JA-SA crosstalk. In *Arabidopsis*, simultaneous induction of JA and ET signalling cancels subsequent suppression effect of SA on JA response, that allows the plants to prioritize JA/ET-mediated defense against pathogens (Leon-Reyes *et al.*, 2010). Another study showed that the ET burst alone can bypass NPR1 dependency of the antagonism of SA on JA and enhanced the expression of SA-related PR genes (Leon-Reyes *et al.*, 2009). These two seemingly contradictory results indicate that the timing and intensity of different signal inductions affect the output of the crosstalk, which might be important to plant defensive strategies against multi-attackers (Pieterse *et al.*, 2012).

2.4. Insect-mediated suppression of plant defense responses
Crosstalk between JA and other phytohormones enable the plant to prioritize its defense to certain attackers or balance its defense reaction in a cost-saving manner (Thaler et al., 2012). However, many pathogens and insects have evolved strategies to hijack the plant defense signalling network for their own benefit. For example, in Arabidopsis and tomato, hemi-biotrophic pathogen Pseudomonas syringae uses the jasmonate mimic coronatine (COR), which is 1000-fold more active than JA-Ile in promoting the interaction between COI1 and JAZs, to stimulate JA pathway and, therefore, inhibit SA-mediated defense responses (Brooks et al., 2005; Katsir et al., 2008; Uppalapati et al., 2007). Similarly, herbivores employ countermeasures to suppress JA-mediated host immunity. Insect pests, such as beet armyworm caterpillars and silverleaf whitefly, amplify the SA-responsive gene expression and attenuate the JA signalling during feeding, probably partially mediated by effectors in their oral secretions such as glucose oxidase (Diezel et al., 2009; Musser et al., 2002; Zarate et al., 2007). Another intriguing study showed that some insects have evolved a way to suppress the induction of plant defense by releasing elicitors from their eggs, augmenting the accumulation of SA while negatively regulating the JA pathway (Bruessow et al., 2010). In caterpillar herbivory, caterpillar salivary-specific JA response was observed in wild-type Arabidopsis but not in ndr1-1 npr1-2 SA pathway double mutant, suggests that effectors in caterpillar’s labial saliva may activate the NPR1 of SA signalling, suppressing the octadecanoid pathway and its related defense responses (Weech et al., 2008).

2.5. Gibberellin biosynthesis and signalling pathway

Gibberellins (GAs) are a family of tetracyclic diterpenoid phytohormones that function as endogenous growth regulators of flowering plants (Sun, 2011). GAs stimulate a wide range of plant physiological processes such as seed germination, stem elongation, leaf expansion, transition from vegetative growth to reproductive growth and flower development (Hauvermale et al., 2012). In recent years, increasing evidence indicates that GAs are also involved in plant response to environmental stresses (Hauvermale et al., 2012; Robert-Seilaniantz et al.,...
Although more than 130 GAs have been discovered in plants, fungi and bacteria, only few of them have biological activity in plant and the four major bioactive GAs are GA₁, GA₃, GA₄, GA₇ (MacMillan, 2001; Sun, 2011). In the GA biosynthesis pathway of higher plants, GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) are key enzymes in the final steps that convert GA intermediates to bioactive GAs (Yamaguchi, 2008). Conversely, bioactive GA can be deactivated by the GA catabolism enzyme GA2-oxidase (GA2ox), which is an essential process for maintaining GA homoeostasis (Schomburg et al., 2003). The production of GA biosynthetic enzymes is controlled through a feedback mechanism by GA signalling (Peter and Stephen, 2012). In Arabidopsis, exogenous GA treatment or knockout of GA signalling repressors is associated with down-regulation of GA20ox and GA3ox transcript levels and up-regulation of GA2ox expression (Zentella et al., 2007).

After being synthesized, bioactive GA is recognized by its soluble receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1). Arabidopsis has three GID1s (AtGID1a, AtGID1b, and AtGID1c), which have functional redundancy and specificity at different growth and developmental stages (Iuchi et al., 2007; Nakajima et al., 2006; Willige et al., 2007). Recently, the crystalline structure of GID1s has been elucidated and shows that GA is trapped in a deep pocket covered by the N-terminal extension of GID1 (Murase et al., 2008; Shimada et al., 2008). GA binding causes a conformation change of the GID1 N-terminal helical lid domain, enabling it to interact with the DELLA domain of GA signalling repressor DELLA proteins and, thus, promote the formation of the GID1–GA–DELLA complex (Murase et al., 2008; Shimada et al., 2008). The GID1–GA–DELLA complex is further recognized by the F-box protein SLEEPY1 (SLY1) in Arabidopsis and triggers subsequent polyubiquitination and degradation of DELLA proteins through a SCF^{SLY1}-dependent 26S proteasome pathway, leading to the derepression of GA responses (Fig. 2.2) (Dill et al., 2004; Fu et al., 2004; McGinnis et al., 2003; Sasaki et al., 2003). The DELLAs’ GRAS domain is required for their interaction with F-box proteins while the DELLA domain is essential for the formation of GID1-GA-DELLA complex (Dill et al., 2004;
Ueguchi-Tanaka et al., 2007; Willige et al., 2007). It was proposed that the conformation change of DELLA proteins due to interaction with GID1 is a prerequisite for the perception of F-box proteins via the GRAS domain (Ariizumi et al., 2011; Hirano et al., 2010). It is important to note that there also is proteasome-independent down-regulation of DELLAs through the direct protein-protein interactions of GA-bound GID1 with DELLAs (Ariizumi et al., 2008; Ueguchi-Tanaka et al., 2008).

DELLA proteins are nuclear-localized, negative GA regulators that act as growth repressors. As explained above, the activation of the GA-mediated growth response is the result of DELLA degradation or inhibition through protein-protein interactions (Sun, 2011). As members of the plant-specific GRAS family proteins, DELLA proteins are named by their N-terminal DELLA domain which contains a conserved sequence Asp-Glu-Leu-Leu-Ala (Hauvermale et al., 2012). Loss-of-function DELLA mutants, like rice slr1 and the Arabidopsis quadruple-della mutant, are usually taller and flowering earlier than their respective wild-type plants; knockout of DELLAs can partially restore the phenotype of the GA biosynthetic Arabidopsis mutant ga1-3 (Cheng et al., 2004; Fu et al., 2002). Conversely, gain-of-function Arabidopsis DELLA mutants, such gai and rga-Δ17, showed dwarf and sometimes sterile phenotype (Dill et al., 2001; Peng et al., 1997). There are five DELLA proteins in Arabidopsis, including GA INSENSITIVE (GAI), REPRESSOR OF ga1-3 (RGA) and RGA-LIKE1 (RGL1), RGL2 and RGL3. The five Arabidopsis DELLAs display overlapping roles but also function specifically in different organs: GAI and RGA in root growth and stem elongation (Dill and Sun, 2001; Fu and Harberd, 2003; King et al., 2001), RGL1, RGL2 and RGA in flower development (Cheng et al., 2004; Tyler et al., 2004), RGL2 in seed germination (Lee et al., 2002; Tyler et al., 2004). Promoter exchange experiments showed that RGL2 and RGA had equivalent growth repressive effect when expressed at the same level, indicating that the functional diversity of DELLAs might be attributed to the transcriptional control under their respective promoters (Gallego-Bartolomé et al., 2010). Although DELLA proteins in many plant species have been studied in great detail, DELLAs’ target
genes or proteins are still largely unknown. According to global expression data and protein interaction studies, DELLLA proteins were proposed to be a convergence node in phytohormone signalling network (Gallego-Bartolomé et al., 2011; Grant and Jones, 2009; Hou et al., 2008). As there are no typical DNA binding domains, it is generally thought that DELLAs exert their functions by interacting with transcriptional factors (Fig. 2.2) (Zentella et al., 2007). Microarray studies showed that DELLAs promote the expression of downstream negative components of GA pathway and help maintain GA homeostasis by feedback regulation on the transcript levels of GA receptors and biosynthetic genes (Zentella et al., 2007). A positive GA signalling regulator, SCARECROW-LIKE3 (SCL3), was identified as a direct target protein of DELLAs. SLC3 and DELLAs antagonize each other by protein-protein interaction in controlling downstream GA responses as well as GA biosynthesis (Heo et al., 2011; Zhang et al., 2011).

In Arabidopsis, root growth inhibition by ethylene or promotion by auxin and the abscisic acid (ABA)-controlled seed dormancy are DELLLA-dependent (Achard et al., 2003; Fu and Harberd, 2003; Penfield et al., 2006). GA and DELLAs are implicated in regulating the abundance of PIN-FORMED (PIN) auxin transporter protein and, thus, affect auxin-dependent growth and development (Willige et al., 2011). DELLAs enhance ABA signalling to antagonize GA effect by promoting the accumulation of the ABA-related gene XERICO (Zentella et al., 2007). In Arabidopsis trichome induction, GA and JA function synergistically to increase trichome number and density (Traw and Bergelson, 2003). During Arabidopsis flower development, GA promotes JA biosynthesis in a DELLLA-dependent manner and, as a result, elevates MYB21, MYB24, and MYB57 expression to stimulate the stamen filament growth (Cheng et al., 2009; Hou et al., 2008). Another study showed that GA and JA have synergistic effect in regulating the transcriptional levels of sesquiterpene synthase genes in Arabidopsis flowers, which is mediated by the interaction between DELLAs and the JA downstream transcription factor MYC2 (Hong et al., 2012).
In ecological situations, DELLA proteins play a role in growth regulation to canopy signals (low R:FR or low blue light) and degradation of DELLAs is the prerequisite for shade-avoidance responses (Djakovic-Petrovic et al., 2007). Further evidence demonstrated that DELLAs interact with bHLH-type transcription factors phytochrome-interacting factor 3 (PIF3) and PIF4 to block their bind activity with their target gene promoters and, therefore, silence PIF3/4-mediated light control of hypocotyl elongation (De Lucas et al., 2008; Feng et al., 2008). The ability of DELLAs to integrate both light and GA signals has been observed in many plant physiological processes, including circadian rhythm, photomorphogenesis and skotomorphogenesis (Achard et al., 2007; Arana et al., 2011; Gallego-Bartolomé et al., 2011). It is surprising that DELLA proteins have interactions with so many proteins. A recent view presents the idea that the N-terminal domain of DELLAs is intrinsically disordered, which provides DELLAs with multiple-protein recognition features (Sun et al., 2010; Xiaolin et al., 2012).

2.6. Growth vs defense response: GA and JA-mediated crosstalk

DELLA proteins play an important role in plant stress response, possibly by integrating stress-related hormones and modulating resource allocation (Grant and Jones, 2009). DELLAs enhance Arabidopsis salt tolerance by integrating salt stress-activated ABA and ET signalling pathways (Achard et al., 2006). It is likely that the ABA and ET-mediated enhancement of DELLA growth restraint function increases the duration of vegetative stage and delays flowering, which contributes to plant survival (Achard et al., 2006). DELLA accumulation prevents cell death and promotes stress tolerance by controlling stress-induced reactive oxygen species (ROS) through the ROS scavenging system (Achard et al., 2008). Under pathogen attack, DELLAs promote the plant susceptibility to biotrophs and resistance to necrotrophs by modulating JA-SA signalling balance (Navarro et al., 2008). After necrotrophic fungus Alternaria brassicicola infection or JA treatment, the induction of JA marker gene PDF1.2 and LOX2 was delayed in the loss-of-function Arabidopsis della mutant while dominant DELLA mutant gai was sensitized for defense gene induction (Navarro et al., 2008). Microarray data
also indicates that DELLAs might attenuate the antagonism effect of SA on JA by inhibiting the activity of pathogen-related WRKY transcriptional regulators (Zentella et al., 2007). Additionally, up-regulation of GA catabolism enzymes and down-regulation of GA biosynthetic enzymes was observed in pathogen-infected rice and pepper, which implies that GA plays a negative role in disease resistance and plant defense requires down-regulation of GA concentration (Lee et al., 2012; Yang et al., 2008).

The molecular mechanism underlying this GA-JA crosstalk was revealed by a recent study showing that DELLAs physically interact with JAZ (Hou et al., 2010). DELLAs compete with MYC2 for binding to JAZ, facilitating the release of MYC2 from JAZ-MYC2 complex, promoting MYC2-dependent JA signalling; on the contrary, GA removes DELLAs from JAZ, which allows JAZ to shut down JA responses (Fig. 2.3A) (Hou et al., 2010; Wild et al., 2012). On the other hand, JAZs compete with growth regulator phytochrome interacting factors (PIFs) for binding to DELLAs; JA triggers the degradation of JAZs and, thus, prioritize plant defense over growth through the COI1-JAZ-DELLA-PIF signalling cascade (Fig. 2.3B) (Yang et al., 2012). In Arabidopsis, the JA downstream regulator MYC2 directly binds to the promoter of DELLA protein RGL3 and induces RGL3 expression, which leads to the suppression of plant growth by DELLA (Wild et al., 2012). Collectively, the GA pathway and JA pathway are connected by DELLAs and JAZs while this crosstalk enable the plant to balance its growth and defense.

2.7. Arabidopsis thaliana and Spodoptera exigua

2.7.1. Arabidopsis thaliana

Arabidopsis thaliana (Brassicaceae) is a relative of many economically crops such as canola (Brassica napus), cabbages (B. oleracea and B. rapa) and mustards (B. juncea, B. nigra and Sinapis alba). As a diploid species (n = 5) with a relatively small genome and a rapid life cycle, Arabidopsis is a popular model organism in plant biology research. Arabidopsis genome sequencing was completed in 2000, which greatly accelerates the study of this organism (The
Arabidopsis Genome Initiative, 2000). Arabidopsis has been widely used in research of plant-insect interactions. As mentioned above, the Arabidopsis defense response can be directly monitored through marker genes, defense proteins and secondary metabolites. With the help of mutant lines, the function of target genes can be determined by comparing the defense responses of mutant lines to wild-type Arabidopsis. In this study, a quadruple-della Arabidopsis mutant (lacks four out of five DELLA proteins in Ler background) was used. It displays slender and pale green leaves phenotype due to the lack of DELLA growth repressors (Lee et al., 2002). Previous studies have shown that this mutant has decreased JA-mediated defense responses against necrotrophic fungus Alternaria brassicicola and increased SA-mediated resistance to hemi-biotroph Pseudomonas syringae pv. tomato strain DC3000 (Navarro et al., 2008). Here, we investigated whether the knockout of DELLA proteins affect the induction of plant defense by herbivory.

2.7.2. Spodoptera exigua

The beet armyworm, Spodoptera exigua, is the generalist pest originated in Southeast Asia and reached North America in late 19th century (Greenberg et al., 2001). Caterpillar of S. exigua can feed on a wide range of crops of more than 18 families and it has become an economically important caterpillar pest in North America and Asia (Greenberg et al., 2001). S. exigua is used in many plant-insect interaction studies as it is easy to maintain and has a rapid generation time. In a suitable environment, S. exigua caterpillar usually has five instars and each instar has its own characteristics which can be identified through color, head capsule size and stripes (Azidah and Sofian-Azirun, 2006).

When chewing plant leaf tissues, elicitors in S. exigua oral secretion (OS) influence plant defense responses. Volicitin, N-(17-hydroxylinolenoyl)-L-glutamine, was the first elicitor identified in S. exigua OS. Application of volicitin to wounded corn seedlings induced OS-specific emission of plant volatiles that attract the nature enemies of S. exigua (Alborn et al., 1997). Interestingly, rather than triggering plant defense response, caterpillar labial saliva has been shown to
have negative effect on *Arabidopsis* induced resistance (Weech *et al.*, 2008). However, in tomato, caterpillar labial saliva induces JA burst and the expression of defense gene *proteinase inhibitor*2 (Tian *et al.*, 2012).

2.8. Summary

Plant growth and defense is dynamically controlled by internal signals and external environmental cues. Plants protect themselves against insect herbivores mainly through the JA defense pathway while the crosstalk between JA and other phytohormone pathways shapes the plant defense output. Some insect species have evolved strategies to hijack the JA-SA crosstalk to suppress JA-mediated defense responses. GA promotes plant growth and development by removing DELLA proteins. As growth repressors, DELLAs promote plant survival in adverse conditions. They are involved in JA-GA crosstalk and have been proved to be important modulators in plant defense-growth tradeoff (Yang *et al.*, 2012).

However, most studies about the defensive role of DELLAs have focused on plant-pathogen interactions. As insects are a major part threat to agricultural output, it is necessary to investigate DELLAs’ role in response to herbivory. In this study, we were trying to address two questions: first, whether DELLAs enhance plant resistance to herbivores; second, whether DELLAs attenuate the caterpillar labial saliva-triggered suppression of JA defense response. Ultimately, understanding the function of DELLAs in plant-insect interactions will give us an idea of how DELLA proteins and GA treatment affect the plant resistance to pests.
**Figure 2.1 Jasmonate signalling pathway and its crosstalk with salicylic acid pathway.** JA biosynthesis is induced in response to chewing herbivory. JA-Ile is perceived by COI1, which triggers the degradation of JAZ and activates the ERF and MYC branches of this pathway (Pieterse et al., 2012). Some caterpillar species use effectors to activate the SA-dependent pathway and, thus, attenuate JA-mediated defense responses (Weech et al., 2008). Arrows indicate activation, truncated lines indicate repression. JA, jasmonate; JA-Ile, jasmonoyl-L-isoleucine; ET, ethylene; SA, salicylic acid; JAZ, JASMONATE ZIM-DOMAIN; NPR1, NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1; PDF1.2, PLANT DEFENSIN1.2; VSP2, VEGETATIVE STORAGE PROTEIN2; PR1, PATHOGENESIS-RELATED GENE1.
Figure 2.2 Gibberellin signalling pathway and DELLAs’ downstream targets. GA triggers the degradation of DELLAs proteins and promotes plant growth. GA affects other hormone signalling through the interactions between DELLAs and different transcriptional regulators (Hauvermale et al., 2012). Arrows indicate activation, truncated lines indicate repression. GA, gibberellin; JA, jasmonate; ABA, abscisic acid; GID1, GIBBERELLIN INSENSITIVE DWARF1; SCL3, SCARECROW-LIKE3; PIF, PHYTOCHROME INTERACTING FACTOR; JAZ, JASMONATE ZIM-DOMAIN; PIN, PIN-FORMED.
Figure 2.3 The crosstalk between gibberellin and jasmonate pathways is mediated by DELLA and JAZ. (A) DELLAAs enhance JA signalling by inhibiting JAZ repressors and promoting the scavenging of ROS (Achard et al., 2008; Hou et al., 2010). (B) JA prioritize defense over growth through the JAZ-DELLA-PIF signaling cascade (Wild et al., 2012; Yang et al., 2012). Arrows indicate activation, truncated lines indicate repression. GA, gibberellin; JA, jasmonate; ROS, reactive oxygen species; JAZ, JASMONATE ZIM-DOMAIN; PIF, PHYTOCHROME INTERACTING FACTOR.
3. Materials and Methods

3.1. Growing Arabidopsis plants

Wild type Arabidopsis Landsberg erecta (Ler) and the quadruple-della mutant (quad-della, gai-t6 rga-t2 rgl1-1 rgl2-1, Ler background) seeds were gifts from Dr. Achard (Achard et al., 2008). The quad-della mutant was generated via cross-pollination using four single DS-insertion mutant lines, gai-t6, rga-t2, rgl1-1 and rgl2-1 (Cheng et al., 2004; Lee et al., 2002). To confirm the DS-insertion in the quad-della mutant, genomic DNA (gDNA) of both Ler and the quad-della mutant was first extracted using DNeasy Plant Mini Kit (Qiagen). Then allele-specific primers (Table 3.1) flanking the Ds region of each insertion were used in polymerase chain reaction (PCR) amplification of the gDNA (Dill and Sun, 2001; Lee et al., 2002). All PCR reactions were run in Mastercycler ep Gradient S (Eppendorf) using Taq DNA Polymerase (BioLabs). The PCR products were separated by electrophoresis on a 1.0% agarose gel. By comparing the PCR results of quad-della mutant and Ler control, all Ds-insertions in quad-della were confirmed and the mutant line was shown to be homozygous.

Arabidopsis seeds were sown in Agro Mix soil (Fafard); soil was pasteurized at 80°C, 2 hours. The seeds were stratified at 4°C for two days and then moved to growth chamber. Seedlings were grown in short day condition (8:16 light:dark schedule, light intensity 250 µE m⁻² s⁻¹ and temperature 23°C) and bottom-watered every 2 days with diluted All Purpose Fertilizer (Plant-Prod; total nitrogen 20%, available phosphoric acid 20%, soluble potash 20%). Three healthy plants were kept per pot. 4-5 week old Arabidopsis plants were used for herbivory experiment. According to Boyes et al., (2001), the plants used for herbivory experiment were between growth stage 1.11 – 1.14 (principal growth stage 1, leaf development: 11 – 14 rosette leaves > 1 mm in length).

3.2. Insect rearing

Spodoptera exigua caterpillars were maintained on a wheat germ-based artificial diet (Bio-Serv) in a growth cabinet under the condition of 22°C, 28-40% relative humidity and 16:8 light to dark cycle. Larvae had five instars under this
growth conditions before pupation. Pupae were collected in a glass jar for reproduction and eggs were moved to a new plastic container to maintain the colony. Fourth instar caterpillars were used in the herbivory experiment.

3.3. Cauterization of caterpillar spinneret

*S. exigua* larvae secrete their labial saliva through a tube-like spinneret (Musser *et al.*, 2002). The secretions can be shut down by cauterizing the spinneret of the caterpillar (Bede *et al.*, 2006). Early fourth instar caterpillars were selected for cauterization. They were placed into ice-cooled cups and then fixed on a Petri dish with plasticine. A pre-heated hot probe was used to burn their spinneret under a dissecting microscope (Olympus). Each cauterized caterpillar was placed in a separate Petri dish with BioServ diet to allow recovery.

Caterpillar labial salivary contains high GOX activity (Merkx-Jacques and Bede, 2005). To test whether the cauterization was successful, a GOX enzyme test was performed (Weech *et al.*, 2008). After the caterpillars recovered, their mouths were cleaned with distilled water to remove remaining food. Then, both cauterized and non-cauterized caterpillars (control) were placed into separate medicine cups which contained glass microfiber filter discs (Whatman). These filter discs were pre-soaked with 100 µl glucose/sucrose solution (50 mg/ml for each sugar, Sigma). The caterpillars were allowed to feed on the discs in the dark for 3-4 hours and then the discs were taken out from the cups to test salivary GOX activity. Each disc was treated with 2.5 Units horseradish peroxidase (HrP, Sigma) and 0.15 mg 3,3’-diaminobenzidine (DAB, Sigma). One positive control (HrP + DAB + H2O2) and five negative controls (HrP + DAB; HrP + H2O2; DAB + H2O2; HrP; DAB) were also included. If there is GOX activity from the caterpillar labial saliva, the glucose in the disc will be oxidized by the enzyme and H2O2 will be generated. HrP catalyzes the oxidation of DAB by H2O2, which forms a dark brown precipitate. Consequently, the biting of the disc and the absence of precipitate indicates cauterization was successful. Both cauterized and normal caterpillars were placed on wild-type *Arabidopsis* (feeder plant) for approximately 12 hours to let them adjust to the plant diet.
3.4. GA treatment

DELLA proteins are rapidly degraded upon application of bioactive GA on plant leaves, therefore, GA treatment might have similar effect as the knockout of DELLA genes (Zentella et al., 2007). GA$_3$ treatment showed the transient degradation of most DELLA proteins in Arabidopsis seedlings 1-2 hours after application (Achard et al., 2003; Silverstone et al., 2001). In this study, Arabidopsis plants were leaf-sprayed with 100 µmol GA$_3$ (Sigma) or mock (0.1% ethanol v/v) 2 hours before herbivory experiment.

3.5. Herbivory experiment

Plants used in herbivory experiment were divided into three groups: the quad-della mutant, Ler and Ler sprayed with GA (Ler + GA). Each plant group had two treatments and one control: caterpillars with intact spinneret (intact), caterpillars with impaired spinneret (cauterized) and control (no caterpillars). In each pot containing three plants, three caterpillars were placed and the pots were enclosed tightly by netting to prevent caterpillar escape. As S. exigua is a Noctuid which is active at night, feeding was conducted in the dark. To minimize the effect of plant volatile signalling in the growth chamber, plexiglass panels were placed between each treatment.

Caterpillars were removed from the plants after 10 hours’ feeding and plant samples were collected in liquid nitrogen-cooled falcon tubes immediately. For gene expression and defense protein analyses, caterpillar-damaged leaves were collected. For hormone analysis, all of the plant above-ground part was collected. Samples from the three plants in the same pot were pooled. All samples were stored in -80°C freezer until analysis.

The herbivory experiment was repeated eight times independently. In gene expression experiment and hormone analysis, four biological replicates were measured (n = 4). In defensive protein assay and estimation of biomass loss, eight biological replicates were analyzed (n = 8).

3.6. Biomass loss due to herbivory
Each genotype and treatment of every experimental repeat contained one replicate to estimate the leaf loss due to herbivory. Plant above-ground parts were collected in an envelope and dried at 70°C for 3 days. By comparing the dry weight of herbivore-damaged plants to the control plants, the biomass loss was estimated.

3.7. Hormone analysis

Plant samples were freeze-dried at -60°C for 24 hours using a Modulyo Freeze Dryer (Thermo Savant). Lyophilized samples were ground by TissueLyser (Qiagen) and sent to the Donald Danforth Plant Science Center for hormone analysis. Plant hormone concentration including SA, ABA, JA, JA-Ile and 12-oxo-phytodienoic acid (OPDA) was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The data was normalized based on the internal standards: deuterium-labeled salicylic acid (D5-SA), abscisic acid (D6-ABA) and jasmonic acid (D2-JA).

3.8. RNA extraction and cDNA synthesis

To extract RNA, *Arabidopsis* leaves were first ground with a sterilized mortar and pestle in liquid nitrogen. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) according to manufacturer’s instruction. RNA quality was tested on a 0.8% agarose gel to check if there was any degradation of RNA. OD$_{260}$/OD$_{280}$ of RNA samples was measured by infinite M200 Pro microplate reader (Tecan) and samples with good RNA quality were used for reverse transcription.

After gDNA wipeout (QuantiTect Reverse Transcription kit, Qiagen) treatment, gDNA contamination was detected by PCR using the primer pair 5’-ATG GGT CGT CAT CAG ATT CAG AGC AGA TAA-3’ and 5’-CAT ATA AGA GGT GTG TTA GAG ACA ATA ATA-3’ which spans an intronic region of the *Arabidopsis laccase-4* gene (Weech *et al.*, 2008). A gDNA positive control was also included. PCR products were run on a 1% agarose gel. 1 µg RNA was used to generate cDNA through the QuantiTect Reverse Transcription Kit.
(Qiagen) following the manufacturer’s protocol. A 1/10 dilution of the cDNA was used in the quantitative real-time polymerase chain reaction.

3.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

Transcript levels of genes of interest were measured by qRT-PCR using a Mx3000p thermocycler (Stratagene) and Absolute Blue qPCR SYBR low Rox Mix (Thermo Scientific) according to the manufacturer’s protocol. Gene-specific primers were obtained from literature (Table 3.2). PDF1.2, LOX2 and VSP2 are marker genes of the JA pathway. PRI is a marker gene of the SA pathway. Reference genes were Actin7 and At4g26410. Primer pairs were first tested by normal PCR to obtain amplicons. After running the PCR products on a 1% agarose gel, the amplicon of each gene was purified using QIAquick Gel Extraction Kit (Qiagen) and sent for sequencing to confirm the amplicon sequence. A standard curve (10^{-2} – 10^{-8} ng/μl) of amplicon was made for each gene. For each standard curve, a 90% - 110% PCR reaction efficiency was achieved and for each sample a dissociation curve was conducted to ensure it was a single peak.

The qRT-PCR was conducted in 0.2 ml semi-skirted 96-well PCR plates and ultra clear caps (Thermo Scientific). On each plate, four biological replicates were analyzed and each sample was analyzed in duplicate. Two technical replicates (two plates) were performed. Each well contained 7.5 μl Absolute Blue qPCR SYBR Low ROX (2X), 1.05 μl forward primer (1 μM or 3 μM), 1.05 μl reverse primer (1 μM or 3 μM), 100 ng cDNA sample (1/10 dilution of cDNA generated by reverse transcription) and 3.4 μl H2O in 15 μl total reaction volume. The following qPCR thermal cycling program was used: enzyme activation at 95°C, 15 min; 40 cycles of 95°C for 15 sec, 58-60°C (according to the annealing temperature of each reaction) for 30 sec and 72°C for 30 sec. Melt curve program was: 95°C for 30 sec and then continuous scan from 60°C to 95°C.

Raw fluorescence data obtained by MxPro v4.10 program (Stratagene) was exported to qPCR miner software to calculate the CT values and amplification efficiency, which were transformed to the initial concentration of the gene
transcripts (Zhao and Fernald, 2005). The expression stability of the two reference genes (Actin7 and At4g26410) in different genotypes and treatments was tested using both Excel-based program Bestkeeper and Brunner’s test (Brunner et al., 2004; Pfaffl et al., 2004). The geometric mean of the two reference genes’ concentrations was used to normalize the target genes. The normalized relative expression values of target genes were used in statistical analysis.

3.10. Protein extraction

Sample tissues were ground to fine powder in liquid nitrogen using a pestle and mortar. For the trypsin inhibitor (TI) assay, ice-cold extraction buffer (0.1 M pH 7.0 sodium phosphate buffer, 0.1% Triton X-100 and 7% polyvinylpyrrolidone) was added to plant sample, followed by vortexing and centrifugation for 10 min at 13,000 rpm. The laccase-like multicopper oxidase (LMCO) assay used the same extraction buffer as in TI assay except that proteinase inhibitor solution (PI, final concentration 0.5x, Sigma) was included to prevent protein degradation. After centrifugation, the supernatant was transferred to a new tube and placed on ice.

3.11. Trypsin inhibitor (TI) assay

The TI assay was performed according to the method described by Lara et al. (2000) with slight modification to optimize it for the infinite M200 Pro microplate reader. Briefly, protein extract and TI standard curve (made by soybean trypsin inhibitor (Sigma), ranging from 0 µg to 5 µg) was prepared in triplicate in a 96-well plate (Costar). Bovine trypsin (0.5 µg, Sigma) as well as no trypsin control was included and the plate was incubated in the microplate reader at 37 °C for 20 min with gentle shaking. Then the trypsin substrate N-benzoyl-DL-arginyl-β-naphthylamine (BANA, final concentration 3 mM, Sigma) was added to the mixture and the plate was incubated for another 80 min. In this reaction, β-naphthalene is released through trypsin-catalyzed hydrolysis of BANA. After 80 min incubation, the reaction was stopped by adding 4% HCl. To measure the product of the reaction, the color reagent p-dimethyl-amino-cinnamaldehyde
(Sigma) was added and the absorbance of the product was read at 540 nm. Inhibitory dose-response curves of TI were drawn using GraphPad Prism 5, based on a Variable Slope model: \( Y = \text{Bottom} + \left( \frac{\text{Top} - \text{Bottom}}{1 + 10^{((\log IC50 - X) \times \text{HillSlope})}} \right) \). Sample raw data were inputted in the software and the corresponding TI concentrations were calculated according to the standard curves.

3.12. Laccase-like multicopper oxidase (LMCO) enzyme activity

After protein extraction, the LMCO assay was performed immediately. The activity of LMCO was measured in a 96-well plate using the method describing by Espín et al. (1997). To 10 µl leaf extract, 10 µl N,N-dimethyl formamide (DMF, final concentration 2%, Sigma), 10 µl 3-methyl-2-benzothiazolinone hydrozone (MBTH, final concentration 2 mM, Sigma) and 170 µl dopamine hydrochloride (final concentration 35 mM, Sigma) were added sequentially. In this reaction, LMCO catalyzes the oxidization of DOPA to dopaquinone. MBTH binds with dopaquinone to form MBTH-quinone adduct which has an absorbance at 476 nm. DMF dissolves MBTH-quinone adduct (Espín et al., 1997). The reaction slope (OD/min) was monitored by the infinite M200 Pro microplate reader as kinetic cycle at 476 nm (35 °C, 3 min with 15 sec interval). All samples were measured in triplicate. Boiled negative controls and tyrosinase positive controls were included. The enzyme activity was calculated using Beer’s Law equation \( (A = \varepsilon cl) \) where the extinction coefficient \( (\varepsilon) \) of MBTH-quinone adduct is 20700 M\(^{-1}\) cm\(^{-1}\).

3.13. Modified Bradford assay

The soluble protein concentration of leaf extract in TI and LMCO assay were measured by modified Bradford assay (Zor and Selinger, 1996; Bradford, 1976). A standard curve was made using bovine serum albumin (BSA, sigma) ranging from 5 – 100 µg/ml. Diluted protein samples and blanks were prepared in 96-well plate (Costar) and Bradford reagent (Thermo Scientific) was added to each well. After incubation in dark for 10 min, the absorbance at 590 nm and 450 nm was measured by the infinite M200 Pro microplate reader. The ratio OD\(_{590}\)/OD\(_{450}\) was
used to calculate the standard curve equation and sample concentrations. After getting the protein concentration of the extracted samples, TI (ng/µg) and LMCO (U/mg) concentration was determined.


Insect performance experiment was performed on a growth bench in the research greenhouse at MacDonald Campus of McGill University. Three types of Arabidopsis plants (Ler, Ler + GA and the quad-della mutant) were grown in short day condition (8 h photoperiod, 23°C, 250 µE m⁻² s⁻¹ light intensity) and 5-6 week old plants were used for this experiment. Third instar S. exigua caterpillars (raised on artificial diet) were collected and weighed. Caterpillars which were either too light (fresh weight < 35.0 mg) or too heavy (fresh weight > 45.0 mg) were discarded. Pre-weighed caterpillars were placed on plants randomly (one caterpillar per pot) and the pots were enclosed tightly by netting to prevent caterpillar escape. Caterpillars were allowed to feed on plant and finish pupation (seven days). Then pupae were collected in 2 ml tubes and dried at 70 °C for three days. Pupa dry weight was measured to closest 0.1 mg. The insect performance experiment was repeated three times independently. For each genotype in every replicate, the growth of twenty caterpillars was measured (total n = 60).

3.15. Statistical analysis

Outliers were detected using Outlier Calculator (GraphPad) and removed. IBM SPSS Statistics Version 20 was used to do the following statistical analysis. Two-way analysis of variance (ANOVA) was performed to analyze the data of Ler and Ler + GA (two fixed factors: GA treatment and caterpillar herbivory) followed by a Tukey post hoc test (significant level, p = 0.05). Within the quad-della genotype, data were analyzed by one-way ANOVA with a Tukey post hoc test (significant level, p = 0.05). For insect performance experiment, pupa dry weight was compared by analysis of covariance (ANCOVA) using the initial fresh weight of caterpillars as a covariate.
Table 3.1 Allele-specific primers for PCR detection of the DS-insertions in the *quad-della* mutant (Dill and Sun, 2001; Lee et al., 2002)

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gai-t6 304</td>
<td>TCGGTACGGGATTTTCGCAT</td>
</tr>
<tr>
<td>gai-t6 300</td>
<td>CTAGATCCGACATTGAAGGA</td>
</tr>
<tr>
<td>gai-t6 302</td>
<td>AGCATCAAGATCAGCTAAAG</td>
</tr>
<tr>
<td>rga-t2 906F</td>
<td>GCCGGAGCTATGAGAAAGTGG</td>
</tr>
<tr>
<td>rga-t2 DS3-2</td>
<td>CCGGTATATCCCGTTTTCG</td>
</tr>
<tr>
<td>rga-t2 2607R</td>
<td>AAGAATTTTTAACAAGTGAACG</td>
</tr>
<tr>
<td>rga-t2 DS5-3</td>
<td>CGGTCCGTCGGAATTTCCC</td>
</tr>
<tr>
<td>rgl1-1 2295R</td>
<td>CCACAGACGCGCTAGAGGATAAC</td>
</tr>
<tr>
<td>rgl1-1 Ds5-P1</td>
<td>CATGGGCTGGGCTCAGTG</td>
</tr>
<tr>
<td>rgl1-1 1670F</td>
<td>AAGCTAGCTCGAAACCCAAAT</td>
</tr>
<tr>
<td>rgl2-1 856F</td>
<td>GCTGGTGAACGCGTGGGAACA</td>
</tr>
<tr>
<td>rgl2-1 1883R</td>
<td>ACGCCCGGTTGTGATGAGT</td>
</tr>
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Table 3.2 Primer sets used for quantitative real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Primer Sequence</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin7</td>
<td>At5g09810</td>
<td>GTATGCTCTTCTCATGCTATCCTTTTCCTCGTTCTG</td>
<td>60</td>
<td>118</td>
<td>Beste et al., 2011</td>
</tr>
<tr>
<td>At4g26410</td>
<td>At4g26410</td>
<td>GAGCTGAAGTGCTCATGACGGTCCGACATACCATGGATCC</td>
<td>60</td>
<td>81</td>
<td>Czechowski et al., 2005</td>
</tr>
<tr>
<td>PDF1.2</td>
<td>At5g44420</td>
<td>CGGCAATGGTGGAAGCAGCATGCATTACTGTTTCGCAA</td>
<td>59</td>
<td>79</td>
<td>Jirage et al., 2001</td>
</tr>
<tr>
<td>LOX2</td>
<td>At3g45140</td>
<td>GTCTACTTGCTTCCACCCGATTCACCATGCATTACTGTTTCGCAA</td>
<td>57</td>
<td>160</td>
<td>Wcech et al., 2008</td>
</tr>
<tr>
<td>VSP2</td>
<td>At5g24770</td>
<td>ATATCCGTACCGAAGACAGAGAAGGCTACGACCATGAGATATCTGCTGCCTCATGCTATCCTTTTCCTCGTTCTG</td>
<td>58</td>
<td>234</td>
<td>Troufflard et al., 2010</td>
</tr>
<tr>
<td>PRI</td>
<td>At2g14610</td>
<td>CACTACACTCAAGTTGTTGGTAGATAGTGCTTCTCGTTTCA</td>
<td>58</td>
<td>131</td>
<td>Kuśnierzycy et al., 2007</td>
</tr>
</tbody>
</table>
4. Results

4.1. Caterpillar damage

20% to 29% plant tissue was consumed by caterpillars, regardless of caterpillar spinneret status or plant genotype. Cauterization of the caterpillar spinneret did not affect feeding.

4.2. Hormone level

Under herbivore attack, plants rapidly increase JA hormone level that leads to the downstream defense reaction. Additionally, ABA and SA pathways are also involved in plant defense response against herbivores (Pieterse et al., 2012).

In Ler plants, caterpillar herbivory induced a JA burst and cauterization of caterpillar spinneret had significant higher JA levels in contrast to normal caterpillar treatment (Fig. 4.1A, Table 4.1). GA treatment significantly increased JA level, but GA treatment and herbivory did not have an interaction effect. In the quad-della mutant, JA burst was also stimulated by caterpillar herbivory while a difference between normal and cauterized caterpillar treatment was not observed.

JA-Ile is the bioactive form of JA (Wasternack, 2007). Compared to controls, caterpillar herbivory caused significant increase in JA-Ile in all genotypes and treatments (Fig. 4.1B, Table 4.1). GA treatment did not have significant effect on JA-Ile level. In Ler and Ler + GA plants, caterpillars with cauterized spinnerets triggered a significant increase of JA-Ile compared to caterpillar with complete salivary secretions. In contrast, this difference was not observed in the quad-della mutant; closure of the caterpillar spinneret did not result in increased level of JA-Ile.

OPDA is the precursor to JA and also has biological functions (Wasternack, 2007). Similar to JA and JA-Ile, caterpillar treatment significantly increased OPDA level in all genotypes except in the intact caterpillar treated quad-della mutant (Fig. 4.1C, Table 4.1). Again, a labial saliva-specific pattern was observed in Ler and Ler + GA plants but not in the quad-della mutant plants.

In addition to JAs, SA and ABA levels were measured. SA levels remained stable irrespective of Arabidopsis genotype or herbivory (Fig. 4.1D, Table 4.1).
Significant increase in ABA level was observed in the quad-*della* mutants subject to herbivory by cauterized caterpillars (Fig. 4.1E, Table 4.1).

4.3. Gene expression

Plant defense responses are associated with global transcript reprogramming (Howe and Jander, 2008). Transcript levels of four target genes, *PDF1.2*, *VSP2*, *LOX2* and *PR1* were analyzed. Two housekeeping genes, *Actin7* and *At4g26410*, were used as reference genes. According to the results calculated by Bestkeeper software and Brunner’s test (ANOVA; *Actin7*, \( F_{(8,27)} = 1.304, p = 0.238 \); *At4g26410*, \( F_{(8,27)} = 0.327, p = 0.948 \)), the stability of the two reference genes was acceptable and they were used to normalize the target genes.

*PDF1.2* is a marker gene of the ERF branch of the JA pathway (Lorenzo et al., 2003). In *Ler* plants, normal caterpillar-treatment showed significant higher expression of *PDF1.2* compared to control (Fig. 4.2A, Table 4.1). *PDF1.2* transcript levels in cauterized caterpillar-treated *Ler* plants were neither significantly different from control nor from intact caterpillar-treated plants. GA-treated plants had significant increase in the expression of *PDF1.2*, but there is no interaction effect between GA treatment and herbivory. In the quad-*della* mutant, control and cauterized caterpillar-treated plants had similar *PDF1.2* level while intact caterpillar-treatment resulted in a >3-fold higher transcript level.

*LOX2* encodes a JA biosynthesis enzyme and is also a marker gene of the MYC branch of JA pathway (Wasternack, 2007). Caterpillar herbivory (both intact and cauterized) triggered a significant increase of *LOX2* transcript level in all genotypes and treatments (Fig. 4.2B, Table 4.1). GA treatment did not show a significant effect on *LOX2* expression. Labial salivary-specific expression pattern was not observed in *Ler*, *Ler* + GA and the quad-*della* mutant.

*VSP2* is another marker gene of the MYC branch of JA pathway (Wasternack, 2007). In *Ler* plants, its expression was significantly increased upon caterpillar feeding while cauterization did not show a significant effect on *VSP2* expression (Fig. 4.2C, Table 4.1). GA treatment did not have a significant effect on *VSP2* transcript level. Compared to control quad-*della* plants, normal caterpillar-
treatment showed about 160-fold higher $VSP2$ level and cauterized caterpillar-
treatment showed about 400-fold higher $VSP2$ level.

$PR1$ is a marker gene of the SA pathway (Dong, 2004). $PR1$ expression in
intact caterpillar-treated $Ler$ plants was significantly higher than that in control
and cauterized caterpillar treatment (Fig. 4.2D, Table 4.1). Application of GA did
not show a significant effect on $PR1$ expression. For the quad-$della$ mutant, intact
caterpillar-treated plant showed similar $PR1$ transcript level as control while
cauterized caterpillar-treated plant had lower $PR1$ levels than control. However,
due to large variation in $PR1$ expression, intact caterpillar treatment did not show
significant difference from cauterized caterpillar treatment. It is of interest that,
when comparing the $Ler$ and quad-$della$ controls, $PR1$ transcript level in the
quad-$della$ mutant was >80-fold higher than that in $Ler$.

4.4. Defensive proteins

The induction of JA-mediated defense signaling results in elevated activity of
defensive proteins (Howe and Jander, 2008). In this study, the defensive proteins
TI and LMCO were analyzed. In $Ler$ and the quad-$della$ mutant, caterpillars with
either intact or impaired labial saliva secretion did not cause significant change of
TI activity as compared to controls (Fig. 4.3A, Table 4.1). GA treatment also did
not affect TI activity. In LMCO assay, normal caterpillar-treated quad-$della$
showed slightly higher LMCO activity than its control while no other significant
difference was observed (Fig. 4.3B, Table 4.1).

4.5. Insect performance

The strength of plant defense is often reflected by the growth of insect that
feed on the plant. In the insect performance experiment, caterpillars had similar
dry pupa weight after growing on the $Ler$, $Ler + GA$ and quad-$della$ plants.
Compared to wild-type $Ler$, no significant effect of DELLA mutation or GA
treatment on pupa biomass was detected while the initial caterpillar weight had
influence on pupa weight (ANCOVA, $F_{(1,154)} = 8.74$, $p = 0.004$ for insect initial
weight and $F_{(2,154)} = 0.19$, $p = 0.889$ for different plant types) (Fig. 4.4).
Table 4.1 Statistical results of plant-insect experiments. Two-way analysis of variance (ANOVA) was performed to analyze the data of Ler and Ler + GA (two fixed factors: GA treatment and caterpillar herbivory) followed by a Tukey post hoc test. Within the quad-della genotype, data were analyzed by one-way ANOVA with a Tukey post hoc test.

<table>
<thead>
<tr>
<th></th>
<th>Ler wildtype (two-way ANOVA)</th>
<th></th>
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<th>quad-della mutant (one-way ANOVA)</th>
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<tbody>
<tr>
<td></td>
<td>Herbivory (Control/Intact/Cauterized)</td>
<td>GA treatment (+ GA$_3$)</td>
<td>Interaction effect (Herbivory × GA treatment)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>F value</td>
<td>P value</td>
<td>F value</td>
<td>P value</td>
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<td>P value</td>
<td>F value</td>
</tr>
<tr>
<td>Jasmonic acid (JA)</td>
<td>F$_{(2, 16)}$ = 76.17 p &lt; 0.001</td>
<td>F$_{(1, 16)}$ = 4.79 p = 0.04</td>
<td>F$_{(2, 16)}$ = 0.69 p = 0.52</td>
<td>F$_{(2, 9)}$ = 169.14 p &lt; 0.001</td>
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<tr>
<td>Jasmonoyl-L-isoleucine (JA-Ile)</td>
<td>F$_{(2, 17)}$ = 26.04 p &lt; 0.001</td>
<td>F$_{(1, 17)}$ = 2.31 p = 0.15</td>
<td>F$_{(2, 17)}$ = 0.21 p = 0.81</td>
<td>F$_{(2, 9)}$ = 13.50 p = 0.002</td>
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<tr>
<td>12-Oxo-phytodienoic acid (OPDA)</td>
<td>F$_{(2, 16)}$ = 40.85 p &lt; 0.001</td>
<td>F$_{(1, 16)}$ = 1.08 p = 0.31</td>
<td>F$_{(2, 16)}$ = 1.16 p = 0.34</td>
<td>F$_{(2, 9)}$ = 8.70 p = 0.01</td>
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<tr>
<td>Salicylic acid (SA)</td>
<td>F$_{(2, 18)}$ = 1.10 p = 0.35</td>
<td>F$_{(1, 18)}$ = 3.05 p = 0.10</td>
<td>F$_{(2, 18)}$ = 2.43 p = 0.12</td>
<td>F$_{(2, 9)}$ = 1.55 p = 0.26</td>
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<tr>
<td>Abscisic acid (ABA)</td>
<td>F$_{(2, 18)}$ = 3.51 p = 0.05</td>
<td>F$_{(1, 18)}$ = 0.03 p = 0.87</td>
<td>F$_{(2, 18)}$ = 0.22 p = 0.81</td>
<td>F$_{(2, 9)}$ = 5.93 p = 0.02</td>
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<tr>
<td>PDF1.2</td>
<td>F$_{(2, 16)}$ = 7.91 p = 0.004</td>
<td>F$_{(1, 16)}$ = 5.39 p = 0.03</td>
<td>F$_{(2, 16)}$ = 1.02 p = 0.39</td>
<td>F$_{(2, 9)}$ = 8.54 p = 0.01</td>
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<td>LOX2</td>
<td>F$_{(2, 18)}$ = 116.42 p &lt; 0.001</td>
<td>F$_{(1, 18)}$ = 0.25 p = 0.62</td>
<td>F$_{(2, 18)}$ = 0.03 p = 0.97</td>
<td>F$_{(2, 9)}$ = 63.53 p &lt; 0.001</td>
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<td>VSP2</td>
<td>F$_{(2, 18)}$ = 20.73 p &lt; 0.001</td>
<td>F$_{(1, 18)}$ = 2.03 p = 0.17</td>
<td>F$_{(2, 18)}$ = 0.55 p = 0.59</td>
<td>F$_{(2, 9)}$ = 5.54 p = 0.03</td>
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<td>PR1</td>
<td>F$_{(2, 16)}$ = 11.43 p = 0.001</td>
<td>F$_{(1, 16)}$ = 2.34 p = 0.15</td>
<td>F$_{(2, 16)}$ = 3.22 p = 0.07</td>
<td>F$_{(2, 9)}$ = 4.933 p = 0.046</td>
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<td>Trypsin inhibitor (TI)</td>
<td>F$_{(2, 41)}$ = 1.80 p = 0.18</td>
<td>F$_{(1, 41)}$ = 1.38 p = 0.25</td>
<td>F$_{(2, 41)}$ = 0.38 p = 0.69</td>
<td>F$_{(2, 20)}$ = 0.01 p = 0.99</td>
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<td>Laccase-like multicopper oxidase (LCMO)</td>
<td>F$_{(2, 42)}$ = 1.08 p = 0.35</td>
<td>F$_{(1, 42)}$ = 0.001 p = 0.98</td>
<td>F$_{(2, 42)}$ = 2.43 p = 0.10</td>
<td>F$_{(2, 21)}$ = 4.57 p = 0.02</td>
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Figure 4.1 Phytohormone levels in response to caterpillar herbivory. *Arabidopsis* plants (Ler, Ler + GA, quad-della) were subject to *S. exigua* caterpillar herbivory (control, no caterpillar; intact, caterpillars with intact salivary secretions; cauterized, caterpillars with impaired salivary secretions) for 10 hours. (A) JA, (B) JA-Ile, (C) OPDA, (D) SA, (E) ABA levels were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Bars represent the means of four biological replicates with standard error of means. The lower case letters indicate significant differences (significant level, p = 0.05).
Figure 4.2 Defense gene expressions in response to caterpillar herbivory. Arabidopsis plants (Ler, Ler + GA, quad-della) were subject to S. exigua caterpillar herbivory (control, no caterpillar; intact, caterpillars with intact salivary secretions; cauterized, caterpillars with impaired salivary secretions) for 10 hours. Transcript levels of (A) PDF1.2, (B) LOX2, (C) VSP2 and (D) PR1 were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Two reference genes, Actin7 and At4g26410, were used to normalize the target genes. Bars represent the means of four biological replicates with standard error of means. The lower case letters indicate significant differences (significant level, p = 0.05).
Figure 4.3 Trypsin inhibitor (TI) levels and laccase-like multicopper oxidase (LMCO) enzyme activity in response to caterpillar herbivory. *Arabidopsis* plants (Ler, Ler + GA, quad-della) were subject to *S. exigua* caterpillar herbivory (control, no caterpillar; intact, caterpillars with intact salivary secretions; cauterized, caterpillars with impaired salivary secretions) for 10 hours. (A) TI levels and (B) LMCO activity were analyzed by enzyme assays. Bars represent the means of eight biological replicates with standard error of means. The lower case letters indicate significant differences (significant level, P = 0.05).
Third instar *S. exigua* caterpillars were allowed to feed on *Arabidopsis* plants (Ler, Ler + GA, quad-della) until pupation (for approximate 7 days). Pupas were collected and their dry weight used to estimate insect performance. Bars represent the means of 60 biological replicates with standard error of means. Insect performances on different types of plants were compared by ANCOVA. The initial larval weight (third instar) was used as the covariate.
5. Discussion

5.1. Growth of the quad-della mutant

GA promotes plant growth and development while DELLA proteins are growth repressors (Sun, 2011). Our preliminary results showed that the quad-della mutant started bolting 3 weeks after germination under long day conditions (16 h photoperiod) while the bolting time of wild-type Ler was 1-2 weeks later. Plants that had started bolting or flowering could not be used in herbivory experiment because entering the reproductive stage results in global hormone level change and gene expression reprogramming that affect plant defense responses (Hennig et al., 2004). Additionally, JA signalling is involved in plant reproductive development and a study in tomato showed that many JA-related defense genes were highly expressed in tomato flowers without insect attack (Browse, 2009; Li et al., 2004b). Therefore, we used short day condition (8 h photoperiod) to grow the plants; this delayed flowering and allowed us to have more plant biomass (vegetative growth stage) before the herbivory experiment. However, we observed that even under short day condition the quad-della mutant start flowering much earlier than wild-type Ler, which has been reported before (Cheng et al., 2004). In our experiment, 5 week old plants were used; quad-della and Ler plants had similar dry weight at this plant age (about 0.12 g for three plants) and suffered similar biomass loss due to herbivory (20 – 29%).

5.2. Herbivory-induced hormone level change

Plants react rapidly to insect attack by increasing their JA biosynthesis, leading to increased levels of JA and related octadecanoid compounds in plant tissues (Kazan and Manners, 2008). In this study, levels of three octadecanoid compounds were measured, which include free JA, JA-Ile (bioactive JA) and OPDA (JA precursor). In addition, ABA and SA levels were also measured. Generally, caterpillar feeding induced similar octadecanoid compounds burst in wild-type Arabidopsis Ler and the quad-della mutant, indicating that DELLA proteins do not have strong impact on herbivory-triggered JA burst (Fig 4.1A, B, C). Previously, DELLAs have been reported to positively regulate JA signalling
by binding to JA signalling repressor JAZs (Hou et al., 2010). In this study, they did not measure hormone levels but instead looked at JA-dependent gene expression. We showed that DELLA proteins do not affect hormone levels, therefore, their effect is downstream. Labial salivary-specific pattern of JA, JA-Ile and OPDA levels were observed in Ler; even though a jasmonate burst was associated with herbivory, this burst is significantly larger in plants treated by caterpillars that did not secrete labial saliva. This supports previous results that show an effector(s) in caterpillar labial saliva negatively impacts jasmonate levels (Weech et al., 2008). This labial salivary-specific difference was not seen in the quad-della mutant, indicating that DELLAs play a role in caterpillar labial saliva-mediated suppression of JA induced defenses. In comparison with wild type, herbivory of the Arabidopsis SA signalling deficient mutant ndr1-1 npr1-2 by caterpillars with intact or impaired labial salivary secretion did not cause a significant change in the JA burst, which is similar to what was observed with the quad-della mutant (Fig. 4.1A, B, C) (Weech et al., 2008). Navarro et al. (2008) showed that DELLAs play an important role in regulating the balance of JA and SA signalling under pathogen stress. Therefore, it appears that DELLA proteins are involved in mediating the crosstalk between JA and SA signalling. GA treatment was shown to have a positive effect on JA level but it did not have an interaction effect with caterpillar treatment. It has been reported that GA promotes JA biosynthesis in a DELLA-dependent manner during Arabidopsis flower development, which may explain the GA treatment effect in our experiment (Cheng et al., 2009). However, salivary-specific pattern in octadecanoid levels was observed in Ler + GA plants but not in the quad-della plants, indicating that DELLA knockout mutations have different effect on plant response to caterpillar labial saliva compared to GA-triggered DELLA degradation.

SA plays a major role in plant resistance to biotrophic pathogens and it also antagonizes JA-mediated defense responses (Pieterse et al., 2012). NPR1 is a downstream component of SA and it regulates a large portion of SA-related defense genes as a transcriptional coactivator (Dong, 2004). Previous study using SA signalling mutant ndr1-1 npr1-2 suggested that the salivary-specific JA
hormone pattern is NPR1-dependent but SA-independent (Weech et al., 2008). In this study, we also observed that the SA level remained unchanged in response to herbivory, which confirms this finding.

We observed a salivary-specific ABA pattern in the quad-della mutant but not in Ler and Ler + GA plants; cauterization of caterpillars results in a 2-fold increase of ABA level in the quad-della mutant (Fig 4.1E). This implies that an effector(s) in the caterpillar labial saliva prevents an increase in ABA levels and this depends on the presence of DELLA proteins. ABA functions synergistically with JA signalling through the MYC2 branch of JA pathway in plant response to biotic and abiotic stresses (Abe et al., 2003; Anderson et al., 2004). DELLAs have been shown to integrate ABA and ET signalling to promote plant salt tolerance (Achard et al., 2006). At a molecular level, DELLA proteins enhance ABA signalling by promoting the accumulation of the ABA-related gene XERICO (Zentella et al., 2007). Taken together, it is possible that DELLA proteins are involved in regulating plant response to caterpillar saliva in an ABA-dependent manner. It will be worthy to measure the expression of ABA marker genes to investigate this.

5.3. Herbivory-induced defense gene expression

In the gene expression study, we analyzed transcript levels of four marker genes: PDF1.2 for the ERF branch of JA pathway, VSP2 and LOX2 for the MYC branch of JA pathway and PRI for the SA pathway.

The plant defensin PDF1.2 is a defense peptide and its gene expression can be induced by incompatible pathogen challenge through the ERF1 branch of JA pathway (Penninckx et al., 1998). In Ler plants, differences of PDF1.2 level between cauterized and noncauterized caterpillar treatment are not statistically significant. The PDF1.2 expression pattern is different from previous research which demonstrated that labial salivary secretions inhibit PDF1.2 expression and cauterization resulted in higher PDF1.2 transcript level (Weech et al., 2008). The difference between that experiment and this one is the time scale as we collected samples after 10 hours of herbivory while Weech et al. (2008) collected samples
after 36 hours of herbivory. TGA transcriptional factors have been shown to positively regulate late PDF1.2 expression (Zander et al., 2010). It is proposed that caterpillar labial saliva interferes with plant defense responses in a NPR1/TGA-dependent way (Paudel et al., 2013; Weech et al., 2008). In this study, early PDF1.2 expression did not show a caterpillar salivary-specific expression. Unexpectedly, although GA treatment did not have interaction effect with caterpillar treatment, it resulted in higher PDF1.2 expression (Fig 4.2A). It has been reported that GA and JA act synergistically in plant trichome induction and sesquiterpene synthase gene expression (Hong et al., 2012; Traw and Bergelson, 2003). In our experiment, GA and JA may have synergistic effect in regulating PDF1.2 expression. In the quad-della mutant, we observed that the caterpillar labial saliva activated PDF1.2 expression while cauterization of spinneret attenuated its expression. In Arabidopsis, the MYC and ERF branches of JA signalling antagonize each other, conferring specific JA responses in an attacker-dependent manner (Dombrecht et al., 2007; Lorenzo et al., 2004). A recent study showed that treating wounded Arabidopsis leaves with P. rapae larval oral secretion activated the ERF branch of JA pathway, which resembles our results that caterpillar labial saliva activated PDF1.2 expression (Verhage et al., 2011). Taken together, this finding indicates that there is an elicitor(s) in caterpillar labial saliva induces PDF1.2 expression, probably in a DELLA-dependent manner. Additionally, Navarro et al. (2008) showed that PDF1.2 induction was delayed in the quad-della mutant infected by A. brassicicola as compared to wild-type Arabidopsis. However, as we only had one sample collection time point, the time pattern of PDF1.2 induction could not be determined. To better investigate DELLAs’ effect on PDF1.2 transcript levels, further experiments are needed to understand the time pattern of gene expression.

LOX2 encodes a lipoxygenase required in octadecanoid biosynthesis which catalyzes the conversion of α-linolenic acid to 13(S)-hydroperoxyoctadecatrienoic acid (Mueller, 1997). LOX2 expression is regulated by JA signalling through a MYC2-dependent, positive-feedback mechanism (Wasternack, 2007). In our study, LOX2 was induced by caterpillar feeding in
both Ler and quad-della plants. However, in Ler, JA levels showed a salivary-specific pattern while this was not observed in terms of LOX2 expression, which might be due to post transcriptional regulation of LOX2 (Thivierge et al., 2010).

Similar to LOX2, VSP2 was induced by herbivory in all genotypes and no salivary specific pattern was observed. VSP2 is positively regulated by MYC2 and DELLAs enhance MYC2 signalling by inhibiting JAZ repressing activity (Yang et al., 2012). However, according to our result, the quad-della plants were not impaired in the induction of VSP2. Again, as we have discussed above, although both the quad-della mutant and Ler had increased VSP2 transcript level under herbivory, VSP2 expression in quad-della might be delayed and further experiments are needed to understand the time pattern of VSP2 expression.

Arabidopsis PR1 is a SA-responsive gene, whose expression can be induced by pathogen infection or exogenous SA treatment (Lebel et al., 2001). In our study, significant increase of PR1 transcript level was observed in Ler plants eaten by intact caterpillar as compared to control and cauterized caterpillar treatment, suggesting that caterpillar saliva activates SA/NPR1 signalling. However, although there was salivary specific pattern of PR1 expression, SA level remained unchanged under herbivory, which implies that the activation of PR1 by caterpillar labial saliva is probably SA-independent but NPR1-dependent (Weech et al., 2008). PR1 displayed much higher expression in the quad-della mutant compared to Ler and it seemed that PR1 is constitutively expressed in the quad-della mutant (Fig 4.2D). This is different from Navarro’s studies which demonstrated that nonpathogen-infected quad-della had low PR1 mRNA level (Navarro et al., 2008). Further experiments are required to test other genes in the SA pathway so that we can have a better understanding of the regulation of the pathway. However, it is still possible that DELLAs play a role in maintaining the homeostasis of SA signalling by repressing its constitutive induction.

5.4. Defense protein activity

We analyzed two types of defense protein, LMCO and TI. LMCO converts plant phenolic compounds to reactive quinones, which bind to dietary proteins
and prevent their absorption in insect gut (Constabel and Barbehenn, 2008). In our experiment, LMCO activity in Ler remained unchanged under herbivory (Fig 4.3A). Unlike the hormone burst and gene expression, the production of defense protein is on a longer time scale (Kessler and Baldwin, 2002). As we collected our samples after ten hours of herbivory, it might be too early for the plants to fully increase their LMCO activity. Additionally, it is reported that Brassica species (Arabidopsis belongs to this family) do not show very strong polyphenol oxidases induction by wounding or MeJA treatment (Constabel and Ryan, 1998).

TI is a proteinase inhibitor and it is also part of the plant’s anti-nutritive defense (Lara et al., 2000). Caterpillar-induced TI activity increase was not seen in both Ler and quad-della (Fig 4.3B). Previous studies have shown that TI had significant higher activity in wild-type Arabidopsis eaten by caterpillars without salivary secretions (Weech et al., 2008). Again, like LMCO, no significant increase in TI activity may be due to our early sample collection time in the herbivory experiment. Overall, TI and LMCO results are not enough for us to judge whether DELLAs are involved in plant defense protein production.

5.5. Insect performance on the Ler, Ler + GA and quad-della plants

Insects gained similar pupa dry weight after feeding on Ler, Ler + GA and quad-della (Fig 4.4). We expected that caterpillars may have better performance on quad-della than Ler and our hypothesis is based on DELLAs’ role in attenuating necrotrophic pathogen infection (Navarro et al., 2008). However, considering that plant defenses against necrotrophic pathogen and herbivory are controlled by different JA signaling branches, it is possible that DELLAs have more positive impact on ERF branch than MYC branch (Hong et al., 2012). In response to herbivory, a similar jasmonate burst, gene expression patterns and defensive protein activity was observed between the wild type and quad-della mutant, therefore, the results from the insect performance experiment are not surprising. Additionally, although some insect grow slower due to defensive compounds, they might be able to overcome the plant defense by simply
consuming more plant tissue (Kessler and Baldwin, 2002; Winterer and Bergelson, 2001).

5.6. General discussion

We observed that in the quad-*della* mutant, a JA burst and the JA-dependent defense gene expression were normally induced by caterpillar herbivory. This quad-*della* mutant lacks four out of five DELLA proteins while there is one DELLA protein RGL3 remaining. A recent study reported that RGL3 expression was induced by JA through a COI1- and MYC2-dependent way and this induction effect by JA was quite strong compared to other DELLA proteins (Wild et al., 2012). As RGL3 also interacts with JAZ and enhance the JA-mediated defense response, the existence of RGL3 in the quad-*della* mutant might attenuate the negative effect caused by mutation of other DELLA proteins. Another recent study showed that GA and JA function synergistically in activating sesquiterpene synthase gene in *Arabidopsis* flowers through the interaction between MYC2 and DELLA proteins (Hong et al., 2012). MYC2 is an important transcriptional regulator in the MYC branch of JA pathway and it is responsible for plant resistance to herbivory (Lorenzo et al., 2004). JA activates MYC2 by removing its repressor JAZs (Chini et al., 2007). If DELLAs inhibit not only JAZs but also MYC2, it is not surprising that DELLAs do not promote plant defense against insect herbivory.

In our experiment, we used GA treatment to mimic the effect loss-of-function DELLA mutation as exogenous GA application can rapidly trigger the degradation of DELLAs. However, no interaction effect between GA treatment and herbivory was observed. This might be because that GA treatment only causes temporary removal of DELLAs and it is not enough to mimic the effect of DELLA mutation. Conversely, knockout of DELLA proteins should have a long-term effect on many physiological processes in plant. It is of interest that GA treatment had positive effect on JA level and *PDF1.2* expression. Considering that GA has been reported to act synergistically with JA in plant trichome induction and flower development, it is possible that GA also promotes part of
JA-mediated defense signalling. However, whether this effect of GA is DELLA-dependent remains unclear.

By cauterizing the caterpillars’ spinneret, we could compare plant responses to caterpillars with labial salivary secretions and those without to tease out the effect of labial saliva on plant responses. Labial salivary-specific octadecanoids (JA, JA-Ile and OPDA) burst pattern was observed in Ler but not in the quad-della mutant, suggesting that DELLA protein are involved in plant response to effector(s) in caterpillar labial saliva. One of the effectors in caterpillar labial saliva that may be responsible for this activity is glucose oxidase (Musser et al., 2002). DELLA proteins enhance the ROS scavenging system in plant cells, so the suppressive effect on JA signalling by caterpillar labial saliva should be stronger in quad-della plants, which is different from what we observed (Achard et al., 2008). This suggests that DELLAs might be involved in linking the caterpillar labial saliva-activated JA-SA crosstalk through other mechanisms. When DELLAs are removed, this link is broken and, thus, no labial salivary-specific pattern will be observed.
5. Conclusion

DELLA proteins are important modulators in the crosstalk between JA-mediated plant defense and GA-dependent plant growth (Yang et al., 2012). We investigated the role of DELLA proteins in plant defense responses against insect herbivores by comparing the *S. exigua* caterpillar-induced defense output in wild-type *Arabidopsis Ler* and the quad-della mutant. To measure the strength of plant defense reaction, hormone level, defense gene expression and defense protein activity were analyzed. Both wild-type *Ler* and the quad-della mutant showed a JA burst in response to herbivory. This was reflected in increased transcript levels of the JA-dependent gene markers, such as *AtPDF1.2*, *AtLOX2* and *AtVSP2*. Consistent with the results of plant defense output, insect had similar performance on *Ler*, *Ler* + GA and the quad-della plants. A caterpillar labial saliva-specific pattern of JA hormone levels were observed in the wild-type background but not in the quad-della mutant, suggesting that DELLAs are involved in plant response to caterpillar labial saliva, probably by mediating the crosstalk between JA and SA pathways. On the other hand, *PDF1.2* transcript levels showed a labial salivary-specific pattern in the quad-della mutant, indicating that an elicitor(s) in caterpillar labial saliva activates the *PDF1.2* expression, probably in a DELLA-dependent manner. Additionally, high constitutive expression of the SA pathway marker gene *AtPR1* was observed in the quad-della mutant but not in wild-type *Arabidopsis*, which indicates that DELLAs play a role in maintaining the homeostasis of SA signalling by repressing its constitutive induction.
6. Recommendations for Future Research

We have investigated the role of DELLA proteins in plant-insect interactions. Although the quad-della mutant showed normal defense response against herbivores, it does not mean that DELLAs are not affecting plant defense or insect growth.

First, as we have mentioned in the discussion part, more sample collection time points are needed so that we will know how fast the JA burst and defense gene expression are induced.

Secondly, as we are not sure if RGL3 is important for plant defense, the use of quintuple Arabidopsis della mutant which lacks all five DELLA proteins will be better for comparison to the quad-della we used (Feng et al., 2008; Wild et al., 2012). Additionally, gain-of-function DELLA mutant, such gai and rga-Δ17, can also be used to see if they have increased defense reaction under caterpillar herbivory.

Gene microarray is a powerful technique that can be used in this study. By investigating the global gene expression profile of Ler and quad-della eaten by either intact or cauterized caterpillar, we will be able to identify the differentially regulated genes between genotypes or treatments. Then, the DELLA-upregulated or downregulated genes can be studied in more details using bioinformatic tools.

Finally, as we found that DELLA proteins are involved in caterpillar saliva-specific response while the mechanism is unclear, it will be interesting to find out the target protein of DELLAs in this crosstalk. A yeast two-hybrid screen can be performed using the five Arabidopsis DELLA proteins and transcriptional regulators in JA and SA pathway. After identifying the direct target of DELLAs, it will be possible to explain the mechanism of this caterpillar saliva-mediated crosstalk.
7. References


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