Diet-dependent regulation of labial salivary genes in the beet armyworm, Spodoptera exigua

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II. ABSTRACT

The beet armyworm, *Spodoptera exigua*, has evolved numerous mechanisms to circumvent plant defense responses. One of these mechanisms is believed to involve the activity of labial salivary glucose oxidase (GOX) which, through the production of hydrogen peroxide, interferes with plant induced defenses. The activity of GOX is diet-dependent. The expression of *SeGox* and another salivary gene, *Se1H,* are differentially regulated in response to diet (plant or transfer from plant to wheat-germ based artificial diet for 4 hours), but not in response to chemically defined artificial diets varying in protein to digestible carbohydrate ratios. However, GOX enzyme activity is dependent on the nutrient quality of the chemically defined artificial diets. This suggests that nutritional quality controls enzyme activity through post-transcriptional regulation. To simulate natural plant diets, *Medicago truncatula* plants were grown under ambient (440 ppm) or elevated (880 ppm) carbon dioxide conditions to alter their C:N ratio. When caterpillars were fed these plants, labial salivary GOX activity was undetectable regardless of the plant treatment. Finally, 5' upstream regions of salivary genes *Se1H, Se2J* and *SeGox* were amplified to determine potential upstream promoter elements, but were unable to be sequenced due to amplicon contamination.

III. RÉSUMÉ

La chenille de *Spodoptera exigua* est capable de nourrir sur les plantes malgré ses défenses anti-herbivores, grâce à des enzymes salivaires. Un de ces enzymes salivaires capable de combattre les defenses, glucose oxidase (GOX), est aussi régulé par la diète avec laquelle la chenille se nourrit. Il est donc important d'identifier la réglementation des produits salivaires comme GOX. Ce projet vise à déterminer les tendances de transcription et d'activité enzymatique de trois gènes salivaires (*Se1H*, *Se2J* et *SeGox*) en réponse de différentes diètes. De plus, les régions en amont des gènes salivaires ont tentées être séquencer afin de trouver des promoteurs putatives. Les résultats ont démontré que les trois gènes ne sont pas régulées transcriptionellement, mais que l'activité enzymatique de GOX dépend de la qualité nutritive des diètes. Cependant, les régions en amont des trois gènes n'ont pas pu être séquencé à cause de raisons de contaminations et d'amplification imprécises.

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VI. General introduction

1. Introduction

Plants are targeted by many organisms for predation. To protect themselves, plants have evolved defense mechanisms against a wide variety of herbivores (Karban and Myers, 1989; Durrant and Dong, 2004). These mechanisms may be either constitutive or induced in response to physiological stress (Leon et al., 2001). Some caterpillar species may be able to circumvent plant induced defense responses, possibly because of labial salivary enzymes, such as glucose oxidase (GOX) (Musser et al., 2002). The focus of this research is to understand the regulation of GOX, as well as other caterpillar salivary gene products, in response to nutritional quality of the diets the caterpillars feed on.

In response to bacterial, fungal and viral pathogens, the plant protects itself by the systemic acquired resistance (SAR) pathway, which has downstream products of pathogenesis-related genes (PR genes) to combat infection (Durrant and Dong, 2004). On the other hand, physical wounding, that happens when leaves are chewed by herbivores, elicits the induced resistance (IR) pathway (Ross, 1961; Karban and Baldwin, 1997). End products of IR are defensive proteins such as proteinase inhibitors (PIs) and laccase-like multicopper oxidases (LMCOs) (Weech et al., 2008). During herbivory, these antinutritive compounds can be found both locally and systemically (Karban et al., 1989). Although SAR and IR involve different elicitors and have different downstream products, an important protein, NPR1, is involved in both these pathways (Spoel et al., 2003). NPR1 is a positive regulator of SAR, but also antagonizes IR. This cross-talk

mediated by NPR1 is believed to have evolved to conserve energy, preventing both SAR and IR being active during plant response to biotic attack (Purrington, 2000; Spoel et al., 2003; Koornneef et al., 2008).

During feeding, caterpillars secrete GOX from their labial salivary gland. This enzyme catalyses the production of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS). H_2O_2 increases the cell's redox potential and activates NPR1, leading to IR shutdown (Spoel et al., 2003). With a weakened IR, plants are unable to protect themselves from caterpillar herbivory (Musser et al., 2002). GOX may also aid caterpillars in coping with the surplus of carbohydrates found in their plant diet (Eichenseer et al., 1999; Bede et al., 2007). Compared to animals, plants have an excess amount of sugar which may be detrimental to caterpillars (Warbrick-Smith et al., 2006). GOX is believed to convert them to a metabolically inaccessible form (Eichenseer et al., 1999, Bede et al., 2007). In choice diet experiments, *Spodoptera exigua* caterpillars were allowed to select from diets which varied in the ratio of protein (P) and digestible carbohydrates (C) to determine that their self-selected ratio was 22p:20c (Merkx-Jacques et al., 2008). Labial salivary GOX enzyme activity also depends on the P to C ratios of the diet (Babic et al., 2008). Since GOX inhibits IR and is dependent on diet, the objective of this research is to understand the effect that dietary nutritional quality has on caterpillar salivary gene transcription and enzyme activity.

2. Hypothesis

Previous work in our laboratory has shown that *S. exigua* GOX enzyme activity is regulated by the P to C of the diet the caterpillars feed on (Babic et al.,

2008). To identify other putative diet-dependent genes in caterpillar labial salivary glands, a differential display experiment performed in Bede's laboratory found that two other genes, *Se1H* and *Se2J*, are also diet-dependent at the transcript level. I hypothesize that the P:C of the diet the caterpillars feed on, affects transcript levels of the caterpillar salivary genes, *SeGox*, *Se1H* and *Se2J*, and also the enzyme activity of GOX.

3. Objectives

1- To measure labial salivary *SeGox*, *Se1H* and *Se2J* transcript levels and GOX enzyme activity in *S. exigua* caterpillars fed chemically-defined diets varying in P:C.

2- To determine if *Medicago truncatula* plants grown under different carbon dioxide (CO_2) levels have altered protein to digestible carbohydrate ratios, which, in turn, will affect labial salivary GOX enzyme activity of caterpillars.

3- To isolate the 5' upstream regions of *Se1H*, *Se2J* and *SeGox* transcription start sites by inverse PCR and to characterize these putative promoter sequences using bioinformatics for regions which may be involved in transcription regulation.

VII. LITERATURE REVIEW

1. Introduction

Plants are vulnerable to pathogen attack and herbivory and have, therefore, evolved mechanisms to protect themselves. These defense response can either be constitutive or induced (i.e. turned on in response to stimulus) (Karban and Myers, 1989) and can be of either of a chemical or physical nature. Although constitutive defenses are expressed during the plant's lifetime, they are often tightly associated with developmental changes. For example, younger leaves often have higher levels of constitutive defenses than older, shaded leaves (Wittstock and Gershenzon, 2002). Physical defenses include trichomes or the tightness of cell walls which may prevent pathogens, such as fungi, from infecting the plant (Israel et al., 1980). Chemical defenses on the other hand can be made up of secondary metabolites and play important roles as antifeedants or toxins in plant chemical defenses (Biere et al., 2004). One example of a constitutive chemical defense is the production of nicotine, a harmful neurotoxin affecting predators. This defense is both constitutive, present in low levels, and induced in response to caterpillar herbivory (Wittstock and Gershenzon, 2002; Steppuhn et al., 2004). Constitutive resistance may be costly to the plant since it is produced even in the absence of a herbivore. Therefore, it may be more energy efficient for the plant if the resistance is induced in response to a stimulus. Constitutive defenses also place selective pressure on attacking organisms to evolve resistance mechanisms (Gershenzon et al., 1985). Therefore, the plant also has defenses that are synthesized in response

to stress (i.e. induced). However, with inducible defense responses, there may be a time lag between the time of attack and full-fledged defense.

2. Induced response

2.1 Definitions

Any kind of physiological change that occurs in plants following damage or stress is referred to as an "induced response." In this thesis, resistance will refer to the plant's ability to decrease the fitness of predators such as herbivores (Karban and Baldwin, 1997). Plants may be subject to different types of assaults (bacteria, viruses, fungi or herbivores) and have evolved a several general mechanism to protect themselves.

2.2 The responses

Three main types of plant defense responses have been extensively studied and are divided into the induced resistance (IR), systemic acquired resistance (SAR) and induced systemic resistance (ISR) pathways, depending on which phytohormones are involved. However, extensive overlap between these signaling pathways shows that plant regulation of defense responses is a sophisticated mechanism involving interactions between multiple hormone-regulated pathways, resulting in the temporal and spatial integration of defense mechanisms geared to target the pest organism. Following pathogen infection, damage or stress, the plant responds to counteract the threat. The plant first acts locally, at the site of damage, but often a signal propagates to other plant tissues to activate defense responses distally, protecting systemic leaves (Ryals et al., 1996; Sticher et al., 1997; Schilmiller and Howe, 2005). It is clear that these defense responses have

elements, which are plant species- and phenology-specific, but also dependent on the nature of the inducer, the signaling pathway elicited, the compartmentalization and the interaction between signaling pathways (cross-talk). In the next sections, a more in-depth overview of these major defense signaling pathways is provided and the nature of cross-talk between these pathways is briefly explained.

2.2.1 Systemic acquired resistance

In recognition of a biotrophic pathogen, the plant mounts a defense response which results in an oxidative burst and localized cell death surrounding the site of infection to prevent propagation of pathogenesis (Glazebrook, 2005). This is followed by a signal which travels systemically through the plant to distal tissues, inducing the expression of pathogenesis-related (PR) genes in nearby leaves. This confers "long term memory" to subsequent infections (Durrant and Dong, 2004).

On the molecular level, salicylic acid (SA) levels are drastically increased at the area of infection as well as distally in neighboring cells (Shulaev et al., 1995). SA is highly correlated with the activation of SAR and, despite active research in this area, it is still unclear whether SA causes SAR to be turned on, or if it is merely a product of SAR activation. At the site of infection, the concentration of reactive oxygen species (ROS) also increases and acts as a trigger for local apoptosis to prevent the infection from spreading throughout the plant (Kombrink and Schmelzer, 2001). ROS increase, apoptosis and cell necrosis, together, are referred to as the oxidative burst. Although it is not completely understood, it is believed that there is feed-forward regulation between the ROS and SA biosynthesis (Alvarez et al., 1998).

Increases in ROS affects the cell's redox potential resulting in an unknown SA-responsive oxidoreductase to reduce the disulfide bonds in the transcription co-factor, NPR1, allowing it to dissociate from its trimeric conformation and enter the nucleus (Despres et al., 2003). Once in the nucleus, another unknown enzyme catalyzes the oxidation of cysteine residues on NPR1 to form a bridge with TGA transcription factors (Mou et al., 2003). TGAs are a family of transcription factors that regulate PR genes as well as genes involved in SA biosynthesis (Durrant and Dong, 2004). The interaction of NPR1 with TGA allows the leucine zipper of TGA to bind DNA with a higher affinity (Despres et al., 2003). In addition to activating TGAs, NPR1 also activates another family of transcription factors known as the WRKY regulators. These proteins regulate certain genes by binding to *cis*-elements known as W-boxes found in several gene promoters responsible for SAR activation (Dong Wang, 2006). In fact, there is one such W-box in the promoter region of *Npr1,* regulating its expression (Yu et al., 2001; Xu et al., 2006).

2.2.2 Induced resistance to wounding

In comparison to SAR, wounding of the plant leaf, for example by caterpillar chewing or simple mechanical damage, elicits IR, a pathway mediated by a group of hormones known as the octadecanoids, which includes jasmonic acid (JA) (Fig. 1) (Beckers and Spoel, 2006). In Solanaceous plants (tomatoes, for example), wounding causes oligosaccharide fragments (*i.e.* galacturonides) of the cell wall to stimulate a signaling pathway that results in the cleavage of the

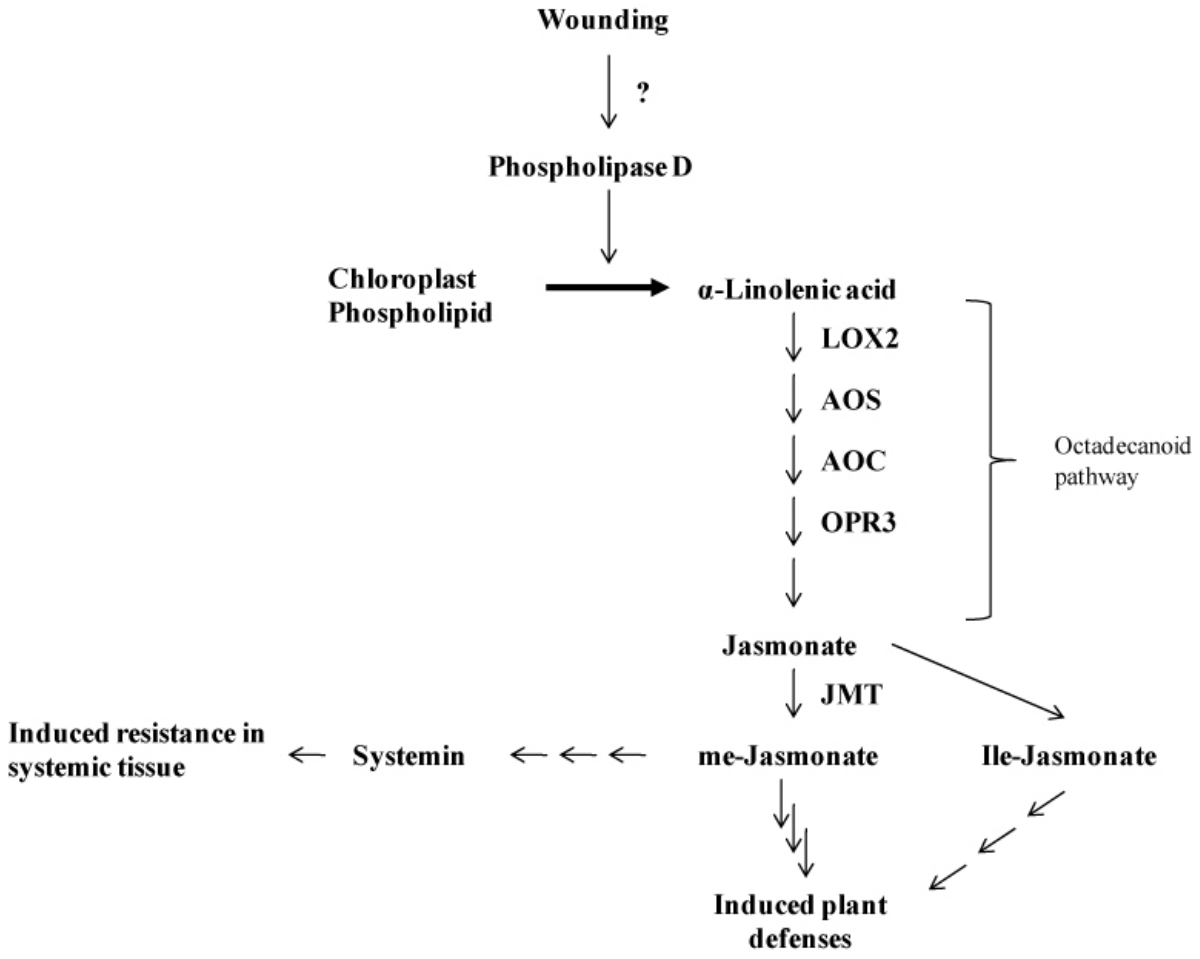

Figure 1. Induced responses in Solanaceous plants. When caterpillars wound leaves by chewing, phospholipases are activated through an unknown mechanism, allowing the hydrolysis of membrane phospholipids to initiate signal transduction of the induced resistance pathway. This pathway includes the activation of the octodecanoid pathway, which produces jasmonate and jasmonate analogs. These metabolites will have downstream effects on transcriptional activation of anti-nutritive genes, as well as induce a systemic response in distal leaves. (Lipoxygenase 2: LOX2; allene oxide synthase: AOS; allene oxide carboxylase: AOC; Oxophytodienoate reductase: OPR3; jasmonate methyl transferase: JMT)

polypeptide prosystemin to the hormone systemin (Ryan and Pearce, 1998; Thaler, 1999). Phospholipase D is activated, which, in turn, hydrolyzes phospholipids from the chloroplast outer membrane to produce the first substrate of the octadecanoid pathway: linolenic acid (Ryu and Wang, 1998). In addition, systemin and octadecanoids, such as JA, activate polygalacturonase to further disrupt the cell wall, releasing more galacturonides (Orozco-Cardenas and Ryan, 1999; Orozco-Cardenas et al., 2001). Though there are similarities in other plants, the signalling events can be species-specific. In Brassicaceous plants (*i.e.* Arabidopsis), for example, the peptide systemin is not involved (Ryan and Pearce, 1998).

Regardless of the plant species, octadecanoid-related compounds, such as 12-oxophytodienoate and JA, play major roles in mediating the plant response. In fact, biosynthetic enzymes of the octadecanoid pathway, such as lipoxygenase 2 (*Lox2*), are up-regulated by JA and wounding, providing an efficient feed-forward mechanism to amplify the signal (Beckers and Spoel, 2006). In systemic responses, JA-isoleucine binds to and activates coronatine insensitive 1 (COI1), a ubiquitin ligase recruiting factor (Katsir et al., 2008) (Fig. 2). COI1 interacts with ubiquitin ligase SCF, having SKP1, cullin and an F-Box proteins as its subunits, which then targets a jasmonate-ZIM (JAZ) protein, a transcriptional repressor, for ubiquitination and proteosome-mediated degradation (Xu et al., 2002; Chung et al., 2008). The JAZ protein strongly interacts with the transcription factor MYC2, which is responsible for IR gene expression. Once the JAZ protein is degraded, MYC2 is free to enter the nucleus and activate transcription (Chung et al., 2008).

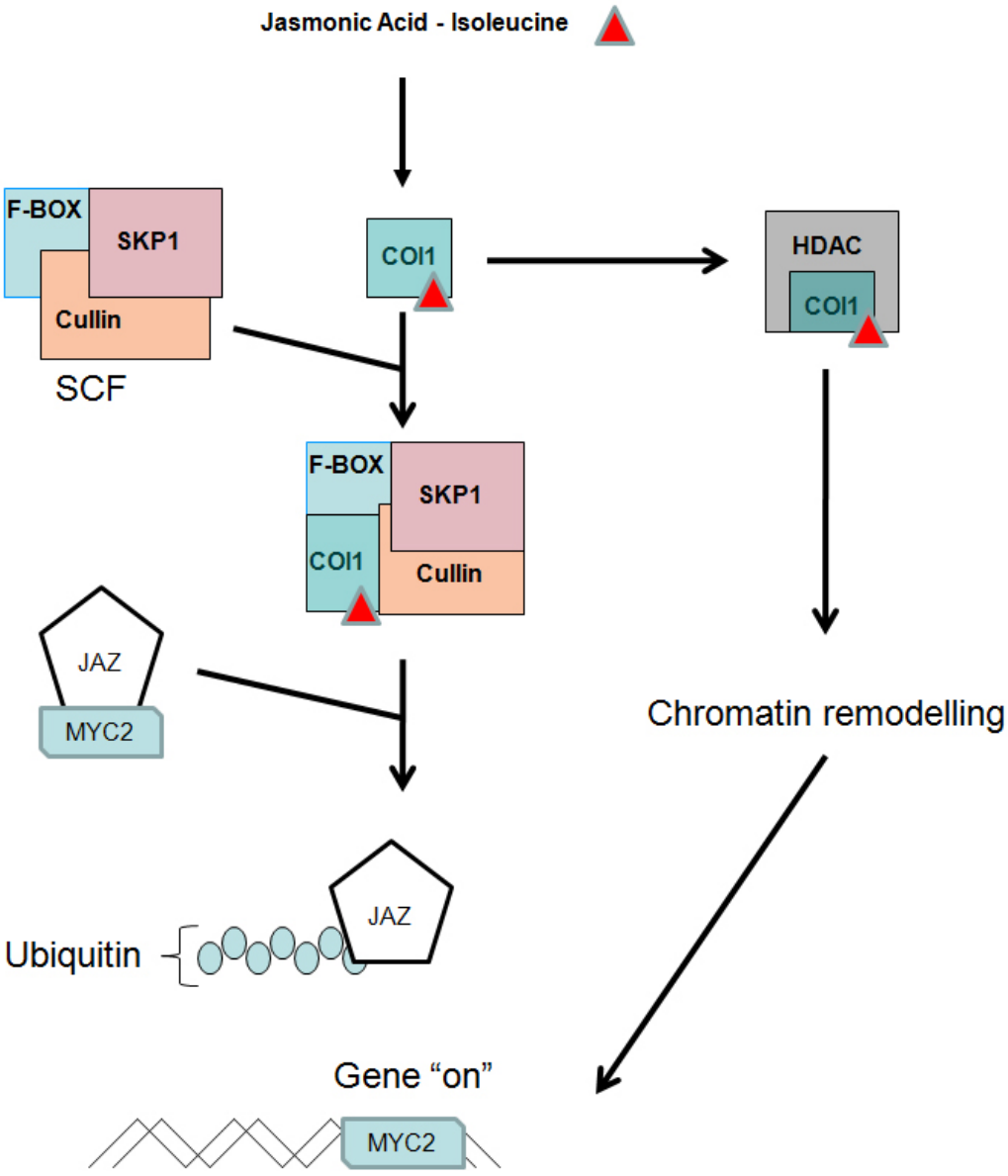

Figure 2. Mechanism of jasmonate-regulated transcription. Jasmonate conjugated with isoleucine is an upstream signal in plant induced resistance. There are two current ideas about the mechanism(s) by which jasmonate regulates transcription. The first is though the recruitment of an ubiquitin ligase to target a JAZ protein, a negative regulator, for proteosome-mediated degradation. This allows MYC2 to translocate to the nucleus to activate the transcription of defense genes. The second mechanism is through the recruitment of a histone deacetylase to target histone moeities leading to chromatin condensation, inhibiting the expression of a repression of JA-dependent gene expression.

COI1 has, also, been shown to recruit a histone deacetylase leading to chromatin condensation and the selective inactivation of genes presumably encoding repressors of JA-dependant gene expression (Fig. 2) by removing histone modifications and triggering chromatin condensation (Devoto et al., 2002).

The end product of the IR pathway are the upregulation of defense-related proteins such proteinase inhibitors (PIs) and laccase-like multicopper oxidases (LMCOs) (Beckers and Spoel, 2006). Although these two proteins have other physiological functions in plants, only their roles in defense against caterpillar herbivory will be discussed (see section 3).

2.2.3 Crosstalk between systemic acquired resistance and induced resistance pathway

SAR and IR, as mentioned above, have several overlapping features and this is referred to as cross-talk (Fig. 3). This was first noted when tomato plants treated with SA and then wounded showed a significant decrease in the gene expression of PIs, an end product of the IR pathway (Doherty et al., 1988). Treatment of *Arabidopsis* plants with SA also showed a noticeable decrease of JA-dependant and systemin-mediated gene expression in response to caterpillar herbivory (Doares et al., 1995; Stout et al., 1996). In another instance of cross-talk, there was a decrease in expression of key enzymes involved in the octadecanoid pathway when plants were subjected to bacterial infection (Spoel et al., 2003). Based on these studies, the potential roles of JA, SA and the protein NPR1 were investigated in this IR-SAR cross-talk. It was shown that NPR1

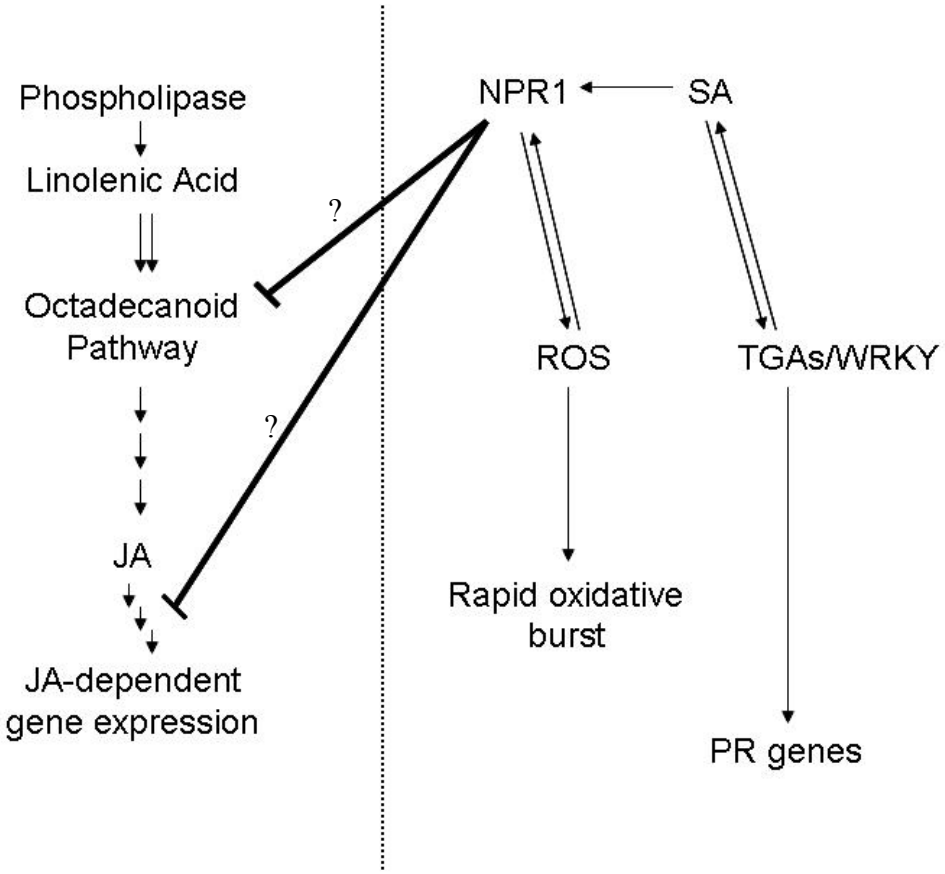

Figure 3. Biochemical pathway depicting the cross-talk between induced resistance and systemic acquired resistance pathways. NPR1 is a positive regulator of plant defense gene expression. It is activated by changes in cellular redox potential and either translocates to the nucleus to regulate SAR gene transcription or remains in the cytosol to inactivate IR by an unknown mechanism. There have been two possible theories regarding the mechanism by which NPR1 is repressing IR. The first is through the inhibition of the octadecanoid pathway and the second is by interfering with the proteosome-mediated degradation of the JAZ protein.

activation correlates with the suppression of JA-responsive genes (Spoel et al., 2003). Transgenic *Arabidopsis* plants with NPR1-glutacorticoid receptor fusion proteins which were constructed to prevent NPR1 nuclear localization and showed that these plants were incapable of turning on their SAR pathway, but SA-mediated suppression of JA-responsive genes was unaffected. These results indicate that although NPR1 negatively affects IR, the mechanism does not involve transcriptional regulation and that the function is cytosolic (Spoel et al., 2003) (Fig. 3). The mechanism underlying the SA-mediated suppression of IR remains to be elucidated. One group proposes that NPR1 prevents the nuclear localization of certain transcription factors responsible for JA-dependent gene expression (Ryals et al., 1997). Another currently held hypothesis regarding NPR1-mediated cross-talk is through the interference of proteosome-mediated degradation of JAZ, disallowing derepression of the MYC2 transcription factor from activating JA-dependent gene expression (Chung et al., 2008).

2.3 Plant-Insect interactions

One of the most agriculturally important pests affecting plant fitness is the caterpillar. Caterpillars are avid leaf eaters and propagate quickly causing a rather serious dilemma for plants. IR pathway is the method by which plants normally defend themselves against herbivores (Karban and Baldwin, 1997). Caterpillars, however, have adapted a mechanism to shut down the IR, allowing itself to feed without being affected by plant defensive strategies (Musser et al., 2002; Weech et al., 2008). As mentioned earlier, NPR1 is an important protein responsible for the activation of the SAR and the repression of the IR (Spoel et al., 2003). However, it

is believed that the SA-mediated antagonism of JA pathways is redox-modulated since cellular glutathione levels rise when plants are pathogen-infected or treated with SA (Koornneef et al., 2008). NPR1 functionality is dependent on the cell's redox potential which affects its ability to dissociate from its oligomeric state and become active. Once activated, it is believed that NPR1 inhibits JA biosynthesis in the octadecanoid pathway or prevents the transcriptional activation of JA-mediated genes by interfering with JAZ degradation (see figure 3). Caterpillars take advantage of this cross-talk by secreting glucose oxidase (GOX) in their saliva. This enzyme catalyzes the oxidation of glucose, producing gluconate and hydrogen peroxide (H_2O_2), an ROS (Eichenseer et al., 1999). The H_2O_2 contributes to the cell's redox protential and activates NPR1 to shut down IR (Musser et al., 2002).

3.1 Caterpillar herbivory

Caterpillars are important crop pests responsible for billions of dollars in losses for farmers. Some caterpillars, like the beet armyworm *Spodoptera exigua*, feed on a number of different plant species (generalists) (Ruberson et al., 1994). These generalist feeders normally have multiple ways to circumvent plant defenses. One example was highlighted above, where caterpillars shut down the plant's induced resistance by secreting GOX in their saliva during feeding (Musser et al., 2002). Lysozyme, another caterpillar salivary enzyme, has also been known to affect plants (Liu et al., 2004). Lysozymes are hydrolytic enzymes capable of breaking down the bacterial peptidoglycans (Johnson, 1966). Lysis of bacteria on the plant leaf by caterpillar salivary lysozyme may result in pieces of

cell wall being released, eliciting the plant SAR, thereby antagonizing the induction of IR (Liu et al., 2004). In another instance, generalist *Trichoplusia ni* caterpillars were able to feed and survive on *Conium maculatum* (poison hemlock), despite the high levels of toxic alkaloids (coniine and γ-coniceine) present in the plant (Castells and Berenbaum, 2008). These caterpillars detoxify the alkaloids by oxidation, using a cytochrome P_{450} and, at the same time, excrete the leftover alkaloids without digesting them. Numerous other examples show that insects can overcome plant defenses and, therefore, understanding how plants defend themselves against caterpillar herbivory and, in turn, how the caterpillar overcomes these defenses, is of considerable interest for pest management strategies.

The insect's ability to circumvent plant defense responses places selective pressure on plants to evolve new mechanisms to protect themselves from herbivory. Nutritional quality is believed to be one such way to discourage insects from feeding. For caterpillars, plants represent a surplus source of carbohydrates but are limited in protein quality and quantity (Mattson, 1980; Merkx-Jacques et al., 2008; Bede et al. 2007). In response to herbivory, plants further accentuate this by upregulating defensive proteins, which will further interfere with caterpillar digestive processes. For example, proteinases are enzymes present in caterpillar gut and are responsible for hydrolyzing dietary proteins allowing proper absorption of amino acids (Amarant et al., 1991). In response to herbivory and wounding, proteinase inhibitors (PIs) are often increased in plant leaf tissues (Green and Ryan, 1972). As the caterpillar feeds on the leaf material, these PIs

bind and inhibit the caterpillar gut proteinases, preventing proper enzymatic activity and absorption of amino acids. Another defense mechanism that plants have is laccase-like multicopper oxidases (LMCOs). These enzymes catalyze the activation of *o*-phenols to reactive *o*-quinones that serve as amino acid cross-linkers, again preventing herbivores from obtaining their nutrients (Kellner et al., 2007). Interfering with the caterpillar's ability to acquire nutrients is an important mechanism for plants to affect insect viability (Merkx-Jacques and Bede, 2005).

3.2 Plants as diets

Although plants are the natural diets of phytophaghous caterpillars, plants provide too little protein (P) and too much carbohydrates (C) for the caterpillar's well being. This places selective pressure on herbivores since, in general, a protein deficient diet causes caterpillars to compensate by consuming more mass to meet their protein requirement, thereby further increasing their carbohydrate intake (Colasurdo, N et al., 2007). In fact, it is hypothesized that the role of GOX is to oxidize glucose to an inaccessible form because of its high abundance in plants. Caterpillars prefer a P-rich diet, whereas, plants are composed primarily of C and are nitrogen-poor (Merkx-Jacques et al., 2008). The P:C of *Medicago truncatula* plants is approximately 7p:67c. This ratio, however, can differ from one plant species to another (for example, *Medicago sativa* nutrient ratio is approximately 17p:28c) and can be altered by fertilization or atmospheric CO_2 levels (Makino and Mae, 1999; Merkx-Jacques, 2006). In this research, plant P:C will be altered by varying the levels of CO_2 the plants are grown under (440 ppm and 880 ppm) to provide nutritionally distinct diets to caterpillars. C3 plants are less efficient at

fixing carbon due to the dual function of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) which can accept either O_2 or CO_2 as a substrate (Raghavendra and Das, 1977). By increasing surrounding levels of CO_2, C3 plants will increase the efficiency of CO_2 fixation (Raghavendra and Das, 1977). However, long-term exposure to the heightened CO_2 will cause the plant to acclimate to surrounding conditions and, thus, reduce photosynthesis by downregulating cellular RuBisCo levels (Tripp et al., 1992; Wolfe et al., 1998; Wang et al., 2008). Since RuBisCo is the most abundant soluble protein in the plant, a significant decrease in RuBisCo levels will decrease the overall nitrogen count of the plant and decrease plant P:C (Schadler et al., 2007). The plant used in this study is *M. truncatula*, a close relative of the important forage crop, alfalfa, *M. sativa*. It has a relatively short life cycle and has recently been chosen as the model organism for legume biology; the genome is approximately 450 Mbp, arranged in 8 pairs of chromosomes (http://www.medicago.org). Compared to other model plant systems, *M. truncatula* harbours few insect parasites and little fungi, allowing for optimal use in the study of plant-insect interactions.

3.3 Nutritional quality for the caterpillar diet

The insect chosen in these experiments is the caterpillar of the beet armyworm, *Spodoptera exigua* (Lepidoptera). An economically important agricultural pest, these caterpillars can be found in southern United States as well as in many East Asian countries (Capinera, 1999). This insect is classified as a generalist herbivore and feeds on a large collection of host organisms that include

vegetables such as asparagus, beet, bean and lettuce crops as well as flowers and weeds.

In choice experiments where *S. exigua* caterpillars were allowed to select their desired amount of P and digestible C, the preferred P:C of the caterpillars vastly differs from that of plant tissue. The artificial diet ratio chosen by the caterpillars was 22p:20c (% w/w). In comparison, the plant diet is typically carbohydrate-biased and protein-poor (Merkx-Jacques et al., 2008). In a similar experiment where caterpillars were restricted to diets with a specific P:C (no choice), caterpillar fecundity was noticeably lower among caterpillars fed a protein-poor and carbohydrate-rich diet than caterpillars feeding on the self-selected 22p:20c diets. Caterpillars fed with nutritionally poor diets had higher mortality rates on carbohydrate-biased diets and delayed development on protein-poor diets. It is clear that diet nutritional quality plays an important role in caterpillar performance.

In fact, the composition of their diet influences GOX enzyme activity and lysozyme expression (Liu et al., 2004; Merkx-Jacques and Bede, 2005). In one experiment, GOX showed diet-dependent enzyme activity in response to different types of diet. Caterpillars fed artificial diet showed much higher GOX enzyme activity compared to caterpillars fed *M. truncatula* plants (Merkx-Jacques et al. 2005). Subsequent experiments assayed GOX enzyme activity in response to artificial diets varying in P:C showed that caterpillar GOX enzyme activity requires a protein-rich diet and that once amino acid requirements have been met, enzyme activity increases with increasing carbohydrate levels (Babic et al., 2008).

Lysozyme, another enzyme from caterpillar labial saliva, has also been linked with diet-dependent expression (Liu et al., 2004). A dot blot was used to visualize lysozyme mRNA expressions when *Helicoverpa zea* caterpillars were fed different plants (Liu et al., 2004). Higher amounts of lysozyme mRNA were observed when the caterpillars were fed cotton and tomato leaves, compared with tobacco leaves. Therefore, caterpillars salivary enzymes that have been implicated in shutting down plant defense responses show diet-dependent expression and activity (Liu et al., 2004; Babic et al., 2008). In a more recent experiment conducted by Bede (unpublished), two genes, *Se1H* and *Se2J*, were identified as having diet-dependent transcript expression by means of the differential display technique cDNA-amplified fragment length polymorphism (cDNA-AFLP). Here, caterpillars were fed either plant diet, artificial diet or transferred from plant to artificial diet for 4 hours. Several fragments, presumably representing different genes, showed different expressions based on the diets. Two differentially-expressed fragments and one constitutively-expressed fragment were chosen for more extensive expression profiling: *Se1H, Se2J* and *Se2M*, respectively. *Se2M* codes for a ubiquitin-like protein (a constitutive reference gene). *Se1H* and *Se2J*'s functions are yet to be determined. Transcript levels, as well as enzyme activity, of caterpillar salivary genes seems to dependent on the diet.

3.2 Analysis of gene expression

Caterpillar labial salivary gene transcript regulation of *HzLys* and *Se1H/Se2J* is dependent on diet (Liu et al., 2004; Bede, unpublished). In this research, I further analyze the potential role that nutrient quality plays in labial

salivary gene regulation and GOX enzyme activity. Gene transcript levels can be measured by a number of different techniques, such as nuclear run off assays coupled with northern blot analysis, competitive reverse transcription PCR or quantitative real-time PCR (qRT-PCR) (Celano et al., 1989; Wang et al., 1989; Bieche et al., 1998). The most robust and reliable of the above mentioned techniques is qRT-PCR.

4.1 Relative quantitative real-time PCR

qRT-PCR is an increasingly popular technique that allows for nucleic acid measurements and regular PCR amplification simultaneously. The method uses the ideas behind fluorescent spectrophotometry and PCR amplification (Bieche et al., 1998). The qRT-PCR technique measures nucleic acid concentrations in real-time during cycles of amplification by polymerase chain reactions. The main requirement for nucleic acid measurements is a dye that will fluoresce in proportion to the amount of double stranded nucleic acids. A very common dye used in qRT-PCR reactions is SYBR green, a polyaromatic compound capable of binding to the minor groove of double stranded DNA with a relatively high affinity and relatively low toxicity (Jin et al., 1994; Ponchel et al., 2003). This compound absorbs light at 487 nm and re-emits it at 522 nm. The intensity of fluorescence is directly proportional to the concentration of double stranded DNA. During a PCR, DNA levels double after each cycle and provide more binding sites for SYBR green, allowing for a brighter fluorescent emission.

During amplification, absolute quantities of the amplicon can be measured by comparing the fluorescence of the sample to that of a standard curve; this

method is known as absolute RT-PCR and is not well accepted within scientific community since there is no real calibration to take into consideration the many variables that may affect the levels of DNA. To correct for uncertainties, it has become common to compare absolute quantities of the fragment of interest to that of a reference gene, such as ubiquitin or tubulin (Livak and Schmittgen, 2001). The reference fragment should provide an indication that, the organism's overall transcript is not being altered; this is known as relative real-time PCR. Typically, real-time PCR is used to measure complimentary DNA (cDNA) as an accurate representative for transcript levels (mRNA) (Boulay et al., 1999). cDNA synthesis is catalyzed by the enzyme reverse transcriptase using mRNA as a template to produce DNA (Baltimore, 1970).

4.2 Inverse Polymerase Chain Reaction

There exists only a limited amount of information regarding diet-dependent transcription regulation of insect genes (Liu et al., 2004; Merkx-Jacques et al., 2005; Bede, unpublished). From the differential display cDNA-AFLP experiment, two genes were shown to be diet-dependent: *Se1H* and *Se2J*. Their regulation, however, is unknown. Therefore studying the promoter regions will shed light on the transcriptional regulation of caterpillar labial salivary genes.

Inverse PCR (iPCR) is a technique used to amplify a region of unknown DNA flanking a region of known DNA (Ochman et al., 1988) (Fig. 4). One prominent uses of iPCR is for the amplification of the 5'upstream region of a transcription start site to locate potential promoters. As long as a short area of the

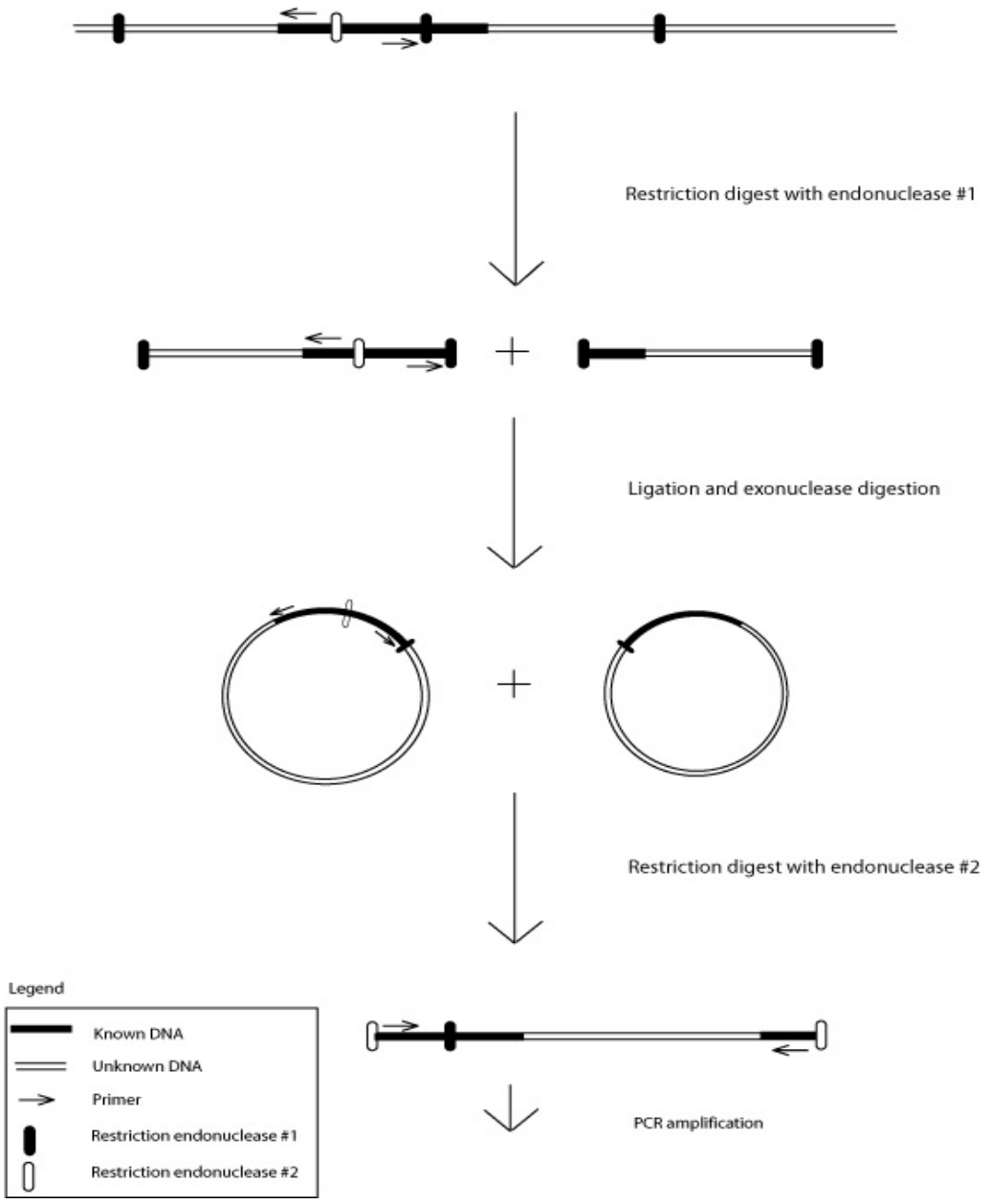

Figure 4. Inverse PCR. Inverse PCR is primarily used to amplify a region of unknown DNA flanking a region of known DNA. First, genomic DNA is digested with a restriction endonuclease, then religated to obtain circular fragments. Linear DNA is removed from the mixture with an exonulease digestion. Before iPCR amplification, samples are purified to remove nucleotides solutions and enzymes. A second run with nested primers is required to increase the specificity of the iPCR. Amplifications are visualized by agarose gel electrophoresis and individual bands are excised and purified to submit for sequencing.

open reading frame of the gene of interest is known, iPCR can be used to isolate the region upstream of the transcription start site.

Genomic DNA is digested using restriction endonucleases to generate fragments of varying sizes surrounded by sticky ends. Each fragment is then circularized using ligase. Now, primers designed against the genes of interest are pointed inwards, surrounding an unknown region, all in a circular piece of DNA. To increase efficiency, circular fragments can be re-digested to become linear, relieving possible hindrance that might limit the polymerase's access to the DNA strand of interest. Subsequent amplification will include fragments containing the 5' region of the known DNA until the first restriction endonuclease site found upstream of the transcription start site. The resulting fragment is amplified by PCR using primers directed towards the region of unknown DNA. To increase specificity of amplification, nested primers are designed to eliminate unspecific PCR products. Sequencing the amplified fragment can allow subsequent bioinformatics analysis to determine putative *cis*-acting promoter elements.

3.3 Summary

In this research, the transcriptional regulation of the labial salivary genes *SeGox, Se1H* and *Se2J* will be studied in caterpillars of the beet armyworm, *Spodoptera exigua*. This caterpillar species was chosen because it is a generalist pest and, therefore, has numerous mechanisms to get around host plant defences. Three salivary genes, including *SeGox*, two of which are differentially expressed in response to artificial versus plant diet, have been chosen for transcript analysis.

It is believed that this transcriptional regulation of salivary genes reflects the nutritional quality and, in particular, the P:C of the diet.

First, *SeGox*, *Se1H* and *Se2J* transcript levels and GOX enzyme activity will be measured as a function of varying P:C of artificial diets. The comparison of *SeGox* transcript levels and GOX enzyme activity will help determine if nutritional quality affects gene expression. Transcript levels of the salivary gene in response to nutritional quality will also reveal which component of the diet (P or C) are the effectors of regulation. Secondly, caterpillars will be fed plants grown under different levels of CO_2, providing nutritionally distinct diets, in an attempt to affect GOX enzyme activity. Finally, the upstream promoter regions of *SeGox, Se1H* and *Se2J* will be sequenced and analyzed bioinformatically for putative *cis*-elements regulating transcription.

VIII. DIET-SPECIFIC SALIVARY GENE EXPRESSION AND ENZYME ACTIVITY IN CATERPILLARS OF THE BEET ARMYWORM, *SPODOPTERA EXIGUA*

1. Abstract

Caterpillar labial salivary enzymes, such as glucose oxidase (GOX) have been shown to play a role in circumventing plant defense responses. Activities of some caterpillar labial salivary enzymes, such as GOX, have also been shown to be diet-dependent and may be transcriptionally regulated. In fact, labial salivary GOX enzyme activity reflects the nutritional quality of the diet, particularly the protein to digestible carbohydrate ratio (P:C) the caterpillar is feeding on (Babic et al., 2008). Transcript levels of *Se1H* and *Se2J* genes are also diet-dependent. In this research, caterpillars were fed artificial diets varying in their P:C levels to determine the roles of nutritional quality on the level of caterpillar labial salivary gene transcription of *Se1H, Se2J* and *SeGox*, as well as GOX enzyme activity. Although, GOX enzyme activity is dependent on dietary P:C, transcription regulation is irresponsive to nutrient quality, suggesting that regulation of these salivary gene products occurs post-transcriptionally. To stimulate natural conditions, the P:C of *Medicago truncatula* plants were attempted to be altered by changing atmospheric CO_2 levels to provide nutritionally distinct plant diets to the caterpillars. Plants grown under ambient or elevated levels of CO_2 (440 ppm vs 880 ppm) did not affect plant P:C levels enough to affect caterpillar labial salivary GOX enzyme activities.

2. Introduction

One strategy used by plants to protect themselves against herbivory is by providing poor nutritional quality to discourage caterpillars from feeding on them (Mattson, 1980; Schadler et al., 2007). To overcome the high levels of carbohydrates in plants, caterpillars increase respiration and may use salivary enzymes, such as glucose oxidase (GOX), to convert glucose to an inaccessible form (Bentley, 1955; Eichenseer et al., 1999; Bede et al., 2007). GOX is believed to circumvent plant defense responses, therefore, studying the regulation of this and other salivary genes is important for further understanding plant-insect interactions (Musser et al., 2002; Liu et al., 2004).

GOX enzyme activity, as well as lysozyme transcript levels, are diet-dependent (Liu et al., 2004; Merkx-Jacques and Bede, 2005; Babic et al., 2008). Dot-blot experiments have shown that lysozyme transcript levels were higher when caterpillars fed on cotton and tomato plants compared to tobacco plants (Liu et al., 2004). Furthermore, GOX studies have demonstrated that enzyme activity is dependent on P:C of artificial diets (Babic et al., 2008). Two other unknown salivary genes, *Se1H* and *Se2J*, were identified by differential display cDNA-AFLP to be differentially regulated in response to the caterpillar's diet (Bede's laboratory). However, it is unclear how salivary enzymes are regulated: transcriptionally or post-transcriptionally. There exist several instances where arthropod salivary gene transcription is regulated in response to external cues. In the tick species, *Dermacentor variabilis*, for example, the salivary gene *DvFerretin* is upregulated in response to mechanical or bacterial stress (Mulenga

et al., 2004). In contrast, regulation may take place post-transcriptionally, such as in the midge species, *Chironomus tentans,* where salivary secretory gene translation is tightly regulated at the mRNA export, splicing and translation level by a serine-arginine ribonuclear protein (Singh et al., 2006).

To understand the role of nutrient quality on caterpillar salivary gene expression and enzyme activity, caterpillars were fed artificial diets varying in P:C. Choice artificial diet experiments have shown that caterpillars select for a diet with a protein to digestible carbohydrate ratio of 22:20 (% w/w) (Merkx-Jacques et al., 2008). In contrast, plants are typically carbohydrate-biased and protein-poor and may not have the nutritional value that caterpillars prefer. Additionally, it seems that nutritional quality, also, plays an important role in caterpillar development, where caterpillars were shown to remain in each larval stage instar for a signigicantly longer time period when caterpillars were fed C-biased diets, in comparison to the self-selected ratio of 22p:20c (Simpson and Abisgold, 1985; Lee, 2007; Merkx-Jacques et al., 2008). To understand the role of dietary nutrient quality on caterpillar salivary gene expression and enzyme activity, caterpillars were fed different artificial diets composed of five different protein to digestible carbohydrate ratios (P:C): 22p:20c, 22p:40c, 30p:20c, 30p:40c and 33p:30c. The 22p:20c and 33p:30c diets have the same P:C ratio, but differ in the total amount of nutrients, whereas 22p:20c and 22p:40c maintain protein levels, but vary in digestible carbohydrate levels. 22p:40c and 30p:40c diets test varying protein levels but constant digestible carbohydrate levels. In this experiment, transcript levels of *Se1H, Se2J* and *SeGox* and GOX enzyme activity

from caterpillar labial salivary glands were measured in response to artificial diets varying in P:C. The results indicated whether nutritional quality plays a role in transcription or post-transcriptional regulation of caterpillar labial salivary genes.

Additionally, *Medicago truncatula* plants were grown under different atmospheric CO_2 conditions to alter the plant P:C ratio to determine the effect of a natural plant diet GOX enzyme activity (Schadler et al., 2007). C3 plants are less efficient efficient at fixing carbon due to the dual function of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) which can accept either O_2 or CO_2 as a substrate (Raghavendra and Das, 1977). By increasing surrounding levels of CO_2, C3 plants will increase the efficiency of CO_2 fixation (Raghavendra and Das, 1977). However, long-term exposure to the heightened CO_2 will cause the plant to acclimate to surrounding conditions and, thus, reduce photosynthesis by downregulating cellular RuBisCo levels (Tripp et al., 1992; Wolfe et al., 1998; Wang et al., 2008). Since RuBisCo is the most abundant soluble protein in the plant, a significant decrease in RuBisCo levels will decrease the overall nitrogen level of the plant and decrease plant P:C (Schadler et al., 2007).

3. Methods and materials

3.1 Rearing caterpillars

Spodoptera exigua caterpillars were obtained from AgriPest Inc. (Zebulon, North Carolina) and were reared in growth cabinets at 22.0 ± 0.1°C with 28-40% ambient humidity and 16:8 light to dark hour cycle. Insects were kept in Tupperware containers, hole-punched, and fed wheat-germ based artificial diets (Bio-Serv).

To test the effects of P:C in caterpillar diets, chemically defined artificial diets were prepared with the following P:C ratios: 22p:20c, 22p:40c, 30p:20c, 30p:40c and 33p:30c (Simpson and Abisgold, 1985). Protein mass is made from a 3:1:1 ratio of casein, albumen and peptone, respectively, and digestible digestible carbohydrate is purely glucose; the bulk of the defined diets is made of cellulose and the following ingredients were added for nutritional quality: cholesterol (0.55%w/w), linoleic acid (0.5%), Wesson salts (2.5%), ascorbate (1%), choline chloride (0.5%), sorbic acid (0.5%), methyl paraben (0.35%), vitamin mix (0.06%). The vitamin supplement was a preparation of thiamine (4.75%w/w of supplement), riboflavin (4.75%), nicotinic acid (19.01%), pyridoxine (4.75%), folic acid (4.75%), myo-inositol (47.53%), calcium panthothenate (9.51%), *p*-aminobenzoic acid (4.75%) and biotin (0.19%) and the diet was suspended in a 2% agar solution. Adult moths were kept in glass jars and were allowed to oviposit on wax paper (Babic et al., 2008).

3.2 Growing *Medicago* plants

Medicago truncatula plants were grown in a growth chamber (16:8 hours light: dark, 22° C). Seeds were scarified in sulphuric acid and planted in pasteurized soil (at 80° C for 1.5 hours). Plants were watered with a dilute fertilizer solution (0.08% of nitrogen, phosphate and potassium) every 48 hours. After six weeks, plants were placed in larger pots and, one week later, were used for caterpillar-feeding experiments. To test the effects of CO_2 on the plant P:C, Medicago truncatula plants were grown at ambient (440 ppm) or elevated CO_2 (880 ppm) conditions.

3.3 Expression levels of caterpillar salivary genes in response to plant and artificial diets

3.3.1 Caterpillars

Caterpillars were reared on Bio-Serv diet until 3rd instars according to Merkx-Jacques et al., 2007, then separated to their diets. The diets chosen for this experiment were Bio-Serv artificial diet, *Medicago truncatula* plants or transferred to artificial diet after 4 hours of feeding on plants. Caterpillars were fed on their respective diet until 4th instar and then analyzed for transcript levels. Labial salivary glands were dissected fifty pairs of glands were transferred to a solution of RNA*later*®, an RNA stabilization solution (Applied Biosystems).

3.3.2 Quantitative real-time PCR

Messenger RNA was extracted from the pooled salivary glands using mTRAP™ mRNA extraction kit midi (Active Motif, California). Genomic DNA contamination was removed by treating the mRNA extracts with 100 units DNAse I (Invitrogen) at room temperature for one hour. The samples were treated with gDNA wipeout from the Quantitect reverse transcription kit (Qiagen) according to the manufacturer's directions. The same kit was used to synthesize cDNA using mRNA as a template and primers provided by the kit. To check for genomic DNA contamination, PCR was performed using primers flanking an intron so that an amplicon size difference may be noted in the case of genomic contamination (Table 1).

Table 1 – PCR program and recipe used to check for gDNA contamination

PCR Program			PCR recipe	
Step	Time	Temperature	Buffer	1X
Denaturation	1:00	94°C	$MgCl_2$	2.5 mM
Annealing	0:30	56°C	dNTPs	0.8 µM
Amplification	1:00	72°C	Forward primer	0.2 µM
30 cycles **F primer** 5'-CTGAAGCCAATAAGCTGTCCA-3' **R primer** 5'-CTCTACACCAAACAGGCTTGC-3'			Reverse primer	0.2 µM
			Taq polymerase	1 Unit
			Template	20 ng

Since bound SYBR molecules fluoresce in proportion to the length of the fragment, amplicon sizes must remain between 100 and 250 base pairs. Complimentary DNA (cDNA) was used to amplify gene specific fragments of approximately 100 to 250 base pairs and amplicons were subsequently diluted by 10 fold increments (10^{-3} to 10^{-7} ng/µL) to be used as standards in the real-time PCR experiment. Gene-specific primers were used (Bede, unpublished data) and the program and recipe used to amplify the standards are located in Table 1. Amplicon concentrations were measured using a Nano-drop ND-1000 spectrophotometer (Thermo Scientific). Every plate included a standard curve of the reference gene, tubulin, and of the genes of interest. Every 20 µL reaction included 1x Brilliant® SYBR® green from Stratagene 0.25 µM of each primer and 1 µL of $1/10^{th}$ diluted cDNA samples. Each sample was carried out in duplicates. Gene expression was analyzed using a Stratagene Mx3000p thermal cycler (Stratagene) with MxPro v3.20 software. The program was set to 95°C melting temperature, 56°C annealing temperature and 72°C temperature

extension, all of which was repeated 40 times. Fluorescence measurements were taken after every annealing step and a final dissociation curve was taken after all the cycles were completed. Dissociation curves were taken to confirm that the primers only amplified a single amplicon. For each treatment, two independent biological replicates and two plate replicates were performed. Each measurement was taken in duplicate. Each run included two negative template controls for each gene, which includes all reagents except the template.

To confirm that qRT-PCR products match the desired fragment, samples were purified and sent to Genome Quebec for sequencing. The sequences obtained were aligned with the original sequence using ClustalW, confirming that the RT-PCR amplicons were the desired gene fragments.

To quantify gene expression, nanograms per millilitres of the genes of interest were divided by the nanograms per millilitres of the reference gene: *Setub*, to obtain a relative expression value against a constitutively transcribed gene.

Caterpillar salivary genes *Se1H* and *Se2J* sequences were compared against the insect genome databases for *Drosophila malanogaster*, *Bombyx mori, Anopheles gambiae* and *Apis melifera* to determine putative functions for these unknown salivary genes.

3.4 Artificial diet experiment

3.4.1 Caterpillars

Caterpillars were reared on Bio-Serv diet until the 3rd instar, at which point they were transferred to Petri dishes to individually feed on their respective chemically defined artificial diets. The diets were one of the following: 22p:20c,

22p:40c, 30p:20c, 33p:30c and 30p:40c. Caterpillars were allowed to grow until 4th instars whereby labial salivary glands were dissected and transferred to RNA*later*. For each biological replicate and each diet, 50 pairs of salivary glands were pooled.

3.4.2 Quantitative real-time PCR

See section 3.3.2

3.5 GOX enzyme activity

3.5.1 Caterpillars

Caterpillars were reared on Bio-Serv diet until 2nd instars, at which point they were individually transferred to Petri dishes and fed the 22p:20c artificial diet. At 3rd instars, 22p:20c diet was removed and the chemically defined diets varying in P:C were added. The diets were one of the following: 22p:20c, 22p:40c, 30p:20c, 33p:30c and 30p:40c. At the 4th instar, caterpillars were weighed and labial salivary glands were dissected. For the enzyme assays, 8 pairs of labial salivary glands were dissected and transferred to a 400 µL of 1 x protease inhibitor (Sigma) diluted with Nathanson's saline solution (150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 10 mM TES, 20 mM $MgCl_2$, pH 6.9 (Christensen et al., 1991; Merkx-Jacques and Bede, 2005)).

3.5.2 Measurement of caterpillar labial salivary glucose oxidase activity

GOX catalyzes the oxidation of glucose to gluconate. This reaction requires O_2 producing hydrogen peroxide (H_2O_2) in the process (Bentley, 1955). GOX activity is measured indirectly using H_2O_2's ability to oxidize *o*-dianisidine to a colored product; this reaction is catalyzed by horseradish peroxidase.

Salivary glands dissected from caterpillars were transferred to a saline solution containing a 1 x protease inhibitor to inhibit the hydrolytic cleavage of peptide bonds catalyzed by endogenous and exogenous proteases. The tissues were homogenized using mechanical shearing. Soluble proteins were separated from insoluble proteins, lipids and nucleic acids by centrifugation at 13,000 rpm for 5 minutes. The supernatant was used for enzymatic activity measurements.

From the soluble protein, 50 µL of the homogenate is mixed with 1.35 mL of reaction cocktail. Reaction cocktail was composed of 8.606 µg/mL horseradish peroxidase, 0.130 g/mL glucose, 0.208 mg/mL *o*-dianisidine and 0.854 M PBS pH 6.9. Samples were mixed, incubated at 37°C and absorbances of the samples were measured every minute for 15 minutes at 460 nm. The positive was 1 mg/ml purified fungal GOX (Sigma). The negative controls for all glucose oxidase enzyme assays consisted of 8 pairs of labial salivary glands, homogenized and boiled in 6 M urea for 5 minutes at 100°C (Babic et al., 2008).

Total soluble protein in the labial salivary gland homogenate were measured by the Bradford assay according to the manufacturer's instructions (Sigma) using bovine serum albumin (BSA) for protein standards (Bradford, 1976). This method employs a dye, Coomassie blue. Native brown Coomassie dye participates in a redox reaction with proteins, disrupting the tertiary structure of the polypeptide and exposing the hydrophobic regions. The dye can then bind to these hydrophobic regions, which stabilizes the blue color of Coomassie. The absorbance of the Coomassie and protein complex can be taken at 595 nm and absorbance is normalized to a BSA derived standard curve.

3.6 Plant diet experiment

3.6.1 Caterpillars

Caterpillars were reared on Bio-Serv artificial diet until the 3rd instar and then transferred to plants grown under either elevated levels or ambient levels of CO_2. Feeding occurred in the plants' respective growth cabinets. At the 4th instar, caterpillars were weighed and labial salivary glands were dissected and transferred to 400 µL of saline solution containing 1 x protease inhibitor solution.

3.6.2 Plants

Medicago truncatula plants were grown in either elevated (880 ppm) or ambient CO_2 (440 ppm) levels and regulated by growth cabinets in the Plant Science phytorium. These plants were not inoculated with Rhizobia. Plant chemical composition analysis was performed by Dr. Arif Mustafa's laboratory according to the Association of Official Analytical Chemists (AOAC). Charred plant ashes were analyzed for P:C, where C includes carbon from soluble and insoluble sugars and P includes nitrogen from proteins. Protein analysis was performed by Valacta using the instrument Leco FP 428 N-Analyzer (Leco Corporation, Michigan). Insoluble sugars were measured according to the method described by Dubois et al. (1956) and was performed using an ANKOM^{2000} fiber analyser (Ankom). Crude proteins were measured from dried plant samples and percent dry matter was used to calculate protein levels in hydrated plants and were calculated using the formula protein = N x 6.25. Acid detergent fibers (cellulose, hemicellulose, lignin) (NDF), ash percentage (ASH), crude protein (CP) and crude fat (CF) contents were used to calculate non-fibrous digestible carbohydrates

(NFC) according to the USDA College of Agriculture and Biological Sciences using the following formula (Sniffen et al., 1992):

$$\%NFC = 0.98\ (100 - \%CP - \%CF - \%ASH - \%ADF)$$

3.6.3 Measurement of caterpillar labial salivary glucose oxidase enzyme activity

GOX enzyme activity was measured as described in section 3.5.2.

3.7 Statistical analysis

Differences in transcript levels and enzyme activity for the artificial diet experiments were analyzed using an analysis of variance (ANOVA) using the Graphpad software v4.0 (Prism). To be considered differentially expressed, there must be a probability of retaining the null hypothesis of 0.05 ($p \leq 0.05$).

4. Results

4.1 Confirmation of cDNA-AFLP results

To show that the data obtained from the cDNA-AFLP studies were significant, labial salivary glands were dissected from caterpillars fed either plants or plants then transferred to artificial diet after 4 hours and transcript levels were measured by qRT-PCR. Transcript levels that showed a 1.8-fold or more difference in expression were considered to be differentially expressed (Geiss G.K. et al., 2000) (Table 2, fig. 5). The dissociation curves for all genes tested showed a single peak and all amplified standard curves were 100% efficient ± 10% with an r^2 value of over 0.95. Caterpillar salivary genes *SeGox* and *Se1H* showed significant differences in transcript levels, whereas *Se2J* and *Se2M* a difference was not seen (Fig. 5). This was expected with *Se2M,* since it was a constitutively expressed gene as seen in the cDNA-AFLP experiment.

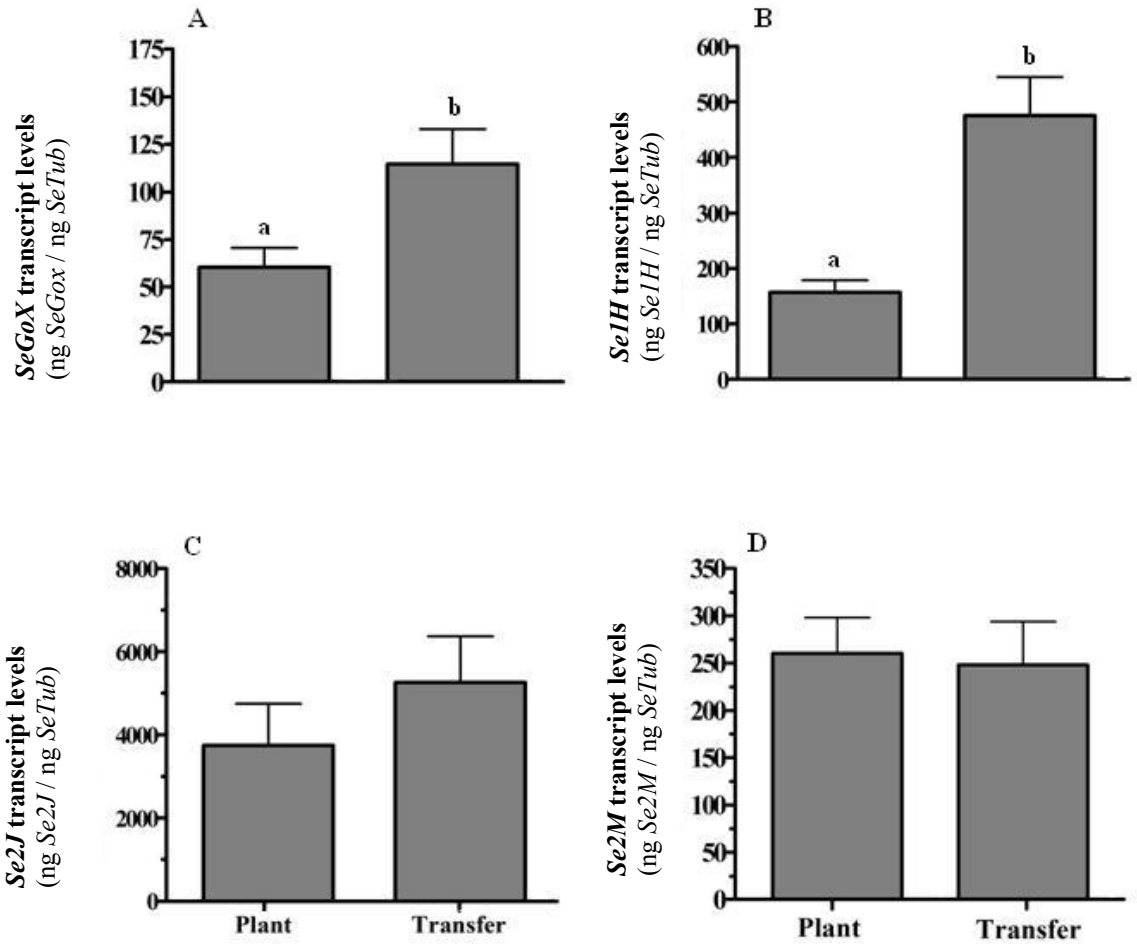

Figure 5. Transcript levels of caterpillar labial salivary genes in response to diet with the standard error of the mean. Caterpillars were reared on Bio-Serv artificial diet until 3rd instars and then transferred to *Medicago truncatula* plants. When caterpillars reached 4th instars, a subset of them were transferred back to Bio-Serv artificial diet for 4 hours and the rest were left on the plant diet. After four hours, 50 pairs of caterpillar labial salivary glands were dissected and pooled. The mRNA was extracted for transcript measurements by qRT-PCR. Transcript levels were calculated as a ratio of the masses of the gene of interest and the reference gene, *Setub*. Transcript levels were considered differentially expressed if fold increase was greater than 1.8: designated by the letters a and b where expression difference is significant. A 1.8 fold increase is considered to be significant since the results are used to confirm previously conducted experiments. **A**. *SeGox* **B**. *Se1H* **C**. *Se2J* **D**. *Se2M*.

Table 2. Fold increase of gene expression when caterpillars were transferred from the plant diet to the BioServ diet

Gene	Fold increase
SeGox	1.9
Se1H	2.8
Se2J	1.5
Se2M	1.1

4.2 RT-PCR and enzyme activity results

In a previous study, caterpillar labial salivary GOX enzyme activity was shown to be P:C dependent (Babic et al. 2008). In this study, the group looked at the following diets: 21p:42c, 22p:20c, 33p:30c, 42p:21c. This suggests that GOX enzyme activity depends on dietary nutritional quality. Therefore, in this study, I have looked at the effects of diets varying in P:C ratios (22p:20c, 22p:40c, 30p:20c, 30p:40c and 33p:30c) of caterpillar labial salivary gene expression and GOX enzyme activity. GOX enzyme activity of caterpillars fed on these artificial diets were significantly different ($p<0.05$) (Fig. 6). GOX showed higher enzyme activity when caterpillars fed on protein- and digestible carbohydrate-rich diets in comparison to protein-poor and digestible carbohydrate-rich diets.

Transcript levels of the caterpillar labial salivary genes, *Se1H, Se2J, Se2M* and *SeGox*, were measured in response to the chemically-defined diets (22p:20c, 22p:40c, 30p:20c, 30p:40c and 33p:30c). Significant differences in gene expression were not observed (*Se1H* $p = 0.43$; *Se2J* $p = 0.46$; *Se2M* $p = 0.50$; *SeGox* $p = 0.37$) (Fig. 7).

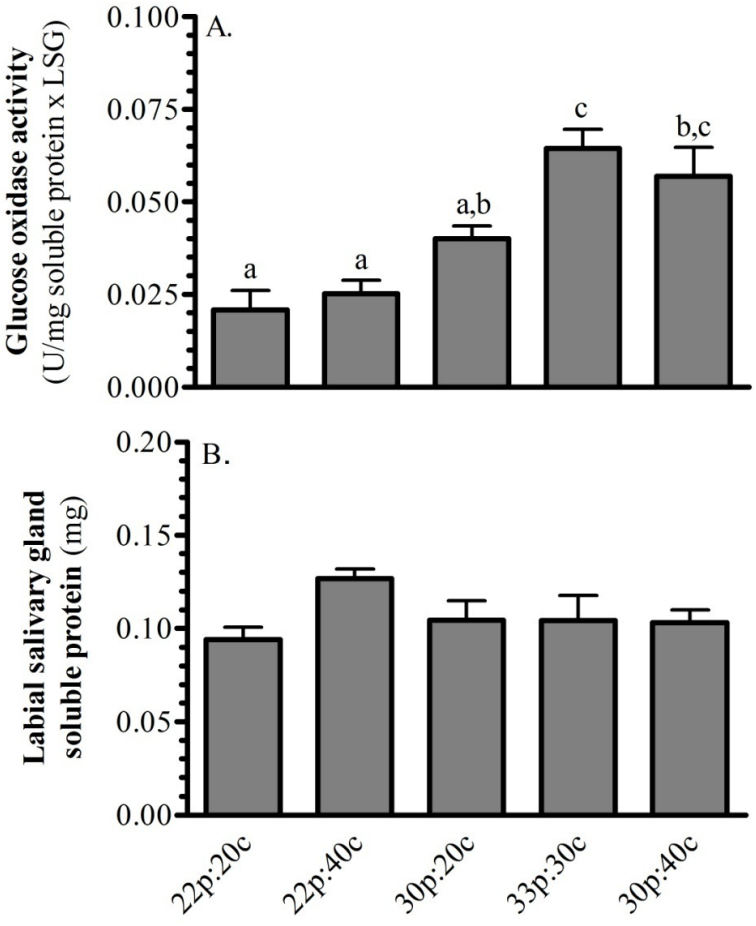

Figure 6. Labial salivary GOX enzyme activity and soluble protein levels with standard error of the mean in caterpillars fed artificial diets containing different protein to digestible carbohydrate . Second instar caterpillars were given 22p:20c artificial diets until 3rd instar and, subsequently, transferred to their respective chemically defined diets until 4th instar. Eight pairs of caterpillar labial salivary glands were pooled for the assay and repeated 6 times. **A.** Caterpillar salivary GOX enzyme activities. Significantly different expression levels are annotated by letter (a, b and c). **B**. Soluble protein concentrations.

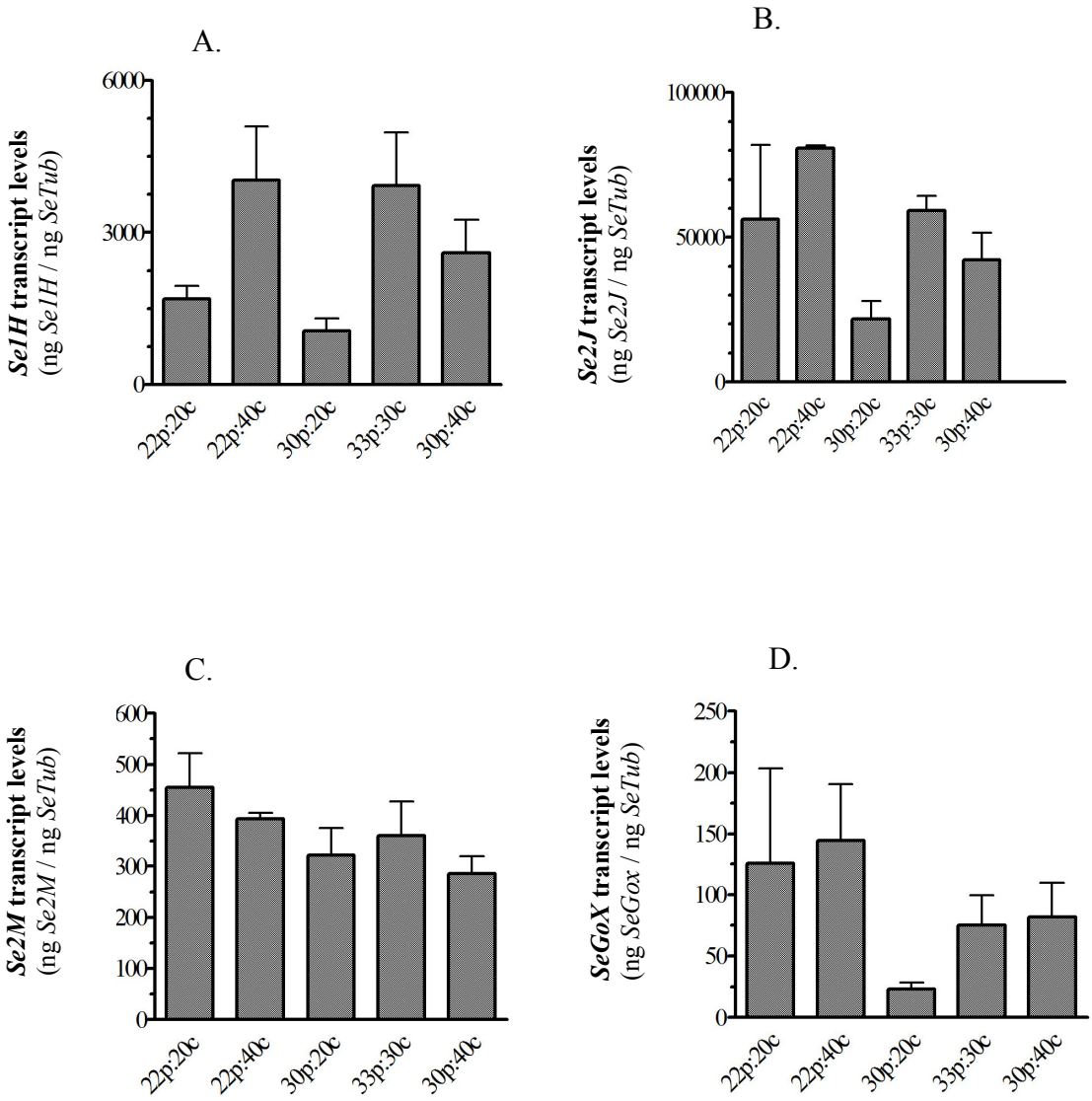

Figure 7. Caterpillar labial salivary gene expression with the standard error of the mean in response to feeding on artificial diets containing different protein to digestible carbohydrate ratios. Caterpillars were reared on BioServ diet until 3rd instar, whereby they were transferred to their respective chemically defined diets. Fifty pairs of labial salivary glands from 4th instar caterpillars were pooled for the assay and repeated for a total of 3 biological replicates. Transcript measurements of labial salivary genes were normalized with *SeTub*. **A.** *Se1H* **B.** *Se2J* **C.** *Se2M* **D.** *SeGox.*

4.3 Plant feeding experiments

The nutritional quality of *Medicago truncatula* plant samples grown at ambient (440 ppm) and elevated (880 ppm) CO_2 levels was analyzed to determine the protein and digestible carbohydrate content (table 3).

Table 3. Nutritional composition of *Medicago truncatula* plants grown at either ambient (440 ppm) or elevated CO_2 (880 ppm)

Plant composition	Plants grown in ambient CO_2 (%)	Plants grown in elevated CO_2 (%)
CP	33.05	33.39
CF	0.57	0.57
ADF	12.13	12.39
ASH	12.89	11.23
NFC	42.53	43.57

From the data shown above, the calculated P:C for plants grown in ambient and elevated CO_2 was approximately 33p:42c and 33p:43c. These values show that *M. truncatula* plants are much richer in P:C when compared *M. sativa* plants. Under these conditions of fertilization, elevated CO_2 levels did not affect the protein to digestible carbohydrate ratio in these plants. Given this P:C ratio, we would expect (in comparison to our articial diet data) that we could detect GOX activity - but we did not which makes us suspect that in the plant, other mechanisms are occuring to lower protein availability.

4.4 Bioinformatics

Sequences for *Se1H* and *Se2J* cDNA were obtained by Bede's laboratory by isolating fragments using genome walking and 5' and 3' rapid amplification of cDNA ends. In this study, these fragments were queried against other insect databases (NCBI, SilkDB, TIGR) against three model organisms, *Drosophila*

malanogaster, Bombyx mori and *Apis mellifera*. For both *Se1H* and *Se2J*, sequence similarities (E-score < 0.1) were not obtained and may suggest that *Se1H* and *Se2J* are novel genes.

5. Discussion

Previous research in Bede's laboratory has, by differential display cDNA-AFLP, identified two caterpillar labial salivary genes, *Se1H* and *Se2J*, whose transcript levels were shown to be diet-dependent. To confirm this pattern, transcript levels of the *Se1H, Se2J*, *SeGox* and the constitutively expressed *Se2M* were quantified by qRT-PCR. Fourth instar caterpillars were grown on plants or transferred from plants to artificial diet for four hours and gene expression was measured. Expression differences were established by taking the ratio of the normalized gene expressions of each diet; ratios greater than 1.8 were considered differentially expressed. Based on these criteria, *Se1H* and *SeGox* were considered to be diet-dependent. *Se2M* was constitutive as established by cDNA-AFLP and thes results confirmed this. *Se2J,* on the other hand, was previously thought to be differentially expressed by cDNA-AFLP, but this verification showed that this was a false positive and this gene is constitutively expressed. An alternative explanation is that caterpillars were not allowed to feed on the artificial diet for a sufficient length of time since a 1.5-fold increase in gene expression is observed. Subsequent studies should include a time course analysis of salivary gene expression in response to diet. *SeGox* transcript levels correlated with previous assays of enzyme activity (Merkx-Jacques and Bede 2005). GOX transcript levels and enzyme activity were higher in caterpillars fed artificial diets compared to

plant diets. There are now three labial salivary genes that seem to be regulated transcriptionally in response to the diet the caterpillar is feeding on: *SeGox* and *Se2J* from this study and *HzLys*, the gene encoding *Helicoverpa zea* lysozyme (Liu et al., 2004).

In the defined artificial diet experiment, five diets varying in protein to digestible carbohydrate ratios were chosen to test the effects of nutritional quality on the regulation of gene transcription and enzyme activity. The diets chosen were: 22p:20c, 22p:40c, 30p:20c, 30p:40c and 33p:30c. Transcript levels of *SeGox*, *Se2J*, *Se1H* and *Se2M* were not affected by diet (Fig. 6). This was different from the first experiment where transcript expression of *SeGox* and *Se2J* increased when caterpillars were placed on artificial diet from plant diet. This suggests that there are factors, other than dietary P:C which influences caterpillar salivary gene expression. In comparison, caterpillar labial salivary GOX enzyme activity did reflect the quality of the diet the caterpillars fed on (Fig. 7). GOX enzyme activity was highly dependent on diet nutritional quality. At low protein levels, GOX enzyme activity is not affected by digestible carbohydrate levels (22p:20c and 22p:40c), whereas it is greatly dependent on the protein level (22p:20c and 30p:20c) (Fig. 6). However, provided that there is enough protein in the diet, the enzyme activity starts to show a relationship to the digestible carbohydrate level as well (22p:20c, 30p:20c and 30p:40c). Therefore, at low protein levels, caterpillars might be restricted by the amount of nutritional amino acids available from their diet, therefore limiting translation of GOX. Amino acids acquired in the diet would be used first for essential protein synthesis, and afterwards, for the

production of GOX. Once protein requirements are met, it becomes feasible to produce more GOX with increasing digestible carbohydrate levels in the diet.

The fact that gene expression levels were not the same as GOX enzyme activity suggests that regulation occurs post-transcriptionally. This is reminiscent of tick translation regulation of salivary proteins where stress and environmental cues were playing a role in the regulation of functional gene product at the translational checkpoints, such as mRNA export, mRNA stability and translation initiation (Singh et al., 2006).

In this experiment, *M. truncatula* were grown at high and low levels of atmospheric CO_2 to alter the C:N ratio. Caterpillars grown on plant diets showed undetectable amounts of glucose oxidase enzyme activity when reared on plants grown at either ambient (440 ppm) or elevated (880 ppm) levels of CO_2 having a composition of 33p:42c and 33p:43c, respectively. In accordance with the artificial diets experiment, it is expected that GOX enzyme activity would be comparable to 30p:40c, but, instead, enzyme activity is undetectable. These results suggest that plants influence herbivore digestive enzyme through mechanisms other than poor nutritional quality, such as protease inhibitors and threonine deaminase (Chen et al., 2007). In this case, when insects digest plant tissue, threonine deaminase degrades threonine residues in the gut, preventing the absorption of the full spectrum of amino acids.

Caterpillar salivary expression of *SeGox* and *Se1H* is regulated in a diet-dependent manner, but not affected by dietary nutritional quality. GOX enzyme activity, however, is diet-dependent and responds to varying P:C. Once dietary

protein intake meets the organisms amino acid requirements, GOX enzyme activity depends on digestible carbohydrate levels.

LINK BETWEEN CHAPTER VIII AND CHAPTER IX

In chapter VIII, caterpillar labial salivary genes *Se1H* and *SeGox* showed similar expression patterns caterpillars were fed either *Medicago truncatula* plant or transferred from *M. truncatula* to artificial diet for 4 hours. This suggests that there may be a common upstream regulator involved in the regulation of these two genes. In the following chapter, the upstream promoter of these caterpillar salivary genes will be sequenced to determine, through bioinformatics, whether the genes share promoter analogy. Regions 5' upstream of the genes if interest will be amplified using inverse PCR and amplicona will be sent to McGill's GenomeQuebec and the Montreal Institute of Clinical Research (IRCM) for sequencing.

IX. PROMOTER SEQUENCES OF CATERPILLAR SALIVARY GENES

1. Abstract

Caterpillar salivary genes *Se1H* and *SeGox* mRNA expression were previously shown to be diet-dependent. These similar patterns in gene expression suggest that there may be similarities in putative consensus sequences in the promoter region of these genes. In the following experiments, the upstream promoter of three genes (*Se1H*, *Se2J* and *SeGox*) were attempted to be amplified and sequenced and analyzed for sequence similarities and the presence of consensus sequences for putative transcription factor binding sites. Fragments were obtained by performing inverse PCR and were then sequenced through Genome Quebec and Montreal Institute on Clinical Research (IRCM).

2. Introduction

Through previously performed cDNA-AFLP and qRT-PCR, two caterpillar salivary genes, *Se1H* and *SeGox*, were found to be differentially expressed in response to diet. Caterpillars fed *Medicago truncatula* plants showed a significantly lower transcript levels of *Se1H* and *SeGox* in comparison to caterpillars that were fed plant diets, followed by a transfer to artificial diet for 4 hours. Transcripts in the above experiments were observed by cDNA-AFLP and confirmed by qRT-PCR. The patterns suggest that these genes are regulated together and possibly share a common upstream consensus sequence element that binds a common transcriptional regulator. A study on pacific white shrimp, *Litopenaeus vannamei,* showed that in response to diets varying in β-1,3-glucan

similar expression patterns were found in genes involved in the same pathway (Wang et al., 2008). A large-scale study of the *cis*-regulatory modules of *Drosophila melanogaster* promoters computationally assessed promoter similarity and found that in 280 instances, when genes are co-regulated, they also function in a common pathway (Li et al., 2007). These two experiments show that regulation similarities between different genes can indicate that their gene products may have roles in a common pathway. In the following experiment, regions 5' upstream of each of the three caterpillar salivary genes (*Se1H, Se2J* and *SeGox*) were sequenced. This was done to perform sequence alignments and bioinformatic analyses to identify protential *cis*-acting elements.

Inverse PCR (iPCR) was used to amplify fragments of genomic DNA 5' upstream of the transcription start site (Chapter VI, fig. 3) (Ochman et al., 1988). To use this technique, previous knowledge about the gene sequence is required to design primers. Genomic DNA is digested using restriction enzymes and, subsequently, religated to obtain circular fragments, allowing for amplification of unknown regions. Primers are designed to amplify unknown regions in these circular plasmids. After ligation, fragments are digested with a carefully chosen restriction enzyme to become linear, giving polymerase access to the DNA strand, therefore relieving hindrance by circular DNA. With the above manipulations, primers now point inwards. The final product should include a region of unknown DNA, flanked by partial fragments of known DNA in which the primers were designed (Wo et al., 2007).

3. Methods and Materials

3.1 Endonuclease digestion

Genomic DNA extracted from 3rd instar *Spodoptera exigua* caterpillars by DNeasy Blood and Tissue kit (Qiagen) was cut by restriction enzymes to yield smaller fragments flanked by sticky ends (Table 4). Before restriction digestion, genomic DNA was visualized by agarose gel electrophoresis to insure that the DNA was not degraded. The gel was prepared with 0.8% w/v agarose suspended in 1 x Tris-Borate-EDTA buffer (TBE).For each 50 µL reaction, 1 µg of genomic DNA was digested with 10 Units of restriction endonuclease at 37°C for 8 hours in a 1 x reaction buffer provided with the enzymes. Restriction endonucleases were all obtained from New England Biolabs (NEB). Enzymes were heat deactivated by incubating samples at 85°C for 10 minutes.

Table 4 – List of NEB restriction endonucleases used for genomic DNA digestion for inverse PCR (enzyme #1) and relinearization (enzyme #2). Each reaction was carried out at 37°C for 8 hours.

Restriction Enzyme #1	Restriction Enzyme #2	Gene
SpeI	HincII	*SeGox*
BamHI	AlwNI	*Se1H*
BbsI	EciI	*Se2J*

3.2 Ligation

Digestion products were ligated using T4 DNA ligase (NEB). Reactions (20 µL) were incubated at 12ºC for 2 hours using 40 Units of enzyme to ligate 100 ng of digested DNA. The reaction was carried out in 1 x ligation buffer. The enzyme was, then, deactivated by incubating the samples at 65°C for 10 minutes.

3.3 Exonuclease digestion

An exonuclease digestion was used to eliminate all linear fragments of DNA. Lambda exonuclease (NEB) eliminated linear DNA fragments due to its 5' to 3' exonuclease activity, leaving circular DNA untouched. Samples were incubated at 37°C for 1 hour with 5 U of enzyme for 100 ng total DNA. Enzyme was, then, deactivated by heating the samples to 75°C for 10 minutes. These samples were purified using a PCR purification kit (Qiagen) and the DNA pellet was resuspended in deionized water.

3.3 Linearization

To avoid spatial hindrance of the polymerase during the iPCR, the circular DNA fragment was relinearized. A second restriction digest was designed to cut between the two primers to insure that amplification directions are conserved (Table 4). Reactions were incubated with 10 U of enzyme at 37°C and allowed to digest for 6 hours. Enzymes were, then, heat deactivated by incubating samples at 85°C for 10 minutes. All restriction enzymes used for this step were obtained from NEB.

3.4 Inverse PCR amplification

Before amplifying the desired fragment, samples were purified using a PCR purification kit (Qiagen). Purified fragments were, then, amplified by PCR using the recipe and program found in Tables 6 and 7, respectively. Primary PCR amplification was carried out with the primers combinations found in Table 8, and then reamplified using nested primers (Table 9). To conserve error-free DNA replication, high fidelity Phusion polymerase was chosen (NEB) instead of

conventional Taq polymerase. Amplicons were vizualized by gel agarose electrophoresis in a 2.0% w/v agarose gel suspended in 1 x TBE. If primers amplified multiple fragments, each band on the gel was excised and purified using the QIAquick Gel Extraction kit (Qiagen) and resuspended in deionized water.

Table 6 – Recipe list for inverse PCR and their respective concentrations (reaction volume 25 µL)

Ingredient	Concentration
Buffer	1 x
Phusion Polymerase	0.5 Units
dNTPs	0.8 mM
F-Primer	0.2 µL
R-Primer	0.2 µL
Template	5 ng

Table 7 – Program used for inverse PCR

PCR step	Temperature (°C)	Time (seconds)
Denaturation	94	20
Annealing	58 (1°) – 59 (nested)	30
Extension	72	105

Table 8 – Primers used for first round inverse PCR

Primer	Gene
5’ – ACAGACTCGATCCGCTTGCAT – 3’ 5’ – ACATATAAGGTCGTGTTGCTGG – 3’	*SeGox*
5’ – TGTACTTCGTGTCACCTGATCG – 3’ 5’ – CGAGGTCTGTTCATTGACTGC – 3’	*Se1H*
5’ – TTGCTCCAGATACAAGGTCGTC – 3’ 5’ – TGCCGTTCAAGATGATGCTGCC – 3’	*Se2J*

Table 9 – Primers used for nested inverse PCR

Primer	Gene
5' – GACATCCACAATCCTCAGGTGC – 3' 5' – ACATATAAGGTCGTGTTGCTGG – 3'	*SeGox*
5' – GACGATAACGTGTACTTCAACAGC – 3' 5' – ACACTGTGTAGTCATCGGTAGG – 3'	*Se1H*
5' – TGGCTCCAGATACAAGGTCGTC – 3' 5' – GATGCAGCCGCTGATACAGCAC – 3'	*Se2J*

3.5 Sequencing

Before sending the samples to be sequenced, absorbance at wavelengths between 230 and 350 nm were taken to determine the purity of samples. For samples extracted from agarose gels, a significant peak at 230 nm was observed. To eliminate the residual agarose causing the peak at 230 nm, samples were purified and resuspended in deionized water. DNA was precipitated in 10% 3 M sodium acetate (v/v) and 3 volumes of 100% ethanol at -70˚C for 3 hours. Samples were, then, centrifuged at 13,000 rpm for 1 hour at 4°C and then washed with 70% ethanol, followed by another 30 minute centrifugation (13,000 rpm) at 4°C. Ethanol was removed and allowed to dry and were finally resuspended in deionized water. Samples were diluted to a final concentration of 25 ng/µL for sequencing. Sequencing was performed by either the Montreal Institute of Clinical Research (IRCM) or Genome Quebec (McGill).

4. Results

Caterpillar genomic DNA extract was visualized by 0.8% w/v gel agarose electrophoresis to test DNA quality (Fig. 8). A band at the top of the gel represents genomic DNA, while light streak marks indicate DNA degradation. The sample with the least degradation (Fig. 8, lane 2) was chosen for iPCR manipulation.

After digestion, ligation and relinearization of circular DNA, samples were purified, amplified by iPCR and reamplified by nested PCR. Samples were visualized on a 0.8% agarose gel (w/v). Bands were then excised and purified from the gel (Fig. 9). Purified fragments were then sent for sequencing. Chromatograms obtained from the sequencing facilities showed, in the case of all three caterpillar salivary genes, overlapping peaks, indicating multiple products (Fig. 10).

5. Discussion

Three caterpillar salivary genes were chosen for promoter analysis. The goal of this experiment was to sequence the 5' upstream region of caterpillar salivary genes and identify, using bioinformatics, putative *cis*-regulatory elements. By iPCR, 5' regions of 3 caterpillar genes, *Se1H, Se2J* and *SeGox*, were amplified. Fragments were isolated and submitted for sequencing to Genome Quebec and the Institut de recherches cliniques de Montréal (IRCM). However, due to problems with DNA purity and multiple amplicons, data obtained from these sequences were inconclusive. Based on the data obtained from the sequencing facilities, it was evident that samples submitted contained at least two fragments, resulting in overlapping peaks in the chromatograms. This problem could have been avoided by running DNA on an acrylamide gel, instead of an agarose gel. This will for a higher resolution on band size differences and will, therefore, increase the purity during the extraction.

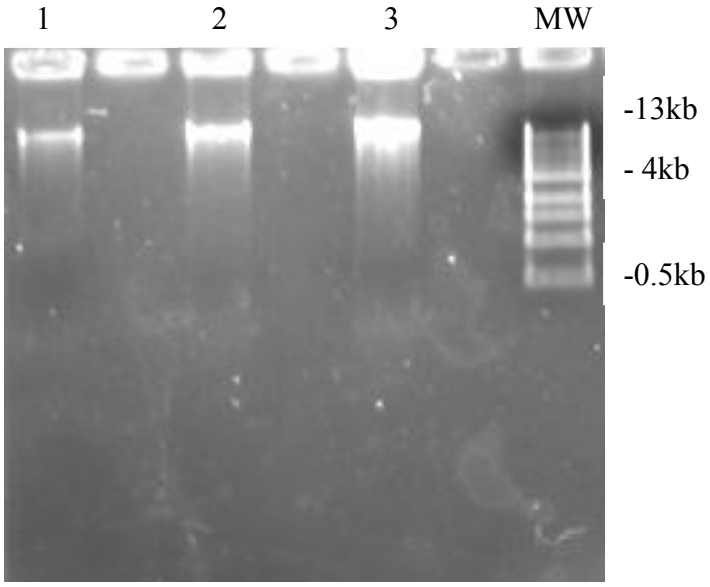

Figure 8 – Genomic DNA. Genomic DNA quality was tested by agarose gel electrophoresis (8% w/v). Lanes 1-3 are genomic extracts from moths using the Qiagen DNeasy Blood and Animal Tissue kit and reconstituted in deoionized water. The three lanes represent three preparations and are repetitions of the same protocol. The sample from lane 2 was chosen for subsequent iPCR manipulations because it showed the least degradation.

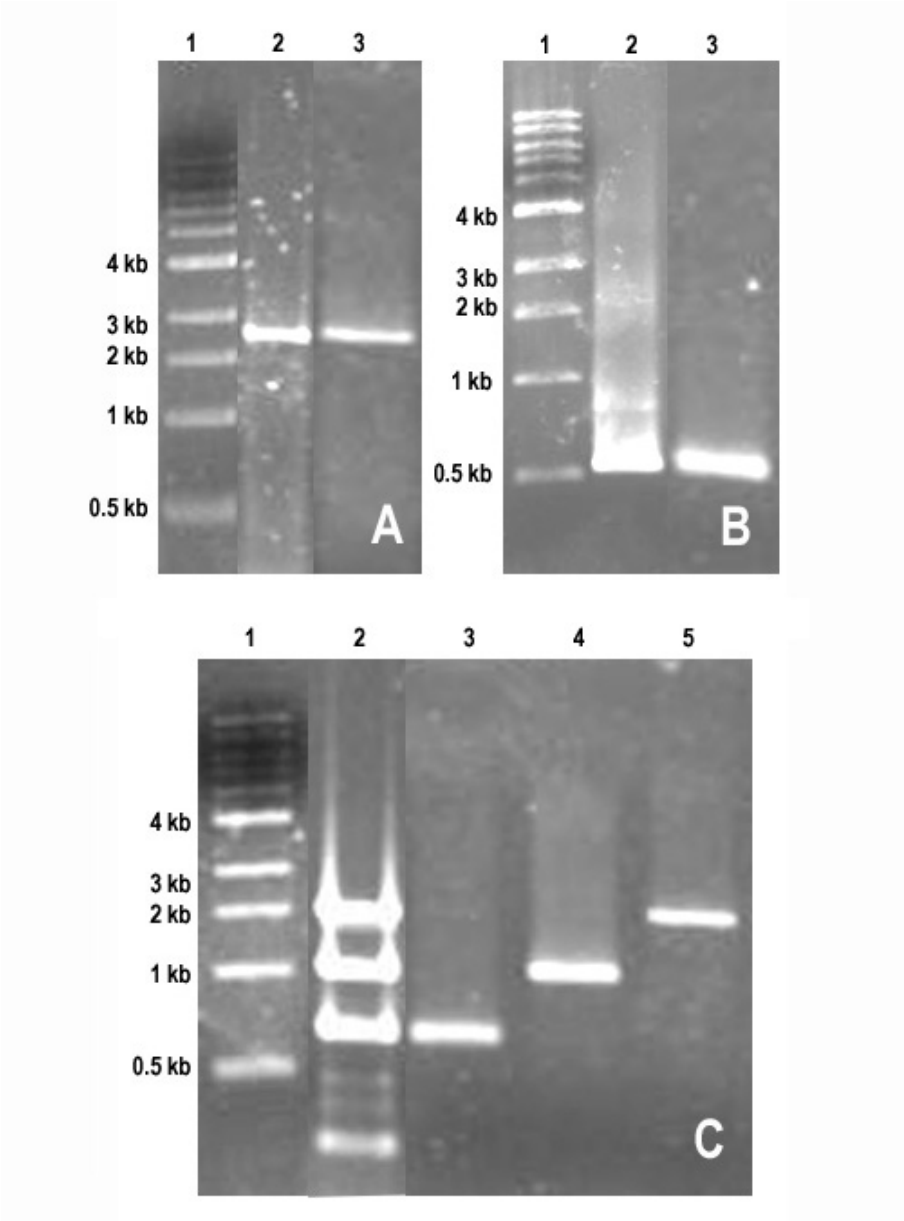

Figure 9 – Agarose gel of iPCR products. **A**. Amplification of *Se1H* gave a single amplicon (lane 2) and was subsequently purified to eliminate smears (lane 3). **B**. Amplification of *Se2J* showed a noticeable smear and one bright band (lane 2). Band was excised and purified for sequencing (lane 3). **C**. *SeGox* amplification showed three distinct bands and further unspecific amplifications (lane 2). Bands were separated and purified to be sent for sequencing (lanes 3-4-5). Negative control samples showed no bands.

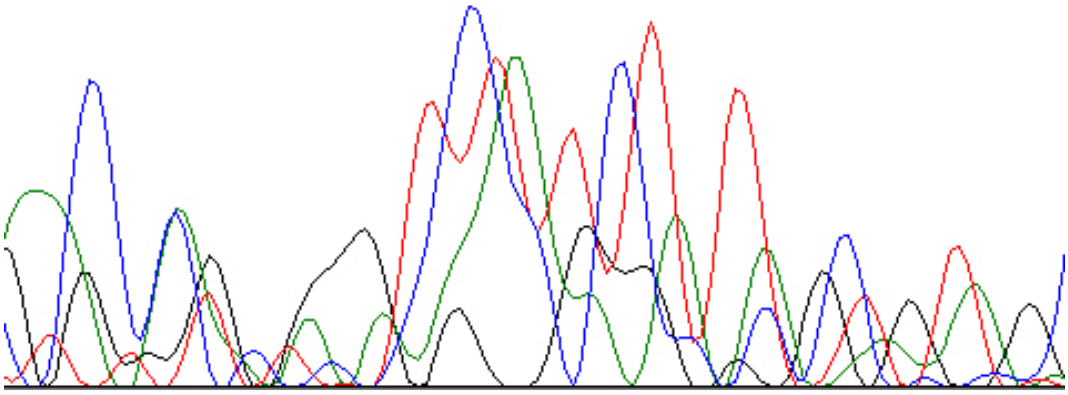

Figure 10 – Sample chromatogram of SeGox iPCR fragment submitted for sequencing. Resulting chromatogram of *SeGox* sequencing. Picture shows approximately 20 nucleotides depicted by overlapping peaks, indicating multiple signals and amplification products.

Furthermore, the quality of genomic DNA is important, as partially degraded DNA can interrupt restriction sites, primer binding sites and the region of interest. In many steps of the protocol, DNA needed to be mixed by pipetting and/or vortexed, potentially shearing the DNA. As a result, DNA degradation took place and was confirmed by agarose gel electrophoresis. When genomic DNA quality was assessed, a bright band was seen, followed by a light streak, indicating that the genomic extract was partially degraded.

Partial degradation of genomic DNA and unspecific amplifications are the most probable obstacles preventing proper sequencing. However, DNA preparation may also be an issue. Inverse PCR amplification of the fragment of interest generated multiple amplicons and needed to be separated and purified on an agarose gel. Purification of the bands resulted in a carryover of trace amounts of agarose, which have a strong absorbance at 230 nm, masking the absorbance of DNA at 260 nm. This contamination by agarose prevents the accurate measurement of nucleic acids concentration and may also interfere with the solubility of the DNA. For agarose to be removed from the mixture, the sample needs to be ethanol precipitated, a step that is likely to decrease the overall yield of the DNA. At each of the multiple steps involved in iPCR, the DNA yield decreases, limiting the amount of DNA available to submit for sequencing.

Although the principle of iPCR may seem feasible, in practice, the DNA is manipulated too frequently to obtain samples with unique amplicons. Other techniques can be used instead that may decrease the risk of degradation and loss, such as genome walking. Genome walking is another PCR-based technique that

does not require as many steps involving nucleases, thus reducing the risk of DNA degradation (Leoni et al., 2008). Genomic DNA is digested with restriction endonucleases and ligated to long suppression adapters. The desired genomic region is then amplified with a primer specific to the outer part of the suppression adapter and a gene-specific primer, followed by a second amplification using nested primers. Contrary to iPCR, this technique cannot generate variable fragments due to unspecific ligation.

Overall, this experiment aimed at isolating the 5' upstream regions of 3 caterpillar salivary gene transcription start sites by iPCR. Chromatograms obtained by the sequencing facilities indicated that in most cases, more than one peak were seen at each nucleotide, indicating two overlapping fragments. A separation of the amplicons on a polyacrylamide gel can resolve small differences in fragment size and can eliminate contamination during the gel extraction step. To increase the yield of amplicons, samples can be cloned in a vector and transfected into *Escherichia coli* cells for amplification.

X. CONCLUSION

Caterpillars salivary enzymes, such as glucose oxidase (GOX) and lysozyme, grant caterpillars the ability to circumvent wounding-induced plant defense responses (Musser et al., 2002; Liu et al., 2004). Moreover, these salivary enzymes are regulated by diet (Liu et al., 2004; Merkx-Jacques and Bede, 2005). For example, GOX enzyme activity is low when caterpillars are fed plants or protein-poor artificial diets (Merkx-Jacques and Bede, 2005; Babic et al., 2008). Furthermore, the Bede laboratory has isolated two novel genes (*Se1H* and *Se2J*) from caterpillar labial salivary glands expressed differentially in response to diet. In this research, transcript levels of caterpillar salivary genes *Se1H, Se2J* and *SeGox* were measured in response to caterpillars feeding on plants or transferred from plant to artificial diets. Results showed that *Se1H* and *SeGox* showed differential expression, confirming the data obtained in a previous differential display cDNA-AFLP experiment and suggesting that these genes are regulated transcriptionally. Furthermore, the same caterpillar salivary genes transcript levels, as well as GOX enzyme activity, were measured in response to caterpillars fed artificial diets varying in protein to digestible carbohydrate ratios (P:C). Diet-dependent regulation at the transcript level was not noted, but significant differences in GOX enzyme activity showed that activity is responsive to nutritional quality. This suggests that protein and digestible carbohydrate levels of the diet might affect gene regulation post-transcriptionally. However, the salivary genes assayed showed diet-dependency when caterpillars were fed plants or

transferred to artificial diets from plants after 4 hours, but this does not appear to be due to differences in P:C, at least at the concentrations tested here.

To simulate the caterpillar's natural diets affected by increasing atmospheric carbon dioxide (CO_2), caterpillars were fed *Medicago truncatula* plants grown at either ambient or elevated CO_2 levels. Plant protein and carbohydrate analyses showed that the P:C of the two plants were comparable to a nutrient-rich artificial diet for which the caterpillars respond with a high GOX enzyme activity when ingested. However, in this case, GOX enzyme activity was undetectable, suggesting that there may be an underlying mechanism the plant employs to inhibit caterpillar digestive enzymes.

In the final experiment, caterpillar labial salivary gene, *Se1H, Se2J* and *SeGox*, 5'-upstream of the transcription start sites were isolated by iPCR. Since little is known about caterpillar salivary transcription regulation, looking for promoter sequences can shed light on potential *trans*-acting elements controling salivary gene regulation. However, sequencing of the fragments could not be completed due to multiple amplicons from the iPCR. To correct for the problems encountered with iPCR, genome walking can provide a more reliable method for isolating the promoter sequence.

XI. FUTURE DIRECTIONS

Based on the cDNA-AFLP experiment and transcript assays, salivary gene expression of *Se1H* and *SeGox* are shown to be diet-dependent. In both experiments, transcript levels were higher in caterpillars reared on a regime containing artificial diets than strictly plant diets. However, the experiment could be repeated by rearing caterpillars on Bio-Serv until 3rd instars and transferring them to the chemically defined diets, caterpillars should be reared on Bio-Serv until 2nd instars, transferred to 22p:20c diet until 3rd instars and finally transferred to their respective diets. This would ensure that no background transcript expression would carry over from the nutrient rich Bio-Serv diet. Secondly, more biological replicates should be performed to eliminate large standard deviations for statistically analyses. For the above experiment, three independent biological replicates were taken and, therefore, a small change in gene expression resulted in a large error bar when comparing transcript levels in response to different treatments. Since standard error is an inverse function of the number of replicates, more biological replicates will solve the problem of large error bars and will help determine the reality of P:C-dependencies of *Se1H*, *Se2J* and *SeGox*.

If P:C-dependency does not occur transcriptionally, an assay can be used to measure translation levels of caterpillars reared on the same diets, further narrowing down the regulatory checkpoint at which P:C-dependency occurs. Taking the ratio of mRNA levels and protein levels of caterpillars fed different diets can give an indication of overall translation efficiency. If mRNA levels

correspond to protein levels, then regulation may occur post-translationally. It is also possible that GOX enzyme activity is being regulated by proteolytic cleavage, producing mature enzymes from inactive zymogens. *Manduca sexta* larvae show that β-1,3-glucan responsive hemolymph serine protease is responsible for proteolytic activation of prophenoloxidase (Wang and Jiang, 2006).

Although the inverse PCR (iPCR) experiment did not show conclusive results, identifying promoters of differentially expressed caterpillar labial salivary genes can lead to understanding the direct effects of diet on gene expression. Since GOX is responsible for plant defense shutdown, caterpillars are able to feed on plants, regardless of induced responses to wounding. The ability of caterpillars to circumvent plant defense responses has cost the agricultural industry millions of dollars in losses every year and, therefore, understanding the regulation of gene expression of these labial salivary genes may be a starting point for new innovations in pest management practices.

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