Red Turpentine *Dendroctonus valens* P450s: Diversity, Midgut Location, and Response to Alfa-pinene Enantiomers

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Introduction

Bark beetle colonization action is a biotic stress to the host, which responds to this action by secreting oleoresin. This mixture of several compounds can be highly toxic for the insects. We hypothesized that the first physiological reaction of the insects to these toxic compounds is to metabolize and subsequently use some of them as pheromone precursors. Epithelial cells from the bark beetle's midgut have been demonstrated to be involved in pheromone production (Nardi and others 2002); however, the site(s) and mechanism(s) where the detoxification process occurs are unknown.

The cytochrome P450-dependent monooxigenases are a metabolic system involved in (1) regulating the titers of endogenous compounds such as hormones, fatty acids, and steroids, and (2) the catabolism and anabolism of xenobiotics such as drugs, pesticides and plant toxins. Monooxygenases are found in virtually all aerobic organisms, including insects. P450's are a multigenic family with a common origin and are found in the endoplasmic reticulum and mitochondria. There are four main gene families in insects: CYP4, CYP6, CYP9, and CYP-mitochondrial (Fereyeisen 1999; Scott 1999; Scott and Wen 2001). Other mechanisms involved in the detoxification process, such as glutathion transferases enzyme (GTS) (Hemingway 2000) and non-specific-esterases, are being widely documented in insects. GTS enzymes are a multigenic family integrated in seven classes, whose function is to metabolize xenobiotic compounds and to protect cells against damage by oxidative stress. Non-specific-esterases (carboxylesterases) also participate in the detoxification process, and only two genes are known.

Sturgeon and Robertson (1985) proposed that cytochrome P45O monoxigenases could be involved in the detoxification process of some toxic monoterpenoids in bark beetles as α-pinene. White and others (1978) demonstrated that cytochrome P450 of gut epithelial cells participate in the transformation of α-pinene to *cis* and *trans*-verbenol in *Dendroctonus terebrans*. Aguilar (2002) showed by inmuno-histochemical techniques that cytochrome P450 and non-specific-esterases of midgut epithelial cells in *D.valens* and *D. mexicanus* are over-expressed when the insects are exposed to

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α-pinene vapors. With respect to GTS enzymes, there are no reports in bark beetles. However, in *Ips typographus*, four esterase isozymes were detected that participate in detoxification processes and/or in the hydrolysis of juvenile hormone (JH) (Stauffer and others 1997). Previous histochemical studies of *D. valens* alimentary canal have shown that non-specific-esterases of midgut increase their activity when insects are exposed to α-pinene (Aguilar 2002).

We are using *D. valens* as a model because it has been demonstrated that the monoterpenoids α -pinene, R-(+)-α-pinene, S-(-)-β-pinene, and S-(+)-α-pinene are highly toxic compounds. They are also very important for their chemical communication system (Hobson and others 1992; White and Hobson 1993). In this species, we are studying the ultra-structural changes in the midgut epithelial cells of *D. valens* after exposure of the insects to α-pinene. We are also studying the localization, diversity, and expression of CYP, non-specific-esterase and glutation–s-transferase genes in this region.

Methods

Ultrastructure

Samples of *D. valens* were collected directly from infested trees and transported alive to our lab. The insects were exposed to α-pinene during 24 hours at 4 °C. The insects were then dissected to obtain the alimentary canal and to extract the midgut. The anterior and posterior midgut were sectionaed and they were fixed in Eppendorf tubes with glutaraldehide 2.5 percent and cacodilate buffer 1M. The samples were then processed with conventional transmission electron microscope techniques.

P450 Complex

The insects were exposed to α-pinene and its enantiomers: R-(+)-α-pinene, S-(-)-β-pinene, and S-(-)-α-pinene during 24 hours at 4 °C. Subsequently, total RNA of midgut was extracted with Trizol method and single-stranded cDNAs for RT-PCR were synthetized from total RNA with MMLV reverse transcriptase. The CYP4, CYP6, and CYP9 (P45O) genes were amplified with degenerated primers (Rose and others 1997; Snyder and others 1996). The expected bands were cut off from the gel and cDNA was isolated using the QIAEX II Gel Extraction Kit. The isolated products were reamplified and sequenced. The nucleotide sequences were compared with sequences deposited in GeneBank.

Results and Discussion

The Midgut Ultra-structure of Insects Without Alpha-pinene

The midgut epithelium is composed of cylindrical cells with microvilli in the apical zone. The cells are limited by a basal membrane named basal lamina that forms invaginations in folds. The cytoplasm is granular and spongy. The nucleus is large-ovoid with one or several electron-dense nucleoli located in the center or mid part of the cell. The primary lysosomes are scarce and mitochondria are found in all the cytoplasm. Their crests have a normal structure and the rough endoplasmic reticulum is widely distributed. Ribosomes are found both free and adhered to the rough endoplasmic reticulum. It is very common to observe empty vacuoles and secretion vesicles irregularly scattered in all cytoplasm.

The Midgut Ultrastructure of Insects With Alpha-pinene

The microvilli, nucleus, and vesicles maintain their original structure; however, it is common to observe a significant increase in the number of mitochondria on the basal and apical zone of the cells. The mitochondria crests are modified, the primary and secondary lysosomes are abundant, the rough endoplasmic reticulum is observed near to the nucleus arranged in parallel packages, and the Golgi complex is evident and maintains its structure with crests in block piles and it is found near of nucleus and the apical zone. The smooth endoplasmic reticulum is very conspicuous. It has a tubular shape with vesicles associated in its extreme with indefinite arrange.

The differences observed in both experiments showed that α-pinene affects the midgut epithelial cells, changing and increasing the number of secondary lysosomes and mitochondria in the apical zone. The rough endoplasmic reticulum shows an overexpression. The Golgi complex and smooth endoplasmic reticulum are clearly evident. These ultrastructural changes suggest that these organelles participate actively in the detoxification process.

P450 Gene Complex

The midgut cDNA of stimulated insects amplify for a CYP4 gene (CYP4 family). The amplification product of this gene exhibited one band of the expected size of ~500bp. These products were sequenced, and Blast analysis showed that amino acid sequences of CYP4 of *D. valens* has a high identity (90, 91 percent) with CYP4G29 from *Leptinotarsa decemliniata*, and CYP4G15 from *Tribolium castaneum*. CYP6 and CYP9 genes have been amplified from total DNA of *D. valens*, and at this moment, we are amplifying these genes from midgut cDNA to obtain their sequences. These results show that three CYP genes are present in the midgut region.

Future Work

- The genes obtained (CYP4, CYP6, CYP9) will be cloned and sequenced to obtain subsequently complete genes using 5' RACE method. We are testing the presence of CYP-mitochondrial (CYP12).
- We are looking at where these CYP genes are located at ultrastructural level in *D. valens* midgut cells.
- We are quantifying the expression levels of CYP genes using Real-Time PCR.
- We are determining the midgut location and expression levels of other enzymes involved in the detoxification process, as esterases and Glutathion-S-Tranferases by histochemical and immunological methods, as well as by molecular techniques.

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