Aggregation Pheromone Biosynthesis and Engineering in Plants for Stinkbug Pest Management

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ABSTRACT

Stinkbugs (Pentatomidae) and other agricultural pests such as bark beetles and flea beetles are known to synthesize terpenoids as aggregation pheromones. Knowledge of the genes and enzymes involved in pheromone biosynthesis may allow engineering of the pheromone biosynthetic pathways in plants to develop new forms of trap crops and agricultural practices for pest management. The harlequin bug, *Murgantia histrionica*, a specialist pest on crucifer crops, produces the sesquiterpene, murgantiol, as a male-specific aggregation pheromone. Similarly, the southern green stink bug, *Nezara viridula*, a generalist pest worldwide on soybean and other crops, releases sesquiterpene *cis*-/*trans*-(*Z*)-α-bisabolene epoxides as male-specific aggregation pheromone. In both species, enzymes called terpene synthases (TPSs) synthesize precursors of the aggregation pheromones, which are sesquipiperitol and (*Z*)-α-bisabolene as the precursor of murgantiol and *cis*-/*trans*-(*Z*)-α-bisabolene epoxide, respectively. We hypothesized that enzymes in the family of cytochrome P450 monooxygenases are involved in the conversion of these precursors to the final epoxide products. This study investigated the tissue specificity and sequence of these conversions by performing crude enzyme assays with protein extracts from male tissues. Furthermore, candidate P450 genes were selected by RNA-sequencing and co-expression analysis and the corresponding recombinant proteins were tested for enzyme activity. To engineer the pheromone biosynthetic enzymes in plants, transient expression of the TPSs of both stink bugs was performed in

Nicotiana benthamiana leaves. Both Sesquipiperitol and (*Z*)-α-bisabolene were found to be produced and emitted from inoculated *N. benthamiana* leaves. Future work will implement stable transformation to engineer murgantiol biosynthesis in crucifer trap crops and develop similar approaches for pheromone engineering of other agricultural pests.

Aggregation Pheromone Biosynthesis and Engineering in Plants for Stinkbug Pest Management

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GENERAL AUDIENCE ABSTRACT

Stinkbugs including the harlequin bug, *Murgantia histrionica* and southern green stinkbug, *Nezara viridula*, are major agricultural pests in the US and worldwide. To control these pests with alternative pest management strategies, we have proposed to develop trap crops that emit pheromones to lure the insects away from crop fields. To establish pheromone biosynthesis in plants, we investigated the corresponding enzymatic steps in both stink bugs. We show that terpene synthase enzyme from both stink bugs can be transformed into plants for the engineering of pheromones in trap crops. With identification of P450 genes in pheromone biosynthesis enhanced trap crops can be made.

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Chapter 1. Introduction and Overview of Research

Semiochemicals in Insect and Plant Interactions

In the natural environment semiochemicals play a strong role in influencing the life of plants and insects (Fig 1.1). Semiochemicals are produced by both plants and insects and often increase fitness for select organisms or are involved in interactions with conspecifics or other species (Aharoni et al., 2005). There is chemical significance for specific structures in specific plant-insect or insect-insect interactions. Insects are highly sensitive to semiochemicals and can differentiate between different blends and isomers (Harrewijn, 2001).

Semiochemicals produced by insects often act as pheromones, mediating intra-species communication, reproduction, feeding, and protection from predators (Agelopoulos et al., 1999). When developmentally mature, insects emit sex and aggregation pheromones that attract potential mates (Landolt, 1997). While sex pheromones are produced mainly by females to attract males, the production of aggregation pheromones is characteristic of males. Aggregation pheromones attract conspecifics in high numbers and are often deployed after finding a source of food (Tittiger, 2003). Pheromones also signal dispersal in the presence of enemies, or trail-forming behavior in some species (Morgan, 2010).

Plant semiochemicals evolved as means to attract insects for reproduction or to increase fitness by reducing feeding pressure by insect herbivores. Semiochemicals in plants include secondary or specialized metabolites of different compound classes such as terpenoids, phenylpropanoids, benzenoids, fatty acid derivatives, and glucosinolates

(Dudareva et al., 2013; Pichersky et al., 2006). Flowers release complex mixtures of volatile compounds to attract pollinators. Plant leaves, especially young nutrient rich leaves, produce volatile and non-volatile semiochemicals to repel insects and/or deter feeding under constitutive or induced conditions (Unsicker et al., 2009). Upon attack by herbivores, plants also release volatile compounds from aboveground tissues or roots in an indirect defense strategy to attract predators or parasitoids (e.g. Rasmann et al. 2005).

Specific semiochemicals do not necessarily have a predetermined function but may rather acquire their function in a particular ecological niche (Reddy and Guerrero, 2004). Therefore, it is possible that the structures of plant and insect-derived compounds resemble each other or are even identical. For example, the sesquiterpene (*E*)-β-farnesene is an aphid alarm pheromone but it is also produced by leaf and flower tissues of many different plants (Harrewijn, 2001). In another example, sexually deceptive orchids mimic mating signals in females of the aculeate *Hymenoptera* order to deceive males into pollination (Schiestl et al., 2003). Volatile compounds produced by the orchids are identical to the female sex pheromones of the pollinator species and when males attempt copulation they pollinate the orchid (Schiestl, 2005).

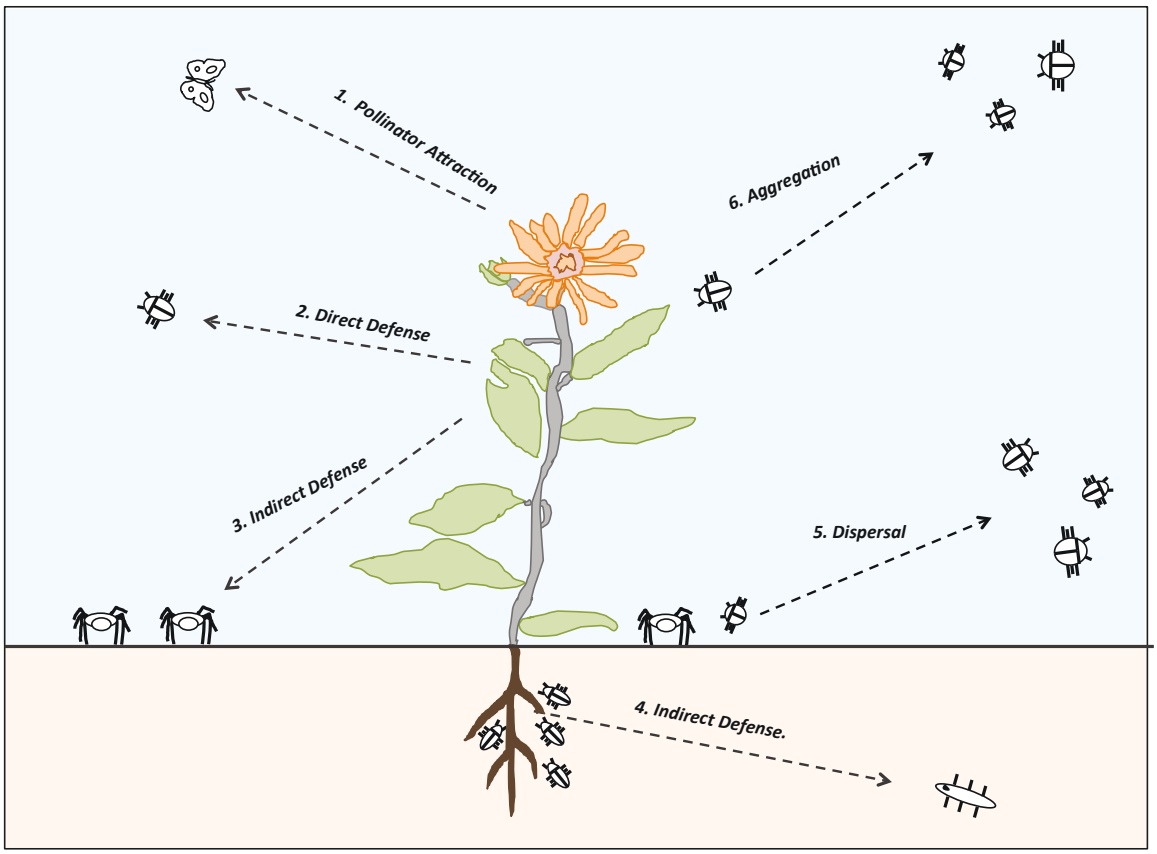


Figure 1.1 Semiochemicals Mediate Interspecies and Intraspecies Communication. 1) pollinator attraction 2) direct defense and 3, 4) indirect defense above and below-ground. Semiochemicals mediate intra-species communication in: 5) dispersal 6) aggregation.

Use of Semiochemicals in Integrated Pest Management

Integrated Pest Management (IPM) mitigates agricultural pest damage through a combination of tactics in chemical, biological, and environmental control. The focus is to keep pest populations below levels that cause economic damage rather than complete pest eradication (Pedigo and Rice, 2009). IPM is a shift away from complete reliance on broad-spectrum insecticides that have potential risks to human health and the environment or are facing use restrictions due to pollinator risks.

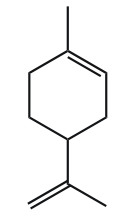
Behavioral manipulation strategies in IPM use semiochemicals such as insect

pheromones or volatile chemical cues released by trap crops to attract insects away from crops (Shelton and Badenes-Perez, 2006). Insect pheromones are chemically synthesized and applied in lures or traps, whereas trap crops are grown around or adjacent to crops to divert insect feeding away from more valuable crops. Such practices require an accurate understanding of the biology of the pest. The most prominent examples of successful trap cropping strategies have been employed in managing cotton boll weevil and various stinkbug pests in soybean fields (Heikki and Hokkanen, 1991)(Pickett et al., 2014). In cotton boll weevil pest management, trap crops are baited with synthetic pheromone, grandlure, greatly enhancing the effectiveness of trap crops (Heikki and Hokkanen, 1991).

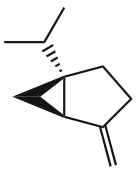
Terpenes in Plants and Insects

Terpenoids or terpenes (also known as isoprenoids) make up the largest class of specialized metabolites, with over 20,000 different chemical structures (Tholl, 2006) and are also important primary metabolites in plant and insects. All terpenes are made from five carbon units and include the hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40). Terpenes can be open chain, cyclic, bicyclic, or polycyclic and roughly 90% of terpenes are oxygenated (Schrader et al., 2015). The compounds often have one or more chiral centers, contributing to their bioactive properties. The lower molecular weight terpene hydrocarbons and

Fig. 1.2. Examples of monoterpene semiochemicals



D-limonene

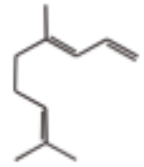


Sabinene

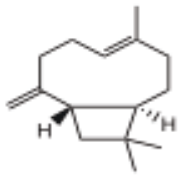
alcohols such as monoterpene(oid)s and sesquiterpen(oid)s are often volatile because they have low vapor pressure at ambient temperatures (Pichersky et al., 2006)

Terpenes do not have prescribed biological functions, but may acquire specific roles within certain ecological niches. In plants, mono- and sesquiterpenes can serve as pollinator attractants or insect deterrents. A good example of a pollinator attractant is D-limonene (Fig. 1.2) from the monkey flower, *Mimulus cardinalis*, which attracts bumblebees for pollination (Byers et al., 2014). Monoterpenes and sesquiterpenes also exhibit antimicrobial activities. For example, conifers produce a bouqet of monoterpenes including α-pinene, myrcene, limonene, cineole, camphene, and santalene in high concentrations to protect against pathogens (Schrader and Bohlmann, 2015). The relative amount of each compound and the isomeric composition varies among species (Aharoni et al., 2005). Furthermore, plant terpenes function in direct or indirect defense against insects. The bicyclic terpene sabinene (Fig. 1.2) emitted by Chinese cabbage, deters feeding and oviposition by the diamond back moth, *Plutella xylostella* (Unsicker et al., 2009). In cucumber, the homoterpene DMNT (Fig. 1.3) is released upon attack by the two-spotted spider mite, *Tetranychus urticae*, to attract the predatory mite *Phytoseiulus persimilis*, a natural predator of *T. urticae* (Bouwmeester et al., 1999). Both above ground tissue and roots have this strategy of chemical defense. In maize, roots produce *(E)*-β-caryophyllene (Fig. 1.3) that attracts parasitic nematodes of the voracious root pest,

Fig. 1.3. Terpene semiochemicals with functions in plant indirect defense



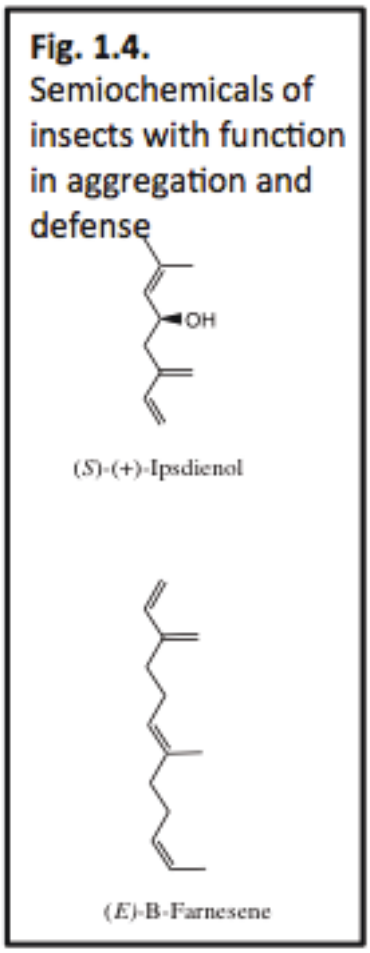
DMNT



(*E*)-B-Caryophyllene

western corn rootworm (Rasmann et al., 2005).

Terpenes of higher molecular weight (C20, C30, C40) and their degradation products have roles in plant primary metabolism and signaling. For instance, the pentacyclic diterpene, gibberelic acid, that is found in plants and fungi is an important phytohormone for seedling germination and plant growth (Schrader and Bohlmann, 2015). Triterpenes and tetraterpenes include sterols, carotenoids, chlorophylls, plastoquinones, and ubiquinones, which are important for cell membrane structure, photosynthesis, and respiration (Aharoni et al., 2005).



Terpenes also seve as primary metabolites in insects. Juvenile hormones, a group of sesquiterpenoids (i.e. oxygenated sesquiterpenes) that are produced in the *corpora allata* of insects regulate polymorphisms within insect colonies and different aspects of growth and development. Juvenile hormones affect embryogenesis, molting, and pheromone production (Noriega, 2014). Ecdysone, a C30 steroid, is also an important hormone for insect development. To produce these hormones insects require plants as a source of sterol precursors since they are unable to produce sterols de novo. Carotenoids (C40) are not biosynthesized by insects, but serve as insect pigments (yellow, green, brown) and include retinal involved in insect vision (Morgan, 2010).

In contrast to the tremendous structural diversity of terpene secondary metabolites

in plants, the occurrence of terpenes with specialized function in insects appears to be more restricted. As one example, volatile terpenes are used as defense compounds in termites and Lac insects (Lacciferinae)(Chakraborty et al., 2016). The major constituent of head secretions within the soldier class of the underground termite species *Reticulitermes lucifugus* is (*E*)-β–farnesene (Harrewijn et al., 2001), which is believed to function as a defense against natural enemies. Within the *Reticulitermes* genus, the monoterpenes myrcene, (*E*)-β-ocimene, (+)-limonene, α-pinene, and β-pinene are also common constituents of head secretions (Morgan, 2010). The larvae of swallowtail butterflies also produce a mixture of defense compounds with (*E*)-β-ocimene and α-pinene, along with β-phellandrene, and (*E*)-β-farnesene (Fig. 1.4). When disturbed, larvae secrete these compounds from a specialized horn like organ, an osmeterium, on the back of their heads and attempt to smear their disturber with these secretions (Chattopadhyay, 2011).

Another important group of volatile terpenes in insects are monoterpenes and sesquiterpenes that serve as major constituents of insect pheromones for beetles, aphids (see above), and weevils. Beetles have been shown to produce a variety of different terpene aggregation pheromones. For example, flea beetles *Phyllotreta cruciferae*, *Phyllotreta striolata*, and *Phyllotreta vitulla* produce himichaladienes as the major components of male-specific aggregation pheromones (Beran et al., 2016). The two major components of *Phyllotreta striolata* pheromone are (6*R*,7*S*)-himachala-9,11-diene and (3S,9R,9aS)-3-hydroxy-3,5,5,9-tetramethyl-5,6,7,8,9,9a-hexahydro-1H-benzo[7]-annulen-2(3H)-one (Gruber et al., 2009). Bark beetles produce ipsdienol as the major component of aggregation pheromones (Morgan, 2010). The entantiomeric composition

varies with species. *Ips pini* pheromone is comprised of 90% (S*)*-(+)-ipsdienol (Fig. 1.4) while *Ips confusus* pheromone is comprised of 95% (R)-(-)-ipsidenol (Wood et al., 1968). Evidence for de novo biosynthesis has been found in these species (Beran et al., 2016; Gilg et al., 2009). Besides the terpene aggregation pheromones found in beetles, terpenes serving as sex or aggregation pheromones have also been reported from stink bugs (Pentatomidae) and will be described in more detail below.

Stink Bug Pests and Terpene Pheromones

Stink bugs (Pentatomidae), shield shaped bugs that are phytophagous (Millar, 2005; Paiero et al., 2013) or predacious eaters (Lundgren, 2011), are named for the peppery scent released upon disturbance that warns away predators. Stink bugs feed on leaves or fruits of crop plants causing significant economic loss each year in major crops (McPherson and McPherson, 2000). Many stink bugs in North America are crop pests, and pest behavior is also prominent abroad such as in the generalist pest southern green stinkbug, *Nezara viridula* (Knight and Gurr, 2007).

The harlequin bug, *Murgantia histrionica* (han) is a specialist pest of vegetable crops in the US and pest status has increased in recent years due to changes in agricultural practices (Wallingford et al., 2011). *M. histrionica* originated from Mexico or central America and migrated north, where it became established in the United States, especially the US south east. *M. histrionica* is a specialist pest of vegetable crops in the family of Brassiceae including collards, kale, broccoli, cauliflower, rapini, and spinach. Damage to leaf tissue results in crop loss each year and unmarketable crops (Leskey et al., 2012; McPherson and McPherson, 2000).

The southern green stinkbug, *Nezara viridula*, feeds on a variety of different crops but is a specialist pest of soybean and recognized as a significant pest in the US and many countries across the world. *N. viridula* is believed to originate from Asia and is found throughout the united states. As with *M. histrionica* population dynamics of *N. viridula* are affected by aggregation behavior, a main source of economic damage from stink bug pests.

Many stink bugs release terpenes as major pheromone components. These compounds often carry a sesquiterpene bisabolane-type skeleton. Bisabolene epoxides are found in sex pheromones of *Chinavia* and *Nezara* stink bug species of African origin such as *N. viridula*, which produces *cis*/*trans*-(*Z*)-bisabolene epoxides. Other bisabolane type pheromones include the zingiberenol pheromone of Brazilian rice stink bugs, *Tibraca limbativentris* and *Oebalus poecilus* (de Oliveira et al., 2013) and the zingiberene pheromone component of red shouldered stink bug, *Thyanta pallidovirens* (Borges et al., 2006). The aggregation pheromone murgantiol (10,11-epoxy-1-bisabolen-3-ol) is produced both by *H. halys* and *M. histrionica* although at different stereoisomeric composition. *M. histrionica* emit two isomers of murgantiol (3*S*,6*S*,7*R*,10*S*) and (3*S*,6*S*,7*R*,10*R*) in a natural ratio of 1.4:1 (Khrimian et al., 2014).

Harlequin Bug Pest Biology and Treatment

Murgantia histrionica, is a specialist pest on crops in the genus *Brassica* including broccoli, Brussels sprouts, cabbage, cauliflower, collard, kale, kohlrabi, rape, Chinese cabbage, turnip, radish, and arugula (Aliabadi et al., 2002). *M. histrionica* has the highest presence in the Southern US and causes white blotches or, under high feeding pressure,

leaf yellowing and wilting that leave crops unmarketable (Wallingford et al., 2011). Adult insects overwinter in the field in leaf litter and become active in early spring. In optimal conditions, males live an average of 68 days and females live 82 days. Nymphs undergo five instars before full maturity and during this time are flightless. A female lays 4-6 egg masses per lifetime (12 eggs per mass). Typically, *M. histrionica* undergo two to three generations per year.

The *M. histrionica* host range is limited to crucifers and species in the caper family (*Capparaceae*) that produce glucosinolates (Wallingford et al., 2013). Glucosinolates likely act as feeding stimulants for *M. histrionica*, playing an important role in host plant selection. Males have preference for volatile blends from *Brassica* species containing glucosinolate break down products over volatile blends from non-host plants without these compounds (Thrift et al., 2018). In addition, glucosinolates are an example of direct semiochemical defense and usually deter insects from feeding; but, *M. histrionica* is resistant to these compounds and uses them for its own defense (Aliabadi et al., 2002). Feeding allows the bugs to consume and sequester glucosinolates in metathoraxic tissues resulting in the formation of chemical deterrents for predators and offering protection from birds and natural enemies.

In addition to host plant volatiles, insect pheromones have a central role in *M. histrionica* biology (Weber et al., 2014). Female behavioral response to host plants is weak and inconsistent, but females are strongly attracted to host plants that have male *M. histrionica* (Wallingford, unpublished) likely due to murgantiol presence. Murgantiol functions in mating and reproduction. The fact that females are not attracted to males on non-host plants suggests that both murgantiol release and host plant volatiles are

important for pest colonization.

Murgantiol and trap crops have been investigated for their use in *M. histrionica* pest management. Field choice tests have shown that mustard, rapeseed, rapini, and arugula are possible trap crop for collards. Particularly, *M. histrionica* has preference for mustard (*Brassica juncea*) over collard (*Brassica oleraceae*) and border rows of mustard can recruit high numbers of *M. histrionica* and decrease feeding injury of collard by approximately 50% (Wallingford et al., 2013). The application of synthetic murgantiol is effective in the field. When applied in lures, murgantiol attracts *M. histrionica* away from crops and when deployed in the presence of the host plant the effectiveness of *M. histrionica* attraction increases 30-fold from attraction solely with pheromone (Weber et al., 2014). Murgantiol is a strong enough attractant to fool harlequin bugs into alighting on non-host plants such as soybean (Thrift et al., 2018).

Terpene Biosynthesis in Plants and Insects

Except for small differences, terpene biosynthesis is widely conserved across all organisms (Lange and Ahkami, 2013) and consists of three main steps (Fig. 1.5): 1) the C5 isoprene units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are formed by the mevanolate (MVA) or methyl-erythritol phosphate (MEP) pathway, 2) condensation of the C5 units by isoprenyl diphosphate synthase enzymes forms prenyl diphosphates of varying length, 3) terpene synthase enzymes convert prenyl diphosphate intermediates to terpene compounds.

IPP and DMAPP are derived from precursors in central carbon metabolism. The eukaryotic MVA pathway converts acetyl-CoA to IPP via mevalonic acid as an

intermediate. In prokaryotes, IPP is produced by the MEP pathway: In this pathway, pyruvate and glyceraldehyde 3-phosphate serve as precursors, which are condensed and undergo six consecutive conversions to IPP and DMAPP (Tholl, 2015) with 2-methyl erythritol 4-phosphate as an intermediate (Wanke et al., 2001). Plants produce IPP and DMAPP via the MVA pathway and MEP pathway, as the plant cell is eukaryotic but contains chloroplasts of prokaryotic evolutionary origin (Tholl, 2015). Insects and other animals produce IPP only through the MVA pathway (Tholl, 2015).

The head to tail condensation of IPP and DMAPP yields prenyl diphosphates of different lengths (Tholl, 2015). One molecule of IPP and one molecule of DMAPP yield geranyl diphosphate (GPP, C10), two molecules of IPP and one molecule of DMAPP give rise to farnesyl diphosphate (FPP, C15), and three molecules of IPP and one molecule of DMAPP form geranyl geranyl diphosphate (GGPP, C20). In plants, the main pool of FPP is localized in the cytoplasm whereas GPP and GGPP pools are predominantly located in the plastid (Tholl, 2015).

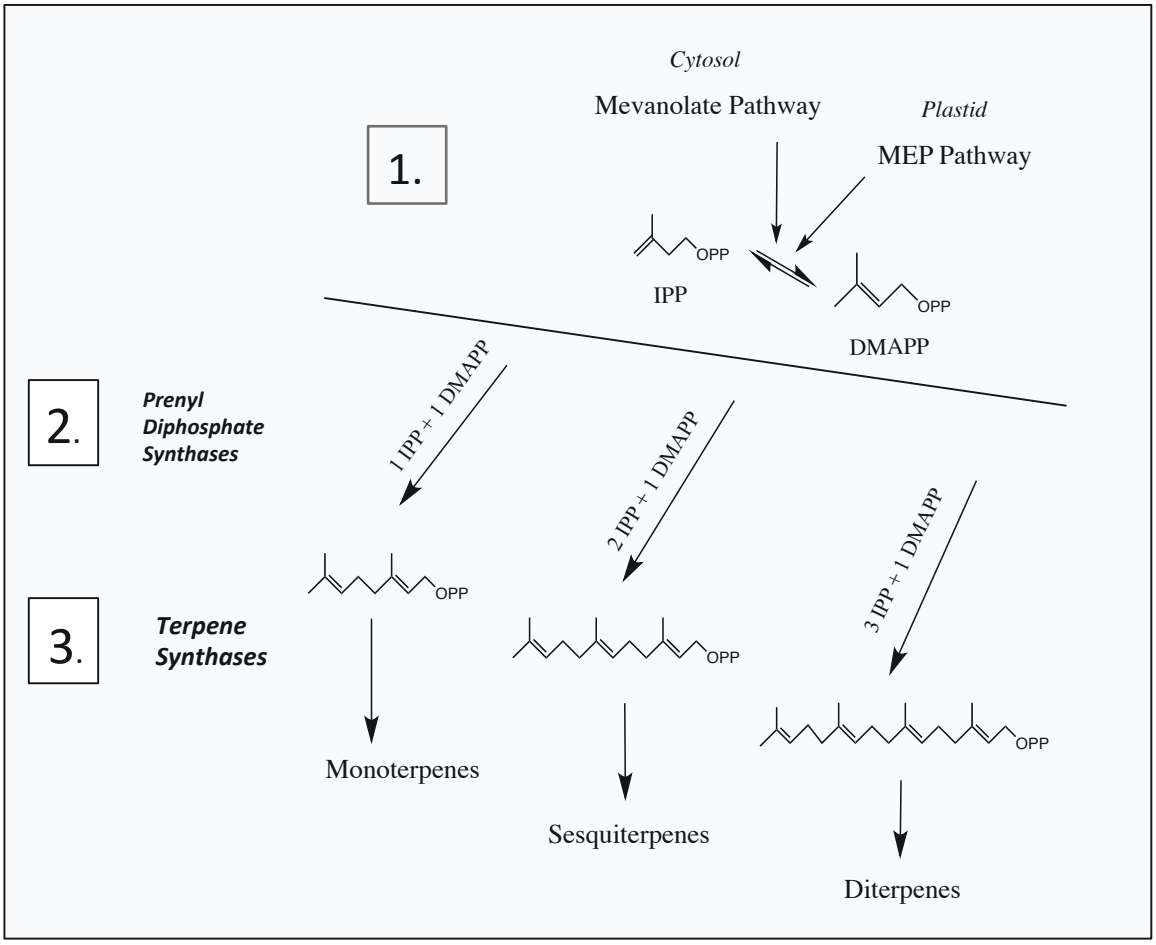


Figure 1.5. Three Core Steps in Terpene Biosynthesis, abridged from (Tholl, 2006). Abbreviations as mentioned in text.

The prenyl diphosphate intermediates are further converted to different terpene compounds by terpene synthase (TPS) enzymes. In plants the vast diversification in the TPS enzyme family is responsible for the diversity of terpene compounds (Tholl, 2006). Terpene synthase-catalyzed reactions all proceed by the formation of a carbocation, which can undergo many different types of reactions including cyclizations, hydride and alkyl shifts leading to diverse molecular structures (Degenhardt et al., 2009).

Following the formation of terpenes by TPS enzymes, further functionalization of

the molecules can occur by oxygenases, dehydrogenases, reductases, glycosyl transferases, or methyl transferases (Lange and Ahkami, 2013). Oxygenation reactions are often catalyzed by enzymes in the cytochrome P450 monooxygenase (CYP) superfamily. Cytochromes P450 are multifunctional enzymes that usually incorporate one atom of oxygen from molecular oxygen into a substrate by using NADH or NADPH as cofactors (Nelson et al., 2013). A cysteine-heme complex at the active site facilitates oxygen transfer, while redox partners, P450 reductases, or phthalate family oxygenase reductases, provide electrons for the reaction. When bound to carbon monoxide the cysteine-heme complex absorbs light at 450 nm, giving the enzyme family its name. P450s are named based on sequence, grouped into families (>40% identity), subfamilies (>55% identity) and given a unique gene identifier; however, the primary sequence is not always indicative of function and a small difference in sequence homology can mean distinct functional differences (Feyereisen, 1999). Of importance for this project, P450 are one of the few types of enzymes that perform epoxidation reactions (Helvig et al., 2004).

Terpene biosynthesis in insects has been studied primarily in the context of primary metabolism such as in the formation of ecdysone (Morgan, 2010) and juvenile hormones (Noriega, 2014). IPP and DMAPP made by the MVA pathway are converted into FPP by prenyl diphosphate synthases (FPP synthases). In juvenile hormone biosynthesis a phosphatase dephosphorylates FPP to farnesol, which undergoes two sequential oxidation reactions to farnesoic acid. Conversion of farnesoic acid to JH involves methylation and epoxidation (Fig. 1.6) and the order of these steps differs between insect species (Noriega, 2014).

While the core pathway in FPP synthesis is conserved in insects, as it is in largely all organisms, insects do not have families of terpene synthases known from plants, bacteria, and fungi (Lancaster et al., 2018a; Lancaster et al., 2018b). However, increasing evidence is showing that insect terpene synthases evolved from a diversified family of isoprenyl diphosphate synthases (Beran et al., 2016; Lancaster et al., 2018a). The first evidence for this came from bark beetle pheromone biosynthesis, where a bi-functinonal enzyme with prenyl tranferase and terpene synthase activity was identified (Gilg et al., 2009). Terpene synthase activity in prenyl diphosphate proteins has also been found in other insect pheromone biosynthetic pathways as shown more recently with the discovery of four TPS enzymes in flea beetle (Beran et al., 2016). Studies of harlequin bug in the Tholl lab have led to the identification of a FPP synthase like terpene synthases as described below.

Pheromone Biosynthetic Pathways in *M. histrionica* and *N. viridula*

M. histrionica and other stinkbugs produce bisabolane type aggregation or sex pheromones. Recently, the Tholl lab has identified FPP synthase like proteins that catalyze the first step in pheromone biosynthesis in *M. histrionica* and *N. viridula* (Lancaster et al., 2018a; Lancaster et al., 2018b). These enzymes convert (*E*,*E*)-FPP into monocyclic intermediates of the pathway (Fig. 1.6). In *N. viridula*, the intermediate is (*Z*)-α-bisabolene, which is converted into the pheromone *cis/trans*-(*Z*)-bisabolene epoxide (Lancaster et al., 2018b). In *M. histrionica*, FPP is converted to the cyclic alcohol, sesquipiperitol, which is then converted further to murgantiol in a one-step or two-step pathway that includes an isomerization and epoxidation reaction. Possibly, this involves a

zingiberenol intermediate (Lancaster et al., 2018a). The final epoxidation step resembles the final step in Juvenile Hormone III biosynthesis. Epoxidation occurs at the C10, C11 position similar to methyl farnesoate epoxidation at the C10, C11 position by a cytochrome P450 (CYP 450). The epoxide is the main feature of juvenile hormone and a potential target for insecticides, so multiple P450s from juvenile hormone biosynthesis have been sequenced and characterized (CYP15A1 from *Diplotera punctata* and *Musca domestica,* CYP6A1 from *Schistocera gregaria,* and CYP9E1 from *Diplotera punctate;* Feyereisen, 2005). It is unclear whether an epoxidase type P450 enzyme catalyzes both isomerization and epoxidation steps or just the final epoxidation on the way from sesquipiperitol to murgantiol.

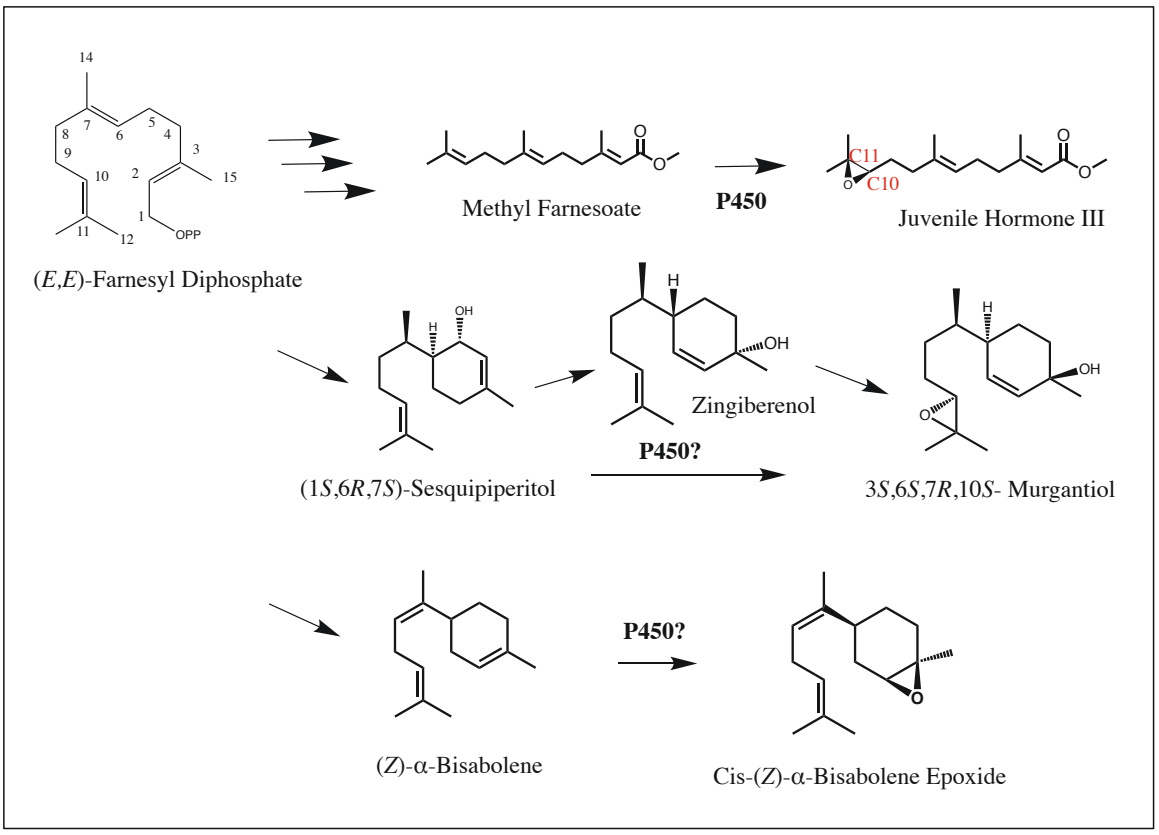


Figure 1.6. Putative Pheromone Biosynthetic Pathways in *M. histrionica* and *N. viridula*, along with the juvenile hormone III biosynthetic pathway. A P450 in JH III biosynthesis epoxidizes the C10,11 position, the same position of the murgantiol epoxide. Zingiberenol is a potential intermediate in the murgantiol biosynthetic pathway.

Engineering Specialized Metabolism and Pheromone Biosynthesis in Plants

Plant genetic engineering is commonly explored as means to develop varieties of crops with improved agronomic traits such as higher nutritional content, increased tolerance to abiotic stress like drought or heat, and resistance to disease or pests (DeVault et al., 1996; Gould, 1988). Increasingly more efforts have evaluated the potential for engineering specialized plant pathways to improve agronomic traits and there are a multitude of studies engineering terpenes into plants to improve the aroma of fruit and flowers or enhance direct and indirect defenses of crops (Dudareva and Pichersky, 2008). Common model plants used for the engineering of terpene biosynthetic pathways are *Nicotiana benthamiana* and *Arabidopsis thaliana* (Pelot et al., 2017; Wu et al., 2006). Both plants have genetically tractable genomes, a short generation time, and they are transformable by *Agrobacterium* infiltration; however, engineering is also readily explored in maize, rice, tomato, *Medicago*, and *Picea* (Tholl, 2006).

As an example, tomato has been used successfully to engineer terpene aroma biosynthesis in fruits. To improve the taste of contemporary tomato cultivars, Davidovich-Rikanati and colleagues (2007) engineered transgenic tomato with geraniol synthase as a single recombinant gene from *Ocimum basilicum* (sweet basil). Ripening tomatoes have a highly active carotenoid pathway in which the monoterpene precursor GPP is an intermediate. By expressing geraniol synthase under a tomato-ripening-specific promotor, they increased the amount of geraniol, along with its oxidized derivative geranial. The resulting fruit had a decrease in red pigmentation and altered fruit aroma, resembling lemons (Davidovich-Rikanati et al., 2007).

Engineering has also been used to bolster crop defense against aphids (Yu et al.,

2012). An initial study in *Nictotiana* showed that suppression of a P450 hydroxylase specific to the trichome gland increased levels of cembatriene-ol, diminishing aphid colonization (Wang et al., 2001). Similarly, integration of a bifunctional monoterpene/sesquiterpene synthase, FaNES1, in *Arabidopsis* increased linalool levels emitted from vegetative tissue (Aharoni et al., 2003) resulting in transgenic lines that significantly repelled the peach aphid *Myzus persicae.* Also, introduction of (*E*)-β-farnesene synthase from *Mentha* x *piperita* increased levels of (*E*)-β-farnesene, the natural aphid alarm pheromone, in *Arabidopsis*. Transgenic plants repelled the peach aphid and also attracted *D. rapea* parasitoids of the aphid, thereby improving defense at two trophic levels (Beale et al., 2006).

While single gene engineering is sufficient to improve plant traits, multiple genes can be used as well. To reduce the hazardous biproducts of organic synthesis of moth pheromones, Ding and colleagues (2014) produced moth pheromones by transient gene expression in *Nicotiana benthamiana.* Starting from *de novo* fatty acid biosynthesis in plants and increasing myristic acid substrate pools with a plastidial thoiesterase (*Cuphea* palustris) the authors engineered a desaturase, multiple fatty acid reductases from moths, and an acetyltransferase (*Euonymus alatus*) to produce alchohols and acetates that are pheromone components of hundreds of moth species. The compounds had distinct length and stereochemistry and matched the same activity of synthetically produced pheromones in trap catches for the small ermine moths, *Yponomeuta evonymella* and *Y. padella.* The work by Ding et al. (2014) has been, so far, the only study showing the successful engineering of insect derived enzymes in plants.

A Stink Bug Pheromone Engineering Strategy for an Enhanced Trap Crop

To date, no studies have explored the genetic engineering of aggregation or sex pheromones for improving trap crops. With what is known in plant terpene engineering and the chemical ecology of *M. histrionica* and other stink bugs, there is an opportunity to experiment with new applications for engineering. In plants, sesquiterpenes are produced in the cytoplasm from pools of FPP (Fig. 1.5). Since stink bug pheromone precursors are produced from FPP, plant derived FPP pools can be used for the production of these compounds. Generation of transgenic plants producing the pheromones would require the engineering of two or three consecutive genes (Fig. 1.6). The P450 step would most likely be localized to the endoplasmic reticulum. After biosynthesis the alcohol product is likely to diffuse or be transported through cell membranes (Pichersky et al., 2006) and volatilize in the headspace around plants. Since the pheromone biosynthetic enzymes are stereospecific, the production of murgantiol along with host plant volatiles could make a highly attractive bouquet of compounds for attracting the harlequin bug away from *Brassica*.

Overview of Research

This study was guided by the hypothesis that stink bugs produce terpene pheromones de novo and that P450s catalyze the final epoxidation steps of pathway intermediates. The overall goal of this work was to identify P450s in stinkbug pheromone biosynthesis and engineer a pheromone biosynthetic pathway into plants

The specific objectives of the study were to:

A. Identify P450 epoxidases in stink bug pheromone biosynthesis

B. Engineer pheromone biosynthesis in model plants

In Chapter II, we present an approach for identifying P450 epoxidases in stink bug pheromone biosynthesis.

In Chapter III, we show that terpene synthase enzymes involved in the first step in pheromone biosynthesis can be engineered into plants for this purpose.

Finally, in Chapter IV the conclusion and significance of this study are discussed, including perspectives in related future research.

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Chapter 2. Identifying a P450 Epoxidase in Stinkbug Pheromone Biosynthesis

Abstract

The stink bugs *Murgantia histrionica* (harlequin bug) and *Nezara viridula* (southern green stink bug) are important agricultural pests in the US and worldwide. Males of both species release terpene aggregation pheromones. Recently, it was shown that the precursors of these pheromones are synthesized de novo by enzymes called terpene synthases (TPS); however, genes and enzymes involved in downstream reactions that include epoxidations have remained elusive. In *M. histrionica*, *Mh*TPS converts (*E,E*)-farnesyl diphosphate to (1*S*,6*R*,7*S*)-Sesquipiperitol, which presumably undergoes isomerization and epoxidation to make the pheromone murgantiol. In *N. viridula*, *Nv*TPS produces (*Z*)-α-bisabolene, which is further converted to the pheromone components *trans*-/-*cis*-(*Z*)-α-bisabolene epoxides. Comparative sex specific transcriptome analysis of *M. histrionica* cuticle-associated tissue, the site of highest *Mh*TPS activity, identified two cytochrome P450 monooxygenases, CYP3229A1 and CYP4G159A1, as possible candidates involved in epoxidation to form murgantiol. In vitro activity of the corresponding recombinant proteins to convert sesquipiperitol or zingiberenol, the putative secondary intermediate to murgantiol, could not be confirmed. Moreover, the biochemical assays for enzymatic activity of these reactions in crude protein extracts of the cuticle and other tissues failed to make murgantiol, which raises questions about the tissue specificity and compartmentalization of the downstream enzymatic steps. No epoxidase activity was found in the crude protein extracts of cuticle tissue from *N. viridula* males. Comparative transcriptome analysis of *N. viridula* male and female cuticle tissue and subsequent RT-PCR analysis suggested two P450s, CYP4G159 and

CYP4HB6, as possible candidate genes involved in pheromone biosynthesis. The functions of these genes and others will be subject to further investigation. Phylogenetic comparison between the cuticle expressed P450 families of *M. histrionica* and *N. viridula* map help identify similar epoxidases in both species.

Contributions: I would like to thank Drs. Don Weber and Ashot Khrimian at the USDA, Beltsville, for providing stinkbugs and authentic standards for gas-chromatography-mass spectrometry analysis. I am grateful to Dr. Claus Tittiger and Sharon Young (University of Nevada, Reno) for collaboration on P450 recombinant protein expression and screening candidate P450s. Thank you to Dr. Thomas Kuhar's lab (Virginia Tech) for maintenance of a harlequin bug colony.

Introduction

Insects communicate with pheromones of diverse chemical structure and composition (Keppner et al., 2017; Müller and Buchbauer, 2011). Many insect pheromones are derived from fatty acids while others arise from terpene (isoprenoid), amino acid, or alkaloid precursors (Blomquist and Vogt, 2003; Jurenka, 2004; Tillman et al., 1999; Yew and Chung, 2015). Among the true bugs (Hemiptera), stinkbugs (Pentatomidae) release sesquiterpene sex and aggregation pheromones with a bisabolane chemical backbone (Millar, 2005). The harlequin bug, *Murgantia histrionica*, releases a mixture of (3*S*,6*S*,7*R*,10*R*) and (3*S*,6*S*,7*R*,10*S*) stereoisomers of 10,11-epoxy-1-bisabolen-3-ol as a male specific aggregation pheromone named murgantiol ((Khrimian et al., 2014; Weber et al., 2014; Zahn et al., 2008). Another stinkbug *Nezara viridula*, a generalist

crop pest worldwide (McPherson and McPherson, 2000), emits a mixture of *trans*-/*cis*-(*Z*)-*α*-bisabolene epoxides as its sex and aggregation pheromone.

Recently, the Tholl lab demonstrated that the precursors of these terpene pheromones are synthesized de novo from the isoprenoid intermediate (*E,E*)-farnesyl diphosphate (FPP) by enzymes called terpene synthases (TPSs) (Lancaster et al., 2018a; Lancaster et al., 2018b). In *M. histrionica, Mh*TPS converts (*E,E*)-FPP to (1*S*,6*R*,7*S*)-sesquipiperitol while in *N. viridula* *Nv*TPS converts (*Z*)-*α*-bisabolene to *trans*-/*cis*-(*Z*)-*α*-bisabolene epoxides (Lancaster et al., 2018b). The genes encoding these enzymes are expressed in specific tissues of the male bugs. In *M. histrionica,* transcript levels of *MhTPS* are highest in the sub-cuticle epithelial tissues of the ventral abdomen. A similar tissue specific expression was observed in *N. viridula* (Lancaster et al., 2018b) with exception that *N. viridula* contains glandular tissue with specialized pheromone storage cells (Cribb et al., 2006). *TPS* transcript abundance correlates with enzyme activity as it could be shown in crude protein extracts of these tissues in both bugs (Lancaster et al., 2018a; Lancaster et al., 2018b) (Fig. 2.1).

To elucidate the entire pheromone biosynthetic pathways in *M. histrionica* and *N. viridula*, we consider that (1*S*,6*R*,7*S*)-sesquipiperitol and (*Z*)-*α*-bisabolene undergo epoxidation to generate the pheromone end products. We assume that the final steps in murgantiol biosynthesis involve the conversion of sesquipiperitol to murgantiol (Fig. 2.2) (Lancaster et al., 2018a). Similarly, we assume that in *N. viridula (Z)*-α-bisabolene is converted to *trans-/cis-(Z)*-α-bisabolene epoxides by an epoxidase-catalyzed reaction. This reaction may occur in the pheromone glands, from which the pheromone is released through ducts onto the surface of the cuticle.

We hypothesize that the described epoxidase reactions are catalyzed by enzymes in the family of cytochrome P450 monooxygenases (P450s). P450s represent a superfamily of enzymes with several clade representations in insects: CYP3, CYP2, CYP4 and the mito-clade (P450s localized to the mitochondria). (Feyereisen, 2006). P450s can be identified by highly conserved motifs such as an N-terminal WxxR motif and C-terminal cysteine residue. Nomenclature of P450s follows such that of number or family designation represents 40 % homology at the amino acid level and the letter or subfamily designation represents 55 % amino acid homology (Nelson, 2013). For example CYP3229A1, CYP3229 is the family, the subfamily is A and a unique gene is designated by the second number, 1. The CYP3 clade which is insect specific is associated with the detoxification of pesticides or plant allelochemicals (Scott and Wen, 2001). P450s are also involved in hormone processes such as ecdysteroid and juvenile hormone biosynthesis (Helvig et al., 2004) as well as cuticle formation (Qiu et al., 2012).

Materials and Methods

Insect Colonies

M. histrionica bugs were collected from a greenhouse colony maintained at Virginia Tech (Blacksburg, VA) as previously described (Lancaster et al., 2018b) Greenhouse conditions included a day/night photoperiod and 79°F temperature. A fresh colony was established from local populations each spring. Bugs were reared on collards. Adult male and females were separated after eclosion and mature virgin male and females were harvested 10-12 days later. To confirm murgantiol emission three mature

male *M. histrionica* were monitored by headspace analysis on a head of organic cauliflower using closed loop stripping as described previously (Huang et al., 2012)

A colony of *N. viridula* was maintained by Don Weber at the USDA, Beltsville. Insects were reared as described previously (Lancaster et al., 2018a) following the following procedure:

Insects were reared in ventilated plastic cylinders (21 cm × 21 cm o.d.) on a diet of organic green beans, shelled raw sunflower seeds and buckwheat seeds (2:1 w/w), glued onto squares of brown wrapping paper with wheat-based wall paper paste Distilled water was supplies in a two-cotton stopped 7 cm × 2 cm o.d. shell vials held together with a rubber band. Insects were reared in a climate controlled growth chamber (25 +/- 5°C, 16:8 h L:D, 65% RH). Eggs were collected weekly and hatched in plastic Petri dishes with a water vial, after molting to second-instars, the nymphs were transferred to the larger rearing cages as described above for the remaining four instars. Newly eclosed adults were removed from cages three times weekly and moved to new cages, isolating males and females. Insects were kept until the mature (14-15 post most) adult stage.

*Mh*TPS1 Activity Analysis in Tissues of *M. histrionica*

Adult harlequin bugs were fixed with hexane vapor. Five male and female tissues were dissected in PBS buffer pH 7 with forceps using a stereo microscope. Cuticle, midgut, fatbody, and head and thorax tissues from males or females were frozen in liquid nitrogen and pulverized using a mortar and pestle. Protein was extracted by resuspension in 50 mM Hepes pH 7 with 5 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 10 % glycerol. Protein concentration was determined with a Bradford assay according to the

manufacturer's instructions. Protein extract from each tissue (50 µg) was brought to a final volume of 200 µL total volume with extraction buffer adding 50 µM (*E,E*)-farnesyl diphosphate and 200 µL of hexane was layered on top of the assay in a 7 mL glass vial. Assay were incubated at 37°C overnight in a water bath. Volatile enzymatic products were collected by 15 sec of mixing using the vortex and separation of the organic phase by centrifugation for 15 min at 7,000×g in a Beckman-Coulter Sorval Centrifuge. GC-MS analysis was performed by liquid injection of organic fractions under Split-2 setting.

P450 Epoxidase Activity Analysis in *M. histrionica* tissues

To detect P450 epoxidase activity in *M. histrionica* tissues additional assay conditions, substrates, tissue preparations, and volatile sampling techniques were used. Epithelial cells, intact cuticle dissected with softbody tissues, and whole abdomen dissected open from individual or pooled bugs (three individuals) were incubated in 500 µL to 1mL of previously described assay buffer with 50 µM (*E,E*)-farnesyl diphosphate in a SPME vial. Tissues were incubated with substrate for at 30°C overnight and volatile compounds were analyzed by SPME-GCMS. Assays were performed by incubating extracts of cuticle or whole-bug tissue, extracted as described previously, with 50 µM sesquipipieritol in 50 mM PBS pH 7 buffer with 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1mM DTT, 0.125 mM sucrose, and 1 mM NADPH. Assays with alcohol substrates were prepared at a final volume of 500 µL in a SPME vial and incubated overnight with gentle shaking at 30°C. Sesquipiperitol was prepared by evaporating off hexane solvent under liquid nitrogen, and resuspending the substrate in 15 µL 2mM Triton-x, and diluting the suspension 3:1 in assay buffer before mixing the substrate with extracts. The same

assays were repeated using (3*S*,6*S*,7*R*)-zingiberenol at a total concentration of the same volume with approximately 50 µg protein extracts.

*Nv*TPS1 Activity Analysis in *N. viridula* Tissues

Five unmated male and female *N. viridula* bugs were dissected and tissues were prepared for enzymatic assays in the same way as described for *M. histrionica*. Crude protein extracts were prepared by tissue pulverization and resuspension in 50 mM Hepes pH 7, 10 mM $MgCL_2$, 5 mM KCL, 1mM DTT and 10 % glycerol and assays were performed with 50 µg protein with 50 µM (*E,E*)-farnesyl diphosphate at a volume of 200 µL. A hexane overlay of equal volume was placed over the preparations and incubated for 6 h at 37°C in a 7 mL glass vials in a water bath. Assay products were collected as previously described and analyzed with GC-MS.

Analysis of P450 Epoxidase Activity in *N. viridula* Cuticle Tissues

Crude protein extracts were prepared from the cuticle tissue of three pooled bugs as previously described and resuspended in PBS pH7 buffer, 1mM $MgCL_2$, 1mM $CaCl_2$. Assays were performed by incubating extracts with 20 µM (*Z*)-α-bisabolene prepared as described for sesquipiperitol at a final volume of 500 µL. Incubations were administered with (*Z*)-α-bisabolene and either 1 mM NADPH or 1 mM NADH together with flavin monooxygenase cofactors FAD, flavin dinucleotide, and FMO, flavin mononucleotide. Preparations were incubated at 30°C overnight in SPME vials and volatile compounds were analyzed by SPME GC-MS headspace sampling the following day. A no-substrate

control was prepared by withholding (*Z*)-α-bisabolene and replacing the volume with added buffer.

Microsomal fractions prepared from cuticle tissue were isolated to enrich for P450 activity in enzymatic assays. Ventral abdominal cuticle from 10 male bugs was removed by dissection as previously described and tissue was pulverized with liquid nitrogen and mortar and pestle. Tissue extracts were prepared (500 mg tissue/4 mL buffer) in 0.1 mM PBS pH 7.4, 10% glycerol, 1mM PMSF, 1mM EDTA, and 0.1 M DTT and centrifugation at 13,000 rpm for 17 min at 4°C with a microcentrifuge. The soluble fraction from 10 bugs (one pooled sample) was combined and centrifuged at 100,000 rpm for 10 min at 4°C in a Beckman micro-ultracentrifuge. The resulting soluble fraction was decanted off and used for an assay by administration of (*Z*)-α-bisabolene. The microsomal pellet was resuspended in 100 µL 50 mM PBS pH 7.4, 0.120 mM sucrose, 2 mM DTT, and 1 mM $MgCl_2$, 2mM $CalCl_2$ resulting in 0.5 mg protein/mL. (*Z*)-α-Bisabolene prepared as previously described was added to the microsomal preparation as a final volume of 100 µM in a 2 mL glass vial. The preparation was incubated at room temperature at 15 min with gentle shaking and 1 mM NADPH was added. A field SPME fiber was used to detect volatile compounds in the headspace of the vial, which was incubated at 30°C overnight. Volatile compounds were analyzed by SPME GC-MS, starting mass detection at 27 min to preclude detector shutdown by substrate due to the sensitivity of SPME analysis.

GC-MS analysis was performed using split injection (5:1) and an injection temperature of 240°C (Lancaster et al., 2018b). Compounds were separated on a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) with a 30 m × 0.25 mm i.d. × 0.25

μm film Zebron ZB-XLB column (Phenomenex, Torrance, CA) coupled to a QP2010S mass spectrometer (Shimadzu). Separation steps were as follows: initial 2 min hold at 40°C, followed by a 5°C/min ramp to 220°C, then a 70 °C/min ramp to 240°C followed by a 2 min hold time at 240°C. Mass spectrometry was performed with a 240°C ion source temperature, 280°C interface temperature, electron ionization (EI) potential of 70 eV, and scan range of 50 to 400 amu. Helium was used as a carrier gas at 1.9 ml/min (Lancaster et al., 2018b).

RNA-seq and De Novo Transcriptome Assembly from *M. histrionica* and *N. viridula*

Different sets of transcriptome data were generated for *M. histrionica* and *N. viridula* prior to this study. For the first RNAseq total RNA was extracted from individual immature (2-3 days post molt) and mature (14-15 days post molt), male and female *M. histrionica* using TRI Reagent (Ambion) according to the manufacturer's protocol. RNA was DNase treated with RQI DNase I (Promega) and purified using the RNeasy Plant Mini Kit (Qiagen). RNA quantity was determined using a Nanodrop ND-1000 spectrophotometer and RNA integrity was analyzed on a Bioanalyzer 2100 (Agilent). RNAseq was performed on an Illumina Genome Analyzer IIx at the Virginia Biocomplexity Institute, Virginia Tech, using paired-end (2x100 bp) reads. Sequences were trimmed using custom Perl scripts for adaptor trimming and Btrim (Kong, 2011), for quality trimming resulting in 97-137 million reads per sample. De novo transcriptomes were assembled using Velvet.

A second set of RNA-seq data were generated from cuticle tissues of mature male and female *M. histrionica*. The procedure was also applied for the same tissues from *N.*

viridula as described (Lancaster et al., 2018b). Total RNA was extracted from pooled abdominal sternites of five mature male and five mature female bugs using Trizol Reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol as described above. RNAseq was performed by Beckman Coulter Genomics (Davers, MA) on an Illumina HiSeq Intstrument using paired-end (2x100 bp) reads resulting in approximately 82M reads per sample. Quality of fastq files was assessed using the publically available software FastQC. Based on quality scores, reads were truncated by 9 bp using Trimmomatic (Bolger et al., 2014) to remove loq quality sequence and the resulting reads were reanalyzed by FastQC. High quality reads were assembled de novo using Trinity (Grabherr et al., 2011) and assembled quality was verified by aligning processed reads to the assembled transcriptome using Bowtie2 (Langmead and Salzberg, 2012).

A third set of transcriptome data was available from different sex and developmental stages of *M. histrionica* as described previously (Sparks et al., 2017).

Transcriptome Analysis for the Identification of Candidate *M. histrionica* and *N. viridula* P450 Epoxidases

Sets of *M. histrionica* transcriptome data were obtained according to sex and tissue specificity and developmental stage as described above. Transcriptome results from collaborators at the USDA Beltsville (Sparks et al., 2017) were mined for P450 genes with higher transcript levels in mature male bugs compared to mature female bugs. Two custom transcriptomes were assembled by de novo assembly combining raw reads

from different sequencing experiments to compare P450 sequences between *M. histrionica* sex and developmental stages using

- Cuticle reads from mature male and mature female tissue
- Whole-bug reads from mature and immature males

To compare P450 transcripts between male and female cuticle tissue and mature and immature adult *M. histrionica* tissues transcripts were identified as P450s using P450 gene sequences from the *M. histrionica* transcriptome (Sparks et al., 2017). P450s from the four main P450 gene families in insects were used to compile a list of non-redundant P450 transcripts from each combined transcriptome. Coding regions in transcripts were positioned by identification of the well the conserved N-terminal WxxxR motif and C-terminal PFxxGxRxCx or filtered out if not encoding open reading frames corresponding to functional genes lengths (380-500 amino acids). Open reading frames from all unique transcripts were extracted and analyzed for the corresponding P450 name.

P450 transcript abundance was compared between sex specific cuticle tissue and male developmental stages in combined transcriptomes by estimation of transcript abundance with Bowtie2. Reads from respective tissue samples (ex. male cuticle and female cuticle) were individually mapped to the combined transcriptome, and transcript abundance was compared by Transcripts mapping per kilobase million (TPM).

The number of male or female transcripts mapping to each P450 transcript was normalized by the number of total reads in the transcriptome (reads per kilobase) and expressed in transcripts per kilobase million (TPM) for male and female cuticle tissue. Read mapping and P450 querying was repeated for a combined database of reads from mature and immature males to identify P450s that were more highly expressed in

sexually mature *M. histrionica* and male cuticle tissue. Transcripts encoding 18S RNA and ribosomal binding protein four (RBS4) were used as control genes for mapping experiments.

Analysis of *M. histrionica* P450 Gene Transcript Abundance

From the non-redundant list of P450s in the combined male and female cuticle transcriptome we identified 37 transcripts encoding unique P450s genes. Transcript abundance of P450 genes previously identified in the *M. histrionica* transcriptome as being male specific (CYP3229A1 and CYP4G159A1) were determined by quantitative Reverse Transcription PCR (qRT-PCR) in the midgut, fatbody, and head and thorax tissues by Sharon Young and Claus Tittiger at the University of Nevada, Reno. cDNA was previously prepared using three biological replicates, one replicate originating from one individual, for all male tissues (Lancaster et al., 2018a). Relative transcript abundance was measured using the ddCt method and normalized to 18S RNA (Livak and Schmittgen, 2001). *Mh*TPS1 transcript levels were used as a positive control. Primers were designed to amplify a fragment of approximately 100 bp and tested for non-specific binding. Reaction plates contained 2 µL cDNA (1 ng/µL), 0.6 µL each primer (300 mM final concentration), 6.8 µL dH_20 and 10 µL *Power*SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) per well. The samples were analyzed using an Applied Biosystems 7300 with default settings (50°C at 2 min, 95°C at 10 min followed by 40 cycles of 95° C at 15 sec, 60°C at 1 min). Primers were tested for non-specific amplification by analyzing the dissociation curve after PCR. Significance was

measured using Student's t-test. Transcript abundance for additional P450s was determined from cuticle tissue or whole male bugs with single biological replicates.

P450 Recombinant Protein Expression and Enzymatic Assay

Recombinant proteins of CYP3229A1 and CYP4G159A1 were expressed by Sharon Young and Claus Tittiger in Sf9 insect cells (Fig. S.2). Open reading frames of candidate P450s were cloned into baculovirus direct expression vectors in fusion with a P450 reductase from *Musca domestica* as performed previously (Sandstrom et al., 2006). Following cloning P450-reductase pairs were expressed by transfection into Sf9 insect cell lines and cell cultures were harvested to extract microsomal P450 fractions.

Epoxidase assays were performed by resuspending microsomal preparations of P450s in 50 mM PBS pH 7, 0.125 mM sucrose, 1mM MgCl2, 1mM CaCl2, and 1 mM DTT (Sandstrom et al., 2006). (1*S*,6*R*,7*S*)-sesquipiperitol and (3*S*,6*S*,7*R*)-zingiberenol substrate were added at a final concentration of 125 µM by evaporating the hexane solvent from substrates under a gentle stream of nitrogen gas, resuspending the substrate in 15 µL 2 mM Triton-x and diluting the mixture 3:1 in with PBS assay buffer (Tittiger, personal communication). Solubilized substrates were allowed to absorb into microsomal fractions by incubation at room temperature for 15 min with gentle shaking. 1 mM NADPH was added to start the reaction prepared in 500 µL volume in a SPME vial. Preparations were incubated with at 30°C overnight with an equal volume of hexane as an overlay. Hexane extraction was performed by vortexing and centrifugation as previously described and GC-MS was used for compound analysis.

Comparative Phylogenetic Analysis of *M. histrionica* and *N. viridula* Cuticle P450s

M. histrionica P450s were used to identify P450s within the *N. viridula* cuticle transcriptome and a list of non-redundant P450 transcripts coding for functional P450 genes was comprised by the method described previously. Putative nomenclature was assigned to *N. viridula* P450s (Fig. S.4) by BlastP search and analysis of amino acid homology to *M. histrionica* P450s (Fig. S.3). All *N. viridula* P450 gene families were identified by alignment to *M. histrionica* P450s. Four P450s belonged to subfamilies not identified in *M. histrionica*, which were assigned new subfamilies (CYP3224C2, CYP3224A2, CYP3224D1, and CYP3224E1). Potentially orthologous genes (80% amino acid homology or greater) were assigned the same gene number as its *M. histrionica* P450 counterpart. Subfamily genes likely representing distinct P450s with 65% amino acid homology or less were assigned a new gene number.

Phylogenies representing cuticle P450 genes in *N. viridula* and *M. histrionica* were assembled by alignment of protein sequences in MUSCLE. A Neighbor joining tree was generated in Genious and reformatted (Newick) in PHyML for analysis in the Interactive tree of life (https://itol.embl.de/). P450 epoxidases from juvenile hormone biosynthesis were included in the analysis.

***N. viridula* Gene Expression Analysis by Semi-quantitative RT-PCR**

Open reading frames encoding full-length P450s were amplified by PCR from cDNA extracted and prepared from RNA of male and female *N. viridula* cuticle tissue. RNA was extracted from the mature male and female cuticle of five 7-10 day old bugs pooled, using Trizol reagent according to the manufacturer's instructions (Qiagen).

cDNA was reverse transcribed from RNA using Superscript reverse transcriptase. P450 genes were amplified from cDNA with primers (melting temperature of 60°C) for amplification of the full open reading frame. PCR with P450 primers and 50 ng cDNA was performed with Taq polymerase (NEB) using 25 amplification cycles and a primer annealing temperature of 55°C. Amplified DNA was analyzed with DNA electrophoresis using ethidium bromide for staining and 1% agarose gel. Expression was compared between male and female cuticle tissue using actin as a housekeeping gene and *NvTPS1* as a positive control.

Results

*Mh*TPS Activity was Specific to *M. histrionica* Cuticle Tissue

*Mh*TPS activity was restricted to mature male *M. histrionica* cuticle tissue (Fig. 2.1). Cuticle tissue from mature male *M. histrionica* biosynthesized sesquipiperitol from (*E,E*)-FPP. Enzymatic conversion of sesquiperitol to zingiberenol or murgantiol was not detected in crude enzyme assays with different *M. histrionica* tissues (Fig. 2.2) or with direct administration of (1*S*,6*S*,7*R*)-sesquipiperitol and (3*S*,6*S*,7*R*)-zingiberenol to tissue extracts of cuticle or whole-bug tissue. Murgantiol was not detected from crude enzyme assays although it was emitted from bugs used for analysis (Fig. 2.2C).

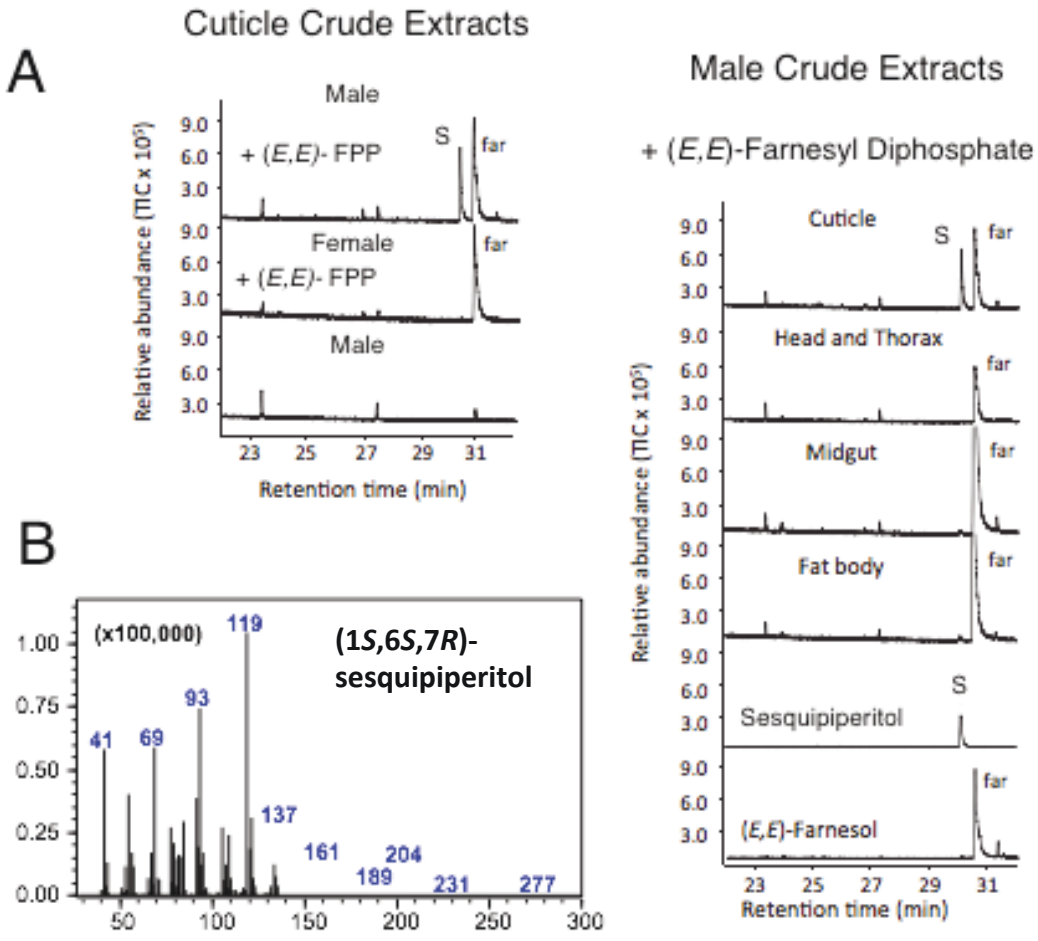

Figure 2.1. ***Mh*TPS activity was restricted to *M. histrionica* mature male cuticle tissue.** Enzyme assays were performed with 50 µM (*E,E*)-FPP and 50 µg protein extract in an overnight incubation at 37°C in a water bath. Volatiles compounds were extracted with hexane and analyzed with GC-MS. **A.** Enzymatic assays with different *M. histrionica* tissues **B.** (1*S*,6*S*,7*R*)-sesquipiperitol mass spectrum.

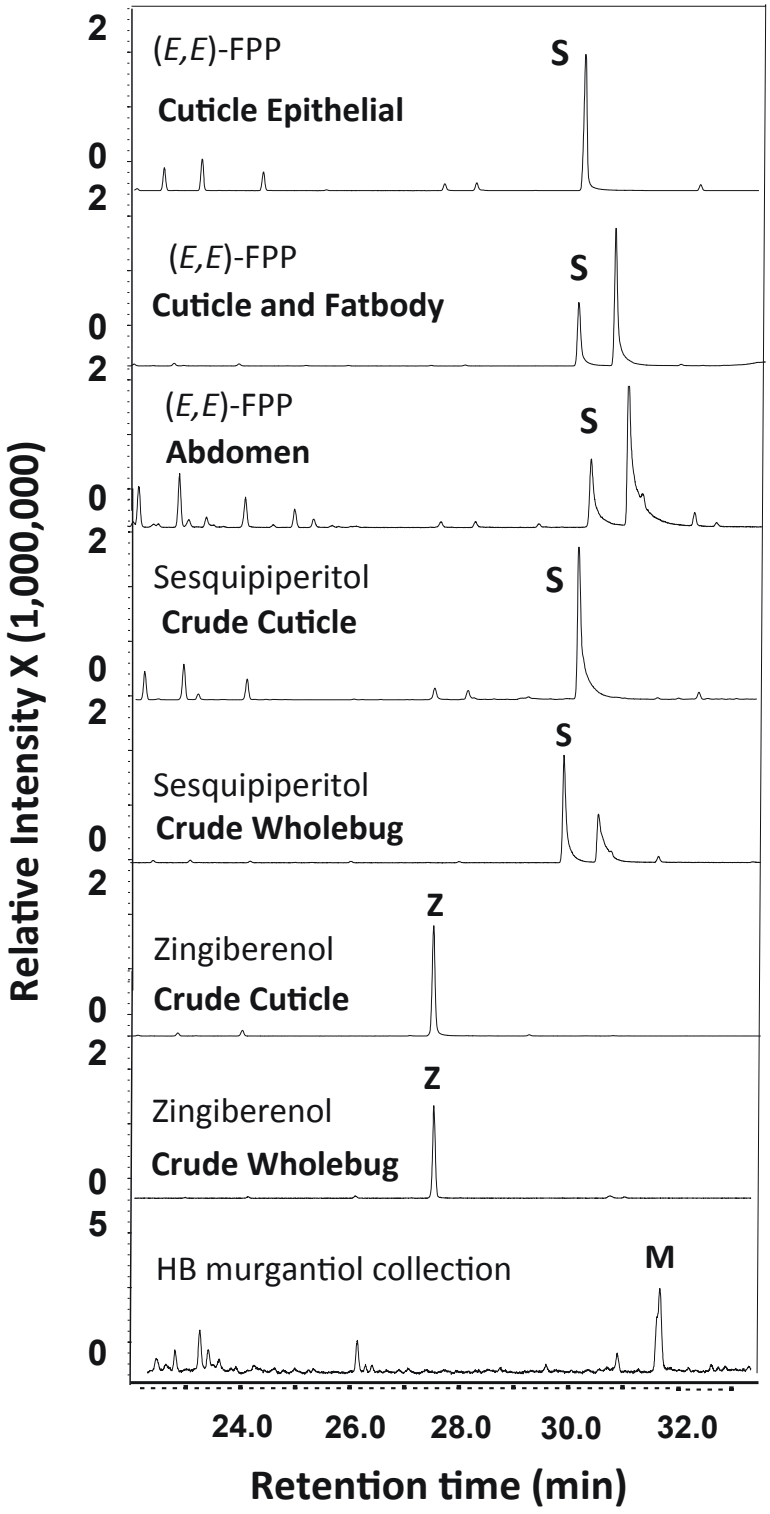

Figure 2.2 P450 epoxidase activity was not detected in *M. histrionica* tissues. Enzymatic assays were performed with (*E,E*)-farnesyl diphosphate (FPP) with intact *M. histrionica* tissues including, cuticle epithelial tissue, cuticle and fatbody, and whole abdomen. Sesquipiperitol and zingiberenol were incubated with crude cuticle or

wholebug extracts. Murgantiol was not detected in enzymatic assays but was collected from *M. histrionica* by insect headspace analysis.

NvTPS Activity was Detected in *N. viridula* Cuticle Tissue

NvTPS activity was detected in cuticle tissue of male bugs (Fig. 2.3). Cuticle protein extracts suppled with (*E,E*)-FPP biosynthesized (*Z*)-α-bisabolene. The product was not detected in cuticle extracts without added substrate and *Nv*TPS activity was much higher in male cuticle tissue than female cuticle tissue.

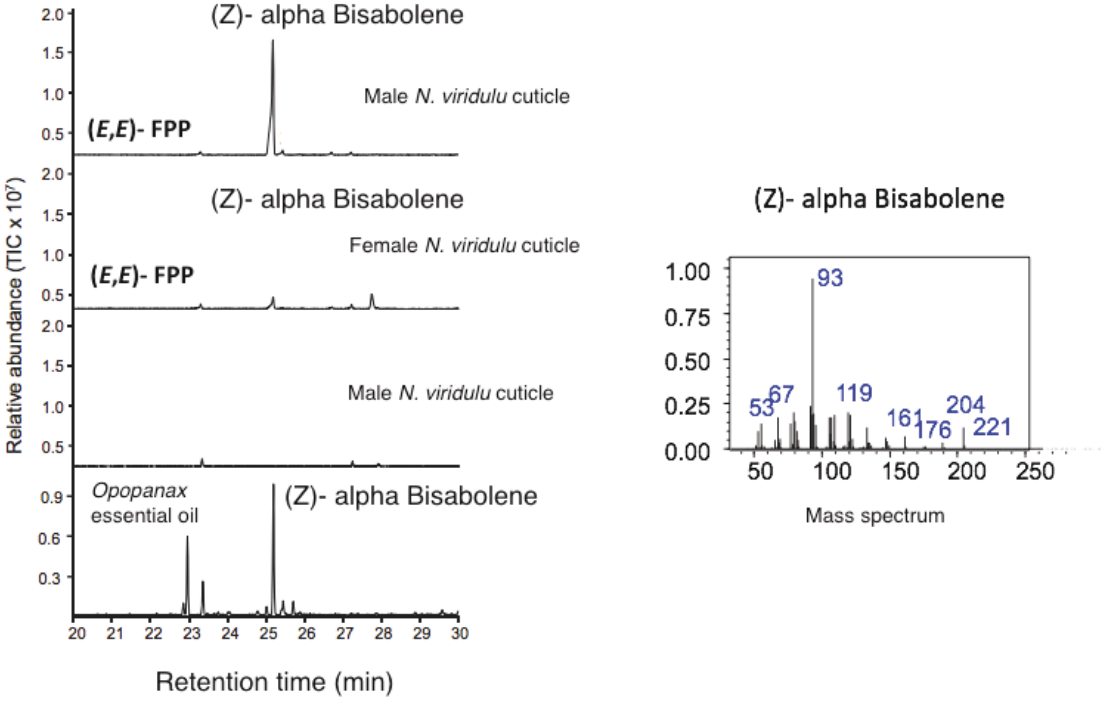

Figure 2.3 *NvTPS* activity was detected in male *N. viridula* tissue. Cuticle protein extracts (50 µg) were supplied with 50 µM (*E,E*)-FPP in 50 mM Hepes assay buffer and incubated for 6h at 37°C. Volatiles were extracted with hexane and analyzed with GC-MS. *Opopanax* essential oil was used as an authentic standard for (*Z*)-α-bisabolene. The mass spectrum of (*Z*)-α-bisabolene standard is depicted.

When (Z)-α-bisabolene was administered to cuticle extracts and SPME was used as a more sensitive form of volatile detection, a compound with a mass spectrum matching that of α-bisabolene epoxide (according to library suggestion) was detected in protein extracts. However, the compound also appeared in extracted without (*Z*)-α-bisabolene and thus appeared to be resident pheromone compound, resulting from storage of pheromones in cuticle tissue (Fig. 2.4). Addition of alternative cofactors (FAD, FMN) to support epoxidase activity did not increase the amount of pheromone epoxides. When administering (*Z*)-α-bisabolene to microsomal fractions of *N. viridula* cuticle tissue, conversion to the bisabolene epoxide was not detected (Fig. 2.4). The soluble fraction isolated from cuticle tissue did not support epoxidase activity *in vitro*.

Identification of Candidate Epoxidases from *M. histrionica* P450s

Comparison of P450s in male and female sexes resulting from previous transcriptome analysis (Sparks et al., 2017) indicated ten *M. histrionica* P450s were male specific including CYP3226B3, CYP307B1_orth, CYP6LT9, and CYP3090C1_orth (Table 2.1). CYP3226B3 had very low expression in male and female cuticle tissue (1 TPM male, 1 TPM female; not shown) and CYP6LT9 sex expression differences were not cuticle specific (University of Nevada qRT-PCR). CYP307B1_orth and CYP3090C1_orth were sex- specific in cuticle tissue (Fig. S.1). CYP4GZ2_orth and CYP3226B3 transcript abundance was higher in mature males compared to immature males (Table 2.1). CYP4GZ2_orth was also sequenced in cuticle tissue and was more highly expressed in male cuticle (Fig. S.1). CYP3226B3 transcripts were lowly expressed in male and female cuticle tissue and not a P450 potential epoxidase.

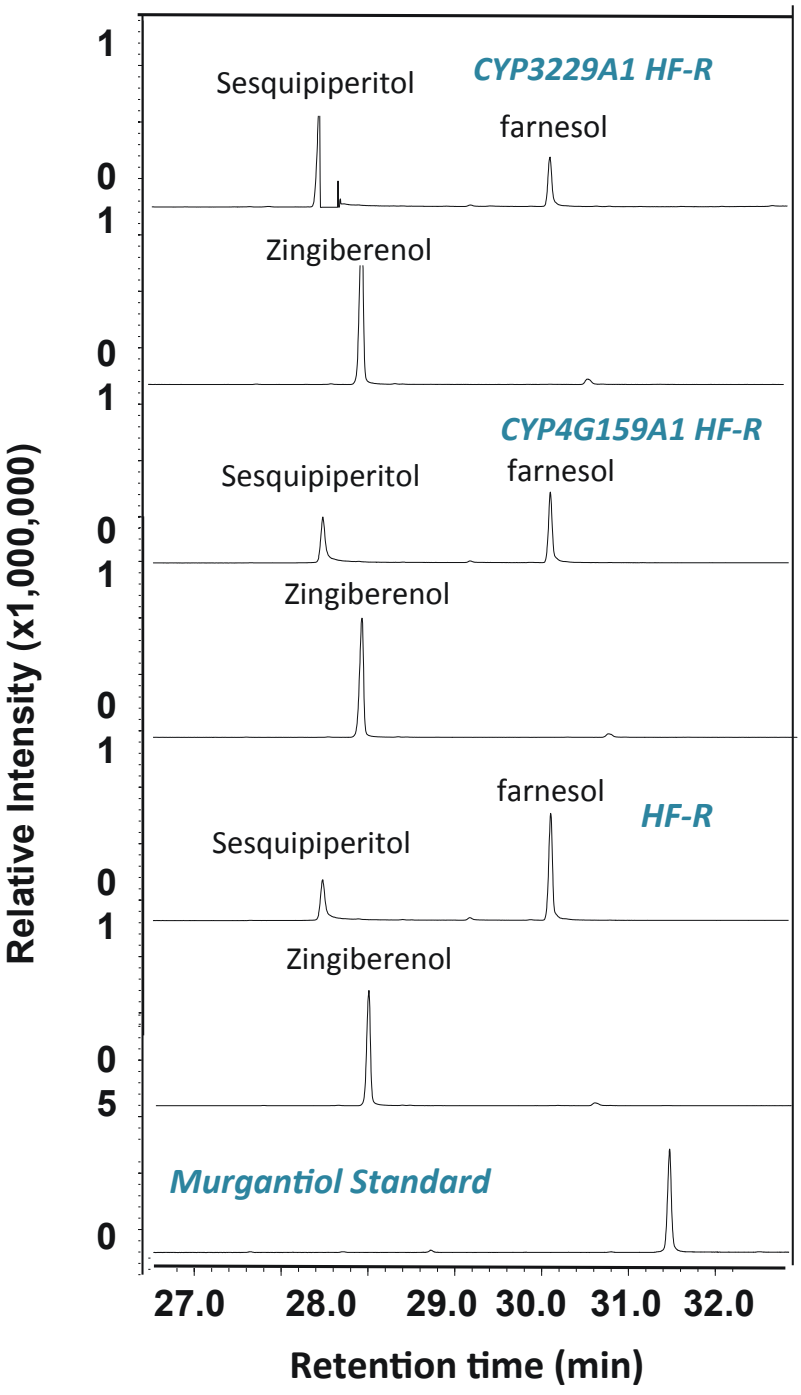

Figure 2.4 ***N. viridula*** **cuticle extracts or microsomes did not support P450 epoxidase activity** ***in vitro*****.** (*Z*)-α-bisabolene substrate was administered to membranous and soluble fractions of cuticle tissue. Crude cuticle protein extracts were incubated with flavin nucleotide cofactors and (*Z*)-α-bisabolene. Detection was performed by SPME-GC-MS in crude assays and exposure to a field SPME fiber to the headspace of a 2 mL glass vial for

microsomal assays. *Trans/cis*-(*Z*)-α-bisabolene epoxide (Trans-BE, Cis-BE) was included as an authentic standard.

CYP3229A1 and CYP4G159A1 had higher transcript abundance in male cuticle compared to female cuticle tissue suggesting potential epoxidase functions (Table 2.1). Gene expression analysis of CYP3229A1 and CYP4G159A1 indicated highest P450 levels in mature male cuticle tissue compared to mature female cuticle tissue and male tissues including midgut, fatbody, and head and thorax (Fig. 2.6). These genes were selected as candidates for subsequent P450 expression and functional analysis. Sampling additional P450 genes expressed in cuticle with qRT-PCR did not indicate further candidate genes or genes more highly expressed in male cuticle tissue compared to female cuticle tissue.

Table 2.1 Comparative transcript abundance of selected P450s with regard to A) *M. histrionica* sex B) development and C) tissue specificity and sex. Transcript abundance of housekeeping genes 18S RNA and RBP4 is presented for custom transcriptomes that were assembled at Virginia Tech.

P450	Male TPM	Female TPM	Fold Change
CYP3226B3	4.71	0.01	undefined
CYP307B1_orth	9.95	0.01	995
CYP3227B3	4.30	0.07	61
CYP3225B3_orth	3.26	0.15	22
CYP3090C1_orth	5.48	0.28	20
CYP3225B1_orth	7.16	0.49	15
CYP302A1_orth	5.85	0.61	10
CYP6LT9	38.09	6.24	6
CYP6LV18_orth	9.37	1.69	6
CYP3224B7	4.65	0.85	5
CYP4KC1	4.33	1.04	4

P450	Mature Male TPM	Immature Male TPM	Fold Change
CYP4GZ2_orth	50.3	5	10.1
CYP3226B3	75.8	19	4.0
CYP4G161_orth	5	1.3	3.8
MhTPS1	212.7	46	4.6
18S	8.6	15.8	0.5
RPBS4	325	497	0.7

P450	Male Cuticle TPM	Female Cuticle TPM	Fold Change
CYP3229A1_orth	8.0	0.	27
CYP4G159_orth	71	8	8
CYP4HC1_orth	23	7	3
CYP4GY1_orth	19	6	3
MhTPS1	316	7	47
18S	10	12	0.9
RBPS4	325	497	0.7

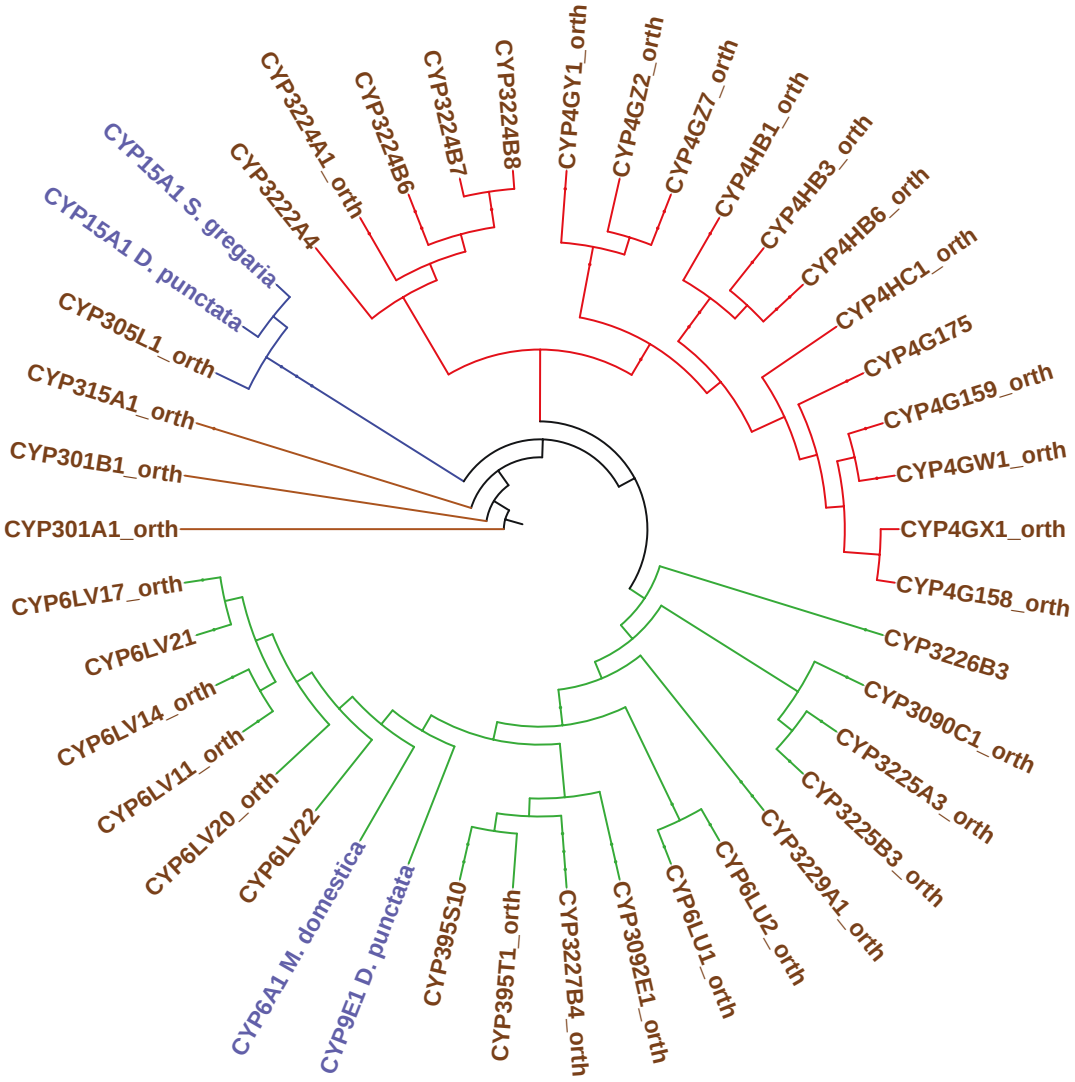

Fig. 2.5. Phylogenetic analysis of P450s identified in transcriptomes of *M. histrionica* cuticle tissue. P450s are associated with clade by color: Green (CYP6), Red (CYP4), Blue (CYP2), and Brown (mito-Clade). Alignment was performed in PHYML using a neighbor joining tree. Characterized P450 epoxidases were included for reference.

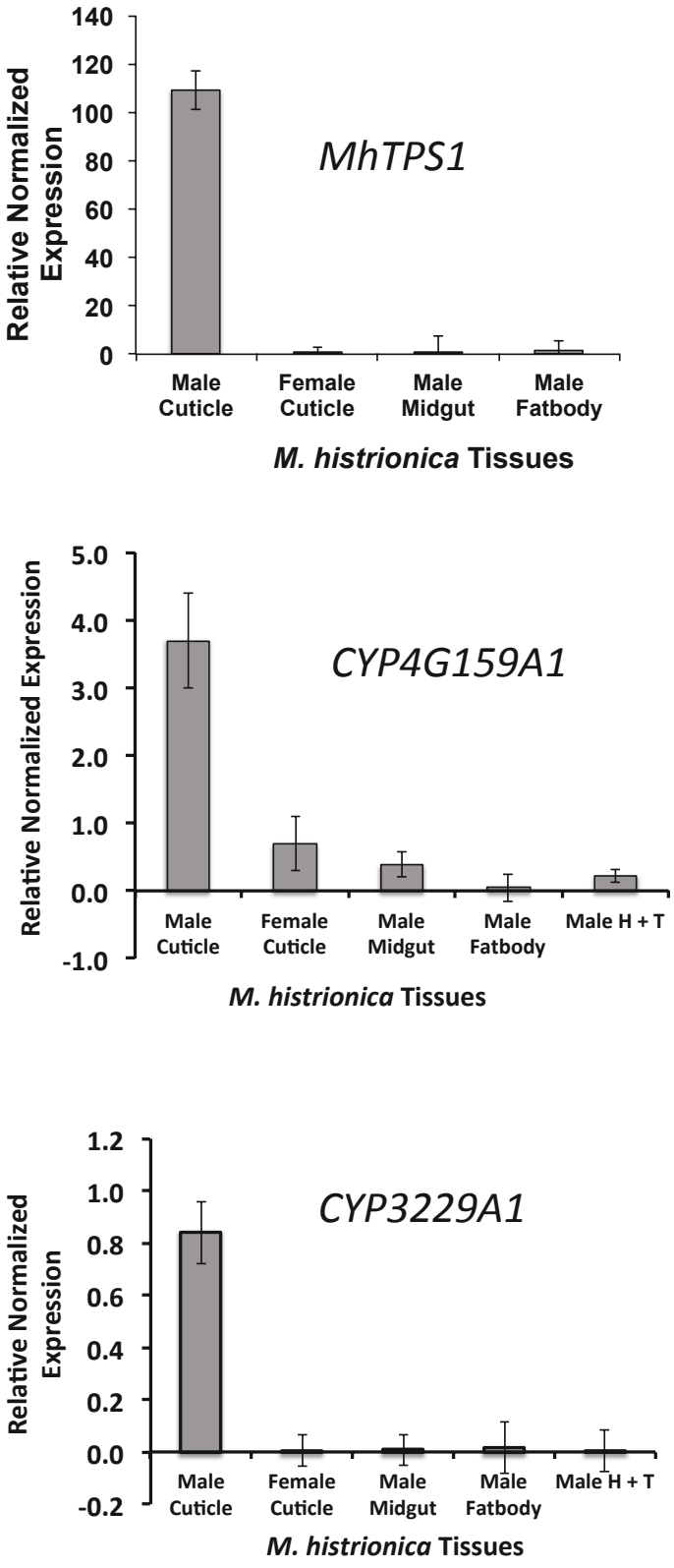

Figure 2.6 Relative expression of P450 epoxidase gene candidates CYP3229A1 and CYP4G159A1 relative to *MhTPS1* in *M. histrionica* tissues. Gene expression was

analyzed in mature male cuticle, female cuticle, male head and thorax, male midgut, and male fatbody tissues. Gene expression was relative and normalized to 18S RNA. Statistical analysis was performed by the ΔΔCT method with three biological replicates.

CYP3229A1 and *CYP4G159A1* from *M. histrionica* Were Not Epoxidases in Murgantiol Biosynthesis

P450s CYP3229A1 and CYP4G159A1 were expressed in Sf9 insect cells as fused proteins to housefly P450 reductases to allow for activity in vitro (Supplemental Fig. 2). Preparations from microsomal fractions were harvested and analyzed for epoxidase activity *in vitro* by administering sesquipiperitol and zingiberenol (Fig. 2.7). Neither of the recombinant proteins converted the substrates into possible epoxide products by performing hexane extraction and analyzing assays GC-MS or by removing hexane from assays and using SPME GC-MS. Hexane extractions are depicted (Fig. 2.7).

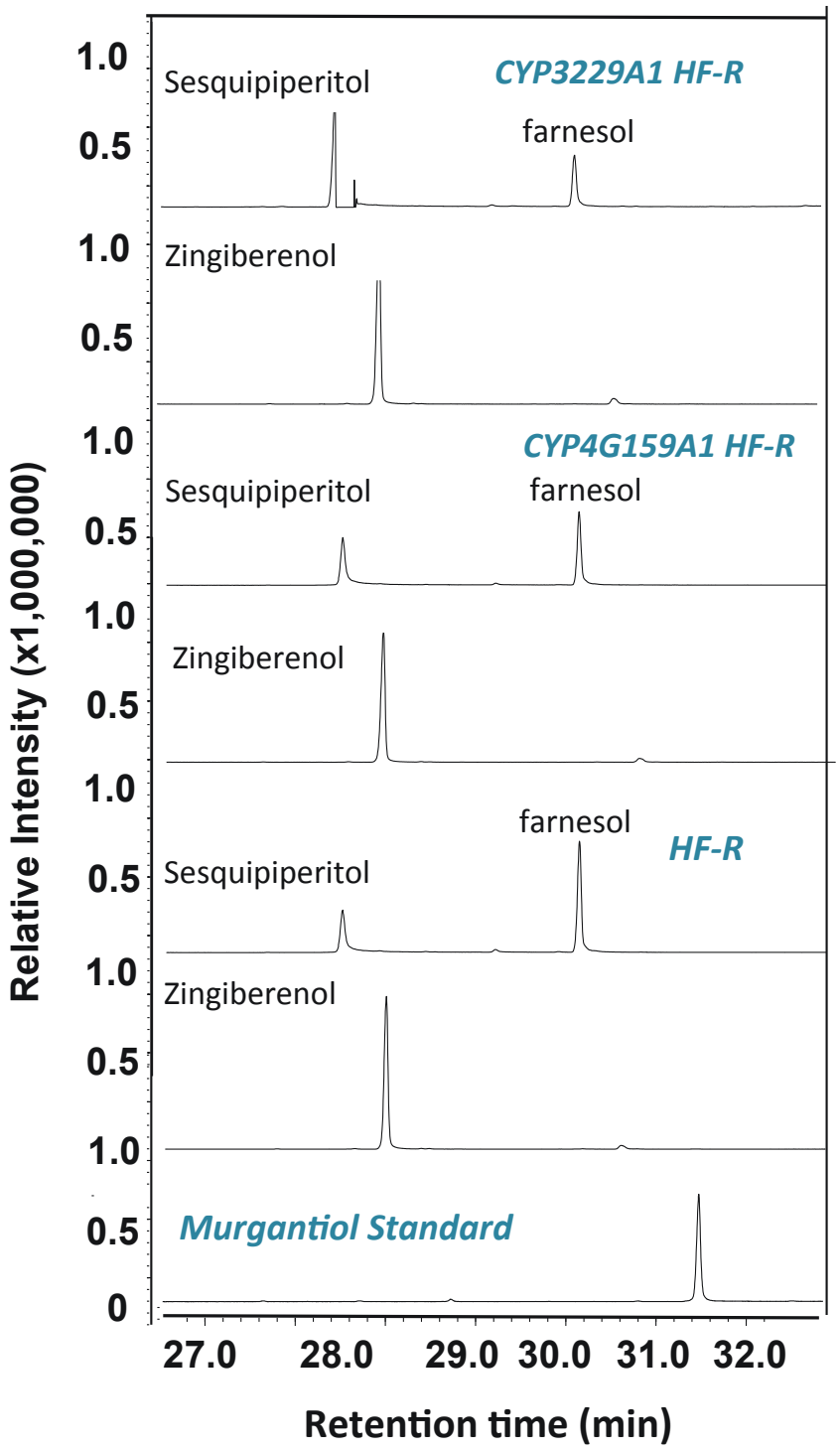

Figure 2.7. Recombinant *M. histrionica* P450s tested for epoxidase activity in vitro by administration of sesquipiperitol and zingiberenol compared to housefly reductase control. Enzymatic assays were performed in SPME vials with solubilized substrate and microsomal fractions. Murgantiol standard was included for comparison and analysis was performed by GC-MS.

Comparative Phylogenetic Analysis of P450s retrieved from *M. histrionica* and *N. viridula* Cuticle Transcriptomes

A total of 37 P450 genes were identified in the cuticle tissue of *M. histrionica* and 42 P450s were identified in the same tissue of *N. viridula*. The overall representation of P450s within the CYP3, CYP4, CYP2, and mito clade were analogous. Phylogenetic comparisons between both species were supported by a combined tree of *M. histrionica* and *N. viridula* P450 sequences (Figure S.3).

CYP3, the largest clade of P450s in both species, is comprised of CYP3 and CYP6 family genes. Approximately half of the CYP3 clade subfamilies including the CYP6LV subfamily was conserved between *M. histrionica* and *N. viridula* (Fig. 2.6 and 2.8). CYP3090C1 and CYP395S10 genes along with CYP3225A and B subfamilies were also conserved in the cuticle tissue of both stinkbugs. A high degree of 1:1 P450 relationships are present in the CYP4G clade including CYP4G175, CYP4G158, and CYP4G159. Genes within the CYP4HB subfamily are present in both species. CYP3224 family genes diverge between *M. histrionica* and *N. viridula* cuticle.

CY2 clade genes CYP301A1 and CYP315A1 were conserved in *M. histrionica* and *N. viridula* cuticle Additional mitochondrial genes were sequenced in *N. viridula* CYP18A1, CYP306A1, CYP307B1. Conserved or associated functions of P450s are listed for reference in Table 2.1.

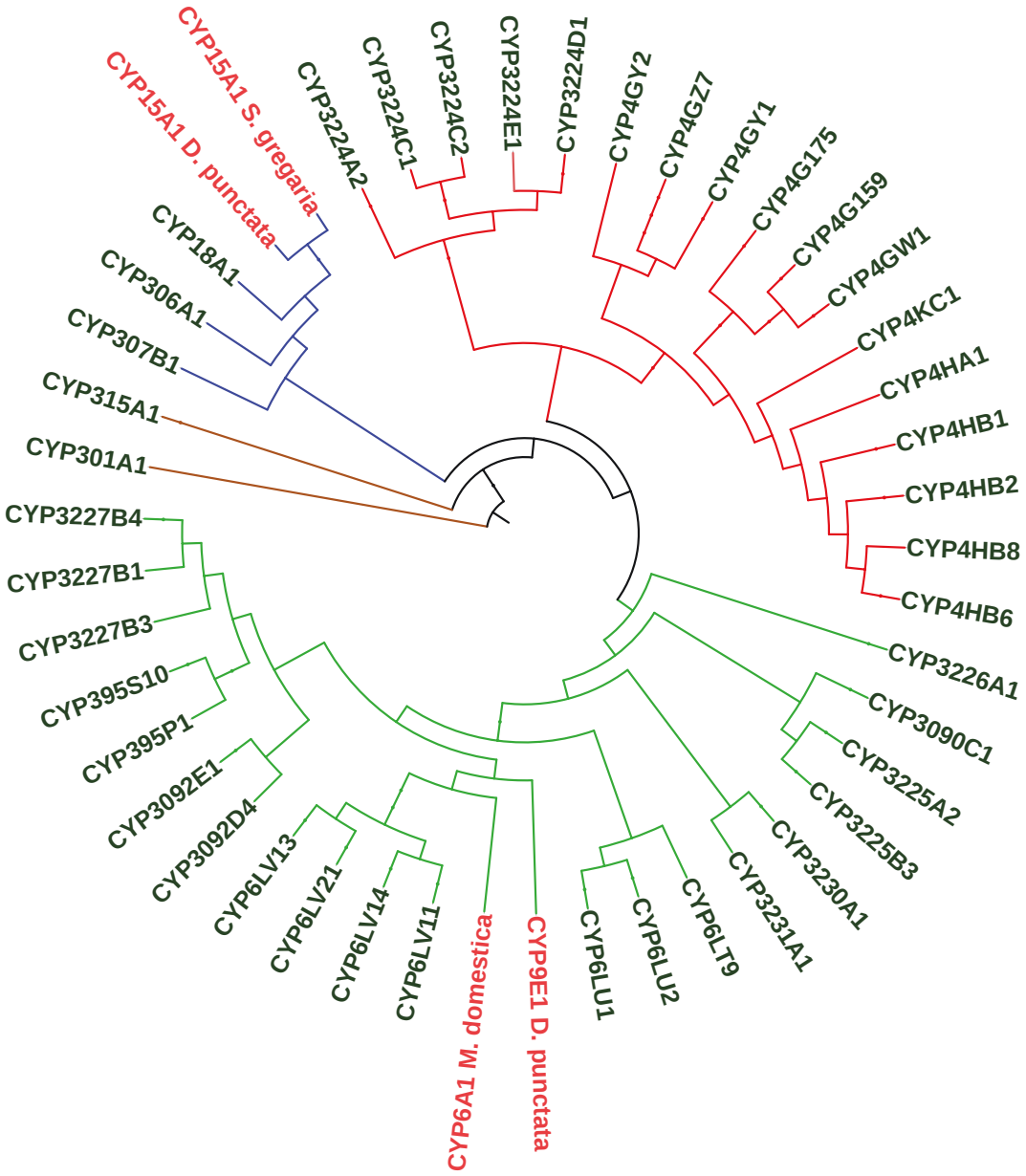

Fig. 2.8. Phylogenetic analysis of P450s identified in the transcriptomes of cuticle tissue from *N. viridula*. P450s are associated with clade by color: Green (CYP6), Red (CYP4), Blue (CYP2), and Brown (mito-Clade). Alignment was performed in PHYML using a neighbor joining tree. Characterized P450 epoxidases were included for reference.

Table 2.2 Conserved and Attributed Roles of Insect P450s (Feyereisen, 1999; Scott and Wen, 2001; Sztal et al., 2012).

P450 Clade	P450 Gene	Associated or conserved functions
CYP3 Clade	-	Lineage- speciifc functions
	-	Xenobiotic detoxification
CYP4 Clade	CYP4G15	Formation of cuticle
	CYP4G1	Ecydteroid metabolism
	CYP15A1	Juvenile hormone epoxidase
CYP2 Clade	CYP18A1	Ecdysteroid metabolism
	CYP306A1	Ecdysteroid metabolism
	CYP307B1	Ecdysteroid metabolism
	CYP301A1	Formation of cuticle
Mito Clade	CYP315A1	Ecdysteroid metabolism

Expression Profiling for Identification of P450 Epoxidases in *N. viridula*

Since the aggregation pheromone of *N. viridula* is released by males from glandular cells, we expected a P450 epoxidase to be more highly expressed in male cuticle tissue compared to female cuticle tissue. We performed semi-quantitative RT-PCR of 23 P450 transcripts based on cuticle specific transcript abundance estimates provided by collaborators at the USDA, Beltsville as part of a whole-bug transcriptome survey (Sparks, unpublished). CYP4G159 and CYP4HB6 were more highly expressed in male cuticle compared to female cuticle tissue (Fig. 2.9). Genes that were more highly expressed in female cuticle compared to male cuticle were CYP3224D1 and CYP3230A1. CYP3071B, CYP4KC1, and CYP3224C1 were specifically expressed in

the female cuticle tissue. Differential gene expression analysis (Sparks, unpublished) suggests CYP395S10, CYP3226A1, and CYP3224A1, which were not investigated, are more highly expressed in male cuticle compared to female cuticle.

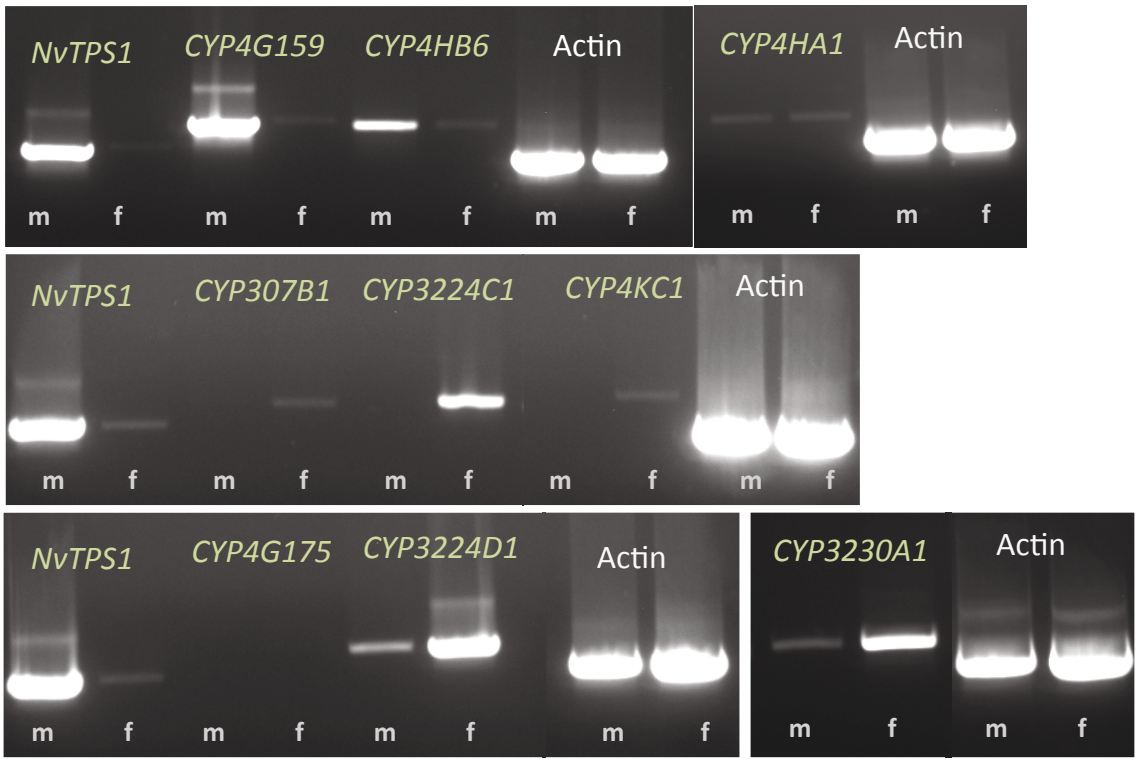

Figure 2.9 Transcript abundance of P450s in the cuticle tissue of *N. viridula*. Comparisons were made between the male cuticle tissue (m) and female cuticle tissue (f). Actin was used as a housekeeping gene for relative expression comparison. *NvTPS1* gene expression was included for comparison between sexes.

Discussion

Identification of P450 Epoxidases in Stinkbug Pheromone Biosynthesis

The *M. histrionica* aggregation pheromone murgantiol is synthesized in a multi-step biosynthetic pathway in which *MhTPS1* converts (*E,E*)-FPP to (1*S*,6*S*,7*R*)-sesquipiperitol in the cuticle of mature males. Further conversion to SSRS:SSRR murgantiol involves isomerization including a potential zingiberenol intermediate and

epoxidation. We found evidence that *Mh*TPS1 activity was specific to the cuticle tissue. However, we were unable to detect a conversion of sesquipiperitol or zingiberenol to murgantiol as evidence of an epoxidase activity. Given the lack of enzymatic evidence for epoxidation it is possible during the final steps in murgantiol biosynthesis pheromone compounds are stored or transported in other *M. histrionica* tissues.

By RNA-sequencing we were not able to identify a P450 that co-expressed at the same levels as *MhTPS1* in the cuticle tissue. CYP3229A1 and CYP4G159A1 were specifically expressed in mature male cuticle tissue but their recombinant proteins were not found to have epoxidase activity with the murgantiol precursors.

Given the similarity in P450s expressed between the *M. histrionica* and *N. viridula* cuticle it may be possible to use *N. viridula* as a model for understanding epoxidation not only in the biosynthesis of bisabolene epoxide but also murgantiol. *N. viridula* has specialized pheromone glands within the abdominal cuticle tissue of males, which restricts epoxidase activity to cuticle epithelial tissue under the assumption that epoxidation occurs in the pheromone glands. Support for a gland or cuticle tissue-specific formation of the pheromone comes from evidence that transcripts of *NvTPS1* are observed in the cuticle.

To investigate activity of a P450 epoxidase within the *N. viridula* cuticle tissue we enriched assays for P450 activity with microsomal fractions and cofactors for P450 epoxidases. Like *M. histrionica*, pheromone could be detected from bugs but epoxidase activity was not detected by tissue extracts. Potentially assay conditions were not optimal for epoxidase activity or epoxidase activity cannot be reconstituted *in vitro* in enzyme assays.

We further applied transcriptome analysis of cuticle tissue for the identification of possible P450 epoxidases. Comparative phylogenetic analysis and sequencing of the cuticle tissue of *M. histrionica* and *N. viridula* showed that 37 P450 and 42 P450s are present in *M. histrionica* and *N. viridula* cuticle respectively. The largest cuticle P450 gene contribution comes from the insect specific CYP3 clade. Perhaps P450s within this clade can be attributed to detoxification of pesticides (Bansal and Michel, 2018). Shared P450 genes such as the CYP6LV subfamily in *M. histrionica* and *N. viridula* are lineage-specific (Sparks et al., 2017) and epithelial tissue lining cuticle provides a first line of defense against insect pesticides (Scott and Wen, 2001). The epoxidase candidate CYP3229A1 within the CYP3 clade had no potential orthologue in *N. viridula* cuticle; the most closely related P450 was CYP3230A1, but this CYP gene is more highly expressed in *N. viridula* female than male cuticle tissue.

CYP4 Clade P450s have conserved roles in cuticle formation or associative processes with hormones (Feyereisen, 1999). CYP4G159A1, which does not have epoxidase activity is male specific in both species. CYP4G159A1 has been characterized for a role in cuticle formation in *M. histrionica* (Claus Tittiger, personal communication). Potentially an epoxidase could be positioned in the CYP4H subfamilies suggesting CYP4HB6 as a more highly expressed P450 in *N. viridula* male cuticle than female cuticle. Gene expression of CYP4HA1 does not suggest a biological function specific to the male cuticle tissue. CYP3224 has different gene sequences in *M. histrionica* and *N. viridula*. Female cuticle gene expression in *N. viridula* suggests CYP3224D1 is not directly involved in pheromone biosynthesis.

One might expect a pair of orthologs between stink bug species within the P450 family to exhibit epoxidase activity. P450 epoxidases produce two stereoisomers of epoxides and perhaps a conserved set of paralogous genes or genes in the same subfamily perform epoxidation in these bugs. P450s with conserved roles in epoxidation such as CYP15A1 in juvenile hormone biosynthesis were not found within *M. histrionica* and *N. viridula* cuticle tissues, suggesting another P450 performs epoxidation in these tissues. Genes that may be excluded in further characterizations are those that we found to be female-specific or that are highly conserved in metabolism of the hormone ecdysteroid biosynthesis. Future approaches for identifying a P450 epoxidase gene involved in *N. viridula* and *M. histrionica* pheromone biosynthesis will likely have to rely upon large scale-functional characterization of male specific cuticle P450s in a heterologous system such as insect cells, yeast, or even tobacco.

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Chapter 3. Engineering Stinkbug Terpene Synthases in *Nicotiana benthamiana* and *Arabidopsis thaliana*

Abstract

Engineering pheromone biosynthetic pathways in trap plants is considered a potential alternative strategy for controlling insect pests. In this study, the expression of terpene synthases involved in stink bug sesquiterpene pheromone biosynthesis was examined by transient expression in *N. benthamiana* and stable transformation in *A. thaliana.*

Stinkbug pheromones such as murgantiol from *M. histrionica* and (Z)-alpha bisabolene epoxide from *Nezara viridula* are biosynthesized from (*E,E*)-farnesyl diphosphate in multiple biosynthetic steps. We found that transient expression of *MhTPS1* in *Nicotiana benthamiana* under control of the CaMV 35S promoter resulted in the production and emission of sesquipiperitol, the murgantiol pheromone precursor. Additionally, (*Z*)-alpha bisabolene, the precursor of the *N. viridula* pheromone, was emitted from *N. benthamiana* leaves by transient expression of *NvTPS1*. To develop a strategy for engineering these genes in trap crops we further transformed them under control of the CaMV35S promotor in *Arabidopsis thaliana*. Transgenic lines did not emit pheromone precursors suggesting an alternative engineering strategy is necessary to produce these compounds from stably transformed plants. Plastidial expression with isoprenyl diphosphate precursors formed from the methylerythritol phosphate pathway offer a potential alternative for the engineering of insect pheromones in plants. Engineering of other pheromones such as that from flea beetles is discussed.

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Introduction

Many stinkbug bugs emit pheromones that belong to the large class of terpene or isoprenoid specialized metabolites. These terpene pheromones can be identical or similar to many terpene volatiles released from plants for the attraction of insect pests, repellence of herbivores or microbial pathogens (Tholl, 2006). Terpene chemicals biosynthesized by stinkbugs share biosynthetic routes with plant terpenes and it has been suggested that strategies using insect derived genes could be extended towards crop improvement by various disciplines (Aharoni et al., 2005; Shelton and Badenes-Perez, 2006). Previously there has been substantial progress in the introduction of terpene volatiles in crops for the improvement of agronomic traits including flavor optimizations and defense against agricultural insect pests (Beale et al., 2006; Schnee et al., 2006).

Murgantia histrionica (harlequin bug) is a substantial pest on crucifer or cabbage crops in the US southeast (Wallingford et al., 2011). Reduction of broad spectrum insecticides to protect beneficial insects has increased the pest pressure by this stink bug leading to the exploration of new strategies for pest control including trap cropping and a combination of pheromone baited traps and trap crops (Wallingford et al., 2013). Another stinkbug, the southern green stinkbug, *Nezara viridula*, is a generalist pest worldwide on several crops including soybean. Both stink bug species emit bisabolane type sesquiterpenes as aggregation pheromones: *M. histrionica* males emit (3*S*,6*S*,7*R*,10*S*)- and

(3*S*,6*S*,7*R*,10*S*)-murgantiol and *N. viridula* males release *trans-/cis*-(*Z*)-α-bisabolene epoxide (Millar, 2005; Zahn et al., 2008).

Previously, the Tholl lab identified enzymes called terpene synthases (TPSs) that produce the precursors of these pheromones. *Mh*TPS converts (*E,E*)-farnesyl diphosphate (FPP) to sesquipiperitol, the precursor to murgantiol and a related enzyme, *Nv*TPS uses the same substrate to make (*Z*)-α-bisabolene as a precursor of its epoxide pheromone (Lancaster et al., 2018a; Lancaster et al., 2018b).We aim to employ these enzymes to engineer trap crops that emit pheromones in a pest management strategy.

Engineering a terpene pheromone biosynthetic pathway in plants takes advantage of the fact that the TPS substrate (*E,E*)-FPP is endogenously produced in plants (Tholl, 2006). In a proof-of-concept study, we have performed transient and stable transformation of the two stink bug derived *TPS* genes in *Nicotiana benthamiana* and *Arabidopsis thaliana* using standard transformation protocols (Reed and Osbourn, 2018) (Clough and Bent, 1998). Transformed plants were examined on *TPS* gene expression and emission of the TPS enzymatic product. The use of *A. thaliana* as a model organism for transformation of *Brassica* trap crops with pheromone biosynthetic genes from *M. histrionica* and other crucifer pests such as *Phyllotreta striolata* (Beran et al., 2016; Gruber et al., 2009) is discussed.

Methods

Codon optimization of *MhTPS1* and *NvTPS1* genes from *M. histrionica* and *N. viridula* and development of T-DNA Binary Vectors

T-DNA binary vectors pMHT33 and pNVT33 were assembled for transformation of *MhTPS1* and *NvTPS1* into plants respectively. *MhTPS1* is a 1.1 KB gene encoding a 385 amino acid sequence without a signal sequence for subcellular localization. *NvTPS1* is a 1.1 KB gene encoding a 378 amino acid protein without a signal sequence. *MhTPS1* and *NvTPS1* were codon optimized for expression in *Nicotiana benthamiana* and inserted downstream the CaMV 35S promotor for assembly of T-DNA binary vectors (Genscript, New Jersey). *TPS* genes were cloned by EZ cloning (Genscript) into pLIFE33, a 10 KB plasmid derived from the pCAMBIA1300 plant transformation vector. Adjacent to the left T-DNA repeat is a double enhanced CaMV 35S promotor, hygromycin resistance gene, and CaMV 35S polyA signal for transcriptional repression (Fig. 3.1). The hygromycin resistance gene provides a selectable marker for genetic transformation in plants. pLIFE33 has an additional CaMV 35S promotor, multiple cloning site, and CaMV 35S terminator adjacent to the right T-DNA where terpene synthase genes were inserted in mHT33 and nVT33 respectively. A kanamycin resistance gene, pUC origin of replication, and pVS1 origin of replication outside the T-DNA borders allows for plasmid selection and replication in multiple microbial hosts including *E. coli* and *Agrobacterium tumefaciens.*

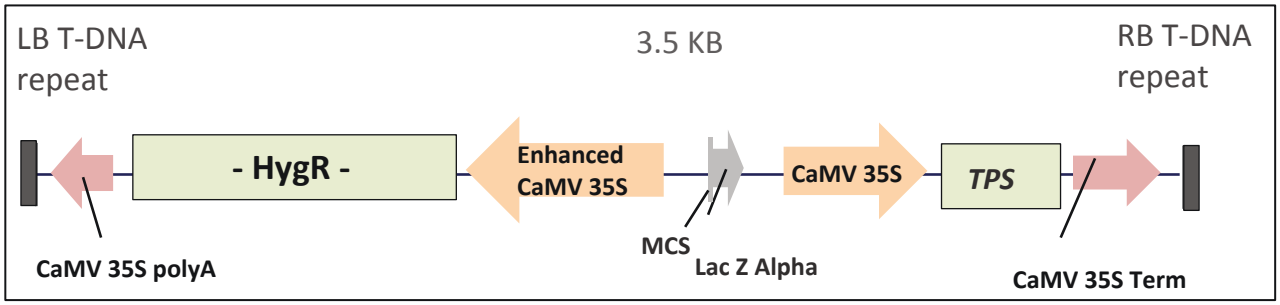


Figure 3.1 Vector map of pLIFE33 plasmid and gene insertion site of *MhTPS1* and *NvTPS1* for the formation of pMHT33 and pNVT33 binary vectors. The pLIFE33 vector contains a 3.5 KB T-DNA region where the TPS genes were inserted. Binary vectors confer resistance to hygromycin and kanamycin (not shown) for plant and microbial selection following transformation.

Co-infiltration of constructs pMHT33 and pNVT33 with P19 in *Nicotiana benthamiana* mediated by T-DNA transformation

MhTPS1 was transiently expressed in *Nicotiana benthamiana* by co-infiltration of GV3101 containing pMHT33 and GV3101 containing P19 in *Nicotiana benthamiana* plant leaves. Four to five-week old plants were infiltrated after expanded leaves had developed. Miracle grow was applied to plants during the first 2-3 weeks of growth to allow leaves to fully expand and plants were watered the night prior to *Agrobacterium* infiltration.

T-DNA binary vectors were transformed into *Agrobacterium tumefaciens GV3101* by freeze thaw transformation. Plasmid DNA (200 ng) was added to 100 µL competent cells and incubated at 4°C for 5 min prior to 5 min flash freeze in liquid nitrogen and heat shock for 5 min at 37°C. SOC medium (0.5 mL) was added and cells were incubated at 30°C for 3 h with 170 rpm shaking. For selection cells with the T-DNA binary vector were plated on LB agar with 50 µg/mL kanamycin, 10 µg/mL rifampcin, and 30 µg/mL gentamycin and grown two days at 28°C. The Ti-plasmid *pMP90* in host strain GV3101 carries a rifampicin resistance gene while the gentamycin resistance gene is located on the bacterial chromosome.

Transient expression was carried out by co-infiltration of TPS-containing GV3101 and a P19 carrying GV3101 strain. The P19 protein allows for suppression of foreign gene silencing (Voinnet et al., 2003). *Agrobacterium* strains were cultured in 10 mL YEP liquid media with antibiotics until saturation at 28°C degrees with 220 rpm shaking. Five mL was transferred to a 500 mL YEP overnight culture with antibiotic selection. Bacterial cells were harvested after centrifugation at 4,000×g and 25°C and the bacterial pellet was resuspended in MES pH 5.6 buffer at an optical density of 0.8 OD. Cells containing the *MhTPS1* and *NvTPS1* genes respectively were mixed with the P19 containing cells and incubated at room temperature for 1 h with gentle shaking. A 2 mL needless syringe was used to infiltrate the cell suspension strains into *N. benthamiana* leaves. Plants were grown and experiments were performed at 70°F under long day photoperiod of 16 h and 150-180 $\mu m/m^2/sec$ light.

Headspace analysis of *N. benthamiana* plants transiently expressing *TPS* genes

Leaf volatiles were collected from *N. benthamiana plants* in regular intervals to observe the effect of *TPS* gene expression on volatile emission. Volatile collections were started three days after infiltration with *Agrobacterium* using a closed loop stripping system with charcoal filters (Huang et al., 2012). Individual plants were placed in glass desiccators. Air was circulated through the desiccator at 9 V with a Fuergut air circulation pump containing the filter. Filters were replaced every 24 h and eluted with 200 µL dichloromethane. One µL of the eluent was analyzed with GC-MS using Split-2 liquid injection. GC-MS analysis was performed as previously described (Chapter 2) (Lancaster et al., 2018b).

For quantification of emitted compounds, a calibration curve with sesquipiperitol and bisabolene standard was generated with concentrations of 6 ng/μL, 12 ng/μL, and 30 ng/μL (duplicate samples per concentration). The calibration curve was determined as follows: Y (ng) = (1/949,746)*Peak Area, R^2 = 0.98. The total amount of sesquipiperitol or (*Z*)-alpha bisabolene collected on the filters was determined based on the total volume of eluent. This number was divided by the fresh weight of the leaves (app. 0.75 mg FW each) and the average emission rate per hour was calculated.

Genetic transformation of *TPS* genes by Agrobacterium-mediated transformation into *A. thaliana*

A. thaliana Columbia ecotype were transformed with the *TPS* genes by vacuum infiltration of pMHT33 and pNVT33 vectors using the floral dip method supported by application of vacuum infiltration (Bechtold and Pelletier, 1998). Prior to transformation, plants were grown under long day conditions with light intensity described above until plants had developed shoots with inflorescences. Approximately eight flowering plants each were vacuum infiltrated with *Agrobacterium* carrying the pMHT33 or pNVT33 binary vector.

Agrobacterium containing pMHT33 or pNVT33 was cultured by inoculating 500 mL cultures of YEP liquid media with 5 mL start cultures containing antibiotics as described above. The cell pellet from 500 mL culture was resuspended in 4.3 g/L MS salts, 5% Sucrose, 1 mg/mL BAP, 2.5 X Gamborg's vitamin solution, and 0.02 % Silwet's solution at a final optical density of 0.8 OD. *Agrobacterium* was inoculated into *Arabidopsis* floral tissue by dipping in a glass desiccator containing the bacterial solution.

A vacuum was pulled to allow the bacterial solution to infiltrate into floral tissue. Next, plants were lightly rinsed with tap water and placed on their side in a tray under low light conditions over night before placing them back upright under the light the next day. Plants were grown until seeds developed by selfing and seeds were harvested by manual collection from siliques in a paper bag.

Selection of *MhTPS1* and *NvTPS1* transgenic seedlings

Transgenic seeds resulting from *Agrobacterium* transformation with the *MhTPS1* and *NvTPS1* constructs were surface sterilized by successive washes with 70% ethanol, 10% bleach and 0.1% Triton-X solution, and sterile H_20, and plated on 10 cm petri dishes with ½ MS agar and 15 µg/ mL hygromycin-B (approximately 200 seeds per plate). Seed batches were stratified by 4°C incubation without light for 2-3 days and transferred to 24°C under long day conditions.

Approximately one hygromycin tolerant seedling was detected per one hundred inoculated seeds when performing transformation with *MhTPS1* (Fig. 3.2A). Four hygromycin tolerant seedlings were transferred to soil for the growing of four *Mh*TPS1 T1 plants (Fig 3.2C). Eight Nv*TPS1* seedlings were identified by tolerance to hygromycin and T1 plants were allowed to self to collect seed. Seeds were replated on ½ MS agar with 15 µg/mL hygromycin. The ratio of seedlings which were tolerant and non-tolerant to hygromycin was analyzed and T2 seedlings segregating 3:1 for hygromycin tolerance were transferred to soil and grown. This was performed to select T2 plants that contained a single genetic insert, for later development of homozygous lines.

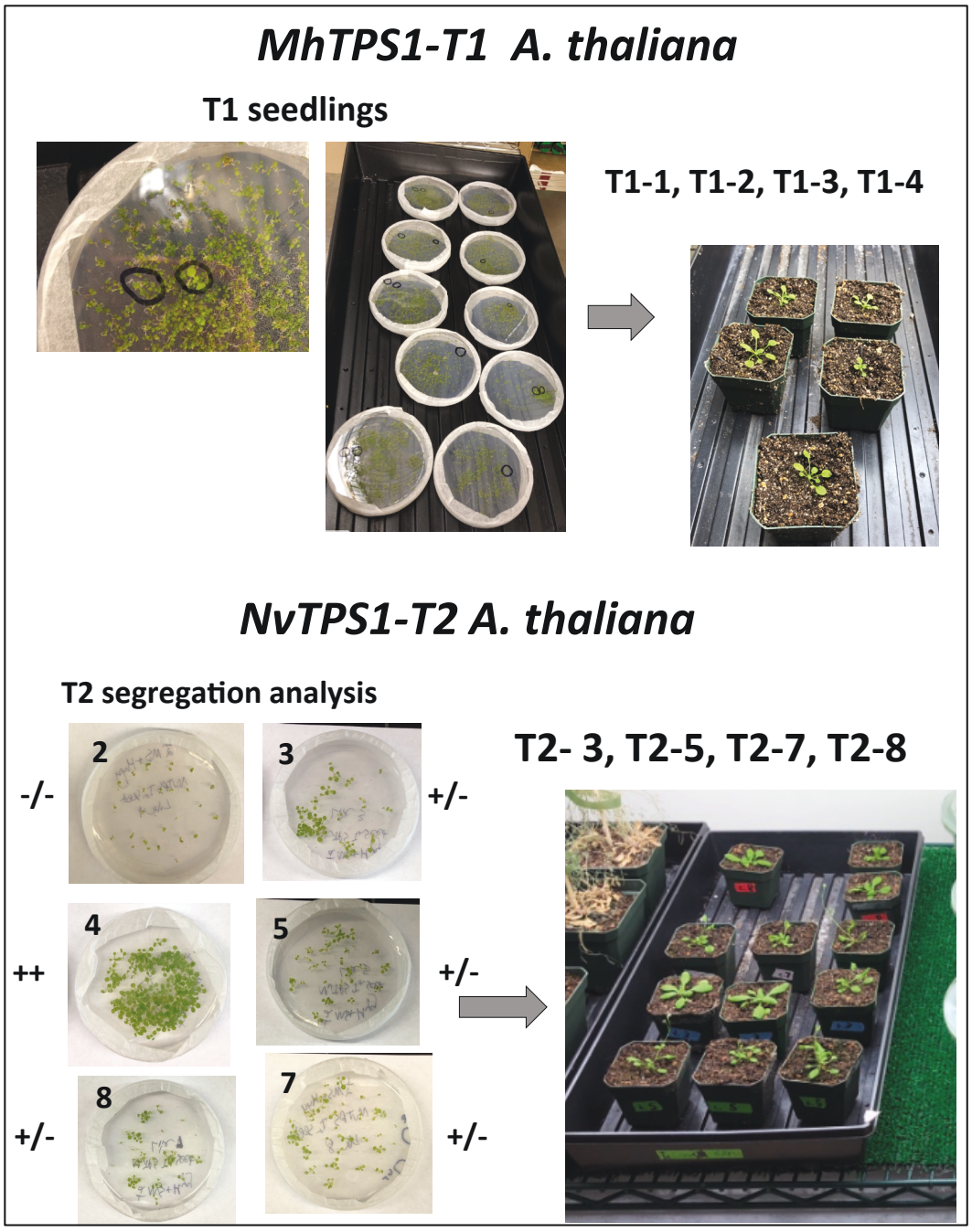


Figure 3.2 Selection of *Arabidopsis thaliana* plants following transformation with *MhTPS1* and *NvTPS1* transgenes. T1 plants resulting from transformation with *NvTPS1* were selfed, seeds were collected, and T2 seedlings were analyzed for *Nv*TPS1 segregation by selection on MS agar with hygromycin-B. (-/-) Wild-type seedlings; (+,+)

seedlings of a hygromycin resistant control; (+/-) *Nv*TPS-T2 seedlings segregating approximately 3:1 for hygromycin-B resistance.

Verification of *MhTPS1* and *NvTPS1* gene insertion and transcription in transgenic *A. thaliana*

Leaves were harvested from transgenic and wild-type *A. thaliana* and pulverized by flash freezing in liquid nitrogen and grinding tissue with a mortar and pestle. Pulverized tissue was resuspended in 0.5 mL 200 mM Tris-HCl pH 7.5 buffer and incubated at room temperature for 2 min. The extract was separated by centrifugation at 13,000 rpm for 3 min with a microcentrifuge and genomic DNA was precipitated from the supernatant with an equal volume of isopropyl alcohol. The crude pellet was resuspended in 100 µL nuclease free water (Edwards et al., 1991) and used as a template for PCR. Gene specific primers for *MhTPS1* and *NvTPS1* codon optimized genes were used to amplify the 1.1 kb genes in 25 µL PCR reaction using Taq polymerase and 25 amplification cycles (NEB standard Taq PCR). Genomic DNA from WT tissues was used as a negative control and actin was amplified from DNA extracts as a positive control.

To verify gene expression leaves from three to five *MhTPS1*-T1 or *NvTPS1*-T2 transgenic plants were pooled and RNA extracted according to the manufacturer's instructions using Trizol RNA extraction reagent (Qiagen). cDNA was prepared from RNA using Superscript reverse transcriptase II (Invitrogen). Gene specific primers were used to amplify *MhTPS1* or *NvTPS1* respectively from 50 ng cDNA template with 25 cycles of PCR. Actin was amplified from cDNA extracted from leaf tissue as a gauge of relative gene expression within prepared cDNA.

Volatile analysis of transgenic plants

MhTPS1-T1 and *NvTPS1*-T2 transgenic plants were analyzed for leaf volatiles once *Arabidopsis thaliana* leaves had developed full rosettes. Blank samples were not collected. Individual transgenic plants were placed in small glass desiccators, exposing a handheld SPME fiber to the plant headspace and trapping volatiles in the desiccator for 16 h. Four *Mh*TPS1-T1 plants and three *Nv*TPS1-T2 plants were used for volatile sampling. SPME fibers were removed after the collection period and compounds were thermally desorbed followed by GC-MS analysis.

For compound analysis by leaf extraction, leaves were collected from *NvTPS1*-T2 plants and pulverized using liquid nitrogen and a mortar and pestle as previously described. Extraction was performed with 85 hexane: 15 ethyl acetate by incubation at room temperature with vigorous shaking (300 rpm) overnight. The organic fraction was drawn off and 1 µL of extract analyzed by GC-MS as described in Chapter 2.

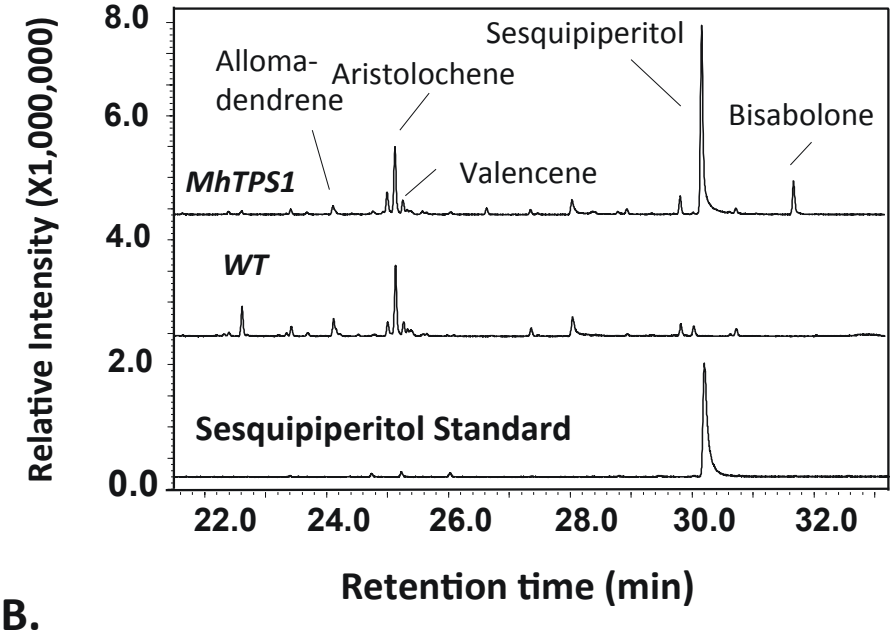
Results

Sesquipiperitol emission from *N. benthamiana* leaves transiently expressing *MhTPS1*

N. benthamiana volatiles were analyzed on Day 4 and Day 6 after infiltration with *Agrobacterium* (Fig. 3.3). (1*S*,6*S*,7*R*)-sesquipiperitol was identified as the predominant volatile. Other detected volatiles were the putative sesquiterpene volatiles allo-aromadendrene, aristolochene, and valencene, which were also found to be emitted from untransformed plants (Fig. 3.3). Another compound that was detected was bisabolone, a likely derivative of sesquipiperitol, either produced by plants or thermal rearrangement

during GC analysis. The average emission rate of sesquipiperitol was approximately 0.8 µg compound per g FW leaves per 24 h.

A.



B.

Sesquipiperitol Average Emission Rate

Sesquipiperitol	Day 4	Day 5	Day 6
Compound mass in 1 µL eluent	5 ng	-	12 ng
Captured from filter	2407 ng	-	1048 ng
Sesquipiperitol/ per g FW leaves	/ 2.25 g FW		
	1.0 µg/ g FW	-	0.5 µg/ g FW
Average	0.8 µg- g FW- 24 h^{-1}		
Standard Curve Peak Area			
Sesquipiperitol	-		
6 ng	4,870,163		
12 ng	9,260,816		
30 ng	27,290,416		

C.

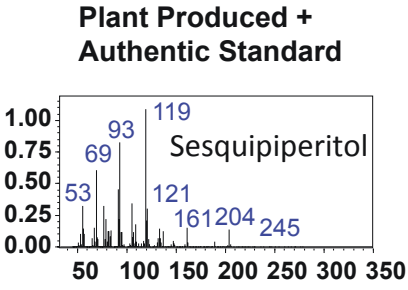
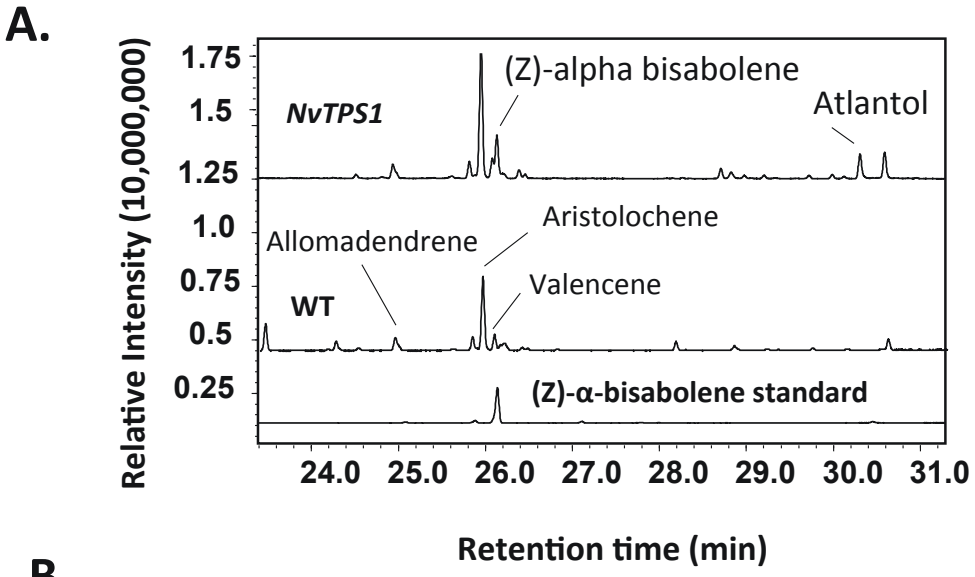


Figure 3.3 Transient formation of sesquipiperitol by transient expression of *MhTPS1* in *N. benthamiana*. A) GC-MS chromatogram of volatiles collected from leaves of transformed and non-transformed plants. B) Average emission of sesquipiperitol calculated from a standard curve as well as day 4 and day 6 volatile collections. C) Mass

spectrum of sesquipiperitol produced from *N. benthamiana* is identical to that of an authentic standard.

Emission of (*Z*)-α-bisabolene in *Nicotiana benthamiana* plants transiently expressing *Nv*TPS1

N. benthamiana leaves transiently expressing *NvTPS1* emitted (*Z*)-α-bisabolene (Fig. 3.4). Similar sesquiterpenes as previously detected were identified in the headspace of plants: allo-aromadendrene, artistolochene, and valencene. (*Z*)-α-Bisabolene was detected with an emission rate of 1.0 µg compound per g FW per 24 h, similar to emission rates of sesquipiperitol. A compound putatively identified as atlantol was also detected in the headspace of transformed plants.



B.

Relative Emission Rate

(Z)- alpha bisabolene	Day 4	Day 5	Day 6
Compound in 1 µL	15 ng	12. 6 ng	9.3 ng
Collected in Filter	3002 ng	2530 ng	1400 ng
Per g FW leaf tissue	/ 2.25 mg	/ 2.25 mg	/ 2.25 mg
	1.3 µg/ g FW	1.1 µg/ g FW	0.6 µg/ g FW
Average	**1.0 µg- g FW- 24 h^{-1}**		

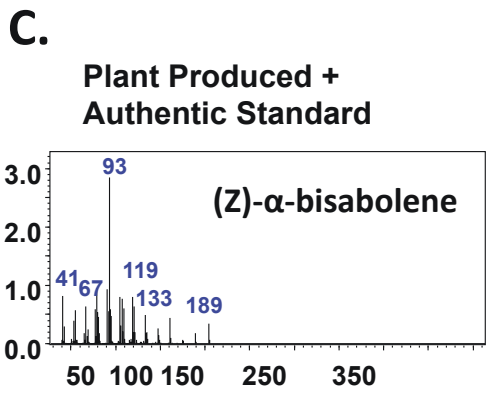


Figure 3.4 Transient formation of (*Z*)-α-bisabolene in *N. benthamiana*. A) GC-MS chromatogram of volatiles produced from *N. benthamiana* transiently expressing *NvTPS1*. B) Average emission rate of (*Z*)-α-bisabolene. C) Mass spectrum of plant produced (Z)-α-bisabolene and that of the authentic (*Z*)-α-bisabolene is identical to that of an authentic standard.

Determination of *TPS* gene insertion and transcription in transgenic *A. thaliana* plants

MhTPS1 was amplified from genomic DNA extracted from *T1 A. thaliana* plants transformed with the *MhTPS1* gene (Figure 3.4). *MhTPS1* transcripts were also was present in pooled tissue of the corresponding lines (Fig. 3.4). Similarly, the presence of the *Nv*TPS1 transgene was demonstrated by genomic PCR in T2 plants (Fig. 3.5). *NvTPS1* gene transcripts could be amplified from combined tissue of those lines (Fig. 3.5 B).

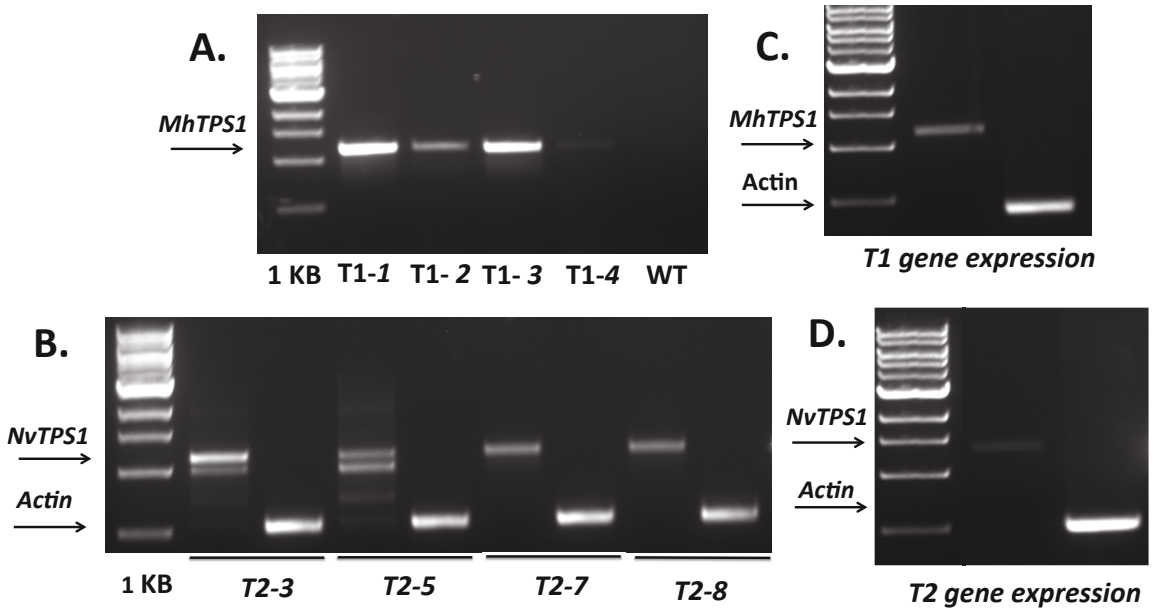


Figure 3.5 Evidence of Insertion and transcription of *MhTPS1* and *NvTPS1* in transgenic *Arabidopsis thaliana*. A) PCR from genomic DNA of *Mh*TPS1-T1 *Arabidopsis* plants. B) Genomic PCR from genomic DNA of *Nv*TPS1-T2 *Arabidopsis* plants. C) RT-PCR from pooled leaf tissue of *Mh*TPS1-T1 *Arabidopsis* plants. D) RT-PCR from pooled leaf tissue of *Nv*TPS1-T2 *Arabidopsis* plants. The housekeeping gene *Actin* was used as a positive control.

Volatile headspace sampling and extraction of transgenic *A. thaliana*

When tested during volatile emission using SPME-GC-MS analysis, no sesquipiperitol was detected in the headspace of four T1 *MhTPS1* plants sampled individually (Fig. 3.6). Instead only volatile organic acids were detected in the background. Similarly, *A. thaliana* plants carrying and expressing the *Nv*TPS1 transgene

did not emit (*Z*)-α-bisabolene (Figure 3.7). Extraction of leaf tissue from these transgenic plants also did not result in the detection of (*Z*)-α-bisabolene (Figure 3.7).

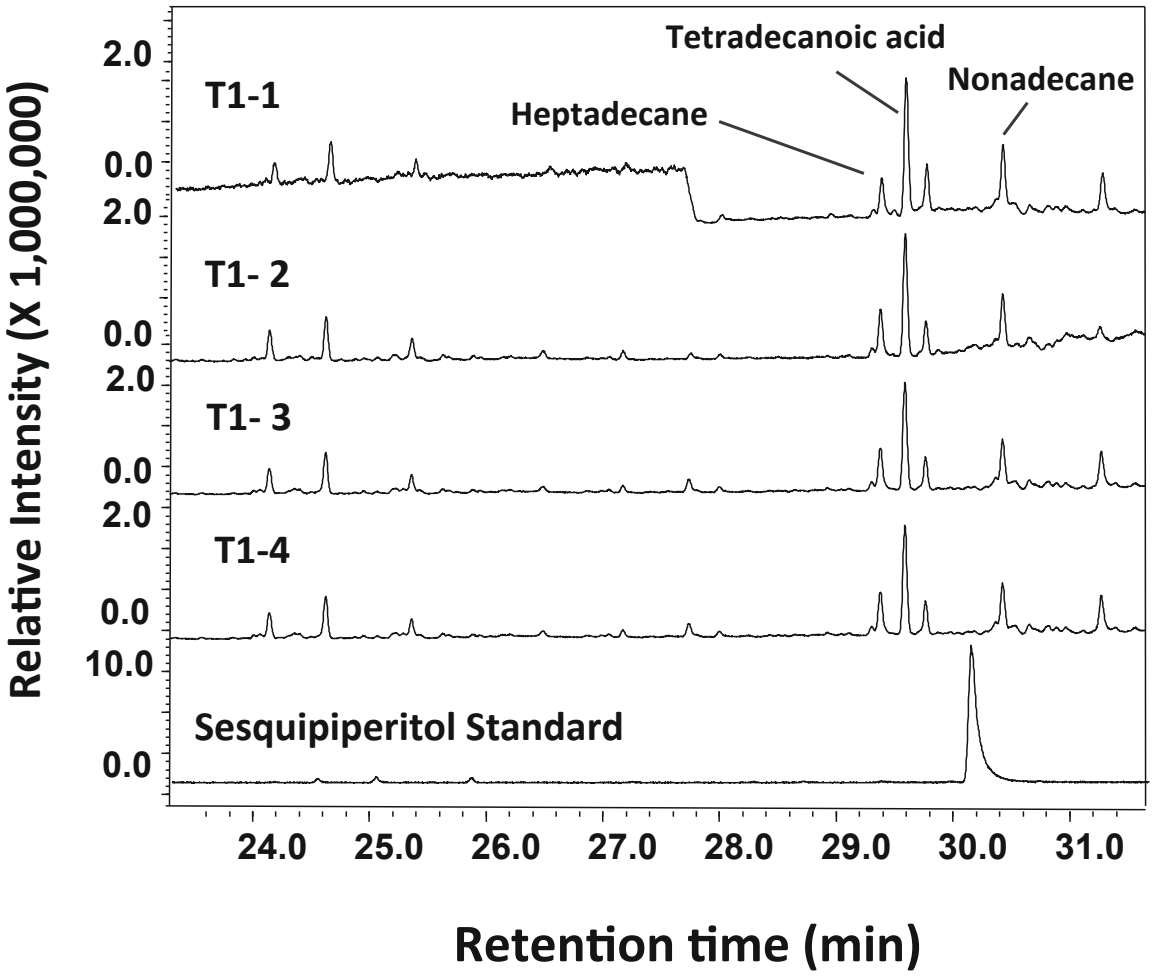


Figure 3.6 Headspace analysis of T1 *Arabidopsis* plants carrying the *Mh*TPS1 transgene. SPME-GC-MS analysis was used to analyze the headspace volatiles of multiple transgenic *Arabidopsis thaliana* plants. A SPME fiber was exposed to the headspace of plants for 16 h. An authentic standard of sesquipiperitol was included for comparison.

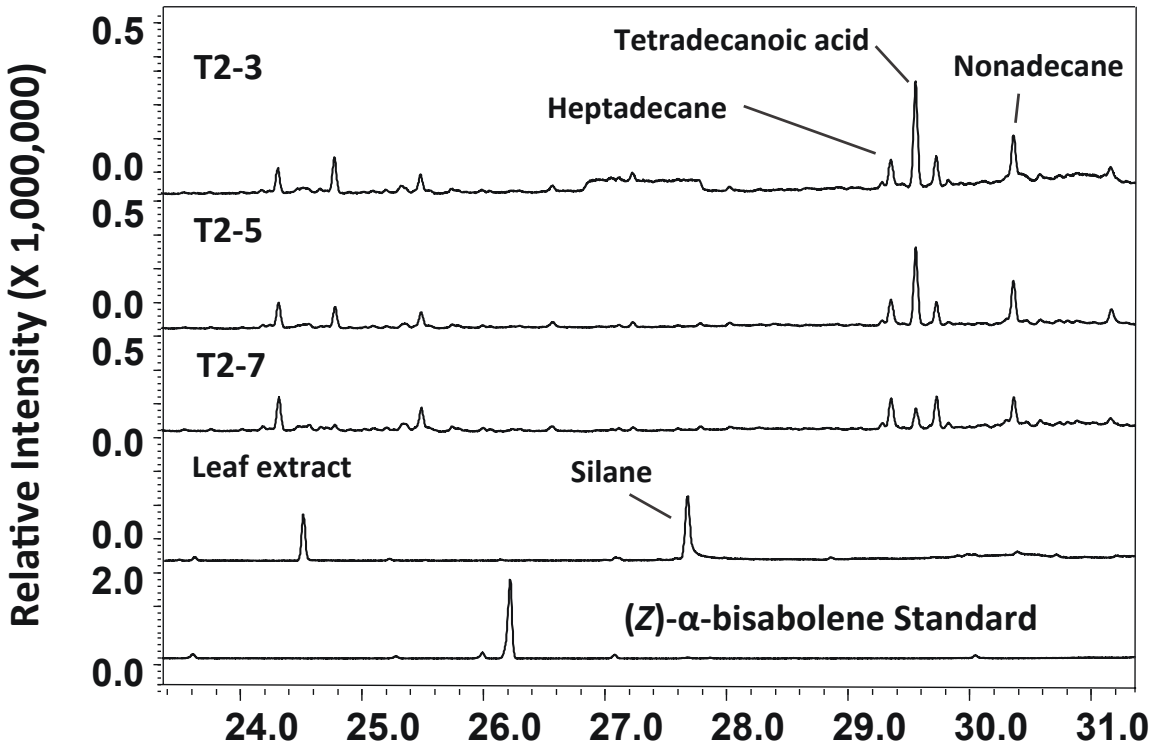


Figure 3.7 Headspace analysis and leaf extraction of T2 *Arabidopsis* plants carrying the *NvTPS1* transgene. Volatiles were sampled with SPME in the headspace of three transgenic *Arabidopsis* plants and analyzed by GC-MS (upper three panels). In addition, pooled leaf tissue from individual plants was extracted with organic solvent and the extracts were analyzed by GC-MS. An authentic standard of (*Z*)-α-bisabolene was included for comparison.

Discussion

Transient expression of stink bug terpene synthases leads to the emission of pheromone precursors

Plants emit a complex mix of volatile compounds that affect plant-interactions. A primary aim of this study was to express terpene synthases from stink bugs in plants with the long term goal to establish pheromone emitting trap plants, which would be specifically attractive for stink bug pests. We found that (1*S*,6*S*,7*R*)-sesquipiperitol, the precursor of murgantiol, was produced and emitted upon transformation with this terpene

synthase (Fig. 3.3) The lack of a targeting peptide led us to assume localization of *Mh*TPS1 in the cytosol, where (*E,E*)-FPP is typically biosynthesized. Compound emission reflects average levels achieved upon transient expression of other terpene synthases in *N. benthamiana* (Reed and Osbourn, 2018).

Transient expression of *NvTPS1* in *N. benthamiana* resulted in the formation of (*Z*)-α-bisabolene and its release in the leaf headspace (Fig. 3.4). Similarly to the *Mh*TPS1 protein, *Nv*TPS1 is presumably mostly localized in the cytosol because of the absence of a transit peptide. The emission rates of (*Z*)-α–bisabolene were comparable to those of sesquipiperitol.

Together, these successful transformation events pave the way for further engineering of the entire pheromone biosynthetic pathways, which would include co-expression with downstream enzymes such as P450 epoxidases investigated in Chapter II. The pathway leading to the formation of *cis*/*trans*-(*Z*)-α–bisabolene epoxides in *N. viridula* would be the simplest starting point for this approach since it requires only two enzymes.

To circumvent the challenge of identifying CYP genes required for pheromone synthesis, an alternative approach could be based on a pathway which leads to the formation of a non-oxygenated terpene pheromone. This is the case for the sesquiterpene himachaladiene, a major pheromone component of the striped flea beetle *Phyllotreta striolata* (Beran et al., 2016). Efforts are underway to transform *N. benthamiana*, and, if successful, also *Arabidopsis thaliana,* with the *TPS* gene responsible for himachaladiene synthesis in combination with a (*Z,E*)-FPP synthase, which provides the specific substrate for the TPS enzyme (Beran et al., 2016). Since (*Z,E*)-FPP synthase requires GPP for the

synthesis of (*Z,E*)-FPP, a plastidial targeting of both the (*Z,E*)-FPP synthase and the TPS protein is desired, since GPP is mostly made in plastids (Tholl, 2015).

Stable transformation of stink bug TPS genes in *Arabidopsis* did not lead to the emission of pheromone precursors

In our attempt to transform *MhTPS1* and *NvTPS1* into *A. thaliana*, four T1 lines and three T2 lines carrying the *MhTPS1* and the *NvTPS1* transgene, respectively, were obtained. Analysis of *MhTPS1* or *NvTPS1* plants with SPME did result in the detection of neither sesquipiperitol nor (*Z*)-α–bisabolene respectively (Fig. 3.6 and 3.7). Moreover, no (*Z*)-α–bisabolene was found in organic extracts of *NvTPS1* transgenics (Fig. 3.7).

One reason why sesquipiperitol was not detected from *MhTPS1* transgenic plants is that glycosylation of the alcohol could occur. A significant reduction of volatile alcohol products was noted with heterologous production of monoterpene alcohols in *Arabidopsis* (Aharoni et al., 2003a). However, glycosylation of (*Z*)-α–bisabolene is less likely unless the compound is further oxidized by endogenous P450 monoxygenases.

Another reason why sesquipiperitol or (*Z*)-α–bisabolene are not emitted from transgenic *Arabidopsis* plants might be the cytosolic localization of the TPS proteins. While (*E,E*)-farnesyl diphosphate is produced predominantly in the cytosol, it is the main precursor for sterol biosynthesis (Tholl, 2015). Competition of sesquiterpene synthases with sterol biosynthetic enzymes is believed to be responsible for low sesquiterpene yields in select cases including the overexpression of an amorphadiene synthase in *A.thaliana* by CaMV 35S expression (Wu et al., 2006). Less than 2 ng amorphadiene per g FW was obtained in *A. thaliana* when overexpressing the enzyme in the cytosol; however, co-

expression of avian FPP synthase in the chloroplast with the amorphadiene synthase achieved 1000 fold higher levels (10 µg amorphadiene per g FW). Potentially such an engineering strategy if necessary could be applied for stinkbug pheromone formation. In an alternative approach, expression of the nerolidol sesquiterpene synthase *Fa*NES1 targeted to the mitochondria has been shown to lead to the production of (*E*)-nerolidol (Aharoni et al., 2003b). by expression of FaNES1 with mitochondrial localization. These strategies have to be tested in future experiments in *Arabidopsis* and subsequently *Brassica* trap crops to optimize metabolic flux in pheromone production.

Other possible reasons why no TPS product could be detected in the transgenic *Arabidopsis* plants could include post-translational modifications or degradation of the TPS protein. Moreover, transgenic plants could be inhibited in growth by the production of the terpene compound and only transgenics with low production rates might have been selected. In this context, it might be possible that successful detection of terpenes by transient transformation is in part caused by expression of the *TPS* genes in *Agrobacterium* and not in plant cells. This scenario will have to be ruled out by measuring terpene emissions in the headspace of *Agrobacterium* cultures prior to leaf infiltration.

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Chapter 4. Trap Crop Development and Future Potential Research

Stink bugs such as *M. histrionica* and *N. viridula* are agricultural pests in the US and worldwide (McPherson and McPherson, 2000). Knowledge about stink bug pheromones and their biosynthesis can be used to develop new strategies in integrated pest management.

In this study we used a combined RNA sequencing and biochemical approach to identify the P450 genes and enzymatic steps involved in the biosynthesis of murgantiol in *M. histrionica* and *trans/cis*-(*Z*)-α-bisabolene epoxide in *N. viridula* requires epoxidation. We hypothesized that a P450 and potentially an isomerase enzyme catalyze the final steps in murgantiol biosynthesis (Lancaster et al., 2018). Similarly we assume that the final step in the biosynthesis of *trans/cis*-(*Z*)-α-bisabolene epoxide in *N. viridula* requires epoxidation. So far, we have been unable to identify a P450 epoxidase involved in these steps in both species. In further work two *N. viridula* P450s that we found to be co-expressed with the *NvTPS1* gene in cuticle tissue (CYP4G159 and CYP4HC1) should be expressed in insect cells and the recombinant proteins tested for epoxidase activity. However, it has to be considered that for both *M. histrionica* and *N. viridula* additional candidate genes have to be tested in a broader-scale approach since the target P450s might not necessarily be male specific.

We consider *N. viridula* to be a more accessible organism for the identification of an epoxidation enzyme since we assume that this protein is exclusively expressed in the unicellular pheromone glands (Cribb et al., 2006) of the mature males prior to the storage and release of the pheromone. In situ localization of the *Nv*TPS protein with specific

antibodies could confirm a localization of the protein in gland tissue. Gland tissue could then be collected more specifically and further RNA-seq applied to narrow the number of P450 epoxidase candidates.

Further research for pheromone biosynthesis can perhaps be guided by a better understanding of *M. histrionica* and *N. viridula* pheromone ecology, physiology, and regulation (Millar, 2005). Murgantiol is emitted in a diurnal rhythm with emission peaking during early afternoon (Zahn et al., 2008). P450 gene expression and/or activity might be regulated by diurnal or circadian rhythms. Regulation of pheromone production by such rhythms as well as hormonal regulation should be better assessed (Jurenka, 2004; Tillman et al., 1999). For example, in bark beetles it has been shown that aggregation pheromone production is stimulated by application of juvenile hormone (Blomquist et al., 2010).

In *N. viridula*, pheromone is emitted by abdominal oscillations during mating, communication, and courtship (Čokl et al., 2007; Millar, 2005). Pheromone biosynthesis and emission are two separate processes, which could also potentially simplify the identification of a P450 epoxidase in this species. In bark beetles it has been shown that terpene pheromones are stored in the form of fatty acid esters prior to their release (Chiu et al., 2018). Overall, a better understanding needs to be obtained about the biosynthetic complexity and molecular regulation of terpene pheromone formation in both stink bugs as it has been obtained in other insects (Boland, 2015; Chiu et al. 2018).

The long term goal of the Tholl lab is to use the identified P450 genes in combination with the already identified terpene synthases for engineering pheromone production in trap crops. Alternatively, the identified genes and proteins could be applied

for a more sustainable synthesis of pheromones in yeast or other microbial platforms (Zhuang and Chappell, 2015).

We transformed stink bug derived terpene synthases in *Nicotiana benthamiana* and found that the precursors to stink bug pheromones can be produced by transient expression. However, further research is necessary to understand an appropriate strategy for stable engineering of these precursors. When *TPS* genes were transformed in *Arabidopsis thaliana* under control of the CaMV 35S promotor using pLIFE33 binary vectors transgenic *Arabidopsis* did not emit aggregation pheromone precursors. The reasons for a lack of volatile production could include insufficient gene expression and perhaps a stronger promoter driving the expression of the transgene could be tested. An alternative explanation is that cytosolic expression of the TPS enzymes is insufficient and compound biosynthesis could be achieved through alternative subcellular localization (Lange and Ahkami, 2013). Furthermore, possible growth inhibitory effects need to be considered during the transgenic selection process. Additional experiments in *Arabidopsis thaliana* and possibly *Brassica* will be necessary to resolve this problem. Volatile terpenes have previously been engineered in a variety of crops including potato, corn, tomato, and wheat (Aharoni et al., 2005; Yu et al., 2012). We are, therefore, confident that it will be possible to transfer knowledge from these successful approaches to the transformation of insect pheromones.

Nevertheless, the lack of P450s currently identified in stink bug pheromone biosynthesis remains a challenge for our engineering efforts. As an alternative proof-of principle approach, we have considered transformation of a terpene synthase involved in aggregation pheromone biosynthesis in the striped flea beetle, *Phyllotreta striolata,* a

specialist herbivore on crucifer crops (Lamb and Palaniswamy, 1990). *P. striolata* produces the sesquiterpene hydrocarbon himachala-9-11-diene (Gruber et al., 2009) as its predominant aggregation pheromone component (Beran et al., 2016), which means that additional enzymes involved in oxidation would hot have to be identified. Himachala-9-11-diene and secondary pheromone components are biosynthesized by *Ps*TPS1 from (*Z,E*)-FPP. *PsTPS1* could be potentially transformed into a *Brassica* trap crop together with the preceding enzyme *Ps*IDS3 producing its substrate (Beran et al., 2016). *Brassica* trap cropping for *P. striolata* has been shown to be successful in field settings and flea beetle pheromones increase pest attraction to trap crop volatiles in a synergistic manner (Lamb and Palaniswamy, 1990).

The testing of modified trap crops in field settings will be important for the overall assessment and success of this approach. Application of pheromones from *M. histrionica* together with *Brassica* host plants has shown to promising for the strong attraction of the pest (Wallingford et al., 2013)(Thrift et al., 2018). Negative aspects of enhanced trap crops might include the inability to retain pests and spill-over effects (Wallingford et al., 2018). However, previous studies show that topical application of systemic pesticides and pesticidal nettings (Wallingford et al., 2018) are effective at reducing problems of pest spillover. Such practices need to be tested in the future once pheromone-enhanced trap crops are available for field experiments.

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Supplemental Figures

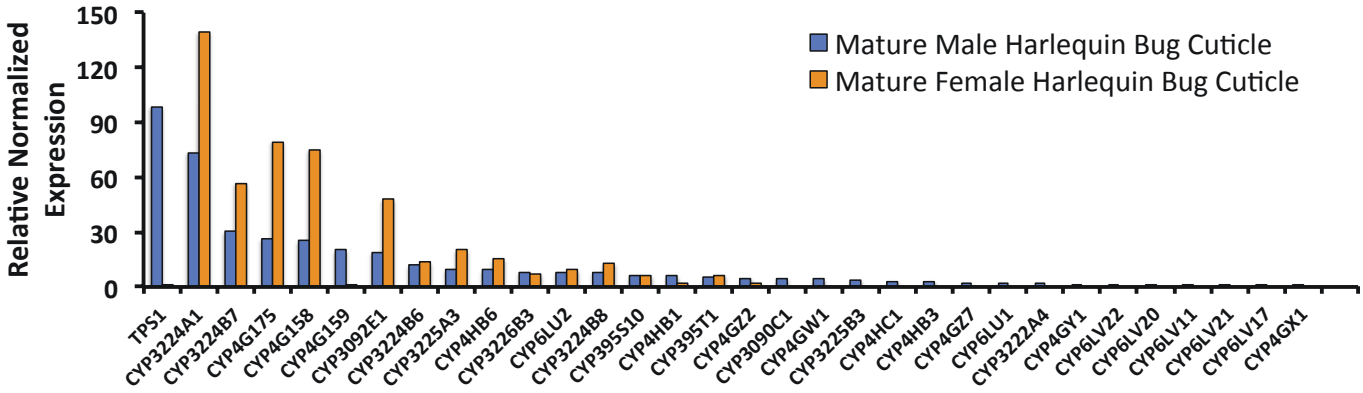

Figure S.1 Expression screening of cuticle P450s in mature male and mature female harlequin bug cuticle tissue. qRT-PCR was performed by extracting RNA from the cuticle of tissue of five male or male bugs, synthesizing cDNA from RNA, and performing qRT-PCR using 18S as a housekeeping gene. The ΔΔCt method was used for gene expression analysis.

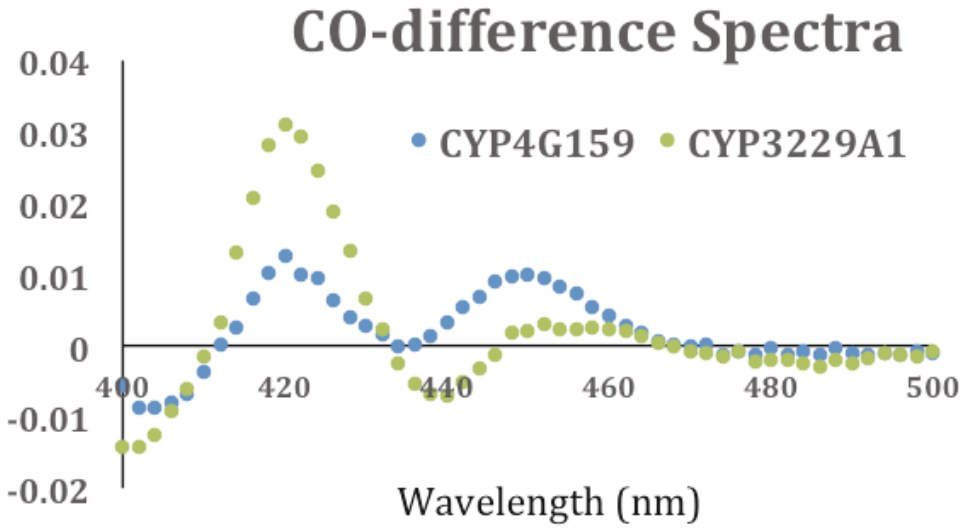

Figure S.2 Activity of recombinant P450s expressed in Sf9 insect cells. A carbon monoxide binding assay was performed with microsomal fractions of recombinant P450s expressed in Sf9 insect cells. The difference in absorbance at 450 nm was used as an indicator for P450 enzyme activity (UNR).

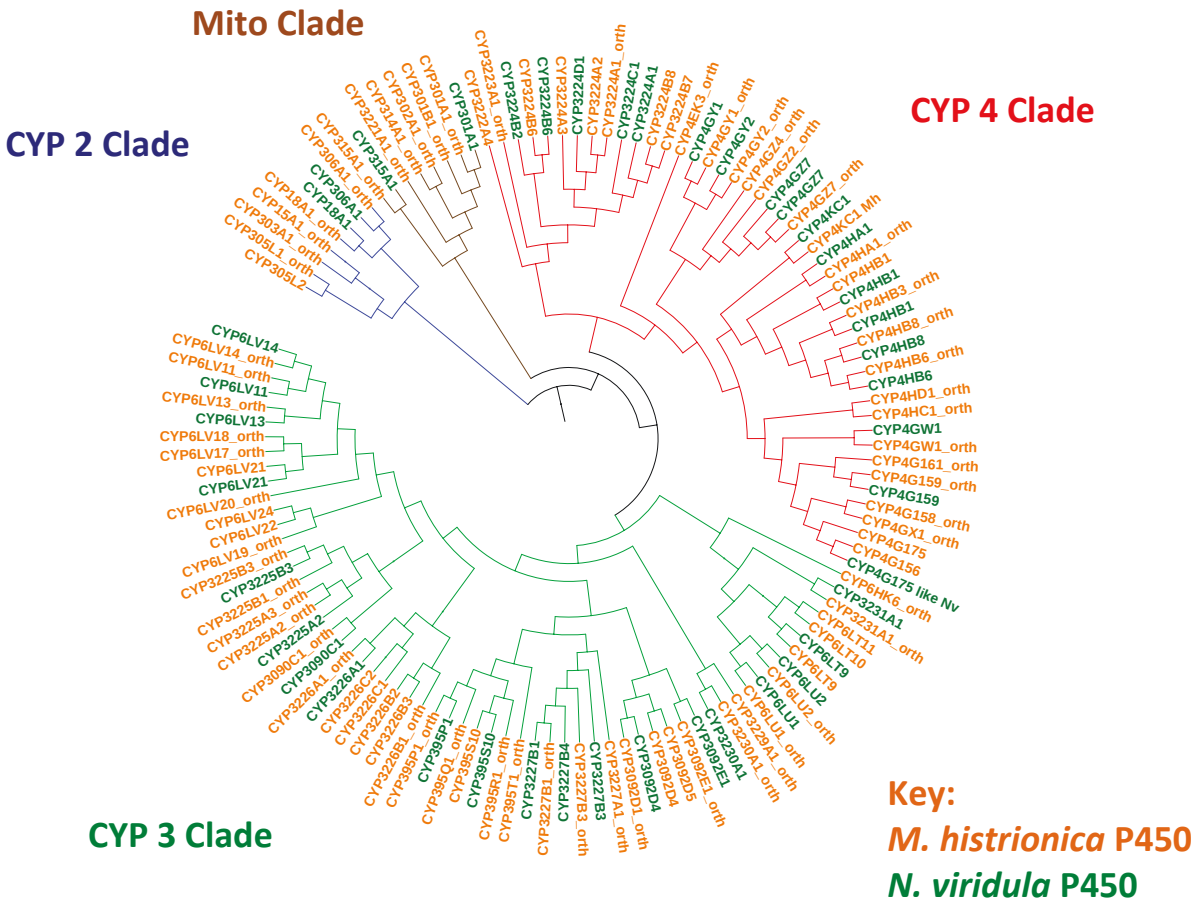

Figure S.3 Phylogenetic analysis of P450s identified in transcriptomes of *M. histrionica* and *N. viridula* cuticle. *M. histrionica* P450s are depicted in orange and *N. viridula* cuticle P450s are depicted in green. P450s are associated with P450 clade by color: Green (CYP6), Red (CYP4), Blue (CYP2), and Brown (mito-Clade). Alignment was performed in PHYML using a neighbor joining tree.

P450	Transcript Name
CYP315A1	TR29686|c0_g1_i1
CYP6LT9	TR11721|c0_g1_i1
CYP6LV14	TR15220|c0_g1_i1
CYP3231A1	TR17905|c0_g1_i1
CYP4HB6	TR20203|c0_g1_i1
CYP3225B3	TR20873|c1_g1_i2
CYP4G175	TR22818|c1_g1_i1
CYP3092E1	TR2448|c0_g1_i1
CYP3224B61	TR24520|c1_g1_i1
CYP4HB1	TR26096|c0_g1_i1
CYP4GZ7	TR26733|c0_g1_i1
CYP18A1	TR26974|c0_g1_i1
CYP395S10	TR28214|c0_g1_i1
CYP6LV11	TR28227|c1_g1_i4
CYP4HA1	TR33962|c0_g1_i1
CYP395P1	TR3459|c2_g2_i1
CYP3225A2	TR37146|c0_g1_i1
CYP3224A1	TR38379|c0_g1_i2
CYP6LU1	TR3886|c0_g1_i1
CYP3090C1	TR41116|c0_g2_i1
CYP4GZ72	TR46227|c3_g6_i4
CYP4KC1	TR4637|c2_g1_i1
CYP4GW1	TR46464|c0_g1_i1
CYP3224AC1	TR4882|c0_g1_i1
CYP4G159	TR50837|c0_g2_i1
CYP6LV21	TR51988|c0_g1_i1
CYP3227B3	TR54098|c1_g1_i1
CYP4HB8	TR56369|c0_g1_i2
CYP4HB1	TR57081|c0_g1_i1
CYP3227B1	TR58551|c0_g1_i1
CYP3230A1	TR61537|c0_g1_i1
CYP4GY1	TR6163|c0_g1_i1
CYP4GY2	TR64694|c25_g2_i1
CYP3227B4	TR64943|c0_g1_i1
CYP3092D4	TR65853|c6_g2_i1
CYP307B1	TR66060|c0_g1_i1
CYP3224B6	TR67054|c1_g3_i1
CYP3224E1	TR67054|c2_g2_i1
CYP306A1	TR67681|c0_g1_i1
CYP301A1	TR9541|c0_g1_i1
CYP6LU2	TR9812|c0_g1_i1
CYP3226A1	TR52366|c0_g1_i1

Figure S.4 P450 Genes expressed in the *N. viridula* cuticle with corresponding transcript identities. P450 nomenclature was assigned by alignment with M. histrionica P450s and transcript names correspond to custom combined cuticle assembly performed at V. Tech with Trinity.