Integrated omics on the physiology of emerald ash borer (*Agrilus planipennis* Fairmaire)

DISSERTATION

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Abstract

Coleoptera comprise one of the most frequently intercepted wood feeders because of their abundance and specialization on live and/or dead trees. Within this order, invasive Buprestids (metallic wood boring beetle) have had devastating effects on the forest ecosystems of North America (NA). Emerald ash borer, *Agrilus planipennis* Fairmaire, is a recently discovered exotic wood boring beetle in NA. It is a native of Northeastern Asia and specializes on ash (*Fraxinus* spp) trees. Since its discovery in 2002 in Michigan, it has killed millions of ash trees and continues to do so at a rapid pace. Asian/native ash (e.g. *F. mandshurica*, Manchurian ash) are resistant to *A.planipennis* attack unless stressed, perhaps by virtue of their co-evolutionary history. Transcriptomic, proteomic and metabolomic studies on NA and Asian ash species have revealed significant qualitative and quantitative differences. Manchurian ash phloem revealed presence of unique phenolics and significantly higher amounts of defense proteins relative to the NA ashes.

During coexistence with plants, herbivorous insects have developed a suite of counter defense mechanisms including avoidance, sequestration, metabolic resistance (antioxidation and detoxification), excretion and target site insensitivity. To date little is known about the metabolic resistance mechanisms in *A. planipennis* during its interaction with ash. Therefore, the overarching goal of this thesis was to understand the
physiological responses of *A. planipennis* on NA ash and its differential survivability on Asian ash via an integrated omics approach. *Chapter 2* deals with standardizing real time quantitative PCR technique to accomplish my further objectives. *Chapters 3 & 4* of my thesis deals with molecular characterization of antioxidant and detoxification enzymes in tissues and developmental stages of larvae feeding on green ash; *chapter 5* deals with the targeted metabolomics of green ash phloem, larval tissues (midgut tissue, midgut content, hindgut-Malpighian tubules) and frass. Lastly, *chapter 6* deals with the comparative transcriptomic and metabolomics of *A. planipennis* larvae feeding on green (NA) and Manchurian (Asian) ash.

I was able to standardize a technique to measure target gene expression levels in *A. planipennis* (*chapter 1*). Expression patterns of antioxidant and detoxification genes reflect a plausible role of these genes in dealing with direct and indirect effects of green ash allelochemicals in diet. Alternatively these enzymes might also be participating in other physiological processes (*chapter 3 & 4*). Targeted metabolomics of larvae feeding on green ash phloem indicated that *A. planipennis* larvae have the ability to metabolize a suite of phenolics present in green ash phloem via biotransformation and/or excretion mechanisms (*chapter 5*). Further, higher expression of peritrophic membrane synthesis genes coupled with general stress response and the higher total phenolic content in frass of larvae feeding on Manchurian ash suggest the prime mechanism of ash resistance is damaging the peritrophic membrane (*chapter 6*). Larvae feeding on green ash had higher levels of digestion genes reflecting a normal digestion metabolism relative to larvae feeding on Manchurian ash. Lastly, results from this research shed light on the wood
boring beetle physiology and also molecular interactions of *A. planipennis* with its Asian and North American host.
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Chapter 1: Literature Review

Insect - Plant Interactions

Interaction between plants and insects determines the structure and diversity of ecological communities through evolutionary times. This phenomenon could be better explained by coevolution theory proposed by Ehrlich and Raven (1964). According to this theory plants develop/synthesize novel chemical defenses to escape from herbivores and increase in abundance whereas the interacting herbivore develops counter defense mechanisms to adapt to these novel defenses. In this review I focus more on the mechanisms evolved by plants and insects during their course of coevolution.

Plants possess several physical and chemical defenses to protect themselves from insects and pathogens. Thorns, cuticle, wax layer are some of the physical barriers whereas secondary metabolites, defensive proteins and non-protein amino acids constitute the chemicals defenses.
**Plant Secondary Metabolites (PSMs)**

Secondary metabolites in plants are classified into various chemical classes among which majority of the compounds belong to terpenoids (>30,000), alkaloids (>12,000) and phenolics (>9,000) (Mithöfer and Boland 2012). Though all these compounds might not have a negative effect on herbivorous insects their mechanism of defense against herbivores would be further discussed in brief.

*Terpenoids:*

Terpenoids are formed from 5 carbon isoprene units; ubiquitously distributed and diverse compounds. They are important for growth and development in plants (Langenheim 1994). Along with these functions, terpenoids also have protective role against herbivores. Terpenoids exhibit antifeedant properties towards a wide range of crop pests such as sunflower moth (Gershenzon and others 1985), corn root worm (Mullin and others 1991) and Colorado potato beetle (Murray and others 1999). At a physiological level, terpenoids impact digestion of herbivores through inhibiting digestive enzymes like α-amylase and proteases (Liu and others 2008; Liu and others 2009). They are also known to increase the levels of factors involved in innate immunity in gypsy moth larvae (Martemyanov and others 2012). Indirect effects of terpenoids include recruiting parasitoids to the infested tree (Buchel and others 2011).
**Alkaloids:**

Alkaloids are nitrogen-containing compounds synthesized from amino acids (Levinson 1976). Detrimental effects of alkaloids on insects include disruption of growth, development and reproduction (Krug and Proksch 1993; Levinson 1976; Sun and others 2012a). These compounds are also known to be neurotoxic and feeding deterrents (Bentley and others 1984; Mao and Henderson 2007; Matsukura and others 2012). Camptomectin, an alkaloid is known to be potential synergist of Bt toxin against Lepidoptera including *Trichoplusia ni* and *Spodoptera exigua* (Sun and others 2012b). Inhibition of α-amylase (digestive enzymes) by alkaloids was observed in Lepidopteran pests (Bouayad and others 2012).

**Phenolics:**

A phenolic compound bears an aromatic ring with one or more hydroxyl groups. Phenolics are constitutively produced in plants and accumulate during growth and development. They are derived from aromatic amino acids through the shikimic acid pathway. Plants synthesize an array of phenolic compounds, ranging from simple to complex in structure. Based on the structure, they are grouped into benzoates, hydroxycinnamates, furanocoumarins, coumarins, stilbenes, flavonoids, hydrolysable tannins, condensed tannins and lignin (Rehman and others 2012). Along with protecting the plants from UV radiation, phenolics provide a first line of defense against herbivory
wherein they act as feeding deterrents, growth reducers, or toxins (Close and McArthur 2002; Degraeve and others 1980; Dreyer and Jones 1981; Mole and others 1993). The biochemical mode of action of phenolics in herbivores depends on the gut pH and the presence of enzymes that can activate them (Appel 1993). At the alkaline pH level found in herbivore guts, phenolic oxidation leads to the formation of reactive oxygen species that readily oxidize vital biomolecules including lipids, proteins, carbohydrates and nucleic acids (Appel 1993; Felton and Summers 1995). Oxidation of phenolics can also occur by plant polyphenol oxidases forming quinones which react with proteins and result in their precipitation (Felton and others 1992). A few phenolics like tannins can bind and precipitate gut proteins effecting digestion (Butler and Rogler 1992). The insecticidal activity of coumarins was observed against fall army-worm, S. frugiperda, wherein larval growth was significantly reduced and a high percentage of mortality was also observed (Vera and others 2006). Similar effects were observed against lepidopteran forest pests such as Plecoptera reflexa, Clostera cupreata and Crypsiptya coclesalis (Sharma and others 2006).

Defense proteins:

Along with PSMs, plants also produce a diverse array of defense proteins that potentially inhibit or degrade insect and pathogen proteins. Protease inhibitors are the most studied in defense proteins in insect-plant interaction. Since the discovery of induction of protease inhibitors in herbivore-attacked tomato, the structure and
mechanism of action of these proteins has been extensively reviewed (Green and Ryan 1972; Rawlings and others 2004; Ryan 1989; Ryan 1990). Protease inhibitors hinder digestion by binding to digestive enzymes and effecting growth and development (Chen 2008; Ishimoto and others 2012; Ryan 1989).

Impairment of digestion is also caused by degradation of peritrophic membrane by plant proteases e.g., cysteine proteases in corn (Pechan and others 2002). Plant lectins or agglutinins are another group of carbohydrate-binding proteins. Negative effect of lectins on the performance of Lepidopterans, Coleopterans, Dipterans and Hemipterans has been recently reviewed (Vandenborre and others 2011). Biochemical mode of action of lectins in insect gut has not been extensively studied. Although, study in European corn borer larvae feeding on diet containing wheat germ agglutinin (lectin) shows hindrance in peritrophic membrane formation (Harper and others 1998).

**Insect Counterdefense Mechanisms**

As plants have evolved chemical defenses, herbivores have developed counterstrategies to deploy these chemical defenses via behavioral and physiological responses (Figure 1.1). Mechanisms employed by phytophagous insects are further discussed in detail.
Feeding preference/ Behavioral avoidance:

Phytophagous insects use various sensory modes of cues including olfactory, gustatory or contact to choose the right host and avoid the toxins (Chapman 2003; Despres and others 2007). Adult oviposition plays an important role in choice of proper hosts for offsprings. This relation between adult preference and larval performance was shown to correlate positively in most of the studies (Mayhew 1997). However, there are studies showing a poor host choice by the adult females (Mayhew 2001). While on host plant, insects can alter their feeding behaviour spatially and temporally (Despres and others 2007; Nealis and Nault 2005). Spatial feeding patterns involve avoiding feeding on plant tissues with high concentration of toxins or exuding the toxins from plant tissues e.g. larvae of *Trichoplusia ni* (Zangerl and Bazzaz 1992), *Bucculatrix thurberiella* (Karban and Agrawal 2002), *Danaus plexippus* (Zalucki and others 2001), *Pygarctia roseicapitis* (Bernays and others 2004)

Sequestration:

Another interesting mechanisms adopted by insects is the use of secondary chemicals acquired from their host for their own benefit (Brower and others 1967; Duffey 1980). Monarch butterflies, *D. plexippus*, are well-studied models for host plant chemical sequestration. Larvae of monarch butterflies sequester cardiac glycosides from milkweed plants rendering the adult butterflies unpalatable to their predators (Brower and
Pyrrolizidine alkaloids, produced mainly by Asteraceae and Leguminaceae are sequestered by Lepidoptera and Coleoptera into non-toxic N-oxide forms in their hemolymph (Morgan 2010). Sequestration of host plant glucosinolates is observed in the sawfly, *Athalia rosea* (Müller and others 2001) and the harlequin bug, *Murgantia histrionica* (Aliabadi and others 2002). Other PSMs sequestered by insects include carotenoids, cycasin, aristocholic acid, tropane alkaloids and grayanoid terpenes (Nishida 2002).

**Metabolic resistance:**

Herbivores are biochemically and physiologically efficient to deploy chemical defenses and acquire nutrients from their hosts. These mechanisms include detoxification, antioxidation, excretion, and target site mutation (Despres and others 2007). Insects can employ multiple counterstrategies to exploit their host plants (Brattsten and others 1977). For example, larvae of Monarch butterfly, *D. plexippus*, use trenching behaviour to inhibit the flow of latex containing sap, adult butterflies sequester few cardiac glycosides while detoxifying others by aldehyde reductase (Brower and others 1967; Marty and Krieger 1984).

There are two major phases of detoxification – phase I and phase II. Enzymes in phase I include Cytochrome P450s (P450s) and phase II enzymes include glutathione-S-transferases (GSTs) and carboxylesterases (CEs). Integration of phase I and phase II is
referred to as detoxification pathway and involves the oxidation and conjugation of non-polar lipophilic molecules to polar hydrophilic forms.

**Cytochrome P450:**

Cytochrome P450s are ubiquitous microsomal enzymes that can oxidatively metabolize a wide array of endogenous and exogenous compounds (Nebert and Gonzalez 1987). Substantial evidence was shown for the involvement of P450s in drug metabolism in mammalian cells (Nebert and Gonzalez 1987). Detoxification of allelochemical in insects was first shown in Lepidoptera larval guts (Krieger and others 1971). Reactions catalyzed by P450s are diverse ranging from hydroxylation to epoxidation, O-, N-, and S-dealkylation, N- and S-oxidations. General reaction stoichiometry of P450 can be represented as

\[
\text{Substrate} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{Substrate-O} + \text{NADP}^+ + \text{H}_2\text{O}
\]

In the instances of a tight enzyme substrate complex efficient conversion of substrate occurs. But, in most cases the reaction uncouples leading to the formation of superoxide and hydrogen peroxide (Feyereisen 1999). A single P450 enzyme can metabolize more than one substrate or can be very specific to single substrate. Induction of one or more P450s is observed in vivo depending on the presence of substrates (Yu 1986). In some instances consumption of diet depends on the induction of P450 enzymes.
(Snyder and Glendinning 1996; Snyder and others 1994). However, in insecticide resistant strains of house fly constitutive expression of P450s occurs (Carino and others 1994). P450 gene superfamily divergently evolved by duplication (Feyereisen 2011). Because of growing numbers of these genes they are classified into different families depending on their similarity at amino acid level. In insects P450 gene family is divided into four clans CYP2, CYP3, CYP4 and a mitochondrial clan (Feyereisen 2006). Among these CYP3 clan is the most diverged subfamily shown to be involved in detoxification of allelochemicals and insecticides (Feyereisen 2006). These enzymes play a crucial role in the adaptation of insect in a naïve/ novel niche (Schuler 2011). Role of P450s in adaptive mechanisms through detoxification is well studied in Depressaria platinicella, Manduca sexta, Helicoverpa zea, Bombyx mori (Cianfrogna and others 2002; Li and others 2002; Stevens and others 2000).

**Glutathione-S-transferases (GST):**

Detoxification mediated by GST is also an important mechanism in mammals as well as insects. These cytosolic enzymes belong to phase II in detoxification pathway (Yu 1992). They catalyze the conjugation of endogenous and exogenous xenobiotics with glutathione converting them into excretable forms (Habig and others 1974). Reactions performed by GSTs include S-alkylation of aryl halides, O-aryl and O-alkyl conjugation of phosphorothioates and phosphates, replacement of nitro group and addition of
glutathione (GSH) to epoxides, $\alpha$, $\beta$ - unsaturated compounds. General reaction performed by GSTs is as follows

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG \text{ (reduced glutathione)}$$

Similar to P450s, GSTs are also known to be induced in presence of their substrates (Yu 1992). Induction of GST was first observed in houseflies exposed to phenobarbital (Ottea and Plapp 1981). Later studies in fall armyworm, *S. frugiperda* feeding on cowpea, mustard and turnip demonstrated an induction of GSTs in response to host allelochemicals (Yu 1982). Several studies showed induction of GSTs in presence of xenobiotics (insecticides and PSMs) in Lepidoptera (Sintim and others 2012; Sonoda and Tsumuki 2005; Ugale and others 2011b; Yamamoto and others 2008; Zhang and others 2011b), Diptera (Gunasekaran and others 2011; Kristensen 2005; Le Goff and others 2006; Lumjuan and others 2005; Mittapalli and others 2007b; Wang and others 2008), a few Orthoptera (Adewale and Afolayan 2006), Ixodida (Dreher-Lesnick and others 2006), and Hemiptera (Francis and others 2005).

**Carboxylesterases:**

Similar to GSTs, carboxylesterases (CEs) also belong to phase II of detoxification pathway. These enzymes hydrolyze ester bonds (C-O and C-N) with the addition of water molecule. General reaction stoichiometry of carboxylesterase is
Hydrolases encompass a wide range of enzymes and are involved in metabolism of pesticides (Ahmad and Forgash 1976). Resistance to malathion in insecticide-resistant strains of houseflies was observed to be provided by the hydrolysis of malathion to its monoacid by CEs (Kao and others 1984). Later there was growing evidence of carboxylesterase-based insecticide resistance in Lepidoptera (Goh and others 1995; Hung and others 1990; Wu and others 2011), Diptera (Whyard and others 1994a; Whyard and others 1994b), Hemiptera (Hung and others 1990) and Coleoptera (Argentine and others 1994). However, there are very few reports on metabolism of PSMs by CEs (Ghumare and others 1989; Lindroth and Weisbrod 1991; Mu and others 2006). Studying the involvement of CEs in allelochemical detoxification is needed.

**Antioxidation:**

Reactive Oxygen Species (ROS) pose a threat to vital biological molecules including proteins, lipids, carbohydrates and nucleic acids. Insects encounter ROS through endogenous and exogenous mechanisms either directly or indirectly. Endogenous mechanisms include incomplete oxidation of oxygen molecule during respiration, leading to ROS formation such as superoxide radical, peroxide radical and hydrogen peroxide (Fridovich 1983). Herbivorous insects encounter ROS as a direct defense from plants or a result of phenolic oxidation (Felton and Summers 1995; Lee and Berenbaum 1989; Miles...
The lipoxygenase pathway is an important source of singlet oxygen in plants (Kanofsky and Axelrod 1986). Another indirect source of ROS in phytophagous insects includes detoxification of PSMs in the tissues (Feyereisen 1999). To overcome the toxic effects of ROS, insects have evolved an array of antioxidant machinery including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). These enzymes act in concert to neutralize the ROS (Larson 1986). The foremost player in the pathway is superoxide dismutase which converts superoxide \((O_2^-)\) to hydrogen peroxide. Toxicity of hydrogen peroxide is higher compared to other ROS (Imlay and others 1988). These highly reactive molecules are readily converted to water molecule by catalase and glutathione peroxidase. The chain of events catalyzed by these enzymes is shown below

\[
\text{O}_2^- \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} \text{H}_2\text{O} \xrightarrow{\text{GPX}} \text{H}_2\text{O}
\]

There are studies demonstrating the role of antioxidant enzymes in insect-plant interactions especially in insects feeding on phototoxic plants (Ahmad 1992; Lee and Berenbaum 1989; Lee and Berenbaum 1990; Mittapalli and others 2007a). Induction of SOD was observed in \(D.\ pastinacella\) due to slower detoxification of xanthotoxin (Lee and Berenbaum 1990). Gut tissues of cotton leaf worm, (\(S.\ littoralis\)) migratory grasshopper (\(Melanoplus sanguinipes\)) and bigheaded grasshopper (\(Aulocara elliotti\)) also showed high levels of antioxidant enzymes while feeding on pro-oxidant diet (Barbehenn 2002; Krishnan and Kodrik 2006). However, induction of antioxidant genes
is also observed during other environmental stress such as temperature and radiation (Church and others 2012; Wang and others 2012; Yang and others 2010).

**Excretion:**

Herbivores can regulate the absorption of PSMs by rapid excretion of these compounds (Sorensen and Dearing 2006). Important players/molecules involved in regulated absorption of PSMs by herbivore gut tissues are ABC transporters. Function of these transporters has been well studied in mammalian cells. This superfamily of transporters is ubiquitously distributed in all the organisms and has a wide range of substrates encompassing endogenous (lipids) and exogenous compounds (drugs, toxins) (Higgins 1992a).

A member of ABC transporter, Permeability glycoprotein or P-glycoprotein is a multidrug resistance transporter protein (MDR) that is abundantly expressed in cancer or tumor cells and rapidly eliminate drugs providing resistance (Marie 1992). A similar scenario was observed in insecticide resistant strains of the tobacco budworm, *Heliothis virescens* which had higher levels of P-glycoprotein (Lanning and others 1996). Additional evidence of MDRs in providing insecticide resistance was drawn from studies in Lepidoptera and Diptera (Bariami and others 2012; Labbe and others 2011b). Interestingly, mutations in ABC transporters are proposed to be linked to Bt resistance in several Lepidopteran pests (Heckel 2012; Hernandez-Martinez and others 2012).

Functional assays in *Tribolium castaneum* has shown the involvement of ABC
transporters in lipid, eye pigment and ecdysteroid transportation also (Broehan and others 2013).

Studies on function of MDRs in herbivores feeding on allelochemically rich hosts are very limited. Sorensen and Dearing (2006) propose the presence of abundant amounts of unmetabolized PSMs present in the feaces of herbivores might be due to their active transportation by the MDRs in the gut of insects or intestines of higher animals. Further experimental evidence of transportation of allelochemicals by these transporters would shed light on interesting and vital mechanisms of herbivore adaptation.

*Target site mutation:*

A cost effective (energy wise), slow and powerful built-in mechanism evolved by insects is target site mutation (Berenbaum 1986). Targets of PSMs or defense proteins are changed at the gene level to escape recognition by plant defense molecule (Berenbaum 1986). Most extensively studied examples are the Lepidoptera adapted on cardenolide rich hosts. Cardenolides are toxic to the herbivores in a dose dependent manner wherein they inhibit the Na\(^+\)-K\(^+\) pump which is important in nerve signal transmission. In response to this insects have developed cardenolide resistant Na\(^+\)-K\(^+\) pumps (Moore and Scudder 1986).

Molecular evidence of amino acid substitution was observed in Na\(^+\)-K\(^+\) pump of Monarch butterfly, *D. plexippus* (Holzinger and others 1992; Holzinger and Wink 1996). Similar adaptation is seen in other Lepidoptera, Coleoptera, Diptera and Heteroptera.
A recent community level study on phylogenetically diverse insects specialized on cardenolide containing plants indicated convergent adaptation of these insects to host plant cardenolide defenses (Dobler and others 2012). Another group of enzymes that are genetically altered by insects include digestive proteins. Plants have a repertoire of defensive protein that can inhibit or degrade insect digestive enzymes as mentioned in previous section. Insects survive these defenses by mutating the target enzymes of plant proteases to sterically insensitive forms (Bown and others 1997; Jongsma and Beekwilder 2011).

Other defenses:

Plants produce a diverse range of defense proteins for example Bowman-birk inhibitor, trypsin, cystatin, cysteine proteases that inhibit herbivore digestive enzyme or peritrophic membrane formation (Ryan 1989). The strategies of herbivorous insects against these defensive proteins is to produce a different class of digestive proteins, an insensitive target and degradation of protease inhibitors (Jongsma and Beekwilder 2011). In diamondback moth, *T. ni* cysteine protease inhibitor are produced to prevent the deleterious effects of cysteine proteases to peritrophic membrane (Li and others 2009).

Another group of detoxification enzymes which are understudied are the β-glucosidases. These enzymes can participate in digestion of cellulose and also in cleaving the glycosidic bonds of PSM glycosides releasing aglycones (Ferreira and others 1997; Lindroth 1988; Tokuda and others 2009). These toxic aglycones are assumed to be
detoxified by the detoxification pathway genes. Apart from biochemical mechanisms, the peritrophic membrane serves as an important physical barrier in insects. The peritrophic membrane is synthesized by midgut epithelial cells and consists of chitin, protein and proteoglycans (Hegedus and others 2009; Lehane 1997). It prevents mechanical abrasion of midgut tissue, compartmentalizes digestion and also prevents the entry of xenobiotics and pathogen (Barbehenn and Martin 1995).

**Era of omics**

Methods of measuring global or specific levels of biomolecules including DNA, RNA, protein and metabolites are collectively termed as Omic studies (Debnath and others 2010). Discovery of these techniques has led to a tremendous advancement in understanding the systems biology. These high-throughput technologies generate enormous data and aid in simultaneous detection of wide range of molecules. Overtime these techniques have become cost effective and automated systems made it easier to process, sequence and analyze the data at a rapid pace. To date genomes of 13 insects belonging to Diptera, Lepidoptera, Coleoptera and Phthiraptera (Hampton 2010; Zdobnov and Bork 2007). Genomics data allows the discovery of molecular markers to understand insecticide resistance, virulence etc (Heckel 2003).

While genomics deals with the structural aspects of genes, function of the gene can be deduced by functional genomics encompassing transcriptomics and proteomics. Each cell or a tissue expresses a specific set of transcripts and proteins influenced by the
environmental conditions reflecting their probable function. Microarray is traditionally used to monitor the transcriptome differences between two conditions. The very first use of microarray was done in *Arabidopsis* (Schena and others 1995). Later on the technique was adapted in biomedical research to understand several disease conditions and effects of drugs in mammals (Brown and Botstein 1999). Microarrays have also gained popularity in studying insect systems. For example it was used to study development, social behaviour and hormone regulation (Blomquist and others 2004; White and others 1999; Whitfield and others 2001). While microarray was used to analyze a group of genes, real time quantitative polymerase chain reaction was extensively used to study individual gene (Heid and others 1996). Validation of microarray data was recommended to be performed by RT-qPCR. Limitations of microarrays include array density (number of spots/chip), poor detection of target mRNAs with lower levels and high background noise (Bier and Kleinjung 2001).

Microarrays are being rapidly replaced by tag based direct sequencing of transcripts. These new generation transcriptomic techniques include serial analysis of gene expression, cap analysis of gene expression and massively parallel sequencing (Brenner and others 2000; Kodzius and others 2006; Velculescu and others 1995). Limitations of these techniques is the difficulty of mapping very short reads to references (Wang and others 2009). The current high-throughput tool overcoming all these limitations is RNA-seq which is based on novel sequencing methods. Advantages of RNA-seq include identification of isoforms, novel transcripts, expression difference > 9000 fold change can also be detected and much less RNA is required (Wang and others 2009).
RNA-seq is widely used in studying diverse physiological mechanisms in insects (Chen and others 2012; Simmons and others 2012). The main limitation of RNA seq is the analysis of massive sequencing data, which requires strong bioinformatic knowledge.

With the increasing transcriptomic information, proteomic is also becoming an important tool to study various biological events. In insects proteomics was used to study the proteins present in peritrophic membrane, salivary glands, hemolymph under various physiological conditions (Celorio-Mancera and others 2012; Hu and others 2012; Li and others 2012; Nicholson and others 2012). Another promising omic technology that is widely used is metabolomics. Metabolites represent a diverse group of compounds including metabolic intermediates, hormones, signaling molecules and secondary metabolites. Qualitative and quantitative changes in cell or tissue metabolites reflect the systemic responses to environmental conditions (Debnath and others 2010). Metabolomics is a widely used technique in insect-plant interactions to study chemical warfare between these two taxa is substantially studied (Jansen and others 2009).

The *Agrilus planipennis* invasion

Invasive herbivorous insects have had devastating effects on North American forest ecosystems either directly or indirectly (Gandhi and Herms 2010). The most destructive invasions recorded to date are by gypsy moth, *Lima*ntria dispers L, beech scale, *Cryptococcus fagisuga*, European elm bark beetle, *Scolytus multistriatus*, hemlock woolly adelgid, *Adelges tsugae*, and balsam woolly adelgid, *Adelges piceae* (Gandhi and
Herms 2010). The recently discovered emerald ash borer, *Agrilus planipennis* has also been recorded as a devastating exotic insect of *Fraxinus* spp (ash) in North America (NA). This alien insect is a native of Northeastern China and specializes on *Fraxinus* genus. Initial *A. planipennis* infestations in China were observed on an introduced NA species, *F. pennsylvania* and a native *F. mandschurica* in early 1960s (Liu 1966). Later *A. planipennis* infestations were recorded on *F. velutina* in 1989 in Tianjin city, China (Liu and others 2003).

Introduction of *A. planipennis* to North America might have occurred in early 1990s via infested ash crates or pallets (Cappaert and others 2005; Herms and others 2004). It was first discovered in 2002 in Detroit, Michigan and Windsor, Ontario. Since then it has killed millions of ash trees in Eastern North America, establishing its populations in 17 U.S. states and 2 Canada Provinces. To date *A. planipennis* is known to infest black, (*F. nigra*), green, (*F. pennsylvania*), white, (*F. americana*), and blue (*F. quadrangulata*) ashes. Infestation in NA are observed on young to matured trees irrespective of whether they are healthy or stressed, whereas Asian ashes (e.g. Manchurian ash) are attacked only under stressed conditions (Rebek and others 2008). This might be due to the co-evolutionary history shared between Asian ash and *A. planipennis*. An infested tree can be diagnosed for the presence of *A. planipennis* only after the first year of attack by the presence of D-shaped adult exit holes, canopy dieback, epicormic shoots and cracked bark over larval galleries (Cappaert and others 2005).
Ash trees are an important source of timber and food for wildlife in forest systems. They also constitute 5-25% landscape trees in urban ecosystems (Haack and others 2002). Significant ecological and economic impacts have been observed since the invasion by *A. planipennis* began, and might be similar to those caused by chestnut blight or Dutch elm disease (Burns and Honkala). With its current rate of infestations/spread *A. planipennis* poses a potential threat to all the NA ash trees.

**Biology of *Agrilus planipennis***

*A. planipennis* is a holometabolous insect with distinct larval, pupal and adult stages (Figure 1.2). Life cycle of *A. planipennis* can vary between 1-2 years. Adults emergence begins during mid- May or early June and peaks in June- July. Following emergence, adults feed on foliage for 5-7 days prior to mating. An adult *A. planipennis* female can lay 60-90 eggs in its life time (Cappaert and others 2005). Eggs are usually laid in the crevice of the bark. Initially eggs are cream colored and later turn reddish brown. Neonates (freshly hatched larvae) chew through the bark and feed on the cambium tissues. There are four larval stages in *A. planipennis* which feed extensively on the phloem and outer xylem from late July through September (Cappaert and others 2005). As they tunnel through the phloem, they form distinctive S-shaped galleries filled with frass. In late September -early October larvae bore through the outer xylem to form a pupal chamber during which they fold themselves into a ‘J’ shaped prepupal larva. They overwinter as prepupae until April and pupate during late April and May. Larvae
that are not fully grown overwinter in the cambial regions and complete their
development in the following year (Cappaert and others 2005). Pupation occur in pupal
chambers in mid-April and adults emerge in May (Poland and McCullough 2006).

Counterdefense mechanisms in wood boring beetles

Wood-boring beetles belong to families Scotylidae, Cerambycidae, Buprestidae
and Curculionidae. Most of these beetles infest weak/stressed, recently cut or dead
tresswhile some infest and kill healthy trees. There has been interest among researchers to
understand nutrition, pheromone production and microbial/fungal associations of these
wood-boring beetles (Aw and others 2010; Burges and others 1979; Calderon and Berkov
2012; Chararas and Courtois 1976; Schloss and others 2006; Serdjukova 1993). But,
studies on the counterstrategies employed by the beetle larvae are very limited. Feeding
behaviour of wood-boring larvae exposes them to the highly defensive chemical
repertoire of the host plant. Given the successful adaptation to the toxic environment of
the host, my research thesis is to dissect the physiologically driven molecular strategies
employed by wood-boring beetle larvae to counter the plant chemical defenses and
successfully adapt on new host. Very little is known about molecular physiology of the
counter defense mechanisms in these beetles.
Transcriptomics of *Agrilus planipennis*

Successful adaption of *A. planipennis* larvae in North America offers a perfect system to study the efficient mechanisms used by wood boring beetles to exploit their host. A transcriptome database for two vital insect tissues including midgut and fatbody was established. Newer-generation Roche-454 pyrosequencing was used to obtain 126,185 reads for the midgut and 240,848 reads for the fat body, which were assembled into 25,173 and 37,661 high quality expressed sequence tags (ESTs) for the midgut and the fat body of *A. planipennis* larvae, respectively. (Mittapalli et al 2010).

Among these ESTs, 36% of the midgut and 38% of the fat body sequences showed similarity to proteins in the GenBank nr database. A high number of the midgut sequences contained chitin-binding peritrophin (248) and trypsin (98) domains; while the fat body sequences showed high occurrence of cytochrome P450s (75) and protein kinase (123) domains. Further, the midgut transcriptome of *A. planipennis* revealed putative microbial transcripts encoding for cell-wall degrading enzymes such as polygalacturonases and endoglucanases. A significant number of SNPs (137 in midgut and 347 in fat body) and microsatellite loci (317 in midgut and 571 in fat body) were predicted in the *A. planipennis* transcripts. Along with the above mentioned abundant sequences, significant number of genes involved in detoxification and antioxidation were also identified both in midgut and fatbody (Table 1.1).
Figure 1.1: Mechanisms of toxin metabolism in insects. Green circles represent allelochemical Pink area represent midgut lumen. Red line represents peritrophic membrane

Figure 1.2 Life cycle of *Agrilus planipennis*
Table 1.1: Occurrences of stress response genes in *A.planipennis* larval midgut and fatbody

<table>
<thead>
<tr>
<th>Response to stress genes</th>
<th>#Occurrence in midgut</th>
<th>#Occurrence in fat body</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytochrome P450</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CYP2 clade</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2, CYP24, CYP49, CYP303, CYP306</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td><em>CYP3 clade</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3, CYP6, CYP9, CYP28</td>
<td>37</td>
<td>45</td>
</tr>
<tr>
<td><em>CYP4 clade</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP4</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>Mitochondrial CYP clade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP12, CYP301, CYP314, CYP315</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td><strong>GST</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Epsilon, Sigma, Omega, Theta, Microsomal</em></td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Catalase</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Carboxylesterases</td>
<td>16</td>
<td>5</td>
</tr>
</tbody>
</table>
References:


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Chapter 2: Validation of reference genes for gene expression studies in emerald ash borer (*Agrilus planipennis*)

Abstract

Emerald ash borer (EAB, *Agrilus planipennis* Fairmaire) an exotic invasive insect pest has killed millions ash trees (*Fraxinus* spp.) across North American and threatens billions more. In this study, we validated six *A. planipennis* reference genes (actin, ACT; beta tubulin β-TUB; glyceraldehyde-3- phosphate dehydrogenase, GAPDH; ribosomal protein, RPL7; translation elongation factor, TEF-1α; and ubiquitin, UBQ) using geNorm, Normfinder and BestKeeper for accurate determination of target mRNA levels in gene expression studies of *A. planipennis*. The stability of the six reference genes was evaluated in different larval tissues, developmental stages and two treatments of *A. planipennis* using quantitative real-time PCR (RT-qPCR). Though there was no consistent ranking observed among the reference genes across the samples, the overall analysis revealed TEF-1α as the most stable reference gene. Given the variation found in the stability of genes in *A. planipennis*, it is essential to validate multiple reference genes prior to performing gene expression analysis of target genes. GAPDH and ACT showed least stability for all the samples studied. Thus, I conclude that TEF1- α is the most appropriate reference gene for gene expression studies in larval tissues, developmental stages and treatments of *A. planipennis*. 
Introduction

Quantitative real-time polymerase chain reaction (RT-qPCR) is the most reliable method for detection and quantification of transcript abundance (Bustin 2002). The sensitivity of RT-qPCR to detect subtle changes in gene expression under different conditions makes it an exceptional and trustworthy technique for quantification of mRNA levels even for low abundant transcripts (compared to Northern blotting). The procurement of accurate results from RT-qPCR is negatively correlated to the background errors i.e. lower the background error, greater the accuracy. Background errors constitute several parameters including quality and integrity of RNA samples, amount of starting material, efficiency of complementary DNA (cDNA) synthesis, transcriptional activity in the biological samples, primer design and retrotranscription efficiency (Andersen et al. 2004). In order to correct for such errors, normalization is required using a reference gene. Usually reference genes are those that are involved in basic cellular processes and are constitutively expressed across treatments. Reference genes are also known as internal control or housekeeping genes. In RT-qPCR, since the target gene is compared to the reference gene, it is necessary to select at least two or three stable reference genes for accurate quantification of target gene expression (Vandesompele et al. 2002).

Emerald ash borer (Agrilus planipennis Fairmaire) has become a major invasive insect of North American ash (Fraxinus spp). Since its discovery in 2002, it has killed millions of ash trees and threatens billions more across the United States (Herms et al.
With the advent of next generation sequencing methods, tissue-specific transcriptomic database for \textit{A. planipennis} using 454 pyrosequencing was developed (Mittapalli et al. 2010). Deciphering gene expression profiles and validation of mRNA levels for candidate genes via RT-qPCR has been crucial to on-going studies; also there is a paucity of knowledge on reference genes in insect systems. Thus far glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin (ACT), translation elongation factor 1α (TEF1-α), phospholipase A2 (PLA2), arginine kinase (AK), ribosomal proteins (RP49, RPS3, RPS18, RPL13a), have been reported to be stable reference genes in insects (Jiang et al. 2010; Van Hiel et al. 2009; Hornakova et al. 2010; Lord et al. 2010; de Boer et al. 2009; Scharlaken et al. 2008). To my knowledge this is the first report on the selection of reference genes for gene expression studies in \textit{A. planipennis}.

**Materials and Methods**

In this study, I selected six candidate reference genes including ACT, β-TUB (beta tubulin), GAPDH, RPL7, TEF-1α and UBQ (ubiquitin). Total RNA was isolated from three larval tissues (midgut, fat body and cuticle of late 3rd-instars) and from six developmental stages (1st, 2nd, 3rd, 4th, prepupal and adults) using Trizol reagent (Invitrogen). Larvae feeding on green (\textit{F. pennsylvanica}) and white (\textit{F. americana}) ash species represented treatments (although both species are susceptible to \textit{A. planipennis}, they respond differently). Approximately 1-2 μg of total RNA was used as the template for first strand cDNA synthesis using Superscript II cDNA synthesis kit (Invitrogen).
Resultant cDNA was diluted to 20 ng/µl concentration and used as template for RT-qPCR.

Primer pairs were designed using the Beacon primer designer 7.0 software (BioRad) with optimal parameters (20-24 bp in length, T_m of 50°C - 60°C and a product size of 90-220 bp). Relative expression value for the genes was calculated using the standard curve method (User Bulletin #2: ABI Prism 7700 Sequence Detection System vide supra (http://www3.appliedbiosystems.com/). Standard curves were constructed with serially diluted pooled cDNA samples. Correlation co-efficient (R^2) and PCR efficiency (E %) was calculated from the standard curve. All the tested primer sets showed an R^2 above 0.99 with E values ranging from 89.28 - 102.07 (Table 2.1). Stability analysis for the six reference genes was done using three different softwares (excel add-ins): geNorm (Vandesompele et al. 2002), Normfinder (Andersen et al. 2004) and Bestkeeper (Pfaffl 2004).

**Results and Discussion:**

The stability values calculated for six candidate reference genes using geNORM revealed TEF-1α as the best reference gene for *A. planipennis* irrespective of the samples (Fig 2.1A, 2.1C, 2.1E). ACT was scored rank #2 in developmental stages; however it was the least stable in tissues and treatments (Table 2.2). The minimum number of genes required for optimal normalization varied across the samples: while 2 genes were suggested for the tissue and treatment samples, 5 genes were suggested for the
developmental samples (Fig. 2.1B, 2.1D, 2.1F). geNORM calculates the average expression stability value (M) and gives the two most stable genes out of the set of genes submitted. The lower the M value, the more stable is the gene with the maximum cut off of 1.5. A normalization factor is calculated based on the geometric mean of the most stable gene and a pairwise variation (V) is calculated between the sequential normalization factors to decipher the optimal number of genes required for precise estimation of target mRNA levels (Vandesompele et al. 2002).

In accordance with geNORM, the stability analysis output obtained with Normfinder revealed TEF-1α as the most stable gene across all samples except for the developmental samples, for which TEF-1α was scored 2nd for stability (Table 2.2B). geNORM and Normfinder operate on the same principle but the latter has an underlying mathematical model which calculates the stability value by combining the inter- and intra-group expression value(s). Variation in expression of the genes is reciprocally related to their stability value, i.e. lower the stability value, lesser is the variation in gene expression among the samples assayed (Andersen et al. 2004).

There was no consistent ranking observed for the reference genes studied across the samples using the BestKeeper software. TEF-1α was observed to be stable only in the treatment samples in contrast to the results obtained with geNORM and Normfinder. However, individual analyses demonstrated that all the genes were stable in tissues, whereas only RPL7 and TEF-1α were verified to be stable in the development samples (Table 2.2C). BestKeeper calculates the descriptive statistics of the given set of genes and obtains a BestKeeper index from the most stable expressed gene (Pfaffl et al. 2004).
Pairwise comparison of all the genes to BestKeeper index identifies the genes with similar expression patterns. Therefore, this software also enables us to monitor the expression levels of target gene in comparison with reference gene. Assessment of expression stability is done based on the calculated variations including standard deviation and coefficient of variance. Ideally, genes that have a standard deviation less than one are considered to be stable (Table 2.2C).

In summary, stability analysis of reference genes in A. planipennis revealed TEF-1α as the potential candidate for gene expression studies. TEF-1α is involved in protein synthesis and is widely used in gene expression studies in plants and animals including insects (Jiang et al 2010; Van Hiel et al. 2009; Tong et al. 2009; Wan et al. 2010; Shen et al. 2010). Another candidate reference gene identified in this study was RPL-7, which showed consistent stability across the samples assayed. Ribosomal proteins are involved in translation and protein synthesis and are reported to be best reference genes in many insects (Scharlaken et al. 2008; Hornakova et al. 2010). Irrespective of the software’s used, β-TUB and UBQ exhibited moderate stability across the samples assayed. While β-TUB plays an important role in cell growth and response to stimuli, UBQ a highly conserved protein is vital for protein degradation and participates in several cellular functions (Hershko & Ciechanover 1998, Nielsen et al. 2010). Among the reference genes analyzed, GAPDH and ACT showed least stability for the samples examined. To conclude: albeit gene expression is highly affected by various physiological conditions such as tissue, age and treatments (Van Hiel 2009; Jiang et al. 2010; Hornakova et al. 2010), expression levels of TEF-1α was found to be most stable across all the samples.
analyzed and thus would be recommended for normalization in gene expression studies
of *A. planipennis*.

**Acknowledgements**

I thank Amy Stone, The Ohio State University Extension, for assisting with collection
of *A. planipennis* larvae. I thank Loren Rivera-Vega (Department of Entomology, The
Ohio State University/OARDC).
Figure 2.1 Ranking, stability and determination of optimal number of reference genes for *Agrilus planipennis* using geNorm. A, C and E represent ranking based on average expression stability value (M) for tissues, developmental stages and treatments; B, D and F display optimal number of genes required for the accurate estimation of the target gene mRNA in tissues, developmental stages and treatments calculated by the pairwise variation (V) of the normalization factors (NFn and NFn+1). Note: *ACT* (actin), *BTUB* (β-tubulin), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *RPL7* (ribosomal protein), *TEF-1α* (translation elongation factor 1α), and *UBQ* (ubiquitin).
Table 2. 2: Summarized output of the three softwares used for selecting best reference gene in *Agrilus planipennis*

<table>
<thead>
<tr>
<th>Rank</th>
<th>Tissues</th>
<th>Development</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>TEF-1α</strong> (0.188)</td>
<td><strong>TEF-1α</strong> (0.182)</td>
<td><strong>TEF-1α</strong> (0.205)</td>
</tr>
<tr>
<td>1</td>
<td>RPL7</td>
<td>ACT</td>
<td>RPL7</td>
</tr>
<tr>
<td></td>
<td>(0.189)</td>
<td>(0.194)</td>
<td>(0.206)</td>
</tr>
<tr>
<td>2</td>
<td>β-TUB</td>
<td>UBQ</td>
<td>UBQ</td>
</tr>
<tr>
<td></td>
<td>(0.205)</td>
<td>(0.194)</td>
<td>(0.230)</td>
</tr>
<tr>
<td>3</td>
<td>GAPDH</td>
<td>GAPDH</td>
<td>β-TUB</td>
</tr>
<tr>
<td></td>
<td>(0.303)</td>
<td>(0.227)</td>
<td>(0.286)</td>
</tr>
<tr>
<td>4</td>
<td>UBQ</td>
<td>β-TUB</td>
<td>GAPDH</td>
</tr>
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<td></td>
<td>(0.312)</td>
<td>(0.228)</td>
<td>(0.334)</td>
</tr>
<tr>
<td>5</td>
<td>ACT</td>
<td>RPL7</td>
<td>ACT</td>
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<tr>
<td></td>
<td>(0.332)</td>
<td>(0.234)</td>
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<td>(0.098)</td>
<td>(0.056)</td>
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<tr>
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<td>(0.062)</td>
<td>(0.109)</td>
<td>(0.085)</td>
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<td>RPL7</td>
<td>β-TUB</td>
</tr>
<tr>
<td></td>
<td>(0.211)</td>
<td>(0.156)</td>
<td>(0.185)</td>
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<td>GAPDH</td>
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<td>GAPDH</td>
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<tr>
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<td>(0.261)</td>
<td>(0.158)</td>
<td>(0.300)</td>
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<tr>
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<td>(0.291)</td>
<td>(0.195)</td>
<td>(0.354)</td>
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<table>
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<th>Tissues</th>
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<th>Treatments</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>RPL7</td>
<td>RPL7</td>
<td>TEF-1α</td>
</tr>
<tr>
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<td>(0.33)</td>
<td>(0.92)</td>
<td>(0.76)</td>
</tr>
<tr>
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<td>UBQ</td>
<td>TEF-1α</td>
<td>RPL7</td>
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<td></td>
<td>(0.41)</td>
<td>(0.99)</td>
<td>(0.833)</td>
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<td>UBQ</td>
<td>UBQ</td>
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<td>(0.60)</td>
<td>(1.03)</td>
<td>(0.845)</td>
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<td>β-TUB</td>
<td>β-TUB</td>
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<td>(1.27)</td>
<td>(0.85)</td>
</tr>
<tr>
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<td>ACT</td>
<td>ACT</td>
<td>GAPDH</td>
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<tr>
<td></td>
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<td>6</td>
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<td>ACT</td>
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<tr>
<td></td>
<td>(0.74)</td>
<td>(1.37)</td>
<td>(1.32)</td>
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Chapter 3: Antioxidant genes of the emerald ash borer (Agrilus planipennis): gene characterization and expression profiles

Abstract

Phytophagous insects frequently encounter reactive oxygen species (ROS) from exogenous and endogenous sources. To overcome the effect of ROS, insects have evolved a suite of antioxidant defense genes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). Emerald ash borer (Agrilus planipennis Fairmaire), an exotic invasive insect pest from Asia has killed millions of ash trees and continues to invade North America at a rapid pace. From an on-going expressed sequence tag (EST) project of A. planipennis larval tissues, we identified ESTs coding for a Cu-Zn SOD (ApSOD1), a CAT (ApCAT1) and a GPX (ApGPX1). A multiple sequence alignment of the derived A. planipennis sequences revealed high homology with other insect sequences at the amino acid level. Phylogenetic analysis of ApSOD1 grouped it with Cu-Zn SODs of other insect taxa. Real time quantitative PCR (RT-qPCR) analysis in different larval tissues (midgut, fat body, Malpighian tubule and integument) revealed higher mRNA levels of ApCAT1 in the midgut. Interestingly, higher mRNA levels for both ApSOD1 and ApGPX1 were observed in the Malpighian tubules. Assay of mRNA levels in developmental stages (larva,
prepupa and adults) by RT-qPCR indicated higher transcript levels of *ApCAT1* and *ApGPX1* in larval and prepupal stages with a decline in adults. On the other hand, the transcript levels of *ApSOD1* were observed to be constitutive in all the developmental stages assayed. However, at the enzyme level activity of CAT, SOD and GPX were similar in all the developmental stages (neonates, larvae and pupae). Results obtained reflect a plausible role of these *A. planipennis* antioxidant genes in quenching ROS from both diet (ash phloem) as well as endogenous sources.
Introduction

Aerobic organisms are continuously exposed to oxidative stress due to the by-products produced during oxygen metabolism and via the metabolism of the encountered toxins including allelochemicals and pesticides (Adamski and others 2003; Barbehenn 2002). These by-products include superoxide radical (\(\bullet O_2^-\)), hydroxyl radical (\(\bullet OH\)) and hydrogen peroxide (\(H_2O_2\)) collectively termed as reactive oxygen species (ROS). Adverse effects of ROS include damage to biologically important macromolecules (DNA, proteins and lipids) leading to programmed cell death (Felton and Summers 1995; Halliwell and Gutteridge 1999; Hanham and others 1983; Imlay and others 1988). However, in insects, ROS are also thought to be involved in innate immunity (Hao and others 2003; Kumar and others 2003).

Phytophagous insects have developed unique defense strategies via detoxification and antioxidant enzymes mainly to combat the deleterious effects of pro-oxidant rich diets obtained from their host plants (Felton and Summers 1995; Krishnan and Kodrik 2006; Mittapalli and others 2007a). Among the antioxidant enzymes, the most studied group of enzymes are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). SOD catalyzes the dismutation of superoxide radical to stable hydrogen (Krishnan and Kodrik 2006) peroxide and oxygen; CAT catalyzes hydrogen peroxide to water and oxygen(Ahmad 1992; Fridovich 1995). On the other hand, APX also catalyzes the conversion of hydrogen peroxide to water but at low concentrations whereas GPX converts hydroperoxides to less reactive alcohols.
in cell membranes (Ahmad, 1989). These antioxidant enzymes are collectively involved in scavenging the exogenous and endogenous ROS to prevent oxidative stress in insects (Krishnan and Kodrik 2006; Mittapalli and others 2007a; Yang and others 2010).

Emerald ash borer (*Agrilus planipennis* Fairmaire), an invasive wood-boring beetle in the family Buprestidae has reached high impact status by killing millions of North American (NA) ash trees (*Fraxinus spp.*) since its discovery in southeast Michigan in 2002 (Poland and McCullough 2006). At the current rate of invasion *A. planipennis* has the potential to decimate NA ash with impacts reminiscent to those of Dutch elm disease and chestnut blight (Herms and others 2004). However, the damage caused by this beetle to its native host (Manchurian ash, *F. mandshucrian*) is insignificant, perhaps a virtue of their co-evolutionary history (Rebek and others 2008). The unique phloem chemistry of Manchurian ash (phenylethanoids and hydroxycoumarins) may contribute to its resistance to *A. planipennis* (Eyles and others 2007). The major damage to ash is caused by the larvae as they feed on the phloem forming typical S-shaped galleries. Adults emerge in late spring or early summer leaving a D-shaped exit hole and feed on foliage (Poland and McCullough 2006). From the *A. planipennis* larval midgut transcriptomic database, sequences of candidate antioxidant genes including a Cu-Zn SOD, a CAT and a GPX, were identified and designated as “*ApSOD1*”, “*ApCAT1*” and “*ApGPXI*”. Further, molecular characterization and expression analysis (via real time quantitative PCR, RT-qPCR) in different tissues (midgut, fat body, Malpighian tubules and integument) and developmental stages (1st-4th instars, prepupa and adult) of *A. planipennis* is reported.
Materials and method

Sequence retrieval, annotation and alignment

The sequences of antioxidant gene were retrieved from the EST database reported per (Mittapalli and others 2010). From the annotated sequences of *A. planipennis* EST database a full length EST of a Cu-Zn SOD (*ApSOD1*) and partial ESTs of a CAT (*ApCAT1*) and a GPX (*ApGPX1*) were recovered. Further identification and annotation of these antioxidant genes was performed by searching against the non-redundant database using the BlastX algorithm at National Center for Biotechnology Information, (NCBI, Bethesda, MD, USA) (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed using an online tool MUSCLE provided by a web service, phylogeny.fr. (www.phylogeny.fr). The protein secondary structure of *ApSOD1* was predicted using the PSIPRED protein structure prediction server at http://bioinf.cs.ucl.ac.uk/psipred/(Jones 1999). For phylogenetic analyses, an unrooted neighbour joining tree was constructed with 1000 bootstrap replicates and excluding positions with gap using MEGA version 5 (Tamura and others 2007).

Insect Material and Dissections

*A. planipennis* larvae were collected from two naturally infested sites of green ash trees at Michigan and Ohio, USA. Larvae of different development stages (1\textsuperscript{st}-4\textsuperscript{th} instar
larvae, prepupa and adults) were collected and maintained in moist conditions on ice till dissections. For tissues, 3\textsuperscript{rd} and 4\textsuperscript{th} instars (7-8 larvae) were dissected in ice cold 1X phosphate buffer saline as described by (Vasanthakumar and others 2008) with few modifications in the dissection technique. Larvae were pinned on a paraffin wax platform at the head and last abdominal segment to perform dissections more efficiently. Tissues including midgut, fat body, Malpighian tubules and integument were collected separately in 1.5ml centrifuge tubes containing pre-chilled Trizol reagent and stored at -80\textdegree C until RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated from tissues and developmental stages including 1\textsuperscript{st}-4\textsuperscript{th} instars, prepupa and adults using Trizol reagent following the manufacturer’s protocol. The total RNA obtained was resuspended in 40µl of DEPC-treated water and the concentration was measured using a Nanodrop (Thermo scientific Nanodrop 2000). About 1-2 µg of total RNA was used for first strand cDNA synthesis using the Superscript II first strand cDNA synthesis kit (Invitrogen). The resultant cDNA was diluted to 20 ng/µl for further use in gene expression analysis using RT-qPCR.
Expression analysis using real-time quantitative PCR (RT-qPCR)

First strand cDNA was used as the template for measuring mRNA levels via RT-qPCR. Reaction mixture consisted of 2 µl cDNA (20ng/µl), 1µl of 5 picomole/µl forward primer, 1µl of 5 picomole/µl of reverse primer, 5 µl of SYBR green (Bio-Rad) and 1µl of nuclease free water making the total reaction volume to 10µl. Concentration of the template and primers were standardized based on the Ct values and melting curves. Target gene was amplified at the following conditions: 95°C for 3 min and then, 40 cycles of 95°C for 10 s and 60°C for 30 s. Primer pairs were designed using the Beacon primer designer 7.0 software (BioRad) with optimal parameters (Table 3.1). Quantitation of target mRNA levels in all the samples (tissues and developmental stages) was analyzed by relative standard curve method as described in the ABI Prism 7700 user bulletin (User Bulletin #2: ABI Prism 7700 Sequence Detection System (http://www3.appliedbiosystems.com/). A. planipennis specific TEF-1α was used as the internal control which was found to be consistent among the samples assayed (Rajarapu and others 2012). Fold change of genes was calculated relative to tissue or developmental stage with lowest expression respectively.

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**Enzyme assays:**

Activity of CAT, SOD and GPX was measured in neonates, larvae and prepupae. Tissues were ground in liquid nitrogen and suspended in sodium phosphate buffer (pH 7, 66mM). Homogenates were centrifuged for 10 minutes at 10,000g. Supernatant was collected and used for assaying the total protein content followed by the activity assays. Total protein content in the extracts was measured using Pierce 660nm against standard curve of serially diluted BSA standards (2-0.13mg/ml). Specific activity of CAT and GPX was measured as described previously with few modifications (Barbehenn 2002). Absorbance of all the assays were measured using Multiscan plate reader (Thermoscientific).

For catalase assay reaction mixture consisted of 10µl of enzyme extract and 5 µl of 3% H₂O₂ and made up to 350µl with phosphate buffer (pH 66mM, pH 7). Change in absorbance was measured at 240nm for 5 minutes. Specific activity of CAT was expressed as micromoles of H₂O₂ reduced per minute per milligram of protein using an extinction coefficient of 39.4M⁻¹cm⁻¹.

Reaction mixture for GPX assay consisted of 20µl of glutathione reductase (1.67 EU/ml), 100 µl of sodium phosphate buffer (pH 7, 66mM containing 1 mM reduced glutathione and 0.2 mM NADPH) and 50µl of Cumene hydroperoxide (1.4 mM in buffer). The rate of decrease in the absorbance at 340 nm was measured for 4 min.

Total SOD activity was measured using Sigma Superoxide dismutase assay kit (Thermoscientific). Briefly, to 200 µl of WST working solution provided in the kit, 20 µl
of dilution buffer and 20µl of enzyme extract were added and incubated at 37°C for 20
minutes. Following incubation absorbance was measured at 240 nm. Specific activity was
calculated as the percentage of inhibition rate of SOD as per the kit protocol. (Sigma
19160 SOD determination kit).

Statistical analysis

Relative expression values (REVs) obtained from standard curve method were
analyzed by PROC MIXED Analysis of Variance (ANOVA) with a significance level (α)
of 0.05 using SAS (SAS Institute Inc. SAS.STAT User’s Guide, Version 9.1). Mean
separation was calculated by Tukey’s method (α = 0.05). For each treatment there were
two biological replicates nested with two technical replicates each. Enzyme activities
were analyzed using general linear model in Minitab 16 on three biological replicates.

Results

Gene characterization of antioxidant genes

Among the annotated antioxidant genes, a full length sequence for ApSOD1 with
an open reading frame consisting of 154 amino acids was retrieved (465 bp; Figure
2.1A). A similarity search of the retrieved ApSOD1 against the non-redundant
nucleotide (nr) database at NCBI using BlastX, revealed 81% similarity to a cytoplasmic
Cu-Zn SOD of *Tribolium castaneum* (XP_968284.1, 2e-68), 77% similarity with *Gryllotalpa orientalis* Cu-Zn SOD (AAV73809.1, 3e-63) and 76% identity with *Camponotus floridanus* Cu-Zn SOD (EFN70085.1, 5e-62) at the amino acid level. A similar search for *ApCAT1* showed 71% identity with catalase of *T. castaneum* (NP_001153712.1, 8e-107), 78% similarity with two different catalase isoforms of *Anopheles gambiae* (ABL09376.1, 8e-107; AAR90327.1, 8e-107) at the amino acid level. Similarity search of *ApGPX1* showed 62% similarity with *Hydra sps.* (ADI56239.1, 2e-07), 56% similarity with GPX of *Aedes aegypti* (XP_001662240.1, 3e-08) and 52% similarity with *T. castaneum* GPX (XP_969937.2, 1e-07).

The deduced amino acid sequence of *ApSOD1* revealed all the important characteristics of a typical Cu-Zn SOD protein including Cu binding site, Zn binding site and two signature motifs (Figure 3.1A). The Cu binding site has three Histidine residues at H46, H48 and H120. Three amino acid residues including Histidine (H63), Histidine (H80) and Aspartate (D83) interact with the Zn ion at the zinc binding site. The two signature motifs were present at the N terminal and C terminal end spanning 9 and 12 amino acids respectively (Figure 3.1A). Secondary structure of the deduced amino acid sequence of *ApSOD1* showed 9 alpha helices and 10 coils. Two SOD specific signature motifs were located within the alpha helices of the deduced protein structure (Figure 3.1B).
Multiple sequence alignment

A partial multiple sequence alignment of the deduced amino acid sequences of \textit{ApSOD1}, \textit{ApCAT1} and \textit{ApGPX1} with other insect sequences revealed a high level of homology among the taxa (Figure 3.2). The percentile of similarity (i.e. identical residues) was 53.3\% for \textit{ApSOD1}, 63.3\% for \textit{ApCAT1} and 34.7\% for \textit{ApGPX1} among the sequences compared.

Phylogenetic analysis

In order to reveal the phylogenetic relationship of \textit{ApSOD1} with other insect sequences and/or the types of SODs, an unrooted neighbor joining tree was constructed with the amino acid sequences of different types of SODs. The dendrogram clearly grouped the SOD sequences into two major clades (i) Cu-Zn SODs and (ii) Mn SODs. \textit{ApSOD1} was included within the Cu-Zn SOD clade (Figure 3.3). Specifically, \textit{ApSOD1} was more closely related to the Diptera Cu-Zn SODs, compared to the Lepidoptera Cu-Zn SODs.

Transcript levels in larval tissues

The transcript levels of \textit{ApSOD1}, \textit{ApCAT1}, and \textit{ApGPX1} were determined in different tissues (midgut, fat body, Malpighian tubule and integument) of 3\textsuperscript{rd} and 4\textsuperscript{th}
instar larvae using RT-qPCR. All the genes assayed showed a tissue specific expression pattern, wherein *ApSOD1* showed higher mRNA levels in the Malpighian tubules with minimal expression levels in fat body and midgut. High mRNA levels of *ApCAT1* were observed in the midgut tissue with low levels in all the other tissues assayed. *ApGPX1* showed high transcript levels in fat body and Malpighian tubules with comparatively low mRNA levels in the midgut and integument (Figure 3.4A). All the genes analyzed showed the least transcript levels in the integument sample. Therefore, the integument sample was taken as the calibrator (1X) (Pfafl, 2001) to calculate the relative fold change among the tissues. *ApCAT1* showed a fold difference of >10,000X (P< 0.05) in the midgut compared to the integument. A fold change of 55.17X (P< 0.05) was observed for *ApSOD1* in the Malpighian tubules compared to the integument. *ApGPX1* showed a fold change of 25X (P< 0.05) in the Malpighian tubules compared to the integument.

*Transcript and enzyme levels during development*

RT-qPCR analysis of *ApSOD1*, *ApCAT1* and *ApGPX1* was also performed in the developmental stages including the four larval instars (1st- 4th), prepupa and adults. Both *ApCAT1* and *ApGPX1* displayed a differential gene expression pattern among the developmental stages assayed, whereas *ApSOD1* had similar mRNA levels throughout the developmental stages assayed (constitutive). High mRNA levels of *ApCAT1* were observed in the 1st and 3rd instars, and prepupal stages with a sudden drop in adults. *ApGPX1* transcript levels were found to be high in the 4th instar and prepupal stage.
compared to other developmental stages (Figure 3.4B). Relative fold change of transcript levels was calculated by taking the adult sample as the calibrator (1X) (Pfaffl, 2001). Significant (p < 0.05) fold change of >10,000 was observed in all stages for \textit{ApCAT1} when compared to the transcript levels in the adult sample. \textit{ApGPX1} showed a significant (p < 0.05) fold change in 4th (3.3X) and prepupal (4.85X) instars when compared to the adult stage. \textit{ApSOD1} had no significant variation among the developmental stages assayed. There were no significant differences in the total enzyme activity of CAT, SOD and GPX during the developmental stages (Neonates, larvae and adults) (Figure 3.5).

**Discussion**

Phytophagous insects have developed a suite of antioxidant defense response in order to deal with the dietary allelochemicals biosynthesized by their host plants. This feature has enabled them to exploit even some of the most noxious and/or naïve ecological niches. \textit{Agrilus planipennis} seems to fit well with the latter, evident from its rampant invasion of NA ash. Resistance of Manchurian ash to \textit{A. planipennis} may result from its unique phloem chemistry with high constitutive levels of phenolics compared to the NA ash species (Eyles and others 2007). Phenolic compounds have the potential to generate ROS and in turn could create oxidative stress within insect tissues (Krishnan and Kodrík 2006). With this knowledge I report the molecular characterization and expression profile of antioxidant gene in \textit{A. planipennis} feeding on green ash.
The tissue specific transcriptomic study of *A. planipennis* revealed a number of antioxidant genes along with other candidate defense genes (Mittapalli and others 2010). In this study the identified antioxidant genes (*ApSOD1, ApCAT1* and *ApGPX1*) shared homology with similar genes from other insect and non-insect species. Specifically, the derived secondary structure of *ApSOD1* revealed several conserved regions of Cu-Zn SOD and the phylogenetic analysis clearly corroborates with previous studies on insect SODs (Yamamoto and others 2007). In general, phytophagous insects overcome oxidative stress during feeding with an up regulation of antioxidant enzymes like SOD, CAT, GPX etc. (Krishnan and Kodrik 2006). Metabolically active tissues like the midgut and fat body provide a platform for major physiological functions which are crucial for insect survival and adaptation (Mittapalli and others 2010; Mittapalli and others 2007a). Tissue specific transcript levels of antioxidant genes in the midgut, fat body and Malpighian tubules in this study were in agreement with other studies (Mittapalli and others 2007a; Munks and others 2005).

Among the antioxidant genes assayed, higher mRNA levels of *ApCAT1* were observed in the midgut compared to *ApSOD1* and *ApGPX1*. This observation probably suggests H$_2$O$_2$ as one of the primary ROS encountered during *A. planipennis*-ash interaction. Putative hypothesis supporting these results include: (i) H$_2$O$_2$ might be one of the primary defense responses of the host plant against insect attack as observed in previous studies (Bi and Felton 1995); (ii) H$_2$O$_2$ might be produced due to phenolic oxidation in the midgut of *A. planipennis* larvae (Felton and others 1989; Kanofsky and
Axelrod 1986). However, these hypotheses with relevance to *A. planipennis* – ash interaction needs to be validated.

A higher level of ApSOD1 transcripts in the Malpighian tubules relative to other issues is intriguing. Recently, it was shown that Malpighian tubules play an important role in detoxification and elimination of toxins (Beyenbach and others 2010b). Although speculative at this point, the function of *ApSOD1* within the Malpighian tubules of *A. planipennis* is to be determined in future studies. Other tissues that revealed higher mRNA levels of *ApSOD1* include the midgut, which is a major interface for digestion and detoxification. Oxidation of dietary phenolics within this vital tissue could lead to the formation of superoxide radicals and therefore be the probable source for *ApSOD1* in the midgut of *A. planipennis* as observed in other studies (Krishnan and Kodrík 2006; Krishnan and others 2007). While the higher transcript levels of *ApGPX1* in the Malpighian tubules of *A. planipennis* is intriguing but unknown at this time. Higher mRNA levels of *ApGPX1* in the fat body suggests their probable role in protecting the tissue from oxidative damage.

Higher mRNA levels of *ApCAT1* in larval and prepupal stages relative to other stages further supports its role in quenching H$_2$O$_2$ derived from the diet and endogenous sources. Negligible *ApCAT1* mRNA levels in adult beetles might be due to (a) low secondary metabolite concentration in the matured leaves on which they feed (Chen and Poland, 2009). (b) presence of an adult specific *ApCAT* gene in dealing with the oxidative toxicity. The sudden decrease of *ApCAT1* transcript levels in adults potentially reflects
the different sources of ROS encountered during the development stages. This latter explanation could suggest the difference in the chemical composition in the phloem and foliage of ash. The constitutive (similar mRNA levels) expression of *ApSOD1* during developmental stages indicates its continuous participation in removal of ROS generated from endogenous sources. Higher levels of *ApGPX1* in late instars (4th and prepupa) might suggest the defense against lipid peroxides stemming from tissue differentiation (Kostaropoulos and others 1996; Mittapalli and others 2007a). Constitutive (similar) enzyme levels of all the antioxidant genes during development might indicate the need for high levels of these enzymes to establish a successful feeding site on green ash. Further feeding bioassays with the presence and absence of phenolics in diet might shed light on the contribution of these genes in encountering the oxidative stress that might be encountered during feeding.

**Acknowledgements**

I thank Loren Rivera-Vega (Department of Entomology, The Ohio State University/OARDC), Kathleen Knight and Kyle Costilow (USDA North Research Station, Delaware), Andrew Boose (Columbus Metro Park), Amy Stone (Ohio State University Extension, Toledo) and Brad Philips (Berlin Heights Metroparks) for assisting with collection of *A. planipennis* larvae. I also appreciate Naresh Kumar and Angela Strock (The Ohio State University, OARDC) for help with driving and sample collection. I thank Therese Poland (USDA, Michigan) for timely supply of EAB adults and eggs.
Table 3.1: Primers used for real time quantitative PCR analysis. *Ap* in the gene name indicates *Agrilus planipennis*. EF-1 α-Elongation factor, SOD- Superoxide dismutase, CAT-catalase, GPX-glutathione peroxidase

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApEF1α - F</td>
<td>CATTGAAACCTACGTGTCGC</td>
<td>21</td>
<td>61.8</td>
<td>97.6</td>
</tr>
<tr>
<td>ApEF1α - R</td>
<td>ACTGGAGTGCTAAACCTGG</td>
<td>20</td>
<td>61.8</td>
<td></td>
</tr>
<tr>
<td>ApCAT- F</td>
<td>GCCCTGTCTCCATCAAAT</td>
<td>18</td>
<td>59.8</td>
<td>96.8</td>
</tr>
<tr>
<td>ApCAT- R</td>
<td>CGGACAGTGACAGGAAT</td>
<td>18</td>
<td>59.5</td>
<td></td>
</tr>
<tr>
<td>ApSOD - F</td>
<td>AAATGGTAGGAAAGCGG</td>
<td>18</td>
<td>59.9</td>
<td>95.7</td>
</tr>
<tr>
<td>ApSOD - R</td>
<td>CTTTACGGGTGCTTGGGA</td>
<td>18</td>
<td>59.2</td>
<td></td>
</tr>
<tr>
<td>ApGPX - F</td>
<td>ACACCCTGTGAAACGTCCGC</td>
<td>21</td>
<td>60.5</td>
<td>100.8</td>
</tr>
<tr>
<td>ApGPX - R</td>
<td>CCTGCGTGAGGGTATCACCAAT</td>
<td>23</td>
<td>59.6</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1 Characterization of a superoxide dismutase (SOD) gene from *Agrilus planipennis* (*ApSOD1*). (A) Nucleotide (first line) and deduced amino acid sequences (second line) of *ApSOD1*. The start codon (ATG) along with its coded amino acid (M) is highlighted in bold and the stop codon is represented as an asterisk (*). Amino acids within the Cu binding site (H46, H48 and H120) are shown with open inverted triangles (▼). Amino acids within the Zn binding site (H63, H80 and D83) are shown with a filled inverted triangle (▲). The two signature motifs are boxed. (B) Predicted secondary structure of *ApSOD1*. Cylinders and solid black lines represent alpha-helices and coils, respectively. Amino acids underlying the alpha-helices are underlined.
Figure 3.2. Multiple sequence alignment of *Agrilus planipennis* antioxidant genes: *ApCAT1* with *Tribolium castaneum* (NP001153712.1), *Hydra vulgaris* (ABC250281), *Homo sapiens* (AAK2918.1); *ApSOD1* with *T. castaneum* (XP968284.1), *Macaca mullata* (NP001027976.1) and *Taenia solium* (AAL66230.1); and *ApGPX1* with sequences of *T. castaneum* (XP969937.2), *Loa loa* (XP003146189.1) and *Hydra sps* (AD156239). Identical residues among all taxa are indicated by “*”*, conserved substitutions by “.” and semi-conserved substitutions by “:” symbols.
Figure 3.3 Neighbour joining phylogenetic tree of *Agrilus planipennis* superoxide dismutase (*ApSOD1*) with other insect SODs. Members of Cu-Zn SOD and Mn SOD pertaining to different insect orders were included: (i) Cu-Zn SODs: *Drosophila melanogaster* (NP 476735.1), *Glossina morsitans morsitans* (ADD20357.1), *Bombyx mori* (NP 001037084.1) and *Hyphantria cunea* (BAF73670.1) (ii) Mn SODs: *D. melanogaster* (NP 476925.1), *G. morsitans morsitans* (ADD18846.1), *B. mori* (NP 001037299.1) and *H. cunea* (ABL63640.1). The numbers on the branch represent bootstrap values.
Figure 3.4. Expression patterns of *Agrilus planipennis* antioxidant genes (*Ap*CAT1, *Ap*SOD1 and *Ap*GPX1) in (A) larval tissues (midgut (MG), fatbody (FB), Malpighian tubules (MT) and integument (CU)) and (B) developmental stages (1st – 2nd larval instars, prepupae (PP) and adult) using real-time quantitative PCR. Relative expression values (REV) were calculated by normalizing the expression with a *A.planipennis* translation elongation factor 1-α (TEF-1α). Error bars represent the pooled standard error for two biological replicates (each with two technical replicates).
Figure 3.5: Specificity of Superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPX). Activity of SOD is determined as the percentage of inhibition rate of the enzyme per mg of protein. Activity of CAT is determined as the micromoles of H₂O₂ reduced per minute per mg of protein. Activity of GPX is determined by the amount of NADPH oxidized per mg of protein.
References:


Yang, LH, Huang, H and Wang, JJ (2010) Antioxidant responses of citrus red mite, 
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Chapter 4: Detoxification enzyme profiles in the emerald ash borer, *Agrilus planipennis* Fairmaire

Abstract

Emerald ash borer, *Agrilus planipennis* Fairmaire is a recently discovered invasive insect of ash, *Fraxinus* spp. in North America. Detoxification is an important mechanism for metabolic resistance against xenobiotics in insects and consists of two phases, phase I and phase II. Key enzymes involved in this process include cytochrome P450 (P450s) and glutathione-S-transferases (GST). In this study, I report the molecular characterization and expression patterns of different classes of P450s and GST in different tissues and developmental stages along with their specific activity during development. Multiple sequence alignment of all five *A. planipennis* P450s (*CYP6FH1, ApCYP6A, ApCYP6B, ApCYP9 and ApCYP12*) and six GSTs (*ApGST-E1, ApGST-E2, ApGST-E3, ApGST-O1, ApGST-S1 and ApGST-µ1*) revealed conserved features of insect P450s and GSTs. Phylogenetic analysis grouped the P450s and GSTs within their respective families and classes. Real time quantitative PCR of field collected larval tissues showed higher mRNA levels for *CYP6FH1* in Malpighian tubules and *ApCYP6A* in midgut. Transcript levels of *ApCYP6B, ApCYP12* were higher in midgut and Malpighian tubules. Transcript levels of *ApCYP9* were higher in midgut and integument. Higher mRNA levels of *ApGST-E1, ApGST-E3* and *ApGST-O1* were observed in the
midgut and Malpighian tubules. On the other hand, ApGST-E2 and ApGST-S1 showed high mRNA levels in fat body and ApGST-µ1 was constitutively expressed in all tissues assayed. During development, all P450s assayed, except ApCYP6A and ApCYP12, were constitutively expressed. Transcripts of ApCYP12 were lower in early larval stages (1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd}). mRNA levels of ApCYP6A were higher in adult stages. ApGST-E2 was observed to be highest in feeding instars, ApGST-S1 in prepupal instars; while the others were constitutively expressed in all developmental stages examined. At the enzyme level, total P450 and GST activity was similar in all the developmental stages assayed. These results suggest that A. planipennis is potentially primed with a suite of detoxification genes whose function is to metabolize ash allelochemicals and that also participate in other physiological functions.
**Introduction**

Emerald ash borer, *Agrilus planipennis* Fairmaire, is a recently introduced invasive insect of high impact status in North America (NA). This wood boring buprestid beetle was accidentally introduced from Asia and was first discovered in the year 2002 (Poland and McCullough 2006). Since its discovery it has killed millions of ash trees (*Fraxinus* spp.) and has a potential to eliminate all the NA ash trees (Herms and others 2004). At the current rate of infestation, *A. planipennis* might have an impact reminiscent to those of Chestnut blight and Dutch elm disease (Herms et al., 2004). Larvae (1<sup>st</sup>-4<sup>th</sup> instars) are the most destructive life stages of *A. planipennis* wherein they girdle ash phloem forming S-shaped galleries and thus obstruct the flow of nutrients eventually killing trees in 3-4 years post-infestation (Cappaert and others 2005). The native Asian host (e.g. Manchurian ash, *F. mandshurica*) is resistant to *A. planipennis* attack perhaps by virtue of their co-evolutionary history (Rebek and others 2008). However, stressed Manchurian ash trees are reported to be colonized by this beetle (Haack and others 2002). Studies on comparative phloem chemistry suggested a unique suite of phenolic compounds present in Manchurian ash that might contribute resistance towards *A. planipennis* colonization (Eyles and others 2007; Whitehill and others 2012). Further, transcriptomic and proteomic level comparison also revealed elevated levels of defense genes/proteins in Manchurian ash as compared to NA ash (Bai and others 2011; Whitehill and others 2011).
Detoxification provides a crucial line of defense through metabolic resistance against a multitude of chemicals including allelochemicals and synthetic pesticides/insecticides (Terriere 1984). It is a two phase process with key players of phase I as cytochrome P450s (P450s), and phase II as glutathione-S-transferases (GSTs) (Terriere, 1984). These genes might also be a key component in the adaptation of phytophagous insects to noxious/naïve host environments (Feyereisen 1999; Schuler 2011).

Cytochrome P450 (P450s):

Cytochrome p450s (p450s) represent highly diverse superfamily of genes involved in biosynthetic, bioactivation and detoxification reactions (Feyereisen 1999). These enzymes have a potential to evolve either by selective mutations of catalytic sites altering substrate specificity or by mutations in the promoter sequences to increase their expression (Schuler 2011). Due to the expanding diversification of P450 genes in insects, they have been classified into four clades comprising several families depending on the percentage identity at their amino acid level. These include CYP3 clade; CYP4 clade; Mitochondrial CYP clade and CYP2 clade (Feyereisen 2006). Among these, CYP3 clade is well studied and known to be involved in detoxification of toxins (Feyereisen, 2006). However, recent studies have reported the involvement of CYP4 clade and a subset of mitochondrial clade also in neutralizing toxins (David and others 2006; Snyder and others 1995).

Unlike the former three clades CYP2 clade encompasses highly conserved genes involved in essential physiological functions (hormone biosynthesis) (Feyereisen, 2006).
Irrespective of the clades, P450 genes share certain universal features at the amino acid level: a heme-binding sequence motif FXXGXXXXCXG, a K-helix EXXRXXP, a C-helix WXXXR and an oxygen binding motif AGXXT. These motifs are highly variable and constitute the substrate recognition sites (SRSs) which participate in substrate binding and recognition (Hlavica 2006).

Glutathione-S-Transferase (GSTs):

GSTs are well known superfamily of enzymes that are involved in detoxifying a wide range of compounds including endogenous and exogenous sources. The mode of action of GSTs is via converting insoluble toxic compounds into less toxic and soluble compounds by an electrophilic addition of the reduced glutathione molecule to the reacting group of the toxin (Habig and others 1974). GSTs also play an important role in protecting the cell against oxidative stress and also in transportation of endogenous lipophilic compounds (Listowsky and others 1988; Weinhold and others 1990).

Depending on their localization in the cell, GSTs are classified as cytosolic and mitochondrial (Enayati and others 2005). Cytosolic GSTs are further classified into six classes including delta, epsilon, omega, sigma, theta and zeta. Irrespective of the class, each subunit of these dimeric proteins has a conserved N-terminal thioredoxin like domain containing 4 β sheaths and 3 α helices (βαβαβα) and a C- terminal domain with α helices (Lumjuan and others 2007). The N-terminal domain has a conserved glutathione binding site, the “G” site, whereas the C-terminal domain has a highly variable substrate binding “H” site. Among all the cytosolic GSTs, delta and epsilon seem most predominant in insect genomes and are known to metabolize insecticides and
plant allelochemicals, thus providing metabolic resistance to insects (Kostaropoulos and others 2001; Ranson and others 2001; Yu 1982). Alternatively omega, sigma, theta and zeta are ubiquitously distributed across taxa and are involved in other essential physiological functions in addition to detoxification. In contrast to other GSTs, microsomal GSTs are not involved in detoxification (Enayati et al., 2005).

From *A. planipennis* larval midgut EST databases, a total of 5 P450s and 6 GSTs belonging to different classes were identified. Expression patterns of these genes were determined in different tissues and developmental stages. Specific activity of total P450 and GST was also determined in neonates, larvae and adults.

**Materials and method**

**Insect Material**

Different developmental stages of *A. planipennis* larvae (1st-4th, prepupal and adults) feeding on green ash (*F. pennsylvanica*) were collected from three naturally infested sites, including Berlin heights, Toledo and Columbus. Newly hatched larvae (neonates) were collected from eggs of adults feeding on green ash in Michigan. Larvae measuring less than 1 cm were identified as first, 1-2 cm as second, 2-3 cm as third, more than 3 cm as fourth instars. Tissues including midgut, fat body, Malpighian tubules and integument were isolated from approximately 10 individuals of 4th instars per replicate in 500 µl Trizol as per (Rajarapu and others 2011). For each developmental stage, 5
individuals per replicate were ground with liquid nitrogen and suspended in 500ul Trizol reagent for total RNA isolation.

**RNA isolation and cDNA synthesis**

Total RNA was isolated from the tissues and developmental stages using 1ml of Trizol reagent (Invitrogen) following the manufacturer’s protocol and treated with Turbo DNase from Ambion. Resultant RNA concentration and purity were measured using a Nanodrop (Thermo scientific Nanodrop 2000). All the samples had $A_{260}/A_{280}$ of 1.8-2.0. About 1-2 µg of total RNA was used for first strand cDNA synthesis using the SuperScript™ First-strand synthesis system for RT-PCR following the manufacture’s protocol (Invitrogen).

**Expression analysis using real-time quantitative PCR (RT-qPCR)**

Sequence retrieval, annotation and phylogenetic analysis were done as mentioned in chapter 3. Primer pairs for the ESTs used in this study are given in Table 4.1. Specificity of the primers was analyzed in silico using BlastN algorithm at NCBI (National center for Biotechnology Information). Expression analysis was performed using Bio-Rad CFX96 Real time System as mentioned earlier (chapter 3). For each primer a no template control (NTC) and a no reverse transcription control (NRT) were
Levels of mRNA or relative expression values (REV) were calculated by Bio-Rad CFX software by comparative Ct method (Livak and Schmittgen 2001). Fold change in expression was calculated relative to neonates in developmental stages and relative to integument in tissues. Translation elongation factor 1- α (TEF-1α) of *A. planipennis* was used as internal control to normalize the expression of the genes across different samples analyzed (Rajarapu and others 2012).

*Extraction and estimation of protein:*

For measuring the enzyme activity, three biological replicates of 10 individuals per stage including neonates (<1day old), larvae (1st – 4th; > 1day old) and adults from green ash were homogenized in liquid nitrogen. Phosphate buffer (pH 7, 100mM) with 100mM EDTA, 10% glycerol, 50mM SDS was added to ground tissues in the ratio of 1:10 (w/v) and were homogenized on ice. Homogenate was centrifuged at 10,000 g for 10 min at 4°C and 10 μl of supernatant was used for activity assay. Protein concentration of the enzyme extract was estimated with Pierce 660 nm reagent over a range of BSA standards (2– 0.13 mg/ml). All the enzyme assays were performed in 96 well plate and the absorbance was measured using MultiScan plate reader (Thermoscientific).
**Cytochrome P450 activity assay:**

Total P450 was measured using P-Nitoanisole-O-Demethylation assay (PNOD) as per Bautista et al. (2009) with small modifications. In brief p-nitroanisole (2mM, 20 µl) and enzyme extract (20µl) were incubated for 10 minutes at room temperature. After incubation, NADPH (4.8mM, 5µl) was added and further incubated for 5min at 30°C. Absorbance of the product formed was measured at 405nm and the concentration was measured using the standard curve generated by serial dilutions of p-nitrophenol (0-100pmol). The PNOD activity was measured as picomole of p-nitrophenol formed per microgram of protein per 5 min.

**Glutathione –S-transferase.1 activity assay:**

Total GST activity was measured using a GST assay kit from Sigma-Aldrich following the manufacturer’s protocol (Habig et al., 1974). Briefly, for 50 assays- 10ml substrate buffer was prepared with 9.8 ml Dulbecco’s phosphate buffer saline, 0.1ml glutathione (100mM) and 0.1ml 1-chloro-2,4-dinitrobenzene (CDNB, 200mM) was prepared. To 10 µl of enzyme extract 190 µl of substrate buffer was added and absorbance was measured for 5 minutes with 30 second interval. Enzyme blank was treated similar to the samples. Specific activity was calculated as micromole of glutathione conjugate formed per milliliter of protein extract per minute per microgram of protein (µmol/ml/min/µg of protein).
2.6 Statistical analysis

Relative expression values and specific activity of enzymes was analyzed using general linear model in Minitab (version 16). Mean separation was done using Tukey’s method ($\alpha = 0.05$). Transformation of observations was done where necessary. For each treatment (tissues and developmental stages), there were three biological replicates.

Results

Sequence retrieval and phylogenetic analysis:

Full and partial length sequences of P450s and GSTs from existing *A. planipennis* larval midgut databases were identified. Five different P450s including *CYP6FH1*, *ApCYP6A*, *ApCYP6B*, *ApCYP9* and *ApCYP12* were mined from the database. Top Blast results showed high similarity of *A. planipennis* P450s to *Tribolium castaneum* P450 sequences (Table 4.2). A multiple sequence alignment of *A. planipennis* P450s with their respective reference sequences from *T. castaneum*, *Aedes aegypti*, *Anopheles gambiae* and *Nassonia vitripennis* revealed two conserved motifs including EXXLXXP and a 10 amino acid residue signature motif FXXGXXXXCXG at the N terminus (Figure 4.1).

Phylogenetic analysis using an unrooted neighbor joining tree clustered *ApCYP6* s including CYP6FH1, *ApCYP6A* and *ApCYP6B* with *T. castaneum* and *A. aegypti* CYP6 with boot strap values of 98 and 82 respectively. Accordingly *ApCYP9* and *ApCYP12*
grouped closely with Hymenopteran *N. vitripennis* CYP9 and Coleopteran *T. castaneum* CYP12 with boot strap values of 61 and 54 respectively (Figure 4.2).

Retrieved GSTs belonged to epsilon, omega, sigma and microsomal classes. They have been named as *ApGST-E1* (654 bp, 217a.a), *ApGST-E2* (657 bp, 218 a.a), *ApGST-E3* (663 bp, 220 a.a), *ApGST-S1* (597 bp, 198 a.a), *ApGST-O1* (762 bp, 253 a.a) and *ApGST-µ1* (453 bp, 150 a.a). Sequence identity search using BlastX algorithms showed highest similarities to the respective reference sequences in the database (Table 4.3). A multiple sequence alignment revealed a conserved glutathione binding site (G-site) for all the classes (Figure 4.3). Epsilon class GSTs including *ApGST-E1*, *ApGST-E2* and *ApGST-E3* have conserved G-site residues Glu 68, Ser 69 and Arg 111. In sigma class member *ApGST-S1*, Tyr 25, Trp 40, Gln 51, Gln 64 and Ser 65 constitute the G-site. *ApGST-O1* has highly conserved residues Cys 28, Pro 29 and Tyr 30 at the N-terminal. Microsomal GST, *ApGST-µ* has the 16 amino acid signature motif VERVRAHLNDVENIL spanning from 63-79 a.a.

An unrooted neighbor joining phylogenetic tree was constructed with the deduced amino acid sequences of *A. planipennis* along with other insect GSTs including Coleoptera, Diptera and Lepidoptera (Figure 4.4). Among the different GSTs of *A. planipennis* *ApGST-E1*, *ApGST-E2* and *ApGST-E3* were grouped with Epsilon GSTs of other insects with bootstrap values of 43-63. *ApGST-S1* was closely related to *D. melanogaster* GST sigma with a bootstrap value of 70. Similarly *ApGST-O1* and *ApGST-µ1* were grouped with other insect GST omega and microsomal class members with a bootstrap value of 73 and 60 respectively.
Expression patterns of detoxification genes in tissues:

The mRNA levels of *ApGSTs* and *ApCYPs* were assessed in different tissues including midgut, fat body, Malpighian tubules and integument using RT-qPCR. Levels of mRNA in tissues were compared to mRNA levels in integument. Among the CYP6 members significantly higher expression of *CYP6FH1* was observed in Malpighian tubule (40X), *ApCYP6A* in midgut (6.6X) and *ApCYP6B* in midgut (9.4X) and Malpighian tubules (11X). Higher mRNA levels of *ApCYP9* were observed in midgut (1.1X) and integument (1X). mRNA levels of *ApCYP12* was significantly higher in midgut (4.4X) and Malpighian tubules (4X) (Figure 4.5A).

The mRNA levels of *ApGST* were assessed in different tissues including midgut, fat body, Malpighian tubules and integument using RT-qPCR with integument as the calibrator (1X). Among the epsilon GSTs *ApGST-E1* showed peak mRNA levels in midgut (91X) and Malpighian tubules (20X); *ApGST-E2* in fat body (6.0X) and *ApGST-E3* in midgut (48X). *ApGST-S1* mRNA levels were high in fat body (4X). Levels of *ApGST-O1* were high in midgut (16X) and Malpighian tubules (11X). Transcript levels of *ApGST-µ1* were high in Malpighian tubules (Figure 4.5B).
Transcript profiles during development:

Transcript levels for all *A.planipennis* P450s and GSTs were determined in all stages of development including neonates, 1<sup>st</sup>-4<sup>th</sup> larval instars, prepupae and adults using RT-qPCR. Transcripts of *CYP6FH1, ApCYP6B* and *ApCYP9* members showed constitutive (similar) levels throughout the developmental stages including neonates, larvae and adults. mRNA levels of *ApCYP6A* showed higher levels in adult stages (4X) (Figure 4.6A).

Among *ApGSTs*, Epsilon member transcripts varied during development wherein levels of *ApGST-E2* were higher in 2<sup>nd</sup> (2.6X) and 3<sup>rd</sup> (4.2X) instars; *ApGST-E3* were lower in prepupal (0.08X) and adult (0.004X) instars and *ApGST-E2* levels were constitutive during development. Interestingly, *ApGST-S1* had higher mRNA levels in prepupal stage (4.5X). Further, mRNA levels of *ApGST-O1* and *ApGST-µ1* were constant during development (Figure 4.6B).

Total enzyme activity:

Specific activity of P450 and GST was determined in neonates, larvae and adults. There was no significant difference (P > 0.05) in the total enzyme activity among the developmental stages assayed (Figure 4.7).
Discussion

Detoxification is a two phase physiological process (phase I and phase II) that occurs in various animal taxa in order to eliminate toxins. These two phases of detoxification either act in co-ordination or independently depending on the type of compound encountered. Phase I enzymes include P450s and among the phase II enzymes, GSTs are the major players in expulsion of toxins (McMahon and others 1987). There have been several studies showing the importance of detoxification enzymesgenes to insects in developing resistance to xenobiotics including chemical insecticides and plant toxins (Huang and others 2011; Schuler 2011; Scott 1999; Ugale and others 2011a; Yu and Huang 2000). In this study I report the characterization and transcript/enzymatic profiles of A. planipennis P450s and GSTs. From the blast results, it was evident that there is a general paucity of molecular knowledge in wood boring beetles. Further, multiple sequence alignment of P450s and GSTs with other insect sequences revealed conserved residues of these sequences (Feyereisen 2006; Mittapalli and others 2007b; Wang and others 2008; Yamamoto and others 2011). The dominance of the recovered epsilon class GST and CYP6 members perhaps implies the importance of these genes during the course of evolution/adaptation of this beetle on its host as observed in other insect groups (Enayati and others 2005; Ketterman and others 2011).

A differential expression pattern was observed for all the assayed P450s in different larval tissues. ApCYP6A and ApCYP6B had higher levels of transcripts in midgut tissue. In insects CYP3 clade members (CYP6 and CYP9) are known to be
induced in presence of xenobiotics including plant secondary chemicals and insecticides (Bass and others 2011; Bautista and others 2009; Cifuentes and others 2012; Jones and others 2011; Mao and others 2011; Niu and others 2011; Zhou and others 2010). Higher expression of ApCYP6A and ApCYP6B in midgut tissue of A. planipennis might be responding to the ash allelochemicals. Several studies of plant-insect interaction have shown the midgut tissue as the major interface for several detoxification enzymes (Hakim and others 2010; Mittapalli and others 2010; Pauchet and others 2010; Rajarapu and others 2011). On the other hand CYP6FH1 and ApCYP6B expressed higher transcripts in Malpighian tubules which is a vital tissue for excretion, but also participates in detoxification (Chahine and O'Donnell 2011). Recent reports demonstrated that Malpighian tubules are also known to play a role in detoxification of xenobiotics (Beyenbach and others 2010a; Chahine and O'Donnell 2011; Dow 2009; Dow and Davies 2006). Higher expression of the P450s in midgut and Malpighian tubules suggests the potential involvement of these genes in detoxifying host secondary compounds.

Another CYP3 clade member, ApCYP9 exhibited an interesting pattern of expression wherein it showed higher transcript levels in midgut and integument. Although many studies have identified CYP6 members as key enzyme in detoxification of xenobiotics, CYP9 members also play a significant role in toxin metabolism (Stevens and others 2000). Higher transcript levels of ApCYP9 in A. planipennis midgut might be induced in presence of ash allelochemicals in diet similar to ApCYP6 members. Interestingly, higher mRNA levels in integument might suggest their involvement in dealing with either endogenous substrates (e.g. cuticular hydrocarbons) or
allelochemicals encountered due to the physical contact of larvae with ash phloem during feeding.

Transcripts of ApCYP12 were higher in midgut and Malpighian tubules which are important tissues of detoxification as mentioned earlier. Members of CYP12 are known to be induced in presence of insecticides and allelochemicals (Bogwitz and others 2005; Feyereisen 1999; Guzov and others 1998). Higher expression of ApCYP12 in midgut and Malpighian tubules with a significantly lower expression in integument suggests the role of ApCYP12 in dealing with the allelochemicals obtained via diet or other endogenous metabolites.

Expression analysis of ApGSTs in tissues showed unique profiles for different class members. Epsilon group members, particularly ApGST-E1 and ApGST-E3 showed peak mRNA levels in the midgut tissue wherein they might be participating in metabolizing dietary toxins ingested from the host plant. ApGST-E2 was also higher in Malpighian tubules, an important tissue studied in dealing with dietary toxins as observed in other insects (Chahine and O'Donnell 2011). On the other hand, ApGST-E2 was highly expressed in fat body which is also an important metabolic tissue for detoxification as demonstrated earlier in A.planipennis (Mittapalli et al., 2010) and in Hessian fly, Mayetiola destructor (Mittapalli et al., 2007).

Higher mRNA levels of ApGST-μ1 were found in midgut and Malpighian tubules. Microsomal GSTs are membrane proteins involved in protecting the cell from oxygen toxicity via lipid peroxidation products (Shi and others 2012). Abundant mRNA levels of this gene in vital tissues including midgut, and Malpighian tubules might be due to higher
stress levels encountered by these tissues during the metabolism of ash toxins and/or the result of other physiological processes. Transcript levels of \textit{ApGST-O1} were higher in midgut and Malpighian tubules. Omega class GST members are known to be induced in the presence of stress factors like bacteria, insecticides and UV light (Yamamoto et al., 2011). Higher levels of \textit{ApGST-O1} in midgut and Malpighian tubules demonstrate that these tissues might encounter some potential toxins via diet. Sigma class member, \textit{ApGST-S1} had higher mRNA levels in fat body as identified in other insects suggesting their possible role in detoxification and other critical physiological functions (Flanagan and Smythe 2011; Huang and others 2011; Kim and others 2011; Yamamoto and others 2006).

Profiling of \textit{ApCYPs} in \textit{A. planipennis} developmental stages revealed interesting patterns. Constitutive higher levels of \textit{CYP6FH1}, \textit{ApCYP6B} and \textit{ApCYP9} was observed in all the instars. Adapted specialist have shown to have a high activity of enzymes involved in detoxifying a compound frequently encountered in its host (Bull and others 1986). Since \textit{A. planipennis} is also a specialist well adapted on green ash, higher number of \textit{ApCYP6} and \textit{ApCYP9} transcripts might be expressed constitutively to the compounds present in green ash phloem. However, there might be other P450s responding to the phloem allelochemicals. Higher levels of \textit{ApCYP6A} in adults might be induced due to the compounds encountered in foliage.

Intriguingly, the developmental profiles of \textit{ApGSTs} revealed constitutive patterns in all the developmental stages assayed except for \textit{ApGST-E2} and \textit{ApGST-S1}. mRNA
levels of *ApGST-E2* was higher in fat body and during feeding stages which might be induced due to the presence of dietary factors. Similar profiles for epsilon GSTs were reported in hoverfly, *Episyrphus balteatus* and diamondback moth, *Plutella xylostella* (Sonoda and others 2006; Vanhaelen and others 2001). On the other hand, mRNA levels for *ApGST-S1* were comparatively higher in the prepupal stage which implies its role in dealing with oxidative stress caused by profound physiological changes during metamorphosis as observed in other insects (Feng and others 2001; Hazelton and Lang 1983; Mittapalli and others 2007b). Additionally, an induction in GST activity due to high hemolymph juvenile hormone (JH) titers was observed in common cutworm, *Spodoptera litura* (Lu and Wu 2007). Presumably, JH titers increase during the prepupal stages of *A. planipennis* as well, which could represent a second factor for the induction of *ApGST-S1*.

I further analyzed the activity of GSTs and P450s in neonates, larvae, and adults. Though there was a differential transcript pattern at mRNA level during development, specific activity of *ApGSTs* towards CDNB was the same in all the stages examined. A poor correlation of enzyme mRNA and actual activity levels has been previously observed for genes with expression regulated by post-transcriptional and post-translational parameters, such as splicing, translational and functional inactivation (Maier et al., 2009). In contrast, specific activity of P450s in developmental stages corroborated the mRNA levels/patterns. Given that total enzyme activities were measured, the contribution of individual isoforms remains unclear. Alternatively, different isoenzymes may target different specific artificial substrates.
In conclusion, expression patterns of *ApCYPs* and *ApGSTs* provide insights into their probable functions in different tissues and development stages. *CYP6, CYP9* and *GST* epsilon class members of *A. planipennis* might be among the key tools used in the successful adaptation of this beetle to its naïve host. Overall, results indicate that midgut and Malpighian tubules might play a significant role in *A. planipennis* ash interactions. Being a specialist, *A. planipennis* might be primed for P450 and GST-based detoxification of green ash secondary compounds, in addition to using these enzymes for other fundamental physiological functions.

**Acknowledgements**

I thank Kathleen Knight and Kyle Costilow (USDA North Research Station, Delaware), Andrew Boose (Columbus Metro Park), Amy Stone (Ohio State University Extension, Toledo) and Brad Philips (Berlin Heights Metroparks) for assisting with collection of *A. planipennis* larvae. I also appreciate Naresh Kumar and Angela Strock (The Ohio State University, OARDC) for help with driving and sample collection. I also thank Therese Poland (USDA, Michigan) for timely supply of *A. planipennis* adults and eggs for collecting neonates.
Table 4.1: Primers used to amplify *Agrilus planipennis* P450s and GSTs

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
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<td>CATTGAAACCTACGTTGTCGC</td>
<td>21</td>
<td>61.8</td>
<td>97.6</td>
</tr>
<tr>
<td><em>ApEF1α</em>-R</td>
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<tr>
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<td><em>ApGST-6A</em>-R</td>
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<tr>
<td><em>ApGST-E2</em>-F</td>
<td>GATGATGACGGATTTGTATTTCC</td>
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<td>TTTGCCCATACCTTTCCATTAG</td>
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<tr>
<td><em>ApGST-E7</em>-F</td>
<td>TGAATAGCGGCACATTAGT</td>
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Table 4.2: Top 3 blast hits for *Agrilus planipennis* Cytochrome P450s (*ApCYP*)

<table>
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<tr>
<th>Subfamily</th>
<th>Gene name</th>
<th>Length (amino acids)</th>
<th>Reference organism with NCBI accession number</th>
<th>Similarity (%)</th>
<th>E value</th>
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<td><em>Harpegnathos saltator</em></td>
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Figure 4.1: Partial alignments of *Agrilus planipennis* P450 amino acid sequences with respective insect P450 family members: CYP6FH1, ApCYP6A and ApCYP6B with *Tribolium castaneum* (TcCYP6, gi|189236550) and *Aedes aegypti* (AaCYP6, gi|157120794); ApCYP9 with *Drosophila mettleri* (DmCYP9, gi|3493155) and *Nisonia vitripennis* (NvCYP9, gi|289177209); ApCYP12 with *T. castaneum* (TcCYP12, gi|91093939) and *Anopheles gambiae* (AgCYP12, gi|27752853). Boxed residues encompass conserved signature motifs EXXRXXP and FXGXXXCXG. Asterisk (*) indicates highly conserved residues, semicolon (;) and period (.) indicate partially conserved residues.
Figure 4.2: Phylogenetic analysis of *Agrilus planipennis* Cytochrome P450 (ApCYP) amino acid sequences with other insect P450 sequences. The topology of the tree was derived by the distance/Neighbour joining criteria with 1000 bootstrap replicates. The numbers on the branch represent bootstrap values. CYP6FH1, ApCYP6A and ApCYP6B with *Tribolium castaneum* ([gi|189236550|](gi|189236550|)) and *Aedes aegypti* ([gi|157120794|](gi|157120794|)); ApCYP9 with *Drosophila mettleri* ([gi|3493155|](gi|3493155|)) and *Nisonia vitripennis* ([gi|289177209|](gi|289177209|)); ApCYP12 with *T. castaneum* ([gi|91093939|](gi|91093939|)) and *Anopheles gambiae* ([gi|27752853|](gi|27752853|)).
Table 4.3: Top 3 blast hits of *Agrilus planipennis* glutathione–S-Transferases (*ApGST*) class members

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Putative identification</th>
<th>Reference organism</th>
<th>Similarity (%)</th>
<th>E value</th>
</tr>
</thead>
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<tr>
<td><em>ApGST</em>-E1 glutathione S-transferase ‘epsilon’</td>
<td><em>Musca domestica</em> AAD54937.1</td>
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<td><em>Stomoxys calcitrans</em> AC083224.1</td>
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<td><em>Tribolium castaneum</em> XP_967313.1</td>
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<td><em>ApGST</em>-E2 glutathione S-transferase’ epsilon</td>
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<td><em>ApGST</em>-μ1 microsomal glutathione s-transferase</td>
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Figure 4.4: Phylogenetic analysis of *Agrilus planipennis* glutathione-S-transferase (ApGST) amino acid sequences with other insect GST sequences. ApGST epsilon members include ApGST-E1, ApGST-E2, ApGST-E3; ApGST-S1 a sigma class member; ApGST-O1, a omega class member and ApGST-µ1, a microsomal GST. The topology of the tree was derived by the distance/Neighbour joining criteria with 1000 bootstrap replicates. The numbers on the branch represent bootstrap values. Accessions of reference sequences used for analysis: *Drosophila melanogaster* GST-E6 gi|199225321|; *Tribolium castaneum* GST-E5 gi|91076572|; *D. melanogaster* GST-D1 gi|7299601|; *Helicoverpa armigera* GST-D gi|16555415|; *T. castaneum* GST-D gi|91080625|; *D. melanogaster* GST-S gi|21627085|; *T. castaneum* GST-S gi|91092852|; *Bombyx mori* GST-O2 (gi|112983952|); *T. castaneum* GST-O gi|270004515|; *D. melanogaster* GST-O gi|268321293|; *T. castaneum* GST-u gi|91086441|; *Aedes aegypti* GST-u (gi|157114362|).
Figure 4.5 mRNA levels of *Agrilus planipennis* detoxification genes in the larval tissues including midgut (MG), fat body (FB), Malpighian tubules (MT) and integument (IN) using real-time quantitative PCR (A) Cytochrome P450 members (CYP6FH1, *ApCYP6A*, *ApCYP6B*, *ApCYP9* and *ApCYP12*); (B) glutathione-S-transferase members (*ApGST-E1*, *ApGST-E2*, *ApGST-E3*, *ApGST-S1*, *ApGST-O1* and *ApGST-μ1*) Transcript levels were normalized with *Agrilus planipennis* translation elongation factor 1-α. Error bars represent the pooled standard error for three biological replicates (each with two technical replicates).
Figure 4.6: Relative fold change in transcript levels of (a) Cytochrome P450s (CYP6FH1, ApCYP6A, ApCYP6B, ApCYP9 and ApCYP12); (b) glutathione-S-transferases (ApGST-E1, ApGST-E2, ApGST-E3, ApGST-S1, ApGST-O1 and ApGST-µ1) and in developmental stages of Agrilus planipennis including larval instars (1st - 4th), prepupa (PP) and adult using real time quantitative PCR. Transcript levels were normalized with Agrilus planipennis translation elongation factor 1-α. Neonate stage was taken as calibrator (1X). Error bars represent the pooled standard error for three biological replicates (each with two technical replicates).
Figure 4.7: Specific activity of (A) P450s and (B) GSTs in different stages including neonates (< 1 day old), larvae (1st-4th instars), and adults. (A) Values represent picomole of p-nitrophenol per microgram of protein per 5 min. (B) Values represent micromole of 1-chloro-2,4-dinitro benzene converted per milliliter per minute. Means are representative of three biological replicates with their respective standard error of the mean.
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Chapter 5: Fate of green ash, *Fraxinus pennsylvanica* phloem phenolics in larval emerald ash borer, *Agrilus planipennis*

Abstract

Phenolics are a diverse group of compounds widely distributed in terrestrial plants. They are known to have deleterious effects on herbivores wherein they act as antifeedants/feeding deterrents, pro-oxidants, antidigestants and/or toxins. Emerald ash borer, *Agrilus planipennis* Fairmaire is an invasive wood boring beetle in North America and a specialist on ash trees (*Fraxinus* spp.). The secondary chemistry of *Fraxinus* spp. is enriched with phenolics. Larvae of *A. planipennis* are well adapted to feed on phenolic-rich phloem tissues and cause extensive damage to the tree. In this study, targeted metabolomics of *F. pennsylvanica* phloem, larval tissues (midgut tissue, hindgut-Malpighian tubules, carcasses), midgut content, and frass revealed differences in phenolic profiles. Presence of new compounds was observed in larval tissues and midgut content indicating participation of these tissues in transforming host phloem phenolics. A few compounds, including syringin, oleuropein hexoside, verbascoside, oleuropein-related compounds, and other unknown peaks were found to be excreted without modification. Results show that *A. planipennis* larvae are metabolically capable to process ash phenolic compounds.
Introduction

Plants synthesize a repertoire of secondary compounds in defense of pathogens and herbivores (Swain 1977). Herbivores have evolved to exploit these chemical defenses via multiple mechanisms such as behavioral avoidance, excretion, metabolic resistance, sequestration and target site mutation (Despres and others 2007). Insects can either use a single mechanism or combination of mechanisms to overcome the host defense barrier. Also, these offense mechanisms differ between specialists and generalists. Specialists tend to have specific mechanisms to deal with allelochemicals as opposed to generalists (Ahmad and others 1986). With the advances in omic techniques countermeasures employed by insects to deploy host defenses are being studied to greater details.

Metabolomics is a well-established technique providing advantage to study the interaction of an organism with its environment at molecular level (Bundy and others 2009). In the chemical warfare between insects and plants, metabolomics is an important tool to identify the chemical phenotype of both the plant and its interacting herbivore. However, they are limited studies conducted in insect-plant interaction to understand the fate of secondary compounds in insects (Glauser and others 2011; Jansen and others 2009; Vihakas and others 2010).

Emerald ash borer, *Agrilus planipennis* Fairmaire is an invasive wood boring beetle specialized on *Fraxinus* spp (ash trees). Larval stages of *A. planipennis* feed on ash phloem forming S-shaped frass filled galleries which obstruct the flow of nutrients leading to the death of tree. Secondary chemistry of ash phloem is dominant with
phenolic compounds including hydroxycoumarins, a monolignol, lignans, phenylethanoids, and secoiridoids (Eyles and others 2007). Phenolics may have direct or indirect impacts on herbivores depending on the physiological conditions of the organism (Appel 1993). Effect of phenolics on herbivores has been well studied in Lepidoptera (Felton and others 1992; Isman and Duffey 1982; Roth and others 1994). Coleoptera, specifically wood boring beetles which have been notorious invasive insects of North America are not well studied for the countermeasures employed against host defenses. Thus, my objective was to study the fate of ash phloem phenolics in *A. planipennis* larvae. Analysis of green ash phloem, *A. planipennis* larval midgut tissue, midgut content and frass revealed distinct and intriguing phenolic patterns. Compounds present in phloem indicated potential mechanisms employed by *A. planipennis* larvae to metabolize the ingested phenolics.

**Materials and methods**

Insects:

Late fourth instar larvae of *A. planipennis* feeding on green ash (*Fraxinus pennsylvanica*) were collected from three trees naturally infested sites in Berlin heights, Toledo and Columbus (N=9). Along with larvae, phloem and frass were also collected from the trees. Approximately 10 larvae per tree were dissected to isolate midgut tissue, midgut content, hindgut and Malpighian tubules. Remaining tissue (carcasses) of the
larvae consisting head, fat body, integument were pooled. Midgut tissue, midgut content, hindgut tissue-Malpighian tubules and carcasses were freeze dried for 24 hrs before extractions.

Data Analysis and statistics:

Total phenolics were measured from UV peak areas and were analyzed using general linear model. Means were compared using Tukey comparisons ($\alpha=0.05$) in Minitab (v.16).

Results

Chromatograms of all the samples including phloem, midgut content (MGC), midgut tissue (MGT), hindgut-Malpighian tubules (HG/MT), carcasses (CC) and frass showed qualitative differences (Figure 5.1). Comparison of total phenolics among phloem, MGC and frass showed significantly higher levels in phloem. There was no significant differences in total phenolics present in tissues (Figure 5.2A; 5.2B).

Further, qualitative analysis of the samples revealed interesting patterns. Syringin, unknown peak 5, 15, 17 and 31 were present in phloem, MGT, MGC and frass. oleuropein hexoside, verbascoside, oleuropein related compound, unknown peak 29 were present in phloem, MGT, MGC, HG-MT and frass. Syringaresinol was present in all the samples analyzed. elenolic acid derivative, nuzhenide, unknown peaks 10, 41, 43
compounds were present only in phloem, MGT and MGC. New compounds formed present in midgut content and frass include peaks 8, 20 and 52. There were several new compounds found only in frass, these include peaks 12, 24, 25, 28, 30, 35, 39, 45, 47 and 50. Phenolic profiles of CC showed an interesting pattern wherein five compounds unique to this tissue were observed including peaks 48, 53, 54, 55, and 1 (Table 5.1).

**Discussion**

Plants synthesize a wide array of compounds to protect against biotic and abiotic stressors. To date there have been approximately 200,000 chemicals known to be produced by plants (Mithöfer and Boland 2012). They are broadly classified into different groups based on their chemical structure. Terpenoids, alkaloids and phenolics are the largest class of compounds encompassing more than fifty thousand compounds (Mithöfer and Boland 2012). These compounds hinder several physiological processes of herbivores effecting their growth and development. Secondary chemical profiles of ash phloem showed presence of phenolics as constitutive defenses (Cipollini and others 2011; Eyles and others 2007; Whitehill and others 2012).

Presence of higher amounts of total phenolics in phloem compared to midgut content and frass indicates the disruption of phenolic structure by the larvae. It also suggests the potential role of foregut tissue in metabolizing the phenolics. Comparison of phenolic profiles of phloem with larval tissues including MGT, MGC, HG-MT, CC and larval frass showed an overall change in the profiles. Presence of syringin, unknown
peaks 5, 15, 17, and 31 in phloem, MGT, MGC and frass indicates that larvae excrete these compounds unmodified. Similarly, oleuropein hexoside, oleuropein related compound, verbascocide, unknown peaks 29 and 32 were also excreted unmodified. However, the quantities excreted are relatively less compared to the amounts present in phloem. Rapid excretion is an efficient mechanisms adapted by specialist insects (Rosenthal and Janzen 1979). Similar observations were also identified in other phytophagous insects feeding on flavonoid and terpene containing hosts (Ferreres and others 2008; Gomez and others 1999; Murray and others 1994). In addition to this, Sorensen and Dearing (2006) has proposed “regulated absorption model” as an important counter mechanisms along with detoxification in specialists herbivores. According to the model, herbivores decrease the amount of plant secondary metabolites circulating the body by limiting the absorption through rapid excretion facilitated by Multidrug resistance transporters (MDR). In mammals, these transporters are found in drug resistant tumor cells (Higgins 1992b). In insects presence of these transporters has been characterized in midgut and Malpighian tubule transporting xenobiotics (Chahine and O'Donnell 2011; Labbe and others 2011a; Labbe and others 2011b). Midgut transcriptome of larval A. planipennis also showed the presence of MDRs. Nevertheless, involvement of A. planipennis MDRs in transportation of syringin, oleuropien hexoside, oleuropein related compound, verbascocide, and others needs to be further evaluated.

Presence of elenoic acid derivative, nuzhenide, unknown peaks 10, 41 and 43 in MGT indicates their uptake and by this tissue. Midgut tissue is the primary interface of the insect-plant interactions involved in vital physiological mechanisms like
detoxification and digestion (Dow 1986). *A. planipennis* MGT has higher mRNA levels of Cytochrome P450s (P450) and glutathione-s-transferase (GSTs) (*chapter 3*). These enzymes can be induced in presence of plant secondary metabolites (Terriere 1984). Formation of new compounds in MGT might indicate the involvement of these enzymes in transforming ash allelochemicals that this tissue might have encountered.

Presence of new compounds in MGC and frass indicates that these compounds might be intermediates and/or end products of few metabolized phenolics in the gut lumen. Oxidation of phenolics depending on the insect gut physiochemical conditions leads to the formation of toxic intermediates like reactive oxygen species and quinones (Barbehenn 2002). Many studies have shown the involvement of antioxidant enzymes in dealing with the active oxygen species in gut lumen, but the fate of quinones is least known (Appel 1993). Presence of new compounds in MGC suggests potential involvement of foregut in metabolizing phenolics. Interestingly, presence of new compounds in CC shows the participation of other tissues also in dealing with the phenolic. Presence of new compounds in frass indicates the metabolism of phenolic compounds by *A. planipennis* larvae and corroborates with the expression of detoxification genes in vital larval tissues as mentioned in previous chapters.

**Conclusions**

Specialist herbivores use multiple resistance mechanism to overcome the detrimental effects of host plant secondary metabolites (PSMs) (Despres et al., 2007).
One of the most efficient counter defense mechanisms is to limit the absorption of PSMs from the diet (Sorensen and Dearing, 2006). In *A. planipennis* larvae, these efflux mechanisms seem to play an important role in excreting the ingested PSMs. Larvae were also able to efficiently metabolize the phenolics present in the diet. In addition, the presence of new compounds in carcasses potentially indicates the role of other tissues in dealing with ash phenolics. From these results it is evident that *A. planipennis* larvae have the ability to metabolize at least part of the suite of phenolics present in ash phloem via various enzyme-based detoxification and/or excretion mechanisms.

**Acknowledgements**

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Figure 5.1 Overlay of chromatograms showing the phenolic profiles of *Fraxinus pennsylvanica*, green ash phloem, larval tissues (midgut content, midgut tissue, hindgut-Malpighian tubules, frass and carcasses. Numbers on the peaks represent retention time of the compound.
Figure 5.2: Total phenolics measured as total UV peak area in chromatograms in (A) phloem, midgut content (MGC), and frass (B) midgut tissue (MGT), hindgut-Malpighian tubule (HG-MT), carcasses (CC). Means are represented with SEM. Letters indicates means that are significantly different.
Table 5.2: Compounds found in *Fraxinus pennsylvanica* (green ash) phloem, midgut tissue (MGT), midgut content (MGC), hindgut-Malpighian tubule (HG-MT), carcasses (CC) and frass. All the compounds were not identified.

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*indicates compounds present in all the samples analyzed.
References:


Abstract

Emerald ash borer, *Agrilus planipennis* is a recently introduced invasive insect pest of ash (*Fraxinus* spp.) in North America (NA). Its invasion has had significant economic and ecological impacts on urban, natural, and managed forest ecosystems and continues to do so. All NA ash species are susceptible to colonization by this beetle whereas Asian species are resistant and colonized significantly only when stressed. Larvae of *A. planipennis* feed on phloem and can kill the trees in 3-4 years post infestation. In this study an integrated approach including transcriptomics, metabolomics, and protein modeling was used to dissect the physiological responses of *A. planipennis* larvae feeding on susceptible North American green ash (*F. pennsylvanica*) and resistant Manchurian ash (*F. mandschurica*). A total of 58,871 high quality expressed sequence tags (ESTs) were obtained from the pooled transcriptome of midgut tissue of larvae feeding on green ash and Manchurian ash. RNA-Seq analysis showed that 380 genes were differentially expressed, with 266 transcripts more highly expressed in *Fm*-MG and 114 transcripts more highly expressed in *Fp*-MG. Validation of candidate genes via real time quantitative PCR corroborated the RNA-seq profiles. Transcripts coding for
digestive enzymes, amino acid transporters, and detoxification genes were present at higher levels in $F_p$-MG whereas transcripts coding for peritrophic membrane synthesis and sugar transporters were present at higher levels in $F_m$-MG. Furthermore, metabolomic studies indicated that $A.\ planipennis$ larvae were capable of modifying both green and Manchurian ash phloem were metabolized to similar extent. Lastly, modeling and docking of a candidate P450 highly expressed in larvae feeding on green ash revealed its efficacy to detoxify an array of phenolic compounds.
Introduction:

Emerald ash borer, *Agrilus planipennis* Fairmaire, is an exotic wood-boring beetle threatening the survival of *Fraxinus* spp (ash) trees in both natural and urban ecosystems in eastern North America (Cappaert and others 2005). *A. planipennis* was discovered in Michigan in 2002 and has rapidly established populations in 17 U.S. states and 2 Canadian provinces, resulting in the loss of millions of ash trees (www.emeraldashborer.info). This Buprestid beetle requires 1-2 years to complete its life cycle (Cappaert and others 2005). Larval feeding creates galleries that girdle the phloem and obstructs the flow of nutrients eventually killing the tree in about 3-4 years. All North American (NA) *Fraxinus* spp. including black (*F. nigra*), green, (*F. pennsylvanica*), white, (*F. Americana*), and blue (*F. quadrangulata*) ashes are susceptible to EAB larval colonization (Herms et al 2004). In contrast, Asian ash (e.g. *F. mandshurica*) are much more resistant by virtue of their co-evolutionary history, and colonization is confined to stressed trees (Rebek and others 2008).

Several studies have aimed to understand the molecular traits underpinning interspecific variation in host resistance. Comparative transcriptomics between the phloem of NA ash spp. and Asian ash (*F. mandshurica* Ruprecht, Manchurian ash) indicated higher transcript levels of biotic and abiotic stress genes in Manchurian ash (Bai et al 2010). Further, chemical and proteomic analyses revealed presence of unique phenolic metabolites and elevated protein levels in Manchurian ash, which is hypothesized to provide defense against *A. planipennis* (Cipollini and others 2011; Eyles
and others 2007; Whitehill and others 2012; Whitehill and others 2011). In contrast to the progress that has been made in understanding ash defenses to *A. planipennis* larvae, relatively little is known about *A. planipennis* defenses and physiological responses that enable larvae to detoxify ash metabolites. An initial transcriptomic study conducted by Mittapalli et al. (2010) and the subsequent gene characterization studies (*chapter 3 and 4*) (Rajarapu and others 2011) suggested potential involvement of the midgut and fat body tissues in detoxification/antioxidation and immune responses. Midgut is the primary interface between the insect and its environment and performs vital physiological functions including digestion and detoxification.

At the genomic and transcriptomic level, next generation sequencing (NGS) techniques have widely replaced traditionally used methods like Sanger sequencing (Schuster 2008). In the recent past plethora of studies in insects using next generation techniques to study various biological processes such as immunity (Chen and others 2012; Choi and others 2012; Pascual and others 2012), toxin resistance (Liu and others 2011; Mamidala and others 2012), and overwintering physiology (Poelchau and others 2011). Among these NGS techniques, RNA-seq is widely used for comparative global gene expression analysis. Advantages of RNA-seq over traditionally used microarrays include identification of splice junctions, alternate splice variants, and novel transcripts (Wang and others 2009). On the other hand metabolomics is a promising approach to integrate with other omic studies for a more holistic understanding of an organism or process (Nadella and others 2012). Targeted metabolomics has been effectively used to
study host plant utilization by Lepidoptera (Ferreres and others 2007; Jansen and others 2009; Lahtinen and others 2006) and Hymenoptera (Salminen and others 2004).

Although enormous data is generated using a single omic technique, discrete functions of genes and proteins are elusive (Fridman and Pichersky 2005; Ge and others 2003). Thus, it is crucial to combine different omic approaches in order to understand the network of events occurring in a cell, tissue or organism in response to internal and external stimuli and to formulate hypotheses (Ge and others 2001). Although to my knowledge no studies exist that have utilized the different omic approaches to study insect-plant interactions, other systems have utilized these approaches. For e.g. Two or more omic approaches were used to identify clusters of co-regulated genes in yeast (e.g., *Saccharomyces cervisea*) and nematodes (e.g. *Caenorhabditis elegans*), to study drug toxicology in mammals; and to understand the phenotypic response to prolonged environmental stresses in plants (*Zea mays, Arabidopsis thaliana*) (Amiour and others 2012; Ge and others 2001; Hirai and others 2005; Lou 2012; Walhout and others 2002).

In the current study, my objective was to understand the adaptation criteria required by *A. planipennis* larvae to survive on two contrasting hosts, i.e. susceptible green ash and resistant Manchurian ash. RNA-seq revealed higher transcript levels coding for digestive enzymes, amino acid transporters, and detoxification genes in larvae feeding on green ash whereas genes coding for peritrophic membrane synthesis and sugar transporters were more highly expressed in larvae feeding on Manchurian ash. Targeted metabolomics of host phloem and frass phenolics revealed differential utilization of the hosts by *A. planipennis* larvae. A protein model derived for a differentially expressed
cytochrome P450 gene (CYP6FH1) revealed a high level of efficiency to detoxify an array of green ash allelochemicals.

**Materials and Methods:**

*Ash common garden*

A common garden plantation consisting of white (*F. americana* L.), green, and Manchurian ash was established at Michigan State University’s Tollgate Research and Education Farm in Novi, MI in April 2004. Each species was replicated 80 times for a total of 240 trees. Trees were arranged in four blocks of 60, with each block consisting of four rows of 15 trees spaced 3 m apart. Each of the three species was replicated 20 times (located randomly) within each block. For this experiment, two trees of green and Manchurian ash per block were randomly selected to be included in the experiment (N=12 trees). Because *A. planipennis* pressure was high around the plantation during its establishment, all trees were protected from *A. planipennis* colonization with two applications of bifenthrin (Onyx™) sprays per year from 2004-2006 and drip-irrigated to facilitate establishment. At the time of this experiment, green and Manchurian ash were 10.49 ± 0.40 and 7.36 ± 0.22 cm in diameter at 50 cm above ground level, respectively.
A. *planipennis* inoculation

Twenty *A. planipennis* eggs were applied to the stems of previously selected green and Manchurian ash. Small pieces of filter paper bearing *A. planipennis* eggs from oviposition in the lab were cut out and secured to the tree bark using cheesecloth. Eggs were applied on 7 July 2011 to coincide with the natural phenology of *A. planipennis* oviposition.

Tree harvest, larval samples, and tissue collection

Trees were harvested on 22 August 2011. Three main stem sections were returned to the lab and stored at 3°C. Stems were debarked using drawknives and early 3rd instar larvae were removed using chisels and forceps. Larvae were maintained on ice until dissections were performed. Phloem tissue encompassing larval feeding sites and frass samples were also collected and stored at -80°C until extractions.

RNA isolation, RNA-seq library preparation, and Illumina sequencing:

Midgut tissue was isolated from 4-5 larvae collected from two trees per species per replicate, as described earlier in chapter 3. Gut contents were removed and tissue was washed thoroughly in phosphate buffer (0.1M, pH 7.00) to remove phloem contaminants. Rinsed tissue was suspended in 1ml Trizol (Invitrogen) for total RNA isolation. Isolated
total RNA was treated with Turbo DNase (Ambion) to remove genomic DNA contamination. Quality of total RNA was measured using Nanodrop and bioanalyzer to assure highest quality for sequencing. Paired-end cDNA libraries for biological replicates per treatment were synthesized using TruSeq RNA prep kit (Illumina) with a small modification in the protocol. Fragmentation of RNA was done for 4 min to obtain longer pair of reads. Libraries were prepared and sequenced by Illumina HiScan SQ at Purdue Genomics Center (http://www.genomics.purdue.edu/~core/).

Denovo sequence assembly and differential expression analysis:

Sequence assembly and preliminary data analysis was performed at Purdue Genomics Center. Low quality sequences and adapters were trimmed using Trimmomatic software (Bolger and Giorgi) with default parameters. Trimmed sequences were assembled using Trinity software (v. r2011-11-26) with default parameters (Grabherr and others 2011). A reference database was created by pooling all the assembled contigs from the two treatments using CD-HIT-EST software. A reference database was annotated using Blast2Go by blasting the sequences against NCBI non-redundant database with BlastX algorithm. Trimmed reads were mapped to the reference database via bowtie (v. 1.x.). Read counts for four libraries were obtained by Sam2Counts. Differentially expressed genes were identified by DESeq, an R based statistical package that follows negative binomial distribution with a False Discovery Rate (FDR) of 5% (Anders and Huber 2010). Genes with a \( P < 0.05 \) were considered differentials and were annotated via
Swissprot and Interproscan in blast2Go. Gene Ontology (GO) enrichment (Fisher exact test) analysis was performed using Blast2GO with a term filter value of 0.05 to identify enriched GO terms between the two transcriptomes. Validation of candidate genes was performed using RT-qPCR as described in chapter 3. Primers used in the study are given in Table 6.1.

**Phenolic-targeted metabolomics:**

Phenolic profiles of phloem and frass samples were characterized from extracts with 70% methanol in water, as described in chapter 4. Phenolics separation, identification, and quantification were conducted using a Waters Alliance 2695 separation module (Milford, MA) with Waters Xterra™ RP18, 5 µm, 4.6 X 150 mm column, 3.9 µm, 3.0 X 20 mm guard column, 996 photodiode array detector, and a Micromass ZQ mass spectrometer. Sample injection volume was 10µl. Samples were eluted at a flow rate of 0.3ml/min. The binary mobile phase consisted of HPLC-grade water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B). ESI mass spectra were collected in both negative and positive mode using the following parameters: capillary voltage of 3.50 kV, cone voltage of 25V, desolvation and source temperatures of 350 and 130°C, respectively, and desolvation and cone gas flow rates of 350 and 50 L/hr, respectively. Quantification of total phenolics was based on peak areas in the UV chromatogram. Compound identification was determined by integrating UV spectra, negative and positive ESI mass spectra, and retention times, and
comparing these data to literature values (Eyles and others 2007; Whitehill and others 2012).

*Molecular characterization of CYP6FH1:*

Differentially expressed P450 was sequenced by Sanger sequencing using two sets of gene-specific PCR primers (Table 4.1). The sequence was further identified and named according to the P450 nomenclature (Nelson 2009). The PCR reaction mixture included 20ng cDNA template (2µl), 10 pmol each of forward and reverse primers (1µl each), and Green Taq DNA polymerase (2X, GeneScript). Thermocycler conditions were 95\(^{0}\)C (3 min); 40 cycle amplification at 95\(^{0}\)C (1 min), 55\(^{0}\)C (1.30 min), 72\(^{0}\)C (1.30 min) and a final step at 72\(^{0}\)C (10 min). The resultant PCR products were separated on 1% agarose gel in 1X TAE buffer. The gel was stained with ethidium bromide solution (10µl/200ml), and the bands were photographed using a Kodak Gel logic 100 imaging system. The resultant PCR products were purified using a Qiagen PCR purification kit. Concentration of purified PCR products was determined with Thermo scientific Nanodrop 2000 and sequenced at Purdue genomics core facility.
Homology modeling and molecular docking of CYP6FH1:

A 3D model of CYP6FH1 was built by homology modeling based on high-resolution crystal structure of homologous proteins. The crystal structure of the closest homolog available in the Brookhaven Protein Data Bank was searched by determining the sequence similarity aided by a BLAST (NCBI) search. Human microsomal cytochrome P450 3A4 (PDB ID: 1TQN) with a resolution of 2.05 Å was determined to be a suitable template based on an identity score of 32% and an E-value of 7e-65. Thereafter, the coordinates of the crystal structure of the P450 3A4 were used as a template to build the model by multiple sequence alignment using ClustalW software. The 3D models of CYP6 were generated by the homology modeling tool, Modeller. The steepest descent energy minimization was performed using the Gromos96 43a1 force field to regularize the protein structure geometry. Automated docking analysis was performed using Autodock 4.0 with the compounds present in green and Manchurian ash phloem. The molecular models of the compounds were built and minimized with the Discovery Studio 3.1 software package (accelrys). To recognize the binding sites in CYP6, blind docking was carried out essentially as described earlier for CYPP397A1V2 (Mamidala and others 2012).
Statistics:

Overall phenolic profiles were compared using principal component analysis (PCA) in Minitab (v. 16). Relative fold change in mRNA levels of candidate genes obtained from RT-qPCR was analyzed using the generalized linear model in Minitab (v. 16). Mean separation was calculated by Tukey’s method ($\alpha = 0.05$). Data was analyzed for normality and homogeneity of variance. For each treatment there were three biological replicates. Total phenolics present in green and Manchurian ash phloem and their corresponding frass was analyzed using the general linear model in Minitab (v. 16).

Results

Transcriptomics (RNA-seq):

Illumina sequencing of midgut tissue of *A. planipennis* larvae feeding on green ash (*Fp*-MG) and Manchurian ash (*Fm*-MG) yielded approximately 36 and 43 million reads each. Further, reads were assembled to 47,580 and 59,528 contigs, respectively. Statistics of the sequencing is described in Table 6.2. Balst2GO results were similar to that observed from 454 pyrosequencing of midgut tissue reported earlier (Mittapalli and others 2010). The reference database combining *Fp*-MG and *Fm*-MG yielded 58,871 high quality expressed sequence tags.

Differential gene expression analysis using DESeq showed 380 differentially expressed genes (Figure 6.1A) with 114 and 266 genes with higher mRNA levels in *Fp*-

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MG and *Fm*-MG, respectively. Among these sequences 94.23% belong to eukaryotes and 0.01% to prokaryotes. Prokaryotic sequences differentially expressed belonged to *Bacillus megaterium*, *Pseudomonas* spp, *Paenibacillus mucilaginosus*, and *Colletotrichum gloeosporioides*. Expression of *Pseudomonas* gene was higher in *Fp*-MG whereas expression of *P. mucilaginosus*, *B. megaterium* and *C. gloeosporioides* coding lyase gene was higher in *Fm*-MG. *Paenibacillus* spp is pathogenic to insects (Enright and Griffin 2004) and higher expression of this bacterial sequence suggests possible infection of *A. planipennis* larvae feeding on Manchurian.

Interproscan analysis of the differentials annotated only 73 genes in *Fm*-MG and 56 genes in *Fp*-MG. The annotated differential genes were categorized into different groups based on the biological processes they participate in (Table 6.3). Biological processes including digestion, transporter activity, and detoxification were differentially represented between *Fp*-MG and *Fm*-MG (Figure 6.1B). The transcriptome of *Fp*-MG was enriched with digestion related genes, including β-glucosidases, which were the most abundant genes, followed by lipases and proteases. Amino acid transporters, a monocarboxylate transporter, and ABC transporters were also present at higher levels in *Fp*-MG than *Fm*-MG. In addition, detoxification genes including cytochrome P450s (P450) and glutathione-S-transferase (GST) were also present in higher levels in *Fp*-MG relative to the *Fm*-MG transcriptome.

In the *Fm*-MG transcriptome, genes involved in chitin metabolism, including peritrophic matrix proteins, chitin deacetylase, and chitinase were more highly expressed than in *Fp*-MG. Additionally, serine protease transcripts were also present in higher
numbers in Fm-MG relative to Fp-MG. Transporters including a sugar transporter, an organic cation transporter, and a trehalose transporter were also present in higher levels. Genes involved in detoxification, including carboxylesterases (CE), sulfotransferase (ST), and lactoylglutathione lyase (LGSH), had higher mRNA levels in Fm-MG compared to Fp-MG. Another candidate gene with higher transcript levels in Fm-MG transcriptome was β-amyloid. A comparative GO enrichment analysis between Fp-MG and Fm-MG showed hydrolases as the enriched GO term in the Fp-MG transcriptome; whereas chitin metabolic process and chitin binding as the enriched GO terms in Fm-MG transcriptome. From the differentially regulated biological processes including digestion, transport, and detoxification, 16 candidate genes were validated using RT-qPCR (Figure 6.1B). Expression patterns between qPCR and RNA-seq were highly correlated ($P < 0.001, r = 0.7$).

**Phenolic targeted metabolomics:**

The four samples analyzed included green and Manchurian ash phloem and their respective larval frass showed unique phenolic profile for each sample. Principal component analysis showed two distinct clusters for Fp and Fm phloem whereas their respective frass samples i.e. Fp and Fm frass showed an overlap (Figure 6.2A). The first two principal components explained 43.5% variation in the data. Compounds present in green and Manchurian ash phloem were identified as per the literature (Table 6.3). There was no significant difference between the phloem of green and Manchurian ash (Figure
Similarly, there was no significant difference between the total phenolics of larval frass feeding on green and Manchurian ash (Figure 6.2B).

Among simple phenolics, tyrosol hexoside and hydroxytyrosol hexoside were detected in both \(Fp\) and \(Fm\)-phloem, but their presence was not detected in \(Fp\) and \(Fm\) frass. Coumarins were found only in \(Fm\)-phloem and were also present in \(Fm\)-frass, except for methylesculin and mandschurin. A monolignol, syringin was found only in \(Fp\)-phloem and was also detected in \(Fp\)-frass. Lignans were found in both \(Fp\) and \(Fm\) phloem. Among phenylethanoids, calceolariosides A, B and C were found only in \(Fm\)-frass and were also identified in \(Fm\)-frass at lower levels. In contrast, verbascoside A, B and C was present in both \(Fp\) and \(Fm\) phloem. Interestingly, only verbascoside A was found in the \(Fp\) and \(Fm\) frass. Secoiridoids including oleuropein hexoside, oleuropein B oleuropein and ligustroside were detected in both \(Fp\) and \(Fm\) phloem. Except for oleuropein hexoside and oleuropein B, other secoiridoids were detected in \(Fp\) and \(Fm\) frass.

Compounds shared between \(Fp\) and \(Fm\) phloem including hydroxytyrosol hexoside, tyrosol hexoside, hydroxyoleuropein, pinoresinol glucoside, verbascocide A and B, oleuropein, ligustroside were metabolized similarly by \(A.\) planipennis. Along with this, there were several new compounds present in the frass of both \(Fp\)-frass and \(Fm\)-frass.
Modeling and docking:

A full-length sequence of a differentially expressed P450 was identified as CYP6FH1. The deduced amino acid sequence of CYP6FH1 showed the following conserved domains: a heme binding sequence motif FGEGPRICIG (443aa - 453aa), a C-helix sequence WKALR (119aa - 123aa), an oxygen binding motif AGFET (310aa - 314aa), a K-helix motif ETLRKYP (368aa - 373aa) and a sequence motif before the heme thiolate ligand PDKFDP (419aa - 427aa) (Figure 6.3). An in silico 3D protein model was constructed using human CYP3A4 as a template (Figure 6.4). An iron-heme group was covalently attached to sulfur atom of the conserved cysteine residue (Cys450). A diagnostic test with Ramachandran plot revealed 12.7 % residues occur in the acceptable region with 2% and 2.3% residues occurring in generally allowed and disallowed regions, confirming the structural fold of CYP6FH1. Docking of CYP6FH1 model with compounds present in green and Manchurian ash showed compounds present in green ash phloem and common compounds between both the species as potential metabolic targets (Table 6.4).

Discussion

Previous studies analyzing the molecular differences underlying interspecific variation in resistance between Asian and North American ash species have focused on comparing ash phloem and have been conducted at the metabolomic and/or proteomic...
level (Cipollini and others 2011; Whitehill and others 2012; Whitehill and others 2011). In this study I used transcriptomics and targeted metabolomics to distinguish the underlying physiological differences in *A. planipennis* larval midgut tissue feeding on resistant Manchurian ash and susceptible green ash in addition to comparing ash phloem and larval frass. Illumina sequencing of *Fp*-MG and *Fm*-MG yielded a higher number of reads compared to the reads yielded by pyrosequencing showing the comprehensiveness of Illumina sequencing (Mittapalli and others 2010). Reference database created by combining the databases of *Fp*-MG and *Fm*-MG yielded two times more (58,871) number of high quality expresses sequence tags (ESTs) than generated by pyrosequencing (25,173), indicating that this study was able to enrich the existing database with new transcripts.

Differential expression yielded 380 midgut specific sequences differentially regulated genes between the *Fp*-MG and *Fm*-MG transcriptomes. Similar to other wood boring beetles *A. planipennis* larval midgut harbor a wide variety of bacteria among which *Pseudomonas* spp are the most abundant species in early fourth instar larvae (Vasanthakumar and others 2008). Hydrolases was the enriched GO term in *Fp*-MG transcriptome. Hydrolases include a large group of enzymes that are involved in hydrolysis of a wide range of chemical bonds like glycosidic, ester, ether, epoxide etc. (Kanehisa and Goto 2000). In *Fp*-MG the major hydrolases included β-glucosidases, enzymes hydrolyzing glycosidic bonds mainly involved in digestion. In contrast *Fm*-MG was enriched with genes involved in chitin binding and chitin metabolism which are key components of peritrophic membrane in insect midgut (Lehane 1997). Though GO
enrichment analysis demonstrated hydrolases and chitin metabolism as the significantly different molecular functions between Fp-MG and Fm-MG, all the annotated differentials were classified in to biological processes to shed light on some candidate genes.

Digestion:

In Fp-MG transcriptome, digestion related genes were present in higher numbers. β- glucosidases were the most abundant genes among the highly expressed genes in Fp-MG. β- glucosidases are midgut epithelial enzymes that can metabolize toxic plant β-glycosides and cellobiose to their respective products (Terra and Ferreira 1994). Phloem feeders encounter high amounts of cellulose in their diet, which requires a complex process for degradation. β- glucosidases play a role in the degradation of cellulose along with other enzymes, but they also hydrolyze plant β-glucosides to aglycones. However, the function of A.planipennis β-glucosidases requires further study, as there is a lack of research on cellulose digestion of wood-boring beetles. Additionally, lipases and a chymotrypsin-like protease were also present in Fp-MG in higher levels. The expression of this suite of digestion-related transcripts, might suggest that A.planipennis larvae might have efficient digestive metabolism on green ash when compared to feeding on Manchurian ash.

Most of the genes with higher mRNA levels in Fm-MG belonged to chitin metabolism and peritrophic membrane proteins, which are principal components of the peritrophic membrane (PM) framework in midgut. In insects, PM plays a critical role
during digestion by compartmentalizing the midgut lumen to prevent the loss of digestive enzymes (Terra and Ferreira 1994). It also protects midgut tissue from abrasion, viruses, plant allelochemicals, and antioxidants (Hegedus and others 2009; Lehane 1997), and thus plays an important role in structural integrity of the midgut. Plants have evolved defense mechanisms against insect PMs by producing secondary chemicals and proteases that cause lesions or holes in PMs, hindering the digestion process (Harper and others 1998; Mohan and others 2006; Pechan and others 2002). Insects counter defend PM damage by overproduction of PM to repair the lesions (Harper and others 1998).

Manchurian ash has very high levels of aspartic protease, which was proposed to damage PM of *A. planipennis* as observed in other insects (Pechan and others 2002; Whitehill and others 2011).

Further, on-going studies on comparative ash transcriptomics revealed another class of proteases (cysteine) that are higher in Manchurian ash compared to NA ash (Mamidala et al., Unpublished). Higher transcript levels of PM metabolism genes might indicate counter defense mechanisms of *A. planipennis* to a damaged PM by Manchurian ash proteases. Higher transcripts of serine proteases in *Fm-MG* might indicate a compensation mechanism for digestion or a counter response to higher amounts of aspartic proteases found in Manchurian ash phloem (Fescemyer and others 2013).
Transporters:

Transporters were highly expressed in both Fp-MG and Fm-MG. However, the classes of transporters differed between the transcriptomes. High expression of amino acid transporters in Fm-MG correlates with the higher amino acid content of green ash phloem relative to Manchurian ash phloem (Hill et al., 2012). Expression of amino acid transporters is regulated by the presence of protein or amino acids in the diet (Erickson et al 1995). Amino acids are building blocks for proteins and enzymes, which are vital for survival and growth. Higher levels of amino acid transporters in Fp-MG suggest an active uptake/utilization of amino acids from the diet. A monocarboxylate transporter was also higher in the Fp-MG transcriptome compared to Fm-MG. Monocarboxylate transporters are present in active tissues and transport low molecular weight substrates (lactate, pyruvate, and branched chain oxo acids) which play a central role in metabolism and cellular communication (Halestrap and Price 1999). A higher mRNA level of monocarboxylate transporter in Fp-MG reflects a highly active state of the tissue (Halestrap and Price, 1999). Another interesting group of transporters highly expressed in Fp-MG relative to Fm-MG is that of the multidrug resistant transporters (MDR). These transporters belong to the family of ABC transporters and handle a wide range of substrates including sugars, peptides, and inorganic acids important for cellular metabolism (Higgins 1992). Along with this function, MDRs confer resistance against drugs to tumor cells, virulent bacteria, and nematodes by allowing for the rapid excretion of the xenobiotics (Higgins 1992, James and Davey 2007). The role of MDRs in insects...
was recently investigated and they are known to be induced in the presence of toxins (Chahine and O'Donnell, 2011; Simmons et al., 2012). Higher mRNA levels of ApMDR1 in Fp-MG may be a response of the midgut to green ash secondary metabolites.

The transcriptome of Fm-MG showed higher levels of a sugar transporter, a trehalose transporter, an organic cation transporter, and an ABC transporter. Sugar transporters include glucose, fructose, trehalose or galactose transporters. BlastX results of ApSuT demonstrated its similarity to either glucose or trehalose transporter. Sugar transporters are important in translocation of energy sources including glucose and trehalose or might play a role in maintaining osmotic stress. Trehalose is an important blood sugar in insects synthesized in the fat body. Overexpression of trehalose transporters is observed during dehydration and salt stress in sleeping chironomid, Polypedilum vanderplanki (Kikawada and others 2007). Higher levels of sugar and trehalose in Fm-MG might indicate osmotic stress or translocation of molecules for energy production of larvae while feeding on Manchurian ash. As discussed earlier, mRNA levels of ApMDR-2 might indicate their role either in dealing with dietary toxins present in Manchurian ash phloem.

*Detoxification / response to stress:*

Higher levels of P450s and GSTs were observed in Fp-MG transcriptome compared to Fm-MG. P450s and GSTs are important players of phase I and phase II stages of the detoxification process. Detoxification is one of the critical defense
mechanisms in insects to deal with xenobiotics, including plant allelochemicals and insecticides (Terriere 1984). Induction of P450s in the presence of xenobiotics in insects and their role in providing resistance to xenobiotics including insecticides and secondary chemicals have been extensively reviewed (Bergé and others 1998; Feyereisen 1999; Schuler 2011; Scott 1999). The differentially expressed GST belongs to class sigma. A spatial and temporal expression analysis of \textit{ApGSTS-1} showed higher expression in the fat body and in prepupal stages, indicating that it might play a role in developmentally derived physiological processes (Rajarapu and Mittapalli 2013).

Larvae feeding on Manchurian ash had higher levels of carboxylesterase (CE) and sulfotransferase (ST) compared to \textit{Fp-MG}. Carboxylesterases are a superfamily of enzymes that hydrolyze carboxyl esters with a broad range of functions. In insects, they are known to play a role in providing insecticide resistance, which has been reviewed by Wheelock et al. (2005). Induction of CEs was also observed in phytophagous insects encountering dietary allelochemicals (Ugale and others 2011a; Zhang and others 2011a; Zhang and others 2012). They are also induced as a non-specific responses to stressors including temperature and insecticides (Ivanovic and Jankovic-Hladni 1991). Thus, we hypothesize that higher levels of CEs in \textit{Fm-MG} might be expressed non-specifically in response to the compounds in Manchurian ash phloem. Sulfotransferases (ST) belong to the phase II detoxification process with a wide range of substrates. They catalyze the conjugation of sulfur to compounds including hormones, drugs, neurotransmitters and xenobiotic compounds thus increasing their solubility for excretion (Weinshilboum and others 1997). Their involvement in sulfating xenobiotic compounds was studied in
several invertebrates including insects (Assem and others 2006; Hattori and others 2007; Hattori and others 2006). Manchurian ash phloem has 30 times the tyramine levels of green ash. Tyramine is a non-amino acid monoamine that is known to be a neuroactive chemical in insects (Lange, 2009). Sulfotransferase is involved in inactivating tyramine by sulfation. Higher expression of ST in Fm-MG might result from encountering higher levels of tyramine in Manchurian ash phloem.

Along with CEs and STs, Fm-MG also had higher mRNA levels of lactoylglutathione lyase (LGSH) a key player in glyoxylase pathway. Cytotoxic methyl glyoxylate ion formed during glycolysis, amino acid metabolism and acetone metabolism is detoxified via glyoxylase pathway (Thornalley 1993). Higher transcript levels of LGSH in Fm-MG relative to Fp-MG indicate either high metabolism of glucose via glycolysis or amino acid metabolism for energy production. Another interesting member with higher transcript levels in Fm-MG is a β-amyloid. In insects β-Amyloids play a protective role in eggs due to their mechanical and biological properties (Iconomidou and others 2000). Higher expression of β-Amyloid in Fm-MG might indicate a possible damage to midgut tissue compensated by the higher expression of structurally robust proteins like amyloids.

**Phenolic profiles and molecular docking:**

To further understand the physiological basis of successful development of *A. planipennis* on susceptible NA ash, targeted metabolomics and molecular docking
approaches were taken. Consistent with previous reports we found significant differences between *Fp*- phloem and *Fm*-phloem (Eyles and others 2007; Whitehill and others 2012). Hydroxycoumarins and phenylethanoids Calceolariosides A and B were found only in *Fm*-phloem whereas syringin was found only in *Fp*-phloem. This difference is evident from the separation of *Fp* and *Fm* phloem into distinct clusters by PCA analysis. Interestingly, similar (52%) phenolic profiles of *Fp* and *Fm* frass indicates similar mechanisms used for dealing with the compounds encountered in the diet. This suggests that *A.planipennis* larvae might have mechanisms to deal with phenolics via potential detoxification enzymes with broader substrate specificities. This is evident from the higher expression of P450s and GSTs both in *Fp* and *Fm* midgut. Alternatively, the larvae might be impaired to synthesize the essential enzymes required for dealing with the compounds present in *Fm*-phloem (Rosenthal and Berenbaum 1979).

Metabolism (excretion and transformation) of compounds present in both *Fp* and *Fm* phloem by the larvae might have evolved during its coexistence with Manchurian ash. Presence of new compounds in *Fp* and *Fm* frass correlates with higher levels of detoxification enzymes including P450s, GSTs, CEs and glucosyl transferases in both *Fp* and *Fm*-MG. These enzymes might potentially be involved in the dealing with phenolics. Pinoresinol dihexoside was found in both green and Manchurian ash phloem which contradicts their role in contributing to the resistance of Manchurian ash (Whitehill and others 2012).

Further evidence for the involvement of *A.planipennis* P450s in dealing with ash phenolic was obtained by modeling and docking of CYP6FH1. Transcripts of CYP6FH1
were higher in \textit{Fp-MG}. From the top 5 compounds (Table 6.3) that have high binding energy with CYP6FH1 in this study I identified only Oleuropein and Syringin. However, these two compounds were also present in the frass of \textit{Fp-MG}. This indicates that larvae might combat these compounds by both detoxification and excretion mechanisms but needs further verification (Rosenthal and Janzen 1979).

In sum, phenolics might not have a potential effect on the \textit{A.planipennis} survival. These specialist insects might have adapted to phenolic rich hosts during their coevolution with their native hosts. Higher levels of peritrophic membrane genes might be induced to the potential damage caused to this structure. This might further synergize the effects of phenolics present in Manchurian ash phloem on \textit{A.planipennis}. Results from this study shed deeper insights into ash phloem defenses and also contributes to the knowledge of molecular physiology of wood boring beetles, a vital group of ecologically important insect species worldwide.

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Table 6.1: Summary statistics of Illumina reads of *Agrilus planipennis* larval midgut tissue

<table>
<thead>
<tr>
<th>Treatment library</th>
<th>Replicate</th>
<th>Total number of reads</th>
<th>Number of contigs</th>
<th>Contig size range (bp)</th>
<th>*N50</th>
<th>Average contig length(bp)</th>
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</thead>
<tbody>
<tr>
<td>Larval midgut feeding on green ash</td>
<td>1</td>
<td>19,160,562</td>
<td>25,079</td>
<td>201-18348</td>
<td>2,022</td>
<td>1,120</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17,152,366</td>
<td>22,501</td>
<td>201-14211</td>
<td>1,831</td>
<td>1,040</td>
</tr>
<tr>
<td>Larval midgut feeding on Manchurian ash</td>
<td>1</td>
<td>20,625,994</td>
<td>31,584</td>
<td>201-13421</td>
<td>2,650</td>
<td>1,393</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23,318,676</td>
<td>27,944</td>
<td>201-25906</td>
<td>2,573</td>
<td>1,328</td>
</tr>
</tbody>
</table>
Table 6.2: Differentially regulated biological process in the midgut of larvae feeding on *Fraxinus pennsylvanica* (green ash) and *F. mandshurica* (Manchurian ash).

<table>
<thead>
<tr>
<th>Functions/Biological process</th>
<th>Fraxinus pennsylvanica (green ash) larval midgut</th>
<th>Number of genes</th>
<th>F. mandshurica (Manchurian ash) larval midgut</th>
<th>Number of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestion*</td>
<td>22</td>
<td><em>Chitin metabolism</em></td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>(β-glucosidase, glycoside hydrolase, lipases)</td>
<td></td>
<td>(Peritrophins, Chitin deacetylase Chitinase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transporter</td>
<td>6</td>
<td><em>Transporter</em></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>(Amino transporters, ABC transporters, Monocarboxylate transporter, Glutathione-s-transferase)</td>
<td></td>
<td>(Sugar transporter, Organic cation transporter, Trehalose transporter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detoxification</td>
<td>5</td>
<td><em>Detoxification</em></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(Cytochrome p450 Glutathione-s-transferase)</td>
<td></td>
<td>(carboxylesterase, sulfotrasferase, lactoylglutathione lyase)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* indicates significantly different functional groups according to GO enrichment analysis
Figure 6.2 Comparison of HPLC-UV chromatographic profiles of (A) Principal component analysis of phloem and frass samples (C) Total phenolics present in Manchurian ash phloem and its corresponding larval frass; green ash phloem and its corresponding larval frass.
Table 6.3 Identification of compounds present in green ash, *F. pennsylvanica* (*Fp*) and Manchurian ash, *F. mandshurica* (*Fm*) phloem

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Phloem sample</th>
<th>RT in HPLC-UV (Retention Time)</th>
<th>Ions</th>
<th>Putative Identification (Eyles et al 2011, Whitehill et al 2012)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fm, Fp</td>
<td>7.68</td>
<td>315, 135</td>
<td>Hydroxytryrosol hexoside</td>
</tr>
<tr>
<td>2</td>
<td>Fm, Fp</td>
<td>9.75</td>
<td>299</td>
<td>Tyrosol hexoside</td>
</tr>
<tr>
<td>3</td>
<td>Fm</td>
<td>10.38</td>
<td>339, 177</td>
<td>Esculin</td>
</tr>
<tr>
<td>4</td>
<td>Fm, Fp</td>
<td>11.52</td>
<td>431</td>
<td>Unknown 1</td>
</tr>
<tr>
<td>5</td>
<td>Fm, Fp</td>
<td>12.45</td>
<td>611, 403, 223</td>
<td>Elenolic acid derivative</td>
</tr>
<tr>
<td>6</td>
<td>Fm</td>
<td>12.67</td>
<td>353, 191</td>
<td>Methyl Esulin</td>
</tr>
<tr>
<td>7</td>
<td>Fp</td>
<td>12.89</td>
<td>417</td>
<td>Syringin</td>
</tr>
<tr>
<td>8</td>
<td>Fm</td>
<td>12.94</td>
<td>389, 501</td>
<td>Oleoside</td>
</tr>
<tr>
<td>9</td>
<td>Fm</td>
<td>13.44</td>
<td>369, 207</td>
<td>Fraxin</td>
</tr>
<tr>
<td>10</td>
<td>Fm</td>
<td>14.14</td>
<td>385*, 223</td>
<td>Mandschurin</td>
</tr>
<tr>
<td>11</td>
<td>Fp</td>
<td>14.87</td>
<td>403, 447, 224</td>
<td>Unknown</td>
</tr>
<tr>
<td>12</td>
<td>Fm</td>
<td>15.6</td>
<td>477, 377, 341, 197</td>
<td>Calceolariside C</td>
</tr>
<tr>
<td>13</td>
<td>Fm</td>
<td>15.9</td>
<td>519, 681</td>
<td>Pinoresinol dihexoside</td>
</tr>
<tr>
<td>14</td>
<td>Fm, Fp</td>
<td>17.2</td>
<td>373, 329</td>
<td>Hydroxy-pinoresinol diglucoside</td>
</tr>
<tr>
<td>15</td>
<td>Fm</td>
<td>18.09</td>
<td>477, 255, 179, 356</td>
<td>Calceolariside A</td>
</tr>
<tr>
<td>16</td>
<td>Fp</td>
<td>18.15</td>
<td>565, 613, 241</td>
<td>Unknown</td>
</tr>
<tr>
<td>17</td>
<td>Fp</td>
<td>18.44</td>
<td>653</td>
<td>Unknown</td>
</tr>
<tr>
<td>18</td>
<td>Fm, Fp</td>
<td>18.82</td>
<td>623</td>
<td>Verbascoside</td>
</tr>
<tr>
<td>19</td>
<td>Fm, Fp</td>
<td>19.18</td>
<td>519, 357, 161</td>
<td>Pinoresinol glucoside</td>
</tr>
<tr>
<td>20</td>
<td>Fm</td>
<td>19.4</td>
<td>477, 316, 281, 161</td>
<td>Calceolariside B</td>
</tr>
<tr>
<td>21</td>
<td>Fm, Fp</td>
<td>20.2</td>
<td>623,</td>
<td>Verbascoside B</td>
</tr>
<tr>
<td>22</td>
<td>Fm, Fp</td>
<td>22.02</td>
<td>539, 377, 307, 275</td>
<td>Oleuropein</td>
</tr>
<tr>
<td>23</td>
<td>Fm, Fp</td>
<td>23.97</td>
<td>523, 361</td>
<td>Ligustroside</td>
</tr>
<tr>
<td>24</td>
<td>Fm</td>
<td>24.04</td>
<td>553, 289</td>
<td>Oleuropein related compound</td>
</tr>
</tbody>
</table>

Bold indicates the dominant ion. * represents compounds identified in ESI positive mode.
Figure 6.2: Characterization of CYP6FH1 (A) Nucleotide (first line) and the deduced amino acid sequence (second line) of CYP6FH1. Start codon ATG and its corresponding amino acid are represented in bold and an * in the amino acid sequence represents a stop. Underlined amino acids are the conserved domains forming catalytic and substrate binding sites of P450.
Figure 6.4: Molecular modeling and docking of CYP6FH1 with *F. pennsylvanica* (green ash) allelochemicals. (A) Representation of the homology model of CYP6FH1. The cartoon model of CYP397A1V2 is colored using a blue to red gradient from N-terminus to C-terminus. The model was created with MODELLER 9v8 using human cytochrome P450 CYP3A4 as a template. (B-E) Green ash allelochemicals docked into the binding site of CYP6FH1. Images show the orientation of the compounds in catalytic site (red ring structure) and interacting amino acids (B) Syringaresinol, (C) Oleuropein (D) Apigenin and (E) syringin.
Table 6.4: Calculated Binding energies, Inhibition constant ($K_i$) and Binding constant ($K_a$) of compounds from *Fraxinus pennsylvanica* allelochemicals against CYP6FH1 ranked in ascending order of calculated $K_a$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Energy</th>
<th>Inhibition Constant</th>
<th>Calculated Binding Constant</th>
<th>Interacting amino acids in CYP6FH1 catalytic pocket</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syraingaresinol</td>
<td>-9.11</td>
<td>210.86 nM</td>
<td>$4.74 \times 10^6$</td>
<td>Lys238, Ala272, Ser139, Asn242</td>
</tr>
<tr>
<td>Oleuropein</td>
<td>-8.27</td>
<td>861.35 nM</td>
<td>$1.16 \times 10^6$</td>
<td>Glu241, Val118, Val116</td>
</tr>
<tr>
<td>Apigenin</td>
<td>-7.29</td>
<td>1.57 µM</td>
<td>$6.36 \times 10^5$</td>
<td>Asn242, Ala360.</td>
</tr>
<tr>
<td>Syringin</td>
<td>-7.47</td>
<td>3.36 µM</td>
<td>$2.97 \times 10^5$</td>
<td>Phe240, Glu119, Val118, Glu121, Ser139, Phe138</td>
</tr>
<tr>
<td>p-coumaryl quinic acid</td>
<td>-7.27</td>
<td>4.67 µM</td>
<td>$2.14 \times 10^5$</td>
<td>Asn242, Leu557, Pro433</td>
</tr>
<tr>
<td>Quercetin</td>
<td>-7.09</td>
<td>6.39 µM</td>
<td>$1.56 \times 10^5$</td>
<td>Ala360, Glu241, Val118, Glu119, Glu121, Phe138, Ser139, Phe133</td>
</tr>
<tr>
<td>Tyrosol hexoside</td>
<td>-7.02</td>
<td>7.19 µM</td>
<td>$1.39 \times 10^5$</td>
<td>Ala360, Glu241, Asn242</td>
</tr>
<tr>
<td>Ligustroside</td>
<td>-5.34</td>
<td>121.71 µM</td>
<td>$8.21 \times 10^2$</td>
<td>Lys238, Phe240, Glu558, Asn554, Glu243</td>
</tr>
<tr>
<td>Nuezhenide</td>
<td>-2.22</td>
<td>23.48 mM</td>
<td>$4.25 \times 10^1$</td>
<td>Glu558, Glu243, Asn242, Glu241</td>
</tr>
</tbody>
</table>

Binding energy -Energy required by the enzyme to position the substrate at the catalytic site for efficient catalysis

Inhibitory Constant- is defined as the concentration required to produced half maximum inhibition. It is estimated using AUTODOCK

Binding constant- Determines the energy of enzyme substrate complex
References:


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Chapter 7 Summary and future directions

Several studies have aimed to understand the molecular traits underpinning interspecific variation in ash resistance. Comparative transcriptomics, proteomics and metabolomics between the phloem of NA ash spp. and Asian ash (F. mandshurica Ruprecht, Manchurian ash) indicated higher levels of biotic and abiotic stress genes, defense proteins and unique compounds in Manchurian ash (Bai and others 2011; Cipollini and others 2011; Eyles and others 2007; Whitehill and others 2012; Whitehill and others 2011). Unique phenolic compounds and high levels of proteases found in Manchurian ash are hypothesized to be the potential defense mechanisms of Manchurian ash. In contrast to the progress that has been made in understanding ash defenses to A. planipennis larvae, relatively little is known about A. planipennis defenses and physiological responses of A. planipennis on ash trees. Thus my objective for this dissertation was to decipher the molecular interactions of A. planipennis larvae with its native (Asian ash) and naïve (North American (NA) ash) hosts. My objectives were accomplished by an integrated omics approach including transcriptomics and metabolomics.
Antioxidation Mechanism:

Phenolics are pro-oxidant compounds, meaning they release reactive oxygen species upon oxidation. This phenomenon is favorable at alkaline pH in the gut of a phytophagous insect (Appel 1993). Woody plants have phenolics associated with cell walls to provide structural integrity. Wood feeding insects encounter these compounds very frequently and need to have efficient mechanisms to deal with these pro-oxidants in their diet. Antioxidation is the potential mechanism in insects to neutralize reactive oxygen species (Larson 1986). Enzymes involved in antioxidation are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Ahmad 1992) An assessment of mRNA levels of antioxidant genes in the larval tissues of *A. planipennis* showed the expression of these genes in midgut, fat body and Malpighian tubule. Distribution of antioxidant genes in these tissues indicates the oxidative stress challenged by these important tissues. In particular, midgut specific expression of catalase indicates its role in encountering hydrogen peroxide formed in this tissue either from diet or endogenous metabolism (Chapter 2). During development, CAT had fluctuating expression patterns in larval stages with a sudden decrease in adults, whereas SOD and GPX had similar levels throughout development. However, specific activities of all the antioxidant enzymes were similar in neonates, larvae and adults. This expression pattern of antioxidant enzymes both at mRNA/protein level indicates that an efficient antioxidant mechanism is essential for *A. planipennis*. This mechanism might be important irrespective of the host they feed, as it is evident in the larval midgut feeding on green, *F.*
*pennsylvanica*, and Manchurian, *F. mandshurica* ash, wherein expression levels of antioxidant genes were higher and similar in larvae feeding on both these hosts.

**Detoxification mechanism:**

Phase I and phase II enzymes are important players of detoxification pathway in herbivorous insects to overcome their host defenses and develop successfully. Cytochrome P450s (P450) are the major group of phase I detoxification enzymes. In larvae feeding on green ash, mRNA levels of P450s were higher in midgut and Malpighian tubule (Chapter 3). Studies have shown the participation of these two tissues in detoxification along with other physiological functions (Dow 1986; Dow and Davies 2006). Modeling of a P450, CYP6FH1, highly expressed in *A.planipennis* larval Malpighian tubule, feeding on green ash, showed an array of compounds unique in green ash and those shared between both the ash species (green and Manchurian ash) as potential substrates. This indicates that *A.planipennis* might have developed P450 derived detoxification mechanisms to encounter phenolics during its coevolution with its Asian hosts. This is further supported by fewer number of P450s differentially expressed between larval midgut of *A.planipennis* feeding on green and Manchurian ash. This suggests that larvae feeding on Manchurian ash might use P450s with broader substrate specificity to detoxify *Fraxinus* spp allelochemicals. Nevertheless, P450s with higher mRNA levels in larval midgut feeding on green ash, relative to those feeding on Manchurian ash, might be an adaptation to the new host. A continuous participation of
these enzymes might also be required throughout development in *A. planipennis* to establish a successful feeding site, as similar mRNA and enzyme levels were observed in all the developmental stages.

Glutathione-S-transferase (GSTs) constitutes major phase II enzymes in detoxification pathway. Among the different classes of GSTs, epsilon class members are known to participate in dealing with allelochemicals. Higher expression of GSTs in metabolically active tissues such as midgut and Malpighian tubule indicates their active role in encountering host or endogenous metabolism derived stress (Chapter 3). Higher expression of an epsilon GST relative to neonates during feeding instars suggests their potential role in dealing with the allelochemicals. However, specific activity of total GSTs was similar in all the developmental stages indicating the importance of higher titers of these enzymes in *A. planipennis*. These observations suggest that *A. planipennis* might require GST derived detoxification mechanisms along with P450 based mechanisms. These two mechanisms might act in parallel to catabolize a single compound or independently, metabolizing different compounds. Similar to P450s, GSTs were also highly expressed in larval midgut of *A. planipennis* feeding on green and Manchurian, indicating that these mechanisms might be well developed in *A. planipennis* larvae to encounter ash allelochemicals. Targeted metabolomic of phloem, larval tissues and frass suggested efficient catabolism of phenolics present in green ash phloem (Chapter 4). In addition to this, presence of new compounds in tissues indicates potential transformation of phloem phenolics. Along with this, few compounds were excreted without modification, although the quantities excreted were lower in frass. Overall,
phenolic flux and their metabolism inside *A. planipennis* larvae can be summarized using a model.

**Hypothetical model:**

According to the model, few ingested phenolics might undergo pH depended oxidation in the foregut of the larvae and pass through the midgut lumen. Phytophagous insect midgut lumen is an active site for digestion and antioxidation (Barbehenn 2002). However, detoxification activities were not measured in the gut lumen of insects. Presence of new compounds in gut lumen indicates potential transformation/detoxification mechanisms. *A. planipennis* larval midgut and Malpighian tubules tissues might be an active site for P450s and GSTs derived detoxification, evident from the presence of new compounds in this tissue. Few compounds might also be absorbed and circulated in the hemolymph, which are dealt by other tissues. Multidrug resistance transporters found in midgut tissue might play a role in reabsorption of compounds present in the hemolymph.
Figure 7.1: Ash allelochemical (phenolic) flux inside *A. planipennis* larvae. Arrows indicate the direction of phenolic flow inside the larvae. Yellow structures indicate potential sites for transformation. P450s – cytochrome P450, GST - Glutathione-S-Transferases.

**Larvae feeding on green, *F. pennsylvanica*, vs Manchurian, *F. mandshurica*, ash:**

In addition to P450s and GSTs, RNA seq of *A. planipennis* larval midgut revealed other phase II detoxification genes including carboxylesterases (CE) and glucosyl transferases, highly expressed in larval midgut feeding on green and Manchurian ash. A comparative expression pattern between the larval midguts feeding on two hosts indicates that *A. planipennis* might have similar mechanisms to deal with allelochemicals encountered in these hosts. This observation is supported by the targeted metabolic
profiles of green ash phloem and Manchurian ash phloem along with the larval frass feeding on them. Phenolic profiles of larval frass feeding on green and Manchurian ash were similar indicating similar catabolism of phenolics corroborating with similar mechanisms of the larvae at gene level. Further, identification of these compounds will shed light on the potential mechanisms used by *A. planipennis* larvae.

Larval midgut feeding on Manchurian ash showed higher expression of sulfotransferases. These enzymes participate in detoxification of phenolic compounds in other insects (Hattori and others 2007). They also deactivate endogenous compounds such as neurotransmitters and proteins (Weinshilboum and others 1997). Higher levels of sulfotransferase in *A. planipennis* feeding on Manchurian ash might indicate their participation in neutralization of unique phenolic compounds or non-protein amino acid, tyramine, in Manchurian ash phloem. Tyramine is 30 times higher in Manchurian ash phloem relative to North American ash hosts (Hill and others 2012). It is a potential neurotransmitter in insects which is deactivated by sulfation (Lange 2009). Tyramine might cause behavioral changes in *A. planipennis* larvae such as inducing excessive feeding leading to increased intake of allelochemicals. It would be interesting to test this hypothesis in *A. planipennis* as the effect of tyramine on insect behavior is unknown.

Peritrophic membrane synthesis genes were highly expressed in *A. planipennis* larvae feeding on Manchurian ash, indicating a possible damage to this important structure in midgut. Damage caused to peritrophic membrane might affect digestion, which is evident from relatively lower levels of digestion genes and might also act in synergizing the effect of Manchurian ash phenolics on *A. planipennis* larvae.
**Future directions:**

Results obtained lay a foundation for studying wood boring beetle physiology in the context of counterdefense mechanisms employed against host defenses and asks new questions about the adaptation of these insects. Induction of detoxification enzymes occur in presence of xenobiotics (Terriere 1984). Though higher transcript levels of detoxification and antioxidant genes was observed in *A. planipennis* larval tissues and developmental stages, contribution of these genes to *A. planipennis* survival is elusive. Studies in other insects have used enzyme inhibitors to deduce their role in xenobiotic resistance (Karatolos and others 2012). Similar bioassays with artificial diet containing inhibitors of enzymes (E.g. Piperonyl butoxide for P450s, Sulfasalizine for GSTs, Bisbenzene sulfonamides) along with phenolic extract would demonstrate the role of these enzymes in adaption of *A. planipennis* on phenolic rich diet. Further, at gene level, knockdown of specific candidate genes via RNA interference (RNAi) would allow to determine the functional importance of the gene for detoxifying putative toxins (Bautista and others 2009). RNAi is a very efficient tool to elucidate the functional properties of genes. This mechanism was efficiently used in members of Lepidoptera, Hemiptera, Diptera and Coleoptera and is recently reviewed (Li and others 2013; Yu and others 2013).

Results from RNA-seq have brought to light other candidate genes that might be playing role in *A.planipennis-* *Fraxinus* spp interactions. β- Glucosidases were the most abundant genes with higher levels in *A. planipennis* larval midgut tissue feeding on green
ash phloem. Studies in insects demonstrate the role of β-glucosidases in digestion and allelochemical detoxification. It would be interesting to analyze the enzymatic properties of β-glucosidases in *A. planipennis* to understand their role in these beetles. Another interesting group of genes were multi drug resistance transporters (MDR). In specialist herbivores they are proposed to expel the toxins from circulation (Sorensen and Dearing 2006). To test this in *A. planipennis*, functional characterization of MDR gene can be achieved by injecting the mRNA into Xenopus oocytes. Developing Xenopus embryos is an efficient system and is widely used for studying gene function (Vize and others 1991). Along with these carboxylesterase (CEs) was also present among the differentially expressed transcripts. CEs are also known to detoxify xenobiotics and have been studied least in insect-plant interaction. Characterizing these genes in *A. planipennis* might give more insights in to CE bases detoxification in this wood boring beetle. Higher expression of peritrophic membrane synthesizing genes in larvae feeding on Manchurian ash is predicted due to the damage caused by the aspartic and cysteine proteases. A confirmation of this observation can be further accomplished by analyzing the microscopic sections of midgut tissue of larvae feeding on Manchurian ash with high level of proteases plus with supplementary artificial diet experiments.

Lastly, modeling studies of a differentially expressed P450 has shown the array of compounds present in green ash phloem as putative substrates. However, metabolomic profiles of phloem and larval samples have shown the presence of these compounds in frass also indicating their expulsion without modification. Therefore, further testing of the model in vitro for its substrate specificity is needed. Integrating this study with
metabolomics or identifying the compounds formed in frass would further contribute to the mechanisms employed by *A. planipennis* larvae dealing with phenolics. Further, it would also be interesting to analyze the response of *A. planipennis* larvae feeding on stressed Manchurian ash. This would complement the on-going studies to understand the metabolomic differences between healthy and stressed ash trees.
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