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CHARACTERIZATION AND IDENTIFICATION OF HOST PLANT-DRIVEN PLASTICITY OF THE CABBAGE LOOPER (*TRICHOPLUSIA NI*) SALIVA

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ABSTRACT

Plant-insect dynamics are a complex network of chemical interactions. How insects are able to adapt to their host plants and how plants can resist or tolerate insects are questions of much importance for evolutionary biology, ecology, physiology, insect behavior, agriculture, food security, and science in general. For example, plants are capable of inducing defenses against insects after detecting insect specific cues. Insects on the other hand might suppress these defenses by releasing molecules present in secretions like saliva. Currently, there is limited information on the saliva composition of chewing insect herbivores and how it might affect plant defenses. The main objectives of this study were to 1. Characterize the saliva of the generalist insect pest, the cabbage looper (*Trichoplusia ni*) and 2. Identify the changes in the composition of insect saliva driven by two of its host plants - cabbage and tomato as compared to artificial diet. These objectives were approached using both transcriptomic (RNAseq) and proteomic techniques (iTraq). A transcriptome of 14,037 genes and a proteome of 434 proteins were established. Feeding on different host plant diets resulted in substantial remodeling of the gland transcriptomes and proteomes, with 4,501 transcripts and 63 proteins significantly differentially expressed across the three treatment groups. Gene expression profiles were most similar between cabbage and artificial diet, which corresponded to the two diets on which larvae perform best. Within these libraries, several interesting enzymes were identified that may play an important role in the cabbage looper's ability to establish on different hosts. Some of these enzymes that were further analyzed are a catalase and three potential myrosinases. Catalase activity was confirmed in the labial glands of the cabbage looper. It was also determined that catalase plays a role in detoxification by reducing the activity of peroxidases as well as herbivore offense by suppressing the induction of trypsin protease inhibitor in tomato. The myrosinase genes identified were differentially expressed in several tissues of the cabbage looper and under different diets.

However, they appear to be broad-spectrum glucosidases rather than specific myrosinases. Finally, as part of the INTAD dual degree, the alternative use of water containing methyl isothiocyanate - a defensive secondary compound in the famine shrub Hanza (*Boscia senegalensis*), was investigated. Hanza waste water has a significant effect on seed germination of several plants and could potentially be used for weed management in small farms of West Africa. This is an example of the study of plant defensive compounds for the use in applied research. This dissertation provides information about caterpillar saliva, which can be used for future functional and ecological studies. Also, it enriches our knowledge about a usually neglected secretion from chewing insects.

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DEDICATION

This dissertation along with all the accomplishments in my life is dedicated to my mother – Eloina Vega Zapata. Your courage, strength, and humility will always accompany me. Here's to remembering you with joy and honoring you by enjoying the wonderful life you worked so hard to give me.

Chapter 1

Introduction

There are approximately 5.5 million insect species in the world[1] – most of these are herbivores[2]. Despite the fact that plants are not necessarily an optimal source of nutrients, herbivory is linked to a radiation in species diversity for both plants and insects[3]. It was in 1964 that Ehrlich and Raven[4] published their seminal paper on the coevolution of butterflies and plants suggesting that coevolution between plants and insects can drive speciation. A paper which was also highly influenced by Fraenkel's 1959 paper [5] on the role of secondary compounds as defense against insects. Since then, there has been an explosion of research attempting to understand the complex interactions between plants and insects. My area of research continues to explore these interactions.

Not only are plants imbalanced in the carbohydrate and protein content required for optimal nutrition of herbivorous insects, but they also contain secondary compounds that can have detrimental effects on insects[6]. Insects on the other hand, have evolved adaptations that allow them to obtain the necessary nutrients from plants as well as overcome their defenses[7]. Simply put, plants and insects are in a delicate balance where plants have developed defenses that will allow them to evade, reduce or tolerate damage caused by herbivore insects. Meanwhile, herbivore insects have developed counter adaptations that allow them to use plants for food, shelter, and even defense.

Plant defenses

Though sessile, plants have developed a number of defenses against herbivores. These defenses can be physical such as thorns, latex, trichomes, resin, etc. as well as chemical such as phenols, alkaloids, proteinase inhibitors, glucosinolates, among others[8]. These defenses can also be classified as *constitutive defenses,* which are always present in the plant or as *induced defenses,* which are triggered under the presence of herbivory. Finally, defenses can also be classified as *direct defenses* – defenses that affect the physiology of the insect directly – and *indirect defenses* – mainly volatiles that attract natural enemies of the herbivore. Because many of these defenses are costly, plants require a mechanism of herbivory recognition that is reliable.

Herbivory recognition

Past studies show that plant responses to insect herbivores differ significantly from those induced by mechanical wounding alone [9], indicating that plants are able to recognize insect specific cues (elicitors) during their interaction. Such elicitors have been identified in the oral secretions (mix of regurgitant and saliva) of several agricultural pests [10]. On the other hand, herbivores may partially avoid detection by stealthy feeding or minimizing release of oral secretions [11]. Some herbivores may even actively suppress defenses by releasing suppressive molecules (effectors) while feeding [12]. To date only 9 classes of elicitors/effectors have been identified, including: beta-glucosidase[13], fatty-acid amino acid conjugates[14], caeliferins[15], inceptins[16], bruchins[17], lipases[18], glucose oxidase[12], ATP-utilizing enzymes[19], and chitinases[20]. Given the large number of plant-insect interactions that exist, it is not difficult to assume that more molecules must be involved in these mechanisms or that the dynamics involved in herbivory recognition are more complex than just presence or absence of such molecules.

The generalist-specialist paradigm

A paradigm or model currently presented and under much research in plant-interaction studies, is the idea of differential responses depending on an insect's host range. Species have been classified as generalist – capable of feeding on plants from different families – and specialists – capable of feeding on only a few species or plants from a single family. In general, the idea is that specialist insects will not be able to use many hosts, but will be capable of tolerating plant defenses; whereas, generalists can feed on many plants but are more susceptible to defensive compounds [21]. Three main predictions have been proposed based on this paradigm. First, specialists are less impacted by plant defensive compounds and may use them for finding their host [22]. Second, induced plant responses to specialists will differ from responses to generalists. Third, generalists should have a 'general' mechanism to tolerate an array of defenses and possess mechanisms to manipulate defenses through conserved plant defensive pathways. Such manipulation has been recently observed through the release of effectors present in saliva during feeding [23].

Insect saliva and its role in plant-insect interactions

Insect saliva plays an important role in plant-insect interactions. Most research on insect saliva has focused on sucking insects. Examples of the role of saliva in host establishment in sucking insects include: digestion [24], anticoagulation or clogging [25–28], and suppression of host defenses [29]. However, these roles have not been thoroughly tested in chewing insects. Because the damage caused during feeding is very different between sucking and chewing insects, the saliva of chewing insects might also play roles in plant-insect interactions not yet identified.

Some of the molecules identified with confirmed role in the saliva of chewing insects include glucose oxidase[12,30], ATP utilizing enzymes[19], lysozymes[31], and the trail finding compound 2-acyl- 1,3 cyclohexadione [32,33]. Also, in the adult of *Heliconius melpomene*, an active protease has been identified [34]. Glucose oxidase has been shown to have antimicrobial properties as well as being involved in eliciting or suppressing plant induced defenses. ATP utilizing enzymes suppress the expression of defensive genes in tomato and glandular trichomes potentially by consuming extracellular ATP, which is involved in plant signaling. Lysozymes from *Helicoverpa zea* are associated with immune defense. Other proteins have been identified in saliva of chewing insects potentially involved in digestion, immunity, detoxification and herbivore offense[35,36]. However, their functions have not been confirmed.

Information on the factors that affect saliva composition and its effect on herbivory recognition are limited. Previous studies have determined that the activity of glucose oxidase in labial glands of caterpillars varies according to diet [37,38]. However, most studies have focused on using artificial diets with different levels of carbohydrate and protein and not on the actual host plants. This dissertation focuses on: 1. Characterizing the saliva composition of a generalist insect; 2. Determining the effect that host plants have on caterpillar saliva composition; and 3. Establishing the role that molecules present in the saliva may play in plant-insect interactions.

System

To answer the question about caterpillar saliva and its role in plant-insect interactions, I used the system of the generalist cabbage looper (*Trichoplusia ni*) and two of its hosts – cabbage

and tomato. The cabbage looper is a generalist that feeds on more than 50 plants from different families[39]. Because of its ability to feed on so many different plant families, it is an excellent system to study host plant driven salivary changes. Cabbage belongs to one of the cabbage loopers' preferred host families – Brassicaceae. Cabbage along with other plants from its family contains glucosinolates as their main defense and to lesser degree trypsin proteinase inhibitors[40,41]. Tomato from the Solanaceae family, on the other hand, also contains trypsin proteinase inhibitors along with alkaloids and phenolic compounds[42]. By exposing cabbage looper larvae to these different hosts, we are able to measure the effect that different secondary compounds can have on its salivary composition.

Chapter overviews

Chapter 2 is a brief review of the genomics of Lepidoptera saliva focusing on plant feeding Lepidoptera and the use of transcriptomic and proteomic techniques to generate hypotheses about the role of saliva in plant-insect interactions. This chapter has been published in the journal of Current Opinion in Insect Science in collaboration with Flor E. Acevedo (https://doi.org/10.1016/j.cois.2017.01.002).

Chapter 3 focuses on the changes in the transcriptome of the salivary glands of the cabbage looper when reared on three diets – cabbage (high quality host), tomato (low quality host), and pinto bean artificial diet (control). Overall, we observed a massive remodeling of the salivary glands at the transcriptomic level depending on its diet. This work was done in collaboration with David A. Galbraith, and it has been published in PlosOne.

Chapter 4 analyzed the same treatments as in Chapter 3; however, here I analyzed changes at the protein level, which is one step downstream from gene expression. Similar to the observations at the transcriptomic level, I observed differences among different diets, although the changes were not as drastic as those in the transcriptome. Also, it provided further evidence about the potential role of saliva in extra-oral detoxification. This section was done in collaboration with Anne Stanley and Bruce Stanley at the Proteomics Core at Penn State Hershey and has been submitted to Journal of Insect Physiology.

A beta-glucosidase with characteristics of a myrosinase was identified in both the transcriptome and proteome from Chapters 3 and 4. Chapter 5 focuses on trying to confirm the role of this enzyme in the cabbage looper. This project was done in collaboration with Daniel Vassao and Jonathan Gershenzon at Max Planck Institute Center for Chemical Ecology. Our results suggest that these genes might be broad-spectrum glucosidases rather than specific myrosinases. However, further research is necessary to confirm this conclusion.

As part of my dual-title degree in International Agriculture and Development, I conducted six months of research at the German Center for Integrative Biodiversity Research (iDiv). Chapter 6 is an expansion of data collected during this time. This chapter focuses on analyzing the potential role of waste water produced during Hanza processing – a famine crop in Niger – as a germination inhibitor. Part of this research was published in the journal Frontiers in Plant Science (https://doi.org/10.3389/fpls.2015.00532).

Finally, Chapter 7 is a summary of the results of the dissertation and how they fit into the broader plant-insect interaction framework as well as future directions.

Chapter 2

Genomics of Lepidoptera saliva reveals function in herbivory

Abstract

Lepidopteran herbivores deposit copious amounts of saliva when feeding. Caterpillar saliva is produced by the paired mandibular and labial glands. Evidence indicates that it may play an important role in allowing an herbivore to establish on its host plant. Genomic studies of Lepidoptera saliva are beginning to reveal the role of saliva in herbivory. Molecules involved in digestion, detoxification, immunity, defense against plant secondary chemicals, chemoreception, etc. have been identified using high throughput genomic tools. These genomic tools have also revealed changes that occur in Lepidoptera saliva when caterpillars feed on different host plants. However, there are other factors either biotic or abiotic (e. g. larval stage, larval health, temperature, water stress, etc.) that might also affect its composition. Though further functional and ecological studies are still necessary to fully understand the role of Lepidoptera saliva on herbivory, here we review current trends.

Introduction

Lepidoptera is a diverse order comprising about 180,000 described species, the majority (99%) phytophagous[43]. During feeding, caterpillars release saliva that comes in direct contact with the plant [37,44]. The salivary components may be recognized by plants and serve as cues to induce or suppress plant defense responses[12,19,35,45,46]. Therefore, saliva likely plays a major role in a caterpillar's ability to successfully feed on a host plant. Insect saliva functions in the

lubrication of mouthparts, digestion, immunity, detoxification, and regulation of host defense responses[47]. Unfortunately, there is a dearth of genomic and proteomic studies on the saliva of Lepidoptera, which makes broad generalizations difficult.

The secretory products of the salivary glands of Lepidoptera include silk and watery saliva. Silk serves different functions including dispersion and escape from natural enemies (ballooning)[48], protection and improvement of food quality (leaf rolling)[49], and communication[50]. Recently, it has been recognized that watery saliva also plays an important role in Lepidoptera-host plant interactions. Further studies using high throughput technology (i.e. microarrays, RNASeq, proteomics, metabolomics) are needed to better understand the process of salivation and the functional roles of specific salivary components. Here we review what is currently known about the composition and function of Lepidoptera saliva.

Structure of glands

Caterpillar saliva originates from the paired mandibular and labial glands. Mandibular glands are tubular-like structures that extend into the larval thorax and their secretions are poured into the lumen of the mandibular adductor apodemes[51] (Fig. 2-1a). Mandibular glands open through numerous pores at the mesal and lateral sides of the mandibles[51]. Labial glands are tubular with a central duct surrounded by a single-cell layer of ectodermal epithelium[52]. Structurally, Lepidoptera labial glands have two regions: the thin and the thick duct (Fig 2-1b). Functionally, at least two regions have been identified: a secretory and a reabsorptive region[52]. The synthesis of salivary proteins and water transport from the hemolymph to the gland lumen occurs in the secretory region while the reabsorptive region specializes in the movement of ions from the saliva into the hemolymph[52]. The labial glands are intercepted by a cluster of glands

referred as the Filippi's or Lyonet's glands (Fig. 2-1b). The paired labial glands release their secretions into the larval spinneret, which opens to the exterior at the hypopharynx (Fig. 2-1b). Here, we refer to mandibular and Lyonet's glands as accessory glands given that the majority of the saliva is produced in the labial glands.

Role of spinnerets and accessory glands in silk and saliva production

Spinnerets have been widely neglected from saliva studies. The fact that spinneret morphology varies among species (Fig 2-1 c-g) indicates to us that these differences might provide information on species specific characteristics of either their silk or saliva. One of the spinnerets best studied is that of the silkworm (*Bombyx mori*) because of their importance in the silk industry. Spinnerets contain muscles, which along with the shape and width of the spinneret influence silk spinning[53]. Also, a recent RNASeq analysis of the *B. mori* spinneret identified not only transcripts encoding cuticular proteins as expected, but also a large number of ion-transporting transcripts[54]. In addition to the mechanical spinning process during silk secretion, the changes in pH along the spinneret and its concentration of ions provide specific characteristics to silk such as elasticity, strength, etc.[54].

Most studies of Lepidoptera saliva have focused on secretions of the labial glands probably because they are easier to collect compared to accessory glands. The function of Lyonet's glands has not been confirmed but histochemical studies in the silkworm larvae, *Antheraea mylitta,* identified lipid granules in their cytoplasm suggesting a role in lubrication[55]. A transcriptomic analysis of the Lyonet's glands in *B. mori* identified several transcripts encoding ion channels and transporters for ions, sugars and amino acids, indicating a role in the transport of small molecules to the labial glands[54]. This correlates with ultrastructure studies, where the

lack of a well developed cytoplasmic membrane system and secretory vesicles also suggests a role in exchange of water and ions and not a secretory one[56].

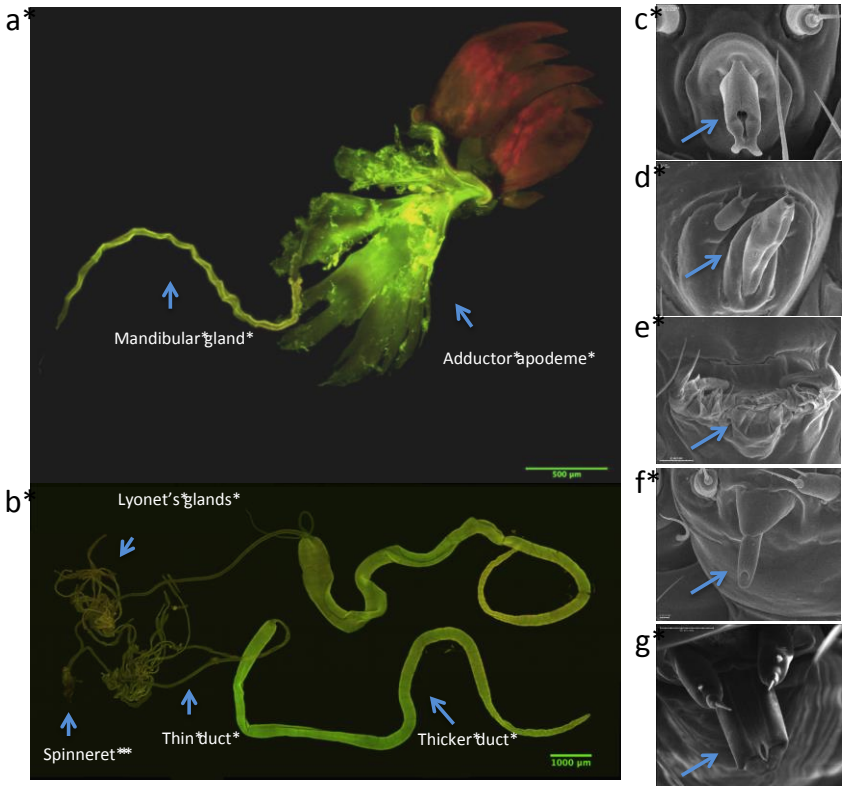

Figure 2-1: Structure of caterpillar salivary glands (a) Confocal Laser Scanning Microscopy (CLSM) image of a mandibular gland attached to the mandibular adductor apodeme in 6th instar Spodoptera frugiperda larva; adductor muscles were manually removed to facilitate the visualization of the gland. (b) CLSM image of labial glands in 6th instar Spodoptera frugiperda larva depicting different morphological regions and accessory glands; in this species the length of these glands is 87.5 % of the caterpillar body length. (c-g) Scanning Electron Micrographs (SEM) showing morphological differences in spinnerets of five Lepidoptera species: (c) European cornborer, Ostrinia nubilalis; (d) corn earworm, Helicoverpa zea; (e) tobacco hornworm, Manduca sexta; (f) cabbage looper, Trichoplusia ni; and (g) fall armyworm, Spodoptera frugiperda. Photo credits: dissections done by Flor E. Acevedo; CLSM images taken by István Mikó.

Mandibular gland secretions are released through pores in the mandibles and not directly into the spinneret, yet they still come in contact with secretions from the spinneret. For example, in *Cactoblastis cactorum* and *Plodia interpunctella*, droplets of mandibular secretions are found

along the silk strands. These secretions contain 2-acyl-1,3 cyclohexanediones used for trail marking[32,33]. Proteomic studies of the mandibular glands of *Vanessa gonerilla* and *V. cardui* also point towards a chemosensory role of the mandibular secretions [57,58]. A high number of odor-binding and chemosensory proteins were identified in the mandibular glands of these two systems.

Saliva composition and function

Despite the diversity and economic importance of phytophagous Lepidoptera, few studies have characterized the composition of their saliva (Table 2-1). There are interspecific differences in the composition of Lepidoptera saliva (Table 2-2). However, despite these differences there are several functional groups that continue to be identified in Lepidoptera saliva studies regardless of species. These include functional groups of transcripts and proteins involved in digestion, detoxification, immunity, herbivore offense, and others (Table 2-3). By studying the composition of saliva, we can hypothesize on its role in herbivory (Figure 2-2). However, these hypotheses are based largely on information available from other orders like Hemiptera[28,47,59,60] and especially aphids, where the research on saliva is much more robust [27]. As more information on the saliva of caterpillars becomes available, differences and similarities between chewing and sucking insect saliva will become clearer.

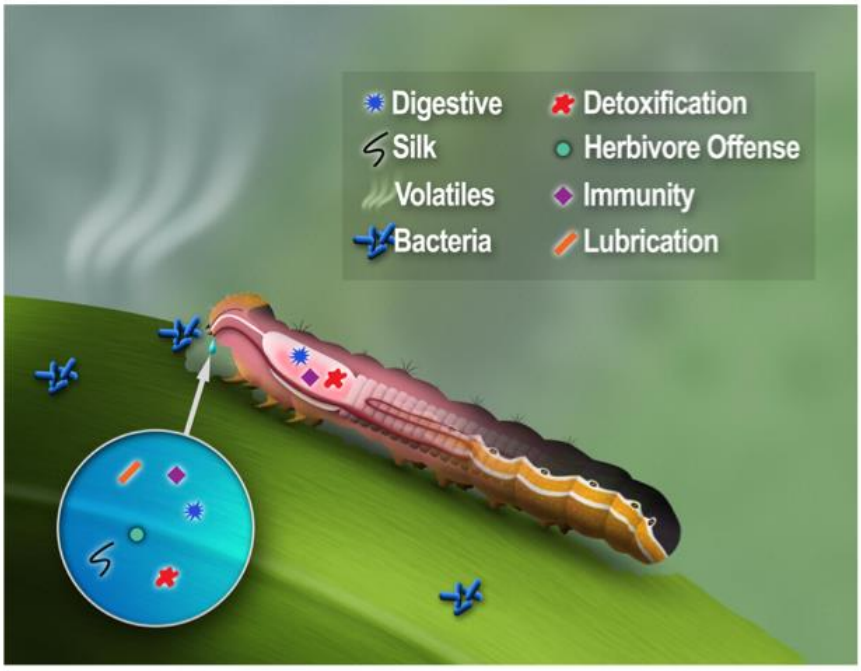

Figure 2-2: Potential dynamics of Lepidoptera saliva. Caterpillars, while feeding on plants release copious amounts of silk and saliva through the spinneret. These secretions contain several molecules potentially involved in lubrication, digestion, detoxification, immunity, herbivore offense, chemoreception, and others. Such molecules allow the insect to establish on its host by breaking down primary and secondary compounds as well as modifying volatile emissions and interacting with plant-associated microbes. These molecules are also ingested with the plant tissue and added to the already present repertoire of enzymes in the digestive system.

Table 2-1: Recent Omic studies of Lepidoptera salivary glands and/or saliva

Species	Family	Technique	Ome	Stage	Tissue	Extraction method	Comparisons
Bombyx mori [28,57]	Bombycidae	Microarray	Transcriptome	5th instar	Labial glands	Homogenized glands	Different tissues (anterior/median vs. posterior labial gland), Drosophila
		2D gel/ MALDI-TOF	Proteome	5th instar	Labial glands	Homogenized glands	Middle vs. posterior labial gland
Helicoverpa armigera [27]	Noctuidae	Sanger & 2-DE/MS	Transcriptome and proteome	3rd and 5th	Labial glands	PBS extraction*	None
Maruca vitrata [54]	Crambidae	454 pyrosequencing	Transcriptome	3rd, 4th, 5th instar	Labial glands	Homogenized glands	None
Vanessa gonerilla [22]	Nymphalidae	LC--MS/MS	Proteome	5th instar	Mandibular and labial	PBS extraction	None
Helicoverpa zea [8]	Noctuidae	NanoLC	Proteome	5th instar	Saliva	Direct collection	None
Spodoptera exigua [34]	Noctuidae	nanoLC/ESI/tandem MS	Proteome	4th instar	Labial glands	Homogenized glands	Artificial diet and plant
Heliconius melpomene [29]	Nymphalidae	LC-MS	Proteome	Adult	Saliva	Direct collection	None
Manduca sexta	Sphingidae	RNASeq	Transcriptome	4th instar	Labial glands	Homogenized glands	Host plants and tissues
Vanessa cardui [21]	Nymphalidae	NanoLC MS/MS	Proteome	5th instar	Mandibular and labial glands	PBS extraction	Artificial diet, host plant, bacteria

*PBS extraction refers to the methods where glands are placed in PBS buffer and content from the lumen is allowed to be released into solution. Proteins in solution are used for analysis.

Table 2-2: Transcripts and proteins potentially involved in digestion, detoxification, herbivore offense, and immunity identified in salivary glands and saliva of phytophagous Lepidoptera

	Functional Groups			
Species	**Digestion**	**Detoxification**	**Herbivore Offense**	**Immunity**
***Bombyx mori* [28,57]**	Proteases	Dehydrogenases Glutathione-S-transferase Superoxide Dismutase Thiol Peroxiredoxin	Protease inhibitors	Lysozyme
***Heliconius melpomene* [29]**	Trypsin protease Cysteine protease Beta-fructofuranosidase Glycerolphosphoryl diester Hydrolase Beta-Hexosaminidase Astacin	?	Protease inhibitors	Lysozymes Beta 1,3 glucanase GMC oxoreductase Hemolin REPEAT gene
***Helicoverpa armigera* [27]**	Carboxypeptidases Lipases Phospholipase Alpha-amylase Maltase Beta fructofuranosidase Fructose-biphosphate aldolase Glucose dehydrogenase Proteases	Carboxyl cholinesterase Oxidase/peroxidase	Protease inhibitors Glucose oxidase	Lysozymes
***Helicoverpa zea* [8]**	Fructosidase Proteases Carboxylesterases	Carboxyl cholinesterase Cytochrome P450	Glucose oxidase Protease inhibitors	Lysozymes [37]
***Manduca sexta* [35]**	?	Glutathione-S-transferase ABC transporters UDP-glucosyl transferases Cytochrome P450s	?	Attacin Cecropin Gallerimycin IMD and TOLL pathway genes
***Spodoptera exigua* [34]**	?	Thiol peroxiredoxin	Protease inhibitors	?
***Vanessa cardui* [21]**	Serine protease	Catalase	Protease inhibitors	Beta-glucan receptors
***Vanessa gonerilla* [22]**	Amylase Proteases Glycolytic proteins	Isocitrate dehydrogenase	Protease inhibitors	Lysozymes Lysosomal glycocerebrosidase

Table 2-3: Compounds with confirmed activity in Lepidoptera saliva

Insect species	Family	Compound	Role in plant	Plant species
Helicoverpa zea	Noctuidae	Glucose oxidase [7]	Suppression of nicotine production*	Tobacco
		ATPases [5]	Suppression of genes from jasmonic acid and ethylene pathways	Tomato
		Lysozyme [37]	?	-
Heliconius melpomene	Nymphalidae	Protease [31]	Digestion of pollen	Pumpkin pollen
Vanessa gonerilla	Nymphalidae	Amylase [22]	?	?
		Lysozyme [22]	?	?
Chilo suppressalis	Crambidae	Amylase [55]	?	?
		Lipase [56]	?	?
		Invertase [56]	?	?
Cactoblastis cactorum	Pyralidae	2-acyl-1,3 cyclohexanedione [19]	Trail finding	?
Plodia interpunctella	Pyralidae	2-acyl-1,3 cyclohexanedione [20]	Trail finding	?

*For a more detailed role of glucose oxidase in other insects, refer to Acevedo et al., 2015

Digestion

Digestive enzymes (i.e. amylases, proteases, etc.) have been identified in basically every Lepidoptera saliva characterized to date [35,36,57,58,61,62]. Saliva is deposited outside of the oral cavity. This fact has led to the hypothesis that saliva is involved in extra-oral digestion. By releasing digestive enzymes before ingestion, saliva could facilitate the breakdown of macromolecules and complement the repertoire of midgut digestive enzymes to increase digestion efficiency. This concept is not new in insects, it has been known for a long time that saliva aids in extra-oral digestion in sucking insects – both predators and phytophagous[24,60]. Given the modified mouthparts (stylets) of sucking insects, the concept was easily accepted. However, the presence of this phenomenon in chewing insects has been ignored or not thoroughly tested. Extra-oral digestion in phytophagous Lepidoptera was observed in the saliva of *Heliconius melpomene* adults. Proteases in their saliva aid with pollen digestion[34,62]. However, this is once again, a special case in which the adult feeds on a non-liquid diet and needs to ingest food through a proboscis. It is possible that even though the saliva of caterpillars has some digestive activity, it is not as significant as in sucking insects.

Detoxification

Plants contain a wide array of secondary compounds that are toxic to herbivores[63]. Because saliva is one of the first secretions to come in contact with the plant during feeding, it may represent the first line of defense against plant defenses. Therefore, it is not surprising that saliva contains detoxification enzymes (Table 2). Detoxification of secondary compounds has been widely studied in the insect midgut[64]. However, although most secondary compounds will

be restricted to the digestive system of the insect, other tissues might also be exposed to these. Detoxification genes in salivary glands include glutathione-S-transferases, cytochrome P450s, UDP-glycosyl transferases, among others[35,36,57,58,61,65,66]. These genes could actively assist in detoxification of plant defenses or detoxify toxic compounds from cellular metabolism. Another hypothesis is the release of detoxification enzymes in the saliva to aid in detoxification of toxins on the leaf surface or during chewing. Some detoxification enzymes such as catalases and dehydrogenases have been identified in the proteome of salivary glands[36,57,58] including a study where the secreted saliva was characterized[35], which means detoxification enzymes are deposited on the wound or add to the already available detoxification enzymes in the midgut. However, the contribution of these enzymes to detoxification on the feeding site needs to be confirmed.

Immunity

Insects and plants do not coexist in a sterile environment and thus caterpillars encounter plant-associated microbes (bacteria, fungi, virus, protozoa, etc.) that may be beneficial or pathogenic. Both transcripts and proteins involved in immunity have been identified in the salivary glands of Lepidoptera. At the mRNA level, transcripts involved in the Toll and IMD pathway are expressed in salivary glands[66]. At the protein level, antimicrobial peptides and beta-glucan receptor proteins have also been identified[21,22,27,29]. There is also evidence of changes in gene expression in the salivary glands when caterpillars were fed diet containing bacteria or bacteria related compounds[57]. Confirmation of active use of saliva as an antimicrobial secretion is limited. Antibacterial activity has been confirmed in the saliva of *Helicoverpa zea*[30]. This activity is attributed to the presence of secreted glucose oxidase and lysozyme [30,31]. Caterpillars with ablated spinnerets (saliva cannot be released) exposed to diet

containing bacteria had a higher mortality than caterpillars with intact spinnerets [30]. Test of this function in other species is necessary to determine how widespread the phenomenon is.

Herbivore offense

Herbivore offense refers to traits that allow herbivores to increase their feeding and exploit their hosts[67]. Lepidoptera saliva contains antioxidant enzymes (e.g., peroxiredoxins, peroxidases, and catalases) (Table 2-2). These compounds could help avoid insect recognition by reducing reactive oxygen species, which are known to be secondary messengers in plant signaling[68]. Saliva also contains several proteinase inhibitors (Table 2-2) that may be involved in reducing the effect of plant proteases on insect digestion[69]. Additionally, Lepidoptera saliva contains molecules that affect volatile emissions and thus could affect the plant's ability to attract natural enemies. For example, in tobacco, feeding by *Heliothis virescens* caterpillars with intact spinneret induced fewer volatile compounds compared to feeding by caterpillars with ablated spinneret[70]. However, how natural enemies respond to these changes in volatiles has not been tested.

The effect of saliva in plant defense regulation is frequently species specific. Plant responses to saliva from different insects can vary, on the other hand, saliva from a particular insect species can have different effects on different host plant[71]. A perfect example of this specificity is the effect of glucose oxidase on plant defenses[71]. Glucose oxidase is one of the most well characterized salivary enzymes involved in herbivore offense in Lepidoptera [12]. It has been identified in the saliva of a wide array of species and is known to suppress herbivore defenses in some hosts, thus increasing insect performance[12,23]. Salivary glucose oxidase from *H. zea* suppresses nicotine production in tobacco[12], but in tomato it induces jasmonic acid-

regulated defenses such as proteinase inhibitor defenses [35]. The saliva from *Ostrinia nubilalis*, induces defenses in both maize and tomato; however, the factors involved in this induction are different between host plants[46]. In tomato, the levels of glucose oxidase in *O. nubilalis* are sufficient to elicit defenses but in maize the induction is due to a yet unknown compound. In Arabidopsis, *Spodoptera exigua* saliva affects the phosphorylation of lipoxygenase 2, a chaperonin and several photosynthesis-associated proteins[72]. However, no information is available on which specific salivary compounds induce these responses.

Furthermore, saliva is composed of multiple proteins that add to the complexity of these interactions. For instance, the saliva of *H. zea* contains glucose oxidase which elicits herbivore defenses in tomato, but it also contains several ATP hydrolyzing enzymes which suppress expression of genes involved in the jasmonic acid and ethylene pathways as well as induction of trichomes in the same plant[19]. In summary, in *H. zea,* salivary ATP hydrolyzing enzymes suppress early defense responses in tomato followed by a delayed induction of defenses due to glucose oxidase. It is clear that we have only begun to unravel the intricate dynamics occurring between plants and insect saliva.

Other

Several other salivary genes and proteins have been identified; some of which probably have a housekeeping function. This means that they are constitutively present and are required for the maintenance of basic cellular function. Among these proteins are several involved in protein synthesis, post-transcriptional and post-translational modification and degradation[36,57,58,61,62]. There is also a consistent presence of proteins involved in storage and vacuolar transport (e.g. arylphorins, apolipophorins, transporters, etc.)[57,58,61,65]. Finally,

as with many other insect secretions and/or tissues, there are many transcripts of unknown identity in need of functional studies.

Changes in Lepidoptera saliva

Studying the overall changes in gene expression of salivary glands or saliva composition can provide information about its role. To date, most studies have focused on changes in saliva due to diet, either on artificial diet with different protein:carbon ratios or different host plants (Table 2-1). There is also some evidence that the saliva of caterpillars changes with exposure to bacteria. The salivary proteome of mandibular and labial glands of *V. cardui* caterpillars changed when caterpillars were challenged with diet containing bacteria or bacterial compounds[57]. Glucose oxidase, because of its role in herbivore offense as well as immunity and ubiquity in Lepidoptera saliva, has been used as a marker to study changes in saliva. It has been shown that glucose oxidase expression changes according to protein:carbon ratio[38,73] as well as when feeding on tobacco and to a lesser degree on diet supplemented with nicotine[74,75]. In *H. armigera*, glucose oxidase activity has a positive correlation with the presence of sugar[75]. In *H. zea*, the amount of glucose oxidase also varies depending on host plant[37] and its expression seems to be upregulated when caterpillars fed on jasmonic acid treated tomato plants[49].

Changes in saliva when insects are feeding on a host plant are expected to be dynamic. However, how quickly saliva responds to external stimuli and how these changes are triggered in the insect still remains to be determined. Also, studies of whether changes in saliva composition benefit the insect or the plant have not been carried out yet. So far, research has focused on determining changes without following up with functional and/or fitness studies. Though there has been an increase in research of the salivary glands and/or saliva of Lepidoptera, these cover a

very small fraction of extant species of Lepidoptera. Also, the activity of only a handful of the salivary compounds discussed in this review has been confirmed (Table 2-3). There is considerable need to continue characterizing sialomes but we must also move to the next phase of functional and ecological studies.

Potential ecological interactions mediated by saliva

Lepidoptera saliva is involved in dynamic and complex ecological interactions (Fig 2-3). We have described how saliva affects host plant chemistry and also discussed how host plants can in turn affect salivary composition. It is known that host plant chemistry can also affect parasitoids and/or microbial communities [77,78], which means that saliva is likely affecting the tritrophic level indirectly. Parasitoids and microbes, on the other hand, could have a direct effect on saliva and indirectly on host plant chemistry. Adding to this complexity is the presence of other herbivores, predators and pollinators, which could also be responding indirectly to salivary compounds. Furthermore, these interactions occur under a large umbrella of abiotic factors (soil nutrition, temperature, water availability, CO_2 levels, etc.), which may affect the biotic components.

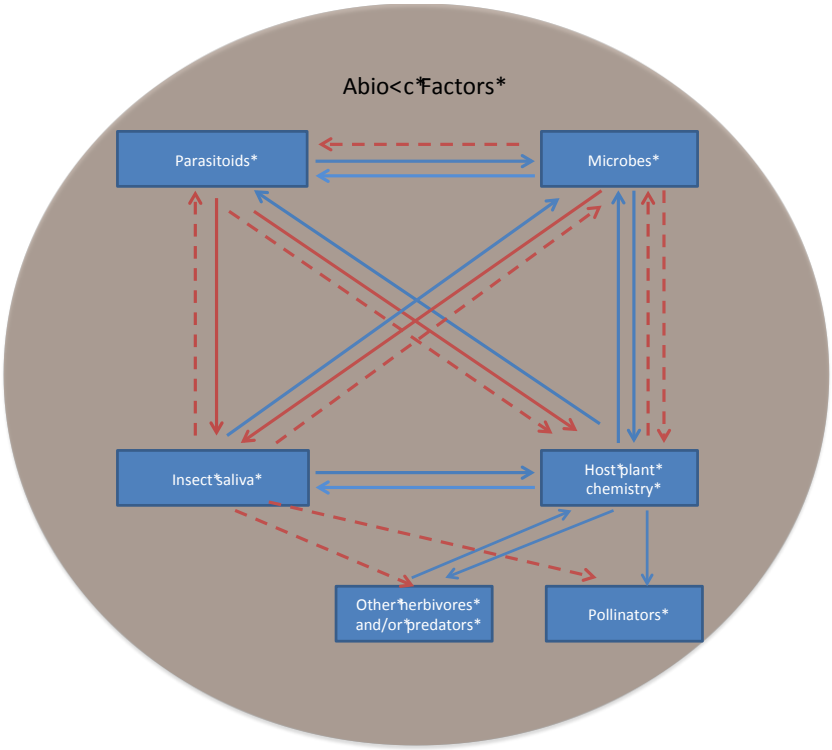

Figure 2-3: Interactions mediated through insect saliva. Blue lines indicate interactions that have been tested, red lines indicate interactions not yet tested. Dashed lines refer to indirect effect through one of the other factors in the interaction (e.g. saliva affecting host plant chemistry or microbes which in turn affect parasitoids).

Conclusions and future directions

Current studies of Lepidoptera saliva remain exploratory due to the paucity of available genomes and the limited number of functional studies using RNAi[79] or genome editing approaches such as CRISPR/Cas9 in Lepidoptera[80]. Testing the myriad of hypotheses about the functions of saliva in herbivory and its ecological relevance is crucial to advance the field. For instance, the role of saliva in carbohydrate hydrolysis, insect immunity, chemosensory, detoxification and lubrication of mandibles remains largely hypothetical as well as changes in

saliva due to interactions with other organisms like bacteria, fungi, virus, and parasitoids, among others. Most studies have only focused on the study of saliva in immature insect stages neglecting its role in their adult forms where diet is often very different. Also, Lepidoptera saliva is likely to contain small molecules with essential biological importance, but most studies have only focused on the identification of proteins in the saliva or gene expression of the glands. Finally, many of the ecological interactions potentially mediated by saliva remain to be tested. These studies would add to the understanding of the undoubtedly complex role of saliva in mediating plant-insect interactions.

Chapter 3

Host plant driven transcriptome plasticity in the salivary glands of the cabbage looper (*Trichoplusia ni*)

Abstract

Generalist herbivores feed on a wide array of plants and need to adapt to varying host qualities and defenses. One of the first insect-derived secretions to come in contact with the plant is the saliva. Insect saliva is potentially involved in both the pre-digestion of the host plant as well as induction/suppression of plant defenses, yet how the salivary glands respond to changes in host plant at the transcriptional level is largely unknown. The objective of this study was to determine how the labial salivary gland transcriptome varies according to the host plant on which the insect is feeding. In order to determine this, cabbage looper (*Trichoplusia ni*) larvae were reared on cabbage, tomato or pinto bean artificial diet. Labial glands were dissected from fifth instar larvae and used to extract RNA for RNASeq analysis. Assembly of the resulting sequencing reads resulted in a transcriptome library for *T. ni* salivary glands consisting of 14,037 expressed genes. Feeding on different host plant diets resulted in substantial remodeling of the gland transcriptomes, with 4,501 transcripts significantly differentially expressed across the three treatment groups. Gene expression profiles were most similar between cabbage and artificial diet, which corresponded to the two diets on which larvae perform best. Expression of several transcripts involved in detoxification processes were differentially expressed, and transcripts involved in the spliceosome pathway were significantly downregulated in tomato-reared larvae. Overall, this study demonstrates that the transcriptome of the salivary glands of the cabbage

looper are strongly responsive to diet. It also provides a foundation for future functional studies that can help us understand the role of saliva of chewing insects in plant-herbivore interactions.

Introduction

Generalist insects feed on a wide array of plants and thus are exposed to a diversity of plant nutritional qualities, as well as different secondary metabolites [81]. Generalist insects have evolved mechanisms to cope with plant defenses, including behavioral avoidance of resistant plants, metabolism of toxic compounds, and even suppression of the induced defenses through the release of suppressive effectors during feeding [7,12,19,82]. Mechanisms by which generalist insects cope with the defensive compounds of different plants have been evaluated. Most of these studies have focused on midgut responses due to its known role in digestion and detoxification [83,84]. However, insects interact with host plant tissue well before digestion is initiated. In fact, the first insect secretion that interacts with plant tissue during feeding is the insect's saliva[44]. In caterpillars, saliva arises from the labial and mandibular glands [85]. Labial saliva is released through the spinneret, which is external to the oral cavity and thus allows saliva to be released on the plant before ingestion. Saliva from chewing insects may play a role in digestion, detoxification, immunity and suppression of plant defenses [36,85] and may represent the insect's first line of defense against plant secondary compounds.

Plants, on the other hand, are able to differentiate between mechanical wounding and herbivore damage [9], which indicates the recognition of insect specific molecules by the plant. Insect oral secretions (mix of regurgitant and saliva) have been found to induce plant defenses [86–88]. Some of the elicitors of plant defense identified in caterpillar oral secretions include: fatty-acid amino acid conjugates[14], a beta-glucosidase[13], inceptin[16], and a porin-like

protein[89]. However, the role of saliva specifically has not been thoroughly studied. To date, salivary molecules from chewing insects known to play a role in plant-insect interactions include: ATP-utilizing enzymes[19] and glucose oxidase[12].

Glucose oxidase is the most well studied salivary enzyme in caterpillars. Glucose oxidase produced by the salivary glands of *Helicoverpa zea* caterpillars suppresses plant defenses in tobacco [12]. Glucose oxidase synthesis and secretion varies according to host plant or diet as well as the dietary carbon to protein ratio [23,38,90]. This provides some evidence of plasticity in the salivary glands of chewing insects in response to different diets and host plants. However, the effect of host plants in the overall salivary composition of chewing insects requires further study. Plasticity is the ability of an organism to express different phenotypes depending on the environment (22, 23). These changes can be either biotic or abiotic: host plant, temperature, light conditions, presence of predators, etc. The environmental changes can be continuous such as in the case of temperature or discrete as in the case of different host plants for a generalist insect [93].

We chose the system of cabbage looper (*Trichoplusia ni*) feeding on cabbage (*Brassica oleracea* var capitata) and tomato (*Solanum lycopersicum*) to study the overall transcriptomic changes that occur in the salivary glands of a generalist, chewing insect when feeding on different host plant species. Cabbage looper is a generalist herbivore from the lepidopteran family Noctuidae. The larvae feed on more than 50 plant species from several families [39]. Although it preferentially feeds on plants of the Brassicaceae family, it is also considered a pest for plants from the Solanaceae, Convolvulaceae, Cucurbitaceae families, among others. Because of its broad host range, the cabbage looper is exposed to a wide range of defensive compounds including general defenses such as phenolics, alkaloids and terpenes, protease inhibitors,

polyphenol oxidases and other defensive proteins, as well as the more specific defenses found in Brassicaceae such as glucosinolates [42,94]. It is critical for the larvae to be able to detoxify each of these defenses, which may involve different mechanisms. Since continuously expressing its full arsenal of defensive responses would presumably be energetically costly, we hypothesize the responses of the cabbage looper larvae must be plastic in order to allow it to utilize a broad range of host plant species. A recent study has shown remodeling of the midgut transcriptome in the cabbage looper when feeding on different hosts [95]; however, whether such plasticity is also present in the salivary glands of this insect is still unknown. We hypothesize the salivary glands of the cabbage looper are plastic and respond to different host plants at the transcriptomic level. To test this hypothesis, we reared the larvae on tomato, cabbage, and artificial diet (control) and then analyzed the overall gene expression in the salivary glands using RNASeq technology.

Materials and Methods

Plants and Insects

Cabbage looper (*Trichoplusia ni*) eggs were purchased from BioServ Inc (Flemington, NJ). Larvae were reared entirely on two host plants: tomato (*Solanum lycopersicum* var. Better Boy) and cabbage (*Brassica oleracea* var capitata 'Platinum Dynasty'), and pinto-bean artificial diet [96]. Whole plants were used instead of clippings and food was never limiting to the insects. Colonies were kept at 23°C in 16:8 Light:Dark conditions. Tomato and cabbage plants were grown in the greenhouse under a 16:8 L:D cycles and fertilized as needed. Days to pupation for 60 caterpillars reared on each treatment were measured.

RNA isolation and sequencing

Fifth instar larvae were allowed to feed for at least 1 day after molt, and then collected from the different treatments (tomato, cabbage and artificial diet). Samples were always collected early in the afternoon from larvae actively feeding. Salivary glands were dissected in chilled PBS buffer and immediately placed in liquid nitrogen and stored at -80^{o}C until further processing. Each sample contained a pool of 10 pairs of salivary glands. Total RNA was extracted using the QIAGEN RNeasy kit (Valencia, CA) following the manufacturer's protocol. Sample quality and quantity were assessed and validated using the Agilent bioanalyzer (Agilent Technologies, Inc; Santa Clara, CA). Samples were submitted to the Genomics Core Facility at The Pennsylvania State University to be sequenced using the Illumina HiSeq 2500 (San Diego, CA). Three biological replicates per treatment were sequenced for a total of nine samples. A barcoded library was made from each sample using the Illumina TruSeq Stranded mRNA Library Prep Kit (#RS-122-2101) according to the manufacturer's protocol. The concentration of each library was determined by RTqPCR and an equimolar pool of the libraries was sequenced on the Illumina HiSeq 2500 in Rapid Run Mode using 100 nt single read.

Transcriptome analysis

Transcriptome sequencing reads were processed with Trimmomatic v0.32 [97], removing low quality reads, adaptor sequences, and reads with more than 5% unknown bases. A set of non-redundant transcripts was generated *de novo* using Trinity v2.1.0 using the default parameters [98]. The processed reads were aligned to this reference transcriptome using Tophat v2.0.10 [99]. Read counts for each transcript were imported into R v3.0.2 for further analyses. Transcripts with low read counts (<10 across all samples) were removed from further analyses. The data were

normalized using a trimmed mean of M-values (TMM) method [100] and tested for differential expression using a generalized linear model in EdgeR v3.4.2 [100]. Transcripts were considered to be significantly differentially expressed when FDR < 0.05. Pairwise comparisons of the three treatments were performed to identify the effects of the three different diets on salivary gland gene expression. The generated transcripts were annotated using a reciprocal best hit BLAST [101] approach to identify *Bombyx mori* orthologs. These orthologs were then used for a gene ontology (GO) analysis using DAVID v6.7 [102]. Functional categories were then clustered by parent GO terms using REVIGO [103]. The same samples used for RNA-Seq were later used for validations using real-time quantitative PCR. Sequence reads and assembled transcripts generated from this study have been deposited in the Gene Expression Omnibus [104] with accession number GSE101549.

Real-time quantitative PCR

Real time quantitative PCR (RTqPCR) was used to validate results from the bioinformatic analysis. One µg of RNA was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription kit following manufacturer's protocol (Applied Biosystems, Inc; Grand Island, NY). Primers were designed for 9 of the differentially expressed genes along with 4 other genes involved in detoxification (Appendix A). All reactions were done using Power SYBR Green PCR Master Mix and ran on a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc; Grand Island, NY). Relative expression for each gene was quantified using the ΔΔCt method [105], and normalized using GAPDH. Actin was also assessed as potential reference gene, but GAPDH was the most stable gene across samples. Standard curves using serial dilutions and melting curves were performed to calculate primer efficiency ($E=10^{\wedge(-1/slope)}-1$) and confirm the presence of single amplicon.

Role of saliva in detoxification

For validation of the role of salivary glands in detoxification, third instar larvae were transferred to tomato plants from *def*-1 (*Defenseless*-1) mutants and Castlemart variety – background for *def*-1 [106]. Def-1 mutants are affected in the octadecanoid pathway and because of this, they do not respond to insect damage. Plants were grown under same conditions as previously described. Salivary glands were dissected from fifth instar larvae allowed to feed for at least 24 h post molting and used for RTqPCR as previously described. Genes analyzed were: catalase, glutathione-S-transferase, protease, proteinase inhibitor, cytochrome P450, and UDP-glycosyl transferase (Appendix A).

Statistics

Differences in days to pupation were determined using ANOVA followed by a separation of means using a Tukey post-hoc test at $p<0.05$. Differences in gene expression using RTqPCR were analyzed using the nonparametric test Mann-Whitney U test. For statistics of transcriptome analyses refer to Transcriptome section described above. Heatmaps were made using the heatmap3 package from R. All statistics were done using R software.

Results and Discussion

Generalist insects feed on a wide array of plants and because of this, they are exposed to a diversity of primary and secondary compounds. Understanding this ability to establish on so many different plants has long been the aim of chemical ecologists. Both behavioral and physiological adaptations have been reported in cabbage loopers that could allow them to cope

with the myriad of plant defenses to which they are exposed [82,107]. For example, changes in midgut transcriptome have been reported in the cabbage looper when feeding on defended and undefended hosts [95]. This plasticity might also be present in other tissues in the insect. Saliva is one of the first secretions to come in contact with the plant during feeding and so it could be playing an important role in an insect's ability to successfully feed on a host. The main goal of this study was to determine the overall transcriptomic changes that occur in the salivary glands of the generalist cabbage looper when it feeds on different hosts.

We established a transcriptome library for the salivary glands of the cabbage looper. We sequenced three biological replicates for three treatments (cabbage fed, tomato fed, and artificial diet fed) for a total of 9 samples. The number of reads generated from each sample ranged from 25,714,877 to 28,381,856. The Trinity assembly generated 38,082 transcripts that corresponded to 30,082 'Trinity genes' (a collection of related transcripts – Appendix B). These were clustered into 14,037 components, out of which, 7,913 corresponded to *Bombyx mori* orthologs based on a reciprocal best-hit blast approach.

Cabbage looper grows at different rates on different host plants

Plant quality can be measured several ways. A common way is by measuring carbon or carbohydrates, nitrogen or protein, and secondary compounds [108]. Though it is normally assumed that higher nitrogen means better quality, the origin of this nitrogen is not specific. This means that it could come from a source that is not beneficial or available for the insect. Furthermore, the effect of protein and secondary compounds is context dependent [109]. For example, some plant defenses might have a toxic effect on insects in the presence of higher protein quality [110], which is counterintuitive. In this respect, a better measurement is to actually

measure the effect the plant has on the insect i.e. growth, fecundity, mortality, etc. Here, we measured how long it took larvae to reach pupation along with mortality when feeding on each host plant (Fig 3-1).

The cabbage looper, even though considered a generalist, commonly grows faster when feeding on plants from the Brassicaceae family. However, it is still able to complete its cycle in plants from other families. There is evidence that this difference in growth is in part due to the different secondary chemistries of the hosts [95]. However, there may also be nutritional differences mediating these responses: Herde and Howe [95] demonstrated reduced growth of caterpillars on mutant tomato plants versus mutant Arabidopsis plants, even though the plants were mutated to reduce defense responses, thus indicating an effect due to something other than induced defenses.

Cabbage loopers were reared on cabbage, tomato and artificial diet. Treatments had a significant effect on the number of days required for larvae to reach pupation. Larvae that were reared on the artificial diet and cabbage reached pupation in approximately 13 and 15 days, respectively; whereas, larvae reared on tomato required an average of 23 days to reach pupation (Fig. 3-1A) ($F_{2,178}$:8.142, pvalue:0.0004). Also, a higher percentage of larvae died when feeding on tomato compared to cabbage and artificial diet (Fig. 3-1B). Because of the differential growth of the caterpillars and percentage mortality on cabbage versus tomato, we refer to cabbage as a "high quality host" and tomato as a "poor quality host".

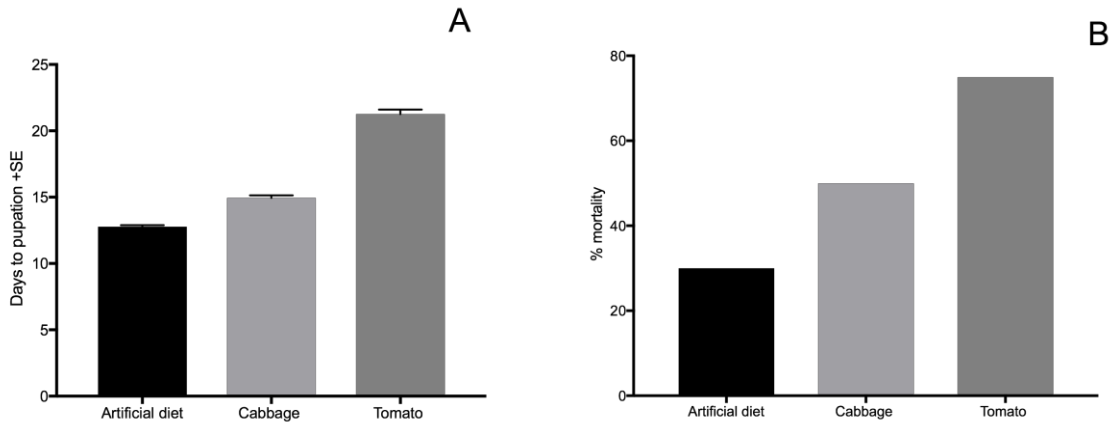

Figure 3-1: Effect of different diets on the growth of cabbage looper (*Trichoplusia ni*). A. Days to pupation of cabbage looper reared on three treatments – cabbage, tomato, and pinto bean artificial diet. B. Percentage mortality of larvae on each diet.

Transcriptomes of cabbage looper salivary glands are extensively remodeled according to host plant species

When comparing gene expression of salivary glands from larvae reared in cabbage (high quality host) against those in artificial diet (control), only 630 (4% of the total transcripts) transcripts were significantly differentially expressed, with 366 transcripts being upregulated and 264 downregulated. A much larger proportion of transcripts were significantly differentially expressed in the glands of larvae reared on tomato (low quality host) compared to larvae reared on artificial diet, where 4,318 transcripts (representing 31% of the total transcripts) were differentially expressed, with 2,386 being upregulated and 1,932 downregulated. Finally, in the cabbage to tomato comparison, 1,100 transcripts were upregulated on cabbage fed larvae and 1,466 downregulated for a total of 2566 differentially expressed transcripts representing 18% of the total transcripts (Fig 3-2). Nine genes were chosen for validations of RNASeq results using RTqPCR. Out of the 27 comparisons made (3 per gene), only 5 did not correlate between the RNASeq and real time qPCR results (Appendix C).

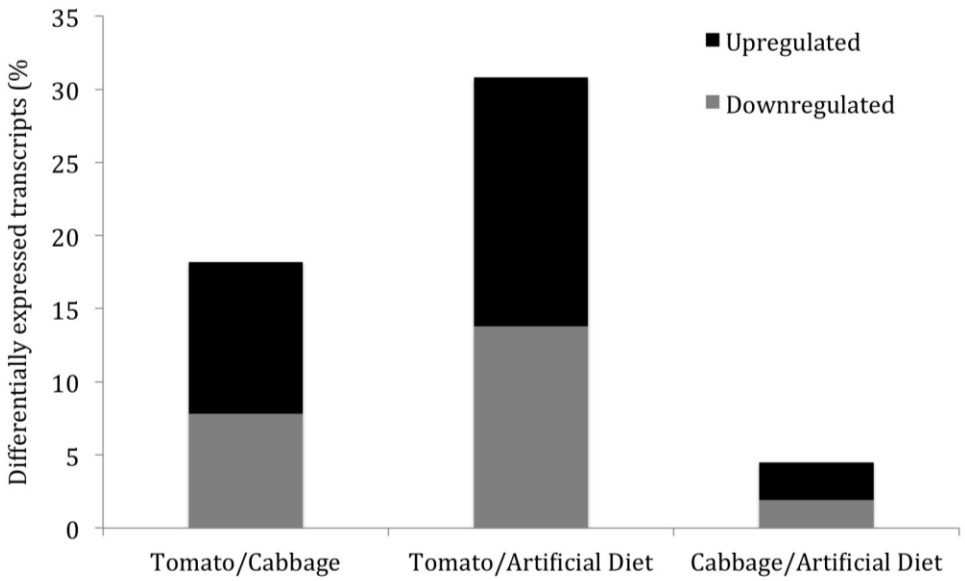

Figure 3-2: Percentage of differentially expressed genes from total transcriptome library for three comparisons – Tomato vs. Cabbage, Tomato vs. Artificial diet, and Cabbage vs. Artificial Diet. Black indicates upregulated genes and grey indicate downregulated genes for each comparison.

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Clustering analysis of all the genes revealed that the transcription profiles of salivary glands of larvae fed on cabbage and artificial diet were similar to each other, while the profile of the glands of tomato-fed larvae were extensively different (Appendix D). The greatest changes were observed in the salivary glands of tomato-fed insects, which were also the insects that had the slowest growth and highest mortality most of which occurred in the early instars. This plasticity in a generalist insect is consistent with a previous study that observed changes in the transcriptome of the generalist two-spotted spidermite (*Tetranychus urticae*) when it fed on tomato plants. In the mite study, a total of 1,275 genes were differentially expressed [111]. In contrast, the salivary glands of the tobacco hornworm (*Manduca sexta*), a specialist of Solanaceae did not exhibit such a dramatic change in gene expression when exposed to different host plants [66].

Spliceosome pathway genes show significant downregulation in larvae reared on tomato

A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified the spliceosome pathway as significantly downregulated (p-value: $1.42X10^{-7}$) in larvae reared on tomato versus artificial diet. The spliceosome is the ribonucleoprotein complex involved in splicing or intron removal of pre-mRNAs, and is involved in both constitutive and alternative splicing of mRNAs [112]. There is little information about the mechanisms and factors that regulate alternative splicing [113], therefore it is difficult to determine the biological significance of differential regulation of this pathway. Also, even though a downregulation of this pathway is observed, transcription change might not translate to protein change. However, we propose at least three hypotheses of what a downregulation of the spliceosome could mean in this system. First, the stress of feeding on tomato could be causing the insect to downregulate transcription in general, including the splicing machinery. In this case, we would expect the proportion of downregulated genes to be higher than the upregulated ones. However, we did not observe this (Fig 3-2). Second, the downregulation of the spliceosome alone could be a response to the stress of feeding on tomato and could be impacting the protein synthesis machinery in general. Finally, the downregulation of this pathway could signify that the salivary glands are switching to different variants of some proteins. Alternative splicing allows eukaryotic organisms to increase their proteome without having to remodel their genome extensively, thus providing an efficient plasticity mechanism [114]. In plants, it has been shown that different stresses have a dramatic effect on alternative splicing, including the spliceosome itself [115]. Insects have been shown to have even higher levels of alternative splicing than plants [116] and thus insects could be utilizing a similar mechanism to cope with stresses due to plant defenses. It remains to be determined whether the plant's defensive mechanism influences this change in the splicing machinery, or if it is an adaptation of the insect. Factors that are involved in suppression of alternative splicing

regulation were identified in our transcriptome [113], including: polypyrimidine tract binding protein (PTB/HNRNP1), heterogeneous nuclear ribonucleoprotein variant A1 (hnRNPA1), sex lethal variant L (SXL) and U2 auxiliary factor 35 kDA (U2AF35). The availability of this library could provide a useful resource for studying different factors that affect alternative splicing and/or the role of alternative splicing in coping with stress in a non-model organism.

Candidate genes may play a role in mediating cabbage looper caterpillar interaction with different host plant species

Digestion

Saliva is involved in digestion in almost every organism that produces it. Because caterpillars release their saliva through an extra-oral structure (spinneret), this could mean that digestion begins before ingestion. The presence of digestive enzymes in the saliva of insects has been widely studied in aphids and other sucking insects [47,117]. Digestive enzymes have also been identified in the salivary glands of a handful of chewing insects (*Helicoverpa armigera, Helicoverpa zea, Manduca sexta*) [35,36,66] but their role and differential expression has not been confirmed. We identified numerous transcripts regularly associated with digestion, including alpha-amylases, lipases, proteases, and glucosidases. Many of these transcripts were differentially expressed including serine proteases, carboxypeptidases, and lipases (Fig 3-3). Most of these were upregulated in the salivary glands of insects reared on tomato plants. Herde and Howe [95] observed upregulation of serine proteases and downregulation of lipases in the midgut when cabbage looper caterpillars fed on tomato. In the midgut, several digestive enzymes are secreted through vesicles[118,119]. This is probably also the case in the salivary glands. These compounds could be functioning to digest the plant before ingestion but they could also be

helping the already present digestive enzymes in the midgut thus allowing for a faster and more efficient digestion. A similar effect has been previously observed in the American cockroach (*Periplaneta americana*): the amylase present in the midgut is produced in the salivary glands and mixed with the food to aid in digestion occurring in the midgut [120]. The presence of digestive transcripts in the salivary glands of the cabbage looper provides more evidence that caterpillars could be depositing these molecules to aide in pre-ingestive or extra-oral digestion. This would be consistent with previous studies in predaceous arthropods [24], where predators will deliver saliva into the prey in order to aid in digestion prior to ingestion. Alternatively, the proteases may be involved in adaptation to dietary proteinase inhibitors [121,122] and not just in the breakdown of macromolecules.

Immunity

Host plants are known to affect an insect's immune response [123,124]. For example, in the autumnal moth (*Epirrita automnata*), differences in encapsulation rate were observed depending on food quality [125]. Because of this, we also screened for transcripts involved in immunity (i.e., attacin, cecropin, BGRP, gloverin, lysozymes). Almost 40% of these transcripts (4 out of 11) were differentially expressed (Fig 3-3). All of these were upregulated when insects were reared on plants compared to artificial diet.

Another possibility is that this differential expression could be due to the microorganisms present on the plant surface. The artificial diet contains antimicrobial compounds, which means it should contain fewer microbes. Differences in immunity related genes in the salivary glands of *Manduca sexta* feeding on different host plants has also been observed [66]. The authors of this study suggest that these differences were probably due to different microbial communities

associated with different host plants. However, this is yet to be tested. The induction of these immune genes in the saliva of insects feeding on plant provides more evidence that it is important to consider the microbiome associated with each plant, as well as the primary and secondary chemistry when studying plant-insect interactions.

Group	Gene	# present	DE
Digestion	Amylase	1	0
	Serine protease	26	5
	Cysteine protease	4	0
	Lipase	12	5
	Phospholipase	16	0
	Maltase	1	0
	Glucosidase	4	1
	Carboxypeptidase	16	3
Immunity	Attacin	1	1
	Gloverin	2	1
	Cecropin	1	1
	Lysozyme	2	2
	BGRP	2	0
	Phenoloxidase	2	0
Response	Aldehyde dehydrogenase	4	2
	Glutathione-S-transferase	14	5
	Glutathione peroxidase	2	0
	Esterase	15	4
	CYP450	19	8
	Thioredoxin	17	5
	Catalase	2	1
	Peroxiredoxin	3	0
	Peroxidase	5	3
	Superoxide dismutase	3	0
	Protease inhibitor	22	10
	Glycosyl transferase	10	5
	Laccase	3	2

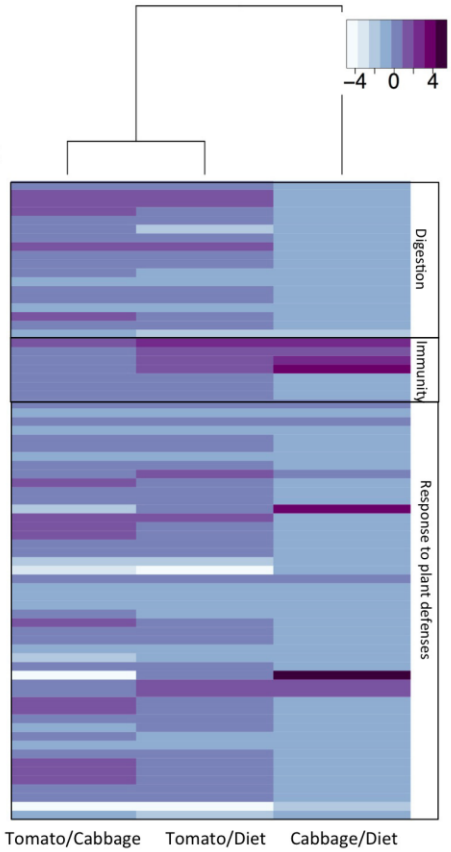

Figure 3-3: Genes of interest and their differential expression (DE). A. List of genes of interest identified in the transcriptome of cabbage looper (*Trichoplusia ni*) salivary glands. #present – indicates number of transcripts identified. DE indicates how many identified transcripts were differentially expressed. B. Heatmap of genes of interest in three comparisons – Tomato vs. Cabbage, Tomato vs. Artificial Diet, and Cabbage vs. Artificial Diet. Darker colors (purple) indicate upregulation in each comparison, light blue indicates no significant differential expression and white indicates downregulation. Only transcripts differentially expressed in at least one of the comparisons included.

Response to plant defenses

We also identified a several detoxification/antioxidant transcripts. These could be having an active role in the detoxification of plant secondary compounds and mitigating oxidative stress. Moreover, some of these proteins could be suppressing or manipulating the signals in the plant to avoid recognition. Hypothetically, the evolution of dietary generalism in insects is associated with the tradeoff of reduced ability to respond to the defensive compounds of different plant families [21]. However, plasticity in expression of the genes involved in detoxification of such compounds could allow a generalist insect to still feed on multiple chemically divergent plant species. Cabbage and tomato rely on different defensive strategies, utilizing different molecules to ward off herbivores. Cabbage, as part of the Brassicaceae family, contains glucosinolates and to some extent proteinase inhibitors. Glucosinolates are hydrolyzed into isothiocyanates, which are toxic and interact with amino groups of proteins and cleave disulfide bonds [126]. Proteinase inhibitors reduce the availability of sulphur-containing amino acids and cause the hyperproduction of trypsin [127]. In contrast, tomato contains high amounts of phenolics and proteinase inhibitors [126,128,129]. Phenolics affect the insect by forming quinones which react with nucleophilic side chains of amino acids making the amino acids unavailable [130]. Differential expression of genes potentially involved in the detoxification of these compounds was observed (Fig 3-4).

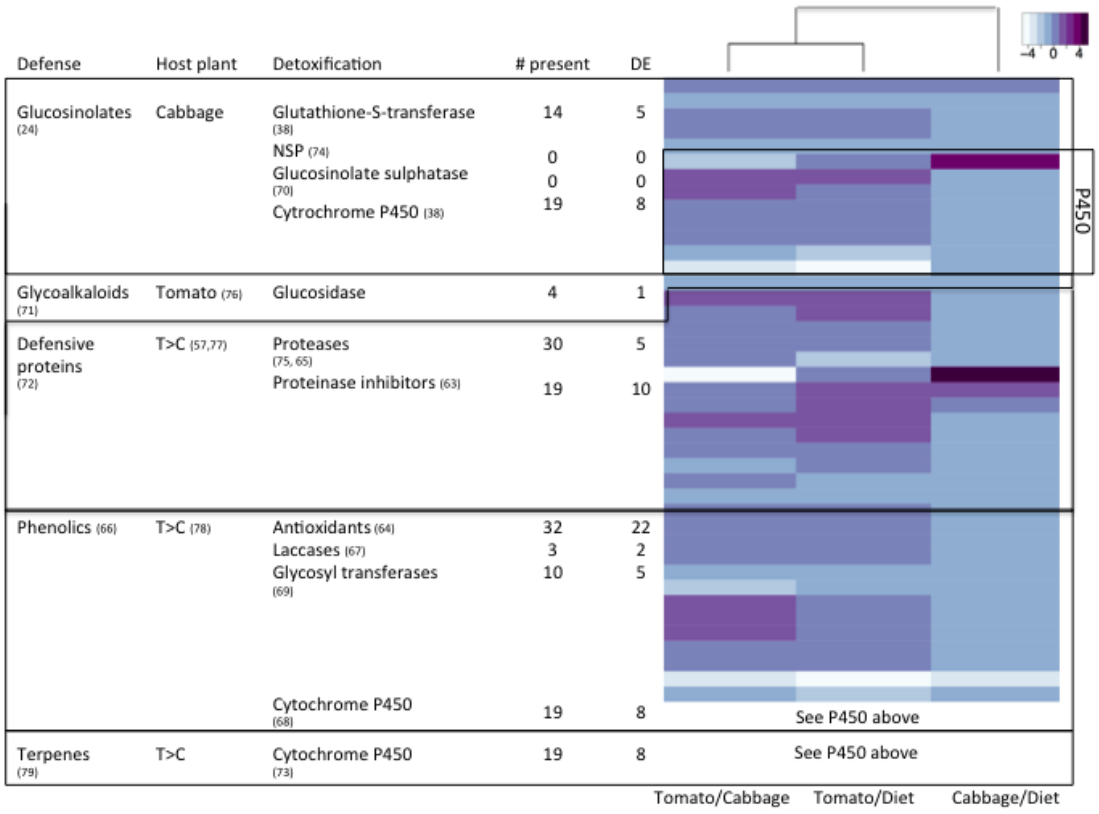

Figure 3-4: Differential expression (DE) of genes involved in the detoxification of cabbage and tomato specific defenses. A. List of defensive compounds associated with different hosts (cabbage and tomato) and insect-related detoxification genes. T>C means that these defenses are found in both host plants but higher in tomato than in cabbage. B. Heatmap of detoxification genes in three comparisons – Tomato vs. Cabbage, Tomato vs. Artificial Diet, and Cabbage vs. Artificial Diet. Darker colors (purple) indicate upregulation in each comparison, light blue indicates no significant differential expression and white indicates downregulation. Only transcripts differentially expressed in at least one of the comparisons were included.

Detoxification of plant defensive compounds is typically divided in two phases. In Phase I, toxic compounds are oxidized, hydrolyzed, or reduced; the resulting compounds are normally nonpolar and cannot be excreted directly. In Phase II the compounds from Phase I are conjugated with other compounds such as sugars, sulfate, phosphate, amino acids or glutathione, and then excreted [131]. Among the proteins involved in detoxification are cytochrome P450s, glutathione-S-transferases, glycosyl transferases, aldehyde reductases, esterases, and others. We

identified 82 genes involved in detoxification processes, with 42 of them being differentially expressed. Most of these were significantly differentially regulated in the tomato vs. artificial diet comparison, where 39 of the 42 differentially expressed genes were present (25 upregulated and 14 downregulated). Protease inhibitors, cytochrome P450s, thioredoxins and glutathione-S-transferases were the most prominent detoxification genes that were differentially expressed in response to feeding on plants.

As a defense mechanism against herbivores, some plants induce protease inhibitors that can affect digestibility in the insect, and also release proteases that can cause damage to the insect itself. For example, maize produces a cysteine protease that damages the peritrophic membrane of caterpillars [132]. A way in which insects could counteract this defense is by producing inhibitors that target plant proteases. In fact, the cabbage looper induces cysteine protease inhibitors to counteract cysteine proteases that might degrade their peritrophic membrane[69]. These results point towards an active role of caterpillar saliva in response to plant induced defenses.

Because insects have an open circulatory system, it is expected that all tissues will have a certain level of detoxification capability in order to overcome harmful compounds found in the hemolymph as well as to detoxify compounds produced during regular cellular metabolism. However, some of the compounds identified here as potentially being involved in response to plant defenses (i.e. proteases, protease inhibitors, carboxylesterases, and others) have been identified in the secreted saliva of *Helicoverpa zea* larvae and *Heliconius melpomene* adults [34,35,62]; thus, pointing towards a potential role in extra-oral detoxification. By releasing detoxifying enzymes prior to ingestion, the cabbage looper could both reduce the pressure of detoxification in the midgut and the exposure of the digestive tract to potential toxins.

Response of detoxification genes when feeding on wild type and mutant tomato

More differences in expression of detoxification genes were observed in the salivary glands of larvae reared on tomato (Fig. 3-3). To confirm the induction of detoxification genes in salivary glands, cabbage looper larvae were reared on artificial diet and then transferred to def1 mutant and wild type tomato. Def1 (Defenseless-1) tomato plants are mutants affected in the octadecanoid pathway and thus, upon herbivore attack, they do not induce wound-related defenses [106]. Comparing the salivary glands of caterpillars raised on wild type and def1 mutant plants, we measured the gene expression of six detoxification genes identified in the RNASeq data as upregulated in tomato versus cabbage and/or artificial diet. The analyzed genes were a catalase, a glutathione-S-transferase, a cytochrome P450, a protease, a protease inhibitor and a UDP-glycosyl-transferase (Fig 3-5). UDP-glycosyl-transferase was the only gene significantly downregulated in larvae feeding on def-1 mutants compared to wild type fed. However, there was a trend for higher expression in larvae feeding on wild type plants for the rest of the genes. Based on this result, we can confirm that detoxification genes are expressed in the salivary glands of cabbage looper, but the expression differences observed in larvae fed on tomato plants versus artificial diets may be due to constitutive differences or the specific defenses associated with the host plants rather than differences in the induced defenses regulated by the octadecanoid pathway. In other words, differences in expression of detoxification genes in the salivary glands will vary more depending on whether the insect encounters a specific type of defense rather than the level of said defense. Also, because higher gene expression does not always translate to higher enzymatic activity [133], future studies assessing changes in enzymatic activity of all these enzymes could provide further evidence of their role in response to plant defenses.

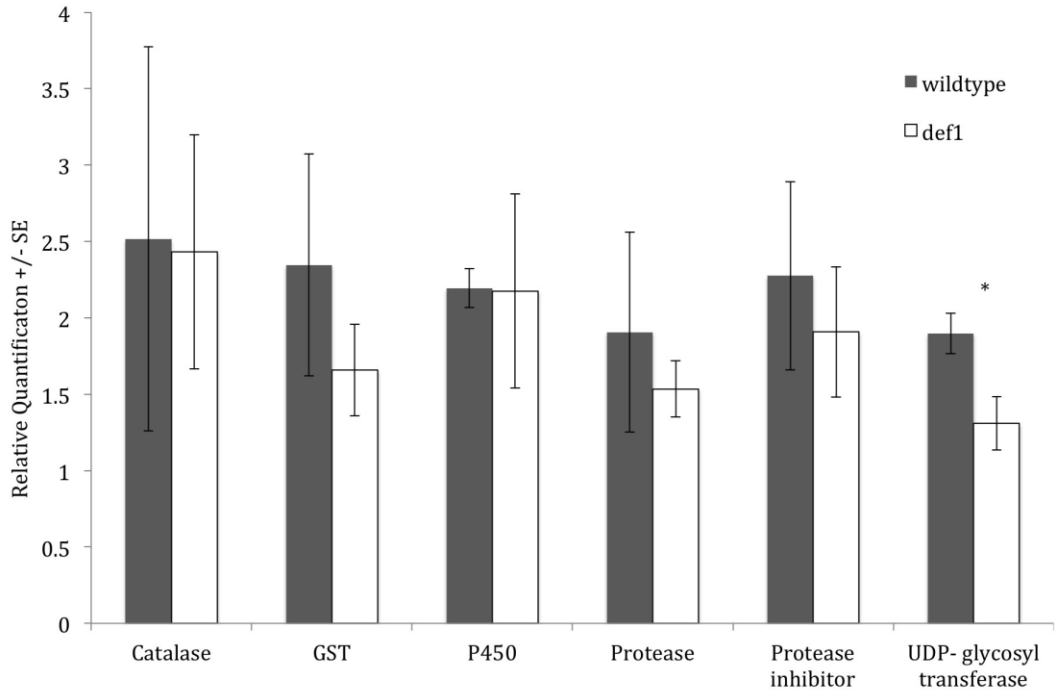

Figure 3-5: Gene expression of detoxification genes in salivary glands of cabbage looper feeding on wildtype and mutant (def-1) tomatoes. Salivary glands were dissected from 5th instar larvae fed on wildtype or mutant tomatoes and used for real time qPCR analyses. Grey and white bars indicate expression of detoxification genes on salivary glands of larvae fed on wildtype and mutant tomatoes, respectively. Bars indicate standard error. Differences were analyzed using the nonparametric test Mann-Whitney U test. * indicates significance at $p<0.05$.

Cabbage looper saliva is plastic and potentially aids in detoxification of plant defensive compounds

In conclusion, transcriptome of the salivary glands of the generalist *Trichoplusia ni*, is plastic. First, diet or host plant has a significant effect on the expression of salivary gland genes. Second, one of the significantly regulated pathways corresponded to the spliceosome pathway. Changes in this pathway may alter splicing patterns and generate diversity in protein activity and

function. Furthermore, we found evidence of expression of digestion and detoxification genes, as well as immune related genes that could be allowing *Trichoplusia ni* to respond not only to differences in host plant, but also to the microbiome associated with the host plants. The availability of this transcriptome library for the cabbage looper salivary glands opens the door to a myriad of research opportunities including using this information for functional studies (i.e. RNAi, CRISPR-Cas9) of specific salivary genes. Using these genomic tools will help elucidate the proximate and ultimate mechanisms that allow generalist insects to establish on a range of hosts.

Acknowledgements

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Chapter 4

Proteomic analysis of labial saliva of the generalist cabbage looper (*Trichoplusia ni*) and its role in interactions with host plants

Abstract

Insect saliva is one of the first secretions to come in contact with plants during feeding and it is potentially involved in host establishment. The composition and role of caterpillar saliva has not been as thoroughly studied as that of sucking insects. This study focuses on characterizing the proteome of the cabbage looper (*Trichoplusia ni*) using iTRAQ labeling and LC-MS/MS and its response in larvae reared on three different diets – cabbage, tomato, and an artificial pinto bean. We identified 254 proteins in the saliva out of which 95 were differentially expressed based on diet. A large percentage (56%) of the proteins identified function in protein metabolism, followed by proteins involved in vesicle transport (6%) and oxidoreductase activity (5%), among other categories. Several of the proteins identified are antioxidant proteins or reactive oxygen species scavengers including catalase, which was expressed significantly higher in the salivary glands of larvae fed on tomato. We further analyzed the gene expression and enzymatic activity of a catalase. We also applied commercial catalase on tomato and measured the activity of defensive proteins – trypsin proteinase inhibitor, polyphenol oxidase and peroxidase activities. Catalase suppressed the induction of trypsin proteinase inhibitor, but not the induction of polyphenol oxidase or peroxidase. These results significantly add to our understanding of phenotypic plasticity in saliva and its role in herbivore offense against plant defenses.

Introduction

Insect saliva is the first secretion to come in contact with the plant during feeding. Its characterization and role has been widely studied in sucking insects [27,59,60,117], but not so much in chewing insects such as caterpillars. Studying insect saliva can help us discover its potential role in host establishment and perhaps even develop tools to control insect pests. Caterpillars deposit copious amounts of saliva on plant wounds during feeding [37,44], which indicates that it might be involved in helping the insect deal with the challenges that come with feeding on plants. Some of those challenges include but are not limited to: suboptimal nutrition, plant defenses, and plant-associated microbes. Caterpillar saliva has digestive, immunity, detoxifying, and even herbivore offense properties [85]. In *Heliconius melpomene* adults, saliva has been shown to contain active proteases which help digest pollen before ingestion [62]. Also, saliva from *Helicoverpa zea* larvae has antimicrobial properties [31,134].

Herbivore offense refers to traits that allow herbivores to increase their feeding of a host plant [67]. An example of saliva's role in herbivore offense is the activity of glucose oxidase (GOX) in the saliva of *H. zea* [134,135]. In tobacco, salivary GOX suppresses the induction of nicotine. The mechanism of this suppression relies on the production of H_2O_2 by GOX during its reaction with glucose[12]. Hydrogen peroxide along with other reactive oxygen species (ROS; i.e. superoxide radical, peroxides, hydroxyl radicals, etc.) are involved in cell signaling and response to biotic and abiotic stresses including: defense against herbivores and pathogens, stomatal closure, plant development, plant hormone cross-talk, etc. [136–138]. Being able to manipulate the presence or absence of ROS in plant wounds can provide insects with a way to indirectly affect plant signaling and thus their ability to feed on a plant.

The objectives of this study were first, to characterize the proteome of saliva from a generalist caterpillar; second, measure host plant mediated proteomic changes in saliva composition; and finally, to study the antioxidant/ROS scavenging properties of caterpillar saliva. To study this, we used the system of the cabbage looper (*Trichoplusia ni*) feeding on two of its hosts – cabbage and tomato. The cabbage looper is a cosmopolitan pest and generalist that feeds on plants from more than 50 families [39]. Because of its generalist feeding habits, cabbage looper larvae may encounter a wide variety of plant primary and secondary compounds. Although, the cabbage looper will feed and complete its cycle on both cabbage and tomato, it prefers and grows better on cabbage than tomato. This difference in preference provides us with a system that includes an optimal and suboptimal host.

Also, because these plants belong to different families, they contain different defensive compounds. For example, tomato relies on phenolic compounds, glycoalkaloids, and defensive proteins such as proteinase inhibitors, polyphenol oxidase, peroxidase, arginase, and threonine deaminase among others [42,130]; whereas cabbage relies mainly on glucosinolates and to a lesser extent on proteinase inhibitors [40,94]. Because of this, we hypothesize that the caterpillars will require a different set of enzymes to deal with these compounds and require a certain level of plasticity. This system provides a good opportunity to measure host plant related salivary changes. Plasticity at the transcriptome level has been identified in the midgut [95] and salivary glands (Chapter 3); however, there is no evidence of whether these changes also occur in the proteome. This study provides evidence of phenotypic plasticity in the labial salivary proteome of the cabbage looper as well as evidence of the role of antioxidant enzymes in the interactions between cabbage looper caterpillars and its hosts.

Materials and Methods

Plants and Insects

Cabbage looper (*Trichoplusia ni*) eggs were purchased from BioServ Inc (Flemington, NJ) and reared on two host plants: tomato (*Solanum lycopersicon* var. Better Boy) and cabbage (*Brassica oleracea* var capitata 'Platinum Dynasty'), and pinto-bean artificial diet [96]. Colonies were kept at 23ºC in 16:8 Light:Dark conditions. Tomato (var. Better Boy) and cabbage (var. Platinum Dynasty) plants were grown in the greenhouse under a 16:8 L:D cycles and fertilized as needed. Four to six week old plants were used to feed larvae.

Protein extraction and sequencing

Fifth instar larvae, allowed to feed on each of the diets for 1 day after molt, were collected from all three treatments. Labial salivary glands were dissected and placed in Ringer's solution (123 mM NaCl, 1.5 mM $CaCl_2$, 5 mM KCl) for two hours to allow proteins to be released into solution[36]. Each sample included at least ten pairs of salivary glands. All samples were collected early afternoon to avoid differences due to the circadian rhythm. Three biological replicates (10 pairs per sample) of insects fed on tomato and cabbage and two replicates of insects fed on artificial diet (8 total) were sent for processing to the Proteomics and Mass Spectrometry Core at Penn State University – Hershey using iTRAQ® (8-plex) proteomics technology. MS spectra were taken from 8 SCX fractions, using a 120 minute gradient from an Eksigent NanoLC-Ultra-2D Plus and Eksigent cHiPLC Nanoflex (AB Sciex; Redwood City, CA) through a 200 µm x 0.5 mm Chrom XP C18-CL 3 µm 120 Å Trap Column and elution through a 75 µm x 15 cm Chrom XP C18-CL 3 µm 120 Å Nano cHIPLC Column. An ABSciex 5600 TripleTOF was used

with the following settings: Parent scan acquired for 250 msec, then up to 50 MS/MS spectra acquired over 2.5 seconds for a total cycle time of 2.8 seconds.

Proteome analysis

Data was analyzed using the Paragon algorithm build 4.5.0.0 contained in the ProteinPilot 4.5 program Build 1654. Local False Discovery Rate (FDR) estimation for the identifications of proteins was based on simultaneously searching a concatenated decoy database which is the exact reverse of each protein sequence in the samples plus 536 common human and lab contaminants, and protein IDs with an estimated Local False Discovery Rate <5% were considered confidently identified. Quantitative differences among treatments were determined using the WHATraq analysis [139] with Benjamini-Hochberg multiple-testing correction. Sample quantitative differences which still showed $p<0.05$ after the multiple testing adjustment were considered significant.

Catalase activity in salivary glands

Salivary glands were dissected from actively feeding fifth instar larvae feeding on cabbage, tomato, or artificial diet. Three pairs of glands were pooled per sample, flash frozen in liquid nitrogen and stored at -80^{o}C until further processing. Frozen glands were homogenized with a pestle in 150 µl of chilled 0.1M phosphate buffer pH 7.0. To determine catalase activity, H_2O_2 breakdown was measured in a 96-well plate at 240 nm ($E=36\ M^{-1}cm^{-1}$)[140]. Each reaction contained 5 µl of salivary gland homogenate plus 200 µl of 1mM H_2O_2. Activity was expressed as µmol/min/mg of protein.

Real time quantitative PCR

Real time quantitative PCR (RTqPCR) was used to measure catalase gene expression in the salivary glands of cabbage looper larvae feeding on artificial diet, cabbage and tomato. Salivary glands from actively feeding fifth instar larvae were dissected and RNA was extracted using Trizol reagent using standard protocol. One μg of RNA was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription kit following manufacturer's protocol (Applied Biosystems, Inc; Grand Island, NY). Primers used were TnCat1F and TnCat1R (Appendix A). All reactions were conducted using Power SYBR Green PCR Master Mix and ran on a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc; Grand Island, NY). Relative expression for each gene was quantified using the ΔΔCt method [105], and normalized using GAPDH.

Effect of saliva on tomato foliar peroxidase activity

To measure the effect of saliva on the activity of peroxidase, a defense in tomato [141], we measured its activity in the presence of salivary gland homogenate. Salivary glands from 5th instar larvae were dissected. Three pairs were pooled per sample and homogenized in 150 μl of chilled 0.1M phosphate buffer pH7.0. Peroxidase activity was measured as described below (section 2.8) with each reaction containing 2 μl of salivary gland homogenate. Controls contained 2.0 μl of buffer in place of gland homogenate.

Effect of catalase on induction of plant defenses

To measure the effect of catalase on plant defenses, we applied 20 μl of catalase (2.5 μg/μl) to wounded tomato plants. The fourth fully expanded leaf from tomato plants were

wounded using a serrated wounding tool. Ten plants were used per treatment. Tissue samples (50 mg) were collected 24 h after wounding to measure trypsin protease inhibitor (TPI) and 48 h after wounding to measure polyphenol oxidase (PPO) and peroxidase (POX) activity. Samples were flash frozen in liquid nitrogen and stored at -80^{o}C until further processing.

Plant defense measurements

Tomato leaf samples were ground in liquid nitrogen, extracted with 1.25 mL of 0.1 M potassium phosphate pH 7.0 with 5% insoluble polyvinyl polypyrrolidone (PVP) for PPO and POX. Five microliters of the extraction supernatant were mixed with 200 µl of 3 mM caffeic acid to measure PPO activity or 190 µl of 3 mM guaiacol plus 10 µl of 3% H_2O_2 for POX. Increase in absorbance was measured at 450 nm and expressed as mOD/min/mg tissue [142]. For TPI activity, extractions were done using 1.25 mL of 0.046 M Tris HCl buffer pH 8.1, 0.0115 M $CaCl_2$ with 5% PVP. For each reaction, 10 µl of leaf extract was mixed with 10 µl of 1mM HCl with 0.4 µg of trypsin and 80 µl of Tris buffer, mixed well and incubated at room temperature for 10 min. Next, 100 µl of 2 mM p-toluene-sulfonyl-L-arginine methyl ester (TAME) were added to each well and measured at 247 nm [143] for 5 min. Trypsin PI activity was calculated as PI (%)=[1-A/C] x 100, where A represents trypsin activity of the sample and C represents the maximum value of tryspin activity in control plants [144].

Statistics

Graphs and statistical analyses were done using GraphPad Prism version 7 for Mac, GraphPad software, La Jolla, CA. Difference among treatments for enzymatic activity assays were analyzed using ANOVA followed by a separation of means using Tukey posthoc test at

$p<0.05$. For statistics of proteome analysis refer to proteome section described above. Trypsin proteinase inhibitor values, which are expressed as percentages were transformed using arcsin(square root) transformation before analyses.

Results and Discussion

Cabbage looper saliva proteome varies with host plant

Salivary glands of 5th instar cabbage looper larvae were dissected and placed in Ringer's solution in order to allow protein secretion into solution. A total of 315119 MS/MS spectra were taken, out of which 11% were identified at a 95% confidence using the NCBInr Ref Seq Lepidoptera database. The total number of proteins searched in database plus contaminants plus concatenated reverse decoy were 33967. ProteinPilot 4.5 identified 434 proteins with confidence >95% (Paragon Algorithm; Appendix E), while orthogonal local FDR estimation [145] yielded 254 protein IDs (Appendix E) with an estimated local FDR <5%. The software WHATraq [139] was used to quantify protein differences among samples. Only the 254 highly confident proteins were considered for further analyses and comparisons.

The majority (56%) of highly confident proteins identified are involved in protein metabolism (i.e. ribosomal proteins, translation elongation factors, etc.). Other categories include vesicle transport, oxidoreductase activity, structural, energy metabolism, heat shock proteins, proteases, etc. (Figure 4-1). The proteomes of larvae reared on three diets – cabbage, tomato, and pinto bean artificial diet – were compared. Out of the 254 proteins, 63 were significantly differentially expressed ($p<0.05$) in at least one of the comparisons (Appendix E). When comparing tomato vs. cabbage, 42 proteins were differentially expressed with 35 upregulated in

tomato. In the tomato vs. artificial diet, 32 proteins were differentially expressed with 30 of them downregulated in tomato. Finally in the cabbage vs. artificial diet comparisons, 22 proteins were differentially expressed, with 6 upregulated in cabbage (Figure 4-2a). Thus indicating diet specific changes in the salivary proteome.

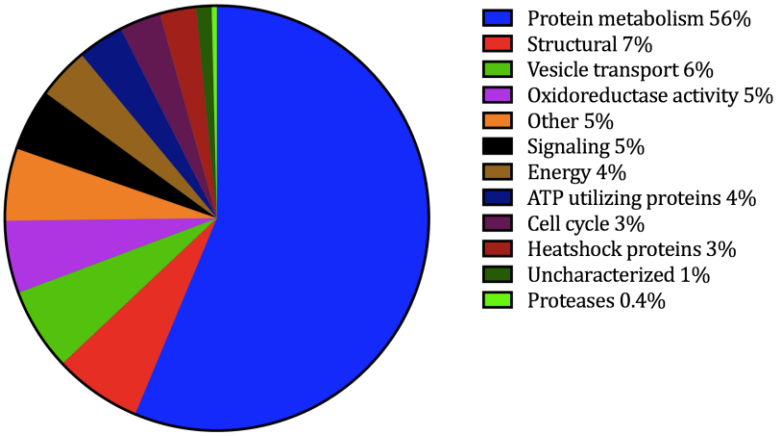

Figure 4-1: Gene ontology of proteins identified in the proteome of the cabbage looper (*Trichoplusia ni*) saliva.

Proteins of interest in the salivary glands of *T. ni*

To date, the saliva of at least 5 noctuid caterpillars has been characterized. These include *Helicoverpa zea*, *H. armigera*, *Spodoptera frugiperda*, *S. exigua*, and now *Trichoplusia ni*. The proteome of *T. ni* is the largest one characterized with 254 proteins followed by *S. exigua* with 131 proteins from homogenized glands and *S. frugiperda* with 98 proteins. The latter used direct collection of saliva from the spinneret, which might explain the lower number, however, this is still higher than 33 proteins identified from *H. zea*, which also used direct collection. Though the collection method of proteins from *H. armigera* was similar to the one of *T. ni*, only 23 proteins

were identified. Both *T. ni* and *S. frugiperda* used iTraq labeling and LC-MS/MS sequencing. This methodology seems to generate the highest number of protein identification.

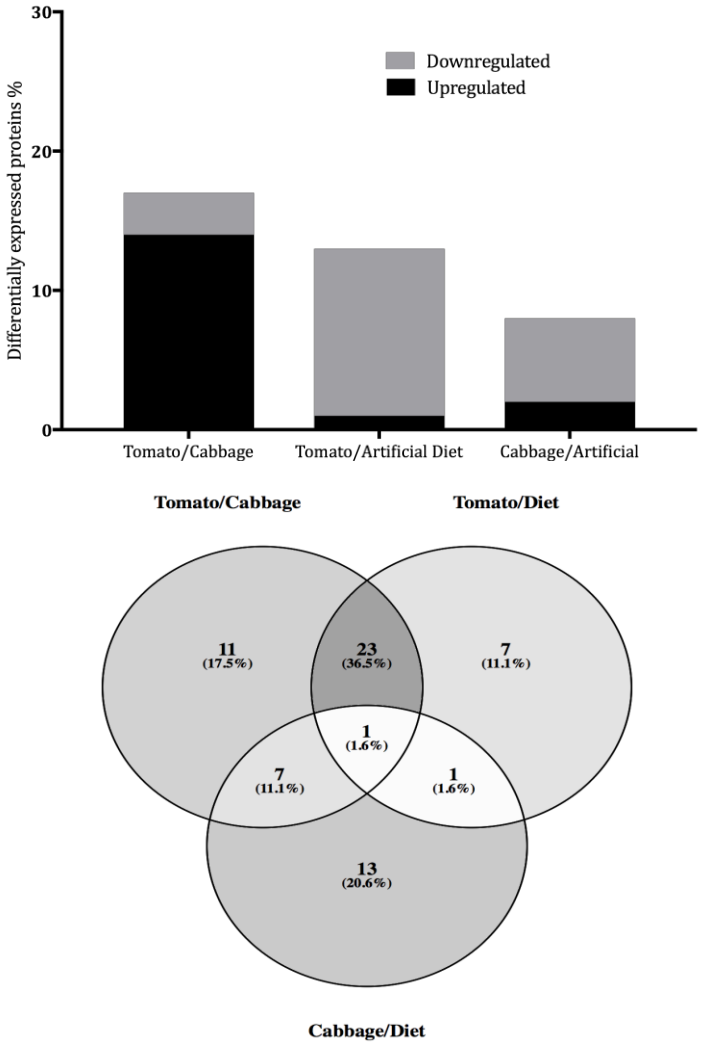

Figure 4-2: **A.** Differentially expressed proteins in the proteome of the cabbage looper saliva for larvae fed on three different diets – cabbage, tomato, and pinto bean artificial diet. Black indicates proteins upregulated in the comparison and grey indicates downregulated proteins. **B.** Venn diagram of proteins differentially expressed for each comparison.

Protein metabolism

A high number of proteins that participate in protein metabolism were identified. These include translation elongation factors, ribosomal proteins, chaperonins, among others. Salivary glands are exocrine glands, which are characterized by a high number of rough endoplasmic reticulum. This organelle contains a high amount of ribosomes, which could explain the abundance of ribosomal proteins in the proteome. These proteins have also been identified in the proteomes of the salivary glands of other Lepidoptera [36,65]. In the comparison of proteomes from larvae reared on tomato vs. cabbage, approximately 62% of the differentially expressed proteins are involved in protein metabolism (Appendix E). Also, the majority of the shared proteins between tomato vs. artificial diet and tomato vs. cabbage comparisons were in this category (65%). This could be in part because the majority of the proteins identified belong to this category, thus creating a bias. However, when comparing cabbage vs. artificial diet, the differentially expressed proteins are across all categories. This indicates that feeding on tomato – a lower quality host – has an effect on protein metabolism in the salivary glands. A similar observation was made at the transcriptome level (Chapter 3), where feeding on tomato had a significant effect in the spliceosome pathway (also involved in protein metabolism)[146].

As previously mentioned, cabbage looper larvae do not grow as fast on tomato compared to cabbage. This difference is mainly attributed to the difference in plant defenses between these hosts. However, we must not disregard the possibility that these changes are due to nutritional differences. For example, in a study by Herde and Howe [95], *T. ni* that grew on mutant Arabidopsis (no glucosinolates), still grew better than larvae reared on a mutant tomato (knockout of JA pathway) indicating that other factors outside of secondary metabolism are also important.

Amino acids respond to stress and modulate translation initiation and elongation factors and thus regulate translation in a global scale causing preferential changes in mRNAs encoding particular proteins [147,148]. The changes in protein metabolism observed in the cabbage looper salivary glands might be a response to differences in amino acid content between the different diets. Changes in amino acid content in the insect could also be the direct effect of plant defenses on amino acid degradation. For example, jasmonic-induced proteins from tomato have been shown to degrade arginine and threonine in the midgut of *Manduca sexta* larvae [149]. This could be a feedback mechanism in the digestive pathway modulated by amino acid changes that indicates the need for a different suite of enzymes in the saliva.

The implications of this phenomenon are still not clear, nor is it clear whether this is an advantage for the plant or the insect. The effect that plant nutrition and/or defenses may have in the transcription and translational machinery itself in an insect hasn't been thoroughly studied. However, the availability of new and cost-effective high-throughput technologies like RNASeq and iTraq will provide the tools to begin exploring this concept.

Vesicle transport

Much of the secretion in exocrine glands such as the salivary glands occurs through exocytosis. Structures normally associated with exocytosis include – rough endoplasmic reticulum, Golgi complex and secretory vesicles. Many proteins involved in transport, including vesicle transport were identified in the proteome of cabbage looper salivary glands. Vesicle transport is a major cellular activity responsible for molecular traffic [150]. The number of significantly different proteins in this category was similar across comparisons. Two of these were significantly downregulated in tomato vs. cabbage comparison, one was upregulated in

tomato vs. diet and 2 were downregulated and 1 upregulated in the cabbage vs. diet (Appendix E). The protein that was significantly different across all comparisons belongs to this category – sex specific storage protein precursor. This protein is involved in storage of amino acids.

Secretion of specific enzymes has not been well-studied extensively in salivary glands. For example, most of what we know about secretion of digestive enzymes has been done in midgut cells. In *Tenebrio molitor*, amylase and trypsin in the midgut are found inside vesicles [119]. Salivary glands are hypothesized to also be involved in digestion, so it is possible that many of these enzymes would also be secreted in a similar way in the salivary glands. Another reason to believe vesicles are important for secretion in salivary glands is that many of the proteins identified in saliva do not have a signal peptide (e.g. glucose oxidase), so these proteins are potentially being released through vesicles. No clear patterns were observed with respect to differential expression of vesicle transport depending on host plants. However, the abundance of transport proteins still indicates a high activity in the salivary glands both in protein metabolism and secretion.

ROS scavengers

Cabbage looper saliva appears to have antioxidant properties. Several reactive oxygen species (ROS) scavengers were identified in the proteome of the cabbage looper saliva, including catalase, thiol peroxiredoxin, thioredoxin, thioredoxin reductase, glutathione peroxidase, among others. Some of these enzymes have been previously identified in the saliva of *Bombyx mori* [151], *Spodoptera exigua* [65] and *Vanessa cardui* [57]. In fact, unlike other Noctuids whose saliva has been characterized, no glucose oxidase was identified in the saliva of cabbage looper. This confirms the very low activity previously reported [23]. No clear correlation between

glucose oxidase activity and phylogeny has been observed. A correlation between host range and oxidative activity was previously hypothesized; however, cabbage looper – a known generalist – does not seem to fit this hypothesis. Unlike other Noctuids with high glucose oxidase that feed mainly on fruit, the cabbage looper is mainly a leaf feeder. Other Noctuid generalists with low glucose oxidase activity include *Agrotis gladiaria, Agrotis ipsilon,* and *Amphipyra pyramidoides*, all of which are defoliators [23]. It would be interesting to determine whether the saliva of these other species is also high in antioxidants.

ROS are chemically reactive species containing oxygen with several functions in plant including as direct defense [152] against herbivores as well as defense signaling [68,153,154]. For example, in tomato, H_2O_2 acts as a secondary messenger for the induction of plant defenses [155]. Also, an oxidative response occurs in plants during herbivory [156,157] including the increase of oxidative enzymes such as peroxidases as well as increase of ROS. A reduction in plant antioxidants has also been observed, thus, the presence of antioxidants and ROS scavengers in the saliva of cabbage looper could be a response to these plant defense mechanisms. We further tested this hypothesis by studying the effect of catalase on plant defenses.

Catalase activity in the cabbage looper salivary glands

A catalase was identified in the proteome of the cabbage looper. Catalases scavenge hydrogen peroxide through the reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

To confirm its presence, gene expression and enzymatic activity were measured in the salivary glands of larvae feeding on cabbage, tomato and artificial diet. Gene expression (Figure 4-3a) and enzymatic activity were highest in larvae feeding on tomato (Figure 4-3b). Although

higher expression of a gene does not always equal higher enzymatic activity, in this case we did find a correlation between these. Among the many defenses in tomato against biotic stress, is the induction of polyphenol oxidase and peroxidases [142], which can cause a high oxidation stress on insect's tissues. Thus, a high activity of an antioxidant like catalase can be of importance to counteract such defenses.

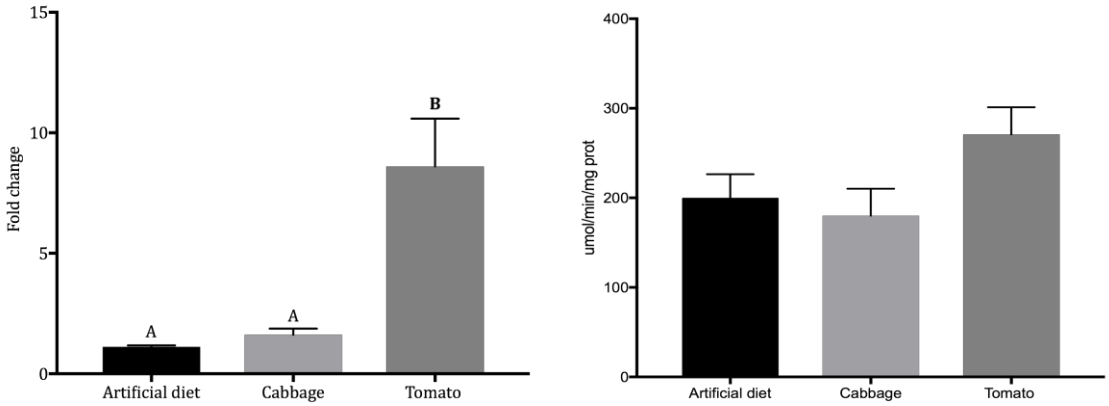

Figure 4-3: Gene expression (**A**) and enzymatic activity (**B**) of a catalase from the saliva of the cabbage looper (*Trichoplusia ni*) feeding on three diets – cabbage, tomato, and pinto bean artificial diet (Mean+SE). An ANOVA was done to determine statistical differences followed for difference of means posthoc Tukey test at $p<0.05$. For A: $F_{(2,9)}=13.12$, pvalue=0.022; B: $F_{(2,27)}=2.718$, pvalue=0.0840. Different letters indicate statistically different treatments.

Saliva proteins reduce activity of plant peroxidase

Tomato peroxidase activity was measured in the presence of cabbage looper homogenized salivary glands. We observed a decrease in peroxidase activity in the presence of homogenized salivary glands (Figure 4-4). Among the hypotheses about the role of saliva in chewing insects is its role in detoxification either extra-orally or by reingestion and thus add to the repertoire of detoxification enzymes already present in the midgut [85]. In tomato, peroxidase is an induced defense against chewing insects [142]. Peroxidase requires the presence of H_2O_2 for its reaction, thus we hypothesize that in the presence of ROS scavengers such as catalase,

glutathione peroxidase and peroxiredoxins, the availability to H_2O_2 would be limited thus affecting its activity. We were able to confirm this concept. A reduction in tomato peroxidase activity has also been observed in the presence of catalase from the regurgitant of *Helicoverpa zea* [141]. The idea that enzymes produced in the salivary glands can aid in reactions occurring in the midgut has been previously documented in cockroaches (*Periplaneta americana*) where the amylase present in the midgut and involved in digestion is produced in their salivary glands [120]. Also, by releasing catalase unto the wound, H_2O_2 can begin to be scavenged even before it is ingested thus reducing the activity of peroxidase early in the interaction and consequently reducing the stress in the insect midgut.

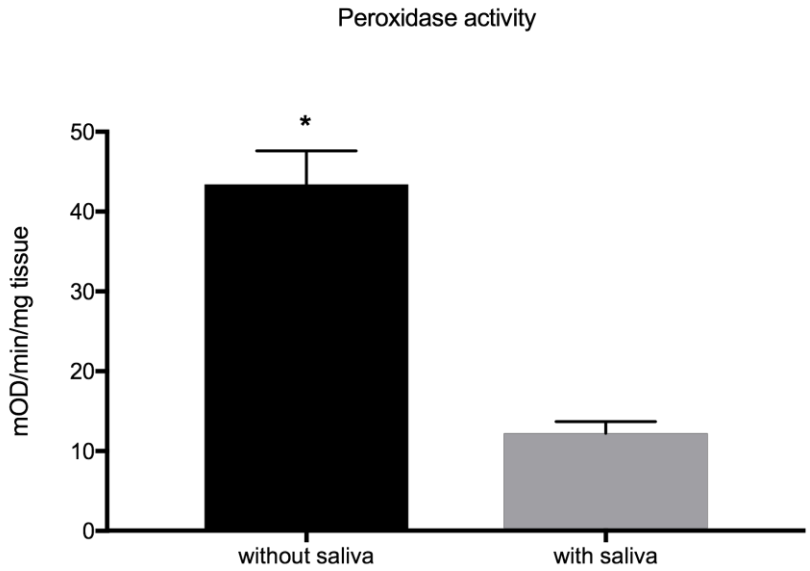

Figure 4-4: Tomato foliar peroxidase activity (Mean+SE) in the presence of cabbage looper saliva. Paired t-test was done to determine statistical difference. $t_{(5)}$=8.128, pvalue=0.0005. *indicates statistical difference between treatment

Catalase suppresses trypsin proteinase inhibitor induction in tomato

Because H_2O_2 is also involved in signaling, we applied purified catalase to wounded tomato plants to test whether application of catalase affects induction of plant defenses. The

treatments included control, wounded plus buffer, and wounded plus catalase. We measured TPI, PPO and POX in tomato. We observed that mechanical wounding induced TPI but in the presence of catalase, TPI was suppressed and not different from control plants (Figure 4-5a). Mechanical wounding also induced PPO and POX activity, but catalase did not seem to have an effect and plants treated with it were not different from wounding alone (Figure 4-5b,c). This is also an example of insect saliva involved in herbivore offense. By reducing the levels of H_2O_2 – a secondary messenger in tomato – through the deposition of ROS scavengers, induction of defenses such as TPI are suppressed and thus reduce the stress in the larvae.

Conclusions

In conclusion, a proteome for the salivary proteins of the cabbage looper is now available. We observed differences in the quantity of proteins depending on host plants. Also, we identified proteins potentially involved in response to plant defenses, in particular, ROS scavengers such as catalase. Catalase was observed to have differential expression and activity when larvae fed on different diets. Also, catalase appears to play a role in both detoxification and herbivore offense in the cabbage looper. The availability of this proteome library opens the door to future functional and ecological studies that could potentially increase our knowledge about the role of caterpillar saliva in plant-insect interactions.

Acknowledgements

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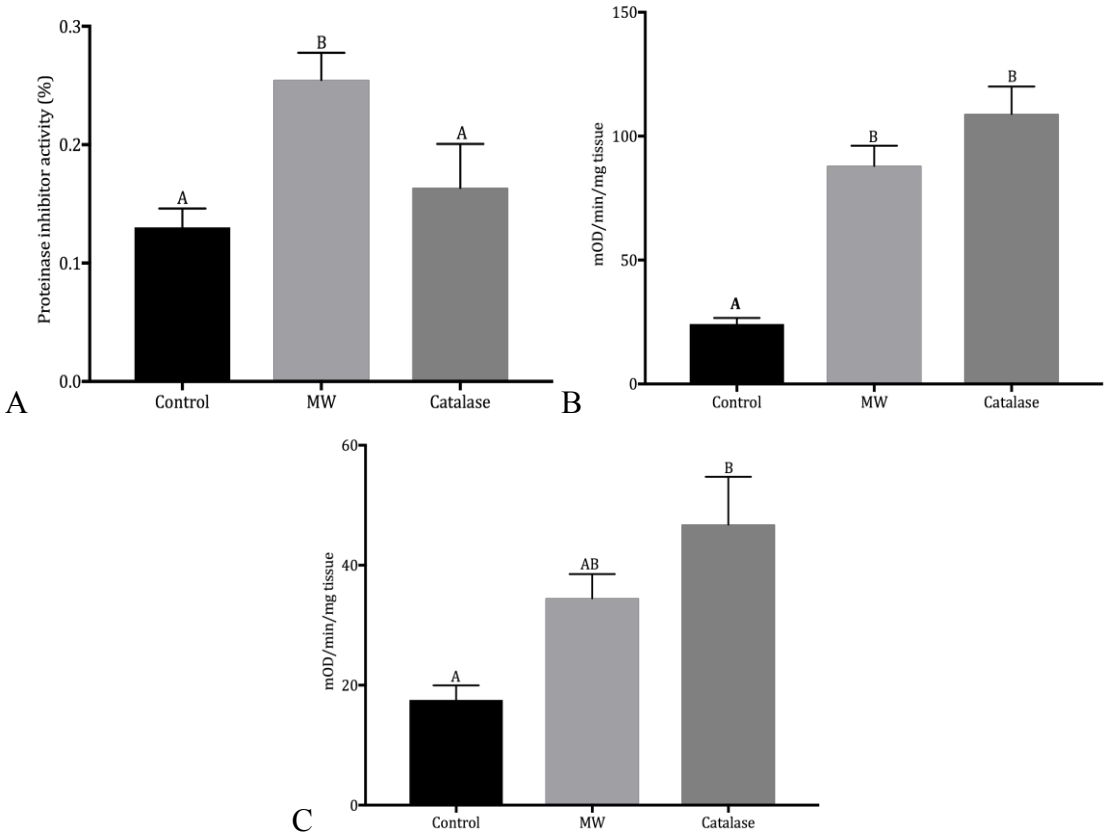

Figure 4-5: Trypsin proteinase inhibitor activity in tomato (A), peroxidase (B) and polyphenol oxidase (C) activity in tomato in the presence of exogenous catalase (Mean+SE). Control are unwounded plants, MW are plants mechanically wounded plus buffer and Catalase are plants mechanically wounded plus catalase. An ANOVA was done to determine statistical differences followed for difference of means posthoc Tukey test at $p<0.05$. For A: $F_{(2,26)}=4.649$, pvalue=0.0188; B: $F_{(2,25)}=33.18$, pvalue<0.0001; C:$F_{(2,26)}=7.546$, pvalue=0.0026. Different letters indicate statistically different treatments.

Chapter 5

Molecular characterization of glucosidases in the cabbage looper (*Trichoplusia ni*)

Abstract

Plants rely on a complex chemical network to defend against herbivores. Insects, on the other hand, have had to develop ways to overcome these defenses. A well-studied plant defense is the mustard-oil bomb, or glucosinolate-myrosinase complex. Plants in the order Brassicales contain glucosinolates, which when hydrolyzed by myrosinases become isothiocyanates. These compounds have deleterious effects on insects. Insects have overcome the mustard-oil bomb by either developing efficient detoxification mechanisms or by having enzymes, which convert glucosinolates into less harmful compounds such as epithionitriles, nitriles, etc. Recently, myrosinases have been confirmed in at least two specialist insects of the Brassicaceae family – *Phyllotreta striolata* and *Brevicoryne brassicae*. Here, we report a third insect species where myrosinase has been identified. Three genes, putatively myrosinases, were identified both at the transcriptome and proteome level in the salivary glands of the generalist *Trichoplusia ni* (cabbage looper). Gene expression of myrosinase was quantified in different tissues of the cabbage looper – salivary glands, fatbody, midgut and Malpighian tubules. We also quantified the expression of these genes on larvae fed on two host plants - cabbage and tomato. Substrate specificity in salivary glands was measured using spectrophotometry. Finally, we test the probability of cabbage looper sequestering intact glucosinolates in their hemolymph. These genes appear to be broad-spectrum glucosidases; however, further research is necessary before this can be confirmed.

Introduction

Plants from the Brassicaceae family contain glucosinolates, which are amino-acid derived compounds that contain a sulphate and thioglucose [158]. There are approximately 100 known glucosinolates, which can be classified as aliphatic, aromatic or indole, depending on the amino acid they are derived from. Glucosinolates and their products have a wide range of functions varying from defense to signaling [41,94,159]. In the presence of enzymes known as myrosinases (glycoside hydrolase family), glucosinolates are hydrolyzed into isothiocyanates, nitriles, thiocyanates, epithionitriles, etc. [160]. Isothiocyanates are the most widely studied byproducts of glucosinolates in part because of their highly reactive nature and role in defense. Glucosinolates are part of the two-component plant defense system. To avoid damage to plant tissues, glucosinolates and myrosinases are compartmentalized in the plant. When plants are ruptured, glucosinolates and myrosinases come into contact and release the different hydrolyzed compounds. This is commonly known as the mustard-oil bomb.

Insects that feed on Brassicas have adapted different ways to overcome negative effects from glucosinolate hydrolysis products. One of the most common ways is through detoxification. Several generalist insects detoxify isothiocyanates by conjugating them to glutathione. The conjugation may occur spontaneously in the midgut or catalyzed by glutathione-S-transferases (GST). GST genes and other detoxifying related genes such as UDP-glycosyltransferases and cytochrome P450s upregulate in the midgut of *Trichoplusia ni* [95]. More specialized strategies have also been identified. Insects from the Pieridae family contain nitrile-specifier proteins, which redirect glucosinolate breakdown products to nitriles, which are less toxic than isothiocyanates [161]. Another example of a specialized strategy is found in the insect species *Plutella xylostella* [162], *Athalia rosae* [163], *Schistocerca gregaria* [164], and *Bemisia tabaci*

[165]. These insects contain a sulphatase (GSS), which disarms the glucosinolate-myrosinase by converting glucosinolates to its desulphated derivative and thus not allowing the hydrolysis by myrosinases.

Brassica specialists have become so adapted to glucosinolates that in many cases these are necessary feeding and oviposition cues [166]. Several specialists also sequester glucosinolates probably as a form of defense against natural enemies. Some insects that have been confirmed to sequester glucosinolates include *A. rosae* [167], *Brevicoryne brassicae* [168], *Murgantia histrionica* [169], and *Phyllotreta striolata* [170]. Finally, some of these specialists are a mustard bomb themselves by containing their own myrosinases. We identified three transcripts and one protein as myrosinases in the transcriptome and proteome of the labial glands of the generalist cabbage looper (*Trichoplusia ni*). Myrosinases, also known as thioglucoside glucohydrolases, are part of the glycosidase family. They possess several properties as glycosidases; however, myrosinases are capable of cleaving the thioglucose found in glucosinolates. Our objective was to characterize these transcripts and determine whether these are indeed myrosinases or if they are broader glucosidases.

Materials and Methods

Plants and insects

Cabbage looper (*Trichoplusia ni*) eggs were purchased from BioServ Inc (Flemington, NJ). Larvae were reared entirely on two host plants: tomato (*Solanum lycopersicum* var. Better Boy) and cabbage (*Brassica oleracea* var capitata 'Platinum Dynasty'). Colonies were kept at

23˚C in 16:8 Light:Dark conditions. Tomato and cabbage plants were grown in the greenhouse under a 16:8 L:D cycles and fertilized as needed.

Full length sequence for three myrosinases

Three sequences with homology to myrosinases were identified in the salivary gland transcriptome of the cabbage looper. Sequences were named MyrA, MyrB and MyrC. Both MyrA and MyrB were full length. MyrC was missing the 3'end. The 3' end gene sequence for MyrC was obtained via 3' Rapid Amplification of cDNA Ends (RACE) using SMARTer RACE 5'/3' kit (CLONTECH, Mountain View, CA) following manufacturer's protocol using gene specific primers (Appendix A) and oligo dT. PCR fragments were gel cleaned using EZNA Omega Gel cleanup kit (Omega, Norcross, GA) and cloned into PGEMT easy vector following standard protocol (Promega, Madison, WI). Samples were sent for sequencing to Penn State Genomics Core facility. Identification was done using homology based identification using BlastX [171]. A multiple alignment of the derived amino acid sequence to other confirmed insect myrosinases (*Brevicoryne brassicae* and *Phyllotreta striolata*) was done using T-Coffee [171]. Identification of potential signal peptides was done using SignalP 4.0 [171].

Changes in gene expression due to host plants

Larvae were reared on cabbage and tomato. Labial glands, midgut, and Malpighian tubules were dissected from fifth instar larvae. To measure gene expression changes due to temporal diet changes, larvae were reared on pinto bean artificial diet [172] until fifth instar. Next, they were starved overnight and later switched to tomato and cabbage. Samples were collected 6 and 12 hours after switch. We also included a treatment in which larvae were placed

on one host for 6 h and then switched to the other for 6 more hours before dissection. All samples were dissected on chilled PBS, flash frozen on liquid nitrogen and stored at -80ºC until further processing.

Gene expression

Real time quantitative PCR (RTqPCR) was used to measure gene expression. RNA was extracted using Trizol reagent using standard protocol. One µg of RNA was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription kit following manufacturer's protocol (Applied Biosystems, Inc; Grand Island, NY). Primers can be found in Table S2. All reactions were conducted using Power SYBR Green PCR Master Mix and ran on a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc; Grand Island, NY). Relative expression for each gene was quantified using the ΔΔCt method [105], and normalized using GAPDH. Standard curves using serial dilutions and melting curves were performed to calculate primer efficiency ($E=10^{\wedge(-1/slope)}-1$) and confirm the presence of single amplicon.

Substrate specificity

Labial glands from 5th instar larvae were dissected. Three pairs were pooled per sample. Glands were then homogenized in 170 µl of citrate buffer pH 6.5. Next, 25 µl of homogenized glands and 25 µl of 5 mM substrate (sinigrin –a glucosinolate, or tomatine –a steroidal glycoalkaloid) were mixed and set at room temperature [170]. Glucose was measured after 2 hours. To measure glucose, 100 µl of assay mix [in 5 mL of buffer, 50 U of glucose oxidase, 12.5 ul of peroxidase (1mg/mL) and 75 ul of O-dianisidine (40 mM)] was added and set at 37ºC for 30

min. Then, 100 µl of 6N HCl was added and measured in a spectrophotometer at 540 nm. A standard curve was done using glucose [173].

Sequestration

To measure whether cabbage looper sequesters glucosinolates from diet, larvae were fed cabbage, pinto bean artificial diet and pinto bean artificial diet with sinigrin. Next, hemolymph, labial glands, midgut, Malpighian tubules and frass were collected from these larvae. Five larvae were pooled per sample. Samples were liophylized and sent for further processing to Max Planck Institute for Chemical Ecology.

Results and Discussion

Sequence analysis

Three genes with homology to *Bombyx mori* myrosinase 1 were identified in the transcriptome of the labial glands of the cabbage looper (*Trichoplusia ni*) according to a blastx search. TnMyrA, TnMyrB and TnMyrC are 499, 492 and 529 amino acids long, respectively (Figure 5-1). TnMyrA and TnMyrB are most similar with 63.6% identity and 77.4% similarity. TnMyrA and TnMyrC shared both approximately 39% similarity and 56% identity with TnMyrC. All three genes contain conserved regions associated with members of the glucohydrolase family. Also, all three of them have signal peptides with TnMyrA at amino acids 18-19, TnMyrB at 19-20 and TnMyrC at 18-19.

Several myrosinases have been deposited in Genbank as potential insect myrosinases. However, only a handful of these have been confirmed including those of *Phyllotreta striolata* and *Brevicoryne brassicae*. TnMyrA, TnMyrB, and TnMyrC have an identity/similarity of 38.2/54.3, 37.3/52.8, and 36.1/51.4 to *P. striolata* and 40/57.8, 42/60, and 39.7/55.6 to *B. brassicae*, respectively. These numbers are similar to comparisons between myrosinases from *P. striolata* and *B. brassicae* (36.6/53.2). Though, homology between sequences can give an idea of the function of a putative gene/protein, this is not enough to confirm that the glucosidases identified in the cabbage looper are indeed myrosinases and not regular broad spectrum glucosidases. We further characterized these genes in the cabbage looper.

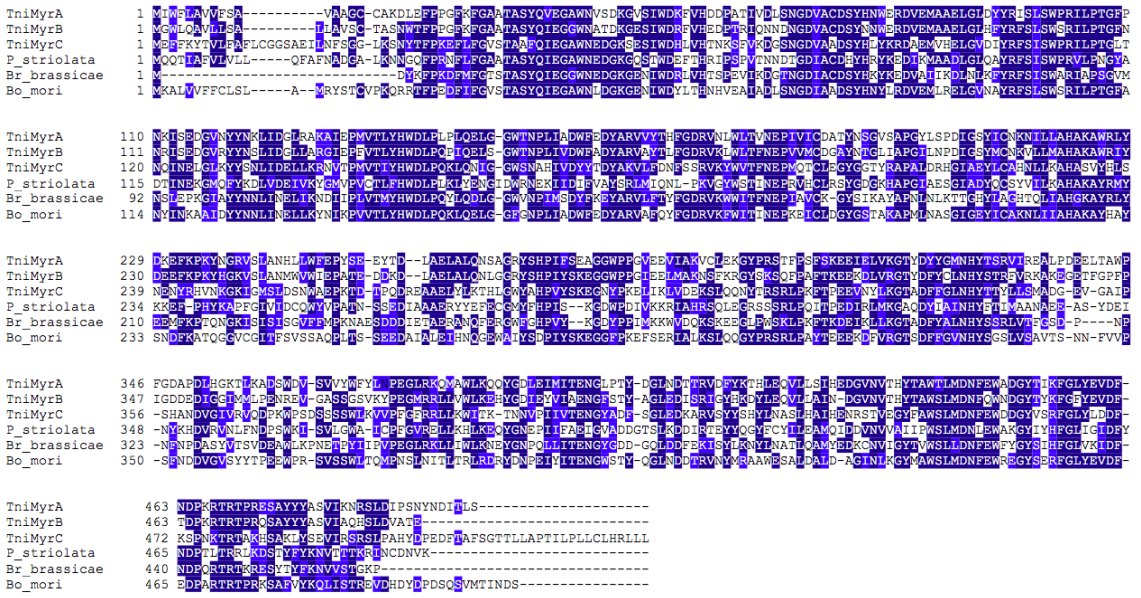

Figure 5-1: Multiple comparisons of amino acid sequences of TnMyrA, TnMyrB, TnMyrC, *Phylotretta striolata* myrosinase (AHZ59651), *Brevicoryne brassicae* myrosinase (Q95X01), and *Bombyx mori* (XP_012545628).

Gene expression changes due to host plant

We measured expression of the three potential myrosinases from different tissues of larvae reared on cabbage. TnMyrA and TnMyrB were significantly higher in labial glands compared to midgut and Malpighian tubules. However, TnMyrC was significantly higher in Malpighian tubules compared to other tissues (Figure 5-2). Because the genes were originally identified in labial glands, we focused on measuring their expression in this tissue when feeding on different hosts. TnMyrA and TnMyrB are expressed significantly higher when larvae are reared on tomato compared to cabbage. TnMyrC was higher on cabbage-reared larvae though not statistically different (Figure 5-3).

We also measured their expression in the salivary glands of larvae after transferring from a different diet. Larvae were reared on artificial diet until fifth instar. Next, they were starved overnight and then transferred to tomato or cabbage. Samples were collected after 6 and 12 h. We also included a treatment of feeding for 6 h on one host and then transfer to the other. TnMyrB was always higher on tomato fed larvae, and different on cabbage->tomato transferred samples, though not significant. TnMyrA and TnMyrC were not statistically different in any of the timepoints (Figure 5-4).

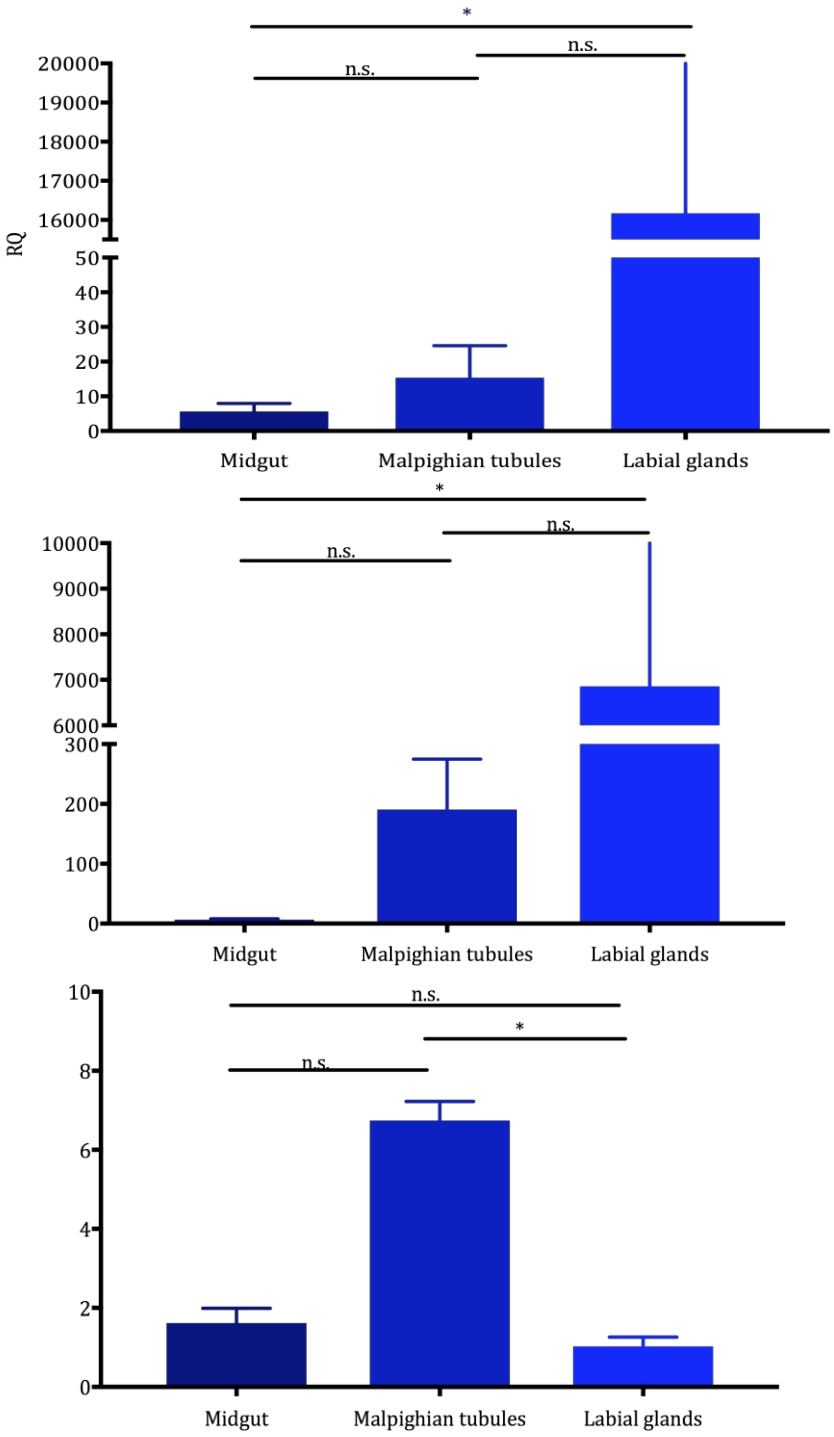

Figure 5-2: Gene expression of three myrosinases (A) TnMyrA, (B) TnMyrB, and (C) TnMyrC in different tissues of the cabbage looper (*Trichoplusia ni*) (Mean Relative quantification +SE). Statistical differences were calculated using Kruskal-Wallis non parametric test. * indicates pvalues<0.05, ** indicates pvalues<0.01.

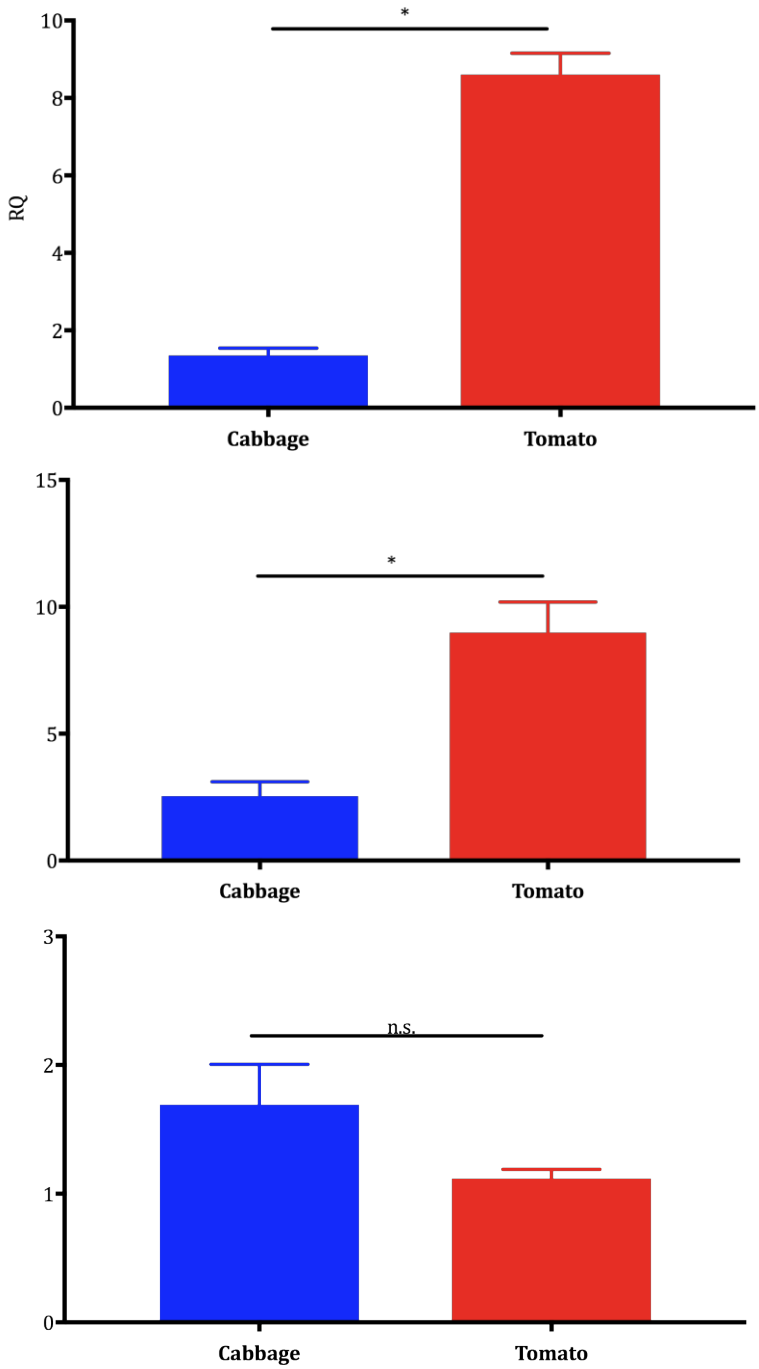

Figure 5-3: Gene expression of three myrosinases (A) TnMyrA, (B) TnMyrB, and (C) TnMyrC in the labial glands of the cabbage looper (*Trichoplusia ni*) feeding on two hosts (Mean Relative quantification +SE). Statistical differences were calculated using Mann-Whitney non parametric test. * indicates pvalues<0.05.

It seems that all three genes are expressed in several tissues; however, TnMyrA and TnMyrB are significantly higher in the labial glands. TnMyrC seems to be expressed more ubiquitously, though it was higher in the Malpighian tubules. Salivary glands and Malpighian tubules are tissues with overlapping functions, thus potentially explaining these similarities [174]. This also indicates that although these are very similar genes, their function and/or substrate specificity might vary.

Substrate specificity

Substrate specificity was measured by quantifying the production of glucose through the hydrolysis of sinigrin and tomatine. No difference between glucosidase activity with sinigrin as a substrate vs. tomatine was observed. Similar rates were identified in the *P. striolata* myrosinase; however, there was a higher activity when glucosinolates were used as substrate compared to other substrates. It is probable that the time we used (2h) was too long, thus reaching saturation.

Because a high expression of TnMyrA and TnMyrB was observed when insects were reared on tomato, we measured potential substrate specificity of these enzymes using tomatine, a glycoalkaloid commonly present in tomato [175], which is also hydrolyzed by beta-glucosidases. When hydrolyzed, tomatine breaks down into the less toxic aglycone tomatidine and the tetrasaccharide lycotetraose. Both of these compounds have been shown to suppress oxidative burst and hypersensitive response in tomato [176]. The use of salivary glucosidases to break down tomatine would mean that the cabbage looper is not only potentially using the saliva for extra-oral detoxification of this compound but also for herbivore offense through the suppression of plant defenses.

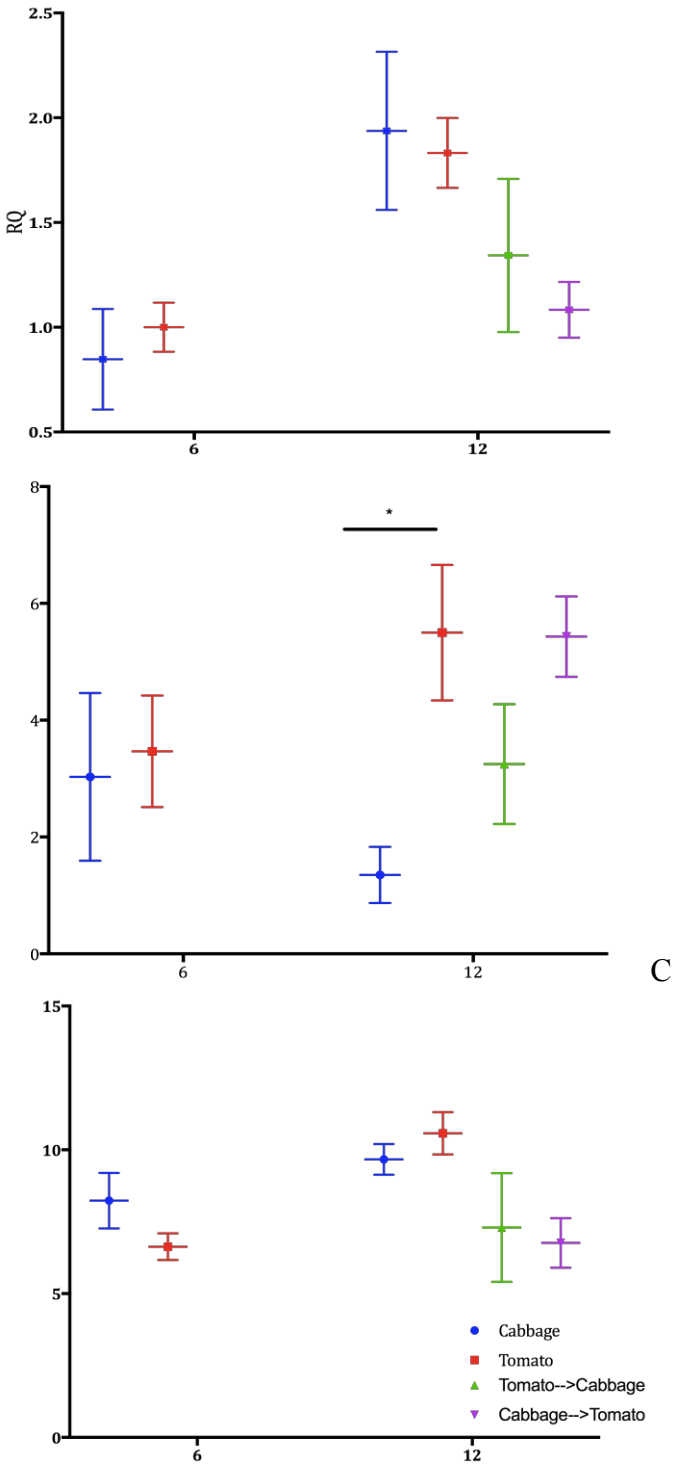

Figure 5-4: Gene expression in the labial glands of cabbage looper (*Trichoplusia ni*) fed on cabbage or tomato after 6 and 12 hours switch from artificial diet (Mean Relative Quantification +SE). Student t-test was used to test for statistical differences at each timepoint. * indicates a pvalue<0.05. A. TnMyrA, B. TnMyrB, C. TnMyrC.

Similar activity using both these substrates was identified in the midgut of cabbage looper and other Lepidoptera [177]. These results suggest that the glucosidases identified in the cabbage looper have a broader spectrum. However, because we cannot separate these enzymes, the activity of one could be masking the others, thus not being able to differentiate. Pure extracts or recombinant expression of each enzyme would allow differentiation among them.

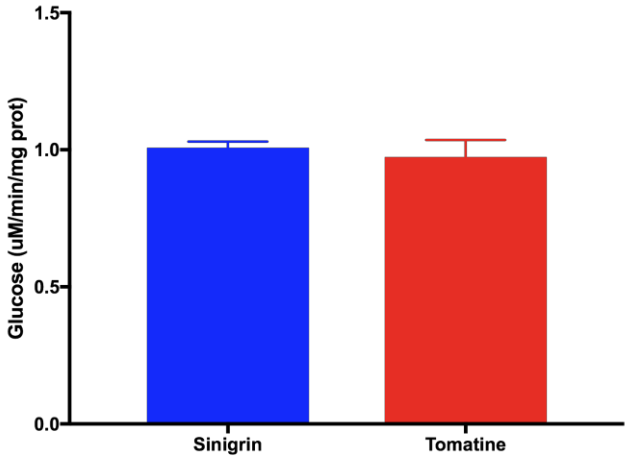

Figure 5-5: Glucose hydrolysis by labial gland homogenate using two substrates (Mean+SE). Student t-test statistical test was used to identify statistical differences. pvalue=0.6316

Glucosinolate sequestration

We measured whether cabbage looper is potentially sequestering glucosinolates. Larvae were fed cabbage, artificial diet and artificial diet plus sinigrin. Then hemolymph, frass and different tissues were analyzed. Only background levels were observed in control samples (diet), significant amounts of sinigrin were identified in the hemolymph and frass of diet plus sinigrin fed larvae (Figure 5-6). Surprisingly, no glucosinolates were identified in cabbage fed larvae. Also, no conjugates of sinigrin hydrolysis with glutathione or derivatives were identified, thus indicating low or no hydrolysis in the midgut. This correlates with the low expression of these genes in the midgut.

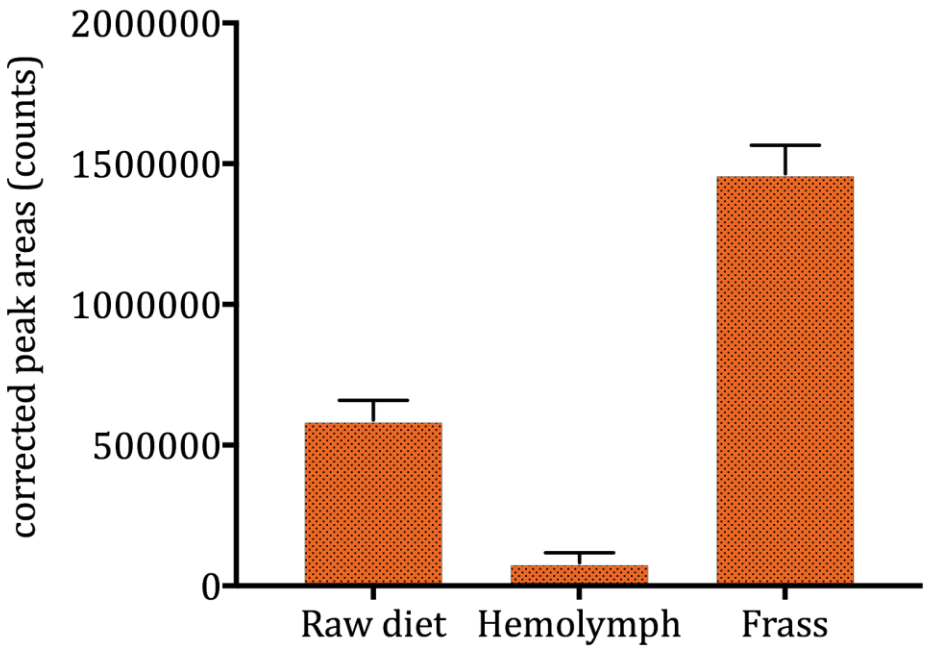

Figure 5-6: Quantification of sinigrin (Mean+SE) accumulated in the hemolymph and frass of cabbage looper (*Trichoplusia ni*) larvae fed on artificial diet + sinigrin.

Given the toxicity of glucosinolate hydrolysis products, the presence of myrosinases within the insects is highly intriguing. To date, the most well accepted hypothesis is that insects have co-opted the mustard-oil bomb as a defense mechanism against natural enemies. This includes the sequestration of glucosinolates from their host which when it comes in contact with their own myrosinases can be toxic to natural enemies. We observed such accumulation in the cabbage looper but only in the diet + sinigrin fed larvae. The absence of intact glucosinolates in the hemolymph of cabbage fed larvae could be due the presence of plant myrosinases, which hydrolyzed the glucosinolates as well as due to lower quantities of the individual glucosinolates in the plant.

Future directions

There is not yet concrete evidence supporting whether the myrosinases identified in the cabbage looper (*Trichoplusia ni*) are either broad-spectrum beta-glucosidases or thioglucosidases. There are however, several experiments that could help clarify this. First, the recombinant expression of the proteins of each gene could provide significant evidence of their function. This was attempted multiple times using *E. coli* as an expression system. However, there were no successful clones. The next step would be to attempt to express these genes in a eukaryotic system. We propose to use the yeast system, Pichia Easy Select (Thermofisher) for future attempts.

Another way to identify the function of these genes is by knocking down or knocking out their expression. We attempted this using RNAi. However, RNAi in Lepidoptera species has been found to be very difficult. We did not have success with this technique either. With the current explosion of gene editing techniques such as CRISPR, this would be a reasonable next step. A short preliminary experiment using CRISPR on *T. ni* done by Duverney Chavarria from the Rasgon lab showed promising results for using this technique (personal communication). Given that we already have full-length sequences for these genes, attempting this delivery should not be too time consuming, at least to determine whether it is a potentially alternative technique.

Also, the easiest and probably least time consuming experiment would be to repeat our substrate specificity experiments using glands collected at the time points where a differential expression among genes has been observed (i.e. tomato reared larvae vs. cabbage reared). However, because higher gene expression does not always correlate with higher protein activity, this confounds previous results.

Finally, whether these are indeed myrosinases or not, it would still be interesting to determine their role in the cabbage looper-host interaction. Some potential roles include, but are not limited to: extra-oral detoxification of plant defensive compounds and defense against natural enemies if they are being used as a mustard-oil bomb as in Brassicaceae plants.

Chapter 6

INTAD: Niger famine crop hanza (*Boscia senegalensis*), glucosinolates in the processing waste water as a germination inhibitor.

Abstract

Niger is a country located in the Sahel region in West Africa. It is one of the most food insecure countries in the world with a population of 20.7 million people out of which 9.5% are undernourished. Hanza (*Boscia senegalensis*) is a perennial, indigenous shrub consumed in times of famine. Before consuming, the seeds need to be "debittered" a process that involves soaking the seeds in water for several days. The water used for this process is then no longer suitable for human consumption. In this chapter, I give a brief overview of the food security situation in Niger as well as present data about the alternate use of hanza processing waste water as a germination inhibitor. I briefly discuss its potential use as a biofumigant or herbicide as well as future research that could enhance our understanding about the impact this technique could have environmentally and socially.

Figure 6-1. A. Hanza seeds post-processing. B. *Zea mays* germination response to hanza waste water.

Food security

Food security is defined as: “when all people at all times have access to sufficient, safe, nutritious food to maintain a healthy and active life”. The concept was first mentioned at the World Food Summit in 1996 [178]. Food security goes beyond the physical aspect of not being hungry. It includes the potential of not having access to food at a certain point in someone’s lifetime.

Food security encompasses food access, utilization, and availability [178]. Food access refers to having the income to purchase appropriate food for adequate nutrition. Food utilization includes the way in which food is processed, stored and eaten. Finally, availability involves the actual consistent availability of appropriate food at all times. In simple terms, a citizen might have the money to purchase high quality food (access) but these foods might not be available at markets (availability). In other cases, the citizen might find good quality food at markets but not have the money to purchase it or appropriate means to process it or store (utilization) such as refrigerators. In all these cases, the citizen is food insecure.

According to the Food and Agriculture Organization (FAO), 795 million people in the world (a little over 1 for every 9) were undernourished in 2014-2016. The total declined by 216 million from 1990-1992. Most of the people undernourished are from developing countries (Figure 6-2) where 780 million are considered undernourished [179].

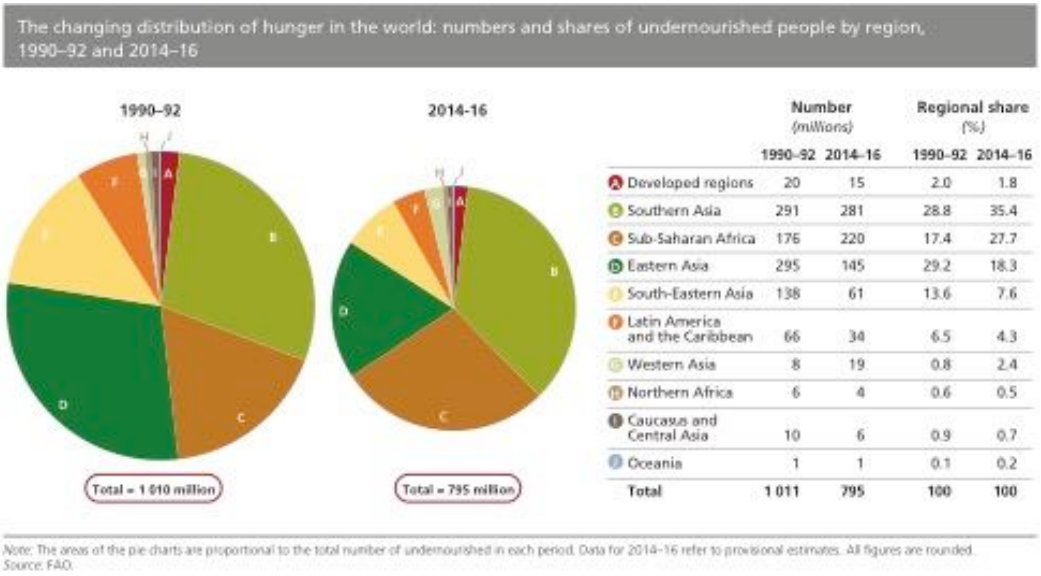

Figure 6-2. World hunger distribution [179]

Niger and famines

Niger is located in the Sahel region in West Africa (Figure 6-3). It has a population of 20.7 million people [180] with a 3.9% increase per year – one of the highest in the world. Reports indicate 45% of the population lives below the poverty line. In 2015, it was the country with the lowest Human Development Index. In 2016, their Global Hunger Index was 33.7%, and although it is lower than their 65% in 1992, it is still considered under the category of serious with 9.5% of the population undernourished and a mortality of 9.6% for children under five [181]. Niger's economy is mainly based on agriculture with approximately 82% of the population relying on farming. Unfavorable agricultural conditions have made it harder for Niger to overcome food insecurity (FAOstat database).

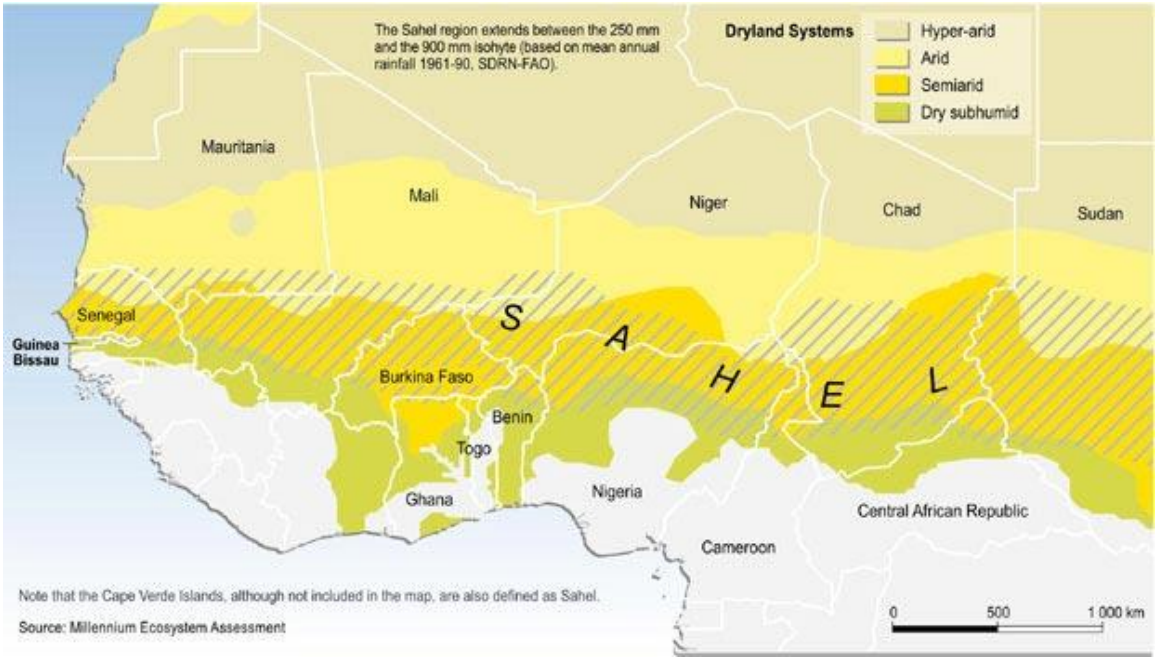

Figure 6-3. Sahel region [182]

Famine is extreme lack of food and it is employed based on the Integrated Food Security Phase Classification (IPC) scale [183]. According to this scale, in order for a lack of food to be considered a famine, at least 20% of the population has to have extreme limited access to food, acute malnutrition exceeds 30% and death rate is 2/10,000 people.

The Sahel region – the region between the Sahara desert and the African Savannah is known for its climatic uncertainty. The region experienced two severe droughts in the early 2000s, which led to food crises (Rubin, 2009; Baro and Deubel, 2006). First in 2005, drought and locust damage reduced food availability and access to critical levels [186]. In the affected area, 24-36 million people were considered food insecure. The crisis had been predicted both by locust devastation the previous year as well as by climatic models. Yet there was controversy about the coverage of the food crisis. Several outlets mentioning that the famine was being overplayed to

encourage donations or that the crisis just wasn't severe. The president at the time also downplayed the famine. Because of this, the help was slow and less than what was actually required. According to Howe and Deverux's famine scales, the 2005 crisis can indeed be considered famine [184].

Table 6-1. Niger in Statistics (data from FAOstat)

	Year	Statistic
Undernourished	2014-2016	1.8 M people
Average protein supply	2014-2016	81 g/capita/day
Food deficit	2014-2017	56 kcal/capita/day
Domestic food price index	2014	7.24
Gross domestic product	2014	894.6 I$
Children under 5	2014	
Underweight		37.90%
Stunted		43%
Wasting		18.70%
Political stability and absence of violence index	2014	1.27
Rural population	2015	81.30%

In 2010, the Supreme Council for the Restoration of Democracy ousted Niger's president Mamadou Tandja in a coup. Unlike the president, the new government was more open about needing help to respond to the famine. In affected areas of Niger, access to staple food is becoming difficult and cases of child malnutrition have increased. Also, lack of water has affected the health of animals. Price spikes in food and fuel have added to the crisis [187].

Table 6-2. Niger top 10 commodities (2013; data from FAOstat)

Production	Import	Export
Millet	Rice	Onions
Cowpeas	Sugar	Rice
Sorghum	Maize	Sugar
Onions	Oil palm	Beans
Milk	Food preps	Oil palm
Groundnuts	Wheat flour	Vegetables
Fruits	Dates	Food prep
Milk (goat)	Sorghum	Dates
Milk (skimmed)	Macaroni	Macaroni
Brassicas	Tea	Cigarettes

The 2017 Global Report on Food Crisis [188] predicts an increase in food insecure people in Niger. The key drivers of this rise in insecurity include constant natural disasters like drought, floods, and locust invasions but more important, the Boko Haram conflict in the area. The conflict in Nigeria has led to population displacement into neighboring countries – including Niger.

Famine foods

Famine food is food normally only consumed during times of extreme hunger or famine [189]. The classification of famine food is mainly a social construct. Most crops defined as famine crops are native crops, which are able to grow in the area even during times of

environmental hardship. Famine foods have been stigmatized. They are normally considered to be lower in calories or quality. However, several studies indicate that several crops considered famine foods are well within the nutritional requirements [190]. A movement towards normalizing the consumption of these foods has begun in countries where famine is common. Because these crops are native, they are better suited for the climate and are readily available. Examples of famine foods in Niger include: Kopto (multispecies leaves), *Borassus acthiopum* Mart., *Manihot esculenta* Crants, *Boscia senegalensis* (Pers.) Lam., *Cassia obtusifolia* L., *Stylochaeton lancifolius* Iotschy & Peyr., *Neocarya macrophylla* (Sabine) Prance, *Zea mays* L., *Crateva adansonii* DC, among others [189].

***Boscia senegalensis* (Pers.) Lam ex Poir**

Boscia senegalensis is a drought resistant shrub from the family Capparaceae, locally known as mukheit, hanza, anza, among other names. It is widely distributed throughout the arid Sahel region. The fruit or hanza can be used to make flour that can be used for baking and porridge. The shrub is usually 1-2 m tall. It flowers in the cool dry season (Oct-Jan) and flowers are probably insect-pollinated. Fruit is a berry about 1.5 cm in diameter, yellow when mature. The fruit pulp is translucent, jelly-like and slightly sweet. It contains two seeds, which are greenish when mature [191].

The shrubs are mainly found under rainfalls fewer than 100-300 mm and in altitudes between 60-1450 m. They grow in arid soils, rocky, clay stony hills, sand dunes, and sand-clay plains. They are also associated with termite mounds. Some of the products derived from it include: food for human and animal consumption, firewood, timber, alcohol, insecticide, and medicine [191].

The processed fruits are used for human consumption; however, they are hard and bitter when mature. Before consumption they need to be “debittered” a process which involves soaking the seeds in water for 4-7 days. Reports about its nutritional quality vary but it is accepted to have a good amount of protein as well as certain amino acids and essential oils. The debittered seed contains 21% protein. It contains high quantities of tryptophan and arginine but it is deficient in lysine and threonine. About 3.68% of its dry weight is fat [192]. It is also high in zinc and iron. It seems these values might vary depending on the region where it is grown [192,193].

Hanza as part of the Capparaceae family contains glucosinolates. Glucosinolates are amino acid-derived secondary plant products that contain a sulphate and a thioglucose [158]. When glucosinolates come in contact with myrosinases (b-thioglucosidases) they are hydrolyzed into isothiocyanates, which are bioactive and known to affect herbivores and pathogens. The glucosinolate in Hanza is glucocapparin, which converts into methyl isothiocyanate (MeITC) [194]. Metham, a commonly used soil fumigant, degrades into MeITC in the soil. MeITC becomes the active compound and has an effect in soil microorganisms including nematodes, Rhyzoctonia, Pythium, Fusarium, Phytophthora, Verticillium, Sclerotinia, club root, etc. Metham has also been shown to control weeds in the field as well as inhibit seed germination [195].

Extracts from different hanza tissues have been previously studied for their potential as insecticide against storage grain pests. It has been observed that seeds hermetically sealed and amended with these extracts caused mortality and reduced emergence of several pests in stored cowpeas and groundnuts [196–199]. However, there is concern that this might affect seed germination [200]. Also, the use of glucosinolate containing plants as green manure – standing crops that are later incorporated into soil – for biofumigation has also been discussed [201].

However, this requires of large amounts of water and equipment to crush the crop and allow release of bioactive compounds. Because hanza is a shrub, it's use as green manure is also unlikely.

Water situation in Niger

The Sahel region suffers from what is considered economic water scarcity, where the infrastructure and investments in water are not enough to meet population demands [202,203]. The rainfall in the area is irregular and unpredictable (Figure 6-4). In 2016, 4 billion people were reported to be in severe water scarcity in the world. Most of these are in Asia and Africa. In Niger, 50-55% of the population face severe water scarcity for at least 4-6 months per year [204].

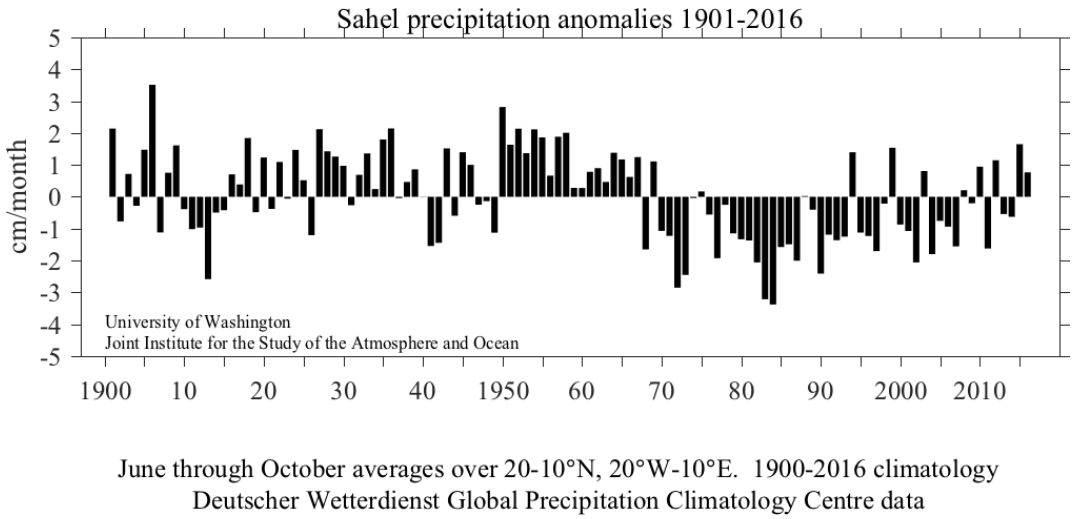

Figure 6-4. Sahel precipitation 1900-2010 [205]

On the other hand, Niger is also exposed to floods. Though normally underreported, damaging rainfall and rain-induced flooding can have devastating consequences in the area. Between 1970-2000, 79 damaging rainfall and floods were reported in the Sahel of Niger [206].

Rain patterns do not explain these floods and most of these are attributed to anthropogenic causes – primarily land use patterns. An agricultural practice in Niger is “flood recession agriculture”. This practice involves planting after flood water has receded in order to use the moisture in the soil. Though this practice allows them to make use of this water, yields from the practice are lower compared to other practices that involve regular irrigation[207]. In conclusion, availability and access to water in Niger is generally uncertain.

Hanza processing

As previously mentioned, the processing of hanza involves soaking the seeds in water. Because the fruit is for consumption, the water used in processing must be clean and of good quality. The process is water consuming which is already a limiting resource, especially during times of famine. The waste water from this process is not suitable for human or animal consumption. A way to reuse this water is required. One of the alternatives discussed was to use for irrigation of standing crops; however, a wilting of crops was observed (personal communication). Because of this, the use of the water as a potential herbicide or germination inhibitor was suggested. Here, we report results from a germination study using hanza waste water to determine its effect on germination inhibition of both common crops and wild plants.

Materials and Methods

Seed sources

Seeds of the following plant species (common names in brackets) were obtained from the following sources: *Brassica nigra* (Black mustard) – personal collection NM van Dam, population "Proefveld Wageningen," The Netherlands, 2009; *Brassica juncea* cv. varuna (Brown mustard) – Division of Genetics, IARI, New Delhi, India (see Mathur et al., 2013); *Zea mays* (corn) "Zuckermays F1, Tasty Gold" and *Solanum lycopersicon* cv. "Hellfrucht Hilmar" (tomato) – Weigelt GmbH & Co, Walluf, Germany; *S. dulcamara* (bittersweet nightshade) – collection Solanaceae Genebank Radboud University Nijmegen, the Netherlands; *Lolium perenne* (perennial ryegrass)– Veevoeder- en Kunstmesthandel J.J. Lamers V.O.F., Heteren, The Netherlands. Seeds of West-African crops species such as *Vigna unguiculata* (local name 'Niébé' or cowpea), *Hibiscus sabdariffa* (local name 'Oseille'), *Sorghum bicolor* (sorghum, abbreviated as *Sor. bicolor* to avoid confusion with *Solanum*), *Pennisetum glaucum* (millet), and *Arachis hypogea* (peanut) were all obtained at a local market in Zinder, Niger, by Renate Garvi and sent to Germany for germination assays.

Production of hanza waste water

Two batches of hanza waste water were produced for germination assays by soaking 400 g of hanza (population Kanya Wamé, Zinder, Niger) each time in deionized water. Batches were kept at 30^{o}C day and night inside a growth chamber. Water was collected daily and replaced with clean water. For days 1 and 2, seeds were soaked in 3.2 L of water, from day 3 onwards the volume of water was cut in half (1.6 L). Wastewater from days 1-3 and 4-6 were pooled and named "High" and "Low", respectively. Seeds imbibed approximately 2 L of water during the

first three days and 0.5 L during the last three days. Two sets of High and Low wastewater pools were made in August and September 2014, respectively.

Germination assay with hanza waste water

For each plant species, ten plastic pots (Teku, 7cm ø, 200 ml volume) were filled with multiplication substrate (Floraton 3, Floragard, Oldenburg, Germany). Each pot was sowed with ten or five (for *Arachis hypogaea*) seeds and lightly covered with a layer of ~5 mm of multiplication mixture. Next, 50 mL of water from each treatment were used to water the pots. This completely saturated the soil in the pots. Treatments included: Control (deionized water), High treatment (water pooled from days 1-3) and Low treatment (water pooled from days 4-6). The pots were covered with clear plastic household wrap, fixed with a rubber band around the pot, and placed in a climate cabinet set to 25°C daytime temperature and 22°C nighttime temperature, 70% R.H. and a 12 h photoperiod to mimic the conditions in Niger. To avoid cross contamination via the water, pots subjected to the same treatment were placed together on one plate (20x20 cm). Number of seeds germinated was counted seven and ten days after sowing to determine germinability. Above ground biomass was collected and air-dried in an oven at 60^{o}C for 24 hours to measure dry weight. The dry biomass per pot was assessed by weighing to the nearest 0.1 mg. One-way ANOVA followed by a Tukey means of separation were used to assess significance.

Results and Discussion

Hanza processing waste water has allelopathic properties

After seven days, watering seeds with waste water of the first three days (High treatment) had a significant negative effect on germination rates for all plant species tested (Figure 4). Hanza wastewater of day 3-6 (Low treatment) had a significant effect (ANOVA, $p<0.05$) on the germination of *L. perenne*, *Sor. bicolor*, *S. lycopersicon*, and *A. hypogaea*, only. After three more days of watering with clean water, the germination percentages in the wastewater treatments had caught up. At that time, the Low treatment had a significant effect on *S. bicolor*, *P. glaucum*, and *S. lycopersicon* germination only. The High treatment still had a significant effect on most plant species tested except for *B. nigra* (Figure 6-4). After ten days the dry weight of the seedlings was measured. The Low treatment had a significant effect on the biomass of *L. perenne*, *S. lycopersicon*, and *Sor. bicolor*. High treatment had a negative effect on the dry mass of all plant species, except for *S. dulcamara* (Figure 6-5). No seedlings from the High treatment were available to assess dry weight for *S. lycopersicon* and *A. hypogaea*.

The allelopathic properties of hanza waste water have been attributed to methyl isothiocyanates (MeITC), a breakdown product of glucoccaparin, the main glucosinolate in hanza plants [209]. Hanza waste water and MeITC had a strong effect on germination even though unsterilized potting soil was used for the germination tests. In many studies allelopathic effects are analyzed with filter paper as the substrate, which may cause an overestimation of the effectiveness of the compound [195]. Moreover, it was shown here that the hanza waste water strongly inhibits germination of almost every plant species analyzed so far, particularly the water from the first three days of processing. The test set included plants from several families –

Solanaceae, Poaceae, Fabacea, Malvaceae and Brassicaceae – both crops and wild plants. No clear indications were found that some families are more susceptible or that crops are more susceptible than weeds. Not surprisingly, the least affected plant species were members of the Brassicaceae (*B. nigra* and *B. juncea*), which themselves contain glucosinolates – albeit different ones – perhaps making them more resistant to glucosinolate breakdown compounds. It would be interesting to assess the effect of the water on the germination of hanza itself. Unfortunately, the viability of dry *B. senegalensis* seeds is very limited and germination rates are low [210] and thus this was not tested.

The MeITC in the wastewater reduces its potential use for other activities after the debittering process, unless the wastewater can be used to control weeds in a sustainable and cost effective manner. Towards this goal, hanza waste water may be used as a natural herbicide. The "Croplife foundation" reports weeding as one of the most taxing and expensive – both physically and economically – labors in crop production in Africa [211]. Most of this weeding is done by hand and by female farmers. Obtaining economically and environmentally sustainable methods of weed control could significantly impact crop yields in smallholder farms and become part of integrated pest management programs. Of course, negative effects on the crop itself should be avoided. This data shows that the germination of crops and weeds were both affected. However, adult crop plants may be less susceptible; field observations in Niger showed that larger sorghum plants are not negatively affected by watering with hanza waste water (Renate Garvi, personal observations). Moreover, the structurally closely related glucosinolate conversion product allyITC, only caused significant effects on plant growth at concentrations that were at least 10-50 times higher than used in this study [212].

Future research

Inhibition of germination was confirmed under laboratory conditions. This experiment should be repeated in the field to test whether the results translate in a more realistic scenario. Weeds normally found in Niger and other parts of Africa where Hanza can be found, should be included in these field studies. For example, Striga or witchweed, is an important weed in the area. The identification of a natural product like hanza waste water to control this weed could be quite impactful.

Before hanza waste water can be incorporated into IPM programs or as a common inhibitor of weed germination, there are several studies that need to be carried out. First of all, it is important to determine the effect that MeITC could have in the soil, both in the physical properties of the soil as well as in the soil microorganisms. Given that MeITC is the main component of Metham, a soil fumigant normally used against nematodes and fungi involved in damping off, it is highly probable that Hanza waste water would also be able to control these pathogens. MeITC fumes could potentially also have effects on humans such as eye or nose irritation [213]. Furthermore, this water contains several microelements [209] that could also affect the soil, microorganisms and potentially cause phytotoxicity to the crops.

It is important to consider the social component. As previously mentioned, famine foods including hanza, are associated with stigmas given that they are only used during famine. Even the director of the WFP has made comments against indigenous foods calling them of “lower quality” and “potentially poisonous”. Before further use can be encouraged, it is necessary to address these concerns. The potential diversification and monetization of these products including the waste water could help shift mentalities in the area. Finally, it is important to remember that this product is used mainly for subsistence, which means that its availability and quantity is

limited and so the amount of waste water that would be available to incorporate as part of a program would also be limited. A way to still make use of the product even if its availability is limited is by using it in the much smaller seedbeds to produce healthy seedlings before transplant as well as using other parts of the plant like leaves, which also contain MeITC. It is also highly probable that people in Niger are already using the waste water in their gardens but this knowledge – like most indigenous knowledge – has not made it into the scientific literature.

The involvement of NGOs and extension agents, where possible, could significantly increase the potential of using this natural product as a biofumigant. They can be involved either in the field experiments, as well as spreading the information about the waste water in the area. In fact, the social enterprise Sahara Sahel Foods has already begun campaigns to reduce the stigma against famine foods like Hanza and others. Because this is a natural product and it is found even in times of extreme climatic changes, I do not foresee many difficulties in the regulatory process and incorporation into IPM programs.

In conclusion, Niger, one of the most food insecure countries in the world continues to struggle with climatic and political challenges. During times of food scarcity and famine, people consume local plants generally referred to as famine foods. One of these famine foods is Hanza (*Boscia senegalensis*). Though a versatile shrub and well adapted to the area, its processing requires and large amount of clean water. This water is no longer available for human or animal consumption after use in the processing of hanza. Its alternative use as germination inhibitor seems promising. However, further studies both biological and sociological are required.

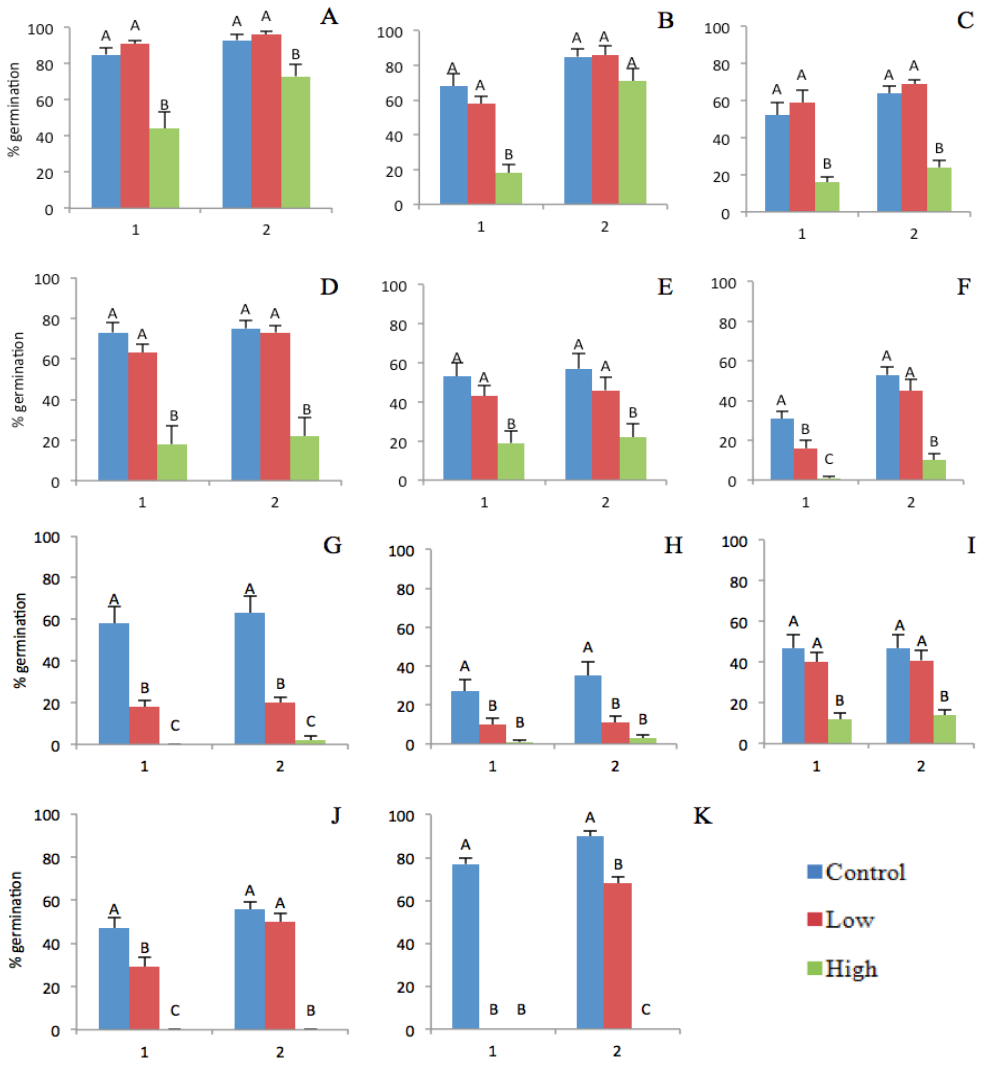

Figure 6-5. Germination percentages on two days 7 and 10 days after sowing per treatment. *Brassica juncea* (A), *B. nigra* (B), *Zea mays* (C), *Solanum dulcamara* (D), *Hirsitum sabdariffa* (E), *Vigna unguiculata* (F), *Lolium perenne* (G), *Sorghum bicolor* (H), *Pennisetum glaucum* (I), *Arachis hypogaea* (J), *S. lycopersicon* (K). Different letters represent statistically significantly different groups ($p<0.05$), Tukey mean separation (Mean+SE).

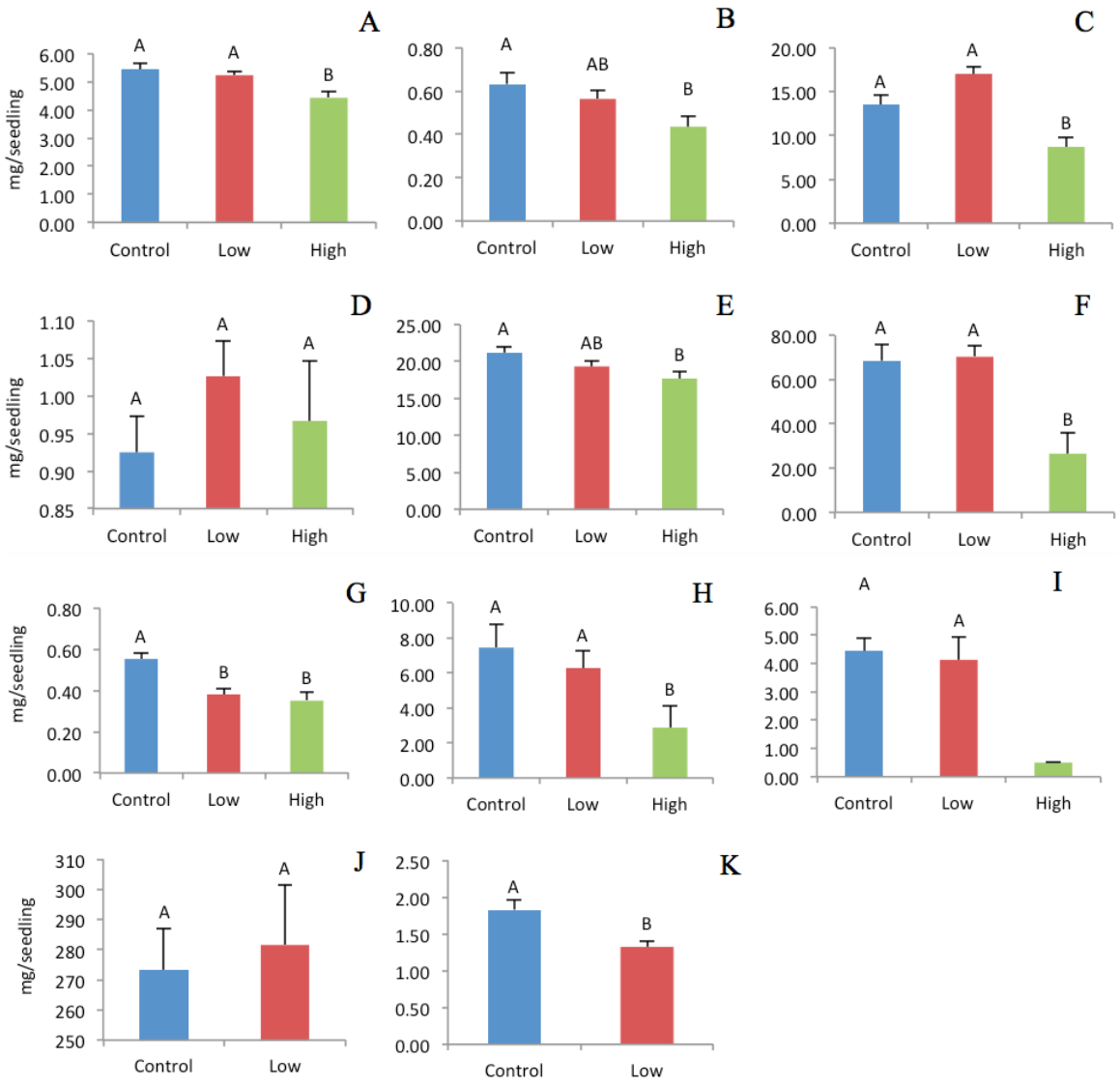

Figure 6-6. Dry weight of seedlings per treatment. *Brassica juncea* (A), *B. nigra* (B), *Zea mays* (C), *Solanum dulcamara* (D), *Hirsitum sabdariffa* (E), *Vigna unguiculata* (F), *Lolium perenne* (G), *Sorghum bicolor* (H), *Pennisetum glaucum* (I), *Arachis hypogaea* (J), *S. lycopersicon* (K). Different letters represent statistically significantly different ($p<0.05$), Tukey mean separation (Mean+SE)

Chapter 7

Conclusions

Diet affects insects' saliva composition, and in turn, saliva affects plant defenses (Fig. 7-1).

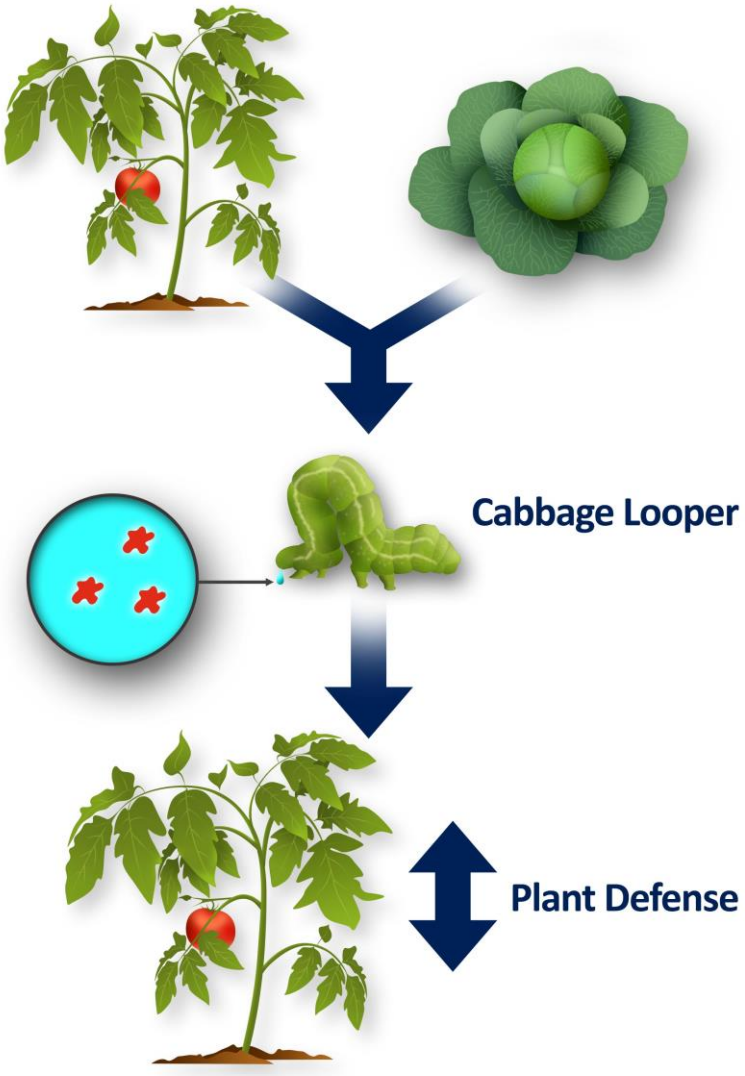

Figure **7-1**. Host plant-driven changes in the saliva of the cabbage looper and its role in plant-insect interactions. Diagram by Nick Sloff.

Hypotheses about the different functions of insect saliva include: digestion, detoxification, immunity, herbivore offense, and probably several others not yet identified. The availability of high throughput techniques such as RNAseq and iTraq are helping scientists begin to test these hypotheses as well as generate new ones. Here, both a transcriptome and a proteome library were established for the labial glands of the cabbage looper. These libraries provide an invaluable tool for future functional studies and to test these hypotheses and others regarding the role of saliva in complex ecological interactions.

Besides establishing libraries for the saliva of the cabbage looper, the hypothesis that saliva of a generalist insect is plastic was also tested. Significant changes due to host plant were observed both in the transcriptome and proteome. A larger effect was observed when comparing suboptimal diets (tomato) versus optimal diets (cabbage and artificial diet). Tomato fed larvae had a significant downregulation in pathways and proteins involved in protein metabolism. Also, evidence of expression of digestion and detoxification genes, as well as immune-related genes that could be allowing the cabbage looper to respond not only to differences in host plant, but also to the microbiome associated with the host plants were observed. These observations were further confirmed in the proteome where several detoxification and antioxidant enzymes were also identified. These enzymes (e.g. catalase) are being used for detoxification as well as herbivore offense.

As with any research, this study answers a few questions but also generates many more. For example, the finding that feeding on tomato has a significant effect in the protein metabolism machinery of the insect requires further analyses. With the information provided here, it is not possible to discern whether the downregulation of the spliceosome pathway and changes in ribosomal proteins is advantageous for the plant, for the insect, both or not advantageous at all.

Because this involves several genes, proteins and entire pathways, it is a complex question to answer. However, it could begin to be tackled by analyzing the changes in alternative splicing in tomato fed larvae.

Also, the identification of several genes and proteins involved in immunity opens the question about how significant is the role of caterpillar saliva in the interaction with microbes. The interaction between saliva and microorganisms has been extensively studied in sucking insects, especially vectors of human diseases. It would be interesting to go beyond the research of pathogens and begin to study mutualistic interactions. Also, here, we have focused on genes and proteins; however, saliva contains several other compounds including metabolites, ions, etc. that could also be important in understanding plant-insect dynamics.

Finally, this dissertation focused mainly in studying the potential effect of the cabbage looper saliva in direct defenses. The role of saliva in indirect defenses has not been as well studied as the role of oral secretions in general (regurgitant plus saliva). Now that individual enzymes have been identified in the proteome, it would be easier to test candidates in wounded plants and measure indirect defenses.

The study of plant-insect interactions continues to be an exciting area of research and the availability of new technology just continues to provide more tools to answer questions that have existed for decades as well as generate new ones. The role of insect saliva in these interactions is a complex and still minimally explored component. It is my firm belief that basic research in this topic will continue to help answer not only ecological concerns but also more applied concerns like pest management and ultimately food security.

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Appendix A

Primers

ID	Contig	Primer name	Sequence 5'-3'
Aquaporin	comp155308	TnAqp1	F-GTCAGCCCATTCGTTAGC
			R-CATCGAATTCTTCCTTGGATTTG
Thiol peroxidase	comp6532	TnThPx1	F-GTGTCGGCATTGGTTTCT
			R-CTCCTTGATGAGGGCTTTG
Peroxidase	comp12985	TnPer2	F-GACCCGGAGAGAGTAGAAAT
			R-GGGTCTGTTAGGTCTCTCATA
Hsp90	comp5870	TnHsp1	F-GATACAACCACCTTCTCAACC
			R-CGAGCCCATTGATGAGTATG
Peroxiredoxin	comp14252	TnPXR3	F-CTTCTACCCGATGGACTTTAC
			R-GTGAGACTTGTCGCTGATG
Glutathione Peroxidase	comp15858	TnGPX1	F-ACGGCTGGACAGTTAAATC
			R-GGATGTCCTTACACCATTCC
Serine/threonine kinase	comp11663	TnSerkin2	F-GCATCTTGCCTTGTCTATCA
			R-TGGTACTCCAGTTCCGTATAA
Catalase	comp27660	TnCat1	F-GAAGTCACCCTTGGCAATAG
			R-CTGATGGTTACAGACACATGAA
Glutathione-S-transferase	comp15433	TnGST1	F-CAAGAAGCAGGAGACCTTAG
			R-GAGTAGACGTTGTCGATGAG
Protease	comp8423	TniProt	F-CTCGATCCCTCGTGAGAATA
			R-CCTACCGCCAGGACTATTA
Proteinase inhibitor	comp877	TniPI	F-ACAAACCCTTCCTGTTCTTC
			R-AACGCCATCTATCGCTTATAC
Cytochrome P450	comp12543	TniP450	F-GAACCGTGCCCGTTATTT
			R-CTGGAACTCCTTCGAGTAAAC
UDP-glycosyl transferase	comp11498	TniGlyT	F-CGTTAGATCGCAACCACTAC R-GCGACACTTCATTCACTATCA
GAPDH	CF259232.1	TniGAPDH1	F-GCCAAGAAGGTCATCATCTC R-GGTCATCAAACCTTCAACAATC
TnMyrA	comp12465	TnMyr1	F-GCAGAACTTGCCCTACAA
			R-GTAGTGGTTCATGCCGTAATA
TnMyrB	comp12458	TnMyr3	F-GAGAGTATCTACCTCCCAGATT R-ACCCAGACATAGGGTCTTAC
TnMyrC	comp10306	TnMyr5	F-GTTCGACCATCCTCCTATATTC R-CTAGGCGTCGACATTTACAG
TnMyrC 3’		MyrRace1F	F-GATTACGCCAAGCTTTGGGCTGAACCAAAGACTGACACACC
		MyrRace2F	F-GATTACGCCAAGCTTGGTCTCGTCTCCCGAAGTTCACACCT

Appendix B

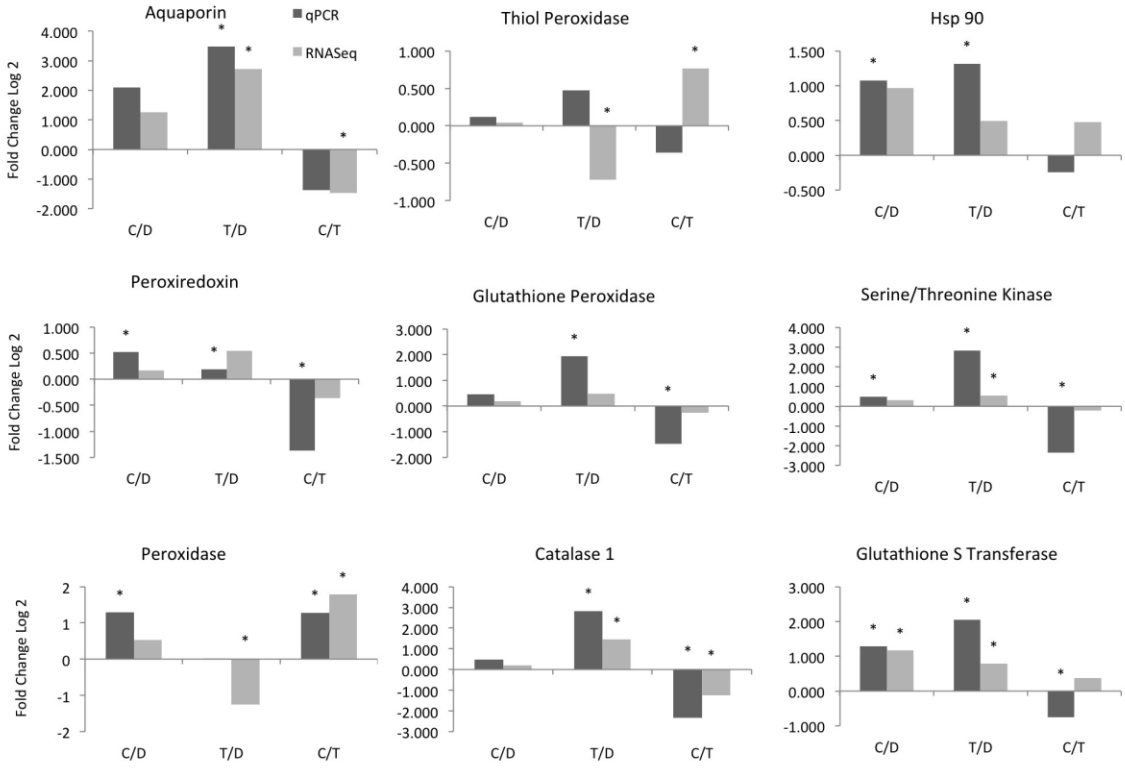
Trinity genes

Total Trinity 'genes'	30,082
Total Trinity transcripts	38,649
Percent GC	41.38

	All transcripts	Longest isoform/gene
Contig N10	5,969	5,163
Contig N20	4,604	3,900
Contig N30	3,684	3,068
Contig N40	2,999	2,422
Contig N50	2,418	1,903
Median contig length	583	451
Average contig length	1,218.82	966.31
Total assembled bases	47,106,062	29,068,458

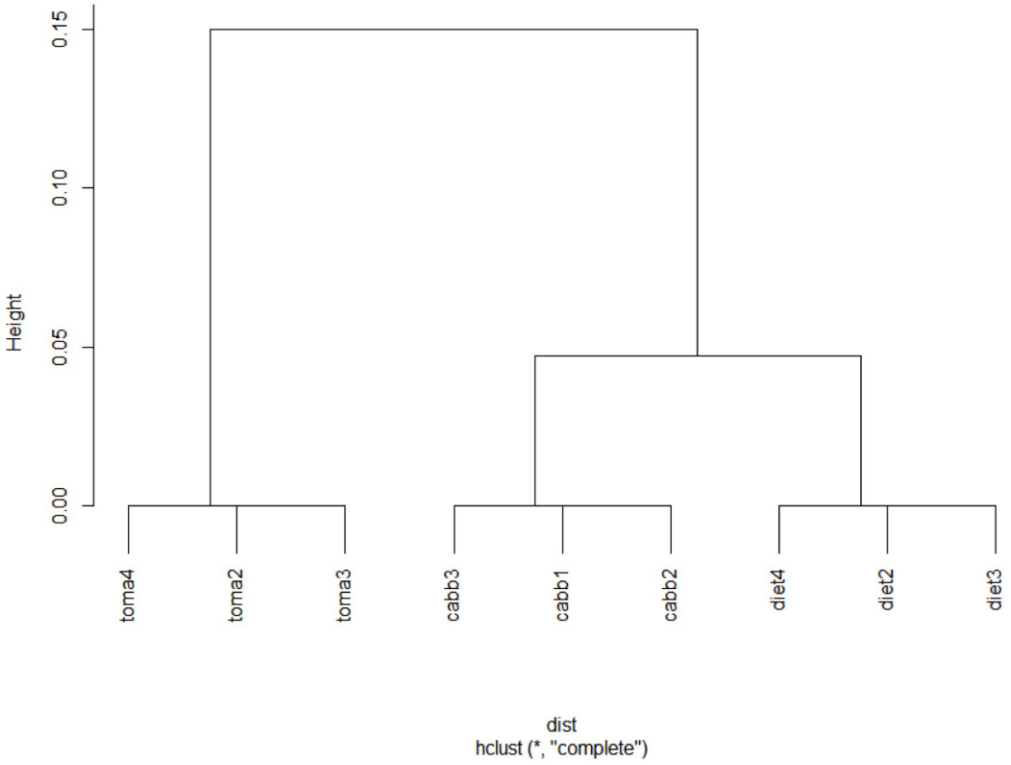
Appendix C

Transcriptome Validations



Appendix D

Transcriptome Biological Samples Clustering



Appendix E

Cabbage looper labial saliva proteome

Protein ID	Accession	Name	GO	Num Peptides	Num Peptides with Quant	Tomato/Cabbage	std	p-value	Tomato/Diet	std	p-value	Cabbage/Diet	std	p-value
1	gi|112983010	translation elongation factor 2 isoform 1	Protein metabolism	428	329	0.721	0.213	0.001	-0.640	0.226	0.005	0.085	0.123	0.487
2	gi|112984390	elongation factor 1-alpha	Protein metabolism	730	301	0.108	0.246	0.660	-0.085	0.261	0.744	0.023	0.142	0.871
3	gi|112982828	heat shock cognate protein	Heat shock	300	221	0.458	0.224	0.041	-0.522	0.240	0.029	-0.058	0.129	0.651
4	gi|112983556	90-kDa heat shock protein	Heat shock	269	169	0.689	0.222	0.002	-0.788	0.237	0.001	-0.103	0.127	0.419
5	gi|304307739	tudor staphylococcus/micrococcal nuclease	Other	133	88	0.315	0.249	0.206	-0.520	0.268	0.052	-0.208	0.144	0.149
6	gi|112983503	beta-tubulin	Structure	231	221	-0.152	0.229	0.507	0.153	0.246	0.534	-0.013	0.134	0.922
7	gi|112983322	transitional endoplasmic reticulum ATPase TER94	Cell cycle	103	50	0.542	0.279	0.052	-0.777	0.305	0.011	-0.212	0.160	0.185
8	gi|112983501	alpha-tubulin	Structure	246	24	0.092	0.346	0.790	0.128	0.389	0.741	0.219	0.210	0.295
9	gi|187281844	"actin, cytoplasmic A4 "	Structure	250	221	-0.228	0.232	0.324	0.070	0.248	0.776	-0.144	0.135	0.286
10	gi|114050901	14-3-3 protein zeta	Signaling	88	59	0.426	0.248	0.086	-0.455	0.268	0.090	-0.004	0.144	0.978
11	gi|148298878	vacuolar ATP synthase catalytic subunit A	ATP utilizing	61	41	0.325	0.276	0.239	-0.363	0.306	0.235	0.020	0.162	0.899
13	gi|112984078	40S ribosomal protein S4	Protein metabolism	127	98	0.477	0.233	0.041	-0.508	0.251	0.043	-0.030	0.134	0.825
14	gi|148298695	poly A binding protein	Protein metabolism	84	61	0.133	0.256	0.603	-0.251	0.276	0.363	-0.130	0.148	0.381
15	gi|148298685	"fructose 1,6-bisphosphate aldolase "	Energy	95	78	0.102	0.271	0.708	-0.048	0.300	0.874	0.068	0.159	0.668

16	gi|114050833	transketolase	Energy	72	42	0.219	0.257	0.396	-0.298	0.280	0.287	-0.072	0.151	0.634
17	gi|112982844	ribosomal protein L7	Protein metabolism	98	62	0.508	0.262	0.052	-0.434	0.286	0.129	0.098	0.151	0.518
18	gi|112982669	ribosomal protein S2	Protein metabolism	64	49	0.602	0.258	0.019	-0.414	0.282	0.142	0.168	0.149	0.260
19	gi|512908279	apolipophorins isoform X1	Vesicle transport	168	6	-0.977	0.434	0.024	0.329	0.474	0.487	-0.647	0.273	0.018
20	gi|112984112	ribosomal protein S3	Protein metabolism	58	50	0.528	0.257	0.040	-0.676	0.278	0.015	-0.155	0.149	0.298
21	gi|148298717	vacuolar ATP synthase subunit B	ATP utilizing	47	28	0.180	0.300	0.547	-0.245	0.324	0.450	-0.105	0.173	0.545
22	gi|112984022	ribosomal protein S9	Protein metabolism	99	48	0.570	0.274	0.038	-0.301	0.303	0.321	0.233	0.159	0.143
23	gi|112983736	eukaryotic translation initiation factor 4A	Protein metabolism	66	34	0.183	0.277	0.510	-0.256	0.303	0.398	-0.027	0.161	0.868
24	gi|219362829	clathrin heavy chain	Vesicle transport	69	23	-0.060	0.317	0.850	-0.003	0.354	0.994	-0.088	0.192	0.645
25	gi|114051866	isocitrate dehydrogenase	Oxidoreductase	53	29	0.493	0.286	0.085	-0.769	0.310	0.013	-0.243	0.164	0.138
26	gi|112983487	ribosomal protein L8	Protein metabolism	52	47	0.554	0.262	0.035	-0.440	0.290	0.129	0.160	0.153	0.295
27	gi|148298752	14-3-3 epsilon protein	Signaling	69	39	-0.083	0.283	0.770	-0.003	0.315	0.993	-0.109	0.170	0.521
28	gi|512917375	phosphatidylinositol transfer protein alpha isoform-like	Vesicle transport	39	21	-0.223	0.299	0.455	0.402	0.336	0.231	0.147	0.184	0.425
29	gi|112983984	polyubiquitin	Protein metabolism	191	20	-0.467	0.447	0.297	-0.031	0.497	0.950	-0.450	0.271	0.097
30	gi|512926925 ; gi|512926913	moesin/ezrin/radixin homolog 1-like isoform X4 ; moesin/ezrin/radixin homolog 1-like isoform X1	Structure	40	22	-0.405	0.287	0.159	0.232	0.316	0.462	-0.187	0.175	0.285
31	gi|512901472	UDP-glucose 6-dehydrogenase-like	Oxidoreductase	53	30	0.468	0.307	0.128	-0.572	0.342	0.094	-0.103	0.177	0.562
32	gi|512917658	eukaryotic translation initiation factor 3 subunit A-like isoform X1	Protein metabolism	61	13	0.109	0.358	0.761	-0.393	0.408	0.335	-0.313	0.218	0.151
33	gi|112983954	ribosomal protein S23	Protein metabolism	54	44	0.453	0.285	0.113	-0.417	0.316	0.187	0.024	0.168	0.884

34	gi|512926720	proliferation-associated protein 2G4-like	Cell cycle	27	20	0.241	0.317	0.447	-0.075	0.357	0.833	0.171	0.191	0.370
35	gi|112982743	elongation factor 1-beta'	Protein metabolism	35	17	0.436	0.368	0.236	-0.229	0.414	0.579	0.237	0.216	0.273
36	gi|112982880	translationally-controlled tumor protein homolog	Cell cycle	52	38	0.265	0.259	0.307	-0.750	0.279	0.007	-0.468	0.150	0.002
37	gi|112982832	translation initiation factor 5A	Protein metabolism	95	86	0.399	0.258	0.121	-0.511	0.277	0.065	-0.102	0.150	0.494
38	gi|112982735	ribosomal protein P0	Protein metabolism	37	24	0.809	0.326	0.013	-0.952	0.354	0.007	-0.141	0.180	0.431
39	gi|112984098	40S ribosomal protein S3a	Protein metabolism	34	24	0.429	0.286	0.134	-0.349	0.319	0.274	0.098	0.169	0.563
40	gi|512920757	T-complex protein 1 subunit gamma-like	Protein metabolism	64	25	0.406	0.299	0.173	-0.314	0.337	0.351	0.132	0.174	0.448
41	gi|112983462	ribosomal protein L7A	Protein metabolism	38	19	0.820	0.328	0.012	-0.756	0.366	0.039	0.087	0.187	0.642
42	gi|148298726	ADP-ribosylation factor	Vesicle transport	41	22	-0.598	0.309	0.053	0.273	0.346	0.430	-0.299	0.187	0.111
43	gi|112984000	ribosomal protein S18	Protein metabolism	68	29	0.381	0.292	0.192	-0.394	0.324	0.224	0.024	0.170	0.888
44	gi|112983898	elongation factor 1 gamma	Protein metabolism	69	46	0.445	0.273	0.102	-0.528	0.298	0.077	-0.085	0.157	0.588
45	gi|112983414	heat shock protein hsp21.4	Heat shock	46	30	-0.558	0.305	0.067	0.572	0.349	0.102	0.039	0.192	0.840
47	gi|114052751	GTP-binding nuclear protein Ran	Vesicle transport	40	22	0.065	0.301	0.828	-0.251	0.331	0.449	-0.156	0.178	0.380
48	gi|112984034	ribosomal protein S8	Protein metabolism	60	50	0.508	0.291	0.081	-0.519	0.328	0.114	0.077	0.172	0.655
49	gi|112983816	glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase	36	11	-0.340	0.378	0.368	0.262	0.437	0.549	-0.098	0.238	0.680
50	gi|112984008	ribosomal protein S17	Protein metabolism	45	28	0.415	0.317	0.191	-0.333	0.356	0.349	0.119	0.185	0.518
51	gi|112984394	ribosomal protein S16	Protein metabolism	89	73	0.574	0.267	0.032	-0.486	0.291	0.095	0.090	0.153	0.557
52	gi|112984266	ribosomal protein L23A	Protein metabolism	35	21	0.598	0.319	0.061	-0.575	0.357	0.107	0.029	0.186	0.877
53	gi|512893346	rRNA 2'-O-methyltransferase fibrillarin-like	Protein metabolism	13	12	0.004	0.333	0.990	-0.149	0.370	0.687	-0.111	0.198	0.575

54	gi|114051357	transgelin	Structure	33	28	-0.722	0.278	0.009	0.426	0.302	0.158	-0.331	0.166	0.046
55	gi|153791817	S-adenosyl-L-homocysteine hydrolase	Other	32	19	0.438	0.327	0.180	-0.780	0.366	0.033	-0.279	0.189	0.139
56	gi|112982800	ribosomal protein L4	Protein metabolism	67	26	0.737	0.304	0.015	-0.831	0.333	0.013	-0.074	0.174	0.670
57	gi|112984310	ribosomal protein L18A	Protein metabolism	43	23	0.782	0.299	0.009	-0.780	0.327	0.017	-0.009	0.173	0.958
58	gi|153791847	abnormal wing disc-like protein	Other	49	35	0.942	0.321	0.003	-0.726	0.352	0.039	0.199	0.186	0.286
59	gi|112983562	ribosomal protein L13	Protein metabolism	41	29	0.935	0.288	0.001	-0.815	0.317	0.010	0.114	0.164	0.488
60	gi|530233623	puromycin-sensitive aminopeptidase-like	Protease	56	20	0.012	0.311	0.970	-0.074	0.344	0.829	-0.040	0.186	0.828
62	gi|512913407	97 kDa heat shock protein-like	Heat shock	36	15	0.234	0.327	0.475	-0.417	0.361	0.248	-0.175	0.192	0.363
63	gi|112983782	cyclophilin A	Other	91	76	0.459	0.256	0.073	-0.527	0.276	0.056	-0.069	0.147	0.639
64	gi|112983418	glycerol-3-phosphate dehydrogenase-1	Oxidoreductase	31	18	0.619	0.330	0.061	-0.678	0.370	0.066	-0.021	0.192	0.912
65	gi|512907480 ; gi|512907476	methionine aminopeptidase 2-like isoform X2	Protein metabolism	41	16	-0.116	0.362	0.748	0.412	0.437	0.345	0.368	0.234	0.116
66	gi|112982792	Y-box protein	Other	19	15	0.344	0.365	0.345	-0.475	0.409	0.245	-0.144	0.210	0.494
67	gi|114052561	cytosolic malate dehydrogenase	Oxidoreductase	26	19	-0.107	0.346	0.757	0.122	0.387	0.753	-0.004	0.207	0.986
68	gi|512896702	26S protease regulatory subunit 7-like isoform X1	Protein metabolism	35	17	0.045	0.316	0.886	-0.216	0.348	0.535	-0.180	0.188	0.338
69	gi|112982661	ribosomal protein S6	Protein metabolism	42	21	0.421	0.336	0.211	-0.303	0.375	0.420	0.127	0.198	0.521
70	gi|112983696	squid protein homologue	Protein metabolism	24	19	0.088	0.348	0.800	-0.071	0.399	0.859	0.067	0.216	0.756
71	gi|112982861	ribosomal protein S11 isoform 2	Protein metabolism	38	25	0.619	0.279	0.026	-0.679	0.305	0.026	-0.043	0.161	0.791
72	gi|512917909	ATP-dependent RNA helicase WM6-like	Protein metabolism	28	20	-0.011	0.347	0.975	-0.000	0.391	1.000	0.026	0.209	0.902
73	gi|512912876	rab GDP dissociation inhibitor alpha-like	Vesicle transport	31	20	-0.014	0.359	0.970	-0.032	0.405	0.936	-0.056	0.217	0.796
74	gi|112984376	ribosomal protein L14	Protein metabolism	22	17	0.245	0.449	0.585	-0.266	0.515	0.605	0.019	0.267	0.944

75	gi|114053313	GTP binding protein	Signaling	22	12	0.269	0.349	0.441	-0.325	0.390	0.405	-0.063	0.208	0.762
76	gi|112984422	60S ribosomal protein L17	Protein metabolism	37	27	0.640	0.330	0.053	-0.722	0.360	0.045	-0.095	0.187	0.613
77	gi|112983886	elongation factor 1 delta	Protein metabolism	34	10	0.918	0.392	0.019	-0.844	0.449	0.060	0.150	0.227	0.509
78	gi|148298697	eukaryotic initiation factor 5C	Protein metabolism	26	10	-0.394	0.377	0.295	0.047	0.409	0.909	-0.361	0.226	0.111
79	gi|112983926	arginine kinase	ATP utilizing	35	13	-0.349	0.338	0.301	0.062	0.373	0.869	-0.281	0.205	0.169
80	gi|512936353	"LOW QUALITY PROTEIN: dynein heavy chain 10, axonemal-like "	ATP utilizing	174	6	-0.271	0.504	0.591	0.220	0.574	0.701	-0.068	0.309	0.826
81	gi|160333861	ribosomal protein L10	Protein metabolism	29	18	0.870	0.322	0.007	-0.803	0.361	0.026	0.105	0.183	0.567
82	gi|148298648	ribosomal protein S13	Protein metabolism	28	26	0.504	0.301	0.094	-0.516	0.334	0.122	0.012	0.174	0.946
83	gi|148298800	enolase	Energy	46	24	0.387	0.285	0.174	-0.046	0.322	0.886	0.392	0.171	0.022
84	gi|512894777	plastin-2-like	Structure	48	25	0.061	0.313	0.846	-0.042	0.350	0.904	-0.018	0.187	0.923
85	gi|112983276	60S ribosomal protein L5	Protein metabolism	43	33	0.724	0.291	0.013	-0.731	0.320	0.022	0.016	0.167	0.923
86	gi|112982798	ribosomal protein L3	Protein metabolism	33	21	0.703	0.306	0.022	-0.616	0.340	0.070	0.051	0.176	0.773
87	gi|112984508	translation initiation factor 2 gamma subunit	Protein metabolism	42	10	0.194	0.446	0.664	-0.578	0.489	0.237	-0.377	0.257	0.143
88	gi|289629216	coatomer protein complex subunit alpha	Vesicle transport	66	8	0.005	0.399	0.990	-0.018	0.449	0.968	-0.012	0.239	0.961
89	gi|512892462	inorganic pyrophosphatase-like	Energy	16	14	0.750	0.351	0.032	-0.796	0.388	0.040	-0.036	0.195	0.853
90	gi|112984062	multiprotein bridging factor 1	Protein metabolism	31	8	-0.100	0.398	0.801	-0.342	0.436	0.432	-0.446	0.233	0.055
91	gi|512895378	valine--tRNA ligase-like isoform X1	Protein metabolism	54	13	0.535	0.347	0.123	-0.351	0.394	0.373	0.145	0.204	0.476
92	gi|112984070	ribosomal protein S5	Protein metabolism	39	30	0.746	0.353	0.035	-0.610	0.398	0.125	0.084	0.202	0.677
93	gi|114051229	microtubule-associated protein RP/EB family member 3	Cell cycle	11	9	0.040	0.392	0.918	-0.247	0.440	0.574	-0.191	0.229	0.404
94	gi|512926768	GTP-binding protein	Vesicle	16	14	-0.012	0.412	0.977	-0.354	0.466	0.448	-0.351	0.251	0.162

	; gi|512926764	SAR1b isoform X2	transport											
95	gi|512934370	transcription factor BTF3 homolog 4-like	Protein metabolism	10	8	-0.313	0.498	0.530	-0.189	0.569	0.739	-0.510	0.313	0.104
96	gi|112983495	ribosomal protein L9	Protein metabolism	29	21	0.600	0.345	0.082	-0.705	0.384	0.067	-0.091	0.194	0.639
97	gi|114051313	chaperonin containing t-complex polypeptide 1 beta subunit	Protein metabolism	44	16	0.073	0.320	0.819	-0.070	0.357	0.844	0.011	0.189	0.951
98	gi|112984200	ribosomal protein L28	Protein metabolism	28	23	0.622	0.286	0.030	-0.717	0.313	0.022	-0.066	0.165	0.688
99	gi|512933725	uncharacterized protein LOC101741548	uncharacterized	252	5	0.036	0.660	0.956	0.428	0.874	0.625	0.664	0.452	0.142
100	gi|112984306	ribosomal protein L19	Protein metabolism	40	23	0.589	0.309	0.057	-0.522	0.346	0.132	0.103	0.182	0.571
101	gi|512921563	insulin-like growth factor 2 mRNA-binding protein 1-like isoform X3	Protein metabolism	19	7	0.263	0.486	0.588	-0.290	0.565	0.607	-0.006	0.284	0.983
102	gi|112984404	ribosomal protein L15	Protein metabolism	16	10	0.515	0.389	0.186	-0.663	0.433	0.126	-0.114	0.225	0.612
103	gi|112984224	"alanine--tRNA ligase, cytoplasmic "	Protein metabolism	45	15	-0.033	0.351	0.926	0.081	0.404	0.841	0.127	0.213	0.550
104	gi|512922422	tumor protein D54-like isoform X2	Protein metabolism	22	16	-0.286	0.378	0.449	0.228	0.433	0.599	-0.154	0.236	0.514
105	gi|112982671	ribosomal protein S12	Protein metabolism	38	26	0.514	0.331	0.121	-0.647	0.377	0.086	-0.104	0.196	0.595
106	gi|148298793	ribosomal protein P1	Protein metabolism	28	23	0.510	0.337	0.130	-0.659	0.367	0.072	-0.149	0.194	0.442
107	gi|112983228	eukaryotic translation initiation factor 3 subunit C	Protein metabolism	36	15	0.550	0.380	0.147	-0.284	0.425	0.503	0.218	0.216	0.313
108	gi|114052088	vacuolar ATP synthase subunit E	ATP utilizing	36	18	0.229	0.331	0.489	-0.424	0.366	0.247	-0.178	0.196	0.362
109	gi|162952033	40S ribosomal protein SA	Protein metabolism	57	39	0.461	0.303	0.128	-0.418	0.335	0.212	0.036	0.176	0.837
110	gi|112984336	ribosomal protein P2	Protein metabolism	16	9	0.150	0.496	0.763	0.581	0.609	0.340	0.705	0.306	0.021
111	gi|151301107	eukaryotic translation initiation factor 1A	Protein metabolism	24	11	-0.103	0.373	0.781	0.147	0.424	0.728	0.043	0.229	0.850

112	gi|112984058	ribosomal protein S7	Protein metabolism	32	17	0.666	0.304	0.028	-0.550	0.339	0.105	0.120	0.179	0.504
113	gi|148298732	ribosomal protein S20	Protein metabolism	14	9	0.712	0.418	0.089	-0.782	0.464	0.092	-0.061	0.234	0.796
114	gi|512911666	eukaryotic peptide chain release factor subunit 1-like isoform X2	Protein metabolism	20	13	-0.002	0.351	0.995	-0.234	0.388	0.547	-0.252	0.209	0.226
115	gi|112984164	ribosomal protein L35	Protein metabolism	22	13	0.635	0.368	0.084	-0.367	0.418	0.380	0.254	0.216	0.240
116	gi|112983314	ras-related GTP-binding protein Rab11	Vesicle transport	20	13	-0.319	0.407	0.433	-0.252	0.447	0.573	-0.507	0.238	0.033
117	gi|512901326	T-complex protein 1 subunit eta-like	Protein metabolism	52	13	-0.026	0.388	0.947	-0.248	0.429	0.563	-0.279	0.216	0.196
118	gi|112984334	ribosomal protein L11	Protein metabolism	29	20	0.368	0.321	0.252	-0.482	0.352	0.172	-0.118	0.185	0.523
119	gi|512924292	heterogeneous nuclear ribonucleoprotein 87F-like	Protein metabolism	18	8	-0.008	0.445	0.985	-0.815	0.485	0.093	-0.753	0.261	0.004
120	gi|525342791	plasminogen activator inhibitor 1 RNA-binding protein-like	Protein metabolism	10	9	0.568	0.430	0.186	-0.797	0.483	0.098	-0.268	0.242	0.267
121	gi|112983184	eukaryotic translation initiation factor 3 subunit B	Protein metabolism	33	10	-0.025	0.399	0.951	-0.196	0.453	0.665	-0.168	0.243	0.491
122	gi|112984274	ribosomal protein L23	Protein metabolism	28	28	0.730	0.337	0.030	-0.307	0.378	0.417	0.394	0.189	0.037
124	gi|112982996	thiol peroxiredoxin	Oxidoreductase	17	16	0.966	0.366	0.008	-1.042	0.403	0.010	-0.070	0.201	0.729
125	gi|512911643	EH domain-containing protein 1-like	Vesicle transport	33	5	0.097	0.577	0.867	-0.210	0.666	0.753	-0.404	0.352	0.252
126	gi|512886672	bifunctional glutamate/proline--tRNA ligase-like	Protein metabolism	62	6	0.838	0.480	0.081	-0.779	0.543	0.151	0.053	0.267	0.844
127	gi|112982910	40S ribosomal protein S28	Protein metabolism	24	23	0.656	0.419	0.117	-0.529	0.468	0.258	0.100	0.226	0.657
128	gi|112982721	zinc finger protein	Other	10	10	0.340	0.502	0.499	-0.287	0.580	0.621	0.134	0.292	0.647
129	gi|512899487	elongation factor 1-alpha 2-like	Protein metabolism	422	10	-0.377	0.556	0.497	0.896	0.654	0.171	0.520	0.332	0.117
130	gi|112984230	ribosomal protein L26	Protein metabolism	25	13	0.889	0.357	0.013	-0.738	0.397	0.063	0.160	0.201	0.426

131	gi|512909262	"asparagine--tRNA ligase, cytoplasmic-like "	Protein metabolism	25	14	-0.229	0.353	0.516	0.427	0.401	0.287	0.196	0.221	0.373
132	gi|112983980	ribosomal protein S19	Protein metabolism	35	15	0.409	0.380	0.283	-0.494	0.425	0.245	-0.065	0.225	0.772
133	gi|148298875	ribosomal protein L31	Protein metabolism	15	8	0.526	0.422	0.213	-0.630	0.474	0.184	-0.079	0.243	0.745
134	gi|112984182	ribosomal protein L30	Protein metabolism	30	13	0.991	0.344	0.004	-0.778	0.388	0.045	0.248	0.198	0.211
135	gi|112982865	profilin	Structure	30	24	0.160	0.314	0.611	-0.092	0.345	0.791	0.080	0.187	0.667
136	gi|512888773	C-terminal-binding protein-like	Protein metabolism	82	6	-0.250	0.540	0.643	-0.178	0.588	0.762	-0.594	0.295	0.044
137	gi|512910560	protein singed-like	Structure	33	6	0.183	0.533	0.731	-0.186	0.578	0.748	-0.047	0.316	0.882
138	gi|512916352	phosphoglycerate kinase-like	Energy	14	9	-0.056	0.444	0.899	0.282	0.512	0.581	0.224	0.275	0.417
139	gi|512920681	"isoleucine--tRNA ligase, cytoplasmic-like "	Protein metabolism	29	5	0.754	0.516	0.144	-0.861	0.571	0.132	-0.074	0.285	0.796
140	gi|112983032	calreticulin precursor	Signaling	14	10	-0.048	0.381	0.901	0.156	0.440	0.722	0.206	0.235	0.379
141	gi|114050729	vacuolar ATPase subunit C	ATP utilizing	17	6	-0.555	0.465	0.232	-0.206	0.497	0.679	-0.702	0.277	0.011
142	gi|151301198	mobility group protein 1B	Protein metabolism	27	15	-0.332	0.397	0.403	0.169	0.448	0.705	-0.200	0.241	0.408
143	gi|151301000	ribosomal protein S24	Protein metabolism	13	12	0.146	0.466	0.755	-0.309	0.514	0.547	-0.147	0.271	0.589
144	gi|114053073	proteasome subunit beta 7	Protein metabolism	21	7	0.261	0.480	0.586	-0.348	0.558	0.533	0.032	0.280	0.908
145	gi|112983862	selenophosphate synthetase 1	Oxidoreductase	47	10	-0.046	0.433	0.916	0.357	0.505	0.480	0.306	0.273	0.262
146	gi|512904754	actin-interacting protein 1-like	Structure	9	8	0.612	0.470	0.192	-0.484	0.542	0.372	0.144	0.265	0.585
147	gi|112984454	protein disulfide isomerase precursor	Oxidoreductase	26	8	0.212	0.544	0.696	0.206	0.641	0.747	0.422	0.317	0.183
148	gi|112983600	cellular retinoic acid binding protein	Other	7	4	1.071	0.654	0.102	-1.570	0.716	0.028	-0.605	0.319	0.058
149	gi|512920944	" tyrosine--tRNA ligase, cytoplasmic-like "	Protein metabolism	35	15	0.024	0.363	0.947	0.102	0.413	0.805	0.156	0.220	0.479
151	gi|512918406	nucleolar protein 58-like isoform X2	Protein metabolism	26	3	-0.786	0.681	0.248	0.242	0.745	0.745	-0.529	0.422	0.210
152	gi|512900540	ryanodine receptor 44F-	Signaling	166	5	-0.109	0.616	0.859	-0.229	0.765	0.765	-0.287	0.405	0.478

		like												
153	gi|114051245	proteasome alpha 3 subunit	Protein metabolism	17	7	0.401	0.431	0.352	-0.585	0.478	0.221	-0.237	0.244	0.331
154	gi|115345341	receptor for activated protein kinase C RACK 1 isoform 1	Signaling	14	13	1.294	0.362	0.000	-1.089	0.403	0.007	0.246	0.201	0.221
155	gi|114051564	glutathione peroxidase	Oxidoreductase	14	6	0.319	0.473	0.500	-0.867	0.515	0.092	-0.563	0.266	0.035
156	gi|512892030	ubiquitin-fold modifier 1-like isoform X1	Protein metabolism	27	13	-0.090	0.427	0.834	-0.301	0.466	0.518	-0.418	0.246	0.090
158	gi|512895440	ubiquitin-like modifier-activating enzyme 1-like	Protein metabolism	18	5	0.119	0.480	0.804	-0.218	0.539	0.686	-0.095	0.287	0.741
159	gi|512890123	"serine--tRNA ligase, cytoplasmic-like "	Protein metabolism	39	6	-0.164	0.726	0.821	-0.238	0.797	0.765	-0.317	0.456	0.486
160	gi|114050993	proteasome zeta subunit	Protein metabolism	16	7	0.404	0.461	0.381	-0.487	0.529	0.357	-0.048	0.271	0.859
161	gi|512894841	twitchin-like	Structure	248	12	0.846	0.378	0.025	-0.573	0.428	0.181	0.303	0.223	0.175
162	gi|114053311	26S protease regulatory subunit 6B	Protein metabolism	18	9	0.621	0.407	0.127	-0.306	0.467	0.512	0.324	0.239	0.175
163	gi|153792659	actin-depolymerizing factor 1	Structure	33	22	0.002	0.324	0.995	-0.252	0.356	0.479	-0.264	0.192	0.170
164	gi|512897336 ; gi|512897332	GTP-binding protein 128up isoform X2	Other	16	3	0.177	0.732	0.809	0.155	0.991	0.876	0.350	0.509	0.491
165	gi|512938594	"histone H3.3-like, partial "	Cell cycle	21	9	0.740	0.449	0.099	-0.220	0.527	0.676	0.579	0.269	0.031
166	gi|112983036	glucosidase precursor	Energy	12	8	0.810	0.394	0.040	-0.177	0.455	0.698	0.664	0.231	0.004
167	gi|114053033	Ef1alpha-like factor isoform 1	Protein metabolism	12	5	-0.120	0.455	0.792	0.030	0.537	0.955	0.044	0.286	0.877
168	gi|512899483	glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1-like	Energy	43	8	-0.361	0.420	0.390	0.306	0.468	0.513	-0.016	0.259	0.951
169	gi|114052645	thymosin isoform 1	Other	23	6	-1.326	0.510	0.009	0.475	0.567	0.401	-0.846	0.344	0.014
170	gi|114051800	eukaryotic translation initiation factor 3 subunit I	Protein metabolism	14	8	0.137	0.441	0.757	-0.388	0.489	0.427	-0.302	0.266	0.257
171	gi|512916235	ATP-binding cassette sub-family F member 1-like	ATP utilizing	18	4	-0.699	0.743	0.347	1.190	0.834	0.154	0.472	0.479	0.325

172	gi|512918255	protein suppressor of white apricot-like	Protein metabolism	37	4	-1.215	0.491	0.013	0.383	0.528	0.468	-0.803	0.306	0.009
173	gi|512917246	ras-related protein Rab-35-like	Vesicle transport	19	1	-1.668	0.949	0.079	0.705	0.996	0.479	-0.963	0.619	0.120
174	gi|512931070	26S proteasome non-ATPase regulatory subunit 2-like	Protein metabolism	23	5	-0.353	0.481	0.464	0.223	0.547	0.683	-0.091	0.297	0.758
175	gi|112984158	ribosomal protein L36	Protein metabolism	9	5	0.309	0.555	0.578	-0.496	0.631	0.432	-0.253	0.323	0.434
176	gi|114052086	proteasome 26S non-ATPase subunit 12	Protein metabolism	16	4	-0.420	0.755	0.578	0.397	0.970	0.683	0.155	0.485	0.750
177	gi|112984362	eukaryotic translation initiation factor 2 alpha subunit	Protein metabolism	19	9	-0.011	0.390	0.977	-0.636	0.419	0.128	-0.670	0.227	0.003
178	gi|112983505	ribosomal protein S10	Protein metabolism	14	12	0.412	0.430	0.338	-0.560	0.509	0.270	0.034	0.249	0.890
179	gi|114052488	alcohol dehydrogenase	Oxidoreductase	10	4	-0.115	0.598	0.847	-0.031	0.671	0.963	-0.114	0.353	0.746
180	gi|120444903	chaperonin	Protein metabolism	59	14	0.593	0.334	0.076	-0.361	0.384	0.347	0.212	0.197	0.284
181	gi|512921130 ; gi|114052663	V-type proton ATPase subunit H-like ; vacuolar ATP synthase subunit H	ATP utilizing	14	3	-0.545	0.849	0.521	0.131	0.927	0.888	-0.417	0.493	0.398
182	gi|114051243	FK506-binding protein	Protein metabolism	22	18	-0.228	0.374	0.543	0.086	0.423	0.839	-0.124	0.226	0.581
183	gi|290560655 ; gi|114052018	nucleosome assembly protein isoform 1 ; nucleosome assembly protein isoform 2	Cell cycle	16	12	0.068	0.382	0.860	0.356	0.438	0.417	0.419	0.233	0.073
184	gi|112983546	ribosomal protein L12	Protein metabolism	33	15	0.948	0.351	0.007	-0.916	0.391	0.019	-0.007	0.201	0.973
185	gi|114052252	calmodulin	Signaling	14	13	0.037	0.358	0.917	-0.508	0.392	0.195	-0.452	0.209	0.031
186	gi|151301158	ribosomal protein L34	Protein metabolism	14	11	0.812	0.346	0.019	-0.921	0.384	0.017	-0.106	0.199	0.594
187	gi|512917455 ; ; gi|512917451	gephyrin-like isoform X2 ; gephyrin-like isoform X1	Structure	31	12	1.666	0.490	0.001	-1.706	0.557	0.002	0.042	0.261	0.872
188	gi|512901637	26S proteasome non-ATPase regulatory subunit	Protein metabolism	23	6	0.082	0.452	0.856	0.468	0.552	0.397	0.558	0.296	0.059

		11-like												
189	gi|187281708	triosephosphate isomerase	Energy	5	5	-0.317	0.433	0.464	0.328	0.501	0.513	0.054	0.273	0.843
190	gi|112983564	45 kDa immunophilin FKBP45	Heat shock	19	5	0.385	0.559	0.491	-0.042	0.647	0.949	0.314	0.340	0.355
191	gi|114053191	glycine--tRNA ligase	Protein metabolism	43	6	0.519	0.505	0.305	-0.788	0.569	0.166	-0.344	0.281	0.220
192	gi|512933487	tubulin alpha-1 chain-like	Structure	260	38	-0.638	0.346	0.065	0.162	0.371	0.661	-0.428	0.216	0.048
193	gi|112983904	ribosomal protein S30	Protein metabolism	5	4	-0.640	0.658	0.330	0.107	0.715	0.882	-0.469	0.394	0.234
194	gi|112983932	ribosomal protein S27	Protein metabolism	7	7	0.620	0.396	0.118	-0.765	0.440	0.082	-0.102	0.229	0.654
195	gi|512926172	probable 26S proteasome non-ATPase regulatory subunit 3-like	Protein metabolism	32	6	-0.430	0.464	0.354	0.072	0.521	0.890	-0.366	0.290	0.207
196	gi|114052545	chaperonin subunit 4 delta	Protein metabolism	32	7	0.472	0.491	0.336	-0.483	0.541	0.372	-0.131	0.280	0.640
197	gi|289629222	coatomer protein complex subunit epsilon	Vesicle transport	10	8	0.537	0.441	0.223	-0.258	0.519	0.619	0.280	0.264	0.288
198	gi|512896698	NEDD8-like	Cell cycle	23	10	-0.133	0.627	0.832	-0.058	0.766	0.939	-0.070	0.411	0.865
199	gi|512934037	SUMO-activating enzyme subunit 2-like	Protein metabolism	26	6	-0.254	0.454	0.576	0.019	0.508	0.971	-0.280	0.275	0.309
200	gi|512908267	LOW QUALITY PROTEIN: protein transport protein Sec24C-like	Vesicle transport	18	1	0.368	1.002	0.713	-0.778	1.102	0.480	-0.410	0.581	0.481
201	gi|112983280	Hsc70/Hsp90-organizing protein HOP	Heat shock	28	5	0.233	0.529	0.660	-0.422	0.611	0.490	-0.178	0.321	0.580
202	gi|114052613	transaldolase	Energy	25	6	0.254	0.513	0.620	-0.058	0.606	0.924	0.211	0.303	0.486
203	gi|124430725	sex-specific storage-protein 2 precursor	Vesicle transport	27	12	-1.979	0.330	0.000	2.685	0.404	0.000	0.730	0.232	0.002
204	gi|134948671	small nuclear ribonucleoprotein sm d2	Protein metabolism	17	5	0.344	0.732	0.639	-0.574	0.770	0.456	-0.229	0.411	0.578
205	gi|512894972	T-complex protein 1 subunit alpha-like	Protein metabolism	57	5	0.153	0.498	0.759	-0.338	0.561	0.547	-0.205	0.287	0.474
206	gi|512936939	aldose reductase-like	Oxidoreductase	36	8	0.339	0.439	0.440	-0.283	0.503	0.574	0.039	0.265	0.882
207	gi|112983000	protein translation factor SUI1 homolog	Protein metabolism	16	8	0.402	0.458	0.380	-0.473	0.534	0.376	0.048	0.276	0.861

209	gi|112984050	proliferating cell nuclear antigen	Protein metabolism	11	4	-0.209	0.614	0.733	0.525	0.690	0.446	0.311	0.388	0.423
210	gi|112983942	ribosomal protein S25	Protein metabolism	26	8	0.747	0.409	0.068	-0.893	0.454	0.049	-0.134	0.233	0.565
211	gi|114053117	eukaryotic translation initiation factor 3 subunit K	Protein metabolism	22	4	-0.561	0.525	0.285	0.107	0.576	0.853	-0.525	0.332	0.114
212	gi|114050749	chaperonin subunit 6a zeta	Vesicle transport	36	3	-0.089	0.708	0.900	0.496	0.838	0.554	0.396	0.466	0.396
213	gi|114052034	proteasome beta-subunit	Protein metabolism	14	2	0.696	0.593	0.241	-0.519	0.689	0.451	0.156	0.352	0.659
214	gi|512924346	glutaredoxin 3-like	Oxidoreductase	34	27	0.337	0.379	0.375	-0.542	0.416	0.193	-0.191	0.210	0.362
216	gi|112983090	eukaryotic translation initiation factor 3 subunit J	Protein metabolism	15	5	0.509	0.495	0.304	-0.720	0.554	0.193	-0.251	0.279	0.368
217	gi|512890860	proteasome subunit beta type-5-like	Protein metabolism	13	3	0.134	0.593	0.821	-0.293	0.653	0.653	-0.142	0.364	0.696
218	gi|112982701	40S ribosomal protein S14	Protein metabolism	8	8	0.569	0.457	0.213	-0.455	0.511	0.374	0.116	0.266	0.663
219	gi|112983936	nonclathrin coat protein gamma1-COP	Other	16	4	-0.033	0.568	0.954	0.408	0.690	0.554	0.402	0.372	0.280
220	gi|512888540	serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform-like isoform X1	Signaling	34	6	-0.204	0.551	0.712	0.030	0.594	0.959	-0.146	0.324	0.653
221	gi|512926411	protein Mo25-like	Other	30	3	-0.919	0.633	0.147	0.576	0.706	0.415	-0.364	0.394	0.355
223	gi|512894321	26S protease regulatory subunit 6A-like	Protein metabolism	30	5	0.095	0.473	0.841	-0.080	0.529	0.880	-0.079	0.286	0.783
224	gi|512935385	T-complex protein 1 subunit epsilon-like	Protein metabolism	35	3	0.018	0.661	0.979	0.026	0.825	0.975	0.007	0.440	0.987
225	gi|114050831	uridine 5'-monophosphate synthase	Protein metabolism	39	6	-0.230	0.429	0.593	-0.388	0.466	0.405	-0.602	0.257	0.019
226	gi|112984156	ribosomal protein L35A	Protein metabolism	15	3	1.059	0.554	0.056	-1.317	0.618	0.033	-0.212	0.299	0.479
227	gi|512919737	ubiquitin-conjugating enzyme E2-17 kDa-like isoform X1	Protein metabolism	17	12	0.174	0.331	0.598	-0.180	0.366	0.623	0.015	0.199	0.941
228	gi|512914087	probable glutamine--tRNA ligase-like	Protein metabolism	21	5	0.423	0.500	0.398	-0.499	0.565	0.377	0.024	0.286	0.934

230	gi|112982916	RNA binding motif protein Y14	Protein metabolism	4	2	1.267	0.972	0.193	-0.999	1.214	0.411	0.386	0.541	0.475
231	gi|512888904	cytoplasmic aconitate hydratase-like	Energy	33	7	0.130	0.471	0.783	0.212	0.559	0.704	0.372	0.299	0.213
232	gi|512920045	titin-like	Structure	89	5	-0.884	0.702	0.208	0.951	0.770	0.217	0.007	0.445	0.988
233	gi|512929539	MATH and LRR domain-containing protein PFE0570w-like	Protein metabolism	29	1	0.000	0.201	1.000	0.000	0.213	1.000	0.000	0.116	1.000
235	gi|261245099	juvenile hormone epoxide hydrolase-like protein 3	Signaling	27	9	-0.087	0.445	0.844	-0.430	0.469	0.359	-0.512	0.256	0.046
236	gi|512899857	signal recognition particle subunit SRP72-like	Signaling	20	4	-0.141	0.674	0.835	-0.273	0.711	0.701	-0.374	0.388	0.335
237	gi|153792609	tropomyosin-2 isoform 4	Structure	10	5	-0.512	0.729	0.483	-0.063	0.770	0.935	-0.542	0.414	0.191
238	gi|112983082	cytosolic juvenile hormone binding protein 36 kDa subunit	Signaling	5	3	0.482	0.629	0.444	-0.847	0.690	0.220	-0.404	0.365	0.268
239	gi|112983958	Annexin IX isoform A	Structure	14	4	-1.019	0.661	0.123	0.581	0.671	0.386	-0.307	0.436	0.481
240	gi|112984248	ribosomal protein L24	Protein metabolism	16	5	0.373	0.597	0.532	-0.722	0.652	0.268	-0.334	0.331	0.313
241	gi|114052609	nucleoplasmin isoform 2	Protein metabolism	37	14	0.333	0.380	0.380	-0.481	0.419	0.251	-0.133	0.222	0.549
243	gi|512903376	26S proteasome non-ATPase regulatory subunit 8-like	Protein metabolism	7	2	0.130	0.791	0.869	0.964	1.057	0.362	1.071	0.574	0.062
244	gi|512896918	acidic leucine-rich nuclear phosphoprotein 32 family member A-like isoform X1	Protein metabolism	4	2	-0.229	0.709	0.747	0.015	0.814	0.985	-0.217	0.449	0.629
245	gi|304571947	phenylalanyl-tRNA synthetase beta subunit	Protein metabolism	23	3	-0.267	0.614	0.663	-0.314	0.757	0.679	-0.689	0.424	0.105
246	gi|112983938	eukaryotic translation initiation factor 3 subunit E	Protein metabolism	10	4	0.769	0.672	0.253	-0.377	0.738	0.610	0.582	0.367	0.113
247	gi|290560891	coatomer protein complex subunit beta 2	Protein metabolism	27	9	0.383	0.442	0.386	-0.456	0.499	0.361	-0.106	0.254	0.676
248	gi|512918634 ; gi|512918630	la protein homolog isoform X2 ; la protein homolog isoform X1	Other	22	4	0.885	0.425	0.037	-0.462	0.489	0.345	0.426	0.254	0.093

250	gi|512921230 ; gi|512921226	nucleobindin-2-like isoform X2 ; nucleobindin-2-like isoform X1	Structure	10	2	-0.113	0.968	0.907	-0.472	1.047	0.652	-0.679	0.593	0.252
251	gi|512885391	"thioredoxin reductase 1, mitochondrial-like isoform X1 "	Oxidoreductase	24	8	0.301	0.420	0.473	-0.366	0.470	0.435	-0.052	0.249	0.834
252	gi|512897194	protein arginine N-methyltransferase 8-like	Other	10	3	0.186	0.695	0.789	-0.537	0.769	0.485	-0.338	0.404	0.403
253	gi|512936747	LOW QUALITY PROTEIN: multiple PDZ domain protein-like	Signaling	67	6	0.718	0.553	0.194	-0.907	0.616	0.141	-0.241	0.316	0.445
254	gi|512913438 ; gi|512913434 ; gi|512913430	DEAD-box helicase Dbp80-like isoform X3	Protein metabolism	26	4	0.066	0.623	0.916	-0.291	0.687	0.672	-0.243	0.354	0.492
256	gi|112982928	eukaryotic translation initiation factor 2 subunit 2	Protein metabolism	12	6	0.006	0.557	0.991	0.106	0.640	0.869	0.115	0.334	0.732
257	gi|112982855	ribosomal protein S15A	Protein metabolism	11	7	-0.263	0.473	0.578	0.108	0.528	0.838	-0.104	0.283	0.713
258	gi|267844871	vacuolar protein sorting 4	ATP utilizing	21	3	0.535	0.692	0.440	-0.681	0.790	0.389	-0.086	0.398	0.828
260	gi|512895472 ; gi|512895468	structural maintenance of chromosomes protein 1A-like	Cell cycle	66	1	-0.063	1.472	0.966	-0.455	1.503	0.762	-0.518	0.824	0.530
261	gi|575771781	alpha-crystallin B chain-like precursor	Heat shock	20	26	-0.835	0.342	0.015	0.984	0.405	0.015	0.384	0.223	0.085
262	gi|512902948	juvenile hormone esterase-like	Other	39	24	-1.290	0.342	0.000	0.429	0.363	0.237	-0.908	0.203	0.000
263	gi|512936951	uncharacterized protein LOC101739986	uncharacterized	49	3	-0.674	0.766	0.379	1.440	0.936	0.124	0.793	0.465	0.088
264	gi|512902688	zinc finger RNA-binding protein-like isoform X1	Protein metabolism	8	2	-0.308	1.111	0.782	-0.415	0.961	0.666	-0.704	0.615	0.252
265	gi|112982970	eukaryotic translation initiation factor 3 subunit G	Protein metabolism	25	2	0.808	0.775	0.297	-0.486	0.903	0.591	0.365	0.446	0.414
266	gi|512885415	uncharacterized protein LOC101738424	uncharacterized	20	1	0.091	1.423	0.949	-0.985	1.544	0.523	-0.894	0.757	0.237
267	gi|112983523	ribosomal protein L10A	Protein metabolism	24	14	0.485	0.372	0.191	-0.534	0.425	0.209	-0.010	0.223	0.966

269	gi|512909791	protein bicaudal D-like	Vesicle transport	6	1	-1.230	0.930	0.186	1.171	1.081	0.279	-0.059	0.601	0.922
270	gi|112982683	catalase	Oxidoreductase	16	4	-0.551	0.577	0.340	1.081	0.696	0.121	0.544	0.391	0.164
271	gi|512901569	trifunctional purine biosynthetic protein adenosine-3-like	Other	8	7	-0.158	0.480	0.742	0.392	0.605	0.517	0.207	0.335	0.536
272	gi|112982812	ribosomal protein L6	Protein metabolism	38	3	0.217	0.643	0.736	-0.114	0.756	0.880	0.118	0.398	0.767
273	gi|512928183	ubiquitin-conjugating enzyme E2 variant 2-like	Protein metabolism	15	6	0.426	0.622	0.493	-0.176	0.636	0.782	0.160	0.326	0.622
274	gi|168823429	kettin protein	Structure	6	3	1.611	0.655	0.014	-1.273	0.758	0.093	0.390	0.354	0.270
275	gi|114052615	nonmuscle myosin essential light chain	Structure	101	3	-0.153	0.558	0.784	-0.456	0.594	0.443	-0.663	0.329	0.044
276	gi|512885572	AP-1 complex subunit gamma-1-like isoform X1	Vesicle transport	7	1	-0.935	1.154	0.418	0.956	1.339	0.475	0.021	0.723	0.977
277	gi|512894099	protein SEC13 homolog	Vesicle transport	27	2	0.050	1.209	0.967	0.816	1.433	0.569	0.602	0.721	0.404
278	gi|512938655	fatty acid-binding protein-like	Lipid	12	3	1.267	0.574	0.027	-1.414	0.638	0.027	-0.129	0.312	0.679
279	gi|512936110 ; gi|512936106	heterogeneous nuclear ribonucleoprotein K-like isoform X2 ; heterogeneous nuclear ribonucleoprotein K-like isoform X1	Protein metabolism	8	4	0.065	0.494	0.895	-0.077	0.560	0.891	0.045	0.295	0.879
280	gi|112983080	repressor splicing factor 1	Protein metabolism	23	8	-0.126	0.453	0.782	-0.171	0.504	0.735	-0.240	0.268	0.370
281	gi|512899104	"26S protease regulatory subunit 4-like, partial "	Protein metabolism	10	2	0.243	0.739	0.742	-0.087	0.868	0.920	0.191	0.430	0.657
282	gi|512926210	26S proteasome non-ATPase regulatory subunit 6-like	Protein metabolism	21	2	0.405	0.779	0.603	0.256	0.933	0.783	0.716	0.454	0.115
283	gi|512899908	protein Spindly-like	Other	25	2	-0.534	0.888	0.548	0.235	1.023	0.818	-0.311	0.578	0.591
284	gi|114053249	vacuolar ATP synthase subunit D	ATP utilizing	13	7	-0.005	0.493	0.992	0.025	0.563	0.964	0.027	0.306	0.930
285	gi|512930286	constitutive coactivator of PPAR-gamma-like protein 1-like	Oxidoreductase	25	2	-0.850	1.231	0.490	0.698	1.459	0.633	0.122	0.904	0.893

286	gi|512927551	mannose-1-phosphate guanyltransferase beta-like	Energy	23	5	0.149	0.543	0.783	0.066	0.653	0.919	0.238	0.345	0.490
287	gi|114053141	exuperantia	Other	14	3	-0.394	0.745	0.597	0.552	0.916	0.546	0.119	0.494	0.810
288	gi|114050835	ubiquitin-conjugating enzyme E2	Protein metabolism	13	1	-0.004	1.168	0.997	-0.469	1.313	0.721	-0.473	0.712	0.506
289	gi|512893009	importin-5-like	Protein metabolism	3	6	-0.393	0.722	0.586	0.488	0.767	0.525	0.068	0.432	0.876
290	gi|112982751	60S ribosomal protein L38	Protein metabolism	32	3	1.228	0.549	0.025	-1.578	0.607	0.009	-0.352	0.298	0.237
291	gi|512889459	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1-like	Other	4	1	0.039	1.169	0.973	0.448	1.431	0.754	0.487	0.785	0.535
292	gi|112983527	ribosomal protein L27A	Protein metabolism	24	5	0.634	0.435	0.145	-0.602	0.491	0.220	0.036	0.251	0.887
293	gi|512903199	putative ATP-dependent RNA helicase me31b-like	Protein metabolism	21	2	-0.271	0.881	0.759	-0.297	0.975	0.760	-0.572	0.495	0.248
294	gi|112984318	60S ribosomal protein L18	Protein metabolism	8	3	-0.444	0.813	0.585	0.610	1.066	0.567	0.157	0.569	0.783
295	gi|512896804	protein bicaudal C homolog 1-B-like	Other	15	1	-0.439	1.142	0.700	0.436	1.209	0.718	-0.003	0.738	0.997
296	gi|512907059	putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15-like isoform X1	Protein metabolism	27	1	-0.273	1.328	0.837	-0.432	1.301	0.740	-0.705	0.794	0.375
297	gi|153791944	farnesyl diphosphate synthase 3	Other	20	12	-0.259	0.390	0.507	0.377	0.441	0.393	0.140	0.239	0.557
298	gi|512898155	probable RNA-binding protein 46-like	Protein metabolism	23	2	-0.356	0.689	0.605	0.315	0.815	0.699	-0.029	0.446	0.948
299	gi|512907580	H/ACA ribonucleoprotein complex subunit 4-like	Protein metabolism	7	5	-0.074	0.600	0.901	-0.274	0.698	0.695	-0.339	0.387	0.382
300	gi|112984216	ribosomal protein L27	Protein metabolism	22	5	-0.022	0.482	0.963	-0.001	0.555	0.999	-0.058	0.286	0.840
301	gi|112983366	protein disulfide-isomerase like protein ERp57 precursor	Oxidoreductase	14	3	0.983	0.765	0.199	-1.115	0.871	0.200	-0.176	0.444	0.692
302	gi|350534642	DJ-1 beta	Oxidoreductase	40	5	0.321	0.406	0.430	-0.305	0.458	0.506	0.030	0.243	0.903
303	gi|512917969	LOW QUALITY PROTEIN: serine/arginine-rich	Protein metabolism	26	2	0.233	1.098	0.832	0.281	1.313	0.831	0.580	0.696	0.405

		splicing factor 1A-like												
304	gi|512914524	LOW QUALITY PROTEIN: 26S proteasome non-ATPase regulatory subunit 1-like	Protein metabolism	7	1	-0.225	1.016	0.825	-0.547	0.992	0.581	-0.772	0.604	0.201
305	gi|512892438	myc box-dependent-interacting protein 1-like	Protein metabolism	27	2	0.394	0.882	0.655	-0.733	0.996	0.461	-0.290	0.483	0.549
306	gi|512895291	microtubule-associated protein futsch-like	Structure	11	6	-0.172	0.648	0.790	0.325	0.709	0.647	0.129	0.379	0.734
307	gi|512893242	uncharacterized protein LOC101739009	uncharacterized	165	1	-0.578	1.586	0.716	-1.315	1.574	0.403	-1.893	0.804	0.019
308	gi|512893176	vacuolar protein sorting-associated protein 26-like	Vesicle transport	41	2	1.190	1.069	0.266	-1.085	1.327	0.414	0.471	0.516	0.360
309	gi|289629218	coatomer protein complex subunit beta	Vesicle transport	13	3	0.438	0.621	0.481	-0.210	0.697	0.764	0.178	0.379	0.638
310	gi|114053203	DnaJ (Hsp40) homolog 2	Heat shock	24	1	0.851	0.926	0.358	-0.541	1.095	0.621	0.310	0.560	0.580
311	gi|289629220	coatomer protein complex subunit delta	Vesicle transport	28	4	0.543	0.502	0.280	-0.306	0.585	0.601	0.206	0.308	0.504
312	gi|112983974	ubiquitin-like protein SMT3	Protein metabolism	11	13	0.441	0.402	0.272	-0.365	0.455	0.423	0.091	0.236	0.701
313	gi|512891911	clavesin-1-like	Vesicle transport	17	4	-2.586	0.618	0.000	1.530	0.604	0.011	-1.077	0.403	0.008
314	gi|512885026	"FK506-binding protein 59-like, partial "	Heat shock	34	10	0.621	0.546	0.256	-0.788	0.582	0.176	-0.237	0.308	0.442
315	gi|512917953	"dynein light chain 2, cytoplasmic-like isoform X1 "	Other	17	4	0.866	0.624	0.165	-1.005	0.689	0.145	-0.159	0.331	0.631
316	gi|512888046	myrosinase 1-like	Other	21	2	-0.476	0.679	0.483	-0.997	0.686	0.146	-1.471	0.389	0.000
317	gi|512926559	uncharacterized protein LOC101740835	uncharacterized	15	2	-0.549	0.956	0.565	-0.550	1.013	0.587	-1.045	0.557	0.061
318	gi|512924855	sorting nexin-12-like isoform X1	Protein metabolism	23	4	0.318	0.783	0.685	0.780	0.955	0.414	0.633	0.451	0.160
320	gi|112983924	40S ribosomal protein S29	Protein metabolism	6	10	0.842	0.387	0.030	-0.729	0.430	0.090	0.132	0.221	0.551
321	gi|512908244	carbonyl reductase [NADPH] 1-like	Oxidoreductase	21	3	-0.074	0.674	0.913	0.212	0.830	0.798	0.178	0.434	0.682
322	gi|112982998	superoxide dismutase [Cu-Zn]	Oxidoreductase	10	7	0.152	0.454	0.738	0.080	0.515	0.876	0.246	0.280	0.380

323	gi|512917049	protein purity of essence-like	Other	11	1	-0.332	1.368	0.808	0.000	0.213	1.000	0.000	0.116	1.000
324	gi|512905034	uncharacterized protein LOC101741149	uncharacterized	8	3	0.066	0.773	0.932	0.519	1.010	0.607	0.483	0.553	0.383
325	gi|114051239	cystathionine gamma-lyase	Signaling	123	2	0.522	0.656	0.426	-0.180	0.776	0.817	0.339	0.410	0.407
326	gi|112983334	methylthioribose-1-phosphate isomerase	Other	21	1	-0.172	1.600	0.914	-0.259	1.787	0.885	-0.430	0.897	0.632
327	gi|512918867	glucose-6-phosphate 1-dehydrogenase-like	Oxidoreductase	7	3	0.486	0.570	0.394	-0.318	0.668	0.634	0.152	0.350	0.665
328	gi|114052242	glutathione S-transferase omega 1	Oxidoreductase	25	3	0.860	0.658	0.191	-1.039	0.735	0.158	-0.192	0.367	0.602
329	gi|512890802	mannose-1-phosphate guanyltransferase alpha-A-like	Energy	21	2	0.391	0.710	0.581	-0.916	0.790	0.247	-0.497	0.410	0.225
330	gi|218505765	death-related protein	Cell cycle	14	6	0.649	0.645	0.314	-0.611	0.739	0.409	0.054	0.376	0.886
331	gi|512901109	LOW QUALITY PROTEIN: probable tRNA (guanine(26)-N(2))-dimethyltransferase-like	Protein metabolism	8	3	0.674	0.503	0.180	-0.677	0.564	0.230	-0.006	0.295	0.984
332	gi|512926635	LOW QUALITY PROTEIN: cell division cycle 5-like protein-like	Cell cycle	7	1	0.000	0.201	1.000	0.000	0.213	1.000	0.167	1.549	0.914
333	gi|148298750	6-phosphogluconolactonase	Energy	12	1	0.296	0.874	0.735	1.378	1.219	0.258	1.674	0.653	0.010
335	gi|512916207	inner centromere protein A-like	Cell cycle	28	2	0.114	0.721	0.874	-0.393	0.824	0.633	-0.278	0.445	0.533
336	gi|284813565	aliphatic nitrilase	Other	25	1	0.490	0.990	0.621	-0.286	1.177	0.808	0.204	0.611	0.738
337	gi|226501798	odorant-binding protein 6	ATP utilizing	23	1	0.498	1.425	0.727	-0.340	1.686	0.840	0.158	0.892	0.859
339	gi|114052925	DnaJ (Hsp40) homolog 13	Heat shock	6	9	0.136	0.428	0.751	-0.855	0.477	0.073	-0.578	0.250	0.021
340	gi|512906668 ; gi|512906664 ; gi|512906660	coiled-coil domain-containing protein 124-like isoform X3	Cell cycle	11	7	0.732	0.584	0.210	-0.779	0.657	0.236	-0.087	0.334	0.795
341	gi|512916289	proline-rich receptor-like protein kinase PERK2-like	Signaling	17	4	-0.153	0.809	0.850	-0.367	0.880	0.676	-0.393	0.459	0.392
342	gi|512939138	histone H4-like ; histone	Cell cycle	9	4	-0.340	0.760	0.654	0.794	0.912	0.384	0.533	0.443	0.230

	;	H4-like												
346	gi|512907757	dipeptidyl peptidase 3-like isoform X1	Lipid	1	2	0.949	0.769	0.217	-0.670	0.877	0.445	0.347	0.406	0.392
347	gi|114052605	26S proteasome regulatory ATPase subunit 10B	Protein metabolism	15	3	0.070	0.668	0.917	-0.239	0.755	0.752	-0.212	0.358	0.554
348	gi|112984128	ribosomal protein L37A	Protein metabolism	82	4	1.097	0.512	0.032	-0.654	0.600	0.275	0.473	0.297	0.111
349	gi|114326261	PTB-associated splicing factor	Other	15	2	0.175	0.946	0.853	0.295	1.298	0.820	0.545	0.662	0.411
350	gi|512893358	regulator of chromosome condensation-like	Cell cycle	4	2	0.742	0.596	0.213	-0.658	0.683	0.336	0.082	0.353	0.816
351	gi|512889113	multiple coagulation factor deficiency protein 2 homolog isoform X2	Vesicle transport	5	2	-0.025	0.785	0.975	1.109	0.990	0.263	0.771	0.496	0.121
352	gi|512890543	tRNA (cytosine(34)-C(5))-methyltransferase-like	Protein metabolism	7	2	-0.243	0.707	0.731	0.024	0.812	0.976	-0.209	0.448	0.641
353	gi|512902638	malignant T-cell-amplified sequence 1-like	Other	10	1	0.424	0.907	0.640	-0.249	1.067	0.815	0.175	0.557	0.753
355	gi|148298804	ribosomal protein L36A	Protein metabolism	33	4	0.771	0.521	0.139	-0.496	0.604	0.411	0.262	0.300	0.383
358	gi|114052058	thioredoxin	Oxidoreductase	40	2	0.224	0.627	0.721	-0.404	0.713	0.571	-0.229	0.371	0.538
360	gi|512900431	uncharacterized protein LOC101746893	uncharacterized	10	2	-0.050	0.794	0.950	0.212	0.943	0.822	0.137	0.517	0.791
362	gi|512915380	indole-3-acetaldehyde oxidase-like	Oxidoreductase	25	4	-0.031	0.516	0.953	0.071	0.594	0.905	0.045	0.319	0.887
363	gi|114053215	eukaryotic translation initiation factor 3 subunit F	Protein metabolism	70	2	0.043	0.712	0.952	-0.190	0.808	0.814	-0.141	0.427	0.741
365	gi|512932511	adenylosuccinate lyase-like	Energy	43	2	0.546	0.768	0.477	-0.135	1.116	0.904	0.773	0.568	0.173
366	gi|512927907 ; gi|512927903	probable ATP-dependent RNA helicase DDX17-like isoform X2	Protein metabolism	5	3	1.173	0.551	0.033	-0.156	0.699	0.824	1.319	0.338	0.000
367	gi|114050771	short-chain dehydrogenase/reductase	Oxidoreductase	33	1	0.639	0.945	0.499	-1.100	1.044	0.292	-0.461	0.530	0.385
370	gi|148298719	ribosomal protein L32	Protein metabolism	12	7	0.934	0.365	0.011	-1.024	0.400	0.011	-0.089	0.209	0.670
372	gi|512913526	eukaryotic translation initiation factor 3 subunit	Protein metabolism	40	2	0.777	0.685	0.257	-0.403	0.832	0.628	0.350	0.430	0.415

		M-like												
373	gi|512906261	26S protease regulatory subunit 8-like	Protein metabolism	14	7	0.151	0.460	0.743	-0.401	0.508	0.431	-0.248	0.275	0.367
374	gi|512935174	uncharacterized protein LOC101742281	uncharacterized	15	2	-0.542	0.707	0.443	-0.251	0.709	0.723	-0.868	0.429	0.043
375	gi|114053231	vacuolar ATP synthase subunit G	ATP utilizing	10	4	0.922	0.661	0.163	-0.856	0.752	0.255	0.105	0.376	0.781
376	gi|512926045	carbonyl reductase [NADPH] 1-like	Oxidoreductase	32	2	-0.386	0.726	0.595	-0.042	0.794	0.958	-0.421	0.445	0.344
377	gi|512926866	methionine aminopeptidase 1-like	Protein metabolism	61	1	-0.517	0.937	0.581	-0.194	1.013	0.848	-0.710	0.575	0.216
380	gi|114053119	chloride intracellular channel isoform 1	Cell cycle	31	3	-0.498	0.851	0.559	0.329	1.147	0.774	0.293	0.552	0.595
381	gi|512936196	"threonine--tRNA ligase, cytoplasmic-like isoform X1 "	Protein metabolism	12	2	-0.059	0.578	0.918	-0.114	0.663	0.864	-0.176	0.361	0.626
383	gi|112983864	ubiquitin-conjugating enzyme E2L	Protein metabolism	14	3	-0.505	0.708	0.475	0.333	0.818	0.684	-0.212	0.464	0.648
384	gi|512925980	"NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial-like "	Oxidoreductase	35	1	1.136	0.709	0.109	-1.181	0.803	0.141	-0.045	0.397	0.910
385	gi|512889134	uncharacterized protein LOC101746930 isoform X1	uncharacterized	136	1	-0.882	1.035	0.394	0.106	1.361	0.938	-0.776	0.783	0.322
386	gi|512893746	NECAP-like protein CG9132-like	Protein metabolism	7	1	-0.554	0.927	0.550	-0.215	1.013	0.832	-0.768	0.573	0.180
387	gi|512911991	uncharacterized protein LOC101739407	uncharacterized	10	4	0.410	0.626	0.513	-0.476	0.717	0.507	-0.044	0.376	0.906
388	gi|512929353	calcyclin-binding protein-like	Protein metabolism	24	1	-1.037	1.028	0.313	-0.555	1.017	0.585	-1.592	0.565	0.005
390	gi|512900795	uncharacterized protein LOC101737906	uncharacterized	4	13	-1.334	0.392	0.001	0.149	0.412	0.717	-1.191	0.236	0.000
392	gi|512932046	prolyl endopeptidase-like	Protease	50	2	-1.158	0.836	0.166	0.695	0.863	0.421	-0.527	0.539	0.328
393	gi|512892807	proteasome subunit beta type-6-like	Protein metabolism	10	3	-0.154	0.652	0.814	0.871	0.797	0.274	0.694	0.419	0.098
394	gi|512917642	bleomycin hydrolase-like isoform X1	Protease	15	2	-0.851	0.934	0.362	-0.124	0.985	0.900	-0.922	0.555	0.097
395	gi|114052765	stromal cell-derived factor	Other	20	1	0.729	1.246	0.558	-0.819	1.433	0.568	-0.089	0.731	0.903

		2 precursor												
396	gi|512939118	histone H2A-like	Protein metabolism	7	2	-0.048	0.668	0.942	0.726	0.835	0.384	0.699	0.456	0.125
398	gi|512926563	bifunctional purine biosynthesis protein PURH-like	Protein metabolism	10	6	-0.235	0.532	0.659	0.060	0.574	0.917	-0.207	0.321	0.520
400	gi|114052186	prefoldin subunit 3	Protein metabolism	9	1	1.149	0.852	0.177	-1.088	0.973	0.263	0.061	0.467	0.896
403	gi|114052412	autophagy related protein Atg8	Cell cycle	23	2	-0.076	0.742	0.918	-0.830	0.798	0.298	-0.907	0.439	0.039
406	gi|512934093	eukaryotic translation initiation factor 3 subunit L-like	Protein metabolism	8	8	0.113	0.463	0.807	-0.050	0.534	0.926	0.233	0.291	0.424
407	gi|512937034	BAH and coiled-coil domain-containing protein 1-like	Cell cycle	39	5	-0.498	0.527	0.345	0.335	0.604	0.579	-0.168	0.336	0.616
408	gi|512907437	uncharacterized protein LOC101739024	uncharacterized	11	4	0.245	0.642	0.703	-0.128	0.721	0.859	-0.084	0.362	0.816
410	gi|148298772	titin2	Structure	17	5	-0.363	0.610	0.551	-0.033	0.690	0.961	-0.341	0.380	0.370
413	gi|512904817	UTP--glucose-1-phosphate uridylyltransferase-like isoform X2	Energy	36	3	0.139	0.736	0.850	-0.424	0.826	0.608	-0.112	0.441	0.800
414	gi|114051277	interleukin enhancer binding factor	Protein metabolism	14	3	0.321	0.715	0.653	-0.790	0.740	0.286	-0.442	0.399	0.268
416	gi|512894144 ; gi|512894140	protein transport protein Sec23A-like isoform X2 ; protein transport protein Sec23A-like isoform X1	Vesicle transport	37	3	-0.268	0.567	0.637	-0.005	0.636	0.994	-0.285	0.340	0.402
417	gi|512912390	zinc finger protein 706-like isoform X5	Protein metabolism	31	2	0.352	0.724	0.626	-0.170	0.855	0.842	0.151	0.444	0.734
418	gi|114053021	ARP1 actin-related protein 1-like protein A	Structure	13	1	0.239	0.810	0.768	-0.659	0.902	0.465	-0.421	0.480	0.381
421	gi|512911130	prolow-density lipoprotein receptor-related protein 1-like	Lipid	14	2	0.000	0.201	1.000	0.613	2.237	0.784	0.152	1.558	0.922
422	gi|114051461	nascent polypeptide associated complex protein alpha subunit	Protein metabolism	13	4	-0.203	0.595	0.733	0.092	0.691	0.895	-0.118	0.351	0.738

424	gi|114052793	ribosomal protein L7Ae	Protein metabolism	21	3	-0.366	0.813	0.653	0.659	1.090	0.546	0.376	0.634	0.553
425	gi|512905006	uncharacterized protein LOC101740187	uncharacterized	24	1	0.522	1.608	0.746	-0.596	1.841	0.746	-0.074	0.885	0.933
426	gi|512888254	LOW QUALITY PROTEIN: rho GDP-dissociation inhibitor 1-like	Protein metabolism	22	4	-0.262	0.589	0.657	-0.195	0.650	0.765	-0.460	0.356	0.197
429	gi|112983906	eukaryotic translation initiation factor 3 subunit H	Protein metabolism	11	7	0.737	0.447	0.099	-0.268	0.519	0.606	0.468	0.260	0.072
430	gi|512898327	LOW QUALITY PROTEIN: cleavage and polyadenylation specificity factor subunit 1-like	Protein metabolism	4	1	-0.452	0.833	0.587	0.957	1.038	0.356	0.505	0.580	0.384
432	gi|512895911	probable serine/threonine-protein kinase kinX-like	Signaling	13	3	1.937	0.615	0.002	-1.860	0.688	0.007	0.078	0.316	0.805
433	gi|114053253	6-phosphogluconate dehydrogenase	Energy	11	5	-0.822	0.556	0.139	0.416	0.616	0.499	-0.385	0.352	0.273
436	gi|512905214 ; gi|512905210	L-xylulose reductase-like isoform X2 ; L-xylulose reductase-like isoform X1	Oxidoreductase	20	5	0.413	0.495	0.404	-0.360	0.559	0.520	0.082	0.293	0.781
438	gi|112983124	eukaryotic translation initiation factor 4H	Protein metabolism	7	4	-0.086	0.503	0.865	0.014	0.551	0.980	-0.037	0.313	0.907
439	gi|512906510	dnaJ homolog subfamily C member 2-like	Heat shock	14	1	0.619	1.062	0.560	1.160	1.484	0.435	1.779	0.774	0.022
441	gi|512935617	"aspartate--tRNA ligase, cytoplasmic-like "	Protein metabolism	15	2	-0.356	0.740	0.631	0.650	1.116	0.560	0.780	0.597	0.191
448	gi|512910256	perilipin-4-like isoform X1	Lipid	11	3	1.019	0.608	0.094	-0.984	0.715	0.169	0.231	0.333	0.488
450	gi|512892149	E3 ubiquitin-protein ligase TRIM33-like	Protein metabolism	143	1	0.013	0.697	0.986	-0.082	0.795	0.918	-0.070	0.435	0.873
452	gi|512892660	choline-phosphate cytidylyltransferase B-like isoform X2	Lipid	112	2	0.314	0.814	0.700	-0.360	0.951	0.705	-0.049	0.506	0.923
453	gi|512893711 ; gi|512893707	probable nuclear transport factor 2-like isoform X2	Protein metabolism	11	1	-0.379	0.744	0.611	-0.031	0.834	0.971	-0.409	0.464	0.377
462	gi|512914417 ;	kinesin light chain-like isoform X3	ATP utilizing	25	2	0.466	0.901	0.605	0.360	1.139	0.752	0.831	0.584	0.155

470	gi|114050793	small nuclear ribonucleoprotein G	Protein metabolism	12	2	-0.258	0.769	0.738	0.290	0.868	0.738	0.090	0.477	0.850
478	gi|148298787	ubiquitin and ribosomal protein S27a	Protein metabolism	75	15	0.524	0.308	0.089	-0.784	0.339	0.021	-0.245	0.179	0.170
481	gi|512925211 ; gi|512925207	sorting nexin-6-like isoform X2	Other	43	3	0.295	0.707	0.677	-0.156	0.817	0.849	0.143	0.403	0.723
485	gi|512921473	eukaryotic translation initiation factor 4 gamma 3-like	Protein metabolism	4	3	0.244	0.709	0.731	-1.073	0.752	0.153	-0.418	0.342	0.222
486	gi|512897265	neurexin-3a-like	Other	2	6	0.158	0.556	0.776	-0.784	0.612	0.200	-0.620	0.330	0.061
488	gi|512915932	cytosolic non-specific dipeptidase-like	Other	25	3	0.349	0.550	0.525	-0.155	0.647	0.811	0.235	0.342	0.491
497	gi|512918819	zinc finger protein Xfin-like	Protein metabolism	33	2	0.276	0.664	0.678	0.013	0.784	0.987	0.297	0.412	0.472
498	gi|512914736	mitochondrial ribonuclease P protein 3-like	Protein metabolism	176	2	-0.186	0.618	0.763	-0.754	0.656	0.250	-0.939	0.361	0.009
501	gi|112983262	small GTP binding protein RAB5	Vesicle transport	22	6	-0.642	0.525	0.222	0.672	0.620	0.278	0.103	0.344	0.766
502	gi|512919719	uncharacterized protein LOC101737592	uncharacterized	15	1	-0.132	1.939	0.946	0.678	2.899	0.815	0.546	1.507	0.717
503	gi|112983834	RNA-binding protein lark	Protein metabolism	30	2	0.381	0.813	0.639	-0.453	0.928	0.625	-0.056	0.436	0.898
527	gi|512894924	heat shock protein 68-like	Heat shock	6	2	0.501	0.920	0.586	-0.046	1.226	0.970	0.688	0.592	0.245
535	gi|512934729	microtubule-associated protein futsch-like	Structure	18	10	0.455	0.375	0.225	-1.289	0.400	0.001	-0.833	0.213	0.000
540	gi|512910396	esterase FE4-like	Other	103	2	-0.445	0.696	0.523	0.748	0.825	0.365	0.365	0.457	0.424
541	gi|512915329	"ribosome-recycling factor, mitochondrial-like "	Protein metabolism	57	15	0.970	0.357	0.007	-0.882	0.388	0.023	0.083	0.204	0.684
542	gi|512887257	putative uncharacterized protein DDB_G0282133-like	uncharacterized	30	3	-1.008	0.609	0.098	0.216	0.701	0.758	-0.614	0.396	0.121
550	gi|512908538	zinc finger protein 594-like	Protein metabolism	27	3	0.672	0.744	0.366	-1.021	0.821	0.214	-0.338	0.422	0.422
553	gi|112984118	ribosomal protein L39	Protein metabolism	17	2	-0.373	0.697	0.592	0.173	0.789	0.827	-0.232	0.430	0.590

564	gi|112983254	eukaryotic translation initiation factor 3 subunit D	Protein metabolism	47	8	-0.089	0.436	0.838	0.183	0.513	0.721	-0.010	0.274	0.970
596	gi|512908335	protein transport protein Sec31A-like	Vesicle transport	64	5	0.522	0.557	0.349	-0.206	0.641	0.748	0.246	0.323	0.446
606	gi|112984026	saposin-related precursor	Lipid	49	2	0.091	0.693	0.895	0.586	0.869	0.500	0.677	0.472	0.152
631	gi|153792009	heterogeneous nuclear ribonucleoprotein A1	Protein metabolism	93	3	0.138	1.059	0.896	-0.257	1.288	0.842	-0.028	0.664	0.966
651	gi|512939211	histone H2B-like	Cell cycle	59	4	0.129	0.571	0.821	0.435	0.673	0.518	0.544	0.360	0.130
652	gi|512926391	LOW QUALITY PROTEIN: hydrocephalus-inducing protein-like	Other	26	2	0.139	0.923	0.880	-1.291	1.151	0.262	-1.124	0.613	0.066
677	gi|512934081	esterase FE4-like	Other	16	3	0.351	0.565	0.534	-1.014	0.597	0.090	-0.679	0.320	0.034
679	gi|512911180	nodal modulator 3-like	Protein metabolism	51	4	0.274	0.509	0.591	-1.195	0.542	0.028	-0.888	0.283	0.002
685	gi|114052675	phosphoribosyl pyrophosphate synthetase	Other	18	6	0.502	0.461	0.276	-0.498	0.520	0.339	0.018	0.269	0.946
697	gi|512890394	golgin subfamily A member 4-like	Vesicle transport	23	3	-0.114	0.766	0.881	0.087	0.918	0.924	0.017	0.501	0.973
704	gi|512900004	pre-rRNA-processing protein TSR1 homolog	Protein metabolism	33	2	0.041	0.868	0.962	-0.048	0.971	0.961	0.026	0.499	0.959
743	gi|114053001	ATP-binding cassette sub-family F member 2	ATP utilizing	14	6	-0.678	0.470	0.150	0.267	0.536	0.619	-0.395	0.298	0.185
745	gi|112984298	ribosomal protein L21	Protein metabolism	73	6	1.049	0.467	0.025	-0.914	0.535	0.087	0.220	0.263	0.403
765	gi|512888265	uncharacterized protein LOC101744489	uncharacterized	48	5	-0.271	0.452	0.549	0.115	0.513	0.823	-0.135	0.281	0.631
876	gi|512900064	spermine synthase-like	Other	33	1	-0.278	0.728	0.703	0.253	0.839	0.763	-0.025	0.468	0.958
896	gi|512937112	"uncharacterized protein LOC101736300, partial "	uncharacterized	36	1	0.386	0.646	0.550	0.014	0.769	0.986	0.399	0.409	0.329
917	gi|512897905	ATP-binding cassette sub-family A member 3-like	ATP utilizing	55	3	0.275	0.652	0.673	-0.696	0.711	0.327	-0.474	0.375	0.206
1080	gi|112984012	heat shock 70 kD protein cognate precursor	Heat shock	87	3	-0.229	0.655	0.727	-0.061	0.757	0.936	-0.294	0.398	0.461
1177	gi|512891488	fibrohexamerin-like	Structure	14	7	0.962	0.474	0.043	-0.955	0.530	0.071	-0.023	0.265	0.931
1182	gi|512906758	neuroblastoma-amplified sequence-like	Vesicle transport	8	5	0.195	0.518	0.706	-0.982	0.556	0.077	-0.826	0.294	0.005

1187	gi|512907285	fat-like cadherin-related tumor suppressor homolog	Cell cycle	5	2	-0.235	0.697	0.736	0.109	0.804	0.892	-0.279	0.421	0.508
1256	gi|512929499	protein QN1 homolog	Cell cycle	22	24	-0.384	0.325	0.238	-0.276	0.346	0.424	-0.651	0.189	0.001
1407	gi|512918346	RNA polymerase II-associated protein 1-like	Protein metabolism	23	5	-0.739	0.471	0.116	0.671	0.534	0.208	-0.089	0.296	0.765
1618	gi|512900327	CAD protein-like	Other	11	2	0.464	0.665	0.486	-0.130	0.792	0.870	0.333	0.416	0.423
1636	gi|112983746	vitellogenin precursor	Vesicle transport	8	6	-1.057	0.743	0.155	-0.415	0.754	0.582	-1.447	0.434	0.001
1675	gi|512915527	tRNA (cytidine(32)-2'-O)-methyltransferase non-catalytic subunit TRM732-like	Protein metabolism	38	1	0.131	1.208	0.914	1.235	1.965	0.530	1.365	1.023	0.182

VITA

Loren J. Rivera-Vega

Education

2012-2017	PhD Candidate – Entomology and International Agriculture & Development The Pennsylvania State University
2009-2011	MSc – Entomology The Ohio State University
2004-2007	BSc –Agricultural Science and Production Escuela Agricola Panamericana (Zamorano)

Experience

2014	Visiting Scholar, iDiv, Leipzig, Germany
2008	Research Assistant, The Ohio State University/ OARDC
2008	Field Technician, MCA-EDA Honduras
2007	Research Assistant, University of Florida

Selected Publications

Rivera-Vega L, Acevedo FE, Felton GW. 2017. Genomics of Lepidoptera Saliva Reveal Function in Herbivory. Current Opinion in Insect Science. doi.org/10.1016/j.cois.2017.01.002

Rivera-Vega L, Krosse S, de Graaf RM, Garvi J, Garvi R, Van Dam NM. 2015. Allelopathic effects of glucosinolate breakdown products in Hanza (*Boscia senegalensis* (pers.) Lam) processing waste water. Frontiers in Plant Science. 6. doi: 10.3389/fpls.2015.00532

Selected Grants and Awards

2017	College of Agricultural Sciences Outstanding Dissertation Award, Penn State University
2016	Michael E. Duke Memorial Award, Penn State University
2015	Monsanto Research Grant, Entomological Society Association
2014	INTAD Competitive Grant, Penn State University
2013	Graduate Student Competitive Grant, Penn State University

Selected Teaching

2017	College Teaching Certificate, Penn State University
2017	International Agriculture (online course), Penn State University
2015	Global Agriculture, Governor's School, Penn State University
2014	Tropical Entomology Graduate Seminar, Penn State University