Plant Cellular Signaling in Response to Wounding or Caterpillar Herbivory

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List of Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACN	Acetonitrile
ACS	1-aminocyclopropane-1-carboxylic acid (ACC) synthase
ALY	Glucoalyssin (5-methylsulphinylpentyl glucosinolate)
ANOVA	Analysis of variance
AOS	Allene oxide synthase
AP2/ERF	APETALA2/Ethylene response factor
APX	Ascorbate peroxidase
ASC	Reduced ascorbate
BANA	*N*-benzoyo-*L*-arginyl-β-nepthylamide hydrochloride
Ca^{2+}	Calcium ion
CDPKs	Calcium-dependent protein kinase
CNBH	Carbon-nutrient balance hypothesis
CO_2	Carbon dioxide
COI1	Coronatine insensitive1
CTR1	Constitutive triple response1
Cu^{2+}	Copper ion
DAMPs	Damage-associated molecular patterns
DHA	Dehydroascorbate (oxidized ascorbate)
DHAR	Dehydroascorbate reductase
EDS	Enhanced disease susceptibility
EIL	Ethylene insensitive-like
EIN2/3/4	Ethylene insensitive2/3/4
ERF	Ethylene response factor
ERS1	Ethylene response sensor1
ERU	4-methylthiobutyl glucosinolate
ESP	Epithionitrile specifier protein
EST	Expressed sequence tag

ET	Ethylene
ETI	Effector triggered immunity
ETR	Ethylene resistant1
FACs	Fatty acid-amino acid conjugates
GA	Giberrelin
GAI	Giberrelin insensitive
GBC	Glucobrassicin (3-indolylmethyl glucosinolate)
GID1	Giberrelin -insensitive1
GOX	Glucose oxidase
GR	Glutathione reductase
GRX	Glutaredoxin
GSH	Reduced glutathione
GSL	Glucosinolate
GSNO	*S*-nitrosoglutathione
GSSG	Oxidized glutathione
GSTs	Glutathione-*S*-transferase
H_2O_2	Hydrogen peroxide
HAMPs	Herbivore-associated molecular patterns
HEL	Hevein-like protein
I3M	Indol-3-ylmethyl glucosinolate
IBE	3-methlysulfylpropyl glucosinolate
ICS	Isochorismate synthase
Ile	Isoleucine
IR	Induced resistance
JA	Jasmonic acid
JAR	Jasmonate resistant1
JAZ	Jasmonic acid-Zim domain
LMCO	Laccase-like multi-copper oxidase
LOX2	Lipoxygenase2
LS	Labial saliva
MAPK	Mitogen-activated protein kinase

MeOGB	Methoxyglucobrassicin (4-methoxy-3-indolylmethyl glucosinolate)
Met	Methionine
MSOB	Methylsulfinylbutyl glucosinolate
MSOP	Methylesulfinylpentyl glucosinolate
MTB	Methyl thiobutyl
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NDR	Non-race-specific disease resistance
NeoGB	Neoglucobrassicin (1-methyoxy-3-indolylmethyl glucosinolate)
NO	Nitric oxide
NPR1	Non-expresser of pathogenesis-related protein1
NSP	Nitrile specifier protein
OPDA	12-*oxo*-phytodienoic acid
ORA59	Octadecanoid responsive factor59
OS	Oral secretion
PAD2.1	Phytoalexin-deficient2.1
PAMPs	Pathogen-associated molecular patterns
PCA	Principal component analysis
PDF1.2	Plant defensin1.2
Phe	Phenylalanine
PIs	Proteinase inhibitors
PPO	Polyphenol oxidase
PR	Pathogenesis-related
RAPH	glucoraphanin (4-methylsulfinylbutyl glucosinolate)
RCA	Rubisco activase
RFP	Rinc zinc finger protein
RGA	Repressor of *ga1-3*
RGL	Repressor of *ga1-3* (RGA)-like
ROS	Reactive oxygen species
RPK	Receptor-like protein kinase
RT	Room temperature

SA	Salicylic acid
SAR	Systemic acquired resistance
SCF^{COI1}	Skp1/Cullin/F-box (SCF^{COI1})
SID2	Salicylic acid induction deficient 2
STR	Strictosidine synthase-like
Trp	Tryptophan
TRX	Thioredoxin
VEGs	Ventral eversible glands
VOCs	Volatile organic compounds
VPD	Vinylpyridine
VSP2	Vegetative storage protein2

Abstract

Plants have complex signaling networks in response to wounding or caterpillar herbivory. The phytohormone jasmonic acid (JA) mediates the principal plant defense response pathway against caterpillar herbivory. However, some larval species manipulate host plant responses leading to the modulation of this induced resistance (IR). Although the exact mechanism of the insect subversion of JA-mediated IR is not clearly understood, effectors in the labial salivary secretions of generalist Noctuid caterpillars, such as *Spodoptera exigua*, are known to activate the salicylic acid (SA)-mediated pathway that antagonize JA responses. Since the caterpillar labial saliva contains significant levels of oxidoreductive enzymes, such as glucose oxidase, it may act by manipulating cellular redox balance in plant tissues. Similarly, these effectors may activate the ethylene (ET) pathway leading to the modulation of the JA pathway. Early plant responses to herbivory by 4th instar *S. exigua* caterpillars with intact or impaired labial saliva secretions was compared. Labial saliva helps maintain a reductive cellular environment in wounded *Arabidopsis thaliana* leaves. Labial saliva-specific expression of marker genes of the JA/ET- and SA-pathways is alleviated in glutathione-compromised *pad2.1* or *tga2/5/6* mutant plants. Also, caterpillar labial saliva modulates the expression of ET-dependent genes in a glutathione-independent manner. Therefore, caterpillar labial saliva acts to modulate the expression of defense-related genes in a SA/NPR1-, glutathione-dependent or an ET-, glutathione-independent manner. In comparison, cellular oxidative stress is elevated in the legume *Medicago truncatula* after caterpillar herbivory. The labial saliva-specific induction of the stress response is alleviated in the ET-insensitive *skl* mutant suggesting that ET is needed for this response. JA- and JA/ET-pathway marker genes are differentially expressed upon caterpillar herbivory in an ET-independent way. However, labial saliva-specific induction of a SA marker gene and suppression of trypsin inhibitor levels require ET perception suggesting that caterpillar labial saliva attenuates the JA-pathway by activating the SA pathway and ET modulates these responses. The role of caterpillar labial saliva in the plant defense signaling network was further explored by identifying differences in the post-translational modifications of nuclear proteins. Of the four proteins identified, the transcription factor, AtABF3, shows labial saliva-specific post-translational modification. In Arabidopsis plants subjected to herbivory by caterpillars with impaired labial saliva secretions, AtABF3 is nitrosylated at Cys_{420} and phosphorylated at Ser_{431}.

Since the expression of its downstream target gene, *AtWRKY40*, is also elevated in this plant, labial saliva-specific post-translational modification may play a role in the modulation of host defense response.

As the levels of atmospheric carbon dioxide (CO_2) are predicted to double in the next fifty years, the current scenario of plant stress response and, thus, the accumulation of defensive metabolites are expected to alter. Along with the increase of photosynthetic efficiency at elevated CO_2, the plant's nitrogen use efficiency will be affected. Thus, plant responses to mechanical wounding at different levels of CO_2 (ambient and elevated) and nitrogen fertilization (nitrate-limited and sufficient) were studied. At ambient CO_2, mechanical wounding induced a jasmonate (JA) burst and increased foliar glucosinolate (GSL) levels in Arabidopsis; however, at elevated CO_2 conditions, this wound-responsive increase of JAs and GSLs are only observed under nitrate-stress conditions. Although MYB transcription factors that regulate both aliphatic or indole GSL biosynthesis are induced in response to wounding, a general shift from aliphatic GSL to indole GSL is observed in wounded Arabidopis leaves.

Résumé

Les plantes répondent aux blessures ou à l'alimentation des larves par des réseaux de signalisation complexes. Lorsqu'une larve s'alimente sur une plante, l'induction d'une phytohormone connue sous le nom d'acide jasmonique (AJ) déclenche l'activation du principal système de défense des plantes. Toutefois, certaines espèces de larves sont en mesure de manipuler le système de défense de leur hôte ce qui atténue le développement de cette résistance. Malgré que cette habileté des insectes soit peu comprise, des effecteurs retrouvés dans la salive labiale des larves généralistes de la famille des Noctuidae, comme *Spodoptera exigua*, peuvent activer la voie de l'acide salicylique (AS) ce qui limite la production d'AJ. La salive contenue dans les glandes labiales des insectes contient des niveaux élevées d'oxydoréductases, comme la glucose oxydase, ce qui perturbe le potentiel redox des cellules végétales. Ces effecteurs peuvent aussi activer la voie de l'éthylène (ET) résultant en une modification de la voie de l'AJ. La première réaction cellulaire de la plante suite aux dommages causés par des larves de quatrième stade de *S. exigua* avec des sécrétions salivaires labiales soit intactes ou dysfonctionnelles a été comparée. La salive labiale aide à maintenir un environnement cellulaire oxydoréduit dans les feuilles d'*Arabidopsis thaliana*. L'expression de gènes marqueurs spécifiques à la salive labiale des voies de l'AJ/ET et de l'AS a été observée dans *A. thaliana* et leur expression était réduite dans les plantes mutantes *pad2.1* ou *tga2/5/6* ne produisant pas de glutathion. Aussi, la salive labiale des larves a modifié l'expression des gènes dépendants de l'ET indépendamment de la présence de glutathion. En conséquence, la salive labiale des larves modifie l'expression des gènes de défense de la voie de l'AS/NPR1- dépendamment du glutathion ou de la voie de l'ET indépendamment du glutathion. En comparaison, le stress oxidatif cellulaire augmente chez la légumineuse *Medicago truncatula* suite à l'alimentation des larves. L'induction de la réponse au stress spécifique à la salive labiale est plus faible dans les mutants *skl* qui sont insensibles à l'ET suggérant que l'ET est nécessaire pour cette réponse. Les gènes marqueurs des voies de l'AJ et de l'AJ/ET sont exprimés différemment suite à l'alimentation des larves indépendamment de l'ET. Toutefois, l'induction du gène marqueur de l'AS par la salive labiale et la suppression de l'inhibiteur de trypsine requièrent la présence de l'ET, suggérant que la salive labiale des larves atténue la voie de l'AJ en activant la voie de l'AS et que l'ET catalyse ces réactions. Le rôle de la salive labiale larvaire dans le réseau de signalisation de la défense des plantes a été exploré

plus en profondeur en identifiant les différences dans les modifications post-traductionnelles des protéines nucléaires. Des quatre protéines identifiées, le facteur de transcription AtABF3, a montré une modification post-traductionnelle spécifique à la salive labiale. En effet, AtABF3 est nitrosylé à Cys_{420} et phosphorylé à Ser_{431} lorsque Arabidopsis est soumise à des sécrétions de salive labiale affaiblies. Puisque l'expression de son gène cible en aval, *AtWRKY40*, augmente dans la plante, les modifications post-traductionnelles spécifiques à la salive labiale pourraient jouer un rôle dans la défense de l'hôte.

Comme on prévoit que la concentration de dioxyde de carbone atmosphérique (CO_2) doublera d'ici cinquante ans, il est possible que le scénario actuel de défense des plantes face au stress ainsi que l'accumulation de métabolites reliés à la défense soient altérés. En plus de l'augmentation de l'efficacité photosynthétique à des niveaux plus élevés de CO_2, l'efficacité de la plante à utiliser l'azote sera affecté. Pour ces raisons, les réponses des plantes aux blessures mécaniques à différents niveaux de CO_2 (actuel et élevé) et à la fertilisation azotée (déficiente et suffisante) ont été étudiées. Au niveau actuel de CO_2, les blessures mécaniques ont induit la production de jasmonates (JAs) et une augmentation des niveaux de glucosinolate (GSL) foliaire chez Arabidopsis. Toutefois, à des niveaux élevés de CO_2, l'augmentation des JAs et du GS suite aux blessures mécaniques est seulement observé lorsque la fertilisation azotée est déficiente. Malgré que les facteurs de transcription MYB qui régularisent autant la biosynthèse du GSL aliphatique que du GSL indole soient activés en réponses aux blessures mécaniques, on retrouve généralement plus de GSL aliphatique que de GSL indole dans les feuilles blessées.

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Preface and Contributions of Authors

This thesis is written in the manuscript format following the "Thesis preparation and submission guidelines" of McGill University. It contains seven chapters; the first two chapters are the introduction and literature review, respectively. Chapters 3-6 contain a published article and three manuscripts in preparation or submitted to peer reviewed journals. The final Chapter 7 contains the general conclusion and recommendations for future reseach. Below is the overview of contribution of authors. Detail contributions of the co-authors for each manuscript are described in the connecting statements before each manuscript.

My role for these manuscsripts was to design and conduct experiments, independently or together with lab mates, sample analysis, data extraction and analysis, and preparation of manuscript together with my thesis supervisor, Dr. Jacqueline Bede. Dr. Bede provided funding, supervision and support and technical assistance for the completion of each project. In Chapter 3, Alexander Amerizian helped me design and conduct the redox experiment. Alberto Prado assisted in carrying out lab protocol (redox assays). The gene expression study was accomplished by the co-author Tanya Copley. In Chapter 4, I, solely, was involved in the experiment design and execution. For Chapter 5, I used plant samples already available (collected by Zhiyi Lan) to conduct protein extraction and analysis. In Chapter 6, Alexander Amerizian and I designed the experiments and collected samples. After sample collection, I performed all the lab procedures and data analysis. Hormone, proteomic, glucosinolate and untargeted metabolites analyses were done in collaboration with different service-providing institutions.

Contributions to Science

Research presented in Chapters 3-6 have contributed to our understanding of how plants respond to wounding and caterpillar herbivory. As well, I investigated how caterpillars manage to overcome these plant defensive mechanisms. These studies have revealed the role of caterpillar labial saliva in the modulation of plant cellular signaling responses. Specifically-

1. This is the first study that has investigated the role of caterpillar labial saliva in plant early responses to herbivory. Caterpillar labial saliva maintains a reduced cellular environment during herbivory of *Arabidopsis thaliana* leaves. In contrast to Arabidopsis, *S. exigua* caterpillar labial saliva increases oxidative stress in the legume *Medicago truncatula*.
2. In Chapter 3, I showed that *S. exigua* caterpillar labial saliva modulates plant defense responses in SA/NPR1-, glutathione-dependent or ET-, glutathione-independent manners.
3. In *M. truncatula*, ET sensitivity is essential for the labial saliva-specific induction of the antagonistic SA-mediated pathway that partially suppresses the full activation of JA-dependent defense responses against caterpillar herbivory.
4. Herbivore- and labial saliva-specific post-translational modifications of nuclear proteins, RABH1C, CAMTA/SR1-like protein, MYB109 and AtABF3 was identified in *Arabidopsis thaliana*. Labial saliva-specific nitrosylation and phosphorylation at Cys_{420} and Ser_{431}, respectively, of the transcription factor AtABF3 and the enhanced expression of its downstream target gene, *AtWRKY40*, in plants fed by caterpillars with impaired labial salivary secretions have shown correlation between this post-translational modification and defense gene induction.
5. Plant response to mechanical wounding at different levels of CO_2 and nitrate-fertilization revealed that wound-responsive jasmonate burst and increase in foliar glucosinolate levels observed at ambient CO_2 was dampened by sufficient nitrate fertilization at elevated CO_2. MYB transcription factors regulating biosynthesis of indole and aliphatic glucosinolates were induced in response to wounding. Indole glucosinolates levels increased in wounded Arabidopsis leaves.

CHAPTER 1. Introduction

Plants have sophisticated mechanisms to defend themselves against pests and pathogens (Kessler and Halitschke, 2007; Wasternack and Hause, 20013). These defenses can be physical or chemical and may directly or indirectly provide protection from attacking herbivores. These defensive mechanisms may either be present constitutively (in the absence of herbivores) or induced upon attack (Gatehouse, 2002; Kessler and Baldwin, 2002). Both strategies, constitutive or induced, have benefits and potential issues. For example, constitutive defenses are present at the time of attack but continuous production and sequestration of toxins can be costly to the plants. As well, it poses selective pressure on herbivores to evolve strategies to adapt or counteract these defenses (Baldwin, 1999; Schwachtje and Baldwin, 2008; Bolton, 2009). Thus, plants also have induced defense responses after recognition of the attack (Wu and Baldwin, 2009). Therefore, plants have different strategies to respond to the diverse range of pathogens and herbivores. Although there are general responses, what is becoming clear is that plants also have specific responses against distinct attackers (Pieterse et al., 2013).

Since caterpillars damage plant tissues during feeding, there is some overlap with plant responses to mechanical damage (Bricchi et al., 2010). Wounding and/or caterpillar feeding initiate a cascade of early signaling events, such as the opening of calcium (Ca^{2+}) channels leading to an increase in intracellular Ca^{2+} levels and the generation of reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) (Orozco-Cárdenas et al., 2001; Maffei et al., 2004; Maffei et al., 2006). At the same time, mechanisms to detoxify ROS are activated to protect cellular components from oxidative damage (Forman et al., 2010; Noctor et al., 2012). Further, redox modulation can lead to post-translational modifications of proteins that may affect enzyme activity, regulation and protein-protein or protein-DNA interactions (Huber and Hardin, 2004; Seo and Lee, 2004; Huber, 2007; Glauser et al., 2008). As second messengers of signaling pathways, H_2O_2 and Ca^{2+}, activate the mitogen activated protein kinase (MAPK) pathway leading to the activation of the jasmonic acid (JA)-mediated pathway (Turner et al., 2002; Maffei et al., 2007b). Enhanced expression of genes encoding enzymes in the JA biosynthetic pathway, such as *allene oxide synthase (AOS)* and *lipoxygenases* (*LOX*), after wounding leads to a burst of JA in local and systemic tissues (Kessler and Baldwin, 2002; Taki et al., 2005). JA conjugates with the amino acid isoleucine (Ile) and, thus, generates the bioactive form (+)7-iso-jasmonyl-L-

isoleucine (JA-Ile) (Fonseca et al., 2009). Binding of JA-Ile to coronatine insensitive 1 (COI1) brings the F-box protein complex SCF^{COI1} and JA-Zim domain (JAZ) proteins in close proximity leading to the proteasome-mediated degradation of the JAZ proteins (Lorenzo et al., 2004; Dombrecht et al., 2007; Kazan and Manners, 2008; Fonseca et al., 2009). Since these JAZ proteins are negative regulators of MYC2/3/4 transcription factors, their degradation leads to the release of these proteins and the expression of JA-dependent defense-related genes, such as *vegetative storage protein2* (*VSP2*) and *lipoxygenase2* (*LOX2*). This results in the accumulation of defensive proteins, such as trypsin inhibitors, and secondary metabolites in wounded plants (Ballare, 2011).

Recognition of the clues from the herbivores modifies these general wound responses and leads to the specificity of plant defense responses to different attackers (Kessler and Baldwin, 2002; Taki et al., 2005; Mithöfer and Boland, 2012). In wild tobacco, *Nicotiana attenuata*, a distinct blend of volatile organic compounds (VOCs) are released when oral secretions (OS) from the caterpillar of the tobacco hornworm, *Manduca sexta*, are applied to mechanical wounds (Halitschke et al., 2001). Thus, the perception of clues from the caterpillar modifies the plant responses (Bonaventure and Baldwin, 2010). Recognition of fatty acid-amino acid conjugates (FACs), such as *N*-linolenoyl glutamate (18:3 Glu) from the OS of the beet armyworm, *Spodoptera exigua* Hübner leads to a JA and ethylene (ET) burst in *N. attenuata* (Halitscke et al., 2004). As well, salicylic acid (SA)-mediated systemic acquired resistance (SAR) pathway is activated in the host plant due to the activity of caterpillar glucose oxidase (GOX), an enzyme abundant in the labial saliva of many noctuid caterpillars (Merkx-Jacques and Bede, 2005; Weech et al., 2008; Diezel et al., 2009; Paudel et al., 2013; Lan et al., 2014). Also, during caterpillar feeding, along with the activation of the JA-dependent MYC pathway, the ethylene response factor1/ octadecanoid responsive AP2/ERF 59 (ERF1/ORA59) branches of the JA pathway are also activated (Kazan and Manners, 2008; Verhage et al., 2011; Pieterse et al., 2012). These two branches integrate ET signaling into the JA pathway. What is important to note is the ERF1 or ORA59 branches may also operate synergistically or antagonistically to the MYC branch of the JA pathway (Spoel et al., 2003; Lorenzo et al., 2004; Koornneef and Pieterse, 2008a; Leon-Reyes et al., 2009; Pieterse et al., 2012). Therefore, the integration of many phytohormone pathways shapes the plant's final defense response to caterpillar herbivory.

Generalist caterpillar herbivores have evolved strategies to counteract the plant's jasmonate-mediated induced resistance (IR) (Zhu-Salzman et al., 2005). Oxidoreductases in the caterpillar labial saliva, for example GOX, have been proposed to be a mechanism used by some Noctuid caterpillar species to prevent the plant from mounting a full defense responses (Musser et al., 2002; Weech et al., 2008; Diezel et al., 2009). In tobacco, a higher level of nicotine was accumulated upon herbivory by caterpillars of the corn earworn, *Helicoverpa zea,* with intact labial salivary secetions compared to caterpillars with impaired labial salivary secretions (Musser et al., 2002). In Arabidopsis, expression of the late JA-mediated genes *plant defensin1.2* (*AtPDF1.2)* and *laccase-like multi-copper oxidase* (*AtLMCO)* are suppressed by caterpillar labial saliva (Weech et al., 2008). Jasmonate (collective name for JA, JA-Ile and 12-*oxo*-phytodienoic acid (OPDA) levels increase in response to caterpillar herbivory but are dampened compared to levels observed if plants are fed upon by caterpillars with impaired labial salivary secretions, indicating that effectors in the labial saliva of some caterpillar species prevent the plant from mounting a full defense response (Weech et al., 2008; Lan et al., 2014). However, this labial saliva-specific suppression of JA-mediated IR is abolished in plants lacking an active SAR pathway (*SAR*$^{-}$ mutant plants) implying that activation of the antagonistic SAR pathway by effectors in caterpillar labial saliva is necessary to interfere with the plant's IR (Weech et al., 2008). Diezel et al. (2009) confirmed this and also showed that GOX activity from the OS of *S. exigua* caterpillars leads to the accumulation of SA that leads to the suppression of JA-mediated IR. However, which actual player, either SA or the transcriptional coactivator Non-expressor of Pathogenesis Related protein1 (NPR1), is involved in this antagonistic interaction is still debated (Van der Does et al., 2013). As well, the phytohormone ET might also modulate NPR1 activity in this crosstalk (Leon-Reyes et al., 2009). Recently, it was shown that the SA-JA antagonism occurs at the level of ORA59; the SA-dependent pathway inhibits the accumulation of ORA59 leading to the attenuation of the JA pathway (Van der Does et al., 2013).

Effectors, such as GOX, present in the labial salivary secretions of some Noctuid generalist caterpillars is one candidate for the suppression of plant induced defenses (Merkx-Jacques and Bede, 2005; Eichenseer et al., 2010). GOX oxidizes glucose to generate H_2O_2 that may lead to the activation of the SA/NPR1 pathway (Musser et al., 2002; Bede et al., 2006; Weech et al., 2008; Paudel et al., 2013; Lan et al., 2014). H_2O_2 may also modulate signal transduction pathways by affecting proteins post-translational modifications, such as

transcription factors (Spoel and Loake, 2011). As well, H_2O_2 leads to the activation of cellular detoxification mechanisms, such as the ascorbate-glutathione cycle, and changes in cellular oxidative state (Quan et al., 2008; Foyer and Noctor, 2011). Increased levels of oxidized glutathione (GSSG) positively affects the JA-mediated IR pathway (Mhamdi et al., 2010). Similarly, changes in the glutathione pool can result in post-translational modification of thiol-based proteins via glutathionylation or *S*-nitrosylation (Leitner et al., 2009; Spoel et al., 2010; Spoel and Loake, 2011). Thus, redox modulation due to caterpillar labial saliva may influence plant signaling networks to impact the plant's final defense response (Mou et al., 2003; Tada et al., 2008; Lindermayr et al., 2010). DELLA proteins scavenge ROS, such as H_2O_2 (Achard et al., 2008). These proteins are negative regulators of the growth-dependent gibberellin (GA) pathway. This pathway is involved in plant-insect interactions since, under caterpillar attack, the plant must determine how to shunt its metabolic flux; into compensatory growth through the GA/DELLA pathway or into induced defenses through the JA pathway (Cipollini, 2010; Wild et al., 2012; Yang et al., 2012). DELLA proteins are required for the caterpillar labial saliva-mediated suppression of the jasmonate burst and downstream responses, such as the expression of *AtPDF1.2*, in the early plant's response to caterpillar herbivory (Lan et al., 2014).

Another important aspect of plant-insect insect interactions is the influence of future predicted climatic conditions and agricultural practices. In the next fifty years, atmospheric carbon-dioxide (CO_2) levels are predicted to double (IPCC, 2014). As well, agricultural fertilization practices can effect plant defense responses (Coviella et al., 2002). As the plant's photosynthetic and nitrogen use efficiency changes, the carbon-nutrient balance hypothesis (CNBH) predicts the reduction of nitrogen-based plant metabolites, particularly defense-related compounds. The focus of the final study is to understand how CO_2 and nitrogen fertilization affect plant stress responses. For simplicity, in this experiment, plant leaves were mechanically damaged rather than subject to caterpillar herbivory.

Rationale of the Study

Plant defense responses to caterpillar herbivory involve complex spatial and temporal integration of multiple phytohormone pathways to shape the plant's final response. This is complicated by insect strategies, such as labial salivary effectors, to interfere with these pathways. We wish to further our understanding of how insect herbivores manipulate plant

defense responses. As well, we wish to understand how environmental conditions and agricultural practices can influence these plant defenses.

1.1. Hypotheses

Plant responses to caterpillar herbivory

Hypothesis 1: Labial salivary secretions of the generalist Noctuid caterpillar *S. exigua* modify plant defense responses by modulating redox balance.

Hypothesis 2: Changes in cellular redox balance in response to caterpillar labial salivary secretions results in labial saliva-specific changes in the post-translational modifications of plant proteins.

Hypothesis 3: Caterpillar labial salivary secretions activate phytohormone pathways that are antagonistic to JA-dependent IR to suppress the plant's ability to mount a defense response.

Plant responses to mechanical wounding under conditions of elevated carbon dioxide levels or nitrogen-stress

Hypothesis 4: Following the CNBH, conditions of elevated CO_2 or nitrogen stress will lead to changes in phytohormone profile and defensive metabolites following mechanical damage.

1.2. Objectives

1. To investigate changes to plant cellular redox status in response to caterpillar herbivory and to determine the effect of caterpillar labial saliva on redox balance.
2. To evaluate the role of phytohormones, such as ET or the GA/DELLA pathway, in response to caterpillar herbivory and determine the effect of caterpillar labial saliva on these pathways.
3. To investigate changes in phytohormones and defense metabolism in response to wounding under conditions of elevated CO_2 or nitrogen-stress.

CHAPTER 2. Literature Review

2.1. Plant Defense Responses

Plant defenses against pest and pathogens involve both direct and indirect mechanisms (Walling, 2000; Kessler and Halitschke, 2007). Apart from physical defenses, such as trichomes, hairs and thorns, chemical compounds, such as cyanogenic glucosides, phenolics, alkaloids, proteinase inhibitors (PIs), glucosinolates (GSL) and saponins, serve as direct defenses (Wu and Baldwin, 2009). These compounds protect the plant by functioning as feeding deterrents, having anti-digestive properties or being toxic to herbivores (Kessler and Halitschke, 2007; Zhu-Salzman et al., 2008). Also, in response to herbivory, plants have indirect defenses such as volatile organic compounds (VOCs) that attract predators and parasitoids to harm the damaging insect herbivore (Kessler and Baldwin, 2002; Heil and Ton, 2008).

If these defenses are present in the absence of stress, they are called "constitutive defenses". For example, in tobacco, basal levels of the neurotoxin nicotine are present in glandular trichomes and may serve as a signal to many species of adult female moths and butterflies to avoid these plants (Steppuhn et al., 2004). These constitutive defenses are expensive to the plant (Hare et al., 2003; Zavala et al., 2004); both in terms of the chemical resources needed to produce these compounds and also in the production of specialized structures often necessary to sequester these compounds. As well, in the absence of strong herbivore pressure, their presence places selective pressure on pests to adapt and evolve strategies to counteract these defenses (Baldwin, 1999). Thus, the plant needs to intelligently regulate their resources to maintain a balance between growth and defense (Wu and Baldwin, 2009).

If the herbivore is able to overcome these defenses, then plants often respond with induced defenses. For example, in tobacco plants, large amounts of nicotine are produced in response to herbivory; therefore, nicotine is both a constitutive and an induced defense in tobacco (Baldwin, 1999). One problem with these induced defenses is that there is a time lag between the start of insect feeding and induced resistance.

2.2. General Plant Responses to Caterpillar Herbivory

In the simplest sense, a plant's response to chewing herbivory is analogous to its response to wounding (Leon et al., 2001). When the plant tissue is mechanically damaged, the plant needs to initiate pathways that are involved in healing and defense responses (Leon et al., 2001; Rhodes et al., 2006). Therefore, following damage, the plant activates a cascade of signaling pathways at both local (eaten) and systemic (distal) tissues (Maffei et al., 2007a; Wu and Baldwin, 2009). When the tissue is broken, there are immediate responses, such as hydraulic and electrical signals, that spread rapidly throughout the plant (Maffei et al., 2007a). Oligogalacturonides break off from the cell wall during insect damage are recognized by receptors leading to the activation of early plant signal transduction pathways (Gatehouse, 2002). The resultant opening of calcium (Ca^{2+}) channels leads to increased intracellular Ca^{2+} levels (Maffei et al., 2007a; Maffei et al., 2007b). As well, cellular levels of reactive oxygen species (ROS) accumulates in many plant species (Orozco-Cárdenas et al., 2001; Maffei et al., 2004; Maffei et al., 2006). Detoxification of excessive ROS and regulation of cellular reduction/oxidation levels precedes the induction of plant responses. Alongside these damage-associated molecular patterns (DAMPs), plants recognize herbivore-associated molecular patterns (HAMPs), that are representative of the attacking herbivore, such as caterpillar footsteps, or insect oral secretions (OSs) (Bown et al., 2002; Felton, 2008; Howe and Jander, 2008; Wu and Baldwin, 2009).

2.2.1. Cellular redox balance

ROS, such as hydrogen peroxide (H_2O_2), are implicated in the plant induced defense responses. Both mechanical wounding and feeding by herbivores lead to the accumulation of ROS in wounded (local) and systemic tissues (Leitner et al., 2005; Maffei et al., 2006). The activation of NADPH oxidase by Ca^{2+}-dependent protein kinases (CDPKs) in wounded potato leaves results in H_2O_2 production (Kobayashi et al., 2007; Wu and Baldwin, 2009). Inhibition of NADPH oxidase results in lower levels of expression of jasmonic acid (JA)-dependent defense genes, such as *Proteinase Inhibitor* (*Pin*) (Sagi et al., 2004). ROS accumulation may also be dependent on levels of the defense-related phytohormone JA (Orozco-Cardenas and Ryan, 1999). Activity of H_2O_2-generating enzymes, such as superoxide dismutase, increases after wounding (Maffei et al., 2006). Since high levels of H_2O_2 are detrimental to cellular components leading to lipid peroxidation and oxidation of proteins, the plant cell mobilizes antioxidant pathways, such as the

activation of peroxidases and catalases, to detoxify the generated ROS (Maffei et al., 2006; Moller et al., 2007; Quan et al., 2008).

To detoxify H_2O_2 and maintain a cellular reduction/oxidation (redox) balance, the Halliwell-Asada (ascorbate/glutathione) cycle is also activated (Quan et al., 2008; Foyer and Noctor, 2011). Cellular levels of reduced glutathione (GSH) and reduced ascorbate (ASC) are generally high (Noctor et al., 2012). If cellular H_2O_2 rise as a result of oxidative stress, ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 to water and the oxidation of ASC to dehydroascorbate (DHA, via the intermediate monodehydroascorbate (MDHA)). Dehydroascorbate reductase (DHAR) reduces DHA back to ASC using GSH to generate oxidized glutathione (GSSG). This last metabolite is reduced back to GSH by glutathione reductase (GR) generating NADPH from $NADP^+$ (Fig. 2.1). Therefore, ROS production is tightly controlled to minimize detrimental reactions. Ascorbate and the small thiol tripeptide, glutathione (ϒ-glutamylcysteinylglycine) are important antioxidants and modulators of redox status in plant cells (Han et al., 2013). Therefore, total glutathione or ascorbate or the ratio of the oxidized-to-reduced metabolite can be used as a measure of cellular oxidative stress. For example, infection by biotrophic pathogens or application of salicylic acid (SA) results in an increase in the foliar glutathione pool in Arabidopsis (Mou et al., 2003; Mateo et al., 2006). Also, the levels of GSSG transiently increase, indicative of oxidative stress and the involvement of ascorbate/glutathione cycle to maintain cellular redox balance. Apart from its function as an anti-oxidant, glutathione participates in signal transduction pathways directly or indirectly by post-translational modification of proteins. Thus, H_2O_2 produced during herbivory acts to change redox potential. This may lead to the activation of the Non-expressor of pathogenesis-related protein1 (NPR1) protein in the systemic acquired resistance (SAR) pathway (Glazebrook, 2005; Mateo et al., 2006; Spoel et al., 2009). NPR1 is constitutively present as an inactive oligomer formed through the disulfide bridges between interacting monomers. Change in cellular oxidative stress results in the activation of glutaredoxin that reduces the disulfide bridges to the monomeric form (Mou et al., 2003; Tada et al., 2008). Monomeric NPR1 enters the nucleus to interact with its transcriptional coactivator, the TGA transcription factors resulting in SA/NPR1-dependent gene expression.

Glutathione is also involved in plant defense responses against insect herbivores (Schlaeppi et al., 2008). Mechanical wounding or treatment with JA leads to the induction of

glutathione biosynthesis and activation of the ascorbate-glutathione cycle (Sasaki-Sekimoto et al., 2005; Chassot et al., 2008). Arabidopsis *pad2.1* has a mutation in the gene encoding the first enzyme in glutathione biosynthesis, ϒ-glutamylcysteine-synthetase (*GSH1*), resulting in low constitutive glutathione levels (Parisy et al., 2007). This mutation makes the plant more susceptible to caterpillar infestation than the wild type (Schlaeppi et al., 2008). Since glutathione is involved in defense signaling and a precursor to GSLs, important defensive compounds, this mutant is susceptible to caterpillar herbivory. As well, a positive correlation between cellular levels of GSSG and the induction of JA-dependent gene expression has been observed in Arabidopsis (Mhamdi et al., 2010).

H_2O_2 or glutathione also modulate signal transduction pathways through the reversible post-translational modification of proteins, such as transcription factors (Spoel and Loake, 2011). For example, as cellular H_2O_2 levels increase, protein cysteine residues (S-H) may be oxidized to S-OH (sulphenic form) that can react with another S-OH losing water to form a disulphide bond between the cysteine residues (Spadaro et al., 2010). As levels of H_2O_2 increase, S-OH may be further oxidized to S-O_2H (sulphinic form) or S-O_3H (sulphonic form). S-O_3H is considered extremely stable, but S-O_2H can be reduced back to the S-OH form by the enzyme sulphiredoxin. S-OH can be further reduced by redox-based enzymes, such as thioredoxin (TRX) or glutaredoxin (GRX) (Biteau et al., 2003; Hancock et al., 2006; Rey et al., 2007). These protein post-translational modifications are important in the regulation of defense signaling proteins (Spoel and Loake, 2011). TRXs, GRXs, glutathione-*S*-transferases (GSTs), glutathione and *S*-nitrosoglutathione (GSNO) participate in the modulation of protein activities in the SA-mediated defense signaling pathway (Tada et al., 2008; Leitner et al., 2009; Spoel et al., 2009; Spoel and Loake, 2011). TRX/GRX- based reduction of cysteine disulphide bonds of oligomeric NPR1 leading to its monomerization is essential for the activation of the SAR pathway, and, conversely, *S*-nitrosylation of active NPR1 using GSNO as the nitric oxide-donor results in the re-oligomerization of NPR1 (Mou et al., 2003; Tada et al., 2008; Lindermayr et al., 2010). GSNO also modulates the activity of TGA transcription factors as well as enzymes involved in ET biosynthesis (Lindermayr et al., 2010); therefore, GSNO-mediated protein-posttranslational modification is an important cellular mechanism of co-ordinating defense-related signaling pathways. Therefore, either directly by modifying proteins or indirectly, through alteration of the cellular glutathione balance, H_2O_2 acts as an important second messenger leading to the

activation of signaling pathways, such as the MAP kinase pathway that results in the activation of octadecanoid biosynthesis and defense responses (Turner et al., 2002; Maffei et al., 2007b).

2.2.2. Jasmonic acid pathway

In response to wounding or assaults that entail wounding, such as feeding by caterpillars, plants accumulate defense-related octadecanoids, such as JA, in local and systemic tissues (Leon and Reyes, et al., 2009). From the chloroplast membrane, phospholipids are cleaved to generate linolenic acid (C18 fatty acid), the precursor to octadecanoid hormones, such as 12-*oxo*-phytodienoic acid (OPDA) and JA (Kessler and Baldwin, 2002; Taki et al., 2005). Jasmonates (JAs, collective name referring to JA, jasmonyl-L-isoleucine (JA-Ile) and OPDA) are upstream signals for the expression of defense-related genes as well as genes encoding JA-biosynthetic enzymes, such as *lipoxygenase2* (*LOX2*) and *allene oxide synthase* (*AOS*) (Halitschke and Baldwin, 2004; Reymond et al., 2004; Schmidt et al., 2005; Wasternack and Hause, 2013). Jasmonate Resistant1 (JAR1) catalyzes the conjugation of JA with the amino acid isoleucine (Ile) to generate the bioactive form, JA-ile. This molecule acts to bring together the F-box protein complex, Skp1/Cullin/F-box (SCF^{COI1}) and jasmonic acid-Zim domain (JAZ) proteins (Staswick and Tiryaki, 2004; Kang et al., 2006; Katsir et al., 2008; Sheard et al., 2010). SCF^{COI1} catalyzes the addition of ubiquitin proteins onto the JAZ protein, targeting it for degradation by the 26 *S*-proteasome (Dombrecht et al., 2007; Yan et al., 2009; Sheard et al., 2010). JAZ proteins are negative regulators of JA pathway that prevent the induction of JA responses by binding to MYC2/3/4 transcription factors (Chini et al., 2007; Thines et al., 2007; Fernandez-Calvo et al., 2011). JAZ proteins maintain a physical interaction with MYC transcription factors by recruiting the co-repressor TOPLESS (TPL) through an interaction with the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Chung et al., 2009; Pauwels et al., 2010; Cheng et al., 2011; Fernández-Calvo et al., 2011; Pauwels and Goossens, 2011; Wasternack and Hause, 2013). Once the JAZ protein is degraded, MYC transcription factors are released and JA-dependent gene expression is observed (Li et al., 2004; Chini et al., 2007; Dombrecht et al., 2007; Thines et al., 2007) (Fig. 2.2).

2.2.3. Jasmonic acid-dependent induced resistance

JA-dependent induced resistance (IR) is a key plant mechanism to deter chewing insect herbivory. Exogenously applied or artificially-induced increases in JA levels positively enhance the resistance of the plant against caterpillar herbivory (Reymond et al., 2004). If a plant is

unable to mount this type of response, they are unable to mount an effective defense response against insect herbivores (Reymond et al., 2004). For example, caterpillars of the cabbage worm, *Pieris rapae*, and the beet armyworm, *Spodoptera exigua*, are significantly larger when feeding on the Arabidopsis coronatine-insensitive1 mutants (*Atcoi1*) that does not have the SCF^{COI1} necessary for the degradation of the JAZ protein (Van Oosten et al., 2008). JA induced by caterpillar herbivory leads to extensive transcriptional reprogramming in the damaged (local) as well as systemic tissues (Reymond et al., 2000; Reymond et al., 2004). This includes the induction of genes related to protection against oxidative stress and cell wall fortification, as well as enhanced expression of genes related to octadecanoid biosynthesis and regulation of defense-related proteins and secondary metabolites. The perception of JA in plant tissues leads to the expression of defense-related genes, followed by the production of defensive metabolites and volatile organic compounds (Howe and Jander, 2008). Induction of JA-mediated defense-related genes, such as *Vegetative Storage Protein2* (*VSP2), Plant Defensin (PDF1.2) and Hevein-Like protein* (*HEL*), are routinely used as the markers of JA-mediated IR in host plants (Weech et al., 2008; Paudel et al., 2013; Lan et al., 2014) (Fig. 2.2). In the legume *Medicago truncatula*, Darwish et al., (2008) also identified genes that were differentially regulated early after herbivory by fourth instar caterpillars of beet armyworm, *S. exigua*. These are genes that encode ribulose-1, 5-bisphosphate carboxylase/oxygenase activase (MtRCA), a strictosidine synthase-like protein (MtSTR like), a ring zinc finger protein (MtRFP), an unknown protein (MtUNK) and a receptor-like protein kinase (MtRPK).

A common defense against caterpillar herbivory is the accumulation of anti-digestive and defensive proteins (Howe and Jander, 2008). PIs are JA-dependent proteins that inhibit dietary proteases in the insect gut, thus limiting availability of amino acid nutrients required for insects growth and development (Zavala et al., 2004). Plant-derived enzymes, such as threonine deaminase and arginase, are induced upon wounding and herbivory and degrade available threonine or arginine amino acids in the insect gut (Chen et al., 2007; Zhu-Salzman et al., 2008). The herbivore and JA-responsive enzymes, such as polyphenol oxidases (PPOs), reduce the nutritional quality of plant tissue by catalyzing the formation of reactive quinones that bind to proteins preventing their absorption by the caterpillar gut (Felton, 2005; Constabel and Barbehenn, 2008). Many plant species, such as *Medicago truncatula*, contain PPOs but Arabidopsis appears to only contain laccase like-multi-copper oxidase (LMCOs) (McCaig et al.,

2005; Schmitz et al., 2007). The activity of these two enzymes can be discriminated based on the substrate used: *o*-diphenol, such as dopamine or pyrocatechol, for PPO activity and *p*-diphenols, such as hydroxyl quinone, for LMCO activity.

Secondary plant metabolites such as GSLs are the main defensive compounds in Arabidopsis. These sulfur-nitrogen rich compounds are compartmentalized separately from the enzyme myrosinase (Halkier and Gershenzon, 2006). Upon tissue damage by caterpillar herbivory, compartmentalization is disrupted. The released myrosinase hydrolyzes the sugar from the GSL followed by secondary arrangements that lead to the production of toxic compounds, such as thiocyanates and iso-thiocyanates (Wittstock and Burow, 2010). In leguminous plants, such as *M. truncatula*, saponins are the important compound that deter herbivores and also increase mortality, retard growth and development, and cause moulting defects of the insects (Geyter et al., 2007; Agrell et al., 2003). *Spodoptera litorallis* larva feeding increased the production of total saponin and the flavonoid apigenin in alfalfa, *Medicago sativa* (Agrell et al., 2003). Larval growth was retarded and the pupal mass was reduced when caterpillars were fed on diets containing high saponin levels (Adel et al., 2000; Agrell et al., 2003; Jones and Dangl, 2006).

2.3. Interactions between the Jasmonate and Other Phytohormone Pathways

2.3.1. Salicylic acid pathway

The plant SA-dependent SAR pathway is normally activated in response to attack by biotrophic pathogens (Glazebrook, 2005). Plants recognize microbes (both pathogen or symbiotes) through pathogen-associated molecular patterns (PAMPs) and, more specifically, microbial effector-triggered immunity (ETI) (Jones and Dangl, 2006). On the plant cellular membrane, receptor-like protein kinases recognize pathogen proteins and sugars associated with highly conserved bacterial and fungal structures, such as flagellin, lipopolysaccharides, chitin or ergosterol (Garcia-Brugger et al., 2006). Recognition of these conserved microbe-associated signals leads to general plant responses, such as a transient increase in Ca^{2+} level and/or the production of ROS, such as H_2O_2, which leads to the induction of SA biosynthesis (Glazebrook, 2005; Mateo et al., 2006). SA inhibits the enzyme catalase and, in some cases, ascorbate peroxidase, cellular enzymes that normally catabolize H_2O_2. This leads to high levels of ROS in a rapid, feedforward mechanism known as the oxidative burst (Chen et al., 1993; Durner and Klessig, 1996). The

oxidative burst and, later, the hypersensitive response are both mediated by rapid increases in SA and H_2O_2. These lead to localization of cell death surrounding the infected area that acts to limit infection as well as strengthening of cell walls by cross-linking phenolic compounds with the goal of restricting pathogens.

Changes in cellular redox potential due to increased cellular H_2O_2 results in the activation of NPR1, a transcriptional co-activator for the induction of downstream defense-related genes in the SAR pathway (Mou et al., 2003; Moore et al., 2011; Peter and Stephen, 2012; Wu et al., 2012) (Fig. 2.2). Normally, NPR1 is cytosolic and associated in a homooligomeric state formed by disulfide bridges between NPR1 monomers (Tada et al., 2008). Changes in cellular redox potential activate redox-responsive oxidoreductases, such as TRX and GRX, that lead to the reduction of disulfide bonds holding the NPR1 oligomers (Tada et al;, 2008). Monomeric NPR1 then enter the nucleus and interacts with TGA transcription factors (Mou et al., 2003; Pieterse and Van Loon, 2004). TGA-NPR1 binds to the promoters of SA-responsive genes leading to the expression of *pathogenesis-related* (*PR*) genes as well as genes encoding enzymes involved in SA biosynthesis (Zhang et al., 1999; Durrant and Dong, 2004; Zhang et al., 2010).

2.3.2. Jasmonic acid-salicylic acid pathways crosstalk

JA- and SA- pathways, which are the backbone of plant defense responses, are antagonistic to each other (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). JA-induced gene expression and JA-mediated defense induced by jasmonates and/or herbivory are suppressed by SA and/or infection by biotrophic pathogens and *visa versa* (Koornneef et al., 2008b; Leon-Reyes et al., 2010a). For example, caterpillars of the cabbage worm, *P. rapae*, perform better on plants pre-infested with the biotrophic pathogen, *Hyaloperonospora arabidopsidis*; the pathogen activates the SA-mediated pathway that antagonizes JA-dependent responses (Koornneef et al., 2008b). This SA-JA antagonism is well documented and allows the plant to prioritize its responses when there is more than one attacker. As mentioned previously, some herbivores can manipulate this antagonism for their own benefit. Effectors in the labial saliva of the caterpillar *S. exigua* cause the activation of the SA-dependent SAR pathway to suppress JA-mediated defenses (Weech et al., 2008; Paudel et al., 2013). Likewise, the reciprocal suppression of the SA-dependent pathway by JA is also observed (Glazebrook, 2005). This can be taken advantage of by pests and pathogens. The biotrophic pathogen *Pseudomonas syringae* produces coronatine, a mimic of JA-Ile, that activates the JA-dependent pathway and suppresses SA-mediated pathogen defensive

pathways (Brooks et al., 2005; Glazebrook, 2005). However, in rare cases, a synergistic effect of JA- and SA-mediated pathways is also reported (Mur et al., 2006). Arabidopsis plants treated concurrently with low concentrations of JA and SA expressed both JA- and SA-responsive genes, *PDF1.2* and *PR1*, respectively. Thus, the relative concentration of hormones along with their temporal production modulate the SA-JA interaction and the resultant "crosstalk" shapes the final outcome of the plant responses (Koornneef et al., 2008a; Leon-Reyes et al., 2010; Erb et al., 2012).

Although the specific players in this crosstalk between SA- and JA-pathway are still being elucidated, redox regulators, such as glutathione, and redox-sensitive proteins, such as NPR1 and TGA transcription factors, are thought to be involved (Ndamukong et al., 2007; Weech et al., 2008). For example, SA treatment increases the glutathione pool and shifts the ratio of reduced-to-oxidized glutathione (GSH/GSSG) towards the reduced state (Mhamdi et al., 2010; Spoel and Loake, 2011). In contrast, high cellular levels of GSSG lead to JA-mediated gene expression (Mhamdi et al., 2010). SA-mediated suppression of JA-dependent gene expression is more pronounced when there is a transient increase in the glutathione pool after SA application (Koornneef et al., 2008b). Thus, SA-mediated redox regulation appears to be important in the attenuation of JA-dependent responses. Additional evidence implicates NPR1 in the JA-SA crosstalk. Arabidopsis *npr1* mutants that are compromised in the SAR pathway were unable to suppress JA-mediated gene expression (Spoel et al., 2003).

SA-mediated antagonism of the JA pathway could be due to the suppression of JA biosynthesis or by repressing JA signaling responses (Fig. 2.2) (Pieterse et al., 2012). Although previous studies suggest that SA may suppress JA biosynthesis, the most recent research suggests that the SA-JA antagonism occurs at the level of octadecanoid responsive element binding factor (ORA59) (van der Does et al., 2013). This JA- and ET-responsive transcription factor belongs to the APETALA2 (AP2)/ ethylene response factor (ERF) family (Lorenzo et al., 2003). Through promoter analysis followed by overexpression studies, van der Does et al. (2013) demonstrated that SA-dependent antagonism of the JA pathway occurs through the attenuation of the ORA59 branch of the JA pathway which shows the integration of JA-, ET- and SA-dependent pathways.

2.3.3. Ethylene pathway

ET is a pleitropic hormone that regulates diverse plant physiological processes from seed germination to fruit ripening (Yoo et al., 2009). Particularly during plant responses to biotic and abiotic stresses, this hormone functions to fine-tune plant responses (von Dahl et al., 2007). The small, gaseous ET can diffuse across the cellular membrane to bind to the endoplasmic reticulum-associated heterodimeric receptors (Hall et al., 2000; Cancel and Larsen, 2002; Chen et al., 2002; Wang et al., 2002). In Arabidopsis, five membrane receptors have been identified: ethylene resistant1 (ETR1), ETR2, ethylene response sensor1 (ERS1), ERS2, and ethylene insensitive4 (EIN4) (Yoo et al., 2009). When ET binds to the receptor, the negative regulator constitutive triple response1 (CTR1) is inhibited, initiating the ET-dependent signaling cascade (Huang et al., 2003). In the absence of ET, CTR1 phosphorylates EIN2 leading to either its inactivation or degradation by the 26*S* proteasome complex (Ju et al., 2012). CTR1 also activates the downstream MAPK signaling cascades that phosphorylates the transcription factor EIN3 leading to its degradation through the 26*S* proteasome system (Guo and Ecker, 2003; Yoo et al., 2008; Hahn and Harter, 2009). In the presence of ET, CTR1 is inactive. Unphosphorylated EIN2 undergoes proteolytic cleavage and its C-terminal domain enters to the nucleus. EIN2 is a putative Nramp metal ion transporter associated with the nuclear envelope that is believed to allow copper ions (Cu^{2+}) needed for EIN3 activity into the nucleus (Hahn and Harter, 2009). EIN3 is a key transcription factor in the ET signaling pathway and its accumulation is enhanced in the presence of ET (Yoo et al., 2009). In concert, inactivation of CTR1 results in the activation of a MAPK cascade involving MKK9 and MPK3/6 that phosphorylate and stabilize EIN3. EIN3 then activates the ethylene response factors (ERFs) that bind to the GCC-box consensus sequence element in the promoter region of ET-dependent genes (Ju et al., 2012; Yoo et al., 2008). Although CTR1 is a MAPKKK and modulates downstream signal transduction through phosphorylation, it does not appear to inactivate MKK9 by phosphorylation but rather by forming a complex with MKK9, preventing its entry into the nucleus (Ecker, 2004).

In response to stresses, such as caterpillar herbivory, a parallel pathway leading to ET biosynthesis and response is induced. Here, activation of the MAPKKK signaling cascade (MAPKKK, MKK4/5/9; MPK3/6) leads to the phosphorylation and activation of 1-aminocyclopropane-1-carboxylic acid synthase2/6 (ACS 2/6), a key enzyme in ET biosynthesis.

This enzyme converts *S*-adenosyl-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor to ET (Liu and Zhang, 2004; Xu et al., 2008).

2.3.4. Modulation of Jasmonic acid-mediated induced resistance by ethylene

ET modifies cellular responses to jasmonates by increasing or attenuating the IR responses (Fig. 2.2) (Spoel, 2003; Koorneef et al 2008a; Leon-Reyes, 2009; Tian et al., 2014). For example, ET has a positive effect on jasmonate-dependent PI activity in tomato, but attenuates jasmonate-induced nicotine biosynthesis in tobacco (Kahl et al., 2000; Onkokesung et al., 2010). Voelckel et al. (2001) showed that ET induced by the OSs of the tobacco hornworm, *Manduca sexta,* suppresses nicotine biosynthesis. In a similar fashion, in Arabidopsis, ET negatively affects GSL accumulation in response to feeding by the specialist caterpillar *P. rapae*, but does not affect JA responses after herbivory by the generalist herbivore, *S. exigua* (Mewis et al., 2006). Such seemingly conflicting biological effects are often observed since hormone concentration and localization as well as the plant-herbivore system influences the final outcome (McCourt, 1999; Onkokesung et al., 2010).

Necrotrophic pathogen infection or herbivore wounding often results in the release of ET together with JA (von Dahl et al., 2007). ET is linked to the modulation of JA-responses and, depending on the blend of hormones produced, activation of the MYC branch and the ERF and/or ORA59 branches of the JA pathway are affected (Fig. 2.2) (Pieterse et al., 2012). In addition to the MYC branch of JA pathway, two ethylene-modulated pathways, the ERF1 branch and the ORA59 branch, integrate spatial and temporal changes in JA- and ET- signaling pathways (Zhu et al., 2011; Leon-Reyes et al., 2013; Song et al., 2014). These complex interactions can be teased apart by using marker genes. For example, *VSP* is a marker for the JA-dependent MYC2 branch while the expression of *PDF1.2* is regulated under the JA/ET ERF1 branch. However, this is complicated by findings that *PDF1.2* is also regulated by TGA transcription factors which implicates the SA/NPR1 pathway in its regulation (Zander et al., 2012). ET production is induced during herbivory by certain species of caterpillar and, thus, the crosstalk between these two branches of the JA pathway is important for the specificity of plant responses to herbivory (Stotz et al., 2000; Robert-Seilaniantz et al., 2011; Verhage et al., 2011; Kazan and Manners, 2012).

Normally, in response to wounding or caterpillar herbivory, the MYC branch of the JA pathway is activated (Lorenzo et al., 2004). While JA plays the dominant role in the MYC branch, ET activates the ERF branch which bifurcates in to the ERF1 and ORA59 branches. These branches modulate the JA-dependent pathway, the SA-dependent pathway and each other. JAZ repressors physically interact with and repress EIN3 and ET insensitive-like (EIL) proteins that are essential for the ET pathway (Zhu et al., 2011). Therefore, by relieving EIN3/EIL repression by JA-mediated JAZ degradation, the ET response is activated. Thus, the activation of EIN3 and EIL requires both JA and ET signaling. Even though JA or ET independently can induce weak expression of *PDF1.2*, together, they synergistically influence transcript expression (Lorenzo et al., 2003). Therefore, ET modulates the ERF branch of the JA pathway by positively enhancing EIN3/EIL but negatively affects the MYC branch (Pieterse et al., 2012). This antagonism is mediated by the physical interaction between MYC TFs and EIN3 leading to the transcriptional inactivation of *EIN3* and, thus, the ERF branch is repressed (Song et al., 2014). As well, EIN3 can interact with MYC transcription factors which leads to the suppression of IR responses. For example, overexpression of *ORA59* leads to the suppression of the MYC branch and, consequently, the plant becomes more susceptible to herbivory by caterpillars of the cabbage butterfly, *P. rapae* (Verhage et al., 2011). Thus, in the presence of ET, these two branches, ERF and ORA59, work antagonistically to each other which might be important to fine tune defense responses against specific intruders (Lorenzo et al., 2003; Anderson et al., 2004; Pré et al., 2008; Song et al., 2014).

ET is also involved in the modulation of the SA-dependent pathway; ET enhances the expression of SA-responsive genes, such as *PR1*, but also may negatively affect the expression of SA biosynthesis enzymes, such as isochorismate synthase (*ICS*) and SA induction deficient 2 (*SID2)* (De Vos et al., 2005; Chen et al., 2009). ET stabilized EIN3 and EIL represses *SID2*, thus, inhibiting SA biosynthesis (Chen et al., 2009). Since JAZ repressors bind and repress EIN3 and EIL, upon degradation of JAZ protein during the activation of the JA-dependent pathway, EIN3 and EIL repression is relieved resulting in the inhibition of SA biosynthesis (Zhu et al., 2011; Kazan and Manners, 2012). Thus, ET is also a key player in modulating the antagonism between SA and JA.

2.3.5. Gibberelin/DELLA pathway

Under stressful conditions, plants allocate resources either to growth or defense (Ballare, 2011; Hou et al., 2013; Huot et al., 2014). For example, in some circumstances, plants subjected to low levels of caterpillar herbivory may engage in overcompensatory growth (Trumble et al., 1993; Arab and Trigo, 2011). However, more commonly, there is a shunt of metabolic flux into defense responses (Hout et al., 2014). Therefore during herbivore attack, plants often divert resources from primary metabolism towards the production of defense metabolites and amplification of resistance pathways (Cipollini et al., 2004). Caterpillar feeding often results in the downregulation of photosynthesis-related genes and plant growth is restricted (Giri et al., 2006; Bolton, 2009; Kerchev et al., 2012). In Arabidopsis, JA treatment or upregulation of JA-dependent responses often hinders leaf expansion, plant growth and delays reproduction (Cipollini, 2010; Wild et al., 2012; Yang et al., 2012). The phytohormone gibberellin (GA) regulates plant growth. Thus, the interaction between JA- and GA-pathways is important to prioritize plant growth *vs* defense during herbivore attack (Wild et al., 2012; Yang et al., 2012; Hou et al., 2013).

GAs, a group of tetracyclic diterpenoid plant hormones, regulate many plant physiological processes, such as seed germination, leaf expansion and development, stem elongation, transition from vegetative to reproductive stage and floral development in Angiosperms (Hauvermale et al., 2012). In the GA biosynthetic pathway, the GA precursor, geranyl-geranyl diphosphate (GGDP) undergoes cyclization and oxidation to produce intermediate GAs. The oxidases, GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox), are key enzymes converting these intermediates to the bioactive forms (GA1, GA3, GA4 and GA7) (Yamaguchi, 2008; Peter and Stephen, 2012). Bioactive GAs are bound by the receptor, GA-insensitive dwarf (GID1), that leads to a conformational changeto enable GID1 to bind to DELLA proteins (Fig. 2.3) (Murase et al., 2008; Shimada et al., 2008). In Arabidopsis, the F-box protein complex SCF^{SLY1} recognizes the GA-GID1-DELLA complex leading to the 26*S* proteasome-mediated degradation of DELLA proteins. DELLA proteins are repressors of the GA signalling pathway (Dill et al., 2004). Therefore, upon their degradation, GA-dependent growth and development occurs. Five DELLA proteins, GA insensitive (GAI), repressor of *ga1-3* (RGA), RGA-like 1 (RGL1), RGL2 and RGL3, are found in *A. thaliana* (Davière and Achard, 2013). Although there is some functional redundancy, DELLA proteins are expressed in different

tissues (Sun, 2011); GAI1 and RGA regulate root growth and stem elongation, RGL1, RLG2 and RGA promote flower development and RGL2 is involved in seed germination (Locascio et al., 2013). The loss-of-function quadruple *della* (quad-*della*) *A. thaliana* mutant has knockouts of four out of the five DELLA proteins*, gai-t6*, *rgat2*, *rgl1-1*, and *rgl2-1* (Achard et al., 2008). This mutant has a constitutive GA-positive phenotype and is taller and flowers earlier than the wild type Landsberg *erecta* (L*er*) genotype.

2.3.6. Jasmonic acid-gibberelin pathways crosstalk

For effective utilization of resources during stress such as caterpillar herbivory, plants have to balance growth and defense, in part, through crosstalk between JA- and GA- signaling pathways (Fig. 2.3). JAZ proteins are negative regulators of the JA-signaling pathway as well as a node for hormonal crosstalk. For example, JAZ proteins interact with transcription factors, such as MYC2/3/4 or EIN3/EIL, that are involved in JA- or ET- signaling pathways, respectively (Chini et al., 2007; Cheng et al., 2011; Fernandez-Calvo et al., 2011; Niu and Figueroa, 2011; Song et al., 2014). In a similar manner, DELLA proteins play an important role in the integration of hormonal signaling pathways (Hauvermale et al., 2012). JAZ and DELLA proteins interact with each other to balance growth and defense (Navarro et al., 2008; Hou et al., 2010; Wild et al., 2012; Yang et al., 2012). Stress-induced JA antagonizes GA-mediated growth (Heinrich et al., 2013). Conversely, GA can inhibit JA-signaling to avoid expensive IR and avoid the waste of resources under favorable growth conditions (Hou et al., 2013).

DELLA proteins interact with JAZ repressors and compete with MYC2 transcription factors for the same binding site, thus, releasing MYC2 to amplify JA-mediated responses (Hou et al., 2010). The combined application of JA and GA leads to the attenuation of JA-dependent response in the GA-deficient mutant*, ga1-3* (Wild et al., 2012). When exogenously applied GA leads to the degradation of DELLA proteins, JAZ is liberated to interact with MYC2, suppressing JA-dependent responses (Hou et al., 2012; Wild et al., 2012). MYC2, on the other hand, binds to the promoter of the *RGL3* gene enhancing its expression (Wild et al., 2012). The increased levels of the DELLA protein RGL3 acts to suppress plant growth during stress.

2.4. Specificity of Plant-Herbivore Interactions

JA-mediated IR is mounted in response to caterpillar herbivory; however, different caterpillar species may elicit distinct plant responses (Bonaventure, 2012). Understanding how the plant

recognizes the specific herbivore and finetunes its defense responses is a major challenge (Erb et al., 2012). Feeding by chewing herbivores, such as caterpillars, is more than mere mechanical damage; it is a combination of wounding, the type of insect feeding and the addition of caterpillar oral secretions that contain effectors that can be detected by the plant and affect its response against the herbivore (Felton, 2008).

When Arabidopsis is fed upon by specialist caterpillar, the small cabbage white, *P. rapae*, an increase in indolyl but not aliphatic GSLs are observed. In comparison, Arabidopsis infested with generalist caterpillars of *S. exigua* accumulate both aliphatic- and indolyl-GSL (Mewis et al., 2006). However, differences in plant responses to herbivory by generalist or specialist caterpillars is not always the case; In Arabidopsis, herbivory by caterpillars of the specialist, *P. rapae*, or the generalist, *S. littoris*, induce similar sets of defense-related genes (Reymond et al., 2004).

In cases when caterpillar-specific differences in plant responses are observed, this may reflect diverse effectors present in insect's oral secretions (Felton et al., 2008). When caterpillars feed on the plant, both regurgitant (from the gut) and saliva (two sources: labial or mandibular) or secretions from the ventral eversible glands (VEGs) are present in the OS (Peiffer and Felton, 2009; Zebelo and Maffei, 2012). In particular, plant responses to fatty acid-amino acid conjugates (FACs), such as volicitin, from the regurgitant and enzymes from the labial saliva have been studied in detail (reviewed by Wu and Baldwin, 2009). FACs are recognized by putative receptors in plant cells and lead to enhanced IR responses (Truitt et al., 2004). For example, when corn is treated with volicitin from *S. exigua* caterpillar regurgitant, volatile compounds are biosynthesized and released (Alborn et al., 1997). These compounds are highly attractive to arthropod predators and parasitoids of the insect herbivores (Wu and Baldwin, 2009). Different forms of FACs are found in the regurgitant of different caterpillar species. For example, the widely studied volicitin, *N*-17-hydroxylinolenoyl-L-glutamine, is found in *S. exigua* regurgitant but regurgitant from the tobacco hornworm, *M. sexta,* contains *N*-linolenoyl-L-glutamic acid (Alborn et al., 1997; Alborn et al., 2003). This diversity in regurgitant effectors may be one mechanism the plant uses to distinguish between caterpillar herbivores.

Other effectors, such as the peptide inceptin that is a proteolytic product of plant chloroplast ATP synthase ϒ-subunit identified in the oral secretions of the fall armyworm

Spodoptera frugiperda, results in elevated levels of JA and SA when applied to corn seedling and increased production of volatile organic compounds (VOCs) (Schmelz et al., 2006). This implies that the caterpillar feeds on the plant and modifies the plant-derived enzyme. The caterpillar then reintroduces this degraded enzyme to the plant as it regurgitates while feeding and the plant recognizes the subunit. However, the frequency of caterpillar regurgitation during feeding is controversial (Peiffer and Felton, 2009). It is believed that caterpillar regurgitation is a defensive response; they do not regurgitate continually on wounded plant tissues, possibly to minimize the release of effectors.

These effectors are used by the host plant to recognize the insect herbivore and induce specific responses; herbivores also have effectors that are used to evade wound responses or induce antagonistic pathways to attenuate JA-mediated responses (Zhu-Salzman et al., 2005). Enzymatic effectors derived from the caterpillar labial salivary secretions, such as glucose oxidase (GOX), have been characterized in insect oral secretions (Felton, 2008; Eichenseer et al., 2010). Labial saliva-derived GOX from caterpillars of *Helicoverpa zea* or *S. exigua* suppress plant induced JA-mediated IR presumably by activating the antagonistic SA-dependent pathway (Musser et al., 2002; Weech et al., 2008; Diezel et al., 2009; Paudel et al., 2013). In a similar manner, phloem sucking insects, such as whitefly and aphids, induce SA responses to antagonize effective JA-dependent defense responses (Zarate et al., 2007; Kamphuis et al., 2013). As well, the ET burst observed during herbivory by specialist caterpillars of the tobacco hornworm, *M. sexta*, reduces the level of induced nicotine in *N. attenuata* (Baldwin, 1999). As phytohormone pathways interact and influence each other, this is probably due to interactions between the JA-dependent IR pathway with the SA/NPR- mediated SAR pathway and/or the ethylene (ET) pathway. Thus, hormonal crosstalk during caterpillar herbivory is important in shaping the final outcome of IR in plants (Erb et al., 2012; Paudel et al., 2013; Pieterse et al., 2013).

2.5. Influence of Caterpillar Labial Saliva on Plant Defense Responses

Similar to specialist caterpillar herbivores that have strategies to cope with the specific defense systems of their host plants, generalist caterpillars have strategies to suppress the general induction of plant defenses. Effectors present in some generalist Noctuid caterpillar species may be a mechanism of these pests to avoid plant defenses. Oxidoreductases in caterpillar labial saliva, for example GOX, is believed to be a mechanism used by some Noctuid species to

prevent plants from mounting their defense response (Musser et al., 2002; Felton 2008; Weech et al., 2008; Paudel et al., 2013). Musser et al. (2002) observed that tobacco plants fed upon by caterpillars of the corn earworm *H. zea* with impaired labial salivary secretions had greater induced nicotine levels than if infested by normal caterpillars or treated with GOX, indicating that GOX in the caterpillar labial saliva acts to attenuate the full extent of plant IR. This may be a common mechanism in generalist Noctuid caterpillars since GOX has been detected in the labial saliva of *H. zea, Helicoverpa armigera, Mamestra configurata* and *S. exigua* but is absent from the Noctuid grass specialist *Pseudaletia unipuncta* (Merkx-Jacques and Bede, 2005; Eichenseer et al., 2010). However, the role of GOX in the labial saliva to suppress plant induced responses is controversial since Tian et al. (2012) have found that salivary GOX results in the induction of late responding *Pin2* gene expression, and triggers trichome development in tomato. This highlights the complexity of these interactions and/or plant specificity. Tian et al. (2012) suggest that the low level of *Pin2* expression observed in response to feeding by the specialist herbivores *M. sexta* and *Trichoplusia ni* could be due to lower levels of labial salivary GOX than in the generalist *H.zea*. Furthermore, the activity of caterpillar labial salivary GOX is dependent upon diet; *S. exigua* caterpillar labial salivary GOX activity is higher when caterpillars fed on artificial diet than on the plant *M. truncatula* (Merkx-Jacques and Bede, 2005).

It is still not clear how caterpillar labial saliva prevents the plant from mounting its IR; however, redox perturbation and the induction of antagonistic pathways are proposed to be involved. Together with the mechanical damage during caterpillar feeding, the presence of the oxidoreductase enzyme GOX in the caterpillar labial saliva leads to the production of H_2O_2 (Eichenseer et al., 2010; Maffei et al 2006). Compared to mechanical wounding alone, the zone of H_2O_2 accumulation around the site of *S. litttoralis* caterpillar feeding was increased (Maffei et al., 2006). Generated H_2O_2 may affect cellular redox balance and H_2O_2 or glutathione can affect protein post-translational modifications (Han et al., 2013; Foyer and Noctor, 2011; Spoel and Loake, 2011). Redox modulation due to labial saliva-mediated increases in H_2O_2 levels may lead to activation and monomerization of NPR1, thus, stimulating the SAR pathway (Spoel et al., 2003; Ndamukong et al., 2007; Koornneef et al., 2008b). GOX present in the labial saliva of *H. zea* induces the production of phytoalexin, a product of the SAR pathway, while inhibiting LOX activity and suppressing JA-mediated plant defense responses (Felton and Korth, 2000). Similarly, effectors in *S. exigua* caterpillar labial saliva require an active SAR pathway to

antagonizes JA-mediated IR in Arabidopsis during herbivory (Weech et al., 2008).Using Arabidopsis mutants, Weech et al. (2008) suggest that NPR1, not SA, mediate caterpillar labial saliva-dependent suppression of plant induced defenses.

As mentioned previously, ET is an important modulator of JA-dependent IR as well as the SA-JA interaction (Van der Does et al., 2013; Song et al., 2014). The ET burst that is linked to the OS of *M. sexta* caterpillars leads to the attenuation of JA-induced nicotine production in *N. attenuata* (Voelckel et al., 2001). However, inhibition of the ET burst during *S. exigua* caterpillar herbivory in tobacco or application of GOX to wounded tissues enhances the SA-dependent SAR pathway and, thus, leads to suppression of JA-mediated responses in tobacco (Diezel et al., 2009). Therefore, caterpillar labial saliva-mediated suppression of JA-dependent IR involves complex interactions between the JA-, SA- and ET-mediated pathways. Furthermore, JA-GA/DELLA pathways that interact during growth *versus* defense signaling and the fact that DELLA proteins participate in ROS scavenging lead to prediction that they might also be involved in the modulatation of plant-insect interactions (Ballare, 2011; Erb et al., 2012; Davière and Achard, 2013). Recently, it was shown that the negative growth regulator DELLA proteins are required for caterpillar labial saliva-mediated suppression of the JA burst and suppression of JA-mediated responses in Arabidopsis (Lan et al., 2014).

2.6. Glucosinolate-Related Defense against Herbivory in *Arabidopsis thaliana*

GSLs are the major defense secondary metabolites found in plants of the Brassicaceae family, which includes the model plant *A. thaliana* (Agerbirk and Olsen, 2012). More than 130 GSLs have been identified and these nitrogen- and sulfur- containing metabolites confer plant resistance against herbivores and pathogens (Agerbirk and Olsen, 2012; Baskar et al., 2012). GSLs are built upon amino acid precursors and classified as indole, aliphatic or aromatic depending on the use of tryptophan (Trp), methione (Met) or phenylalanine (Phe), respectively, as the starting block (Sonderby et al., 2010a). In *A. thaliana,* nearly 40 GSLs have been identified; most of them are aliphatic or indole GSLs derived from Met or Trp, respectively (Kliebenstein et al., 2001). In *Arabidopsis*, these chemically stable and water soluble thioglucosides are stored in the vacuoles of special S-cells near the phloem tissues (Wittstock and Burow, 2010; Winde and Wittstock, 2011). During seed germination and plant development or upon tissue damage, GSLs may come into contact with myrosinase, a β-thioglucosidase (Rask

et al., 2000; Baskar et al., 2012). Myrosinases are stored in myrosin cells to prevent auto-toxicity but, once released, these enzymes cleave the thioglycosidic bond of the GSL releasing their sugar moiety (Textor and Gershenzon, 2009). The resulting aglycone is highly unstable and undergoes spontaneous structural rearrangement leading to the production of potentially toxic isothiocyanates, nitriles, epinitriles or thiocyanates (Winde and Wittstock, 2011; Basker et al., 2012).

When a caterpillar feeds on the leaf, cells are broken, allowing contact between the GSL and myrosinase leading to toxic deterrent compounds (Lambrix et al., 2001; Hopkins et al., 2009). As well, caterpillar feeding induces GSL accumulation, particularly indole GSLs (Textor and Gershenzon, 2009; Kos et al., 2012). Although, indole GSLs are considered a more effective plant defense against caterpillar herbivory, aliphatic GSLs also confer resistance (Kliebenstein et al., 2001; Gigolashvili et al., 2007a; Textor and Gershenzon, 2009; Agerbirk and Olsen, 2012; Kos et al., 2012). High accumulation of indole GSLs in the *MYB51* overexpressing Arabidopsis line as well as the accumulation of aliphatic GSLs in the *MYB28* overexpressing Arabidopsis line both have higher resistance against caterpillars of *S. exigua* compared to wild type plants (Gigolashvili et al., 2007a; Gigolashvili et al., 2007b; Kos et al., 2012). In contrast, caterpillars of specialist herbivores, such as *P. rapae* and *Plutella xylostella*, are not affected by either type of GSL (Mueller et al., 2010).

Certain GSL-producing plants or the attacking herbivore may have strategies to modify GSL breakdown (Wittstock et al., 2004; Winde and Wittstock, 2011). Nitrile-specifier protein (NSP) and/or epithiospecifier-like protein (ESP) present in certain Arabidopsis ecotypes leads to the production of simple nitriles or epithionitriles rather than the toxic (iso)thiocyanate (Lambrix et al., 2001; Wittstock and Burrow, 2010). Caterpillars of the generalist herbivore *S. litoralis* perform better on the nitrile-producing *A. thaliana* mutant (Burow et al., 2006). However, production of nitrile by damaged plants attracts adult females of the parasitoid wasp *Cotesis rebecula* (Mumm et al., 2008). Therefore, this might be a strategy of the plant to induce indirect defenses to protect themselves against specialist herbivore, such as the cabbage looper *P. rapae* that uses GSLs and isothiocyanates as feeding and oviposition attractants (Hopkins et al., 2009).

Defense-related phytohormones, such as JA and SA, affect GSL biosynthesis (Baskar et al., 2012). Application of JA induces the expression of genes that encode enzymes in GSL

biosynthesis and leads to an increase in constitutive GSLs (Mikkelsen et al., 2003). The Arabidopsis JA-insensitive *coi1* mutant has lower GSL concentration (Mewis et al., 2006). As well, GSL induction by herbivore feeding was not observed in these plants. Mechanical wounding leads to the expression of genes encoding important regulators in GSL biosynthesis, such as *MYB28* and *MYB51* (Gigolashvili et al., 2007a; Gigolashvili et al., 2007b). The stress hormone SA, on the other hand, suppresses the expression of *MYB28/29* and negatively affects aliphatic GSL biosynthesis (Gigolashvili et al., 2007a; Li et al., 2013). In Arabidopsis, there also appears to be antagonism between the two major branches of the pathway leading to indole or aliphatic GSLs (Gigolashvili et al., 2008).

2.7. Experimental Organisms

2.7.1. *Arabidopsis thaliana*

Arabidopsis thaliana is a member of Brassicaceae family and a close relative to economically important crops such as canola (*Brassica napus*), and cabbages (*Brassica oleraceae* and *Brassica rapa*). Because of its small diploid genome, short life cycle and known genome sequence, it is one of the most extensively investigated model plants (The Arabidipsis genome Initiative, 2000). In response to caterpillar herbivory, Arabidopsis defenses include induction and activation of GSLs, LMCOs and PIs (Wasternack and Hause, 2013; Zhu-Salzman et al., 2008). The availability of well-characterized mutants has been useful for the research. For example, the *pad2.1* mutant has a mutation in the gene encoding ϒ-glutamylcysteine synthetase, the enzyme that catalyzes the first step of glutathione biosynthesis (Parisy et al., 2007). The *tga2/5/6* line is deficient in TGA transcription factors involved in the SA-dependent SAR pathway (Zhang et al., 2003). The quad-*della* mutant lacks four of the five DELLA proteins that are negative regulators of the GA pathway (Archard et al., 2008). Therefore, these mutants were used to elucidate phytohormonal crosstalk in plant-insect interactions.

2.7.2. *Medicago truncatula*

Medicago truncatula belongs to the Fabaceae family. Plants in this family form a symbiotic relationships with nitrogen-fixing rhizobia. It is a close relative of the important forage crop, alfalfa, and a model to study legume-rhizobial interactions (Jones et al., 2007). *M. truncatula* has a comparatively small diploid genome (~500 Mbp), is self-fertile and the sequencing of its euchromatic region has been completed covering 94% of the genes (Young et al., 2011). In this

research, the ethylene-insensitive mutant *skl* which has a mutation in the nuclear Nramp transporter EIN2 was used to investigate the role of ET in plant-insect interactions (Penmetsa et al., 2003). EIN2 is believed to allow the transport of Cu^{2+} ions into the nucleus which is needed for the transcription factor EIN3 to bind to the promoter region of ET-responsive genes (Alonso et al., 1999).

2.7.3. *Spodoptera exigua*

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a generalist pest. These caterpillars are voracious feeders that can attack a wide range of crops representing more than 18 plant families (Greenberg et al., 2001). Thus, it is an economically important agricultural pests. *S. exigua* caterpillars are used in many plant-insect interaction studies and a number of effectors involved in the modulation of plant IR have been identified in this insect (Eichenseer et al., 2010). For example, the first FAC, volicitin, was identified from *S. exigua* regurgitant (Alborne, 1999). Application of volicitin to wounded corn seedling leads to the biosynthesis and release of plant volatiles that attract natural enemies of the caterpillar pest. In contrast, *S. exigua* caterpillar labial saliva is believed to suppress host plant IR (Alborn et al., 1997; Weech et al., 2008; Diezel et al., 2009; Paudel et al., 2013; Lan et al., 2014); however, it was shown that labial salivary GOX induces the JA burst and late expression of the JA-dependent *Pin2* in tomato (Tian et al., 2012). Therefore, the role of caterpillar labial saliva in induced plant defenses is controversial and plant-specific differences may represent differences in plant responses (i.e. early vs late) or host plant nutritional quality. Dietary quality, in particular the ratio of protein-to-digestible carbohydrate (p:c), affects the labial salivary GOX activity (Merkx-Jacques and Bede, 2006). By cauterizing the spinneret, the organ through which labial saliva is secrected, two populations of caterpillars are created; those with intact or those with impaired labial salivary secretions (Musser et al., 2002). This technique was used to tease apart the role of *S. exigua* labial saliva in these plant-insect interactions.

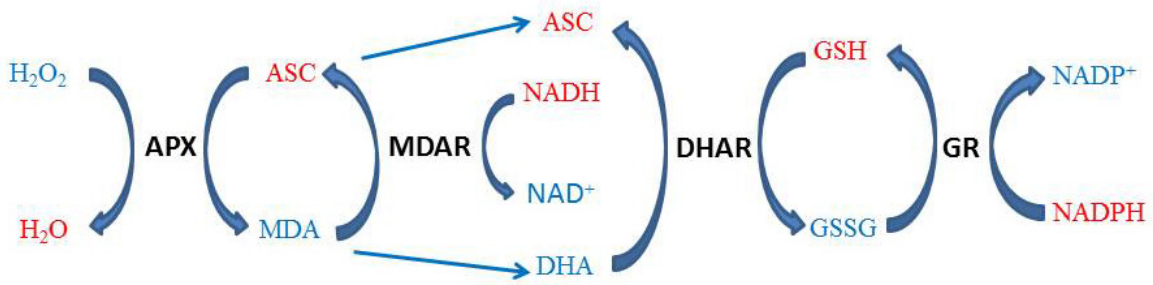

Figure 2.1. Halliwell-Asada (ascorbate-glutathione) cycle. Increased hydrogen peroxide (H_2O_2) is reduced to water by ascorbate peroxidase (APX). Reduced ascorbate (ASC) is oxidized to dehydroascorbate (DHA) via the intermediate monodehyroascorbate (MDA). Dehydroascorbate reductase (DHAR) reduces DHA back to ASC using reduced glutathione (GSH) generating oxidized glutathione (GSSG). This last metabolite is reduced back to GSH by glutathione reductase (GR) using $NADP^+$ to generate NADPH.

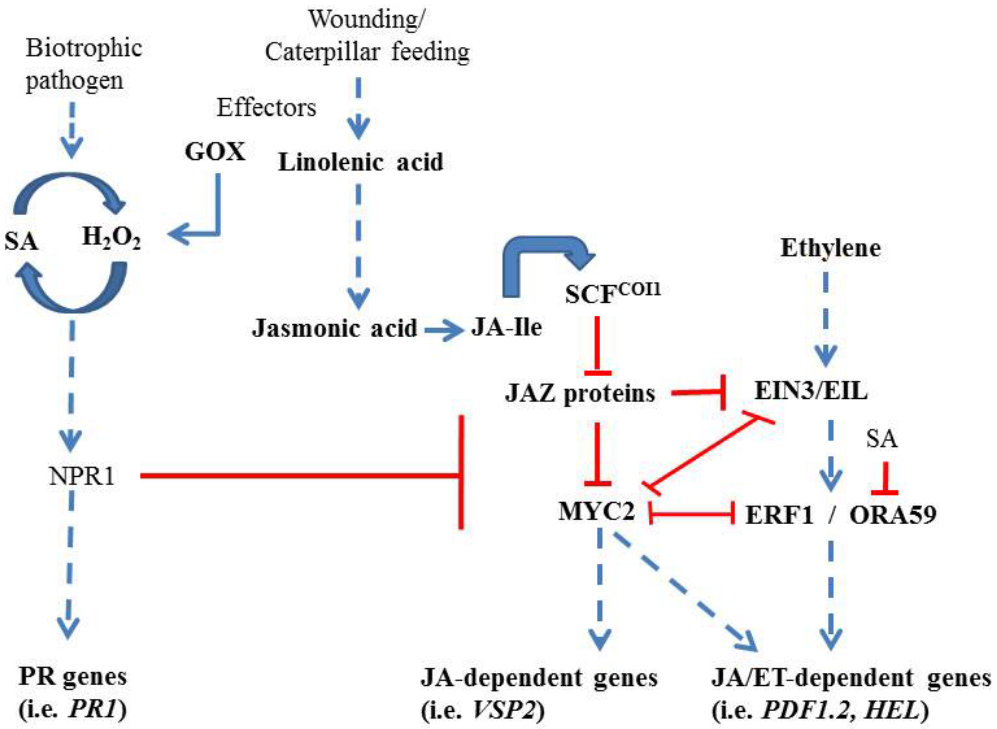

Figure 2.2. Crosstalk between phytohormone-dependent pathways. In response to wounding or caterpillar herbivory, jasmonic acid (JA) is biosynthesized and the bioactive jasmonate-isoleucine (JA-Ile) leads to the activation of the JA-response pathway. JA-Ile bridges SCF^{COI1} to jasmonate zim-domain (JAZ) proteins leading to JAZ protein degradation and release of MYC transcription factors (Pieterse et al., 2012). The salicylic acid (SA)-dependent pathway is activated by biotrophic pathogen or effectors such as glucose oxidase (GOX) present in the caterpillar labial saliva (Felton et al., 2008; Weech et al., 2008, Diezel et al., 2009). Activation of the SA-dependent SAR pathway interferes with the JA-dependent IR pathway. Some evidence suggests that NPR1 interferes with SCF^{COI1} degradation of JAZ proteins (Mou et al., 2003). However, other studies show that the antagonism lies downstream possibly due to SA pathway antagonism of the ORA59 branch of the JA/ET pathway (van der Does et al., 2013). ET-dependent responses are activated in response to herbivory. ET inhibits the repressor CTR1 that leads to stabilization of EIN3 and EIL. Downstream, there is an activation of ERF1/ORA59 branches. There are a number of potential signalling nodes between these pathways: is JAZ-EIN3/EIL, MYC2-ERF1/ORA59, SA-ORA59 andMYC2-EIN3/EIL (Pieterse et al., 2012; van der Does et al., 2013; Song et al., 2014). Solid blue lines indicate direct effects, dotted blue lines show downstream responses and red truncated lines show antagonistic relationships.

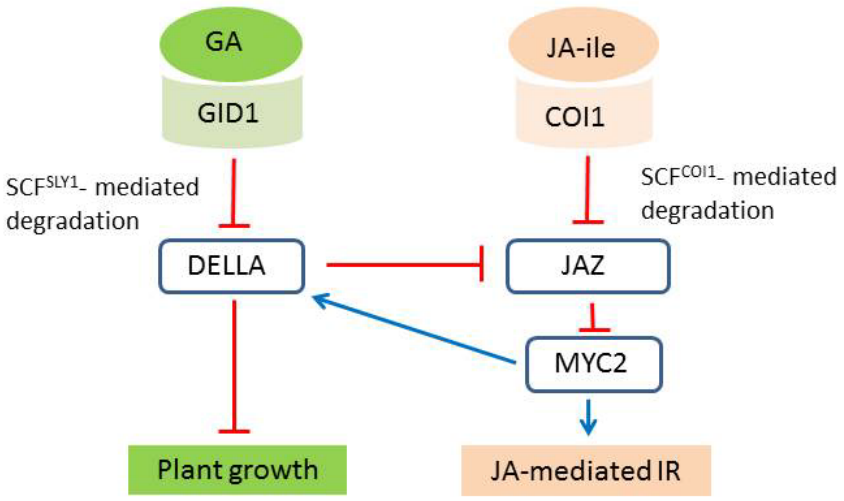

Figure 2.3. Gibberellin (GA)-Jasmonate (JA) crosstalk. GA binds to the GID1 receptor and triggers SCF^{SLY1}-mediated degradation of DELLA proteins leading to plant growth (Wild et al., 2013, Hou et al., 2010). Binding of bioactive JA-Ile to the COI receptor leads to SCF^{COI1}-mediated degradation of JAZ and releases MYC transcription factors. Once released, MYC transcription factors lead to the induction of JA-mediated induced resistance (IR) (Pieterse et al., 2012). DELLA proteins interact with JAZ proteins and compete with MYC transcription factors for the same binding site (Hou et al., 2010). Thus, released MYC transcription factors leads to activation of JA-mediated plant responses. At the same time, MYC transcription factors enhance the level of DELLA proteins that leads to the suppression of plant growth during stress (Wild et al., 2013). Red truncated lines show negative regulation and blue lines shows positive regulation.

Connecting Statement to Chapter 3

S. exigua caterpillar labial saliva is rich in oxidoreductase enzymes, such as GOX, that may effect cellular redox balance (Merkx-Jacques and Bede, 2005; Maffei et al., 2006; Eichenseer et al., 2010). H_2O_2 is produced during caterpillar herbivory either due to wounding or GOX present in caterpillar labial saliva. While excessive ROS are detrimental to cellular components, at controlled level ROS, such as H_2O_2, are the second messengers that lead to activation of downstream plant responses (Schröder et al., 2008; Forman et al., 2010). Plants activate enzymatic and non-enzymatic antioxidant responses, such as the ascorbate-glutathione cycle, to detoxify excessive ROS (Noctor et al., 2012). In Chapter 3, early redox responses of the host plant after herbivory by *S. exigua* caterpillars with intact or impaired labial salivary secretions was investigated. At the same time, expression levels of JA-, SA, and ET-dependent genes were evaluated.

I conducted the experiment to measure redox metabolites while the gene expression study was performed by the co-author Tanya Copley. For the redox metabolites, I was assisted in plant sample collection by Alexander Amerizian. Alberto Prado helped with metabolite analysis. I am responsible for data analysis and manuscript preparation together with my supervisor, Dr. Jacqueline Bede. She funded this project through her NSERC grant and provided guidance and supervision throughout the experiment. This chapter has been published in Frontiers in Plant Science (Paudel et al., 2013).

CHAPTER 3. Arabidopsis Redox Status in Response to Caterpillar Herbivory

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3.1 Abstract

Plant responses to insect herbivory are regulated through complex, hormone-mediated interactions. Some caterpillar species have evolved strategies to manipulate this system by inducing specific pathways that suppress plant defense responses. Effectors in the labial saliva (LS) secretions of *Spodoptera exigua* caterpillars are believed to induce the salicylic acid (SA) pathway to interfere with the jasmonic acid (JA) defense pathway; however, the mechanism underlying this subversion is unknown. Since Noctuid caterpillar LS contains enzymes that may affect cellular redox balance, this study investigated rapid changes in cellular redox metabolites within 45 min after herbivory. Caterpillar LS is involved in suppressing the increase in oxidative stress that was observed in plants fed upon by caterpillars with impaired LS secretions. To further understand the link between cellular redox balance and plant defense responses, marker genes of SA, JA and ethylene (ET) pathways were compared in wildtype, the glutathione-compromised *pad2-1* mutant and the *tga2/5/6* triple mutant plants. *AtPR1* and *AtPDF1.2* showed LS-dependent expression that was alleviated in the *pad2-1* and *tga2/5/6* triple mutants. In comparison, the ET-dependent genes *ERF1* expression showed LS-associated changes in both wildtype and *pad2-1* mutant plants and the ORA 59 marker *AtHEL* had increased expression in response to herbivory, but a LS-dependent difference was not noted. These data support the model that there are SA/NPR1-, glutathione-dependent and ET-, glutathione-independent mechanisms leading to LS-associated suppression of plant induced defenses.

3.2 Introduction

As plants interact with multiple organisms, they need to prioritize their actions to respond appropriately. Plants manage this through synergistic or antagonistic interactions mediated through growth and defense hormones: A process known as crosstalk (Spoel and Dong, 2008; Robert-Seilaniantz et al., 2011). In plant-pathogen interactions, activation of the systemic

acquired resistance (SAR) pathway by biotrophic pathogens may render the plant more susceptible to nectrotrophic pathogens that elicit jasmonate (JA)- and ethylene (ET)-mediated responses (Glazebrook, 2005). Insect herbivores also exploit this plant hormone crosstalk to prevent the induction of defensive pathways (Felton and Korth, 2000); however, the mechanisms underlying this are not fully understood.

When tissues are damaged during caterpillar feeding, rapid changes in calcium signatures and the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), leads to the induction of the JA pathway and plant defense responses (Lou and Baldwin, 2006; Arimura et al., 2011). At low, regulated concentrations, H_2O_2 is an important signaling molecule, however, uncontrolled levels are destructive as H_2O_2 readily reacts with cellular components (Schröder and Eaton, 2008; Forman et al., 2010). ROS is generated by mechanical damage but also by enzymes, such as glucose oxidase (GOX), present in the caterpillar's labial saliva (LS; Eichenseer et al., 2010). In lima bean, the zone of H_2O_2 accumulation around the site of leaf damage is widened by ~500 μm by *Spodoptera littoralis* caterpillar herbivory compared to mechanical wounding (Maffei et al., 2006). This caterpillar LS-associated production of H_2O_2 is proposed to be a strategum of some insect species to interfere with induced plant defenses (Musser et al., 2002; Bede et al., 2006).

To avoid the detrimental effects of ROS, antioxidant proteins, such as superoxide dismutase, catalase, peroxidase, and the Halliwell-Asada (ascorbate/glutathione) cycle are activated to maintain cellular redox homeostasis (Noctor et al., 2012). The Halliwell-Asada cycle lowers cellular H_2O_2 levels through a series of redox reactions involving ascorbate and glutathione. Therefore, in response to stress, plants often alter the total glutathione pool or the ratio between oxidized to reduced glutathione (GSSG:GSH) to maintain low H_2O_2 levels. Recognition of biotrophic pathogen attack or SA mimic treatment may result in an increase in total glutathione levels (Fodor et al., 1997; Mou et al., 2003; Mateo et al., 2006; Mur et al., 2006) Infiltration of SA into *Arabidopsis* leaves initiates a transient oxidation of the glutathione pool 6 hr after the time of injection (Mou et al., 2003; Mateo et al., 2006). In response to mechanical damage, the ratio of GSSG/total glutathione increases, reflecting an oxidized cellular environment, with oxidized glutathione (GSSG) positively linked to JA signaling (Mhamdi et al., 2010; Gfeller et al., 2011). *Arabidopsis* glutathione mutants are more susceptible to microorganism and insect attack (Ball et al., 2004; Parisy et al., 2007; Schlaeppi et al., 2008).

Arabidopsis *pad2-1* mutant lacks γ-glutamylcysteine synthetase that catalyzes the first step in glutathione biosynthesis (Parisy et al., 2007); therefore, glutathione levels are approximately one-fifth wildtype levels. This line is more vulnerable to *S. littoralis* herbivory (Schlaeppi et al., 2008; Mhamdi et al., 2010; Leon-Reyes et al., 2010a; Dubreuil-Maurizi et al., 2011). As well, as glutathione pools and ratio change, related processes, such as protein glutathionylation or *S*-nitrosylation that are also implicated in the regulation of defense against pathogens and herbivores, are affected (Wünsche et al., 2011; Espunya et al., 2012).

In response to caterpillar herbivory, the active form of JA, JA-isoleucine, bridges a jasmonate ZIM-domain (JAZ) proteins with the E3 ubiquitin ligase SCF^{COI1} complex, resulting in the proteasome-mediated degradation of JAZ and release of the basic helix-loop-helix transcription factor MYC2, responsible for the expression of JA-associated genes, such as *VSP2* and *LOX2* (Lorenzo et al., 2004; Dombrecht et al., 2007; Kazan and Manners, 2008; Robert-Seilaniantz et al., 2011). Caterpillar herbivory-related increases in ET biosynthesis may modulate these JA responses through cross-talk between the JA-dependent MYC2-branch and ET-dependent branches (Stotz et al., 2000; Bodenhausen and Reymond, 2007; Kazan and Manners, 2008; Diezel et al., 2009; Robert-Seilaniantz et al., 2011; Verhage et al., 2011). Two AP2/ERF transcription factors, ethylene response factor1 (ERF1) and ORA59 integrate ET crosstalk with the JA pathway (Penninckx et al., 1998; Lorenzo et al., 2003; Pré et al., 2008); though both these branches are induced by ET, evidence points to them being parallel and, perhaps, functionally redundant. Together, the MYC2 and ET-pathways, ORA59/ERF1, act synergistically or antagonistically allowing the integration of temporal and spatial hormone concentrations and localization to generate a specific signal signature (Kazan and Manners, 2008; Robert-Seilaniantz et al., 2011).

Effectors in the caterpillar LS may also activate the SAR pathway leading to the attenuation of JA-dependent responses (Kazan and Manners, 2008; Weech et al., 2008; Leon-Reyes et al., 2010a; Verhage et al., 2011). In recognition of attack by biotrophic pathogens, plants mount the systemic defense response, SAR, initiated by increases in cellular SA and H_2O_2 that positively impact each other's production (Rao et al., 1997; Glazebrook, 2005; Mateo et al., 2006). The resultant change in glutathione redox balance results in the activation of the Nonexpressor of PR-genes1 (NPR1) through thioredoxin-catalyzed reduction of the disulfide bridges, changing the protein from its cytosolic oligomer form to the monomer that enters the

nucleus (Spoel et al., 2009; Noctor et al., 2012). Association of NPR1 with TGA transcription factors leads to the expression of pathogenesis-related genes, such as *PR1*. The mechanistic basis of the antagonism between SA- and JA-pathways is still debated (Lorenzo and Solano, 2005). Early evidence suggests that SA interferes directly with JA biosynthesis (Doares et al., 1995; Rayapuram and Baldwin, 2007). However, NPR1 has been shown to be interfere with JA signaling downstream of JA biosynthesis (Mou et al., 2003; Spoel et al., 2003; Ndamukong et al., 2007; Spoel and Dong, 2008; Tada et al., 2008; Koornneef and Pieterse, 2008a; Leon-Reyes et al., 2010a). This may reflect the observation that ET modifies SA/NPR1 inhibition of JA responses such that in the presence of ET, the attenuation of JA-dependent gene expression is NPR1-independent; however, in the absence of ET, NPR1 is necessary to interfere with these responses (Leon-Reyes et al., 2009). Weech et al. (2008) used *Arabidopsis* mutants to show that caterpillar LS interference of JA-dependent plant defenses by activation of the SAR pathway requires an active NPR1. In addition, Diezel et al. (2009) showed that damage of wild tobacco by caterpillars of the tobacco hornworm, *Manduca sexta*, results in an ethylene burst that attenuates the SA-mediated suppression of plant defense responses. Therefore, in plant-caterpillar interactions, there appears to be extensive interplay between JA-, SA- and ET-pathways.

The present research is designed to understand the potential role of cellular redox balance in the ability of caterpillar LS to interfere with host plant defense responses. Since caterpillar LS contains redox enzymes, such as GOX that generate H_2O_2, caterpillar saliva should perturb the redox state or balance even more than mere wounding (Eichenseer et al., 2010; Noctor et al., 2012). By using normal caterpillars with intact LS secretions or insects where LS secretions have been impaired by cauterization of the spinneret, one can tease out the effect of LS on the modulation of plant responses. Therefore, in response to herbivory by caterpillars with intact or impaired LS secretions, the redox metabolites glutathione and ascorbate were measured to identify the impact of LS on cellular redox balance. As well, transcript responses of JA-, ET- and SA-dependent marker genes were compared in wildtype plants and two mutant lines, *pad2-1*, compromised in glutathione biosynthesis, and a *tga2/5/6* triple mutant that is deficient in the basic leucine zipper TGA transcription factors that interact with NPR1 (Zhang et al., 2003; Parisy et al., 2007).

3.3 Materials and Methods

3.3.1 Plants

Arabidopsis seeds ecotype Col-0 (TAIR CS3749) and the *pad2-1* mutant (TAIR CS3804) were obtained from the *Arabidopsis* Biological Resource Centre (Ohio State University). Seeds of the *Arabidopsis tga2/5/6* triple mutant were a generous gift from Dr. Li (University of British Columbia).

For redox metabolite experiments, wildtype plant seeds were surface-sterilized by soaking them for 2 min in 70% ethanol, followed by 5 min in 50% bleach. Seeds were rinsed 3 times in sterile distilled water and sown in Premier Promix BS (Premier Horticulture Inc). After cold treatment at 4°C for three days, seeds were transferred into a growth cabinet (light intensity 140 $\mu Em^{-2}s^{-1}$, 12:12 light:dark at 22°C). Plants were bottom-watered as needed, about 3 times per week with dilute 0.15 g/L N-P-K fertilizer.

For gene expression experiments, seeds were surface-sterilized as described above and germinated on half-strength MS media with 1% agar. After cold treatment for 3 days at 4°C, seeds were placed in the growth cabinet and transferred to Agro-Mix at germination. At 5 weeks post-germination, one plant from each genotype (Col-0, *pad2-1* and *tga2/5/6*) were transplanted into a 12.5 x 12 cm^2 pot.

Approximately 7-week old plants in the late vegetative growth stage, between growth stages 3.7 to 3.9 according to Boyes et al. (2001), were used in redox metabolite or gene expression experiments.

3.3.2 Caterpillars

Beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), insects were reared for multiple generations from eggs purchased from Bio-Serv (Frenchtown, NJ). Insects were reared under defined conditions in a growth cabinet (16:8 light:dark, RH 28-40%, temperature 28.5°C) and fed a wheat germ-based artificial diet (Bio-Serv). Adult moths were allowed to mate and the eggs collected to maintain the colony.

3.3.3 Impairment of caterpillar labial salivary secretions

Caterpillar LS is secreted through a specialized organ, the spinneret (Musser et al., 2002). To impair LS secretions, this spinneret was cauterized as previously described (Musser et al., 2002;

Bede *et al*., 2006). Prior to the experiment, caterpillars were allowed to feed >12 hr on *Arabidopsis* plants to allow the insects to adjust to the plant diet.

3.3.4 Measurement of redox metabolites

Leaf H_2O_2 levels were not measured directly due to the high variability associated with the instable nature of this compound and confounding effects by high leaf phenolic content and ascorbate (Queval et al., 2008). Therefore, other metabolites associated with the ascorbate/glutathione cycle were measured since they closely correlate with H_2O_2 levels (Ng et al., 2007). Six week old *Arabidopsis* plants were subject to one of three treatments: untouched (control) or subject to herbivory by 3 x 4^{th} instar *S. exigua* caterpillars with intact or impaired salivary secretions. As *S. exigua* caterpillars feed most actively at night, experiments were performed during the dark to more accurately simulate an ecological scenario. Rosette leaves showing signs of herbivory were harvested at 5, 15, 25, 35 and 45 min and immediately frozen in N_2. The experiment was repeated thrice.

At each time point, ascorbate and glutathione were measured in 3-4 independent samples. Plant samples were finely ground in liquid nitrogen and extracted in 0.2 N HCl at a ratio of 100 mg leaf/1 mL acid. This was followed by neutralization with NaOH as described in Queval and Noctor (2007). Chemicals used in redox metabolite assays were purchased from Sigma Chemical Company.

Ascorbate

Total, oxidized and reduced ascorbate from the leaf extract supernatant were determined by measuring reduced ascorbate levels spectrophotometrically at A_{265} using an Infinite M200 Pro microplate reader (Tecan) according to Queval and Noctor (2007). Total ascorbate was measured by converting dehydroascorbate (DHA) to the reduced form by incubating the supernatant in dithiothreitol (0.4% v/v) in 67.2 mM sodium phosphate (NaH_2PO_4) buffer, pH 7.5 for 30 min at room temperature. Triplicates of each sample were incubated with ascorbate oxidase (0.2 U) and reduced ascorbate was measured after 8 min incubation. Reduced ascorbate (ASA) levels were measured by adding ascorbate oxidase to the neutralized leaf extract supernatant in 0.1 M sodium phosphate buffer, pH 5.6, incubating at room temperature for 30 min and analyzing as above. Concentrations were determined from a six-point L-ascorbate standard curve (40 to 240 μM). Oxidized ascorbate levels were calculated by subtracting reduced from total ascorbate.

Glutathione

Measurement of glutathione is based on a recycling assay (Rahman et al., 2006; Queval and Noctor, 2007); glutathione reductase, in the presence of NADPH, catalyzes the reduction of GSSG to GSH that reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) forming 5-thio-2-nitrobenzoic acid (TNB) that can be measured spectrophotmetrically at $A_{412.}$ Total glutathione was measured by incubating leaf supernatant in 0.6 mM DTNB and glutathione reductase (0.015 Units) in 0.1 M sodium phosphate (NaH_2PO_4) buffer, pH 7.5. After the addition of 0.5 mM β-NADPH, the TNB chromophore was monitored at A_{412} at 5 sec intervals for the first 2 min. Total glutathione concentration was calculated based on triplicate 8-point standard curve (100 nM to 60 μM). Oxidized glutathione (GSSG) was measured by removing any reduced GSH from the sample by precipitation with 2-vinylpyridine followed by conversion of GSSG to GSH and measurement using the glutathione reductase/β-NADPH/DTNB method as described above (Griffith, 1980; Rahman et al., 2006; Queval and Noctor, 2007). Briefly, leaf supernatant was incubated with 1 μl 2-vinylpyridine (approx. 10-fold above GSH levels) for 30 min at room temperature. After centrifugation at 13,000 rpm for 5 min to remove excess 2-vinylpyridine, samples were diluted in 0.1 M sodium phosphate buffer, pH 7.5 and assayed in triplicate. GSSG levels were determined from a triplicate 8-point GSSG standard curve ranging from 100 nM to 3.2 μM. Reduced GSH was calculated by subtracting 2 x GSSG from total glutathione.

3.3.5 Gene expression

Three days before the herbivory experiment, clear plastic bottles were placed around the plants with mesh covering the tops. *Arabidopsis* plants were subject to one of three treatments: untouched (control) or subject to herbivory for 36 hours by 6 x 4^{th} instar *S. exigua* caterpillars with intact or impaired salivary secretions. The experiment was repeated twice; at each time point, two independent samples were taken for gene expression analysis (total n = 3-4). Dry weights of the vegetative tissue from two replicates of each genotype and treatment for each experimental replication (total n = 4) were measured to determine the approximate leaf mass eaten by caterpillars or removed by mechanical damage.

RNA extraction, cDNA synthesis and quantitative real time-polymerase chain reaction

Plants were finely ground in liquid nitrogen and total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocols. After DNase treatment (Wipeout,

QuantiTect Reverse Transcription kit (Qiagen)), the absence of genomic contamination was confirmed using 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' primers which spanned an intronic region of the *AtLMCO* gene (Weech et al., 2008). One µg of RNA was converted to cDNA using a QuantiTect Reverse Transcription Kit following the manufacturer's instructions.

Gene-specific primers were identified from the literature or designed using Primer3 (Table 3.1). Transcript expression was analyzed in duplicate using the Brilliant One-Step quantitative RT-PCR kit (Stratagene), according to the manufacturer's protocol, in a Mx3000p thermocycler (Stratagene). Gene amplicon products were verified by sequencing. Each 96-well plate, contained a standard curve of the gene-of-interest, a non-template control and each sample in duplicate. Each reaction contained 1 x SYBR green I, 0.375 nM ROX, 100 nM of the forward and reverse primer, mastermix that contained dNTPs, $MgSO_4$ and *Taq* polymerase, and either water (non-template control), serial dilutions of PCR amplicon (standard curve) or 85 ng cDNA sample. Standard curves ensured an efficiency of between 90-110%. Thermocycler conditions are as follows: 95ºC for 10 min; 40 cycles of denaturation at 95ºC for 45 sec, annealing for 1 min, and elongation at 70°C for 45 sec. The annealing temperature was dependent on the primers used (Table 3.1). Dissociation curves were performed to ensure amplicon purity. Two technical plate replicates were performed.

From the standard curve, gene copy numbers were estimated and normalized against the constitutive reference gene *AtACT2* (At3g18780). *Arabidopsis AtACT2* expression was not affected by osmotic stress or when plants were treated with viral pathogens or stress-related hormones, such as methyl jasmonate or salicylic acid, or caterpillar herbivory (Stotz et al., 2000; Dufresne et al., 2008; Weech et al., 2008). In the current study, *AtACT* was stably expressed within a genotype and not affected by treatment (+/+: $F_{(2,9)} = 0.26$, $p = 0.77$; *pad2-1*: $F_{(2,9)} = 1.10$, $p = 0.37$; *tga2/5/6*: $F_{(2,7)} = 0.42$, $p = 0.68$) (Brunner et al., 2004).

3.3.6 Statistical analysis

For the redox experiment (repeated independently three times, n=5-10), statistical differences ($p \leq 0.05$) in metabolite levels were determined using a two-way analysis of variance (ANOVA) using SPSS version 20 (SPSS Inc.). If a significant time x treatment factor was observed, a one-way ANOVA followed by a Tukey HSD *post hoc* test was conducted to identify the significant

difference. The gene expression experiment was repeated twice with two independent biological samples analyzed at each time (total n = 3-4). Within each genotype, transcript expression was analyzed by a one-way ANOVA. Statistical differences ($p < 0.05$) were determined using a Tukey HSD *post hoc* test (Rieu and Powers, 2009). Alternatively, because of the variation inherent with insect feeding studies, a greater than 5-fold change in gene expression with respect to control plants was also considered significantly different. Results from statistical analyses are shown in Appendix 3.1.

3.4 Results and Discussion

3.4.1 Ascorbate-glutathione cycle

The ascorbate-glutathione cycle is critical to enable the plant to maintain cellular redox status during stresses, such as insect herbivory (Noctor et al., 2012). Oxidative stress, such as increased H_2O_2 levels, may result in either an increase in the levels of total glutathione (glutathione pool) or increased levels of GSSG relative to GSH (redox balance) (Noctor et al. 2012). Total ascorbate levels were within the reported physiological range and did not change over the 45 minute time course and was independent of treatment (Fig. 3.1A, Appendix 3.1) (Queval and Noctor, 2007). Oxidized and reduced ascorbate levels and the ratio of oxidized ascorbate/dehydroascorbate did not change in response to caterpillar herbivory. Total glutathione levels were within the expected physiological range and affected by treatment (Fig. 3.1B, Appendix 3.1) (Queval and Noctor, 2007). Caterpillar herbivory did not affect the oxidized GSSG/reduced GSH ratio but total glutathione levels are lower in plants infested with caterpillars with impaired salivary secretions compared to the control. This likely reflects the reduced glutathione levels found in this treatment. Caterpillar herbivory also had significantly lower oxidized GSSG levels at 35 min post-herbivory; this effect was not salivary-dependent.

Cellular glutathione-ascorbate metabolites levels and/or redox balance are involved in plant defense against pathogens or herbivores (Mou et al., 2003; Ball et al., 2004; Parisy et al., 2007; Schlaeppi et al., 2008; Wünsche et al., 2011; Espunya et al., 2012). The majority of experiments investigating changes in redox metabolites in response to stress (wound, herbivory, pathogens) characterize long term changes in the cellular oxidative status (Fodor et al., 1997; Mou et al., 2003; Ball et al., 2004; Mateo et al., 2006; Schlaeppi et al., 2008; Gfeller et al., 2011). In this study, we are interested in early changes in cellular antioxidant levels or redox

balance (ratio) to caterpillar herbivory that may lead to changes in gene expression. The difficulty in this short term experiment is to synchronize the initiation and intensity of insect herbivory. Lou and Baldwin (2006) and this study monitored redox metabolites within the first 45 minutes after the initiation or simulation of herbivory. Lou and Baldwin (2006) noted an increase in H_2O_2 levels 30 minutes after wounding and application of *Manduca sexta* caterpillar regurgitant on *Nicotiana attenuata* leaves. In response to biotrophic pathogens, an increase in total or reduced glutathione levels leads to reduction and activation of NPR1 (Fodor et al., 1997; Mou et al., 2003; Fobert and Després, 2005; Mateo et al., 2006); even though SA injection into leaves shows a transient oxidation of the glutathione pool. In comparison, after wounding, the GSSG/total glutathione ratio increased leading to an activation of the JA pathway (Mhamdi et al., 2010; Gfeller et al., 2011).

Cellular redox changes occur in response to mechanical damage during insect feeding. However, Noctuid caterpillar LS, that has been implicated as a stratagem to delay the induction of plant defenses, contains numerous enzymes that may affect cellular redox balance, most notably the H_2O_2-producing enzyme GOX (Musser et al., 2002; Weech et al., 2008; Eichenseer et al., 2010). Compared to controls, herbivory by caterpillars with intact salivary secretions did not affect cellular redox balance except for a transient decrease in oxidized GSSG at 35 min (Fig. 3.1B). In comparison, reduced glutathione levels were lower in leaves subject to herbivory by caterpillars with impaired salivary secretion compared to controls, indicating oxidative stress. This suggests that the production of H_2O_2 by enzymes in the caterpillar LS may act to maintain cellular GSH levels so glutathione does not act further as a signaling molecule (Szalai et al., 2009).

3.4.2 Transcript expression in response to caterpillar herbivory

To explore the link between cellular redox balance and plant responses to caterpillar LS, expression of JA-, ET- and SA-dependent gene markers were analyzed in wildtype, *pad2-1* mutants, that contain only about 20% of normal glutathione levels, and the *tga 2/5/6* triple mutant (Zhang et al., 2003; Parisy et al., 2007). Together with NPR1, TGA transcription factors are activated by a change in redox balance and responsible for SA-dependent gene expression (Després et al., 2003; Mou et al., 2003; Lindermayr et al., 2010). It must, however, be noted that the TGA transcription factors have also been shown to regulate a subset of oxylipin-dependent defensive gene expression (Mueller et al., 2008; Zander et al. 2010).

JA, SA and ET play central roles in mediating the plant's response to caterpillar herbivory (Weech et al., 2008; Diezel et al., 2009; Onkokesung et al., 2010). Pré et al. (2008) recently suggested that the transcription factors ORA59 and ERF1 act in parallel pathways to integrate these JA/ET responses. How caterpillar LS manages to manipulate these JA/ET pathways is unknown, but Weech et al. (2008) proposed that caterpillar LS requires an active SA/NPR1 pathway for this strategem. To further complicate issues, recent evidence suggests that ET potentiates SA antagonism with JA and renders it NPR1-independent (Leon-Reyes et al., 2009).

Pathogenesis-related 1 (*AtPR1*, At2g14610) is a SA-responsive, NPR1-dependent gene marker induced in response to biotrophic pathogen attack and aphid feeding (Glazebrook, 2005; Mur et al., 2006; Kusnierczyk et al., 2007; Walling, 2008). In our study, *AtPR1* gene expression was greater than 5-fold higher in plants infested by caterpillars with intact LS secretions compared to caterpillars with cauterized spinnerets and control plants, indicating that caterpillar LS secretions result in the activation of SA/NPR1-dependent gene expression (Fig. 3.2A, Appendix 3.1). Through activation of the SA pathway by effectors in their LS secretions, *S. exigua* caterpillars are believed to impair the plant's ability to fully mount a JA-dependent defense response (Weech et al., 2008). Mewis et al. (2006) also observed *AtPR1* expression in *Arabidopsis* response to herbivory by caterpillars *of P. rapae* and *S. exigua*; both these caterpillar LS glands contain redox enzymes, such as GOX (Eichenseer et al., 2010). The increase in *AtPR1* expression was alleviated in *pad2-1* and *tga 2/5/6* mutant plants, in line with previous studies showing that glutathione and the TGA transcription factors are upstream signals in *AtPR1* expression (Després et al., 2003; Mou et al., 2003; Lindermayr et al., 2010).

Expression of the gene encoding plant defensin, *AtPDF1.2b* (At2g26020), is induced by treatment of plants with JA and ET working synergistically through ORA59 (Penninckx et al., 1998; Pré et al., 2008); however, antagonism between MYC2 and ERF1 regulation of *AtPDF1.2* is proposed to reflect MYC2 regulation of *ERF1* expression (Dombrecht et al., 2007). As well, SA-dependent suppression of *AtPDF1.2* expression requires active NPR1 and TGA transcription factors (Spoel et al., 2003; Ndamukong et al., 2007; Koornneef et al., 2008). ET modulates this SA-JA antagonism; NPR1-dependent antagonism of the expression of JA-dependent genes, such as *AtPDF1.2*, becomes NPR1-independent in the presence of ET (Leon-Reyes et al., 2009).

In wildtype plants, an 18-fold increase in *AtPDF1.2* transcript expression is observed in response to herbivory by caterpillars with impaired salivary secretions compared to normal caterpillars or control plants, in agreement with previous studies that caterpillar LS suppresses JA-dependent plant defenses (Fig. 3.2B, Appendix 3.1) (Musser et al., 2002; Weech et al., 2008). In *pad2-1* and *tga2/5/6* mutants, LS-mediated restraint of *AtPDF1.2* expression is not observed, indicating that glutathione and TGA transcription factors are required for the suppression of plant induced defenses by caterpillar herbivory. In *pad2-1* mutants, a 12.5-fold increase in *AtPDF1.2* levels is seen in plants infested by caterpillars compared to controls. The lower glutathione levels in the *pad2-1* mutant may impair the activation of a pathway, such as the reduction of NPR1 and/or TGA transcription factors, which are needed for the LS-mediated suppression of plant defenses (Mou et al., 2003; Fobert and Després, 2005). A 5-fold increase in *AtPDF1.2* expression is seen in plants fed upon by caterpillars compared to controls in the *tga 2/5/6* mutant plants. However, it must be noted that TGA transcription factors also regulate the late expression (~48 hr) of a subset of JA-dependent genes, such as *AtPDF1.2* (Zander et al., 2010). Perhaps, a strong difference in gene expression between normal and cauterized caterpillars is not observed because of the requirement for TGA transcription factors, although a 5-fold increase in expression is observed in caterpillar-infested *tga 2/5/6* mutants compared to controls. These results suggest that caterpillar LS-dependent suppression of JA-mediated activation of *AtPDF1.2* gene expression is dependent on glutathione levels and, perhaps, the activation of TGA transcription factors.

In wildtype plants, results correlate with previous observations that glutathione negatively regulates *AtPDF1.2* expression (Koornneef et al., 2008); we also observed that wildtype plants infested by caterpillars with impaired salivary secretions had lower reduced glutathione compared to controls and, consequently, higher *AtPDF1.2* expression (Fig. 3.1B and 3.2B). Also, the LS-associated negative regulation of *AtPDF1.2* is alleviated in the *pad2-1* mutant. Our observation that this LS-mediated suppression of *AtPDF1.2* is lessened in the *tga 2/5/6* triple mutant supports observations that suppression of *AtPDF1.2* gene expression requires the interaction of glutaredoxin480 with TGA transcription factors (Ndamakong et al., 2007; Koornneef and Pieterse, 2008; Zander et al., 2012). ET also plays a role in modulating the mechanism of SA/NPR1 inhibition of JA-dependent responses (Leon-Reyes et al., 2009); in the presence of ET, this suppression becomes NPR1-independent. However, given the links to

glutathione and, possibly TGA transcription factors, and previous research, our data points to a LS mediated NPR1-dependent inhibition of *AtPDF1.2* gene expression (Weech et al., 2008).

Alternatively, current models propose that JA-dependent inhibition of *AtPDF1.2* expression may be mediated through the negative regulation of *ERF1* (At3g23240) by MYC2 (Dombrecht et al., 2007; Zander et al., 2010). Therefore, *ERF1* expression was measured to determine if it was mirrored by *AtPDF1.2* expression. As seen with *AtPDF1.2*, a significant increase in *Arabidopsis ERF1* transcript expression is observed in response to herbivory by caterpillars with impaired LS secretions compared to normal caterpillars or control plants (Fig. 3.2B and C, Appendix 3.1); however, this LS-mediated suppression of *ERF1* is also observed in the *pad2-1* mutants. The distinct patterns between *AtPDF1.2* and *ERF1* expression suggest LS-mediated regulation is likely not reflective of MYC2 antagonism of *ERF1*; however, they suggest that there may be LS-linked, an ET, glutathione-independent mechanism of suppression. LS-suppression of *ERF1* is alleviated in the *tga2/5/6* triple mutant. Zander et al. (2010) found that TGA transcription factors may suppress *ERF1* expression.

Hevein-like (*AtHEL*, PR4, At304720) gene expression is a marker of the ORA59 branch of the JA/ET-signaling pathways (Potter et al., 1993; Dombrecht et al., 2007; Pré et al., 2008; Verhage et al., 2011; Zarei et al., 2011). In comparison to *AtPDF1.2*, suppression of JA-linked *AtHEL* expression by the SA pathway is NPR1-independent (Ndamukong et al., 2007). In wildtype and *pad2-1* mutant plants, over a 5-fold increase in gene expression is observed in plants infested by caterpillars compared with controls (Fig. 3.2D); however, a LS effect is not observed (Appendix 3.1). These results support the argument that caterpillar LS-mediated suppression of induced plant defenses is glutathione- and NPR1-dependent. Unexpectedly, this caterpillar-mediated *AtHEL* expression was at basal levels in the *tga 2/5/6* triple mutant plants, suggesting that these transcription factors may be involved in regulation of *AtHEL* expression.

The gene encoding *lipoxygenase2* (*AtLOX2*, At3g45410) is an early expression marker of the JA-responsive MYC2 branch (Bell et al., 1995; Dombrecht et al., 2007). As has been observed previously, *AtLOX2* levels are induced 7-fold in response to insect herbivory and a LS-gland specific difference in gene expression is not observed (Fig. 3.2E, Appendix 3.1) (Weech et al., 2008). This same pattern was observed in *pad2-1* and *tga 2/5/6* mutant plants. Though regulated by MYC2, the strong upregulation of this early gene occurs before SA/NPR-1

mediated crosstalk (Mou et al., 2003; Spoel et al., 2003; Ndamukong et al., 2007; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Tada et al., 2008; Leon-Reyes et al., 2010). As well, LS-associated post-transcriptional modifications of LOX2 may regulate activity rather than gene expression (Thivierge et al., 2010).

The stress-associated *AtSAP6* (At3g52800) was induced in plants fed upon by caterpillars with impaired LS secretions compared to controls (Fig. 3.2F, Appendix 3.1). This difference was alleviated in the *pad2-1* and the *tga* 2/5/6 triple mutants indicating the possible involvement of glutathione and TGA transcription factors in the regulation of expression of this gene. *AtSAP6* is strongly induced in response to numerous stresses, such as wounding and herbivory by caterpillars of the specialist *P. rapae* (Reymond et al., 2004; Ströher et al., 2009); however, in response to herbivory, this transcript was induced in both the wildtype and the JA-perception impaired *coi1-1 gl1* mutant implying that JA signaling is not required for the expression of this gene.

3.5 Conclusion

Plant responses to insect herbivory are mediated through carefully regulated, complex hormone-mediated interactions. Herbivory by *S. exigua* caterpillars attenuate these JA-dependent plant defense responses; a mechanism believed to be related to LS-associated secretions (Musser et al., 2002; Weech et al., 2008). Given the presence of GOX in the LS of this caterpillar, the relationship between LS secretions and changes in cellular redox potential was investigated. Changes in cellular oxidative stress and, in particular, the GSSG/total glutathione ratio are signals for the induction of JA-dependent defenses (Gfeller et al., 2011; Szalai et al., 2009). Herbivory by caterpillars with intact salivary secretions did not affect cellular redox balance, except for a transient decrease in oxidized GSSG at 35 min (Fig. 3.1B, Fig. 3.3). In comparison, herbivory by caterpillars with impaired salivary secretions resulted in an increase in cellular oxidative status through a decrease in reduced glutathione levels. In support of this, genes, such as *AtPR1* and *AtPDF1.2*, showed LS-dependent transcript expression that was alleviated in the *pad2-1* and *tga2/5/6* triple mutant (Fig. 3.2A, B, Fig. 3.3).

Increased expression of *AtPR1* by herbivory using caterpillars with intact salivary secretions supports the notion that LS-mediated attenuation of JA responses acts through crosstalk with the SA/NPR1 pathway (Fig. 3.3). LS-associated modulation by the ET pathway is

less likely since *ERF1* expression and *AtPDF1.2* expression in the *pad2-1* mutant are disparate and LS-dependent changes are not observed in the expression of the ORA59 marker *AtHEL*. However, there is some evidence in our results that a LS-mediated ET, glutathione-independent pathway leading to JA suppression may exist. *P. rapae* caterpillar oral secretions, which are a mixture of gut-derived regurgitant, secretions from the ventral eversible gland and salivary secretions from the mandibular and labial glands, activate the ORA59 branch of the JA/ET pathway leading to the suppression of MYC2-dependent defenses (Felton, 2008; Hogenhout and Bos, 2011; Verhage et al., 2011; Zebelo and Maffei, 2012). These caterpillars also show a feeding preference for plants that overexpress ORA59. Taken together, these data support a model where caterpillar herbivores utilize multiple strategies to interfere with JA-dependent responses.

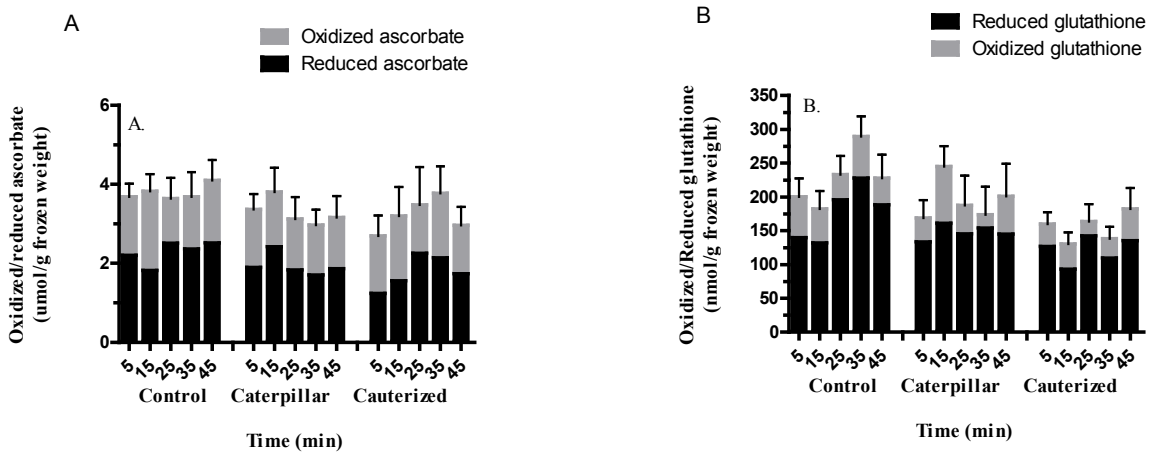

Figure 3.1: Time course of redox metabolites in *Arabidopsis* plants subjected to herbivory by caterpillars with normal (caterpillar) or impaired salivary secretion (cauterized). A) Foliar ascorbate levels. Solid bars represent reduced ascorbate (ASc) and open bars represent oxidized ascorbate (DHA). Bars represent means of 3-4 independent samples ± SE. Significant differences in level of ascorbate was not observed in response to caterpillar heribivory. B) Foliar levels of glutathione. Solid bars represent reduced glutathione (GSH) and open bars represent GSH equivalent of oxidized glutathione (GSSG). Bars represent means of 3-4 independent samples ± SE. Significant differences were determined by two-way ANOVA (Appendix 3.1).

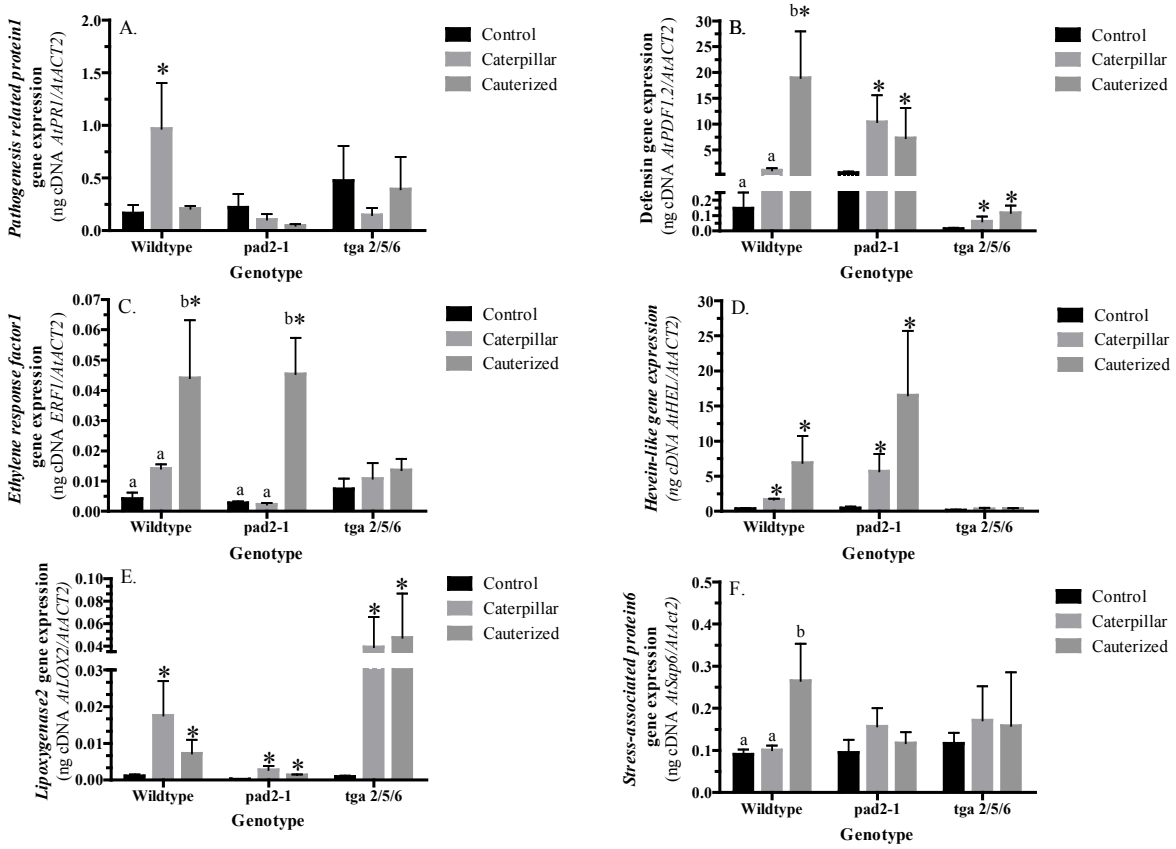

Figure 3.2: Arabidopsis transcript expression in response to caterpillar herbivory analyzed by quantitative real-time reverse transcription PCR (qRT-PCR). Seven-week-old Arabidopsis plants (Col, +/+), *pad2-1* and *tga2/5/6* mutants were subjected to herbivory by caterpillars with intact (caterpillar) and impaired (cauterized) labial salivary secretion for 36 hrs. cDNA were generated from total RNA and gene specific primers were used to determine relative expression levels of A) *AtPR1*, B) *AtPDF1.2*, C) *ERF1*, D) *At HEL*, E) *AtLOX2*, and F) *AtSAP6*. Bars represent mean values of 3-4 independent biological repliactes normalized with the reference gene *AtACT2* ± SE. Different letters above bars indicate significant difference between treatments identified by ANOVA followed by Tukey HSD test at $p \leq 0.05$ (Appendix 3.1). An asterisk denotes a fivefold or higher gene expression from control levels.

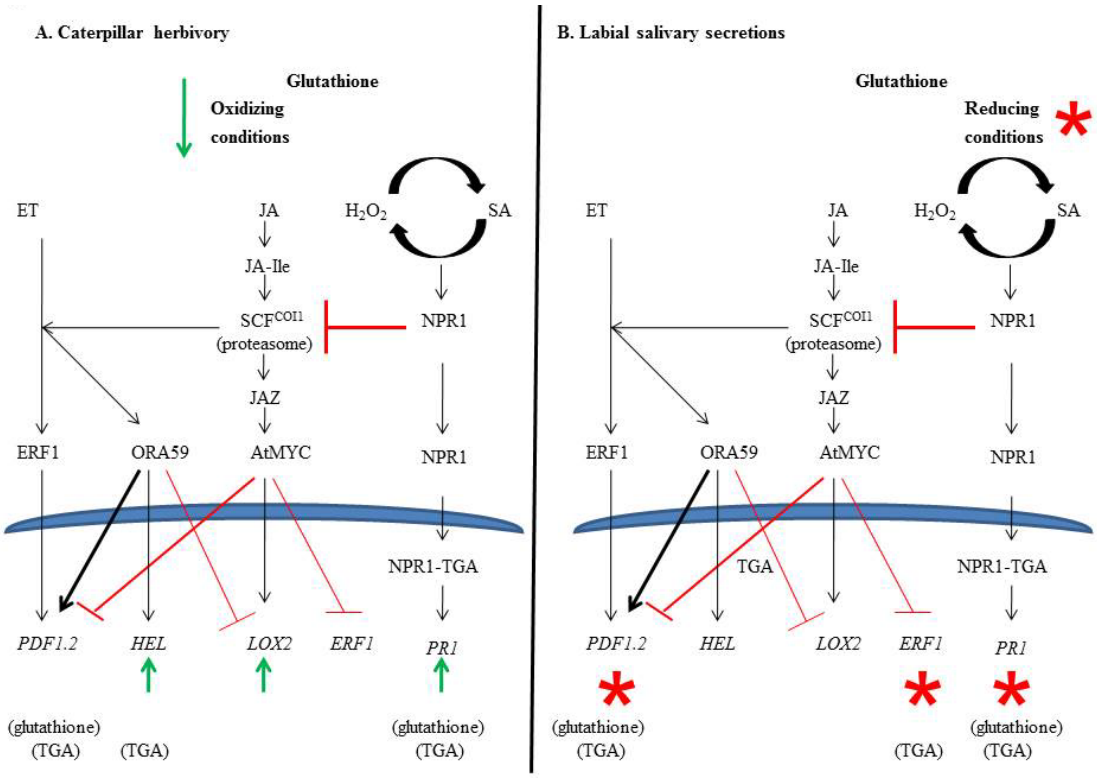

Figure 3.3. Model of ethylene-, jasmonate-, and salicylate-dependent pathway illustrating major cross-talk signaling nodes and marker genes. A) Changes in redox metabolites and gene expression in response to caterpillar herbivory. Illustrates caterpillar herbivory-dependent changes compared to control plants. Cellular GSSG, which is linked to the induction of JA-defenses, decreases transiently 35 min after caterpillar herbivory. Markers of SA, ORA59 and MtMYC2 pathway, respectively *AtPR1*, *AtHEL* and *AtLOX2*, are induced in response to herbivory. B) Proposed model for caterpillar labial saliva (LS) mediated suppression of jasmonate-dependent responses. Illustrates LS-associated changes between plants fed on by caterpillars with intact vs impaired salivary secretions. Asterisks indicate LS associated changes. Herbivory by caterpillars with impaired salivary secretions result in oxidative stress (lower total and reduced cellular GSH levels) compared to controls. Induction of *AtPR1* and suppression of *AtPDF1.2* and *ERF1* are LS dependent. Involvement of glutathione and TGA TFs are indicated in brackets underneath the marker gene.

Table 3.1. Primers used in quantitative real-time polymerase chain reaction (qRT-PCR).

Gene/ Accession number	Annealing temperature (°C)	Forward (5'-3')	Reverse (5'-3)	Ref.
ERF1 At3g23240	62	GAC GGA GAA TGA CCA ATA AGA AG	CCC AAA TCC TCA AAG ACA ACT AC	Swarup *et al*. (2007)
AtHEL At304720	57	CAA GTG TTT AAG GGT GAA GA	CGG TGT CTA TTT GAT TGA AC	Conn *et al*. (2008)
AtLOX2 At3g45410	57	GTC CTA CTT GCC TTC CCA AAC	ATT GTC AGG GTC ACC AAC ATC	Weech *et al*. (2008)
AtPDF1.2b At2g26020	59	CGG CAA TGG TGG AAG CA	CAT GCA TTA CTG TTT CCG CAA	Jirage *et al*. (2001)
AtPR1 At2g14610	62	CAC TAC ACT CAA GTT GTT TGG A	CAT GCA TTA CTG TTT CCG CAA A	Primer3
AtSAP6 At3g52800	63	TCA ACG CAT CGA ACG GCT CTG A	GCG AAA GCG AAT CCG TTG GTG AAA	Primer3
AtACT2 At3g18780		ACC AGC TCT TCC ATC GAG AA	GAA CCA CCG ATC CAG ACA CT	Dufresne *et al*. (2008)

Connecting Statement to Chapter 4

In Chapter 3, we showed that effectors in the labial saliva of *S. exigua* caterpillar helped maintain a reduced cellular environment as an early response to caterpillar herbivory in *A. thaliana*. Labial saliva could mediate the suppression of JA-mediated IR by activating the antagonisitic SA/NPR1 pathway and this antagonism could be glutathione-dependent or -independent. ET may also be involved in this mechanism. Leon-Reyes et al. (2009) have shown that ET signaling is important in the mediation of SA-JA crosstalk. In Chapter 4, the study is designed to understand the role of ET in plant-insect interactions. In this study, the response of *M. truncatula* (Jemalong A17) to *S. exigua* caterpillar herbivory was compared to the responses of the ET-insensitive (*skl*) mutant that is not responsive to ET due to mutation of *MtSkl*, the ortholog of Arabidopsis *EIN2* (Penmetsa et al., 2008). Early response of these plants to caterpillar feeding is evaluated by measuring ascorbate and glutathione levels. Changes in defense hormones, expression of defense-related genes, and levels and activity of defense-related proteins in the host plant upon caterpillar feeding were analyzed.

I designed the experiment, conducted the laboratory work, analyzed the data, and prepared the manuscript. Hormones were analyzed at the Danforth Plant Science Center (Missouri, USA). Dr. Bede provided supervision, funded the project and corrected the manuscript. This chapter will be submitted to the journal of "Molecular Plant-Microbe Interactions".

CHAPTER 4. Role of Ethylene in Herbivore-Induced Defense Responses in the Model Legume, *Medicago truncatula*

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4.1. Abstract

In response to caterpillar herbivory, changes in cellular oxidative state results in a jasmonate burst leading to plant defense responses; however, the oral secretions of some caterpillar species modulate this induced resistance. In particular, the labial saliva of generalist noctuid caterpillars contain effector(s) that manipulate plant defense responses by activating signaling pathways that attenuate the jasmonate-dependent defense-related responses; however, the exact mechanism has yet to be elucidated. A potential candidate involved in this cross-talk is the ethylene pathway. In this study, we compared the biochemical and molecular responses of the model legume *Medicago truncatula* and an ethylene-insensitive mutant, *skl*, to herbivory by 4th instar *Spodoptera exigua* (Hübner) caterpillars with intact or impaired labial salivary secretions. Within 45 minutes after herbivory, cellular oxidative stress levels increase, as evidenced by changes in the ratios of oxidized-to reduced ascorbate and glutathione. In the *skl* mutant, the labial saliva-specific increase in ascorbate ratio is not observed, suggesting that ethylene perception is required. Ten hr post-herbivory, gene markers of the jasmonate and jasmonate/ethylene pathways are differentially expressed; *MtVSP* is induced and *MtHEL* repressed in a caterpillar labial saliva- and ethylene-independent manner. In contrast, expression of a classic marker of the systemic acquired resistance pathway, *MtPR1*, is caterpillar labial saliva-dependent and requires ethylene perception. Caterpillar labial saliva also suppresses the induction of jasmonate-related trypsin inhibitor activity in an ethylene-dependent manner. Together, these findings suggest that caterpillar labial saliva activates the systemic acquired resistance pathway to interfere with jasmonate-dependent induced resistance; as well, ethylene modulates the crosstalk between these pathways.

4.2. Introduction

Although the jasmonate (JA)-mediated signaling pathway primarily governs plant induced resistance (IR) in response to chewing insect herbivores, ethylene (ET)-dependent signaling

cascades modulate this response in either a positive or negative manner (Glazebrook, 2005). ET-induced by oral secretions (OS) of the tobacco hornworm *Manduca sexta* suppress nicotine biosynthesis in *Nicotiana attenuata* (Voelckel et al., 2001); therefore, some insect herbivore species have evolved mechanism(s) to counteract plant IR by exploiting this hormonal crosstalk (Felton and Korth, 2000). Effector(s) in insect saliva may delay or prevent the plant from mounting a full JA-mediated response (Musser et al., 2006; Walling, 2008; Weech et al., 2008; Diezel et al., 2009; Eichenseer et al., 2010; Paudel et al., 2013); this is believed to be a strategy evolved by some generalist Noctuid caterpillar species to circumvent plant defenses.

As caterpillars feed, plant tissues are macerated and mandibular and labial saliva released from the caterpillar onto the wounded tissues. Caterpillar herbivory leads to the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), around the wound site (Orozco-Cárdenas et al., 2001; Maffei et al., 2006; Arimura et al., 2011; Maffei et al., 2012). At low, controlled levels, H_2O_2 is an important second messenger that leads to the activation of JA-mediated signaling pathways (Forman et al., 2010; Arimura et al., 2011). However, at higher levels, H_2O_2 is detrimental to cellular function as it can lead to lipid peroxidation and protein oxidation as well as reacting with nucleic acids (Moller et al., 2007). Thus, the induction and activation of enzymes, such as peroxidases and catalases, are critical to detoxify ROS (Maffei et al., 2006; Quan et al., 2008). Also, to balance the cellular redox levels, the Halliwell-Asada (ascorbate/glutathione) cycle is activated in plant cells (Quan et al., 2008; Noctor et al., 2012). In this cycle, cellular H_2O_2 level is lowered due to series of oxido-reductive reactions that involve ascorbate and glutathione; ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 to water and the subsequent oxidation of ascorbate (ASC) to dehydroascorbate (DHA, via the intermediate monodehydroascorbate (MDHA)). Dehydroascorbate reductase (DHAR) reduces DHA back to ASC using GSH to generate oxidized glutathione (GSSG). This last metabolite is reduced back to GSH by glutathione reductase (GR) producing NADPH from $NADP^+$ (Foyer and Noctor, 2011). Besides maintaining cellular redox homeostasis, glutathione is also involved in defense signaling cascades directly or as a result of protein post-translational modifications, such as glutathionylation or *S*-nitrosylation (Spoel and Loake, 2011; Han et al., 2013).

Therefore, in response to biotic stresses, changes in oxidative stress are observed either by increases in the cellular redox metabolic pool or by shifting the ratio of oxidized-to-reduced redox metabolites (Noctor et al., 2012). Total glutathione levels often increase in plants infected

by pathogens or by salicylic acid (SA) treatment leading to the activation of the systemic acquired resistance (SAR) pathway (Mou et al., 2003; Mateo et al., 2006). The *Arabidopisis* mutant, *mpk4*, accumulates high SA levels due to mutation in the gene encoding mitogen activated protein kinase 4 (MPK4), a negative regulator of SA biosynthesis (Petersen et al., 2000; Mateo et al., 2006); this mutant has higher constitutive glutathione levels and is more resistant to pathogen infection. On the other hand, plants compromised in their ability to synthesize or accumulate glutathione are more susceptible to pathogens and insect herbivores (Ball et al., 2004; Parisy et al., 2007; Schlaeppi et al., 2008; Maughan et al., 2010). For example, the *Arabidopsis pad2*.1 mutant, that accumulates one-fifth of wild type glutathione levels due to mutation in the gene encoding ϒ-glutamylcysteine-synthatase (ϒ-ECS), the enzyme that catalyzes the first step of glutathione biosynthesis, is more vulnerable to herbivory by caterpillars of the Egyptian cotton leafworm, *Spodoptera littoralis* (Parisy et al., 2007; Schlaeppi et al., 2008). Apart from changes in the total glutathione pool, a shift in the ratio of oxidized-to-reduced glutathione (GSSG/GSH) is also important in stress responses (Noctor et al., 2012). An increased ratio of oxidized-to-total glutathione and, in particular, an increase in GSSG levels is correlated with the activation of the JA pathway (Mhamdi et al., 2010).

It was unexpected, therefore, that in Arabidopsis, foliar herbivory by caterpillars of the beet armyworm, *Spodoptera exigua*, did not affect cellular total glutathione levels or the oxidized-to-reduced ratio within the first 45 minutes (Paudel et al., 2013). As well, at 35 min., GSSG levels are reduced. In contrast, if caterpillar labial saliva secretion is impaired, the total glutathione pool is lower compared to control plants or plants infested by caterpillars with normal labial salivary secretions. This suggests that caterpillar labial saliva plays a role in modulating plant cellular oxidative state in response to insect feeding. In fact, generalist noctuid caterpillars, such as *S. exigua*, have abundant oxidoreductases in their labial saliva, such as glucose oxidase (GOX) that oxidizes glucose to produce hydrogen peroxide (H_2O_2) and gluconate, that may affect cellular ROS levels (Musser et al., 2002; Bede et al., 2006; Eichenseer et al., 2010). For example, mechanical damage of plant leaves generates a H_2O_2 zone at the wound site (Maffei et al., 2006); however, this area is significantly increased when the damage results from caterpillar feeding. Therefore, caterpillar labial salivary enzymes, such as GOX, may affect H_2O_2 produced at the wound site that then affects the cellular oxidative state and downstream responses. In fact, evidence suggests that the labial saliva of some generalist noctuid

caterpillar species may suppress the plant's ability to mount an effective IR response (Musser et al., 2002; Bede et al., 2006; Weech et al., 2008; Paudel et al., 2013; Lan et al., 2014). Tobacco plants wounded by caterpillars of the corn earworm, *Helicoverpa zea*, with impaired labial salivary secretions have greater induced nicotine levels than if infested by normal caterpillars suggesting that these noctuids have effector(s) in their labial saliva that prevent the plant from mounting effective induced defense responses (Musser et al., 2002).

Plant IR in response to caterpillar herbivory is primarily mediated by the jasmonate-dependent pathway; however, changes in cellular oxidative state may lead to the activation of signaling pathways that stimulate or attenuate the JA signaling pathway. For example, an increase in reduced glutathione levels leads to the activation of the nonexpressor of pathogenesis-related protein1 (NPR1) in the systemic acquired resistance (SAR) pathway that leads to the attenuation of the JA-dependent IR (Spoel et al., 2003; Ndamukong et al., 2007; Koornneef et al., 2008b). Increased H_2O_2 also leads to the induction of ET biosynthesis genes (Chamnongpol et al., 1998; Vandenabeele et al., 2003). As well, enhanced glutathione levels in *NtGp* lines of tobacco overexpressing the ϒ-ECS gene leads to the accumulation of 1-aminocyclopropane-1-carboxylate (ACC), an immediate precursor of ET (Xu et al., 2008; Ghanta et al., 2014). Thus, glutathione is involved in activating the ET-pathway.

Changes in plant cellular oxidative stress may also lead to the activation of the JA pathway (Mhamdi et al., 2010). JA is converted to the bioactive form, (+)-*7*-iso-jasmonyl-L-isoleucine (JA-Ile), that acts to bring together the E3-ubiquitin ligase protein, SCF^{COI1}, and JA-zim domain (JAZ) proteins that are in complex with MYC2/3/4 transcription factors (Xu et al., 2002; Lorenzo et al., 2004; Staswick and Tiryaki, 2004; Chini et al., 2007; Dombrecht et al., 2007; Thines et al., 2007; Yan et al., 2009; Pauwels et al., 2010; Cheng et al., 2011; Fernandez-Calvo et al., 2011). Degradation of the JAZ proteins through the ubiquitin-proteasome pathway releases the MYC transcription factors leading to JA-dependent gene expression (Kazan and Manners, 2008). Thus, the JA-mediated IR becomes active, resulting in the induced expression of JA-related defense genes, such as polyphenol oxidases (*PPO*). These enzymes reduce dietary nutritional quality by catalyzing the formation of quinones from leaf polyphenolics (Constabel and Barbehenn, 2008); these highly reactive compounds bind to proteins preventing their absorption by the caterpillar gut (Felton, 2005). Other JA-dependent defenses include proteinase

inhibitors (PIs) that inhibit dietary proteases, such as trypsin, in the insect gut, thus, limiting the availability of amino acid nutrients needed for insect growth (Zhu-Salzman et al., 2008).

In the JA-dependent signaling pathway, input from other phytohormones modifies downstream signal cascades to shape the plant's final response. For example, the phytohormone ET can play an important role in JA-induced IR, either enhancing or attenuating plant defense (Pieterse et al., 2009). ET is a small gaseous molecule able to diffuse across plant cellular membranes. Binding of ET to the heterodimeric receptor on the endoplasmic reticulum formed by the interaction between ethylene resistant1 (ETR1) and ethylene response sensor1 (ERS1) inactivates the negative regulatory protein constitutive triple response1 (CTR1) (Hall et al., 2000; Cancel and Larsen, 2002; Chen et al., 2002; Wang et al., 2002). Therefore, in the absence of ET, CTR1 phosphorylates ethylene-insensitive 2 (EIN2) preventing it from entering the nucleus (Ju et al., 2012). However, upon CTR1 inactivation, EIN2 is released and the mitogen activated protein kinase (MAPK) cascade MKK9-MPK3/6 is activated leading to the stabilization of EIN3 and EIN3-like protein1 (EIL1) in the nucleus (Fujimoto et al., 2000; Yoo et al., 2009). Associated with the nuclear envelope, EIN2 is a putative metal ion transporter that transports Cu^{2+} needed for EIN3 activation (Alonso et al., 1999). EIN3 and EIL1 function downstream of EIN2 and regulate downstream transcription factors, such as ethylene response factor1 (ERF1) and ERF2, that bind to the GCC-box consensus sequence element in the promoter region of ET-dependent genes (Fujimoto et al., 2000; Yoo et al., 2009; Yoo et al., 2008).

ERF1and another ET-dependent transcription factor, the octadecanoid-responsive *Arabidopsis* AP2/ERF protein (ORA59) form the two branches of the ET-signaling pathway and integrate ET-responses with the JA pathway (Lorenzo et al., 2003; Pré et al., 2008). Caterpillar herbivory generally activates the MYC branch of the JA pathway; however, ET signals from herbivore feeding are integrated into these ERF1/ORA59 branches to activate or attenuate the MYC pathway (Spoel et al., 2003; Lorenzo et al., 2004; Koornneef and Pieterse, 2008a; Leon-Reyes et al., 2009; Pieterse et al., 2012; Pieterse et al., 2013). For example, ET has a positive effect on JA-dependent proteinase inhibitor activity in tomato but attenuates JA-induced nicotine biosynthesis in tobacco (Kahl et al., 2000; Winz and Baldwin, 2001). Attenuation of nicotine biosynthesis in tobacco plants infested by *M. sexta* caterpillars is caused by an ET burst linked to caterpillar oral secretions (Voelckel et al., 2001; Diezel et al., 2009).

There are multiple putative signaling nodes between the JA- and ET-pathways mediated by the MYC and ERF1 or ORA59 branches, respectively (Zhu et al., 2011). JAZ proteins repress the transcription factors MYC and EIN3/EIL; MYC directly and EIN3 and EIL through recruitment of the co-repressor histone deacetylase (Zhu et al., 2011). Therefore, JAZ protein degradation in response to the jasmonate burst leads to the proliferation of these branches (Pieterse et al., 2012). However, MYC2 represses *EIN3/EIL* expression (Pré et al., 2008; Leon-Reyes et al., 2009; Pieterse et al., 2012). Caterpillar oral secretions activate the ET-responsive pathways to antagonize the JA-dependent pathways; however, plants prioritize the JA-pathways to antagonize ET-dependent pathways making the plant less attractive to the herbivore (Verhage et al., 2011).

In this study, we investigated the potential role of ET in caterpillar labial saliva-mediated suppression of plant IR. Oxidative stress, hormone levels and gene and protein markers of JA-dependent IR were compared in wildtype *Medicago truncatula* and the ET-insensitive *skl* mutant. ET-insensitivity in *skl* mutant results from a mutation in *MtSkl1*, an ortholog of the Arabidiosis *EIN2* (Penmetsa and Cook, 1997; Penmetsa et al., 2008). The legume *M. truncatula* may develop a symbiotic relationship with rhizobia that assist the plant by fixing atmospheric nitrogen (Oldroyd and Downie, 2008); this unique symbiosis may lead to hormonal interactions that are distinct from other plant species, such as Arabidopsis (Anderson and Singh, 2011). Plants were infested by 4th instar caterpillars of *S. exigua*, a generalist pest species that has high levels of GOX associated with its labial saliva (Merkx-Jacques and Bede, 2005). To understand the potential role of labial saliva in these interactions, plant responses to caterpillars with normal versus impaired labial salivary secretions (cauterized) were compared.

4.3. Materials and Methods

4.3.1. Chemicals

All chemicals were purchased from Sigma Chemical Co. unless otherwise noted.

4.3.2. Plant growth conditions

M. truncatula cv. Jemalong A17 seeds were scarified in concentrated sulfuric acid (12 min) and thoroughly washed with sterile distilled water. Two genotypes were planted; wild type and the ET-insensitive *skl* mutant. After stratification at 4 °C for two days followed by two days at room temperature (RT), seedlings were sown in pasteurized Agro mix (Fafard). Plants were kept in

growth cabinets (light intensity 260 $\mu Em^{-2}s^{-1}$, 16:8 hours light:dark, 22 °C) and watered twice weekly with dilute 20:20:20 NPK fertilizer (1.05 g NPK/ 7 L H_2O). At three weeks, seedlings were transplanted to one liter pots.

4.3.3. Caterpillar growth conditions

Caterpillars of the beet armyworm, *S. exigua*, were reared on a wheat germ-based artificial diet (Bio-Serv). The colony was maintained in growth cabinets (28 °C, 60% RH, 16:8 light:dark). Fourth instar caterpillars were used in the experiments. Caterpillars were fed on *M. truncatula* cv. jemalong (wild type "feeder" plants) for 24 hours before being transferred to experimental plants.

4.3.4. Ablation of caterpillar spinneret (cauterization)

To evaluate the role of caterpillar labial saliva in the modulation of plant IR, two populations of caterpillars were used; one with normal (mock) and the other with impaired (cauterized) labial salivary secretions. In the cauterized caterpillars, the spinneret was burnt shut with a hot probe to prevent the secretion of labial saliva (Musser et al., 2006). Since GOX is a key enzyme in the caterpillar labial saliva, a horseradish peroxidase (Hrp)-*o*-diaminobenzidine (DAB)-coupled reaction, that detects GOX activity, was performed to evaluate the success of the cauterization. Caterpillars, both mock and cauterized, feed on glass fiber discs saturated with a glucose:sucrose solution (1:1; 50 mg/ml each). After visible feeding, *o*-diaminobenzidine tetrahydrochloride and Hrp (2.5 U) were added to the disc. The presence of GOX is indicated by a brown precipitation.

4.3.5. Sample collection

Five to six week old *M. truncatula* (wild type or *skl* mutant) plants received one of the following three treatments: either caterpillars with normal salivary secretions or cauterized caterpillars or remained untouched (control). Treatments were performed at night (*i.e.* one hour after light are off in the growth cabinets) since these caterpillars exhibit a predominantly nocturnal feeding behaviour. For herbivore treatments, three 4^{th} instar caterpillars were introduced per plant. To contain the caterpillars, a modified transparent bottle was fitted to the pot for all the plants one week before the experiment with netting used to cover the open tops. For the redox metabolite analysis, leaf samples were collected at 15 min intervals within the first 45 min following herbivory. For hormone analysis, whole plants cut at the crown were obtained after 10 hour of herbivory. Caterpillar-fed leaves were collected for gene expression and defense protein analysis

at 10 and 34 hours after herbivory, respectively. Samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. For ascorbate and glutathione analysis, samples were collected from three independent plants and the experiment was repeated three times to generate nine biological replicates. Samples were collected from two to three independent plants and the experiment was repeated three to five times to generate at least ten biological replicates for analysis of phytohormones and defense related proteins or five biological replicates for gene expression studies.

4.3.6. Analysis of redox metabolites: ascorbate and glutathione

Ascorbate and glutathione content of the leaf samples was analyzed following the protocol described in (Queval and Noctor, 2007). Frozen leaf samples were ground in liquid nitrogen and extracted in acidic medium (0.2 N HCl) followed by neutralization with 0.2 N NaOH. Spectrophotometric analyses of total, oxidized and reduced forms of ascorbate and glutathione were performed in a 96-well plate format using the Tecan Infinite M200 Pro microplate reader as described below.

Ascorbate content

In triplicate, samples were measured at 265 nm to determine levels of reduced ascorbate (ASC). Samples were then incubated with ascorbate oxidase (AO, 0.2 U) in 0.1 M sodium phosphate buffer (NaH_2PO_4), pH 5.6 for 8 min at room temperature (RT). The difference in A_{265} was used to calculate the level of reduced ASC in the sample (Queval and Noctor, 2007). To determine total ascorbate levels, samples were incubated in 1 mM dithiothreitol (DTT) in 67.2 nM sodium phosphate buffer, pH 7.5 for 30 min at RT. DTT reduces dehydroascorbate to ASC. An ASC standard curve (ranging from 40-240 μM) and blanks were included in each plate.

Glutathione content

Glutathione content was measured by the recycling assay method described in Rahman et al., (2006) and Queval and Noctor (2007). For total glutathione (GSH and GSSG), sample extracts were incubated with 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB, 0.6 mM) and 0.1 M sodium phosphate buffer, pH 7.5. GSH reacts with DTNB to produce GS-TNB that then converts into TNB (5-thio-2-nitrobenzoic acid) that can be measured at 412 nm. After the addition of glutathione reductase (GR, 0.015 U) and NADPH (0.75 mM), both GSSG and GS-TNB are converted to GSH. TNB levels were measured at A_{412} every 5 s intervals for 2 minutes. The

slope of the first 90 s was used to calculate GSH concentration using a standard curve of free GSH (ranging from 1-50 μM).

To measure GSSG, the neutralized sample extract was incubated with 1 μl of 2-vinylpyridine (VPD) for 30 minutes at RT. VPD precipitates GSH that is then removed by centrifugation at 13,000 rpm for 5 minutes. The VPD-treated extract was diluted with 0.1 M sodium phosphate buffer, pH 7.5 and the concentration of GSSG was measured by the recycling assay as described above. A standard curve generated using free GSSG (ranging from 0.1-2.5 μM) was used to calculate GSSG concentration. Reduced GSH in the sample was calculated by subtracting 2 x GSSG from the total glutathione.

4.3.7. Gene expression

RNA extraction and reverse transcription

Total RNA was extracted from samples using the RNeasy kit (Qiagen) following the manufacturer's instructions. After determining RNA concentration and quality (260/280 of ~2), cDNA was generated using a QuantiTect reverse transcription kit (Qiagen). Before reverse transcription, genomic DNA in the samples was degraded using DNase included in the kit. The absence of genomic DNA contamination was confirmed using the primers (5'-CTCTCCTGCATTTCCACTTTC -3' and 5'- TTCTTGACCCTACCAAACATCA -3') that amplify an introgenic region by PCR (Darwish et al., 2008). As a positive control, genomic DNA was used as template. cDNA samples were diluted 1:10 and transcript abundance was measured by quantitative real time-polymerase chain reaction (qRT-PCR).

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

Gene expression was analyzed by SYBR green-based qRT-PCR using the MX3000p thermo-cycler (Stratagene). Reactions contained 1 x SYBR green with low ROX (Absolute Blue, Thermo Scientific), cDNA and 1 μM of forward and reverse primers. Thermocycler conditions were as follows: 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s (55 °C - 60 °C for the different gene-of-interest (GOI), and elongation at 70 °C for 30 s. Primers for the GOI and the reference gene, *MtGADPH*, are listed in Table 4.1. Five biological replicates (n = 5) for each treatment from the two genotypes were analyzed in an opaque white 96-well plate. Samples were spotted in duplicate and non-template controls were included in each plate. Two technical plate replicates were analyzed.

The relative expression ratio of the target gene *versus* the reference gene was calculated as $R0_{GOI}/R0_{REF}$, where R0 is the initial template concentration in each reaction. R0 was calculated using the formula $R0=1/(1+E)^{Ct}$, were E is the average efficiency of gene in the exponential phase and Ct is the threshold cycle (Zhao and Fernald, 2005).

4.3.8. Hormone analysis

JA, JA-Ile, 12-oxo-phytodienoic acid (OPDA), SA, and abscisic acid (ABA) levels were analyzed at the Danforth Plant Science Center (Missouri, USA) by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were ground using a TissueLyser for 2 min at a frequency of 15 Hz s^{-1}. Hormones were extracted from lyophilized leaf samples in ice cold methanol:acetonitrile (MeOH:ACN, 1:1, v/v). Samples were spiked with deuterium-labelled internal standards of SA (D^5-SA), ABA (D^6-ABA), and JA (D^2-JA). Supernatants were collected after centrifugation at 16,000 g and extraction of pellets repeated. Pooled supernatants were evaporated using a speedvac before re-dissolving the pellets in 30% MeOH for hormone analysis. Hormones separation was accomplished on a monolithic C_{18} column (Onyx, 4.6 mm × 100 mm, Phenomenex) using a binary solvent of 0.1% acetic acid in HPLC-grade water (v/v) (solvent A) and 90% ACN with 0.1% acetic acid (v/v) (solvent B) with a flow rate of 1 ml min^{-1}. After 2 minutes of 40% solvent A, a gradient to 100% solvent B was accomplished over the next 5 minutes and then held at 100% solvent B for 2 minutes. Analytes were ionized in the negative mode by electron spray ionization (TurboIonSpray, TIS) and MS data was acquired by a QTRAP mass spectrometer (AB Sciex).

4.3.9. Analysis of defense metabolites

Trypsin inhibitor (TI) levels and polyphenol oxidase (PPO) activity were measured in the leaf extracts following protocols described in Weech et al. (2008). For soluble protein extraction, ice-cold extraction buffer (0.1 M sodium phosphate buffer, NaH_2PO_4, pH 7, containing 0.1% triton-X and 7% polyvinylpyrrolidone (PVP)) was added to the finely ground leaf samples. After centrifugation at 13,000 rpm for 10 min., the supernatant was transferred to a fresh tube. For the PPO assay, proteinase inhibitor cocktail (final concentration: 0.5 x) was added to the extraction buffer to inhibit protein proteolysis.

The Bradford assay was performed to calculate the total soluble protein in the sample. A standard curve was generated using bovine serum albumin (BSA) ranging from 5-100 µg.

Standards and samples were incubated with Bradford reagent (Fisher Scientific) for 10 min followed by measuring the absorbance at 590 nm (Bradford, 1976).

Trypsin inhibitor (TI) assay

Leaf soluble protein extracts were incubated with bovine trypsin (0.5 µg) at 37 °C for 20 min with constant shaking. The trypsin substrate, *N*-benzoyo-*L*-arginyl-β-nepthylamide hydrochloride (BANA, 2.6 mM), was added. Samples were incubated for an additional 80 min with constant shaking. Trypsin cleaved BANA to release β-naphthalene. After the reaction was stopped by adding HCl (0.47 %), *p*-dimethyl-amino-cinnamaldehyde (0.025%) that reacts with β-naphthalene was added and the absorbance read at 540 nm. Samples were analyzed in triplicate. As well, a standard curve of soybean trypsin inhibitor, type 1S (range 1 ng – 5 µg), negative controls with no trypsin and blanks were included on each plate.

Polyphenol oxidase (PPO) assay

Dimethyl formamide (DMF, 0.1%) and 3-methyl-2-benzothiazolinone hydrazine (MBTH, 0.2 mM) were added to triplicate soluble protein extracts. These compounds stabilize the reactive quinones that are produced by PPO. After the addition of the substrate dopamine hydrochloride (35 mM), reaction kinetics was measured at 476 nm every 10 second for 3 min. The rate for the first 90s was used for the analysis. In addition, sodium dodecyl sulphate (SDS)-treated and boiled samples (negative controls), tyrosinase (positive control) and blanks were included in each plate.

4.3.10. Statistical analysis

A 2-way analysis of variance (ANOVA) was used to analyze redox metabolites using SPSS version 20 using a statistical significance value of $p \leq 0.05$ (Appendix 4.1). Since ET may affect plant growth and development, genotypes were analyzed separately (Benavente and Alonso, 2006). When an interaction was detected (*i.e*. time x treatment), samples within each treatment were analyzed by 1-way ANOVA followed by a Tukey HSD *post-hoc* test to identify the significant effect.

Gene expression, hormone levels, TI levels and PPO activity were analyzed by 1-way ANOVA within each genotype followed by a Tukey HSD *post-hoc* test to determine statistical differences ($p \leq 0.05$) between treatments (Appendix 4.2) (Rieu and Powers, 2009).

4.4 Results and Discussion

4.4.1. Caterpillar labial saliva induces an oxidative response in *Medicago truncatula*

In the root nodules of legumes, the ascorbate-glutathione cycle is present to suppress the detrimental effects of ROS associated with nitrogen fixation (Matamoros et al., 1999; Matamoros et al., 2003). In this cycle, ascorbate and glutathione are used as substrates in a series of oxido-reductive reactions catalyzed by the enzymes APX, DHAR and GR to lower the level of cellular H_2O_2 (Foyer and Noctor, 2011). In comparison, foliar ascorbate and glutathione levels are low and variable compared to root nodules (Rellan-Alvarez et al., 2006). As cellular redox balance is important in herbivore-induced stress responses, levels of total, reduced, and oxidized forms of ascorbate and glutathione were measured in *M. truncatula* leaves after larval *S. exigua* herbivory. Caterpillars had either intact or impaired (cauterized) labial salivary secretions to determine the role of labial saliva, if any, on plant responses. In both wild type and the ET-insensitive *skl* mutant, total ascorbate does not change over the initial 45 min after herbivory (Fig. 4.1A, B, Appendix 4.1). As well the ratio of oxidized (DHA) to reduced (Asc) ascorbate does not change in control plants or in plants subject to herbivory by caterpillars with impaired labial salivary secretions over the 45 min time course. In comparison, herbivory by caterpillars with normal labial salivary secretions results in an increase in the cellular DHA/ASC ratio at 45 min (Fig. 4.1A, Appendix 4.1). This labial saliva-specific response is not seen in the *skl* mutant, suggesting that caterpillar labial saliva-associated changes in plant redox metabolites requires ET perception.

Total and reduced glutathione (GSH) levels do not change in response to caterpillar herbivory over the first 45 min (Fig. 4.1C, D, Appendix 4.1). In comparison, in response to herbivory by caterpillars with normal labial salivary secretions, a transient increase in GSSG levels and, therefore, in the GSSG/GSH ratio is observed in wounded leaves of wild type *M. truncatula* within15 to 30 min after the initiation of herbivory compared to control plants (Fig. 4.1C, Appendix 4.1). GSSG decreases to basal levels at 45 min. In contrast, GSH, GSSG or total glutathione levels do not change in the *skl* mutant in response to caterpillar feeding (Fig. 4.1D, Appendix 4.1). However, an increase in the ratio of GSSG/GSH is also observed after 15 minutes in plants infested by caterpillars with intact labial salivary secretions (Appendix 4.1).

Activation of the ascorbate-glutathione cycle in response to pathogen and herbivore attack is critical to maintain cellular redox status (Ball et al., 2004; Parisy et al., 2007; Schlaeppi et al., 2008; Espunya et al., 2012). In fact, *pad2* mutants that have lower glutathione levels are more susceptible to caterpillar herbivory highlighting the important role of the ascorbate-glutathione cycle in plant resistance (Parisy et al., 2007). Increased total and reduced glutathione in response to biotrophic pathogen infection activates NPR1, an important modulator protein of the SAR pathway (Mou et al., 2003; Mateo et al., 2006). GSH may also be involved in regulating signal transduction pathways by affecting protein post-translational modification (Spoel and Loake, 2011; Han et al., 2013). In the current study and previously, we investigated early redox response of two model plants, *M. truncatula* and *A. thaliana,* to caterpillar herbivory. In *Arabidopsis*, *S. exigua* caterpillar labial saliva helps retain a reductive cellular environment by maintaining the level of total and reduced glutathione in wounded tissues since these levels are lower during infestation by caterpillars with impaired labial salivary secretions (Paudel et al., 2013). In contrast, even though the current study shows no effect on the total level of either ascorbate or glutathione, the ratio of DHA/ASC as well as the level of GSSG and, hence, the ratio of GSSG/GSH, increases in *M. truncatula* as an early response to caterpillar feeding (Fig. 4.1A, C). These changes are not observed in plants fed upon by insects with impaired labial salivary secretions. Thus, the leguminous plant seems to perceive a signal(s) from the caterpillar labial salivary secretion that acts as an inducer of oxidative stress. These caterpillar labial saliva-dependent changes are not observed in the *skl* mutant indicating that, in *M. truncatula*, ET perception is needed for the activation of the ascorbate-glutathione cycle in response to caterpillar herbivory.

4.4.2. JA burst upon caterpillar herbivory is not affected by ethylene insensitivity

As expected, a jasmonate burst is observed in response to caterpillar herbivory (Fig. 4.2A, B, C, Appendix 4.2). Free JA and bioactive JA-Ile are strongly elevated in both genotypes of *M. truncatula*, wild type and *skl* mutants, upon *S. exigua* caterpillar feeding (Fig. 4.2A, B). Therefore, in *M. truncatula*, ET does not play a role in mediating the early jasmonate burst in response to caterpillar feeding. A labial saliva-specific difference in jasmonate levels is not observed in *M. truncatula* unlike previous observations in *Arabidopsis* (Weech et al., 2008; Lan et al., 2014). In *Arabidopsis*, labial saliva impairs the plant's ability to mount full jasmonate burst. In *M. truncatula*, caterpillar-specific induction of OPDA, a precursor in the JA pathway

and bioactive molecule, and SA is not observed (Fig. 4.2 C, SA data not shown). ABA levels also remain unchanged after feeding of caterpillars on wild type plants but are higher in *skl* mutants fed upon by caterpillars with intact labial salivary secretions (Fig. 4.2 D, Appendix 4.2). Ethylene is known to reduce ABA sensitivity for stomata closure during drought/heat stress (Tanaka et al., 2005). Exposure of plant tissues upon wounding by caterpillars results in drought stress (Tang et al., 2006). Here we show that caterpillars maintain the level of drought-related hormone ABA in wildtype plants but the level is increased in *skl* mutant in a labial saliva-specific manner (Fig. 4.2 D, Appendix 4.2).

4.4.3. Gene expression analysis

In *M. truncatula*, expression of seven genes was monitored in response to caterpillar herbivory: four of these genes were previously identified to be differentially expressed in response to *S. exigua* herbivory (*MtRCA*, *MtSTR*, *MtRPK* and *MtRFP*) (Darwish et al., 2008). *He*vein-*l*ike *protein* (*MtHEL, PR4*) is a recognized marker of the ERF1/ORA59 pathway whereas *v*egetative *s*torage *p*rotein (*MtVSP*) is a marker for the MYC branch of the pathway (Pieterse et al., 2012). Pathogenesis-related protein 1 (*MtPR1*) is a marker of the NPR1-dependent SAR pathway (Glazebrook, 2005; Pré et al., 2008; Pieterse et al., 2012).

As expected, expression of *MtRCA*, *MtSTR like* and *MtRFP* are suppressed in response to caterpillar herbivory (Fig. 4.3A, B, D, Appendix 4.2); a labial saliva-specific difference is not observed. Expression of *MtRPK* is also lower in response to insect feeding (Fig. 4.3C); this is contrary to what was observed by Darwish et al. (2008) and likely reflects differences between early (1 hr; Darwish et al., 2008) and later (10 hr, this study) gene expression. Caterpillar herbivory-dependent suppression of *MtSTR*, *MtRPK* and *MtRFP* is not seen in the *skl* mutants. For *MtSTR* and *MtRFP*, this possibly reflects their low constitutive expression in the ET-insensitive *skl* mutant and points to the possible involvement of ET in the regulation of these genes.

Expression of *MtVSP* and *MtHEL* are regulated by JA and JA/ET, respectively. Similar to the jasmonate profile (Fig. 4.2A, B, Appendix 4.2), caterpillar feeding enhanced the expression of *MtVSP* which confirmed the induction of the MYC branch of the JA-mediated defense pathway (Fig. 4.3E). A caterpillar labial saliva-dependent regulation is not observed which is in agreement with previous observation of MYC-dependent gene expression in *Arabidopsis* (Lan et

al., 2014). The same expression pattern is observed in the ET-insensitive *skl* mutant which was expected since JA but not ET plays central role in the induction of the *VSP* gene (Pré et al., 2008; Verhage et al., 2011). In contrast, *MtHEL* transcripts were suppressed in response to *S. exigua* herbivory in both plant genotypes (Fig. 4.3F). The suppression of *AtHEL* by the antagonistic SA-mediated pathway is independent of NPR1 (Ndamukong et al., 2007). Therefore, the observed suppression of *MtHEL* could be due to the antagonistic effect of the caterpillar-induced MYC branch on the AP2/ERF branch of the JA pathway (Lorenzo et al., 2004; Pieterse et al., 2012). In contrast, this antagonism mediated by the MYC branch on *AtERF1* expression is not observed in *Arabidopsis* subject to *S. exigua* caterpillar herbivory (Paudel et al., 2013). Therefore, this may represent the differential regulation between the ORA59 and ERF1 branches of the JA/ET pathway in different plant species in response to caterpillar herbivory (Verhage et al., 2011).

Labial saliva-specific induction of *MtPR1* is observed in response to caterpillar feeding. The gene encoding PR1, an important marker of SAR pathway, is induced in plants fed on by caterpillars with intact labial salivary secretions but not by cauterized caterpillars; this suggest that effector(s) in caterpillar labial saliva activate the SAR pathway (Fig. 4.3G, Appendix 4.2). In *Arabidopsis*, the labial salivary-specific induction of the *AtPR1* gene is also observed late (36 hr) but not early (10 hr) after the initiation of *S. exigua* herbivory (Paudel et al., 2013; Lan et al., 2014).

4.4.4. Defense proteins

In wild type *M. truncatula*, trypsin inhibitor (TI) is significantly higher in plants infested by caterpillars with impaired labial salivary secretions than by normal caterpillars or control plants suggesting that effector(s) in *S. exigua* caterpillar labial saliva prevents the plant from mounting full JA-mediated defense responses (Fig. 4.4A, Appendix 4.2).The labial saliva-specific suppression of TI is abolished in ET-insensitive *skl* mutant. Therefore, ET plays a role in this suppression. The activity of the defense-related protein, polyphenol oxidase (PPO), is not affected by caterpillar herbivory in either genotype (Fig. 4.4B, Appendix 4.2).

4.5. Conclusions

In addition to Ca^{2+} and membrane potential changes, one of the first plant responses to caterpillar herbivory is increased oxidative stress and changes in cellular redox balance (Maffei et al., 2006). This may manifest itself by increases in total ascorbate or glutathione levels or shifts in

the balance of oxidized-to-reduced metabolites (Noctor et al., 2012). In *M. truncatula*, total ascorbate and glutathione levels do not change in response to caterpillar herbivory. Instead, changes in the oxidized-to-reduced ascorbate and glutathione ratios occur, indicating oxidative stress are observed within the first 45 minutes after caterpillar herbivory (Fig. 4.1A, C). With ascorbate, the labial saliva-specific increase in ascorbate ratio is not observed in the *skl* mutant, suggesting that this caterpillar labial saliva-dependent difference requires ET perception (Fig. 4.1B). As well, compared to controls, a transient increase in GSSG and GSSG/GSH is observed within the first half an hour in plants infested by caterpillars with normal labial salivary secretions (Fig. 4.1C). In *Arabidopsis*, GSSG levels are positively correlated with JA-dependent responses (Mhamdi et al., 2010). However, a labial saliva-dependent change in glutathione ratio is not observed in wildtype or *skl* mutant plants (Fig. 4.1C, D).

In *M. truncatula*, caterpillar herbivory results in increases in oxidative stress within the first hour. This is in contrast with *Arabidopsis*, where caterpillar labial saliva is involved in suppressing changes in oxidative stress associated with herbivory by caterpillars with impaired labial salivary secretions (Paudel et al., 2013). These apparent contradictory results may reflect the impact of host plant nutritional quality (i.e. *Arabidopsis vs M. truncatula*) on effector(s) levels in the caterpillar labial saliva (Bede, unpublished data) or plant species-specific differences.

Later responses to caterpillar herbivory involve changes in phytohormones, gene expression and defense-related proteins. Ten hours after the initiation of herbivory, a strong jasmonate burst is observed. Unlike Arabidopsis, a labial salivary-specific difference in jasmonate phytohormones, *i.e*. jasmonic acid or JA-Ile, is not observed (Fig. 4.2A, B) (Paudel et al., 2013). As with previous studies in *Arabidopsis*, caterpillar labial saliva-related differences in SA hormone levels are not observed (Weech et al., 2008; Lan et al., 2014).

As has been reported previously, caterpillar herbivory results in the suppression of *MtRCA*, *MtSTR like*, and *MtRFP* expression (Darwish et al., 2008). *MtVSP*, a marker of the JA pathway, is induced in response to caterpillar herbivory in a labial saliva- and ET-independent manner (Fig. 4.3E). In contrast, levels of *MtHEL*, a marker of the JA/ET pathway, is lower in response to caterpillar herbivory (Fig. 4.3F). Since this pattern of expression is also labial saliva- and ET-independent, this may reflect the involvement of antagonistic phytohormone pathways,

such as the gibberellin/della pathway, in these plant-insect interactions. Of key interest is the expression pattern of the SA marker gene, *MtPR1*. As in *Arabidopsis*, caterpillar labial saliva-dependent *PR1* expression is observed in *M. truncatula* (Fig. 4.3G) (Paudel et al., 2013). This is alleviated in the *skl* mutant illustrating the potential role of ET in *MtPR1* gene expression. In *Arabidopsis*, SA-inducible *PR1* expression has been shown to be enhanced by ET (Lawton et al., 1995).

Finally, caterpillar labial saliva suppresses the induction of the important defensive protein TI in an ET-dependent manner. Together, these results support the model that caterpillar labial saliva activates the SA/NPR1 pathway to interfere with plant induced resistance and points to the importance of ET in modulating this crosstalk.

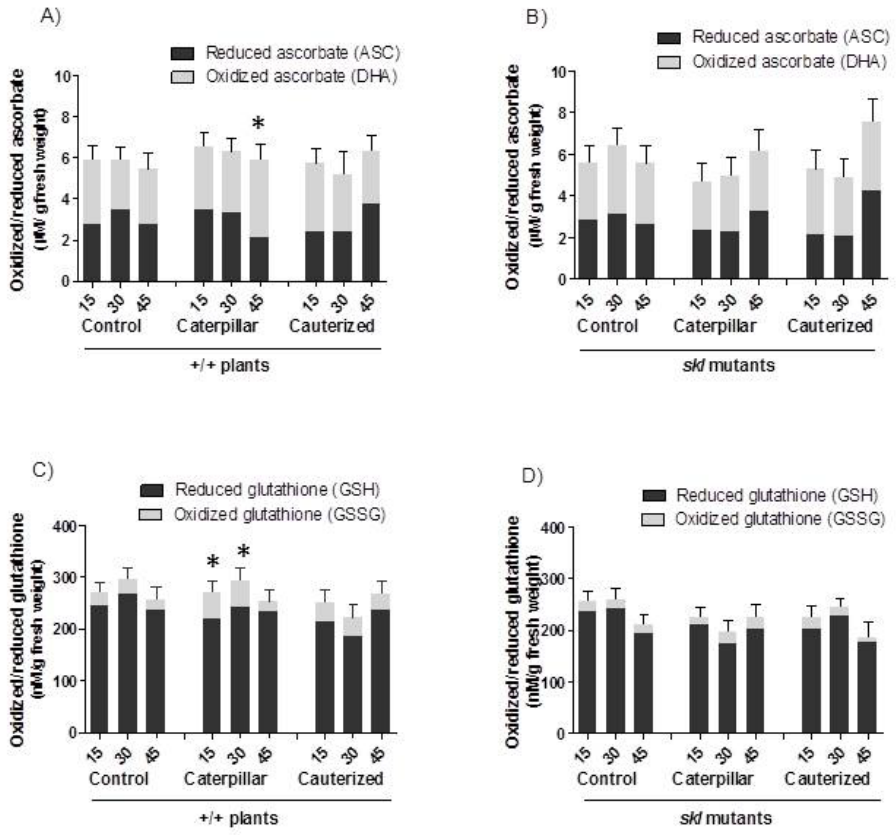

Figure 4.1. Time course of redox metabolites in *Medicago truncatula* plants subjected to *Spodoptera exigua* caterpillar herbivory. 6 week old *M. truncatula* wildtype or *skl* mutant plants were subjected to herbivory by 4th instar *S. exigua* caterpillars. To assess the involvement of caterpillar labial saliva in plant responses, two populations of caterpillars were used: those with intact (caterpillar) or impaired (cauterized) labial salivary secretion. Ascorbate levels in *M. truncatula* wildtype plants **(A)** or *skl* mutants **(B)**. Solid bars represent reduced ascorbate (ASC) and open bars represent oxidized ascorbate (DHA). At 45 min, the ratio of DHA/ASC is significantly increased in wildtype plants infested by caterpillars with intact labial salivary secretions. Glutathione levels in wild type plants **(C)** and *skl* mutants **(D).** Solid bars represent reduced glutathione (GSH) and open bars represent oxidized glutathione (GSSG). Bars represent the means of 5-8 independent biological replicates ± SE. In the wild type plants, GSSG and, thus, the ratio of GSSG/GSH is significantly increased in wild type plants subjected to caterpillar herbivory with intact salivary secretion at 15 to 30 min after herbivory compared to plants infested by caterpillar with impaired labial salivary secretions or controls. Bars represent the means of 5-8 independent biological replicates ± SE. Significant differences are represented by an asterix ($p \leq 0.05$) (Appendix 4.1).

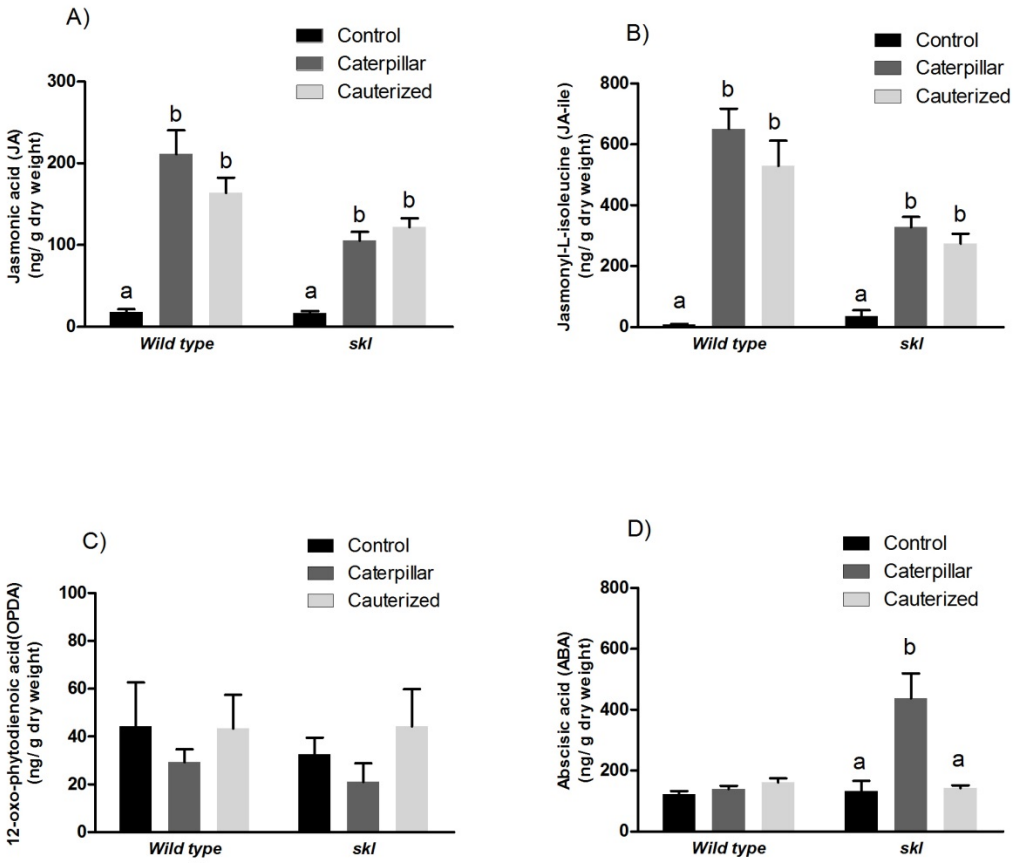

Figure 4.2. ***Medicago truncatula*** **phytohormone levels after infestation by** ***Spodoptera exigua*** **caterpillars.** 6 week old *M. truncatula* wildtype or *skl* mutant plants were subjected to herbivory by 4th instar *S. exigua* caterpillars for 10 hr. To assess the involvement of caterpillar labial saliva in plant responses, two populations of caterpillars were used: those with intact (caterpillar, dark grey bars) or impaired (catuterized, light grey bars). Aerial levels of **A)** jasmonic acid (JA), **B)** jasmonyl-isoleucine (JA-Ile), **C)** 12-*oxo*-phytodienoic acid (OPDA) and **D)** abscisic acid (ABA) were compared. Bars represent the means of three independent biological replicates ± SE. Letters indicate significant difference between treatments ($p \leq 0.05$) (Appendix 4.2).

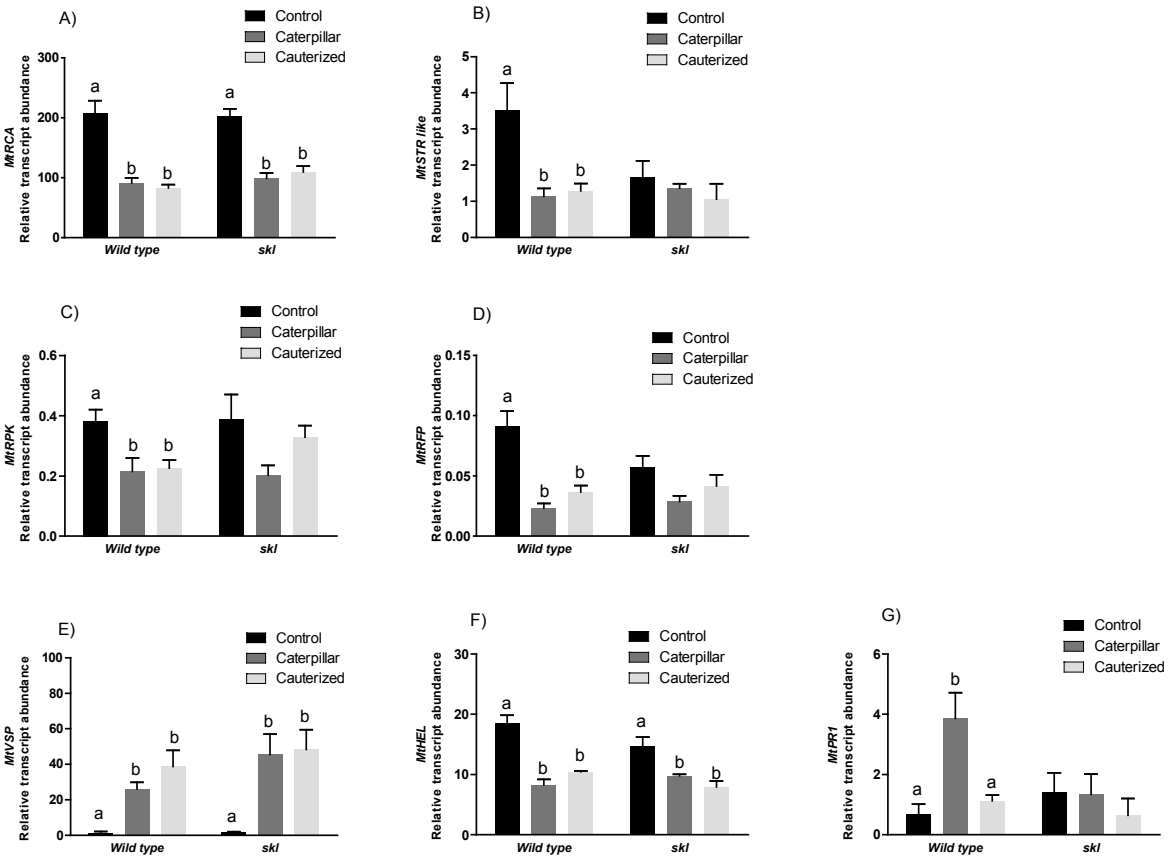

Figure: 4.3. ***Medicago truncatula*** **defense-related gene expression in response to** ***Spodoptera exigua*** **caterpillar herbivory.** 6 week old *M. truncatula* wildtype or *skl* mutant plants were subjected to herbivory by 4th instar *S. exigua* caterpillars for 10 hr. To assess the involvement of caterpillar labial saliva in plant responses, two populations of caterpillars were used: those with intact (caterpillar, dark grey bars) or impaired (catuterized, light grey bars). Relative expression of **A)** *MtRCA*, **B)** *MtSTR like*, **C)** *MtRPK*, **D)** *MtRFP*, **E)** *MtVSP*, **F)** *MtHEL*, and **G)** *MtPR1* genes were compared in caterpillar-wounded or undamaged leaves from control plants. Expression levels were normalized to the constitutively expressed *MtGADPH* gene. Bars represent the means of 4-5 independent biological replicates ± SE. Letters indicate statistical significant differences between treatments ($p \leq 0.05$) (Appendix 4.2).

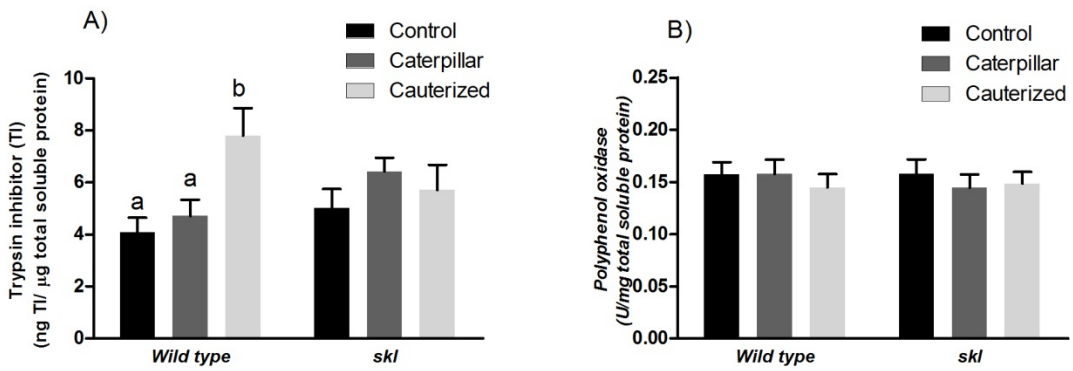

Figure 4.4. ***Medicago truncatula*** **defense-related protein levels and activity in response to** ***Spodoptera exigua*** **caterpillar herbivory.** 6 week old *M. truncatula* wildtype or *skl* mutant plants were subjected to herbivory by 4th instar *S. exigua* caterpillars for 34 hr. To assess the involvement of caterpillar labial saliva in plant responses, two populations of caterpillars were used: those with intact (caterpillar, dark grey bars) or impaired (catuterized, light grey bars). **A)** Trypsin inhibitor (TI) levels and **B)** polyphenol oxidase (PPO) activity were compared in caterpillar-wounded or undamaged leaves from control plants. Bars represent the means of 10-12 independent biological replicates ± SE. Letters indicate significant difference between treatments ($p \leq 0.05$) (Appendix 4.2).

Table 4.1. Primers used for quantitative real time-polymerase chain reaction (qRT-PCR).

Gene	Annealing temperature (°C)	Amplicon length	Forward (5'-3')	Reverse (5'-3)	Ref.
Reference Gene:					
MtGADPH	55	100	TGCCTACCGTCGATGTTTCAGT	TTGCCCTCTGATTCCTCCTTG	**Kakar et al., 2008**
Genes-of-interest:					
MtRFP (TC188467)	55	116	AGCAGCTTCAAGAGTTTGTCA	CCAGCAATCATCAAGACTTAAG	**Primer3**
MtRPK (CA919558)	58	168	CAATTCAGCCAGCACTGAAGAT	GGCCTATATTACAGGGGTGAAA	**Primer3**
MtSTR (TC144098)	55	162	GTTACTCCACGTCACTAGAGC	CATTGTGACCTATTGGAACTC	**Primer3**
MtRCA (TC168053)	55	127	CAGACAAGTATTTGGAAGGTG	GAGGCATTTGAATCAAGAAC	**Primer3**
MtHEL (TC191726)	58	114	AATGGCAAGCATGAAGGTAGCA	ATGCATGGAGCAAGAGAACCAG	**Primer3**
MtPR1 (TC148927)	58	187	TGAGGTTGTGGGTGAATGAGAA	ACATAGTTGCCTGGTGGATCGT	**Primer3**
MtVSP (TC177677)	58	221	GACCTTTGGGTGTTTGACATTGA	TCCTTCTGTTTGAGTGGTCTTCCT	**Primer3**

Connecting Statement to Chapter 5

In Chapters 3 and 4, we showed that cellular redox balances are modified in host plants less than an hour after caterpillar feeding. Caterpillar herbivory also leads to a JA burst soon after herbivory. These rapid changes in metabolic flux and modulation of responses are often mediated by post-translational modification of proteins, such as transcription factors, that can affect enzyme activity, regulation or protein-protein or protein-DNA interaction (Seo and Lee, 2004; Huber, 2007). In Chapter 5, I explored protein post-translational modification(s) in *A. thaliana* after *S. exigua* caterpillar herbivory. Since caterpillar labial saliva contains enzymes that may affect protein post-translational modifications, I determined the role of caterpillar labial saliva in these protein modifications. As well, I used an Arabidopsis quad-*della* mutant to investigate differences in protein post-translational modifications that may contribute to JA/GA crosstalk.

For this experiment, I used plant samples already available in the lab from previous experiments conducted by Zhiyi Lan. I performed the nuclear isolation and soluble protein extraction. Shortgun proteomics was performed at the Institute for Research in Immunology and Cancer (IRIC), Université de Montréal. I performed the proteomic analysis and selected proteins for further characterization. I performed the gene expression studies and prepared the manuscript together with Dr. Bede. In addition, she provided overall supervision and funding for the project (NSERC).

CHAPTER 5. Post-Translational Modifications of Arabidopsis Nuclear Proteins in response to caterpillar herbivory

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5.1 Abstract

Plant responses to caterpillar herbivory begin within minutes of the attack. Therefore, protein post-translational modifications must be involved in the signaling responses to caterpillar herbivory. In this study, we identified changes to the *Arabidopsis thaliana* (L.) Heynh. proteome, focusing on post-translational modification of nuclear transcription factors, in response to herbivory by caterpillars of the beet armyworm, *Spodoptera exigua* Hübner. As caterpillar labial saliva has been implicated in a stratagem to suppress plant induced defenses, we cauterized the spinneret of a subset of caterpillars to allow the identification of caterpillar labial saliva-specific differences. Post-translational modifications of four nuclear proteins were identified. Modification of RabH1c, including a Cys-sulfenic oxidation, was found in response to caterpillar herbivory. Cys-nitrosylation of a CAMTA/SR1-like protein was also observed in herbivore-attacked plants. In the L*er* ecotype, target genes of the transcription factor CAMTA/SR1, such as *AtNDR1* and *AtEIN3*, also showed an increased expression in response to caterpillar herbivory. The phosphorylation site of MYB109 at Thr_{184} was identified. Lastly, caterpillar labial saliva-dependent modification of the transcription factor ABF3 was identified. This protein is nitrosylated at Cys_{420} and phosphorylated at Ser_{431} in Arabidopsis subject to herbivory by caterpillars with impaired labial salivary secretions. These post-translational modifications correlated with the expression of the ABF3-marker gene At*WRKY40* that was also expressed at highest levels in these plants. Therefore, we identified the post-translational modifications of four nuclear proteins, of which three were transcription factors. Further studies are being conducted to validate the relationship between modifications of these proteins and downstream defense pathways.

5.2 Introduction

Within minutes of caterpillar attack, rapid influx of intracellular calcium and increased levels of reactive oxygen species leads to the biosynthesis of the jasmonate (JA)-related defense hormones

(Maffei et al., 2007). This JA burst occurs too rapidly to involve gene expression and, thus, must involve protein post-translational modifications, such as phosphorylation, nitrosylation *etc*., that can affect enzyme activity, regulation and protein-protein or protein-DNA interactions (Huber and Hardin, 2004; Seo and Lee, 2004; Huber, 2007; Glauser et al., 2008). These dynamic, reversible changes allow plants to rapidly modify their metabolic flux and allow the integration of information from multiple phytohormone pathways. A common protein target of post-translational modifications is transcription factors that can influence gene expression over a long time scale (Moore et al*.,* 2011).

One of the first "decisions" a plant under caterpillar attack must make is how to divert resources: into overcompensatory growth or defense (Mole, 1994). This trade-off between growth and defense is critical to the survival of the plant and often referred to as the "dilemma of plants" (Herms and Mattson, 1992); these two processes are mediated by two main phytohormone pathways, gibberellins or JA, respectively (Ballare, 2011; Davière and Achard, 2013; Erb et al., 2012). Gibberellins are tetracyclic diterpenoid compounds that regulate diverse physiological functions from seed germination to organ and fruit development (Sun, 2011; Hauvermale et al., 2012; Davière and Achard, 2013). Gibberellins function by initiating a signalling cascade that leads to the 26*S* proteosome-mediated degradation of negative growth regulator DELLA proteins, thereby, initiating growth-related pathways (Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004; Hartweck and Olszewski, 2006; Murase et al., 2008; Shimada et al., 2008). When a plant is wounded by caterpillars, activation of the JA biosynthesis pathway results in activation of JA biosynthesis and the accumulation of 7-jasmonyl-L-isoleucine (JA-Ile) (Fonseca et al., 2009). Through binding its receptor, JA-Ile promotes the degradation of jasmonate ZIM-domain (JAZ) proteins through the 26S proteasome, thereby releasing MYC2/3/4 transcription factors leading to induced plant responses (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008; Yan et al., 2009; Sheard et al., 2010; Fernández-Calvo et al., 2011; Erb et al., 2012). There is crosstalk between these two pathways and in response to many stresses, the JA-mediated pathway takes priority over GA-dependent growth process in *Arabidopsis thaliana* (L.) Heynh. (Hou et al., 2010; Wild et al., 2012; Yang et al., 2012; Heinrich et al., 2013; Lan et al., 2014). However, there also are links between the GA/DELLA pathway and caterpillar strategies to suppress JA-mediated induction of host plant defenses (Lan et al.,

2014); we recently showed that negative growth regulator DELLA proteins are required for the caterpillar labial saliva-mediated suppression of the JA burst (Lan et al., 2014).

Plant use clues from insect oral secretions (regurgitant, saliva) to recognize and respond appropriately to caterpillar attack (Reymond et al., 2000; Stotz et al., 2000; Weech et al., 2008; Diezel et al., 2009; Tian et al., 2012; Paudel et al., 2013; Lan et al., 2014). As expected, insects, also, have strategies to impair the plant's ability to mount a defense response. The labial salivary secretions of caterpillar species, including the beet armyworm, *Spodoptera exigua* Hübner, contain oxidoreductase enzymes, such as glucose oxidase (GOX), that are believed to interfere with JA-dependent signaling pathways (Weech et al., 2008; Eichenseer et al., 2010; Tian et al., 2012; Paudel et al., 2013; Lan et al., 2014). The mechanism underlying this subversion is not fully understood but evidence suggests that labial saliva activation of salicylic acid (SA)-dependent and ethylene-dependent pathways attenuate JA responses (Weech et al., 2008; Diezel et al., 2009; Paudel et al., 2013). As well, the gibberellin/DELLA-pathway is also involved (Lan et al., 2014).

Rapid changes in response to caterpillar herbivory occur through protein post-translational modifications that co-ordinate plant phytohormone signaling. Typically in response to caterpillar herbivory, the JA pathway mediates defense responses (Ballare, 2011; Erb et al., 2012); however, insects have strategies to avoid, delay or suppress these defenses (Musser et al., 2002; Weech et al., 2008; Walling, 2009; Verhage et al., 2011; Consales et al., 2012; Maffei et al., 2012). In particular, the labial saliva of some caterpillar species contains effector(s) that impair the plant's ability to mount a defense response (Musser et al., 2002; Bede et al., 2006; Weech et al., 2008; Paudel et al., 2013; Lan et al., 2014). The mechanism underlying this is still being elucidated, but may involve SA, ethylene and gibberellins/DELLA pathways (Diezel et al., 2009; Paudel et al., 2013; Lan et al., 2014). In this study, we identified nuclear proteins that exhibited post-translational changes in response to caterpillar herbivory. For transcription factors, expression of target gene(s) was also monitored to determine if protein post-translational modifications could potentially affect the binding of these proteins to promoter regions of marker genes. In addition, attention was paid to caterpillar labial saliva-dependent protein post-translational and gene expression differences. Lastly, to further understand the involvement of the gibberellins/DELLA pathway in plant-insect interactions, protein post-translational modifications were investigated in the loss-of-function quadruple-*della* (quad-*della*) mutant

Arabidopsis plants; these plants have knockouts in four of the five DELLA proteins, *gai-t6, rga-t2, rgl1-1* and *rgl2-1*, resulting in constitutively elevated giberellin responses (Cheng et al., 2004).

5.3 Materials and Methods

5.3.1 Chemicals

Chemicals were obtained from Sigma Chemical Company unless otherwise specified.

5.3.2 Plant Cultivation

Arabidopsis thaliana (L.) Heynh ecotype Landsberg erecta (L*er*) or quadruple-*della* mutants (quad-*della*, loss-of-function mutations in *gai-t6, rga-t2, rgl1-1* and *rgl2-1* in a L*er* background) seeds were stratified for 48 hr at 4°C then sown in Agromix (Farfard) that had been pasteurized (80°C for 2 hrs). Plants were placed in growth cabinets under short day length conditions (8:16 light:dark, light intensity 250 $\mu E\ m^{-2}\ s^{-1}$, 23°C). Plants were watered with dilute nutrient solution (20:20:20 NPK, 0.1 gm/5L water) every two days. At two weeks, plants were thinned to three plants per pot.

5.3.3 Caterpillar maintenance and cauterization

Caterpillars of the beet armyworm, *Spodoptera exigua* Hübner, were reared on a wheat germ-based artificial diet (Bio-Serv). The colony was maintained in a growth cabinet (16:8 light:dark, 28-40% RH, 22°C). Adult moths are allowed to mate and eggs collected to maintain the colony (> 2 years).

Early 4^{th} instar larvae were used in herbivory experiments. Labial salivary secretions of a subset of caterpillars was impaired by cauterizing their spinneret, the organ through which labial saliva is secreted (Musser et al., 2002; Bede et al., 2006). Briefly, the day before the experiment, caterpillars were placed in plastic cups on ice for 5 min. Sedentary insects were immobilized on a dissecting tray with plastercine. The spinneret was then quickly burned by a hot probe. After a time for recouperation (approx. 3 hr), the success of cauterization was confirmed by allowing them to feed on a glass disc and testing for the presence of the enzyme glucose oxidase (GOX), an enzyme prevalent in *S. exigua* labial salivary secretions, by the peroxidase/3,3'-diaminobenzidine (DAB) assay (Bergmeyer, 1974; Eichenseer et al., 2010). Both cauterized and mock cauterized caterpillars feed on a glass filter disk soaked with glucose/sucrose solution (50 mg/mL each sugar). Since *S. exigua* caterpillars produce abundant GOX in their labial salivary

secretions (Eichenseer et al., 2010), insects with normal salivary secretions will exude this enzyme onto the filter disc. After several hours of feeding, horseradish peroxidase (2.5 U) and DAB (0.15 mg) are added to the disks and the presence of GOX indicated by a brown precipitate. Both groups then fed separately on Arabidopsis plants (feeder plants) for at least 12 hrs to allow them to adjust to a plant diet.

5.3.4 Herbivory experiment

Three 4th instar caterpillars, either cauterized or mock, were placed in a pot containing 5 week old Arabidopsis (growth stages 1.11-1.14 (Boyes et al., 2001)) during the dark phase. With the general observation that Noctuid caterpillars are more active at night, caterpillars were allowed to feed on the plants at dark period. As well, control plants without insects were included. To restrict larvae on the experimental plants, pots with caterpillars as well as controls were covered with a nylon mesh. A plexi-glass panel was placed to physically separate treatments to minimize the effects of any plant volatiles. After 10 hours of feeding, caterpillars were removed. For proteomic experiments, whole aerial tissues from the three plants per pot were pooled, immediately frozen in liquid nitrogen and stored at -80°C until analysis. For gene expression experiments, wounded rosette leaves from the three plants in each pot were pooled, flash-frozen in liquid nitrogen and stored at -80°C until analysis. All rosette leaves were collected for the undamaged controls. Two biological replicates were collected for each treatment and the experiment was repeated five times.

5.3.5 Nuclear isolation and protein extraction

Due to the amount of material required for the extraction of nuclear protein, samples from the five experimental dates were pooled. Nuclei were isolated from leaves according to Allen et al. (1989) with minor modifications. Briefly, 5 g of frozen leaf tissue was ground in liquid nitrogen and suspended in ice-cold nuclei homogenization buffer (which consists of basal buffer (2.5 M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM KCl, 2.5 M NaCl) also containing 40% glycerol, 0.6 M sucrose and 2 mM dithiothreitol (DTT)) at a final concentration of 1 gm plant material/5 mL homogenization buffer. The homogenate was filtered through two layers of miracloth (125 μM pore size, Fisher Scientific). The filtrate was centrifuged at 2,000 g for 20 min at 4°C. The pellet was gently resuspended in ice-cold wash buffer (10 mL, 1 x basal buffer containing 25% glycerol, 0.5 M sucrose and 0.01% triton X-100).

After the second centrifugation at 2000 g for 20 min at 4°C, the pellet was resuspended in ice cold wash buffer (2 mL, 1 x basal buffer containing 0.5 M sucrose, 0.001% triton X-100 and 2 mM DTT). The resuspended nuclei was layered on an ice cold percoll gradient (80%, 50%, 35% and 20% percoll in 1 x basal buffer containing 0.5 M sucrose, 0.001% triton X-100 and 2 mM DTT). After centrifugation at 3200 g for 30 min at 4°C, the nuclei band at the interface of 80%/50% percoll was collected. Nuclei were washed with ice cold wash buffer using twice the sample volume. After resuspending the pellet in wash buffer , nuclei were pelleted by centrifugation at 5,000 g for 20 min at 4°C. The presence of nuclei was confirmed by visualization after 4',6-diamindino-2-phenylindole (DAPI) staining.

Nuclear proteins were extracted using the CelLytic PN Isolation/Extraction kit (Sigma). Samples were suspended in nuclear protein extraction buffer containing protease inhibitor cocktail and 1% DTT for 30 min at 4°C. Soluble proteins were pelleted by centrifugation at 12,000 g for 15 min at 4°C.

Protein concentration was measured by a modified Bradford assay using a bovine serum albumin (BSA) standard curve (5-100 μg/mL) (Bradford, 1976; Zor and Selinger, 1996). Samples and BSA were incubated with Bradford reagent for 10 min followed by measurement of absorbance at 590 nm and 450 nm. The ratio of OD_{590}/OD_{450} was used to calculate soluble protein concentration. Samples were sent to the Institute for Research in Immunology and Cancer (IRIC), Université de Montréal for proteomic analysis.

5.3.6 Proteomic analysis

Proteins were digested by trypsin (1700 Units in 50 mM ammonium bicarbonate) for 8 hr at 37°C under orbital rotation (600 rpm). Samples were loaded onto a C_{18} precolumn (0.3 mm i.d. x 5 mm) followed by a C_{18} column (150 mm x 10 cm) and separated using on a Nano liquid chromatography (nanoLC)-two dimensional (2D) system (Eksigent) connected to a LTQ-Orbitrap Elite (ThermoFisher Scientific). Peptides were eluted using a linear gradient from 10 to 60% acetonitrile (ACN) containing 0.2% formic acid (FA) at a flow rate of 0.6 μL/min.

Each full MS spectrum acquired with a 60,000 resolution was followed by 12 MS/MS spectrum, where the 12 most abundant multiply-charged ions were selected for MS/MS sequencing. Tandem MS experiments were performed using collision-induced dissociation in the linear ion trap. Peaks were identified using a Mascot version 2.4 (Matrix science) and peptide

sequence were searched against the Arabidopsis non-redundant protein database as well as Arabidopsis Expressed Sequence Tag (EST) database at National Center for Biotechnology Information (NCBI). Tolerance was set at 15 ppm for precursor and 0.5 Da for fragment ions during data processing. For the post-translational modification of proteins, occurence of oxidation, deamination, phosphorylation, nitrosylation, carbamidomethylation, and sulfonation were considered.Corresponding proteins of the identified peptide sequences with post-translational modifications are listed in the Table 5.1.

5.3.7 Gene expression: quantitative real time-polymerase chain reaction (qRT-PCR)

Leaf samples were ground in liquid nitrogen using a cold, sterile mortar and pestle. Total RNA was extracted from the fine powder (100 mg) using the RNeasy Plant Mini kit (Qiagen) following the manufacturer's protocol. RNA quality was assessed by visualization after separation on a 0.8% agarose gel and spectrophotometrically (260/280).

The complimentary DNA (cDNA) copy was generated from total RNA using the QuantiTect Reverse Transcription kit (Qiagen). After treatment to remove genomic DNA (gDNA), the absence of contaminating gDNA was confirmed by amplifying the sample using a primer pair that spans an intronic region of Arabidopsis *laccase4* gene (*AtLMCO4*, Supplemental Table I) (Weech *et al.,* 2008). A gDNA positive control was included. PCR products were separated on a 1% agarose gel.

cDNA was generated from total uncontaminated RNA following the manufacturer's instructions. A dilution (1/10) of the cDNA samples were used in qRT-PCR to analyze gene expression. Relative transcript abundance was analyzed using SYBR green-based qRT-PCR using a MX3000p thermocycler (Stratagene). Reactions contained cDNA, primer mix (70 nM each of forward and reverse primers, Table 5.1) and SYBR green with low ROX (Absolute Blue, ThermoScientific) in opaque white 96-well plates and were analyzed in duplicates. For qRT-PCR, the following thermocycle program was used: 95°C for 15 min followed by 40 cycles of 95°C for 15 sec, annealing temperature for 30 sec (Table 5.1), 72°C for 30 sec. Dissociation curves confirmed amplicon purity. Two technical plates were performed. For each gene (reference genes and genes-of-interests), PCR was used to amplify the gene and the extracted bands were sent for sequencing at the Institut de Recherché Cliniques de Montréal, Université de Montréal.

Raw fluorescence data was exported to qPCR miner software. The initial concentration of gene transcript (R_0) was calculated by $R_0 = 1/(1+E)^{Ct}$, where E is the average efficiency of the gene in the exponential phase and Ct is the threshold cycle (Zhao and Fernald, 2005). Expression of the three references genes (*AtACT2, AtUnk, AtUBQ*) were not affected by treatment (Brunner et al., 2004). The geometric mean of these three references genes was used to normalize expression of the genes-of-interest (Vandesompele et al., 2002; Pfaffl et al., 2004).

5.3.8 Statistical analysis

Gibberellins regulate pleitrophic processes during plant development (Davière and Achard, 2013); therefore, to avoid potentially confounding effects due to phenological differences, wildtype L*er* and quad-*della* mutant plants were analyzed separately. Statistical differences ($p \leq 0.05$) in gene expression were determined within each genotype by one-factor analysis of variance (ANOVA) using SPSS version 20 (SPSS Inc.) followed by a Tukey HSD *post hoc* test. Results from statistical analyses are shown in Appendix 5.1.

5.4 Results and Discussion

5.3.1 Post-translational modifications of nuclear proteins

5.3.1.1 RabH1c

The Arabidopsis genome encodes 57 putative RAB GTPases, which are further divided into 8 subclasses based on their structures (A-H) (Rutherford and Moore, 2002; Asaoka et al., 2013). The functions of these small Ras-like GTP-binding proteins include intracellular membrane trafficking. The general paradigm is that the cytosolic, GDP-bound form of the Rab protein, once activated and C-terminal prenylated, localize to the membrane for their activity (Wollard and Moore, 2008). RabH1c, a homologue of the animal Rab6 and yeast Ypt6, is present in the roots, root hairs and leaves (Rutherford and Moore, 2002; Johansen, 2009). YFP-fusion studies in Arabidopsis and tobacco have shown that RabH1C localizes to the Golgi apparatus where it associates with the coiled-coil protein golgin GC5 (Johansen et al., 2009). The animal homologue, Rab6, though predominantly associated with the Golgi, is also found in the nucleus (Fridmann-Sirkis et al., 2004).

In yeast and plants, Rab expression and activity is associated with stress responses (Cyert 2003; Agrawal et al., 2009). *AtRab7* is induced in response to salt and drought stresses and confers enhanced tolerance that may partially be attributed to its ability to modulate reactive

oxygen species levels (Gorvin and Levine 2000; Mazel et al., 2004). Overexpression of rice *OsRGP1* in tobacco plants unexpectedly resulted in increased salicylic acid (SA) levels and SA-dependent gene expression in response to wounding (Sano et al., 1994). Though less well studied, RabH1c is also involved in stress responses and implicated in the maintenance of redox balance (Khandelwal et al., 2008).

A modified peptide **<u>S</u>DDMVDVNLKTTSNSSQGEQQGGAGGGGG<u>CSC</u>** was identified in L*er* and quad-*della* mutants plants; the peptide was phosphorylated on Ser_{183} and Ser_{213}, nitrosylated on Cys_{212} and the Cys_{214} was oxidized to the sulfenic form ($\Delta m = +16$ Da) (Fig. 5.1A-C). These C-terminus modifications may negatively affect protein prenylation and, hence, localization and activity. In L*er* plants, the modified peptide was present in the highest levels in plants attacked by caterpillars with impaired labial salivary secretions, suggesting that caterpillar labial saliva interferes with the post-translational modification of this protein. Cysteine sulfenic acids (Cys-SOH) are highly reactive; they may undergo further oxidation to form the sulfinic (Cys-SO_2H) or sulfonic (Cys-SO_3H) acids or with an accessible thiol to form a disulfide bridge (Spadaro et al., 2010). In contrast, in the quad-*della* mutant, this modification was predominantly found in plants subject to herbivory by caterpillars with intact labial salivary secretions, implicating the gibberellin/DELLA pathway in the post-translational modification of this protein.

5.3.2 Post-translational modifications of putative transcription factors

5.3.2.1 Putative calmodulin-binding transcriptional activator 3 (CAMTA3)/Signal responsive 1 (SR1)

In our study, two peptides were identified that matched an EST putatively encoding the transcriptional regulator calmodulin-binding transcriptional activator 3 (CAMTA3)/Signal responsive 1 (SR1) (At2g22300). This protein positively regulates the JA burst and glucosinolate biosynthesis in response to herbivory by generalist caterpillars of the cabbage looper, *Trichoplusia ni* (Laluk et al., 2012; Qiu et al., 2012). Therefore, the calmodulin domain of this protein may be involved in coupling intracellular calcium changes in response to herbivore attack with the induction of JA defense responses (Poovaiah et al., 2013). In contrast, CAMTA3/SR1 is a negative regulator of plant immune responses and binds to the promoter region of key genes in the SA- and ethylene-signaling pathways, such as *AtEDS1, AtNDR1* and

AtEIN3, thereby co-ordinating these pathways (Galon et al., 2008; Du 2009; Nei, 2012; Qiu et al., 2012). Caterpillar labial saliva has been proposed to interfere with JA-associated defenses by stimulating the SA-responsive pathway (Kunkel and Brooks, 2002; Beckers and Spoel, 2006; Weech et al., 2008). Therefore, CAMTA3/SR1 suppression of the SA pathway may impair the caterpillar's ability to suppress induced plant defenses. In contrast, caterpillar regurgitant-associated effectors and CAMTA3/SR1 positively activate the ethylene pathway that antagonizes the SA pathway (Diezel et al., 2009). CAMTA/SR1 is also involved in cold acclimation and positively regulates *AtCBF2* expression (Dohrety, 2009). In flower development, *AtSR1* expression is induced by the DELLA protein RGA (Hou et al., 2008); however, in the quad-*della* mutant, the DELLA protein RGA is not expressed (Cheng et al., 2004

In our study, the peptide, **ELVCAGLSQKHLLR**, is nitrosylated on the Cys residue in response to caterpillar herbivory in the L*er* background and in response to herbivory by caterpillars with intact salivary secretions in the quad-*della* mutant (Fig. 5.2A-C); this implies that the putative CAMTA3/SR1-like protein is nitrosylated in response to caterpillar herbivory but this requires DELLA proteins when labial saliva is not present.

CAMTA3/SR1 is a negative regulator of *AtEIN3* and *AtNDR1* (Galon et al., 2008; Du, 2009; Nei 2012). It was, therefore, surprising that expression of these two genes increased in response to caterpillar herbivory (Fig. 5.2D, E, Appendix 5.1). NDR1, a positive regulator of SA signaling during pathogen infection, forms a multiprotein complex scaffold (NDR1-RIN4-RPM1-RPS2) to co-ordinate defense signaling at the plasma membrane (Day et al., 2006). Knepper et al. (2011) have also shown that this integral membrane protein helps to maintain cellular integrity by maintaining plasma membrane-cell wall connectivity and, thus, preventing fluid loss. Thus, this protein may be involved in the perception of mechanical stress, such as that associated with caterpillar herbivory. EIN3 transcription factor is required for ethylene signaling (Potuschak et al., 2003). Activation of the ethylene pathway may lead to impaired induced defenses in Arabidopsis (Stolz et al., 2000; Diezel et al., 2009). If caterpillar-associated Cys-nitrosylation of ELVCAGLSQKHLLR interferes with binding of this transcription factor to the promoter region of these two genes then this may explain the observed pattern of gene expression. Activation of the SA or ethylene pathway may then contribute to the attenuation of JA-dependent defenses (Weech et al., 2008; Paudel et al., 2013; Lan et al., 2014).

In comparison, in the quad-*della* mutant, this peptide is only nitrosylated in plants subject to herbivory by caterpillars with intact salivary secretions (Fig. 5.2C); expression of *AtNDR1* and *AtEIN3* is increased in these plants (Fig. 5.2D, E, Appendix 5.1). In contrast, the peptide is unmodified in plants infested by caterpillars with impaired labial salivary secretions but *AtEIN3* expression is still induced; this implies that either DELLA proteins are required in this signaling pathway or nitrosylation of this peptide only has weak or no effect on transcriptional regulation.

5.3.2.2 MYB109

The R2R3 transcription factor MYB109 (At3g557730) regulates many caterpillar-associated, defense-related genes, including those involved in trichome initiation and glucosinolate biosynthesis (Guan et al., 2011; Tan et al., 2012). Trichomes, either as a mechanical barrier or as sources of detrimental chemical compounds, are an important antiherbivore defense (Tian et al, 2012). In many plant species, such as Arabidopsis, this is a developmentally plastic trait [(Van Poecke, 2007); trichome number and/or density may be increased in newly formed leaves in plants under insect feeding pressure. Traw and Bergelson (2003) found that artificial damage by forceps-pinching of rosette leaves in Columbia (Col-0) and L*er* Arabidopsis ecotypes resulted in increased trichome number and density on newly developed leaves. The JA-dependent induction of trichome number and density was modulated by other phytohormones. In particular, SA had a negative impact and gibberellins a positive effect on trichome formation.

AtMYB109 expression is positively regulated by ethylene response factor2 (ERF2) and negatively regulated by WRKY26 (Dombrecht et al., 2007). In our experiment, the MYB109 peptide **YADLWNNGQWMANSVTTASVKNENVDE<u>T</u>TNPPSSK** is phosphorylated twice with a clear phosphorylation site at Thr_{184} (Fig. 5.3A). The second phosphorylation site is more cryptic and may occur on Tyr_{157} or Ser_{190}. In the L*er* background, this protein is predominantly unphosphorylated (Fig. 5.3B). In comparison, the quad-*della* mutant plants, MYB109 is phosphorylated in untouched control plants and plants subject to herbivory by caterpillars with intact labial salivary secretions. This indicates a potential tie in the post-translational modification of this protein to the gibberellin/DELLA pathway.

MYB109 positive regulates the expression of *AtESP*, the gene encoding epithiospecifier protein (ESP) (Dombrecht et al., 2007). This protein influences the hydrolysis of glucosinolates in response to insect herbivory: Binding of EPS to myrosinase shifts glucosinolate hydrolysis

from forming isothiocyanates to nitriles and epithionitriles (Lambrix et al., 2001; Kissen, 2012; Wittstock and Burow, 2010). Compared to nitriles, isothiocyanates negatively impact caterpillar growth and development of generalist *Spodoptera littoralis* (Burow et al., 2006). In contrast, generalist *Trichoplusia ni* caterpillars preferred Arabidopsis plants that produced nitriles rather than isothiocyanates (Lambrix et al., 2001). In comparison, caterpillars of the crucifer specialist caterpillars, *Pieris rapae*, did not distinguish between the two compounds even though adult females preferentially laid their eggs on plants that produce isothiocyanates compared to nitriles (Mumm et al., 2008).

Arabidopsis ecotypes exhibit different *ESP* expression and activity (Kissen et al., 2012); in the nitrile-producing L*er* ecotype, ESP is found in all tissues with the exception of roots (Burow et al., 2007). Expression of the *AtESP* transcript is induced by methyl jasmonate treatment and is MYC2-dependent (Dombrecht et al., 2007). Two splice variants have been identified in the non-nitrile producing Col-0 ecotype (Kissen et al., 2012). In our experiment, primers used in qRT-PCR to monitor gene expression recognized both transcript variants.

In L*er*, the MYB109 peptide YADLWNNGQWMANSVTTASVKNENVDETTNPPSSK was generally present in its unphosphorylated form (Fig. 5.3B). Expression of the target gene, *AtESP*, was strongly induced in response to caterpillar herbivory (Fig. 5.3D, Annex 5.1); greater than a 3-fold increase in expression was observed but a labial saliva-dependent difference was not seen. In contrast, in the quad-*della* mutant plants, the protein was phosphorylated twice on Thr_{184} and either Tyr_{157} or Ser_{190} in control plants as well as plants fed upon by normal caterpillars. In these plants, again, basal levels of *AtESP* expression increased in response to caterpillar herbivory.

In the quad-*della* mutant plants, MYB109 is phosphorylated on Thr_{184} suggesting that gibberellin signaling may lead to the post-translational modifications of this protein. This may partially explain previous observations that gibberellin treatment had synergistic effects on trichome formation (Traw and Bergelson, 2003). However, it does not appear that Thr_{184} phosphorylation affects expression of downstream genes, such as *AtESP*.

5.3.2.3 ABA response element binding factor 3 (ABF3)

As a result of wounding during caterpillar feeding, foliar ABA levels may lead to the expression of bZIP transcription factors *ABF3, AREB1* and *AREB2* that encode the ABA response element

binding factor 3 (ABF3) and ABA-dependent response factor binding proteins 1 and 2 (AREB1 and AREB2), respectively (Reymond et al., 2000; Fujita et al., 2005). ABF3, AREB1 and AREB2 exhibit partial functional overlap and form homo- or heterodimers for full activity as transcriptional regulators (Yoshida et al., 2010). In our study, we identified two post-translational modifications of ABF3 (At4g34000) in response to herbivory by caterpillars with impaired labial salivary secretions; nitrosylation of Cys_{420} and phosphorylation of Ser_{431} (Fig. 5.4A). This protein has been shown to have multiple site phosphorylations (Chen et al., 2010; Kline et al 2010; Sirichandra et al., 2010). In fact, phosphorylation of Thr_{451} by the Snf1-related kinase 2 Open Stomata 1 (OST1) enhances protein stability (Sirichandra et al., 2010).

ABF3 regulates the expression of a number of ABA-dependent genes including those encoding LEA proteins and transcriptional regulators, such as WRKY40 (Yoshida et al., 2010). The transcription factor WRKY40 integrates information from many phytohormone signaling pathways to fine-tune plant responses to changing environmental inputs (Rushton et al., 2011). For example, *AtWRKY40* is induced in response to ABA and negatively regulates ABA-responsive genes, suggesting an important role in a feedback regulatory loop when the ABA signal is absent (Chen et al., 2010; Liu, et al., 2012; Rushton et al., 2012). WRKY40 is also a negative regulator of SA-mediated plant immune responses (Pandey et al., 2010). As well, *AtWRKY40* expression is rapidly induced in response to wounding of leaves through the COI1/JA-dependent pathway (Wang et al., 2008). This transcription factor then negatively regulates the expression of the JA-pathway repressor *jasmonic acid ZIM-domain protein8* (*AtJAZ8*) (Pandey, 2010). Thus, WRKY40 co-ordinates plant responses to multiple phytohormones, including ABA, SA and JA, acting as a negative regulator of ABA and SA signaling and a positive regulator of JA signaling pathways (Rushton et al., 2012; Schweizer et al., 2013). Thus, caterpillar performance, for example larva Egyptian cotton leafworm, *Spodoptera litorallis* (Boisduval), is negatively affected by WRKY40 (Schweizer et al., 2013).

In our experiment, *AtWRKY40* expression was induced in response to caterpillar herbivory (Fig. 5.4 D, Appendix 5.1); however, a 2-fold increase in transcript levels is observed in response to attack by insects with impaired labial salivary secretions compared to herbivory by normal caterpillars. As AtWRKY40 has a positive effect on JA-dependent defenses through its negative regulation of the SA pathway and the JA repressor AtJAZ8, this suggests that a factor in the caterpillar labial saliva interferes with *AtWRKY40* expression to suppress JA-dependent

responses. In contrast, in quad-*della* mutant plants, the nitrosylated, phosphorylated form of this protein is predominantly found in plants attacked by caterpillars with impaired labial salivary secretions (Fig. 5.4C). Even so, expression of the downstream target gene, *AtWRKY40*, was induced in response to caterpillar herbivory (Fig. 5.4D); a labial saliva-dependent difference was not observed.

Thus, there may be a link between the post-translational modifications of the bZIP transcription factor AtABF3 in response to caterpillar herbivory and expression of the downstream target gene *AtWRKY40* (Fig. 5.4D). In L*er*, AtABF3 is phosphorylated and nitrosylated in plants attacked by caterpillars with impaired labial salivary secretions. As well, expression of the AtABF3 target gene, *AtWRKY40*, was highest in these plants. However, the caterpillar labial saliva-dependent difference in gene expression observed in L*er* plants was alleviated in the quad-*della* mutants (Fig. 5.4D); therefore, DELLA proteins may be involved in the regulation of this gene in response to caterpillar herbivory.

5.5 Conclusions

Caterpillar herbivory-associated post-translational modification of AtRabH1c found in L*er* plants was only observed in quad-*della* mutant plants attacked by caterpillars with normal labial salivary secretions, suggesting the involvement of the gibberellins/DELLA pathway in the post-translational regulation of this protein (Fig. 5.1A-C). As well, post-translational modifications of AtMYB109 were predominantly found in quad-*della* mutant plants, indicating the input of this pathway (Fig. 5.3A-C); however, post-translational modifications did not affect downstream expression of the target gene *AtESP1* that was expressed in response to caterpillar herbivory in L*er* and quad-*della* plants (Fig. 5.3D).

We also identified caterpillar-specific nitrosylation of the transcriptional regulator AtCAMTA/SR1-like protein. Even though this protein is a negative regulator of SA- and ethylene-signaling pathways, increased expression of *AtNDR1* and *AtEIN3* is observed in plants fed upon by caterpillars with normal salivary secretions, suggesting induction of these pathways which may lead to the attenuation of JA-dependent defense responses (Fig. 5.2A-E) (Kunkel et al., 2002; Beckers et al., 2006; Weech et al., 2008; Diezel et al., 2009). There also is a caterpillar labial saliva-specific post-translational modification of AtABF3 (Fig. 5.4A-C). Here, we see lower transcript levels of *AtWRKY40*, a positive regulator of the JA-pathway, in plants fed upon

by caterpillars with intact salivary secretions compared to those whose spinneret had been cauterized shut. Therefore, it is tempting to speculate that caterpillar labial saliva leads to protein post-translational modifications that suppress JA-dependent defense responses and activate pathways, such as the SA pathway, that may further attenuate these defenses. However, the potential implications of these post-translational modifications in plant-insect interactions must be validated through further studies.

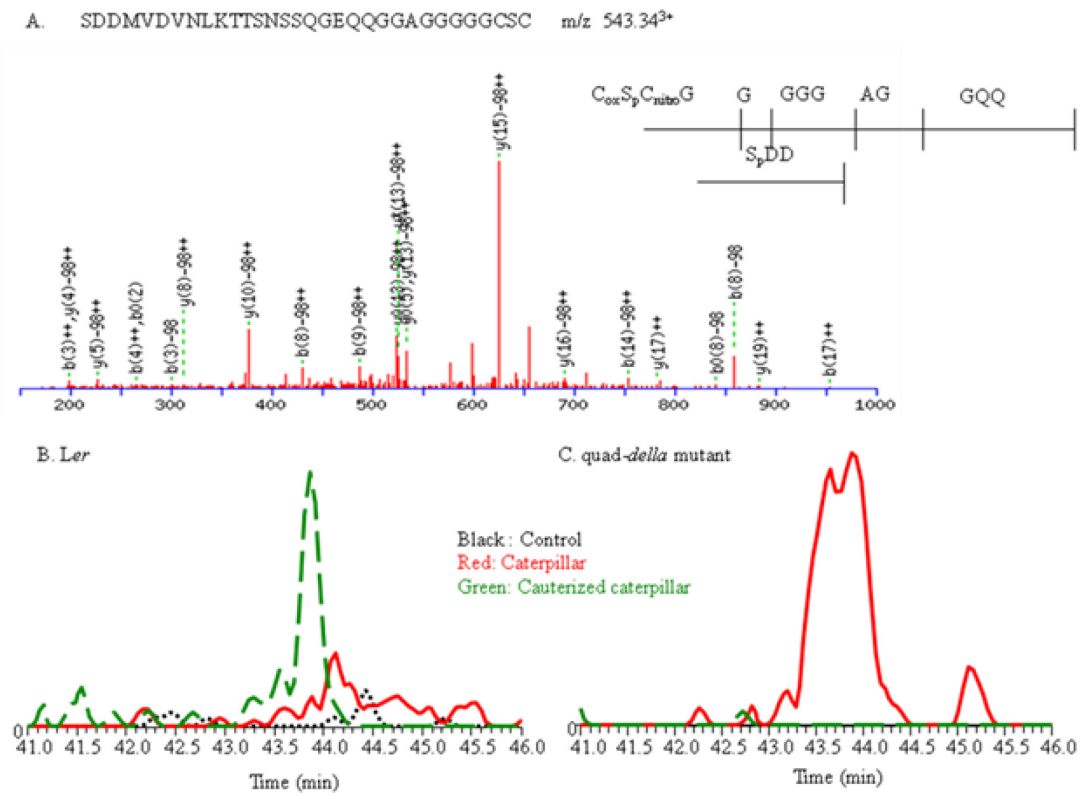

Figure 5.1. Post-translational modification of the SDDMVDVNLKTTSNSSQGEQQGGAGGGGGCSC peptide of the GTPase RabH1C.

A) MS/MS spectrum indicating phosphorylation on Ser_{183} and Ser_{213}, nitrosylation on Cys_{212} and oxidation of Cys_{214}. **B)** Extracted ion chromatogram for m/z 543.34^{3+} in L*er* plants. **C)** Extracted ion chromatograph for m/z 543.34^{3+} in quad-*della* mutant plants. Dotted black line indicates undamaged control plants. Solid red line indicates plants subject to caterpillar herbivory. Dashed green line indicates plants subject to herbivory by caterpillars with impaired labial salivary secretions.

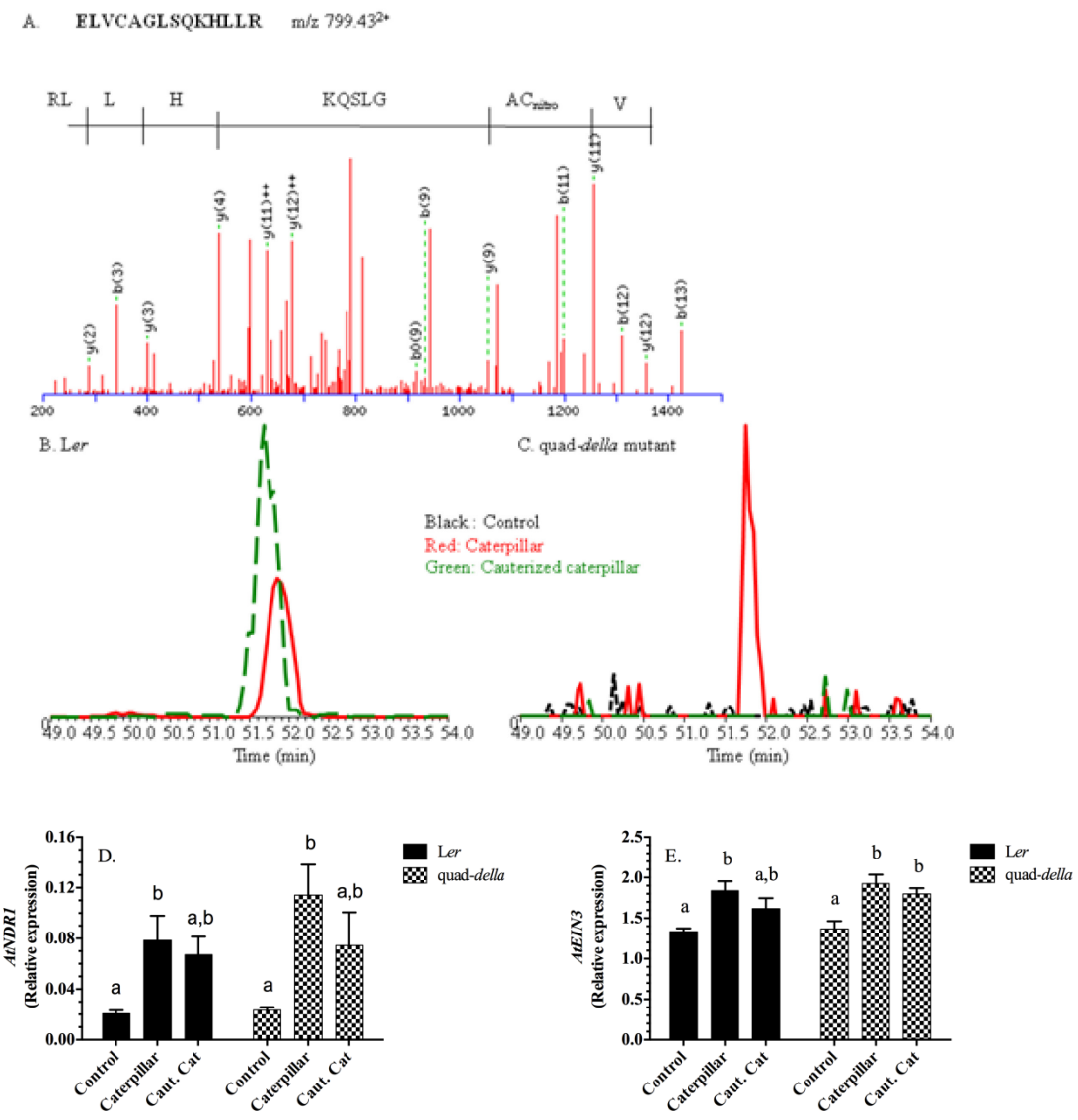

Figure 5.2. Post-translational modification of the ELVCAGLSQKHLLR peptide of a CAMTA/SR1-like transcription factor and gene expression of its downstream target genes. **A)** MS/MS spectrum indicating nitrosylation of the Cys residue. **B) and C)** Extracted ion chromatogram for m/z 799.43^{2+} in L*er* plants and quad-*della* mutant plants. Dotted black line indicates undamaged control plants, and solid red line and dashed green line indicate plants subject to caterpillar herbivory by caterpillars with intact and impaired labial salivary secretions respectively. Expression of the CAMTA/SR1 transcription factor target genes **D)** *AtNDR1* and **E)** *AtEIN3* in L*er* (solid bars) and quad-*della* mutant (hatched bars) plants. Bars represent the means of five independent biological replications ± SE and different letters indicate significant differences between treatments at $p \leq 0.05$ (Appendix 5.1).

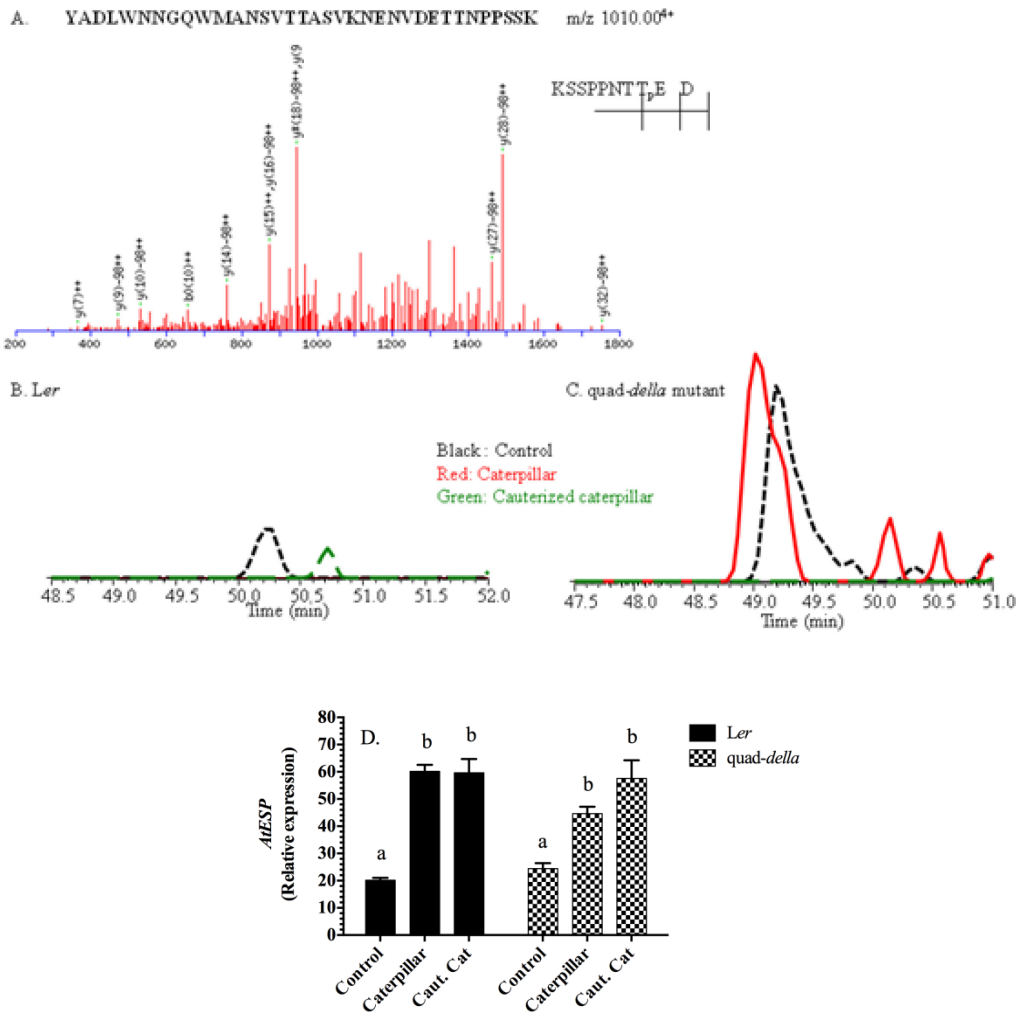

Figure 5.3. Post-translational modification of the YADLWNNGQWMANSVTTASVKNENVDETTNPPSSK peptide of MYB109 transcription factor and gene expression of its downstream target gene. A) MS/MS spectrum indicating phosphorylation of Thr_{184}. Extracted ion chromatogram for m/z 1010.00^{4+} in **B)** L*er* and **C)** quad-*della* mutant plants. Dotted black line indicates undamaged control plants, and solid red line and dashed green line indicates plants subject to herbivory by caterpillars with intact and impaired labial salivary secretions, respectively. **D)** Expression of the MYB109 transcription factor target gene *AtESP* in L*er* (solid bars) and quad-*della* mutant (hatched bars) plants. Bars represent the means of five independent biological replications ± SE. Different letters indicate significant differences between treatments at $p \leq 0.05$. (Appendix 5.1).

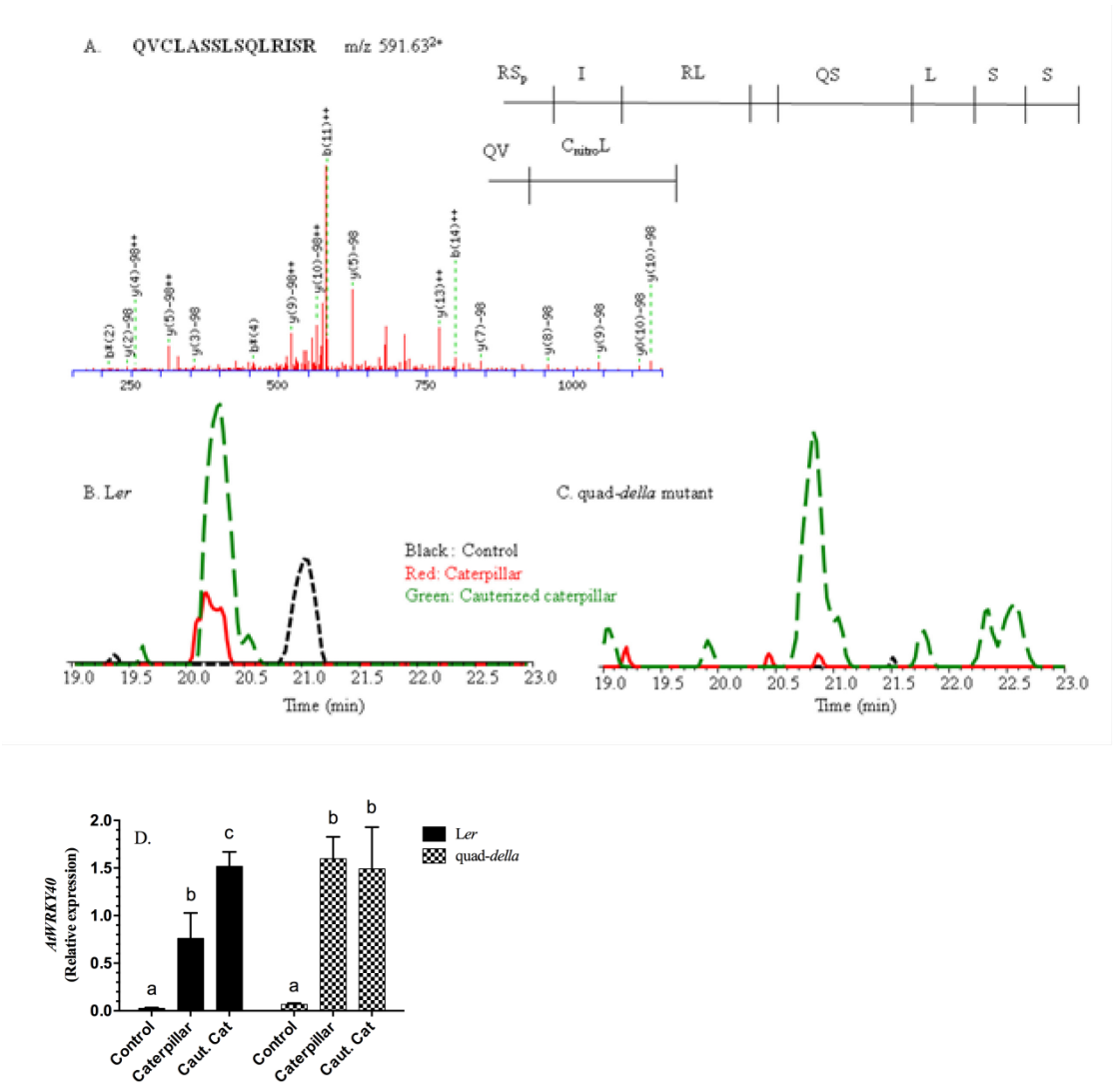

Figure 5.4. Post-translational modification of the QVCLASSLSQLRISR peptide of the ABF3 transcription factor and gene expression of its downstream target gene. A) MS/MS spectrum indicating nitrosylation of Cys_{420} and phosphorylation of Ser_{431}. Extracted ion chromatogram for m/z 591.63^{2+} in **B)** L*er* plants and **C** in quad-*della* mutant plants. Dotted black line indicates undamaged control plants, and solid red line and dashed green line indicate plants subject to herbivory by caterpillars with intact and impaired labial salivary secretions, respectively. **D)** Expression of the ABF3 transcription factor target gene *AtWRKY40* in L*er* (solid bars) and quad-*della* mutant (hatched bars) plants. Bars represent the means of three to four independent biological replications ± SE. Different letters indicate significant differences between treatments at $p \leq 0.05$ (Appendix 5.1).

Table 5.1. Corresponding proteins to the identified post-translationally modified peptide sequences. Peptides were searched against the *Arabidopsis* non-redundant protein database or expressed sequence tag (EST) database of NCBI using the blast algorithm (October, 2013) (Boratyn et al., 2013).

Peptide sequence	Corresponding protein	E-value	Identity %	Algorithm
SDDMVDVNLKTTSNSSQGEQQGG AGGGGGCSC	AtRabH1c	$1e^{-15}$	100%	blastp
ELVCAGLSQKHLLR	AtSR1-like			pblastn
YADLWNNGQWMANSVTTASVKN ENVDETTNPPSSK	AtMYB109	$1e^{-17}$	100%	blastp
QVCLASSLSQLRISR	AtABF3	$3e^{-8}$	100%	blastp

Table 5.2. Primers used for quantitative real time-polymerase chain reaction (qRT-PCR).

Gene	Annealing temperature (°C)	Forward (5'-3')	Reverse (5'-3)	Ref.
AtNDR1 (AT3G20600)	60	CTTTTCTTATGGCTTAGTCTCCGTG	ATCTTGGTCGTGTTGATGGTGG	Nie et al., 2012
AtEIN3 (AT3G20770)	60	GACAGAACCGTTTTCACCTGCGAGA	CTGAGGAAATCCAACTACAGGCTTA	Nie et al., 2012
AtESP (AT1G54040)	60	CTACAGGAGCGAAACCTTCC	GATCAGGCCATACCTCACCT	Dombrecht et al., 2007
AtWRKY40 (AT1G80840)	60	TGCGAGTTGAAGAAGATCCACCGA	TCCGAGAGCTTCTTGTTCTCAGCA	Czechowski et al., 2004
AtActin2 (At3g18780)	60	ACCAGCTCTTCCATCGAGAA	GAACCACCGATCCAGACACT	Dufresne et al. (2008)
AtUBC (AT5G25760)	58	GCAGTTGACAATTCGTTCTCT	GAGCGGTCCATTTGAATATGTT	Primer3
AtUnk2 (AT4G33380)	60	TTGAAAATTGGAGTACCGTACCAA	TCCCTCGTATACATCTGGCCA	Czechowski et al. (2005)
AtLMCO4 (NM_129364)	58	ATGGGTCGTCATCAGATTCAGAGCAGATAA	CATATAAGAGGTGTGTTAGAGACAATAATA	Weech et al., 2008

Connecting Statement to Chapter 6

In the next 50 years, atmospheric CO_2 is predicted to double (IPCC, 2014). This will undoubtedly affect plant defense responses. Thus, in Chapter 6, plant stress responses at different CO_2 and nitrate levels were investigated. Arabidopsis leaves were mechanically wounded to measure the levels of defense-related hormones, glucosinolates (GSL) and the expression of *MYB* transcription factors that regulate GS biosynthesis. Unbiased metabolic profiling of Arabidopsis by LC-qTOF mass spectrometry was performed to identify candidates that were affected at elevated CO_2 and nitrogen-stress conditions.

I am responsible for designing the experiment and conducting the laboratory work. Alexender Amirizian contributed to the literature review and sample collection. I performed the gene expression studies; Jessica Giddings and Shoieb Akaram prepared the cDNA for this transcript analysis. Phytohormones were analyzed at the Danforth Plant Science Center (Missouri, USA). Targeted glucosinolates were analyzed at the Radboud University Nijmegen, Netherlands. I performed the untargeted metabolite analysis at the Mass spectrometry facility at the University of Iowa, USA. I am responsible for data analysis and manuscript preparation together with my supervisor, Dr. Jacqueline Bede. Agriculture and Agri-Food Canada and a NSERC grant to Dr. Bede funded this project. This paper will be submitted to the journal “Plant Physiology”.

CHAPTER 6. Arabidopsis Metabolic Response to Wounding: Effect of Carbon *vs* Nitrogen fertilization

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6.1 Abstract

The increased atmospheric carbon dioxide (CO_2) levels predicted to occur before the end of the century will impact plant photosynthesis and, thus, metabolic flux into defensive secondary compounds. As soil nitrogen levels may affect plant responses to CO_2 enrichment, these two factors must be investigated together to understand how climate change will impact plant defense strategies. We compared Arabidopsis foliar metabolic profile in plants grown under different CO_2 regimes (440 pm vs 880 ppm), nitrogen fertilization (nitrate-limited vs nitrate-sufficient) and in response to mechanical damage of rosette leaves. Nitrate fertilization dampened the jasmonate burst in response to wounding in plants grown at elevated CO_2 levels. The leaf glucosinolate (GSL) profile strongly mirrored this jasmonate burst; total foliar GSL levels increase in response to damage in plants grown at ambient CO_2 but only increase in nitrate-stressed plants grown under elevated CO_2 conditions. Foliar damage resulted in the induction of a number of MYB transcription factors that regulate GSL biosynthesis; elevated levels of *AtMYB29, AtMYB76* and *AtMYB34* were observed in mechanically damaged rosette leaves though *AtMYB28* was only induced in response to wounding at ambient CO_2 levels. *AtMYB51* expression reflects phytohormone levels; *AtMYB51* transcription was positively regulated by salicyclic acid and suppressed by jasmonate. Though, there is a general upregulation of MYB transcription factors that regulate both aliphatic and indole GSL pathways, there is a general shift in wounded Arabidopsis leaves from aliphatic to indole GSLs.

6.2 Introduction

In response to environmental stresses, plants have evolved an impressive diversity of chemical defenses (Rausher, 2001). In particular, plant secondary metabolites involved in protection against insect herbivores can function as feeding deterrents, antinutritive factors or toxins to protect plant tissues or act as cues to attract natural enemies of plant pests (Baldwin et al., 2001). Synthesizing these defense metabolites is costly and plants must efficiently balance the tradeoff between growth and defense (Coley, 1988; Bidart-Bouzat et al., 2005). However, the current picture might change as atmospheric carbon dioxide (CO_2) levels are predicted to rise dramatically by the end of this century (Weaver et al., 2007; IPCC, 2014). CO_2 enrichment is known to promote photosynthetic and nitrogen use efficiency, particularly in C_3 plants, through an increase in the rate of CO_2 fixation. Even though plants are predicted to be more tolerant to nitrogen deficiency, there also may be limitations in nitrogen fertilizers due to increasing production costs. This problem may be exacerbated in countries with limited access to costly farming inputs (Edgerton, 2009). Therefore, global changes in atmospheric CO_2 levels combined with potential limitations of nitrogen fertilizers will alter plant nutrient patterns in agricultural fields. Understanding how plants adapt to these rapidly changing environmental conditions still remains a challenge (Leakey et al., 2009).

To explain the relationship between nutrient availability and plant defenses, Bryant *et al.* (1983) proposed the carbon-nutrient balance hypothesis (CNBH) that predicts that growth processes are most limited by nitrogen shortage under elevated CO_2, resulting in an excess of carbon. Consequently, carbon-rich metabolites push metabolic flux towards the synthesis of carbon-based defenses, such as phenolics and terpenoids, while nitrogen-based defenses, such as alkaloids and cyanogenic glycosides, are expected to decline as nitrogen is preferentially allocated to growth processes. Since its formation in the early 1980s, the CNBH has been tested in a large number of plant species. While it successfully predicted the shifts in secondary metabolism in some cases (Agrell et al., 2000; Coley et al., 2002; Agrell et al., 2004; Wassner and Ravetta, 2007), other studies have been inconclusive. For instance, carbon-based condensed tannins levels dropped in *Betula pendula* (Betulaceae) saplings receiving high N treatments (Keinänen et al., 1999). As well, carbon-based diterpenoids were more abundant in the resin of *Pinus sylvestris* (Pinaceae) after nitrogen application (Bjorkman et al., 1998) and phenolics increased in adequately fertilized *Solanum carolinense* (Solanaceae) (Cipollini et al., 2002).

Moreover, *Datura stramonium* (Solanaceae) plants accumulated lower levels of the alkaloid scopolamine under elevated CO_2 conditions whereas atropine levels increased during CO_2 enrichment (Ziska et al., 2005). These studies have called into question the ability of CNBH to accurately predict how C- and N-supply affect plant secondary metabolite levels (Hamilton et al., 2001).

Proponents of the CNBH have criticized these studies for focusing on only one class of carbon- or nitrogen-based secondary metabolites; this theory should only be used to predict overall changes in the relative abundance of metabolite subgroups in relation to the plant tissue carbon-to-nitrogen ratio (Stamp, 2003). Also, CNBH should only be used to formulate predictions on defense allocation in plants with phenotypic flexibility as several species do not respond to changing environmental conditions through shifts in secondary metabolism (Stamp, 2003). Finally, Haukioja et al. (1998) argue that the terms carbon- and nitrogen-based compounds provided by Bryant et al. (1983) were poorly defined as phenylpropanoids were classified as carbon-based metabolites despite being derived either from phenylalanine (Phe) or dehydroshikimate, a nitrogen-free organic acid. Therefore, solely classifying these compounds as carbon- or nitrogen-based defenses might be problematic (Koricheva, 2002). Therefore, it is important to assess overall changes in metabolic profiles to accurately determine the impact of nutrient availability on plant defenses (Stamp, 2003). The recent use of unbiased metabolite profiling to simultaneously measure hundreds of metabolites in plant tissues could be a valuable tool to evaluate these metabolic shifts (Schauer and Fernie, 2006).

Thale cress, *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae), is a fast-growing herbaceous plant (Graham and May, 2011). Like most plants in the order Brassicales, the main defense metabolites produced by *A. thaliana* are glucosinolates (GSLs). These nitrogen- and sulphur-rich compounds are constitutively present in plant tissue and their synthesis is stimulated by biotic stress and mechanical wounding (Mewis et al., 2005). More than 120 GSLs have been identified in plant species from the order Brassicales (Fahey et al., 2001). The basic skeleton of these compounds is a *S*-glycosylated thiohydroximate sulfate ester linked to an amino acid derived side chain (Winde and Wittstock, 2011). *A. thaliana* produces ≈40 different aliphatic, aromatic and indole GSLs derived from glucose and methionine (Met), Phe or tryptophan (Trp), respectively (Reichelt et al., 2002; Pfalz et al., 2011). Unlike indole GSLs, the core structure of

aliphatic GSLs is not directly derived from Met, but from homo-Met, a chain-elongated amino acid (Fig. 6.1).

The impact of elevated atmospheric CO_2 on GSL accumulation has been assessed in several Brassicaceae species. Karowe et al. (1997) found that a shift in GSL levels was not correlated with the carbon-to-nitrogen ratio of plant tissue as it increased in all species under CO_2 enrichment while total foliar GSLs increased, decreased or remained unchanged in a stage- and species-specific manner. Other studies have either found no difference in GSL content or slight changes in the concentration of a few compounds between plants grown under ambient and elevated CO_2 levels (Karowe et al., 1997; Bidart-Bouzat et al., 2005; Schonhof et al., 2007; La et al., 2009; Klaiber et al., 2013). Bidart-Bouzat et al. (2005) reported a CO_2 x herbivory interaction: *A. thaliana* plants with lower constitutive defenses accumulated significantly more GSLs after damage by caterpillars of the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), under elevated CO_2 levels. On the other hand, feeding by *P. xylostella* caterpillars on *B. napus* subjected to CO_2 enrichment resulted in lower levels of the most predominant foliar indole GSL 3-indolylmethyl GSL (glucobrassicin, GBC) as well as total GSL levels. However, as *B. napus* plants produced more biomass, these results could reflect a dilution effect rather than a direct CO_2 x herbivory interaction. Overall, CO_2 enrichment does not appear to have a consistent effect on GSL biosynthesis.

Nitrogen availability influence on GSL synthesis has mainly been studied in relation to plant sulphur status in Brassicaceous plants. *B. oleracea* var. *capitata* accumulated more GSLs when grown in nitrogen-limited conditions (Rosen et al., 2005). Overall, a change in the aliphatic to indole GSL ratio was not observed as individual GSL levels uniformly increased, with a few minor exceptions. These results are inconsistent with observations in *Brassica rapa* where nitrogen stress led to an overall reduction in GSL accumulation in plants receiving adequate sulfur fertilization (Kim et al., 2002). In *B. oleracea* var. *italica* changing nitrogen fertilization rates had a non-linear effect on foliar GSL profiles, suggesting that nitrogen stress favours the synthesis of 3-indolylemethyl GSL (GBC) to stimulate plant growth as it is a precursor for the growth regulator indole-3-acetic acid (Aires et al., 2006). In contrast, *B. oleracea* var. *alboglabra* plants subjected to nitrogen stress had higher total GSL levels than those receiving an adequate or excessive N dose under ambient CO_2 conditions (350 ppm) (La et al., 2009). Elevating CO_2 levels to 800 ppm results in a greater increase in aliphatic and total GSLs in nitrogen-deprived

plants than in adequately fertilized plants suggesting a significant CO_2 x nitrogen interaction. Moreover, the increase in the carbon-to-nitrogen ratio under CO_2 enrichment did not lead to depressed GSL levels in bolting stems (La et al., 2009).

Six R2R3-type MYB transcription factors regulate GSL biosynthesis (Hirai, 2009) (Fig. 6.1). MYB 34, MYB51 and MYB122 are responsible for the biosynthesis of indole GSLs (Frerigmann and Gigolashvili, 2014). These MYB transcription factors show some functional redundancy and tissue-specific expression patterns (Gigolashvili et al., 2009); MYB34 and MYB122 are predominantly associated with root tissues whereas MYB51 is found in leaves (Gigolashvili et al., 2007; Gigolashvili et al., 2009). MYB34 positively regulates genes involved in the biosynthesis of Trp and indole-3-acetic acid as well as the genes encoding cytochrome P450 CYP79B2/3 and CYP83B1 that catalyze enzymes in the GSL biosynthetic pathway. Overexpression of *AtMYB34* leads to the accumulation of 3-indolylmethyl GSL (GBC), the most abundant indole GSL (Celenza et al., 2005). Overexpression of *AtMYB51* results in the accumulation of indole alkaloids and reduced leaf consumption by caterpillars of the beet armyworm, *Spodoptera exigua* Hübner (Gigolashvili et al., 2007b). In comparison, MYB122 has a minor but complementary role in indole GSL biosynthesis (Frerigmann and Gigolashvili, 2014).

In contrast, MYB28, MYB29 and MYB76 positively regulate aliphatic GSL biosynthesis (Gigolashvili et al., 2007a; Gigolashvili et al., 2008). MYB28 induces the expression of *MAM1/3*, *CYP79F2* and *ST5b/c* transcripts which encode enzymes in the aliphatic GSL pathway. MYB29 induces the accumulation of short-chain GSLs and may serve as an integrator of signals from MYB26 and MYB76 as it is upregulated by both these transcription factors and has a direct inhibitory effect on MYB28 (Hirai et al., 2007; Sonderby et al., 2010a). MYB76 is not considered a major regulator of aliphatic GSL biosynthesis as *Atmyb76* mutants have similar GSL profiles to wildtype plants (Gigolashvili et al., 2009), but Sønderby et al. (2010) reported that MYB76 overexpression leads to an increase in long-chained GSLs. It is of interest that MYB28, MYB29 and MYB76 function antagonistically and repress expression of *MYB34, MYB51* and *MYB122* transcripts (Gigolashvili et al., 2009). How expression of these six MYB factors that are directly involved in the regulation of GSL biosynthesis are affected by elevated atmospheric CO_2 conditions and nitrogen stress is unknown.

Mechanical damage activates multiple signalling pathways which modulate gene expression and lead to the production of defense metabolites (Howe, 2004). Early signalling events include increased calcium (Ca^{2+}) and reactive oxygen species (ROS) fluxes that lead to octadecanoid signalling and downstream defense responses (Bonaventure and Baldwin, 2010; Maruta et al., 2011). Key octadecanoid signalling molecules are 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA) and the biologically active form of JA, 7-jasmonoyl-L-isoleucine (JA-Ile)(Fonseca et al., 2009). Increases in abscisic acid (ABA) are also often observed in response to wounding, possibly as a response to water losses at the site of damage (Erb et al., 2012). In contrast, salicylic acid (SA) increases as part of the hypersensitive response to pathogens as part of the systemic acquired resistance (Jones and Dangl, 2006). Mechanical wounding often results in the induction and activation of secondary metabolic pathways resulting in, for example, increased production of anthocyanins in *A. thaliana* (Morker and Roberts, 2011), alkaloids in *Lupinus polyphyllus* (Fabaceae) (Wink, 1983) and terpenoids in *Gossypium hirsutum* (Malvaceae) (Opitz et al., 2008). At the transcriptional level, in GSL biosynthesis, mechanical wounding activates the expression of *MYB28, MYB29, MYB51, MYB76* and *CYP79B2/3* (Mikkelsen et al., 2003; Schuster et al., 2006; Gigolashvili et al., 2009; Sonderby et al., 2010a). However, there is limited information on the effect of wounding on GSL biosynthesis. Higher levels of indole GSLs, 3-indolylmethyl-GSL (glucobrassicin, GBC) and 4-hydroxy-3-indolylmethyl-GSL (4HO3IM), were found after wounding or feeding by the crucifer specialist flea beetle *Phyllotreta cruciferae* damage of *B. napus* cv. 'Tobin' and *B. juncea* cv. 'Cutlass' compared to *B. napus* cv. 'Westar' where only 3-indolylmethyl-GSL levels increase (Bodnaryk, 1992). In contrast, wounding did not affect GSL levels in *Sinapis alba* cv. 'Ochre' cotyledons, but this species has higher constitutive levels of GSLs compared to other plant species. In *Arabidopsis thaliana*, foliar indole GSLs (*i.e.* 4-hydroxy-3-indolylmethyl-GSL) increased 24 hr after damage by ribbed forceps (Mikkelsen et al., 2003). In comparison, increases in levels of aliphatic GSL (*i.e.* 8-methylthiooctyl GSL and 8-sulphinyloctyl GSL) and indole GSL (*i.e.* 3-indolylmethyl-GSL and N-methyloxy-3-indolylmethyl GSL) increased after methyl jasmonate treatment.

Bidart-Bouzat and Imeh-Nathaniel, (2008) stress the need to study CO_2-dependent changes in stress-induced foliar defense metabolite profiles as they could provide valuable predictions on future plant-herbivore interaction patterns. As nitrogen supply is known to affect

plant responses to CO_2 enrichment (Ainsworth and Rogers, 2007), these two factors need to be studied simultaneously to accurately predict the outcome of global climate change on plant defense mechanisms. The CNBH predicts that a shift in the carbon-to-nitrogen ratio of plant tissues is reflected in the carbon- and nitrogen-based defense metabolite profile. Based on that assertion, CO_2 enrichment and nitrogen stress should favour the production of carbon-based compounds (*i.e*. phenolics) at the expense of nitrogenous metabolites (*i.e*. GSLs) as these environmental factors are known to increase plant carbon-to-nitrogen ratio (Bryant et al., 1983). The aim of this project is to evaluate the effect of CO_2 enrichment and nitrogen deficiency on metabolite levels in mechanically wounded *A. thaliana* leaves. Phytohormone and plant metabolites were analyzed by both targeted and unbiased strategies. GSL levels as well as expression of MYB transcription factors regulating GSL biosynthesis will be analyzed. As well, a non-targeted approach was used to identify metabolites through liquid chromatography-quadrupole time-of-flight-mass spectrometry (LC-qTOF-MS) (De Vos et al., 2007).

6.3 Materials and Methods

6.3.1 Plant growth conditions

Arabidopsis thaliana (Col-0) seeds were cold stratified at 4 °C for two days to obtain a constant germination rate (Zhang et al., 2006). After sowing, trays were transferred to one of two growth cabinets (16:8 h light:dark, 250 μE m^{-2} s^{-1}, 23 °C) under ambient (440 ppm) or elevated (880 ppm) CO_2. After two weeks, seedlings were transferred to pots and randomly assigned to one of the two fertilization groups; the first set was subjected to nitrogen stress (1 mM nitrate) and the second group was given sufficient nitrate (10 mM nitrate). To make up these fertilizers, concentrations of all other components were the same with the exception of Cl^-; the difference in Cl^- concentration is considered insignificant as it is at a supra-optimal level and below potentially toxic levels (Marschner, 1995; Loudet et al., 2003); Cl^- was approximately 9 mM higher in the N-stressed plants. Plants were fertilized every two days with watering.

Wound treatment and sample collection

At approximately 6 weeks (stage 3.9 (Boyes et al., 2001)), half of the plants for each treatment were randomly selected to be mechanically damaged; approximately 20% of each rosette leaves in the mechanically wounded treatment was removed using a hole punch. To minimize volatile signalling between different groups of plants, a plexiglass panel was placed between wounded

and control plants. After 24 hours, the entire rosette was harvested and flash frozen in liquid nitrogen and stored at -80°C until subsequent analysis. For hormone, gene expression, GSL and untargeted metabolomic analyses, two biological replicates were taken and the experiment was temporally repeated (total n = 4 independent biological replicates for each analysis).

6.3.2 Phytohormone extraction and analysis

Phytohormone (JA, JA-Ile, OPDA, SA, and ABA) levels were analyzed by the Proteomics and Mass Spectrometry facility at the Danforth Plant Science Center (St Louis, Missouri, USA) by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Lyophilized plant samples were ground with a TissueLyser (Qiagen) for 2 min at 15 Hz s^{-1}. Hormones were extracted in ice-cold methanol:acetonitrile (MeOH:ACN, 1:1, v/v) from leaf samples spiked with deuterium-labelled internal standards of SA (D5-SA), ABA (D6-ABA), and JA (D2-JA). After centrifugation at 16,000 g, the supernatants were collected and pellet extraction repeated. The pooled supernatants were evaporated and the resulting pellet redissolved in 200 µL of 30% MeOH.

Chromatographic separation of metabolites was accomplished using a monolithic C_{18} column (Onyx, 4.6 mm × 100 mm, Phenomenex) using a mobile gradient of 40% solvent A (0.1% acetic acid in HPLC-grade water, v/v) to 100% solvent B (0.1% acetic acid in 90% acetonitrile (ACN), v/v) in 5 min at a flow rate of 1 mL min^{-1}. A 4000-QTRAP (AB Sciex) was used to acquire MS spectra. Parameters for analysis were set as follows: ESI in the negative mode (TurboIonSpray), capillary voltage -4500, nebulizer gas (N_2) 50 arbitrary units (a.u.), heater gas 50 a.u., curtain gas 25 a.u., collision activation dissociation high, temperature 550 °C. Compounds were detected using multiple reaction monitoring (MRM) transitions that were optimized for each phytohormone and deuterium-labelled standard. Concentrations were determined from standard curves of known phytohormone concentrations.

6.3.3 Glucosinolate extraction and analysis

GSLs were extracted and analyzed following (Kliebenstein et al., 2001). Fifty mg of lyophilized leaf material was ground using a TissueLyser. Following incubation at 90 °C for 10 min to inactivate plant myrosinases, samples were ultra-sonicated for 15 min in 70% MeOH. After centrifugation at 2,975 g for 10 min, the supernatant was transferred to a clean tube and the pellet was re-extracted. Supernatants were pooled and cleaned up using a diethylaminoenthyl Sephadex

A-25 ion exchange column preconditioned with sterile MilliQ water. After washing with 70% MeOH (2 × 1 mL), MilliQ water (2 × 1 mL) and 20 mM sodium acetate buffer, pH 5.5 (1 × 1 mL), GSLs were treated with 10 U of arylsulphatase and incubated at RT for 12 hrs. The desulfated GSLs were eluted in sterile MilliQ water (2 × 0.75 mL) and the eluants were lyophilized.

GSL extracts were separated by high performance liquid chromatography (DIONEX summit HPLC). Compounds were separated on a reverse-phase C18 column (Alltima C18, 150 × 4.6 mm, 3 μm, Alltech) using a mobile gradient from 2% ACN to 35% ACN for 30 min at a flow rate of 0.75 mL min^{-1}. Compounds were detected by a photodiode array detector (DAD) at 229 nm (EC, 1990). GSLs were identified based on retention time, UV spectra and MS spectra. Reference standards of GSLs (glucoiberin (3-methylsulphenylpropyl GSL), glucoerucin (4-methylthiobutyl GSL), progoitrin (2-hydroxy-3-butenyl GSL), sinigrin (2-propenyl GSL), gluconapin (3-butenyl GSL), glucobrassicanapin (4-pentenyl GSL), glucobrassicin (indol-3-ylmethyl GSL), sinalbin (4-hydoxybenzyl GSL), glucotropaeolin (benzyl GSL), and gluconasturtiin (2-phenylethyl GSL)) and sinigrin were included in the HPLC analysis. Correction factors were used to calculate GSL concentrations from the sinigrin standard curve (Buchner, 1987; EC, 1990; Brown et al., 2003).

6.3.4 Gene expression analysis

Total RNA was extracted from leaf tissue samples using an RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. RNA quality and concentration were determined spectrophotometrically (Infinite M200 Pro plate reader, Tecan). The success of DNA degradation was verified by polymerase chain reaction (PCR) using primers designed against an intronic region of ETHYLENE-INSENSITIVE-LIKE2 (*EIL2*) (5'-CAGATTCTATGGATATGTATAACAACAA-3' and 5'-GTAAAGAGCAGCGAGCCATAAAG-3') (Proietti et al., 2010). PCR amplicons were separated on a 1% gel. Genomic DNA was included as a positive control.

The relative transcript expression of MYB transcription factors involved in GSL biosynthesis (*MYB28*, *MYB29*, *MYB76*, *MYB34*, *MYB51* and *MYB122*) was measured by quantitative real time-PCR (qRT-PCR, MX3000p thermo-cycler, Stratagene) using absolute blue SYBR green with low ROX (Fisher Scientific). The qRT-PCR reaction contained 1 x SYBR

green, cDNA (1/10 dilution) and 80 nM of gene-specific forward and reverse primers (Supplemental Table 1). The thermal cycling program was: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec, 58-60 °C for 30 sec (temperature dependent on primer pair, Table 6.1) and 72 °C for 30 sec. The presence of single amplicon was confirmed by a sharp dissociation curve. Samples were analyzed in duplicate and two technical plates were performed.

The relative expression of the target genes was calculated as a ratio ($R0_{GOI}/R0_{REF}$) to the geometric mean of three reference genes (*AtACT2/7, AtUnk, AtUBQ*), where the initial template concentration, R0, is calculated using the formula $R0 = 1/(1+E)^{Ct}$ where E is the average efficiency of gene in the exponential phase and Ct is the threshold cycle (Zhao and Fernald, 2005).

6.3.5 Untargeted metabolite extraction and mass spectroscopy

Metabolites extraction and analysis was conducted as described by De Vos et al. (2007). Lyophilized leaf samples were finely ground using a TissueLyzer (15 Hz s^{-1}) and metabolites extracted in 75% aqueous methanol (MeOH) acidified with 0.125% formic acid (v/v). Following vigorous vortexing (10 s) and sonication (40 kHz, 20 minutes in a water bath maintained at 20 °C), samples were centrifuged at 20,000 g for 10 min and the supernatants transferred to clean tubes. Supernatants were then filtered through 0.2 μm PTFE syringe filters and transferred to HPLC vials.

Metabolites separation and identification was performed by ultra-performance liquid chromatography (UPLC) interfaced with a quadrupole time-of-flight hybrid mass spectrometer (Q-ToF-MS)(Waters) at the Mass Spectrometry Facility at the University of Iowa. Randomized samples were separated on a C_{18} (Waters Acquity BEH, 2.1 x 100 mm, 1.7 μm) column using a gradient solvent at a flow rate of 0.2 mL/min; the mobile phase was increased from 5% acetonitrile (ACN) with 0.1% formic acid to 75% ACN with 0.1% formic acid over 20 min, 75% ACN with 0.1 formic acid was maintained for 5 minutes and then ACN levels were lowered to initial conditions over 1 min and re-equilibrated for 4 min. Column temperature was maintained at 40 °C. For MS detection, negative mode electrospray ionization (ESI) was used and data was collected in the centroid mode by following the procedure described by de Vos et al. (2007). Full scan MS spectra for the ions in the mass range of 100-1500 Da were collected every 900 ms with an interscan delay of 100 ms.

Liquid chromatography-mass spectrometry (LC-MS) processing and metabolite identification

Data pre-processing and alignment was performed with MzMine program (version 2.10) (Pluskal et al., 2010). Briefly, raw data from the Waters Q-ToF-MS was converted to .cdf format and imported to MzMine. Data was filtered using Savitzky-Gravity filter in MzMine, base-corrected and signals were detected in the centroid mode. The chromatogram was built using a m/z tolerance of 0.05 m/z and deconvoluted by using the algorithm Local Minimum Search. Peaks were aligned using the Join aligner algorithm with a m/z tolerance of 100 ppm followed by gap filling. After gap filling, filtration was used to retain the peaks that are present in three out of four biological replicates. The peaks were identified by searching the metabolites against the in-house database built by downloading the metabolites from the PlantCyc database. The m/z tolerance of 5 ppm was allowed during metabolite search and identification. With this tolerance limit, when more than one metabolite was identified per peak, it was removed from further analysis.

6.3.6 Statistical analysis

For hormone, gene expression and GSL analyses, statistically significant differences ($p \leq 0.05$) were identified by conducting a 3-factorial analysis of variance (ANOVA) using SPSS (vers. 20). When significant interactions between CO_2, nitrate- and mechanical damage were detected, the effects of nitrate and wounding were tested separated under each CO_2 regime by 2-way ANOVA followed by Tukey HSD *post-hoc* test (Appendix 6.1).

For the untargeted metabolomic analyses, principal component analysis (PCA) was performed to identify the major factors responsible for metabolite variation in the different treatments. Visible observation of PCA showed tight clustering of the replicates. Covariance PCA was performed to identify the factors contributing to variation; PCA1 and PCA2 together accounted for more than 73.4 % of data variability. To identify metabolites that contribute significantly to this variation, metabolites at the extreme (highest and lowest) values in the loading matrix of PCA1 and 2 were extracted and analyzed by 2-factorial ANOVA to identify the effects of CO_2 and nitrate on metabolite levels under constitutive and induced conditions. To further understand the effects of wounding, metabolites were compared between wounded and control plants at each condition (high *vs* low nitrate at ambient *vs* high CO_2) by a Student's T-test ($p \leq 0.05$) (Appendix 6.2).

6.4 Result and Discussion

6.4.1 Under elevated CO_2 conditions, the jasmonate burst is limited by nitrogen excess.

Under ambient CO_2 levels, JA and JA-Ile levels, representing the strong jasmonate burst, increase in response to wounding (Fig. 6.2A, B; Appendix 6.1). Unexpectedly, however, under conditions of elevated CO_2, this jasmonate burst is not observed when plants are nitrogen fertilized (Fig. 6.2A, B, C), particularly when plants are grown in elevated CO_2 conditions. In a similar fashion, increases in the signaling molecule and biosynthetic precursor to JA, OPDA, are not observed in nitrate-fertilized plants regardless of the CO_2 environment (Fig. 6.2C). Therefore, nitrate fertilization dampens the jasmonate response under conditions of elevated CO_2. Sun et al. (2013) also observed that Arabidopsis plants grown at elevated atmospheric CO_2 levels showed a decline in jasmonate-dependent defenses in response to attack by the peach aphid, *Myzus persicae*. Under ambient CO_2 conditions, ABA levels are higher in response to wounding in plants that were nitrate-fertilized (Fig. 6.2D). Arabidopsis SA levels are higher under elevated CO_2 and not induced in response to mechanical damage (Fig. 6.2E).

6.4.2 Glucosinolate biosynthesis and levels

In response to plant damage, a shift from aliphatic to indoyl GSLs is often observed (Mikkelsen et al., 2003; Reymond et al., 2004; Kos et al., 2012). Therefore, we measured the expression of key MYB transcription factors involved in the regulation of GSL biosynthesis and GSL levels in Arabidopsis grown under different environmental/fertilization conditions in response to wounding. In Arabidopsis, mechanical damage induces the expression of *MYB28* and *MYB29*, responsible for the regulation of genes encoding enzymes in the aliphatic GSL pathway, as well as *MYB 51*, responsible for the regulation of genes encoding enzymes in the indole GSL pathway (Mikkelsen et al., 2003; Schuster et al., 2006; Gigolashvili et al., 2009; Sonderby et al., 2010b). In addition, these two pathways are antagonistic; MYB factors in the indole pathway are believed to downregulate the aliphatic pathway and *visa versa* (Gigolashvili et al., 2008).

Focusing on MYB transcription factors that regulate aliphatic GSL biosynthesis, mechanical wounding induced expression of *AtMYB28, AtMYB29* and *AtMYB76*; however, depending on the MYB factor, this reflected nitrate and atmospheric CO_2 conditions (Fig. 6.3C, Appendix 6.1). For example, wound-dependent *AtMYB28* induction is only observed in plants grown under ambient CO_2 conditions (Fig. 6.3C). Constitutive *AtMYB29* expression levels are

lower under nitrate stress (Fig. 6.3B). With sufficient nitrate fertilization, higher *AtMYB28* levels are found in plants grown at elevated CO_2 levels (Fig. 6.3A). Overall, these results suggest that nitrate-stress negatively affects the constitutive expression of MYB transcription factors that may result in lower aliphatic GSL levels.

MYB51 and MYB34 regulate indole GSL biosynthesis (Frerigmann and Gigolashvili, 2014). Constitutive *AtMYB51* expression is higher under elevated CO_2 conditions and under nitrate-sufficient conditions (Fig. 6.3A, B); however, in comparison with previous reports *AtMYB51* is not induced in response to mechanical damage (Gigolashvili et al., 2007b). However, this result is consistent with Millet et al. (2010) and Frerigmann and Gigolashvili, (2014) who show that *AtMYB51* expression is suppressed by JA. *MYB34* expression is upregulated in response to wounding (Fig. 6.3C). *AtMYB122* expression levels are below the detection limits.

GSLs are sulfur- and nitrogen-rich secondary metabolites. Therefore, the CNB hypothesis predicts that GSL levels are lower under nitrogen-limiting or CO_2-rich conditions (Bryant et al., 1983). Overall, foliar levels of aliphatic GSLs are slightly elevated in plants grown nitrate-stress conditions (Fig. 6.4; Appendix 6.1); for example, levels of the aliphatic 3C class 3-methylsulfinylpropyl GSL (glucoiberin (IBE)), under ambient CO_2 conditions, and the aliphatic 5C class 5-methylsulphinylpentyl GSL (glucoalyssin (ALY)) increase under nitrate stress at both levels of CO_2 (Fig. 6.4 A, C). In contrast, in plants grown under enriched CO_2 conditions, constitutive foliar levels of the aliphatic 4C class of GSL 4-methylthiobutyl GSL (glucoerucin (ERU)) are lower under nitrate-limiting conditions (Fig. 6.4B), supporting the CNB hypothesis. Nitrate levels do not affect indole GSL levels (Fig. 6.5; Appendix 6.1). Unlike aliphatic GSLs, atmospheric CO_2 rather than nitrate appears to play a greater role in influencing indole GSL levels. Increased foliar levels of 4-methyoxy-3-indolylmethyl GSL (4-methoxyglucobrassicin (4MeOGB)) and 1-methoxy-3-indolylmethyl GSL (neo-glucobrassicin (NeoGB)) levels and lower levels of their biosynthetic precursor 3-indolylmethyl GSL (glucobrassicin (GBC)) are observed in Arabidopsis grown under elevated CO_2 conditions (Fig. 6.4A, B, C); this suggests increased flux toward later indole GSLs under enriched CO_2 levels.

Overall, total foliar GSL levels increased in response to mechanical damage (Fig. 6.6A; Appendix 6.1). Under ambient CO_2 conditions, this increase reflects higher indole GSL levels. In

contrast, in plants grown in elevated CO_2 conditions, wounding only increases total GSL levels in the leaves of nitrate-stressed plants; this reflects an increase in both aliphatic and indole GSLs. When individual GSLs are analyzed, in *Arabidopsis* plants grown under ambient CO_2 conditions, levels of the aliphatic GSLs, 4-methylsulfinylbutyl GSL (glucoraphanin (RAPH)) and 5-methylsulphinylpentyl GSL (glucoalyssin (ALY)) increase when plants are damaged, glucoalyssin being increased only at nitrogen-stress condition (Fig. 6.4C, D; Appendix 6.1). However, in plants grown at elevated CO_2 levels, mechanical wound-induced increase in the levels of both glucoalyssin and glucoraphanin is only seen in nitrate-stressed plants (Fig. 6.4C, D). Indole GSLs, such as 3-indolylmethyl GSL (GBC) and 1-methyoxy-3-indolylmethyl GSL (neoglucobrassicin (NeoGB)) are induced in response to wounding (Fig. 6.5 A, C; Appendix 6.1). Unexpectedly, levels of neoglucobrassicun are only increased in nitrate-stressed plants. This likely represents different indole GSLs upregulated in response to different conditions. At all CO_2 levels tested, levels of Meothoxyglucobrassin do not increase in response to mechanical damage (Fig. 6.5B).

6.4.3 Untargeted metabolomics

Metabolites identified under different treatment conditions are listed in Appendix 6.2. Principal component analysis (PCA) was used to analyze identified compounds to determine the effect of CO_2 x nitrate x wounding on constitutive and wound-induced metabolites (Fig. 6.7). PCA1 and PCA2 together accounted for 73.4 % of the variability. Atmospheric CO_2 levels are a main contributing factor accounting for this variance. In further analysis, the 10 metabolites representing the extremes (positive and negative) of the PCA loading matrix were compared (Appendix 6.3).

Constitutive metabolites

Two long-chain aliphatic GSLs, 8-methylthiooctyl GSL and 7-methylthioheptyl GSL, are significantly higher in plants grown at elevated CO_2. The flavonoid isovitexin-7-*O*-glucosyl-2"-*O*-rhamnoside is also elevated under this condition (Appendix 6.3). Isovitexin metabolites are associated with pest and pathogen resistance and elevated levels have previously been found in wheat leaves grown under elevated CO_2 (Estiarte et al., 1999). Two precursors to important plant hormones, the cytokinin *trans*-zeatin precursor *trans*-zeatin riboside monophosphate and a lipid

precursor in JA biosynthesis, (*9Z*, 11*E*, 15*Z*)-(13*S*) hydroperoxyoctadeca-9,11,15-trienoate, are identified in plants grown under high CO_2 conditions (Appendix 6.3).

In contrast, the GSLs 3-indolylmethyl-GSL, 3-butenyl GSL and 8-methylsulfinyloctyl GSL and the flavanoids kaempferol-3-*O*-gentiobioside-*O*-rhamnoside and kaempferol-3-*O*-[6-(4-coumaroyl)-β-D-glycosyl-(1-2)-β-D-glucoside are higher in plants grown at ambient CO_2 (Appendix 6.3). In the aliphatic GSL biosynthetic pathway, thioalkyl GSL are oxidized to the sulphinyl form and then converted to the alkyl form (Halkier and Du, 1997), therefore, our results suggest that under elevated CO_2, flux through the GSL aliphatic pathway is slowed down at the oxidation step and as a result there is an accumulation of the thioalkyl GSLs. These results also suggest that there may be a shunt at naringenin from kaempferol-related flavanols to apigenin-derived vitexin-type compounds (i.e. isovitexin) as CO_2 levels increase. Sirohydrochlorin, a precursor in sulfur and iron metabolism (Tripathy et al., 2010; Saha et al., 2012), is only identified in plants grown at ambient CO_2 levels.

Other metabolites showed a C x N interaction. At ambient CO_2 levels, a number of metabolites exhibit changes in response to nitrogen levels (Appendix 6.3). 5-methylthiopentyl GSL, (*1R,6R*) 6-hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate and 5-amino-6-(5-phospho-D-ribosylamino) uracil levels are higher at elevated CO_2, however, these metabolites are responsive to high nitrate conditions under ambient CO_2 . In contrast, levels of 4-methylsulfinylbutyl GSL, 2-phenylethyl GSL and 6-methylsulfinylhexyl GSL are responsive to nitrate-stress conditions. GSL increases under nitrate-stress were unexpected since GSLs are recognized as N-rich compounds, therefore, one would predict that levels would be lower. (*1R,6R*) 6-hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate is involved in quinone biosynthesis and 5-amino-6-(5-phospho-D-ribosylamino) uracil is a precursor in flavin biosynthesis (Nowicka and Kruk, 2010; Klein et al., 2013). Under ambient CO_2 levels, higher levels of the cofactor flavin mononucleotide and (-)-3,4-dihydroxyphenylacetate are detected; however, they are responsive to nitrate-stress condition under elevated CO_2 (Appendix 6.3).

Wound-regulated metabolites

The effect of CO_2 and nitrate fertilization on wound-induced metabolites will be described in two parts. Firstly, for the different sets of treatments, identification of induced compounds will be presented (Appendix 6.2). Then, a comparison of treatment effects on wound-induced

metabolites will be discussed (Appendix 6.3). Under conditions of ambient CO_2 and sufficient nitrate fertilization, noteworthy changes in GSL levels are observed in response to mechanical damage (Appendix 6.2); flux is redirected from constitutive aliphatic GLSs, such as 3-butenyl GLS, 3-methylthiopropyl GSL, 5-methylthiopentylhydroximoyl-cysteinylglycine and 8-methylthiooctyl GSL, into indole GSLs in mechanically-damaged plants.

We would predict that if plants are subjected to nitrate-stress under conditions of ambient CO_2, there would be a shift to carbon-based compounds, particularly in response to stress. In undamaged plants, diverse secondary metabolites are present representing phenylpropanoids (i.e. 5-hydroxycaffeate, (-)-medicarpin-3-O-glucoside-6"-malonate, diprenylphlorisovalerophenone) as well as GSLs (6-methylthiohexyl GSL and indolylmethyl-desulfoGSL) (Appendix 6.2). In response to wounding, there is a strong downregulation of the aliphatic GSL pathway.

Under elevated CO_2 and nitrate-sufficient conditions, lower numbers of constitutive GSL metabolites are detected. As well, increased levels of the lignin precursor coniferaldehyde glucoside is found which may indicate higher carbon-based compound levels. In wounded plants, a strong presence of GSL-related compounds suggested an upregulation of this pathway. In nitrate-stressed plants, there are comparatively more metabolites related to carbon-based secondary metabolites, such as terpenoids. As well, a number of precursors in the jasmonate biosynthetic pathway are identified in these plants. When the plants are mechanically damaged, an increase in terpenoid and kaempferol biosynthetic intermediates are identified and, compared to wound-induced metabolites in the elevated CO_2, nitrate-sufficient plants, less GSL intermediates are detected in these nitrate-stressed plants.

Statistical comparison of wound-inducible metabolites between the four treatments indicated that CO_2 levels affected the presence of four metabolites (Appendix 6.3): Higher levels of the 8-methysulfinyloctyl GSL and the flavonoid isovitexin-7-*O*-glucosyl-2"*O*-rhamoside and lower levels of two metabolites in the flavonoid kaempferol biosynthetic pathway, kaempferol-3-gentiobioside-7-*O*-rhamnoside and kaempferol 3-*O*-[6-(4-coumaroyl)-β-(1→2)-glycosyl (1→2)glucosyl-β-glucoside are found in mechanically-damaged plants grown in elevated CO_2 conditions. Elevated atmospheric CO_2 or nitrate-levels had a positive effect on 5-methylthiopentyl GSL levels. Four wound-inducible GSL metabolites showed a significant C x N interaction. Levels of 7-methylthioheptyl GSL, 8-methylthiooctyl GSL and 3-

methylsulfinylpropyl GSL are positively affected by elevated CO_2 and high nitrate conditions. In contrast, 6-methylsulfinylhexyl GSL is not detected in wounded plants grown under ambient CO_2 and nitrate-sufficient levels.

6.5 Conclusions

As expected, growth conditions such as elevated CO_2 levels and/or nitrate-stress affected the constitutive Arabidopsis metabolite profile; however, as illustrated by the PCA, CO_2 levels had a greater effect than nitrogen-limitation (Fig. 6.7). The GSL profile of plants grown in ambient CO_2 conditions (440 ppm) contained more sulfinylalkyl GSLs compared to those grown at double the CO_2 concentration (880 ppm) that had more thioalkyl GSLs; this suggests that plants grown at elevated CO_2 levels slow down aliphatic GSL flux at the oxidation step (Fig. 6.1, Appendix 6.3). The metabolomic profile also suggests that there is a shunt at naringenin from kaempferol-based flavanols into apigenin-type vitexin-related compounds under these elevated CO_2 conditions. Unexpectedly, at ambient CO_2 levels, constitutive levels of a number of aliphatic GSLs are higher in plants under nitrate-limitation, particularly sufinylalkyl GSLs (Fig. 6.4A, C; Appendix 6.2). As well, even though overall indole GSL levels do not change, under conditions of elevated CO_2 levels, the 3-indolylmethyl GSL (glucobrassicin) is converted to 1-methyoxy-3-indolylmethyl GSL (neo-glucobrassicin) and 4-methoxy-3-indolylmethyl GSL (4-methyoxyglucobrassicin) (Fig. 6.5A, B, C).

In Arabidopsis, a complex interplay of phytohormone pathways hone plant responses to stresses, such as wounding, by affecting MYB transcription factors that regulate aliphatic and indole GSL biosynthesis (Gigolashvili et al., 2009; Sonderby et al., 2010a; Frerigmann and Gigolashvili, 2014). In the aliphatic GSL pathway, mechanical damage and MeJA treatment positively regulate *AtMYB29* expression, while SA treatment has an antagonistic effect (Gigolashvili et al., 2008). Wounding also induces *AtMYB76* expression (Gigolashvili et al., 2008). In the indole GSL pathway, mechanical damage of Arabidopsis induces *AtMYB51* expression (Gigolashvili et al., 2007b); *AtMYB51* transcription is inducible by ethylene and SA and suppressed by JA (Millet et al., 2010; Frerigmann and Gigolashvili, 2014). In contrast, in this pathway, JA, ABA and MYB51 positively regulated *AtMYB34* expression whereas transcription is suppressed by the ethylene-dependent ERF1 transcription factor (Frerigmann and

Gigolashvili, 2014). *AtMYB122* expression is positively regulated by MYB51 and negatively regulated by MYB34; in our experiment, *AtMYB122* expression is below detection limits.

Foliar damage of Arabidopsis resulted in a rapid jasmonate burst, but this increase in JA and JA-Ile levels is stronger in nitrate-stressed plants grown under elevated CO_2 conditions (880 ppm) (Fig. 6.2A, B). As well, under these conditions (CO_2 880 ppm, limited nitrate), constitutive levels of JA biosynthetic precursors 13(*S*)-hydroperoxy-9(*Z*), 11(*E*), 15 (*Z*)-octadecatrienoate and OPC6-3-hydroxyacyl-CoA are also elevated (Appendix 6.2). As expected, foliar GSL levels closely mirrored the jasmonate burst (Fig 6.2A, Fig. 6.6A). At ambient CO_2 levels, the increase in total GSL in wounded plants reflected an increase in indole GSLs. At elevated CO_2 levels, an increase in total GSL is only observed in nitrate-stressed plants and reflected by an increase in both aliphatic- and indole-related GSL compounds. MYB transcription factors regulate GSL biosynthesis: Aliphatic GSLs by AtMYB28, AtMYB29 and AtMYB76 and indole GSLs by AtMYB34, AtMYB51 and AtMYB122. As expected from the literature, wounding induced expression of *AtMYB29*, *AtMYB76, AtMYB34* as well as *AtMYB28* but induction of this last gene is only observed in plants grown at ambient CO_2 levels (440 ppm) (Fig. 6.3C) (Gigolashvili et al., 2007a; Gigolashvili et al., 2007b; Gigolashvili et al., 2008). Suppression of *AtMYB29* is observed in nitrate-limited plants (Fig. 6.3B); this is unexpected since wounding and MeJA treatments are thought to positively regulate *AtMYB29* expression and a higher jasmonate burst is observed in nitrate-limited plants (Fig. 6.2A-C) (Gigolashvili et al., 2008). Since ethylene and SA positively regulate *AtMYB51*, increased constitutive SA levels may explain the enhanced expression of *AtMYB51* in plants grown at elevated CO_2 levels (880 ppm) (Fig. 6.2E, 6.3A).

Even though there is a wound-induced increase in expression of MYB factors that regulate both aliphatic and indole GSLs, overall, in response to wounding, there is a shift from aliphatic to indole GSLs. Under conditions of ambient CO_2 (440 ppm), the increase in total GSLs in response to wounding reflects an increase in indole GSLs (Fig 6.6A). Untargeted metabolomic analysis shows that plants grown under sufficient nitrate levels have lower 3-butenyl GSL and elevated secologanin levels when damaged. If plants are nitrate-stressed, even though an increase in 4-methylsulfinylbutyl GSL (RAPH) levels is observed (Fig. 6.4D), a decrease in the levels of a number of aliphatic GSLs in response to wounding was detected by untargeted metabolomics (Appendix 6.2 and 6.3). The increase in indole GSLs is primarily reflected in increased levels of the biosynthetically related GSLs 3-indolylmethyl GSL (GBC) and 1-methyoxy-3-indolylmethyl

GS (NeoGB) in response to foliar damage (Fig. 6.5A, C); the increase in 1-methyoxy-3-indolylmethyl GS is only observed in nitrate-stressed plants. In plants grown under elevated CO_2 (880 ppm), a wound-induced increase in aliphatic and indolyl GSLs is observed in nitrate-stressed plants (Fig. 6.4C and 6.5C); even though under nitrate-sufficient fertilization, an increase in the number of aliphatic and indoyl GSLs is noted (Fig. 6.5A, Appendix 6.2, Appendix 6.3B). Therefore, results indicate that at ambient CO_2, there is a suppression of the aliphatic pathway that may be mediated by MYB34; this suppression is alleviated in plants grown at higher CO_2 levels that are under nitrate-stress.

Evaluating the CNB hypothesis is inherently difficult based on defining carbon-based vs nitrogen-based metabolites. Plant cellular metabolism depends on photosynthesis and, hence, nitrogen for chlorophyll and enzymes, such as RUBISCO. That said, secondary metabolites such as alkaloids and GSLs are regarded as nitrogen-based whereas phenolics and terpenoids are considered carbon-based. With this crude delineation, in response to wounding of plants grown at ambient CO_2 (440 ppm), though total indole GSLs increase, lower numbers of aliphatic GSLs and more intermediates in carbon-based flavonol pathways are found in plants that are nitrate-stressed (Fig. 6.4, Appendix 6.2). At elevated CO_2 levels, a wound-induced increase in aliphatic and indole GSLs is only observed in nitrate-limited plants (Fig. 6.6A). In parallel to findings at ambient CO_2, the diversity of wound-induced GSL-related compounds is less and more metabolites in terpenoid and flavanol pathways are observed in nitrate-stressed plants (Appendix 6.2). This is somewhat consistent with the CNB hypothesis and illustrates the trade-offs that must be taken into account. Indole GSLs are effective against caterpillar herbivores that wound the plant during attack (Gigolashvili et al., 2007b; Agerbirk et al., 2009). Therefore, the plant has a strong selective pressure to maintain this defensive strategy even if it "steals" resources from other pathways. However, when plants are nitrate-stressed, though GSLs still increase, the diversity in compounds is not maintained.

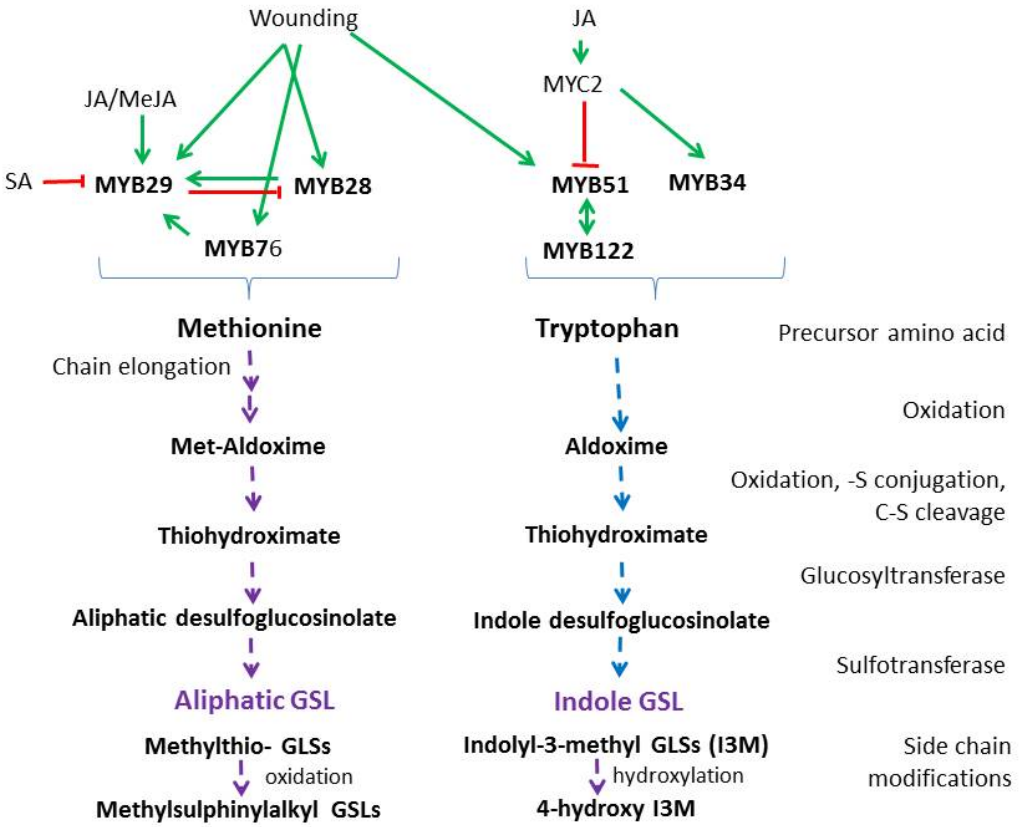

Figure 6.1. Regulation of glucosinolate biosynthesis by MYB transcription factors. Wounding as well as induction of stress hormones, jasmonic acid (JA), methyl-JA (MeJA) and salicylic acid (SA) modulate the activity of MYB transcriptions factors (TFs) that regulate glucosinolate biosynthesis (Gigolashvili et al., 2009). Activated MYB28/29/76 regulate genes that are involved in aliphatic GSLs biosynthesis, while MYB51/34/122 regulate indole GSL biosynthesis pathway genes. In the first two lanes, the purple and blue dotted lines show steps leading to biosynthesis of aliphatic and indole GSLs, respectively. Different enzymatic reactions involved in GSL biosynthesis process are outlined at the right lane. (Adapted from Gigolashvili et al., 2009)

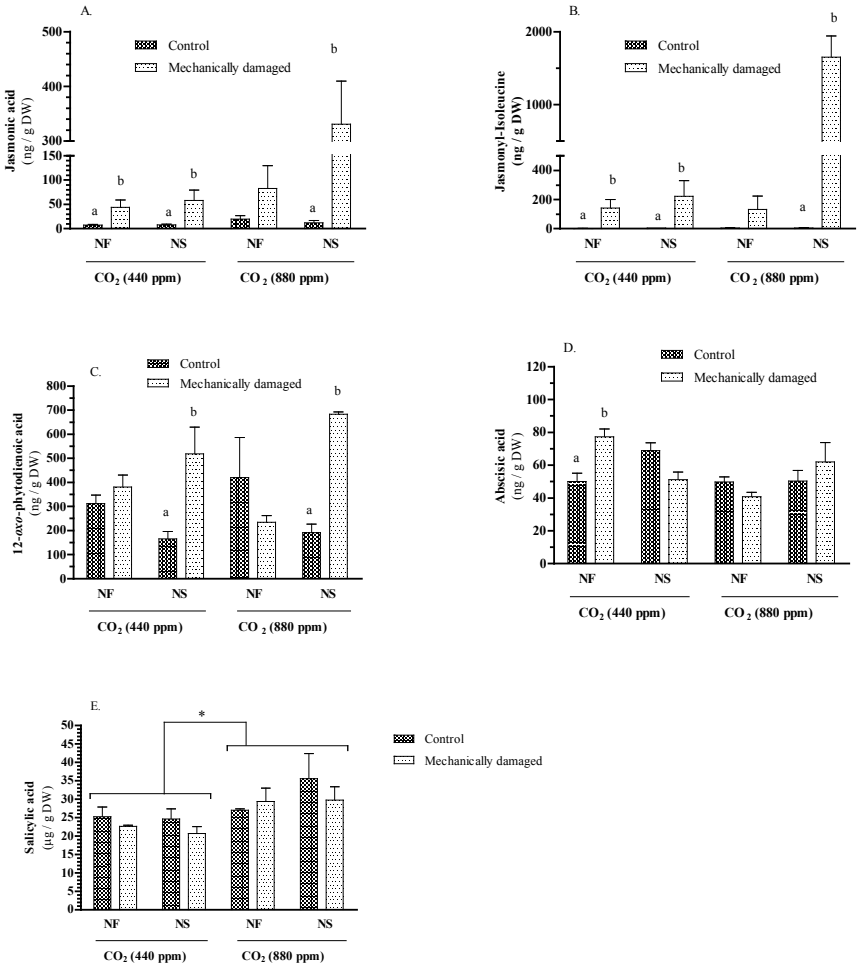

Figure 6.2. Effect of atmospheric carbon dioxide levels, nitrate-limitation and wounding on *Arabidopsis* foliar phytohormones. 6 week old Arabidopsis plants were grown under ambient (440 ppm) or elevated (880 ppm) carbon dioxide (CO_2) levels and limited (nitrate-stressed; NS) or sufficient (nitrate-fertilized; NF) nitrogen fertilization. Phytohormone levels were compared in rosette leaves collected from control and wounded plants. **A)** Jasmonic acid (JA) **B)** 7-Jasmonoyl-L-isoleucine (JA-Ile) **C)** 12-*oxo*-phytodienoic acid (OPDA) **D)** Abscisic acid (ABA) **E)** Salicylic acid (SA). The experiment was repeated 2 times with two biological replicates analyzed for each experimental replicate. Statistical differences were determined by 3-factor Analysis of Variance (ANOVA) (Appendix 6.1A). When interactions were significant, data were separated to show treatment effects. Significant difference between control and mechanical wounding is indicated by alphatical letters. An asterix indicates significant difference between the grouped variables.

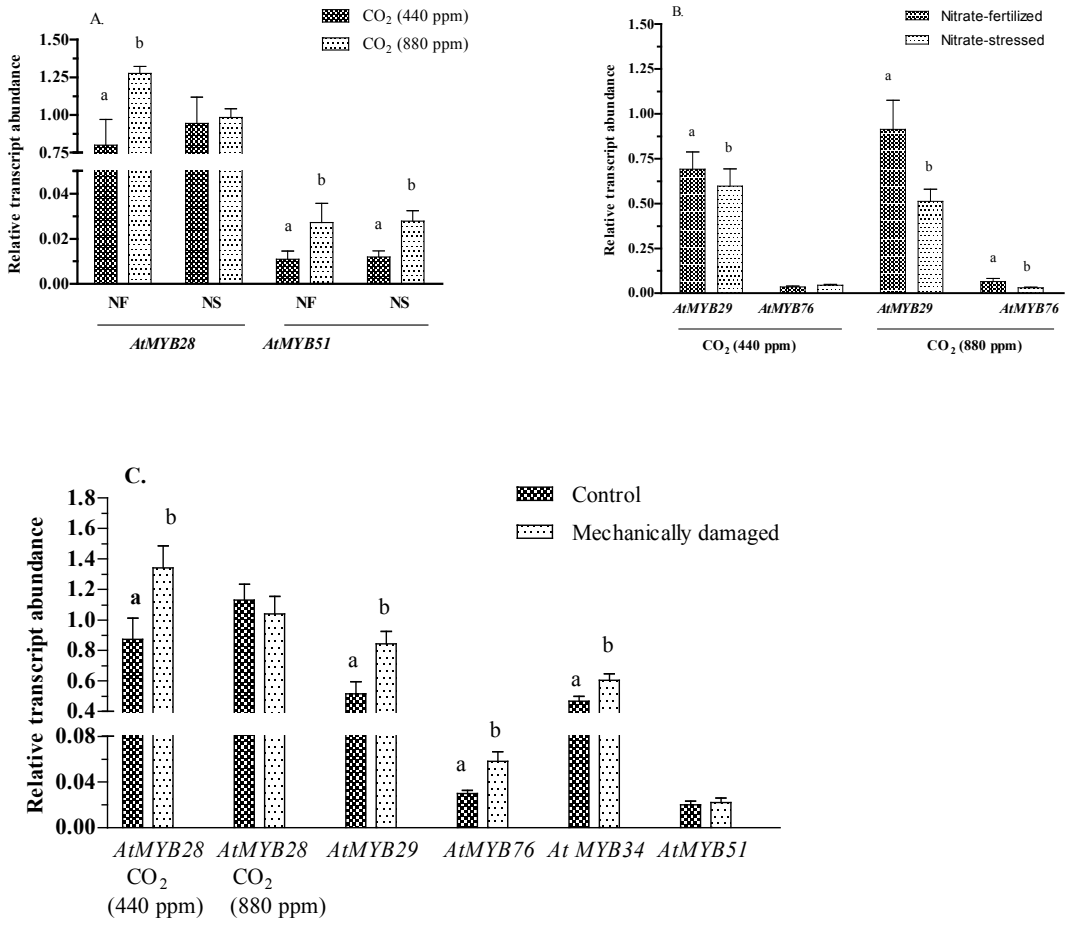

Figure 6.3. Effect of atmospheric carbon dioxide levels, nitrate-limitation and wounding on foliar *Arabidopsis* MYB transcription factor gene expression. MYB28, MYB29, MYB34 MYB51 and MYB76 transcription factors regulate glucosinolate biosynthesis in Arabidopsis. 6 week old Arabidopsis plants were grown under ambient (440 ppm) or elevated (880 ppm) carbon dioxide (CO_2) levels and limited (nitrate-stressed; NS) or sufficient (nitrate-fertilized; NF) nitrogen fertilization. *AtMYB* gene expression was compared in rosette leaves collected from control and wounded plants (n=5). The geometric mean of three reference genes, *AtACT2, AtUNK2* and *AtUBQ*, was used to normalize the expression of the genes-of-interest. **A)** CO_2 levels **B)** nitrate fertilization and **C)** mechanical damage. Statistical differences were determined by 3-factor Analysis of Variance (ANOVA) (Appendix 6.1B). When interactions were significant, data were separated to show treatment effects and significant differences noted by alphabetical letters.

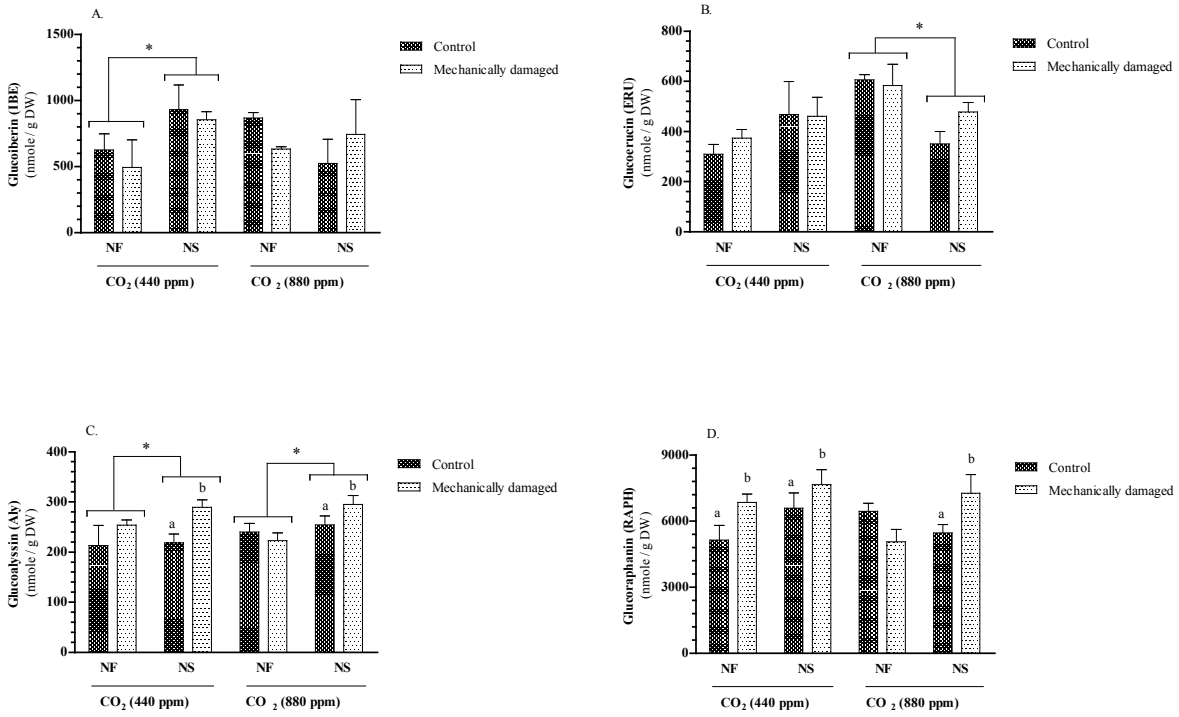

Figure 6.4. Effect of elevated carbon dioxide, nitrogen-limitation and wounding on *Arabidopsis* foliar aliphatic glucosinolate levels. 6 week old *Arabidopsis thaliana* plants were grown under ambient (440 ppm) or elevated (880 ppm) carbon dioxide (CO_2) levels and limited (nitrate-stressed; NS) or sufficient (nitrate-fertilized; NF) nitrogen fertilization. Aliphatic glucosinolate (GSL) levels were compared in rosette leaves collected from control and wounded plants. **A)** Glucoiberin (IBE), **B)** Glucoerucin (ERU), **C)** Glucoalyssin (Aly) and **D)** Glucoraphinin (RAPH). The experiment was repeated 2 times with two biological replicates analyzed for each experimental replicate. Statistical differences were determined by 3-factor Analysis of Variance (ANOVA) (Appendix 6.1C). When interactions were significant, data were separated to show treatment effects. Significant difference between control and mechanical wounding is indicated by alphatical letters. An asterix indicate significant difference between the grouped variables.

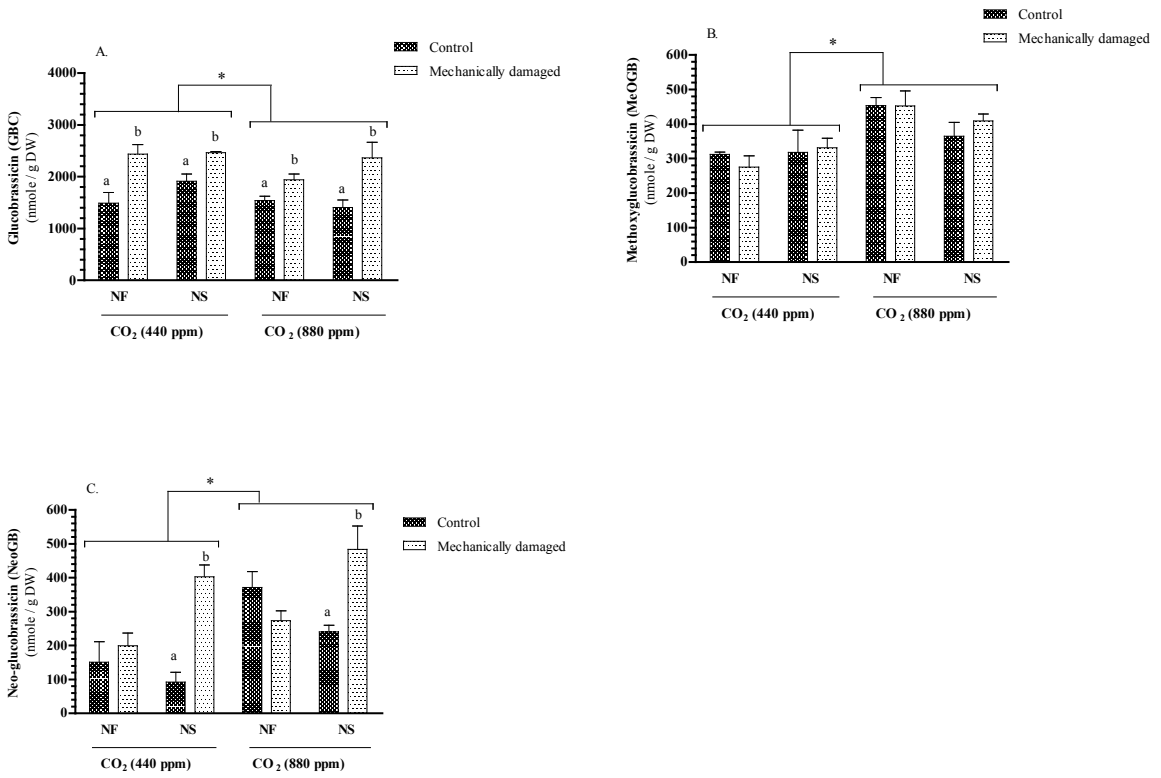

Figure 6.5. Effect of elevated carbon dioxide, nitrogen-limitation and wounding on *Arabidopsis* foliar indole glucosinolate levels. 6 week old *Arabidopsis thaliana* plants were grown under ambient (440 ppm) or elevated (880 ppm) carbon dioxide (CO2) levels and limited (nitrate-stressed; NS) or sufficient (nitrate-fertilized; NF) nitrogen fertilization. Indole glucosinolate (GSL) levels were compared in rosette leaves collected from control and wounded plants. **A)** Glucobrassicin (GBC), **B)** Methoxyglucobrassicin (MeOGB) and **C)** Neo-glucobrassicin (NeoGBC). The experiment was repeated 2 times with two biological replicates analyzed for each experimental replicate. Statistical differences were determined by 3-factor Analysis of Variance (ANOVA) (Appendix 6.1C). When interactions were significant, data were separated to show treatment effects. Significant difference between control and mechanical wounding is indicated by alphatical letters. An asterix indicate significant difference between the grouped variables.

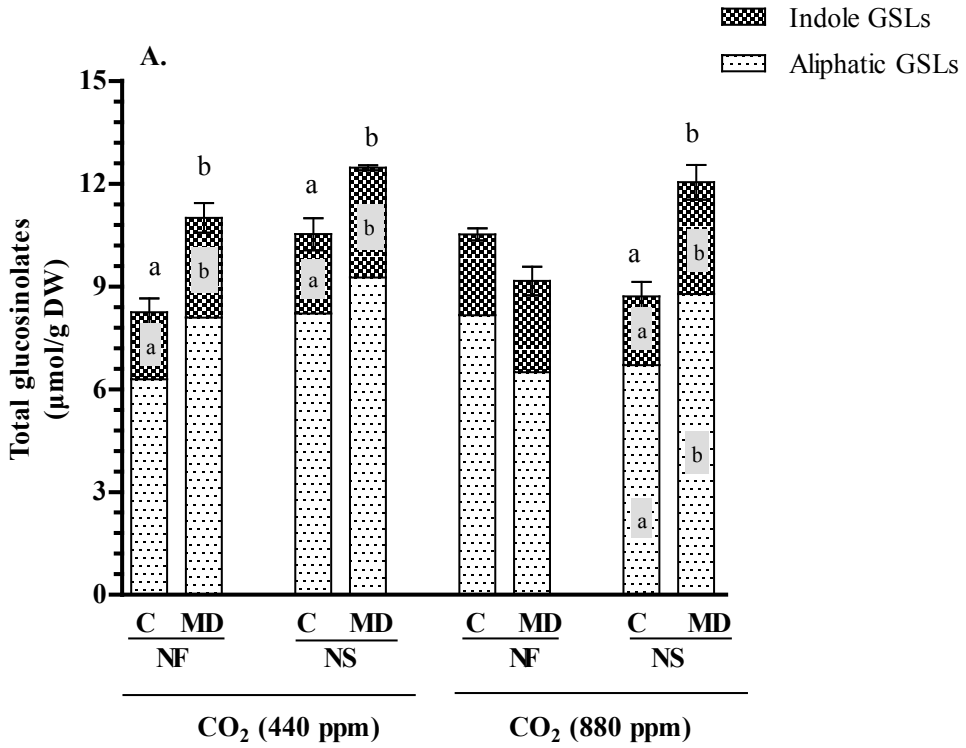

Figure 6.6. Effect of elevated carbon dioxide levels, nitrogen-limitation and wounding on *Arabidopsis* total foliar glucosinolate levels. 6 week old *Arabidopsis thaliana* plants were grown under ambient (440 ppm) or elevated (880 ppm) carbon dioxide (CO_2) levels and limited (nitrate-stressed; NS) or sufficient (nitrate-fertilized; NF) nitrogen fertilization. Glucosinolate (GSL) levels were measured in rosette leaves of the control (untouched, C) and mechanically damaged plants (MD). **A)** Total GSL are represented by aliphatic GSL (dotted bar) and indole GSL (hatched bar). The experiment was repeated 2 times with two biological replicates analyzed for each experimental replicate. Statistical differences were determined by 3-factor Analysis of Variance (ANOVA) (Appendix 6.1C). When interactions were significant, data were separated to show treatment effects and significant differences noted by alphabetical letters (alphabetical letters inserted into to the bars represent significance for aliphatic GSL (dotted bar) or indole GSL (hatched bar)).

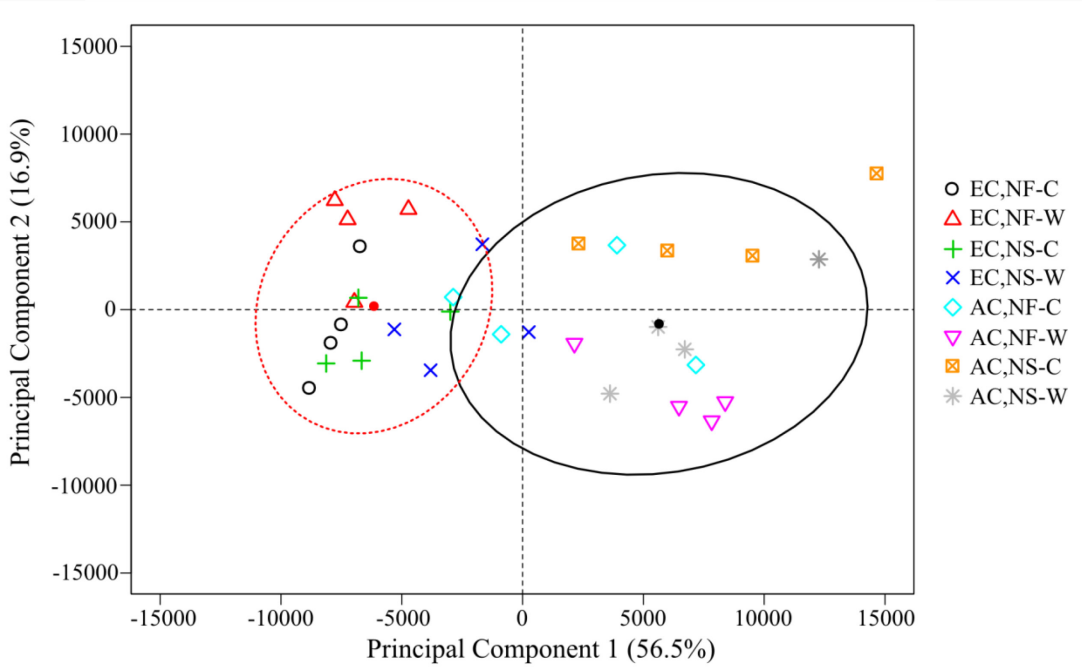

Figure 6.7. Principal component analysis biplot of untargeted metabolites identified in *Arabidopsis thaliana* grown at different levels of carbon dioxide- and nitrate-fertilization and subject to mechanical damage. 6 week old Arabidopsis plants were grown under ambient (440 ppm) or elevated (880 ppm) carbon dioxide (CO_2) levels and limited (nitrate-stressed) or sufficient (nitrate-fertilized) nitrogen fertilization. Metabolite profile of rosette leaves collected from control and wounded plants were compared by high performance liquid chromatography-quantitative time-of-flight mass spectroscopy (LC-qToF). Principal component analysis (PCA) plot illustrates the profile of 132 annotated metabolite peaks. PCA1 and PCA2 together accounted for 73.4 % of the variability. A 90% probability density ellipse, including centroids, have been drawn around the data belonging to elevated (red circle) and ambient CO_2 (black circle). EC: elevated CO_2; AC: ambient CO_2; NF: nitrate-fertilized; NS: nitrate-stressed; C: control; W: mechanically wounded.

Table 6.1. Primers for quantitative real time-polymerase chain reaction (qRT-PCR).

Gene	Annealing temperature (°C)	Forward (5'-3')	Reverse (5'-3)	Ref.
Detection of genomic contamination:				
AtEIL2		CAGATTCTATGGATATGTATAACAACAA	GTAAAGAGCAGCGAGCCATAAAG	**Proietti et al. (2011)**
Reference Genes:				
*AtACT2 (*At5g09810)	60	GTATGCTCTTCCTCATGCTATCCTT	TTCCCGTTCTGCGGTAGTG	**Beste *et al*. (2011)**
AtUnk (At4g26410)	60	GAGCTGAAGTGGCTT CCATGAC	GGTCCGACATACCCATGATCC	**Czechowski et al. (2005)**
AtUBC (At5g25760)	58	GCAGTTGACAATTCG TTCTCT	GAGCGGTCCATTTGAATATGTT	**Primer3**
Genes-of-interest:				
AtMYB28	57	TCTGATTAGGGTTGAAACGGTGTGG	CGACCACTTGTTGCCACGAGA	**Primer3**
MYB29	58	GGCAACAAGTGGTCAGTCATAGCG	TTGAGTCATAGGCAAGTGGCTTGTG	**Primer3**
MYB76	57	TCGTGGCAATAAGTGGTCTGTCATA	GGGTTAGAAGAAGCTAGTGGCTTGT	**Primer3**
MYB34	57	TAAGGGTAACAAGTGGGCCGC	GATGCCTTTTTGCTTCAACCGCT	**Primer3**
MYB51	57	TCACGGCAACAAATGGTCTGCT	CGGTACCGGAGGTTATGCCC	**Primer3**
MYB122	58	CATGGCAACAAATGGTCGGCC	CCGGCTCCATCGAGAAGGGAT	**Primer3**

CHAPTER 7. General Conclusions and Future Directions

7.1 General Conclusions

Plants induce complex networks of phytohormone signaling pathways in response to caterpillar herbivory (Pieterse et al., 2012). Caterpillar wounding or enzymes, such as glucose oxidase (GOX), present in the caterpillar labial saliva lead to the production of reactive oxygen species (ROS) (Maffei et al., 2006; Eichenseer et al., 2010). At controlled level, ROS function as second messengers for the activation of downstream JA-biosynthesis and JA-dependent signaling pathways (Howe and Jander, 2008; Wu and Baldwin, 2009). However, at higher level, ROS is detrimental to cellular components; thus, plants activate enzymatic and non-enzymatic antioxidant systems, such as the ascorbate-glutathione cycle, to maintain the cellular redox balance (Schröder et al., 2008; Forman et al., 2010; Noctor et al., 2012). In response to pathogen infection and caterpillar herbivory, changes in the glutathione pool and a shift in the ratio of oxidized-to-reduced glutathione are often observed (Schlaeppi et al., 2008; Mhamdi et al., 2010; Noctor et al., 2012). Thus, glutathione is linked to regulating defense responses directly or indirectly by modulating signaling responses via post-translational modification of proteins, such as glutathionylation or *S*-nitrosylation (Spoel and Loake, 2011; Han et al., 2013). To study the early redox response of the host plant, reduced and oxidized levels of ascorbate and glutathione were measured in *Spodoptera exigua* caterpillar infested *Arabidopsis thaliana* (Chapter 3) and *Medicago truncatula* (Chapter 4) plants. In Chapter 3, *S. exigua* caterpillar feeding did not modify the levels of ascorbate in *A. thaliana*; however, there was a decrease in the level of oxidized glutathione (GSSG) in Arabidopsis leaves upon caterpillar feeding and the level of total glutathione was reduced when caterpillar labial saliva was impaired (Paudel et al., 2013). Thus, labial saliva of the caterpillar is shown to be involved in maintaining a reduced cellular environment in wounded plant tissues. On the other hand, the ratio of oxidized-to-reduced ascorbate (DHA:ASC) and the level of GSSG increases in *M. truncatula* leaves infested by caterpillar with intact labial salivary secretions (Chapter 4). Therefore, as an early response to caterpillar feeding, labial saliva secretions leads to the induction of oxidative stress in the legume *M. truncatula*. Thus, the responses of these two plants to caterpillar feeding lead to distinct responses. Caterpillar mediated induction of oxidative stress is alleviated in the ethylene-

insensitive *skl* mutant suggesting that ethylene is involved in the modulation of early plant responses to caterpillar herbivory.

JA-mediated IR is the principal defense mechanism in host plants against caterpillar herbivory, although the ET-dependent pathway functions in parallel either to enhance or attenuate JA responses (Leon-Reyes et al., 2013; Wasternack and Hause, 2013). Meanwhile, caterpillars have also evolved mechanisms to circumvent host IR (Zhu-Salzman et al., 2005). Although, there are different views regarding how effectors present in caterpillar oral secretions (OS) lead to suppression of IR, labial saliva-specific production of H_2O_2 due to caterpillar feeding is believed to be one of the mechanisms (Musser et al., 2002; Diezel et al., 2009; Weech et al., 2008, Paudel et al., 2013, Lan et al., 2014). Activation of the antagonistic SAR pathway or the inhibition of the ethylene burst by GOX present in the labial saliva of *S. exigua* caterpillar is responsible for the suppression of JA-mediated IR in *A. thaliana* and *N. tabacum*, respectively (Weech et al., 2008; Diezel et al., 2009). In Arabidopsis, the expression of *AtPR1,* a marker gene of the SAR pathway, was induced upon feeding by caterpillar with intact salivary secretions, while a marker gene of the JA/ET pathway, *AtPDF1.2,* was only induced in response to caterpillars with impaired labial salivary secretions. Thus, caterpillar labial saliva is involved in the subversion of JA-mediated plant defense by activating the antagonistic SA pathway. However, this labial saliva specific modulation of genes was abolished in the glutathione-deficient *pad2.1* and *tga2/5/6* mutants of *A. thaliana*. Also, ET-dependent genes, *ERF1* and *AtHEL*, were modulated by caterpillar labial saliva in a glutathione-independent way. Thus, labial saliva of caterpillar modulates the expression of defense-related genes in SA/NPR1-, glutathione-dependent and ET-, glutathione-independent manners.

In Chapter 4, by using wild type and the ET-insensitive *skl* mutant of *M. truncatula*, we showed that ET-insensitivity does not affect the induction of the jasmonate burst and the expression of the JA-dependent *MtVSP* gene in response to caterpillar herbivory. However, caterpillar labial saliva-specific expression of *MtPR1* in the wild type plant was abolished in the *skl* mutant. This showed the potential role of ET in enhancing the induction of the antagonistic SAR pathway that affects the full induction of JA-dependent defense responses upon caterpillar feeding. It is further supported by labial saliva-specific suppression of the defense protein TI in an ET-dependent manner. Thus, the study elucidated the importance of ET in the modulation of host response during caterpillar herbivory.

Since plant responses to caterpillars occurs within minutes of herbivory, post-translational modifications of proteins may be responsible for dynamic changes in metabolic flux and integration of hormonal network in signaling cascades (Seo and Lee, 2004; Huber, 2007; Spoel and Loake, 2011; Erb et al., 2012;). Labial saliva-specific post-translational modifications of nuclear protein in response to caterpillar herbivory were identified in wildtype and the quad-*della* mutant of *A. thaliana* ecotype L*er*. Since DELLA proteins, which are the negative regulators of the Gibberelin (GA) pathway, integrate crosstalk between JA- and GA-pathways to modulate growth *vs.* defense in plants during stress conditions, we concentrated our effort to identify post-translational modification of nuclear proteins in response to caterpillar feeding in these two plants (Davière and Achard, 2013; Hou et al., 2013; Hout et al., 2014). Furthermore, DELLAs are involved in scavenging ROS produced during caterpillar herbivory (Achard et al., 2008). Herbivore- and/or labial saliva-specific modifications of RABH1C, CAMTA/SR1-like protein, MYB109 and AtABF3 were identified in these plants. Labial saliva-specific nitrosylation and phosphorylation of AtABF3, and the differential expression of its downstream gene, *AtWRKY40*, was observed upon herbivory by caterpillar with intact or impaired labial saliva secretion in L*er* plants. Further work is needed to confirm the labial saliva-specific post-translational modification of host plant ABF3 and its putative role in plant-insect interactions.

In the next 50 years, it is predicted that atmospheric CO_2 levels will double (Weaver et al., 2007; IPCC, 2014). As the photosynthetic- and nitrogen use efficiency of the plant increases, plant metabolic flux is expected to alter, particularly for defensive compounds (Leaky et al., 2009; Klaiber et al., 2013). It is hypothesized that at higher levels of CO_2, plants will invest more in carbon-based defense compounds and nitrogen-based defenses will decline as plants allocate nitrogen towards growth (Bryant et al., 1983). We showed that the induced jamonate burst and the increased level of foliar GSLs in response to wounding is only observed under nitrogen-limited conditions at elevated CO_2. Though, mechanical wounding induces expression of MYB transcription factors that regulate both indole and aliphatic GSL, indole GSL biosynthesis seems to prevail in wounded Arabidopsis leaves under ambient CO_2. On the other hand, under elevated CO_2, wound-induced accumulation of indole and aliphatic GSL occurs in the nitrogen-stressed condition. It was also shown that CO_2, more than nitrogen-limited condition, affects the metabolic profile in *A. thaliana*.

7.2 Future Directions

To further our understanding of phytohormone pathways involved in plant-insect interaction and the role of caterpillar labial saliva in the manipulation of host plant defense, the following studies are suggested:

a. Confirm the role of ethylene in plant-insect interaction

In Chapter 4, we showed differential expression of marker genes of the JA-, SA- and ET-pathways in wildtype and ethylene-insensitive *skl* mutant of *M. truncatula* in response to caterpillar herbivory with intact or impaired labial saliva secretions. Further studies should consider enhancing the endogenous ET levels in the plants by exogenous application of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or inhibiting ET biosynthesis by using the Aminoethoxyvinylglycine. Then by investigating the modulation of JA-mediated defense response by caterpillar herbivory should help confirm our observations that the ET is essential to enhance the labial saliva-specific activation of the SA-dependent pathway that attenuates host's IR.

b. Further characterization of labial saliva-specific post-translational modifications of proteins during caterpillar herbivory

Herbivore-specific and labial saliva-specific post-translational modification of CAMTA/SR1-like protein and AtABF3, respectively, were identified in *A. thaliana* after *S. exigua* caterpillar herbivory. Gene expression studies of the target genes of these transcription factors suggest that the activation of the antagonistic SA pathway could lead to subversion of plant's JA-mediated defense response against caterpillars. Further studies to conduct site-directed mutagenesis of the transcription factor, AtAFB3, is proposed; the identified phosphorylation at serine residue (S_{431}) can be mutated to alanine (S431A) or aspartate (S431D) that eliminate or mimic phosphorylation, respectively. By transforming the mutated constructs into a loss-of-function *Atabf3* Arabidopsis mutant background, further characterization of caterpillar labial saliva-specific modulation of plant response upon herbivory can be performed.

c. Study of plant metabolic profile in response to caterpillar herbivory under elevated CO_2

Wounding and caterpillar herbivory share common aspects regarding the regulation of plant defense responses (Koo and Howe, 2009). In Chapter 6, we studied the metabolic flux in

mechanically wounded Arabidopsis plants under elevated CO_2 and nitrogen stress; in this experiment, leaves were mechanically damaged. In further studies, it is suggested to investigate plant defense responses aginst caterpillar herbivory under the elevated atmospheric CO_2 level and nitrogen stress conditions.

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Appendices

Appendix 3.1. Statistical analysis of redox metabolites and defense gene expression in *Arabidopsis thaliana*. A two-way ANOVA followed by a Tukey HSD *post-hoc* was used to evaluate redox metabolite levels over a 45 minute time course. A one-way ANOVA was used to evaluate differences in gene expression within each genotype. A five-fold or higher difference in gene expression is also indicated.

Ascorbate		
Total	Range: 2.69 to 3.98 µmol/g FW	Effect of treatment, $F_{(2,103)} = 1.33$, $p = 0.27$; Effect of time, $F_{(4,103)} = 0.16$, $p = 0.96$, Interaction, $F_{(8,103)} = 0.66$, $p = 0.73$
Oxidized (Asc)	Range: 1.22 to 1.83 µmol/g FW	Effect of treatment, $F_{(2,100)} = 0.41$, $p = 0.66$; Effect of time, $F_{(4,100)} = 0.90$, $p = 0.47$, Interaction, $F_{(8,100)} = 0.43$, $p = 0.90$
Reduced (DHA)	Range: 1.23 to 2.81 µmol/g FW	Effect of treatment, $F_{(2,105)} = 2.50$, $p = 0.09$; Effect of time, $F_{(4,105)} = 0.58$, $p = 0.68$, Interaction, $F_{(8,105)} = 0.97$, $p = 0.47$
Oxidized/reduced (Asc/DHA)		Effect of treatment, $F_{(2,100)} = 1.62$, $p = 0.21$; Effect of time, $F_{(4,100)} = 0.1.30$, $p = 0.28$, Interaction, $F_{(8,100)} = 1.11$, $p = 0.36$
Glutathione		
Total	Range: 151 to 226 nmol/g FW	Effect of treatment, $F_{(2,109)} = 3.35$, $p = 0.04$; Effect of time, $F_{(4,109)} = 0.32$, $p = 0.86$, Interaction, $F_{(8,109)} = 0.16$, $p = 0.99$) (Fig 2A);
Oxidized (GSSG)	Range: 9.9 to 42.6 nmol/g FW	Effect of treatment, $F_{(2,89)} = 3.31$, $p = 0.04$; Effect of time, $F_{(4,89)} = 2.10$, $p = 0.09$, Interaction, $F_{(8,89)} = 2.24$, $p = 0.03$. Since interaction was significant, this was followed by a 1-way ANOVA to determine the time point where there was a significant difference: 5 min: $F_{(2,19)} = 2.69$, $p = 0.09$; 15 min: $F_{(2,14)} = 1.91$, $p = 0.18$; 25 min: $F_{(2,19)} = 2.64$, $p = 0.10$; 35 min: $F_{(2,20)} = 3.76$, $p = 0.04$; 45 min: $F_{(2,19)} = 0.53$, $p = 0.60$
Reduced (GSH)	Range: 93.2 to 227.9 nmol/g FW	Effect of treatment, $F_{(2,87)} = 3.42$, $p = 0.04$; Effect of time, $F_{(4,87)} = 0.67$, $p = 0.62$, Interaction, $F_{(8,87)} = 0.56$, $p = 0.81$
Oxidized/reduced (GSSG/GSH)		Effect of treatment, $F_{(2,87)} = 0.99$, $p = 0.37$; Effect of time, $F_{(4,87)} = 2.18$, $p = 0.08$, Interaction, $F_{(8,87)} = 1.07$, $p = 0.39$
Gene expression	**Genotype**	
AtPR1	wildtype	$F_{(2,8)} = 4.44$, $p = 0.05$; 5-fold increase in gene expression in plants attacked by caterpillars with intact labial salivary secretions compared to control plants or between control plants or plants infested by caterpillars with impaired salivary secretions.
	pad2-1	$F_{(2,9)} = 1.23$, $p = 0.32$
	tga 2/5/6	$F_{(2,7)} = 0.35$, $p = 0.72$

AtPDF1.2	wildtype	$F_{(2,8)} = 6.00$, $p = 0.03$; 18-fold increase in gene expression in response to herbivory by caterpillars with impaired salivary secretions compared to normal caterpillars or controls
	pad2-1	$F_{(2,8)} = 1.50$, $p = 0.28$; 12.5-fold increase in gene expression is seen between plants infested by caterpillars compared to controls
	tga 2/5/6	$F_{(2,7)} = 3.31$, $p = 0.10$; 5-fold increase in gene expression is seen in plants fed upon by caterpillars compared to controls
ERF1	wildtype	$F_{(2,8)} = 5.07$, $p = 0.04$; 10-fold increase in gene expression seen in plants fed upon by caterpillars with impaired salivary secretions compared to control plants.
	pad2-1	$F_{(2,9)} = 12.83$, $p = 0.002$; 20-fold increase in gene expression seen in plants fed upon by caterpillars with impaired salivary secretions compared to control plants or plants attacked by caterpillars with labial salivary secretions.
	tga 2/5/6	$F_{(2,7)} = 0.61$, $p = 0.57$
AtHEL	wildtype	$F_{(2,9)} = 2.50$, $p = 0.14$; 5-fold increase in gene expression is observed in plants infested by caterpillars compared with control.
	pad2-1	$F_{(2,9)} = 2.22$, $p = 0.17$; 10-fold increase in gene expression is observed in plants infested by caterpillars compared with controls
	tga 2/5/6	$F_{(2,7)} = 3.31$, $p = 0.10$
AtLOX2	wildtype	$F_{(2,7)} = 1.48$, $p = 0.29$; 7-fold increase in gene expression is observed in plants infested by caterpillars compared with controls
	pad2-1	$F_{(2,9)} = 3.68$, $p = 0.07$; 7-fold increase in gene expression is observed in plants infested by caterpillars compared with controls
	tga 2/5/6	$F_{(2,7)} = 1.16$, $p = 0.37$; ; 40-fold increase in gene expression is observed in plants infested by caterpillars compared with controls
AtSAP6	wildtype	$F_{(2,8)} = 5.02$, $p = 0.04$
	pad2-1	$F_{(2,9)} = 0.85$, $p = 0.46$
	tga 2/5/6	$F_{(2,7)} = 0.14$, $p = 0.87$

Appendix 4.1. Statistical analysis of redox metabolites in *Medicago truncatula*. Significant differences in redox metabolites were analyzed by 2-factorial ANOVA ($p \leq 0.05$) followed by Tukey HSD *post hoc* test. Significant differences were analyzed within each plant genotype.

Ascorbate wild	**Range**	
Total	2.26 to 11.12 µmol/g FW	Effect of treatment, $F_{(2,55)} = 0.478$, $p = 0.623$; Effect of time, $F_{(2, 55)} = 0.089$, $p = 0.91$, Interaction, $F_{(4, 55)} = 0.309$, $p = 0.871$
Oxidized (DHA)	0.92 to 5.3 µmol/g FW	Effect of treatment, $F_{(2,55)} = 1.66$, $p = 0.19$; Effect of time, $F_{(2, 55)} = 0.87$, $p = 0.42$, Interaction, $F_{(4, 55)} = 0.913$, $p = 0.463$
Reduced (Asc)	0.538 to 7.12 µmol/g FW	Effect of treatment, $F(_{2,55}) = 0.03$, $p = 0.967$; Effect of time, $F_{(2, 55)} = 0.07$, $p = 0.93$, Interaction, $F_{(4, 55)} = 1.4$, $p = 0.24$
Oxidized/reduced (DHA/Asc)	0.19 to 7.67	Effect of treatment, $F_{(2,55)} = 2.76$, $p = 0.07$; Effect of time, $F_{(2, 55)} = 1.86$, $p = 0.16$, Interaction, $F_{(4, 55)} = 2.5$, $p = 0.049$
Ascorbate *skl*		
Total	2.4 to 13.18 µmol/g FW	Effect of treatment, $F_{(2,49)} = 0.448$, $p = 0.64$; Effect of time, $F_{(2, 49)} = 1.34$, $p = 0.27$, Interaction, $F_{(4, 49)} = 0.96$, $p = 0.437$
Oxidized (DHA)	0.66 to 6.08 µmol/g FW	Effect of treatment, $F_{(2,49)} = 0.54$, $p = 0.58$; Effect of time, $F_{(2, 49)} = 0.16$, $p = 0.84$, Interaction, $F_{(4, 49)} = 0.29$, $p = 0.88$
Reduced (Asc)	0.906 to 7.57 µmol/g FW	Effect of treatment, $F_{(2,49)} = 0.103$, $p = 0.902$; Effect of time, $F_{(2, 49)} = 1.75$, $p = 0.18$, Interaction, $F_{(4, 49)} = 1.06$, $p = 0.38$
Oxidized/reduced (DHA/Asc)	0.11 to 4.64	Effect of treatment, $F_{(2,49)} = 0.19$, $p = 0.82$; Effect of time, $F_{(2, 49)} = 0.127$, $p = 0.88$, Interaction, $F_{(4, 49)} = 0.23$, $p = 0.91$
Glutathione wild	**Range**	
Total	129.8 to 401.2 nmol/g FW	Effect of treatment, $F_{(2,49)} = 1.12$, $p = 0.33$; Effect of time, $F_{(2,49)} = 0.145$, $p = 0.86$, Interaction, $F_{(4,49)} = 1.01$, $p = 0.409$
Oxidized (GSSG)	1.87 to 43.48 nmol/g FW	Effect of treatment, $F_{(2,49)} = 4.32$, $p = 0.019$; Effect of time, $F_{(2,49)} = 4.09$, $p = 0.02$, Interaction, $F_{(4,49)} = 1.98$, $p = 0.11$
Reduced (GSH)	97.4 to 368.25 nmol/g FW	Effect of treatment, $F_{(2,49)} = 2.12$, $p = 0.131$; Effect of time, $F_{(2,49)} = 0.157$, $p = 0.855$, Interaction, $F_{(4,49)} = 0.88$, $p = 0.47$

Oxidized/reduced (GSSG/GSH)	0.014 to 0.265	Effect of treatment, $F_{(2,49)} = 5.08$, $p = 0.01$; Effect of time, $F_{(2,49)} = 4.47$, $p = 0.016$, Interaction, $F_{(4,49)} = 1.38$, $p = 0.25$
Glutathione *skl*	**Range**	
Total	104.78 to 374.31 nmol/g FW	Effect of treatment, $F_{(2,48)} = 1.52$, $p = 0.22$; Effect of time, $F_{(2,48)} = 1.3$, $p = 0.28$, Interaction, $F_{(4,48)} = 1.13$, $p = 0.36$
Oxidized (GSSG)	2.3 to 19.86 nmol/g FW	Effect of treatment, $F_{(2,48)} = .67$, $p = 0.51$; Effect of time, $F_{(2,48)} = 0.48$, $p = 0.61$, Interaction, $F_{(4,48)} = 1.93$, $p = 0.12$
Reduced (GSH)	87.68 to 345.52 nmol/g FW	Effect of treatment, $F_{(2,48)} = 1.79$, $p = 0.176$; Effect of time, $F_{(2,48)} = 1.23$, $p = 0.30$, Interaction, $F_{(4,48)} = 1.22$, $p = 0.312$
Oxidized/reduced (GSSG/GSH)	0.02 to 0.11	Effect of treatment, $F_{(2,48)} = 1.84$, $p = 0.169$; Effect of time, $F_{(2,48)} = 0.027$, $p = 0.97$, Interaction, $F_{(4,48)} = 3.15$, $p = 0.02$ Since interaction was significant, this was followed by a 1-way ANOVA to determine the time point where there was a significant difference: 15 min: $F_{(2,19)} = 3.9$, $p = 0.03$; 30 min: $F_{(2,18)} = 2.5$, $p = 0.10$; 45 min: $F_{(2,13)} = 1.4$, $p = 0.27$

Appendix 4.2. Statistical analysis of phytohormones, gene expression and defensive proteins in *Medicago truncatula*. Significant differences in phytohormones, gene expression and defensive proteins were analyzed by 1-factorial ANOVA ($p \leq 0.05$) followed by Tukey HSD *post hoc* test. Significant differences were analyzed within each plant genotype.

	wildtype		***skl* mutant**	
Hormones	**F-value**	**p-value**	**F-value**	**p-value**
JA	$F_{(2, 7)} = 34.39$	$p < 0.001$	$F_{(2, 6)} = 41.84$	$p < 0.001$
JA-ile	$F_{(2, 7)} = 43.06$	$p < 0.001$	$F_{(2, 6)} = 28.12$	$p = 0.001$
OPDA	$F_{(2, 6)} = 0.38$	$p = 0.669$	$F_{(2, 7)} = 0.992$	$p = 0.44$
ABA	$F_{(2, 6)} = 2.96$	$p = 0.109$	$F_{(2, 7)} = 11.49$	$p = 0.009$
Gene Expression	**F-value**	**p-value**	**F-value**	**p-value**
MtPR1	$F_{(2,8)} = 10.34$	$p = 0.011$	$F_{(2,9)} = .443$	$p = 0.655$
MtVSP	$F_{(2,9)} = 11.18$	$p = 0.004$	$F_{(2,9)} = 7.58$	$p = 0.012$
MtHEL	$F_{(2,11)} = 35.68$	$p < 0.0001$	$F_{(2,10)} = 9.63$	$p = 0.005$
MtRPK	$F_{(2,11)} = 5.76$	$p = 0.019$	$F_{(2,12)} = 2.75$	$p = 0.104$
MtRFP	$F_{(2,11)} = 20.97$	$p <0.0001$	$F_{(2,10)} = 3.72$	$p = 0.019$
MtRCA	$F_{(2,12)} = 27.95$	$p <0.0001$	$F_{(2,11)} = 3.72$	$p <0.0001$
MtSTR	$F_{(2,12)} = 8.13$	$p = 0.006$	$F_{(2,9)} = .783$	$p = 0.505$
Defense proteins	**F-value**	**p-value**	**F-value**	**p-value**
Trypsin inhibitor	$F_{(2,29)} = 6.46$	$p = 0.05$	$F_{(2,25)} = .864$	$p = 0.434$
PPO	$F_{(2,29)} = 0.34$	$p = 0.714$	$F_{(2,28)} = 0.273$	$p = 0.763$

Appendix 5.1. Statistical analysis of gene expression data in *Arabidopsis thaliana*. Significant differences were analyzed within each plant genotype. A one-factor analysis of variance (ANOVA) followed by a Tukey HSD *post hoc* was used to compare caterpillar treatments within each genotype (L*er* and quad-*della* mutant).

Gene Expression	**L*er* wildtype**		**quad-*della* mutant**	
	F value	p-value	F value	p-value
AtNDR1	$F_{(2, 14)} = 4.75$	$p = 0.03$	$F_{(2, 14)} = 4.90$	$p = 0.03$
AtEIN3	$F_{(2, 14)} = 5.92$	$p = 0.02$	$F_{(2, 14)} = 9.73$	$p = 0.003$
AtESP	$F_{(2, 14)} = 48.53$	$p < 0.001$	$F_{(2, 14)} = 15.4$	$p < 0.001$
AtWRKY40	$F_{(2, 11)} = 5.53$	$p = 0.03$	$F_{(2, 11)} = 8.91$	$p = 0.007$

Appendix 6.1. Statistical analysis of A) *AtMYB* gene expression, B) phytohormones, and C) glucosinolates in *Arabidopsis thaliana*. Significant differences in gene expression and levels of measured metabolites were analyzed by 3-factorial ANOVA (significance level, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$). When a 3-way interaction existed between CO_2, nitrate and mechanical stress (wounding), it was followed by 2-way ANOVA between nitrate and wound treatments at ambient and elevated CO_2. When a significant nitrate x wounding interaction was observed, the effect of mechanical damage was further analyzed by 1-way ANOVA.

A) *AtMYB* gene expression

Genes	Interaction/ Treatment effect		Ambient CO_2 (440 ppm)	Elevated CO_2 (880 ppm)
		F-value (Sig.)		
MYB29	C x N x W	$F_{(1, 25)} = 1.8$ (NS)		
	Nitrogen effect	$F_{(1, 25)} = 4.8$ (*)		
	Wounding effect	$F_{(1, 25)} = 8.5$ (**)		
MYB28	C x N x W	$F_{(1, 24)} = 0.002$ (NS)		
	C x W	$F_{(1, 24)} = 4.56$ (*)		
	Wounding effect		$F_{(1, 13)} = 5.4$ (*)	$F_{(1, 11)} = 0.32$ (NS)
MYB76	C x N x W	$F_{(1, 24)} = 3.8$ (NS)		
	C x N	$F_{(1, 24)} = 4.8$ (**)		
	Nitrogen effect		$F_{(1, 12)} = 1.76$ (NS)	$F_{(1, 12)} = 5.4$ (*)
	Wounding effect	$F_{(1, 24)} = 11.1$ (**)		
MYB51	C x N x W	$F_{(1, 28)} = 0.13$ (NS)		
	CO_2 effect	$F_{(1, 28)} = 9.9$ (**)		
MYB34	C x N x W	$F_{(1, 26)} = 0.001$ (NS)		
	Wounding effect	$F_{(1, 26)} = 5.2$ (*)		

B) Phytohormones

Hormones	Interaction/ Treatment effect		Ambient CO_2 (440 ppm)	Elevated CO_2 (880 ppm)
		F-value (Sig.)		
SA	C x N x W	$F_{(1, 22)} = 0.42$ (NS)		
	CO_2 effect	$F_{(1, 22)} = 7.5$ (**)		
JA	C x N x W	$F_{(1, 22)} = 7.0$ (**)		
	N x W		$F_{(1, 11)} = 0.22$ (NS)	$F_{(1, 11)} = 8.68$ (**)
	Wounding effect	Nitrate-stressed		$F_{(1, 11)} = 25.1$ (**)
		Nitrate-fertilized	$F_{(1, 11)} = 9.18$ (**)	$F_{(1, 11)} = 1.15$ (NS)

JA-Ile	C x N x W	$F_{(1, 22)} = 4.8$ (*)		
	N x W		$F_{(1, 11)} = 0.22$ (NS)	$F_{(1, 11)} = 5.94$ (*)
	Wounding effect	Nitrate-stressed	$F_{(1, 11)} = 7.14$ (*)	$F_{(1, 11)} = 25.6$ (**)
		Nitrate- fertilized		$F_{(1, 11)} = 0.15$ (NS)
OPDA	C x N x W	$F_{(1, 22)} = 2.8$ (NS)		
	N x W	$F_{(1, 22)} = 16.88$ (***)		
	Wounding effect	Nitrate-stressed		$F_{(1, 11)} = 41.2$ (***)
		Nitrate- fertilized		$F_{(1, 11)} = 0.37$ (NS)
ABA	C x N x W	$F_{(1, 20)} = 11.67$ (**)		
	N x W		$F_{(1, 9)} = 17.88$**	$F_{(1, 11)} = 1.828$ (NS)
	Wounding effect	Nitrate-stressed	$F_{(1, 9)} = 5.96$ (NS)	
		Nitrate- fertilized	$F_{(1, 9)} = 12.29$ (**)	

C) Glucosinolates

Glucosinolates (GS)	**Interaction/ Treatment effect**		**Ambient CO_2 (440 ppm)**	**Elevated CO_2 (880 ppm)**
		F-value (Sig.)		
Total	C x N x W	$F_{(1, 24)} = 5.6$ (*)		
	N x W		$F_{(1, 12)} = 0.2$ (NS)	$F_{(1, 12)} = 9.2$ (**)
	Wounding effect	Nitrate-stressed	$F_{(1, 12)} = 7.3$ (*)	$F_{(1, 12)} = 8.2$ (*)
		Nitrate- fertilized		$F_{(1, 12)} = 1.3$ (NS)
Indole	C x N x W	$F_{(1, 24)} = 4.3$ (*)		
	N x W		$F_{(1, 12)} = 0.06$ (NS)	$F_{(1, 12)} = 7.3$ (*)
	Wounding effect	Nitrate-stressed	$F_{(1, 12)} = 26.9$ (***)	$F_{(1, 12)} = 25.9$ (**)
		Nitrate- fertilized		$F_{(1, 12)} = 1.5$ (NS)
Aliphatic	C x N x W	$F_{(1, 24)} = 5.1$ (*)		
	N x W		$F_{(1, 12)} = 0.24$ (NS)	$F_{(1, 12)} = 8.4$ (*)
	Wounding effect	Nitrate-stressed		$F_{(1, 12)} = 4.4$ (*)
		Nitrate- fertilized		$F_{(1, 12)} = 2.8$ (NS)

Indole GS	**Interaction/ Treatment effect**	**F-value (Sig.)**
Glucobrassicin (GBC) 3-indolylmethyl GS	C x N x W	$F_{(1, 24)} = 3.9$ (NS)
	CO_2 effect	$F_{(1, 24)} = 4.9$ (*)
	Wounding effect	$F_{(1, 24)} = 36.1$ (***)
Methoxygluco-brassicin (4MeOGB) 4-methoxy-3-indolylmethyl GSL	C x N x W	$F_{(1, 24)} = 0.003$ (NS)
	CO_2 effect	$F_{(1, 24)} = 18.7$ (***)

Neo-glucobrassicin (NeoGB) 1-methyoxy-3-indolylmethyl GSL	C x N x W	$F_{(1, 24)} = 0.4$ (NS)		
	CO2 effect	$F_{(1, 24)} = 18$ (***)		
	N x W	$F_{(1, 24)} = 24$ (***)		
	Wounding effect	Nitrate-stressed	$F_{(1, 12)} = 43.3$ (***)	
		Nitrate-fertilized	$F_{(1, 12)} = 0.3$ (NS)	
Aliphatic GS	**Interaction/ Treatment effect**		**Ambient CO_2 (440 ppm)**	**Elevated CO_2 (880 ppm)**
			F-value (Sig.)	
Glucoiberin (IBE) 3-methylsulfinylpropyl GSL	C x N x W	$F_{(1, 24)} = 0.37$ (NS)		
	C x N	$F_{(1, 24)} = 5.2$ (*)		
	Nitrogen effect		$F_{(1, 12)} = 6.3$ (*)	$F_{(1, 12)} = 0.53$ (NS)
Glucoerucin (ERU) 4-methylthiobutyl GSL	C x N x W	$F_{(1, 24)} = 1.3$ (NS)		
	C x N	$F_{(1, 24)} = 9.9$ (**)		
	Nitrogen effect		$F_{(1, 12)} = 2.3$ (NS)	$F_{(1, 12)} = 11.4$ (**)
Glucoraphanin (RAPH) 4-methylsulfinylbutyl GSL	C x N x W	$F_{(1, 24)} = 5.7$ (*)		
	N x W		$F_{(1, 24)} = 0.26$ (NS)	$F_{(1, 12)} = 9.9$ (**)
	Wounding effect	Nitrate-stressed	$F_{(1, 12)} = 5.04$ (*)	$F_{(1, 12)} = 6.3$ (*)
		Nitrate-fertilized		$F_{(1, 12)} = 3.7$ (NS)
Glucoalyssin (ALY) 5-methylsulphinylpentyl	C x N x W	$F_{(1, 24)} = 0.21$ (NS)		
	Nitrogen effect	$F_{(1, 24)} = 4.7$ (*)		
	Wounding effect	$F_{(1, 24)} = 5.4$ (*)		

Appendix 6.2. M**etabolites identified in *Arabidopsis thaliana* grown at different regimes of carbondioxide- and nitrogen-fertilization and subjected to mechanical damage.** Metabolites were identified by untargeted LC-qTOF mass spectrometry and evaluated in control *vs* mechanically damaged *A. thaliana* plants within each regime of CO_2 (ambient or elevated CO_2) and nitrate-fertilization (nitrate-sufficient or -stress) to produce the list of constitutive metabolites (present in control plants only) and those present in mechanically damaged plants. Levels of metabolites present in both control and mechanically damaged plants were evaluated by student t-test ($p \leq 0.05$) and differentially regulated metabolites in response to mechanical damage are presented as elevated or suppressed.

Ambient CO_2, nitrate-sufficient		
Constitutive only		
ADP-α-D-glucose		
5-amino-6-(5-phospho-D-ribosylamino)uracil	Flavin biosynthesis	
flavin mononucleotide	Flavin biosynthesis	
2-(2'-methylthio)ethylmalate	Glucosinolate biosynthesis	
3-methylthiopropyl glucosinolate	Glucosinolate biosynthesis	
5-methylthiopentylhydroximoyl-cysteinylglycine	Glucosinolate biosynthesis; aliphatic	
8-methylthiooctyl glucosinolate	Glucosinolate biosynthesis	
indole-3-acetyl-*myo*-inositol-D-galactoside	IAA storage and transport	
1-18:1-2-16:0-phosphatidylglycerol	Phosphoglycerol biosynthesis	
1-18:2-2-*trans*-16:1-phosphatidylglycerol	Phosphoglycerol biosynthesis	
1-18:3-2-*trans*-16:1-phosphatidylglycerol	Phosphoglycerol biosynthesis	
7^1-hydroxychlorophyll *a*	Photosynthesis	
acetylenedicarboxylate	Pyruvate metabolism	
(1*R*,6*R*)-6-hydroxy-2-succinylcyclo-hexa-2,4-diene-1-carboxylate	Quinone biosynthesis	
(2*S*,5*R*)-2-(2-hydroxypropan-2-yl)-5,9-dimethyl-1-oxaspiro[5.5]undec-8-ene-7,10-dione	Terpenoid biosynthesis	
diprenylphlorisovalerophenone	Terpenoid biosynthesis	
Mechanically damaged only		
myo-inositol 1,2,3,4,5,6-hexakisphosphate	Cellular signaling	
7^1-hydroxychlorophyllide *a*	Chlorophyll degradation	
flavin adenine dinucleotide	Flavin biosynthesis	
isovitexin-7-*O*-glucosyl-2"*O*-rhamnoside	Flavone biosynthesis	
(-)-phaseollidin	Flavonoid biosynthesis; phytoalexin	
1-18:3-2-18:3-digalactosyldiacylglycerol	Galactolipid	

5-methylsulfinylpentyl glucosinolate	Glucosinolate biosynthesis	
6-hydroxyindole-3-carboxylic acid 6-*O*-β-D-glucopyranoside	Glucosinolate biosynthesis; indole	
coniferaldehyde glucoside	Monolignol glucoside biosynthesis	
UDP-*N*-acetyl-α-D-glucosamine	Nucleic acid biosynthesis	
1-*O*-feruloyl-β-D-glucose	Phenylpropanoid pathway	
allicin	Phytoanticipin; organosulfur compound	
Differentially regulated in response to wounding		
trans-heptaprenyl diphosphate	Terpenoid biosynthesis; dolichol	Suppressed
3-butenylglucosinolate	GSL biosynthesis	Suppressed
secologanin	Terpenoid biosynthesis	Elevated
acetylenedicarboxylate	Pyruvate metabolism	Elevated
Ambient CO2, nitrate-stressed		
Constitutive only		
uridine-5'-phosphate		
S-5-methylthiopentylhydroximoyl-L-cysteine	Cysteine metabolism	
flavin mononucleotide	Flavin biosynthesis	
6-methylthiohexylglucosinolate	Glucosinate biosynthesis	
indolylmethyl-desulfoglucosinolate	Glucosinate biosynthesis	
5-*O*-(indol-3-yl acetyl-*myo*-inositol) D-galactoside	IAA transport and storage	
(-)-medicarpin-3-*O*-glucoside-6"-malonate	Isoflavonoid biosynthesis	
UDP-*N*-acetyl-α-D-glucosamine	Lipid biosynthesis	
5-hydroxycaffeate	Phenylpropanoid biosynthesis	
1-18:2-2-*trans*-16:1-phosphatidylglycerol	Phosphoglycerol biosynthesis	
1-18:3-2-*trans*-16:1-phosphatidylglycerol	Phosphoglycerol biosynthesis	
dihydrogeranylgeranyl-chlorophyll *a*	Photosynthesis	
magnesium-protoporphyrin IX 13-monomethyl ester	Photosynthesis	
diprenylphlorisovalerophenone	Terpenoid/phenolic secondary metabolite	
Mechanically damaged only		
N,N'-diacetylchitobiose	Carbohydrate	
β-D-gentiobiosyl D-glucosyl crocetin	Carotenoid biosynthesis	
6,9-octadecadienedioic acid	Fatty acid biosynthesis	
palmatine	Fatty acid biosynthesis	
kaempferol 3-*O*-β-D-glucosyl (1→2)-glucosyl-(1→2)-β-D-glucoside	Flavonol biosynthesis	
1-18:2-2-18:3-digalactosyldiacylglycerol	Galactolipid biosynthesis	
1-hydroxy-3-indolylmethyl glucosinolate	Glucosinolate biosynthesis	
coniferaldehyde glucoside	Lignin biosynthesis	

deoxypodophyllotoxin	Lignin biosynthesis	
trans-cinnamoyl-β-D-glucoside	Phenylpropanoid biosynthesis	
7[1]-hydroxychlorophyll *a*	Photosynthesis	
geranylgeranyl-chlorophyll *a*	Photosynthesis	
allicin	Phytoanticipin; organosulfur compound	
Differentially regulated in response to wounding		
3-methylthiopropyl-glucosinolate	Glucosinolate biosynthesis	Suppressed
6-methylthiohexylglucosinolate	Glucosinolate biosynthesis	Suppressed
3-methylsulfinylpropyl-glucosinolate	Glucosinolate biosynthesis	Suppressed
8-methylsulfinyloctyl glucosinolate	Glucosinolate biosynthesis	Suppressed
2-phenylethylglucosinolate	Glucosinolate biosynthesis	Suppressed
3-butenylglucosinolate	Glucosinolate biosynthesis	Suppressed
indole-3-acetyl-*myo*-inositol-D-galactoside	IAA storage and transport	Suppressed
dTDP-4-dehydro-6-deoxy-α-D-glucose	Nucleic acid biosynthesis	Suppressed
1-18:2-2-*trans*-16:1-phosphatidylglycerol	Phosphoglycerol biosynthesis	Suppressed
trans-heptaprenyl diphosphate	Terpenoid biosynthesis; dolichol	Suppressed
kaempferol 3-*O*-[6-(4-coumaroyl)-β-glucosyl-(1→2)-glucosyl (1→2) β-D-glucoside	Flavonol biosynthesis	Induced
Elevated CO2, nitrate-sufficient		
Constitutive only		
5-amino-6-(5-phospho-D-ribitylamino) uracil	Flavin biosynthesis	
(-)-phaseollidin	Flavonoid biosynthesis; phytoalexin	
sinapoyl-CoA	Glucosinolate biosynthesis	
6-methylthiohexylglucosinolate	Glucosinolate biosynthesis	
coniferaldehyde glucoside	Lignin biosynthesis	
dihydrogeranylgeranyl-chlorophyll *a*	Photosynthesis	
7[1]-hydroxychlorophyll *a*	Photosynthesis	
trans-heptaprenyl diphosphate	Terpenoid biosynthesis; dolichol	
Mechanically damaged only		
S-adenosyl-L-methionine		
ADP-glucose		
N-acetylglutamyl-phosphate	Amino acid biosynthesis	
crocin	Carotenoid biosynthesis	
flavin mononucleotide	Flavin biosynthesis	
O-β-D-glucosyl-apigenin	Flavonoid biosynthesis	
kaempferol 3-*O*-β-D-glucosyl (1→2)-glucosyl-(1→2)-β-D-glucoside	Flavonol biosynthesis	
5-methylsulfinylpentyl glucosinolate	Glucosinolate biosynthesis	

6-hydroxyindole-3-carboxylic acid 6-O-beta-D-glucopyranoside	Glucosinolate biosynthesis: indole	
malonyldaidzin	Isoflavone	
(-)-medicarpin-3-*O*-glucoside-6"-malonate	Isoflavonoid biosynthesis	
2,3-bis[(3*R*)-3-hydroxymyristoyl]-α-D-glucosaminyl 1-phosphate	Lipid biosynthesis	
xylogalacturonan	Pectic polysaccharide	
2-coumarate	Phenylpropanoid biosynthesis	
cis-coumarinic acid-β-D-glucoside	Phenylpropanoid biosynthesis	
1-18:3-2-*trans*-16:1-phosphatidylglycerol	Phosphoglycerol biosynthesis	
geranylgeranyl-chlorophyll *a*	Photosynthesis	
allicin	Phytoanticipin; organosulfur compound	
1-methylxanthine	Purine alkaloid	
acetylenedicarboxylate	Pyruvate metabolism	
chorismate	Shikimate biosynthesis	
Differentially regulated in response to wounding		
dTDP-4-dehydro-6-deoxy-α-D-glucose	Nucleic acid biosynthesis	Elevated
L-arogenate	Phenylalanine biosynthesis	Elevated
propionyl-AMP	Fatty acid biosynthesis	Elevated
2-phenylethylglucosinolate	Glucosinolate biosynthesis	Elevated
8-methylthiooctyl glucosinolate	Glucosinolate biosynthesis	Elevated
indolylmethyl-glucosinolate	Glucosinolate biosynthesis	Elevated
4-thiazolidine carboxylic acid (raphanusamic acid)	Glucosinolate breakdown	Elevated
Elevated CO2, nitrate-stressed		
Constitutive only		
ADP-α-D-glucose		
guanosine diphosphate		
oxaloacetate		
brassinolide-23-*O*-glucoside	Brassinosteroid inactivation	
5-fluoro-5-deoxy-d-ribulose-1-phosphate	Cysteine and methionine metabolism	
5-amino-6-(5-phospho-D-ribosylamino)uracil	Flavin biosynthesis	
flavin mononucleotide	Flavin biosynthesis	
sinapoyl-CoA	Glucosinolate biosynthesis	
3-methylthiopropyl-glucosinolate	Glucosinolate biosynthesis	
6-methylthiohexylglucosinolate	Glucosinolate biosynthesis	
6-hydroxyindole-3-carboxylic acid 6-*O*-β-D-glucopyranoside	Indole metabolism	
13(*S*)-hydroperoxy-9(*Z*), 11(*E*), 15 (*Z*)-octadecatrienoate	Jasmonic acid biosynthesis/Fatty acid biosynthesis	

OPC6-3-hydroxyacyl-CoA	Jasmonic acid biosynthesis/Fatty acid biosynthesis	
coniferaldehyde glucoside	Lignin biosynthesis	
7[1]-hydroxychlorophyll *a*	Photosynthesis	
dihydrogeranylgeranyl-chlorophyll *a*	Photosynthesis	
acetylenedicarboxylate	Pyruvate metabolism	
mevalonate diphosphate	Terpenoid biosynthesis: mevalonate pathway	
geranyl hydroquinone	Terpenoid biosynthesis	
diprenylphlorisovalerophenone	Terpenoid biosynthesis	
Mechanically damaged only		
4-hydroxy-3-indolylmethyl glucosinolate	Glucosinolate biosynthesis	
DIBOA-β-glucoside	Indole compounds	
palmatine	Isoquinoline alkaloid biosynthesis	
(+)-sesamolinol	Lignan biosynthesis	
UDP-*N*-acetyl-α-D-glucosamine	Lipid biosynthesis	
coniferaldehyde glucoside	Monolignol glucoside biosynthesis	
1-*O*-feruloyl-β-D-glucose	Phenylpropanoid biosynthesis	
1-18:3-2-16:3-monogalactosyldiacylglycerol	Phosphoglycerol biosynthesis	
1-18:3-2-18:3-digalactosyldiacylglycerol	Phosphoglycerol biosynthesis	
acetylenedicarboxylate	Pyruvate metabolism	
Differentially regulated in response to wounding		
(*S*)-malate		Suppressed
6-methylthiohexylglucosinolate	Glucosinolate biosynthesis	Suppressed
inosine monophosphate	Nucleic acid biosynthesis	Suppressed
1-18:3-2-*trans*-16:1-phosphatidylglycerol	Phosphoglycerol biosynthesis	Suppressed
16α, 17-epoxy gibberellin A4	Phytohormone	Suppressed
pheophorbide *a*	Chlorophyll breakdown	Elevated
pheophorbide *b*	Chlorophyll breakdown	Elevated
secologanin	Terpenoid biosynthesis	Elevated
kaempferol-3-*O*-gentiobioside-7-*O*-rhamnoside	Flavonol biosynthesis	Elevated

Appendix 6.3. Statistical analysis of treatment effects on A) constitutive and B) wound-induced metabolites in *Arabidopsis thaliana*. Significant differences in levels of metabolites contributing higher variability in principle component analysis were analyzed by 2-factorial ANOVA (significance level, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$). When a 2-way interaction existed between CO_2 and nitrate-fertilization, effects of nitrate-fertilization were evaluated at ambient and elevated CO_2.

A) CO_2 and nitrate-fertilization effects on constitutive metabolites

Metabolites	C x N (Sig.)	CO_2 effect (Sig.)	
8-methylthiooctyl glucosinolate-*D*-glucoside	$F_{(1,12)} = 0.08$ (NS)	$F_{(1,12)} = 6.8$ (*)	Higher level at elevated CO_2
7-methylthioheptyl glucosinolate	$F_{(1,12)} = 0.14$ (NS)	$F_{(1,12)} = 5.7$ (*)	Higher level at elevated CO_2
isovitexin-7-*O*-glucosyl-2"O-rhamnoside	$F_{(1,12)} = 1.06$ (NS)	$F_{(1,12)} = 67.1$ (***)	Higher level at elevated CO_2
kaempferol-3-*O*-gentiobioside-7-*O*-rhamnoside	$F_{(1,12)} = 1.9$ (NS)	$F_{(1,12)} = 27.9$ (***)	Higher level at ambient CO_2
kaempferol 3-*O*-[6-(4-coumaroyl)-β-*D*-glucosyl-(1->2)-glucosyl-(1->2)-β-*D*-glucoside	$F_{(1,12)} = 0.04$ (NS)	$F_{(1,12)} = 14.1$ (**)	Higher level at ambient CO_2
3-indolylmethyl-glucosinolate	$F_{(1,12)} = 2.9$ (NS)	$F_{(1,12)} = 9.06$ (**)	Higher level at ambient CO_2
3-butenylglucosinolate	$F_{(1,12)} = 3.2$ (NS)	$F_{(1,12)} = 28.6$ (***)	Higher level at ambient CO_2
8-methylsulfinyloctyl glucosinolate	$F_{(1,12)} = 0.57$ (NS)	$F_{(1,12)} = 6.1$ (*)	Higher level at ambient CO_2
sirohydrochlorin	$F_{(1,12)} = 3.4$ (NS)	$F_{(1,12)} = 178.8$ (***)	Identified in ambient CO_2
(9Z,11E,15Z)-(13*S*)-hydroperoxyoctadeca-9,11,15-trienoate	$F_{(1,12)} = 1.4$ (NS)	$F_{(1,12)} = 30.1$ (***)	Identified in elevated CO_2
trans-Zeatin riboside monophosphate	$F_{(1,12)} = 2.5$ (NS)	$F_{(1,12)} = 645$ (***)	Identified in elevated CO_2
4-methylsulfinylbutyl glucosinolate	$F_{(1,12)} = 41.0$ (*)	- higher level at ambient CO_2 - responsive to nitrate-stress condition under ambient CO_2	
2-Phenylethylglucosinolate	$F_{(1,12)} = 10.5$ (**)	- higher level at ambient CO_2 - responsive to nitrate-stress condition under ambient CO_2	
5-methylthiopentylglucosinolate	$F_{(1,12)} = 9.8$ (**)	- higher level at elevated CO_2 - responsive to nitrate-sufficient condition under ambient CO_2	
6-methylsulfinylhexyl glucosinolate	$F_{(1,12)} = 31.9$ (***)	- responsive to nitrate-stress condition under ambient CO_2	
(1*R*,6*R*)-6-hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate	$F_{(1,12)} = 205.3$ (***)	- higher level at elevated CO_2 - responsive to nitrate-sufficient condition under ambient CO_2	
5-amino-6-(5-phospho-D-ribosylamino)uracil	$F_{(1,12)} = 119.8$ (***)	- higher level at elevated CO_2 - responsive to nitrate-sufficient	

		condition under ambient CO_2
riboflavin-5'-phosphate (FMN)	$F_{(1,12)}$ = 332 (***)	- higher level at ambient CO_2 - responsive to nitrate-stress condition under elevated CO_2
R(-)-3,4-dihydroxyphenyllactate	$F_{(1,12)}$ = 41.0 (***)	- higher level at ambient CO_2 - responsive to nitrate-stress condition under elevated CO_2

B) CO_2 and nitrate-fertilization effects on wound-induced metabolites

Metabolites	**C x N (Sig.)**	**CO_2 effect (Sig.)**	**Remark**
8-methylsulfinyloctyl glucosinolate	$F_{(1,12)}$ = 4.1 (NS)	$F_{(1,12)}$ = 4.8 (*)	Higher level at elevated CO_2
TDP-glucose	$F_{(1,12)}$ = 1.4 (NS)	$F_{(1,12)}$ = 8.3 (**)	Higher level at elevated CO_2
isovitexin-7-*O*-glucosyl-2"*O*-rhamnoside	$F_{(1,12)}$ = 0.8 (NS)	$F_{(1,12)}$ = 67.11 (***)	Higher level at elevated CO_2
kaempferol-3-O-gentiobioside-7-*O*-rhamnoside	$F_{(1,12)}$ = 0.29 (NS)	$F_{(1,12)}$ = 15.5 (**)	Higher level at ambient CO_2
kaempferol 3-*O*-[6-(4-coumaroyl)-β-(1→2)-glucosyl-(1→2)glucosyl-β-glucoside	$F_{(1,12)}$ = 1.3 (NS)	$F_{(1,12)}$ = 56.5 (**)	Higher level at ambient CO_2
5-methylthiopentylglucosinolate	$F_{(1,12)}$ = 0.29 (NS)	- higher level at elevated CO_2 - responsive to nitrate-sufficient condition	
3-methylsulfinylpropyl-glucosinolate	$F_{(1,12)}$ = 11.7 (**)	- higher level at elevated CO_2 - responsive to nitrate-sufficient condition under elevated CO_2	
8-methylthiooctyl glucosinolate	$F_{(1,12)}$ = 13.6 (**)	- higher level at elevated CO_2 - responsive to nitrate-sufficient condition under elevated CO_2	
7-methylthioheptyl glucosinolate	$F_{(1,12)}$ = 21.6 (***)	- higher level at elevated CO_2 - responsive to nitrate-sufficient condition under elevated CO_2	
6-methylsulfinylhexyl glucosinolate	$F_{(1,12)}$ = 16.9 (***)	- higher level at elevated CO_2 - responsive to nitrate-stress condition under ambient CO_2	