Time-Course of CYP450 Genes Expression From *Dendroctonus rhizophagus* (Curculionidae: Scolytinae) During Early Hours of Drilling Bark and Settling Into the Host Tree

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Abstract

*Dendroctonus* bark beetles (Scolytinae) are one of the most important disturbance agents of coniferous forests in North and Central America. These beetles spend their lives almost entirely under the tree bark, and their survival and reproductive success depend on their ability to overcome the toxic effect of the trees’ oleoresin. The cytochromes P450 (CYPs) are associated with the detoxification process of xenobiotics, as well as other physiological processes. Different cytochromes (families 4, 6, and 9) in the *Dendroctonus* species have been expressed under several experimental conditions; nevertheless, the expression time-course of these genes is unknown. To explore the induction speed of CYPs, we evaluated the relative expression of the CYP6BW5, CYP6DG1, CYP6DJ2, CYP9Z18, and CYP9Z20 genes at the early hours of drilling and settling into a tree (1, 2, 4, 6, 8, 12, 18 h) both in females and males, solitary or paired, of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright. Our findings show that the five genes were rapidly overexpressed in the early hours (1 to 6 h) in both sexes and in solitary and paired conditions, suggesting their participation in the detoxification process. Additionally, the CYPs expression shows up- and down-regulation patterns through these short times, suggesting their probable participation in other physiological processes as the biosynthesis of hormones, pheromones or compounds related to reproduction.

Key words: monoterpene, detoxification, CYP450, bark beetle

Bark beetles of the genus *Dendroctonus* Erichson (Curculionidae: Scolytinae) are natural components of coniferous forests, which contribute to their regeneration by eliminating injured, diseased, or weakened trees. Nevertheless, under disturbed environmental conditions, they can become pests whose outbreaks can kill millions of trees; this can cause alterations to many ecological processes in coniferous forests and economic losses to the forestry industry (Raffa et al. 2008, Weed et al. 2013).

Host colonization is the initial stage of the *Dendroctonus* bark beetles’ life cycle because reproduction is conditional on the successful infestation of the host by the progenitors (Byers et al. 1985). During tree colonization, the pioneering sex (the female) locates an appropriate and susceptible host (Borden et al. 1982, Franceschi et al. 2005, Kroken et al. 2015), initiates a gallery entrance, and, by releasing a pheromone (typically), attracts a mate. In aggregating species, the pair jointly overcomes the host’s chemical defenses by releasing pheromone components that attract new attacks that further weaken the host. Once the defensive response ceases, the pair extends the gallery while the female oviposits into the gallery walls (Seybold et al. 2006). Each step is energetically demanding, and during this time insect physiology should rapidly shift from addressing the unique demands of host localization to addressing detoxification and pheromone production and finally to mating and oviposition (Borden et al. 1982; Seybold et al. 2006; Keeling et al. 2006; Pitt et al. 2014; Tittiger and Blomquist 2016, 2017).

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As soon as insects drill through the bark, the host releases primary oleoresin (a dense and viscous fluid composed of monoterpene and sesquiterpene hydrocarbons and diterpene acids) in response to physical damage to the living tissue, and this substance forms a chemical and physical barrier that prevents or hinders the insects’ penetration into the phloem (Faldt 2000, Keeling et al. 2006). The monoterpene, α-pinene, β-pinene, myrcene, limonene, and 3-carene—typically major constituents of conifer oleoresin—are generally highly toxic to insects, and can produce cellular damage in various tissues; at sufficient concentrations and durations of exposure they can cause the death of bark beetles (Smith 1965, López et al. 2011, Chiu et al. 2017). To respond to the threat that these toxins pose to initially attacking individuals, the detoxification metabolism of conifer-attacking Dendroctonus bark beetles should respond immediately through the genetic machinery, which includes genes such as cytochromes P450 (CYPs), carboxylesterases, and glutathione-S-transferases (Després et al. 2007). In herbivorous insects, CYPs are of particular interest since they appear to have an important role in plant-insect interactions, as they are specialized on the metabolism of endogenous and exogenous compounds (Berendse 2002, Feyereisen 2011, Schuler 2011).

In general, CYP genes can reach peak expression levels just a few hours after the exposure to a xenobiotic (Cariño et al. 1994, Poupardin et al. 2010, Misra et al. 2011, Li et al. 2016). For bark beetles, in particular, some CYP genes have demonstrated a capacity to metabolize different toxic monoterpene, such as α-pinene, and myrcene, through hydroxylation reactions; these genes may also participate in the de novo synthesis of pheromones (Sandstrom et al. 2006, 2008; Song et al. 2013; Nadeau et al. 2017). Several studies have analyzed the expression of specific CYPs under diverse experimental conditions (e.g., insects fed and unfed; solitary and paired; different exposure times; single monoterpenes, racemic mixtures, and combinations; with assays of individuals in both laboratory and field) and documented and evaluated their expression through transcriptome analysis following exposure to stimuli (Huber et al. 2007, Keeling et al. 2012, Cano-Ramírez et al. 2013, López et al. 2013, Dai et al. 2015, Obregón-Molina et al. 2015, Nadeau et al. 2017). Some CYP genes from families 4, 6, and 9 in Dendroctonus rhizophagus Thomas and Bright and their respective orthologues in Dendroctonus valens have been characterized molecularly and their expression evaluated in vitro in pre-emergent insects exposed to different monoterpane vapors at 8 and 24 h (Cano-Ramírez et al. 2013, López et al. 2013), as well as adults at different colonization stages in the field: solitary females, pairs before oviposition, pairs after oviposition, and solitary females once the male has left the gallery (Obregón-Molina et al. 2015). In these studies, CYP6BW5, CYP6DG1, CYP6DJ2, CYP9Z18, and CYP9Z20 genes showed high expression levels after the monoterpane exposure and phloem-feeding in both species.

Dendroctonus rhizophagus is an aggressive species endemic to the Sierra Madre Occidental, Mexico (Mendoza et al. 2011). This species is univoltine, synchronous, and colonizes only seedlings and young saplings (trees ≤3 m height and 10 cm diameter) of 11 pine species, mainly Pinus engelmannii Carr., P. durangensis Martínez, and P. arizonica Engelm (Pinales, Pinaceae) (Salinas-Moreno et al. 2004). Unlike other species of the genus that produces mass attacks, only one or two pairs of Dendroctonus rhizophagus colonize an individual pine tree (Cibrían-Tovar et al. 1995).

Although previous studies in CYP genes have demonstrated their induction by several stimuli, none of them has evaluated the expression of these genes during the first hours after insect exposure to monoterpenes and/or oleoresin. Documentation of the rapidity of induction is fundamental to understanding the response capacity of the insect to the chemical environment of the host trees. Thus, the aim of this study was to evaluate the expression profile of the early-induced response of CYP6BW5, CYP6DG1, CYP6DJ2, CYP9Z18, and CYP9Z20, genes probably involved in monoterpane detoxification in D. rhizophagus during the initial hours of drilling and settling into the tree’s bark.

Materials and Methods

In this study, pre-emerged, unfed adult females and males of D. rhizophagus were collected on June 2017, from naturally-infested young trees in the locality of La Ciudad (23°43′53.58″ N, 105°40′10.57″ W) in Durango State, Mexico. Insects were separated by sex (Armandariz-Toledo and Zúñiga 2017) and stored in polycarbonate Magenta vessels GA-7 (Magenta Corp., Sigma-Aldrich-Merck, Darmstadt, Germany) filled with moist paper towels (average humidity 60%) up to 72 h in the dark at 4°C.

Induction

Insects of each sex were introduced into noninfested healthy young trees logs (30 cm height, 10 cm diameter) in order to feed beetles with phloem during 1, 2, 4, 6, 8, 12, and 18 h. Briefly, the beetles were placed, solitary or paired (female was inserted first, followed by a male), in small holes drilled into the phloem of young pines. Holes were covered with a metal wire mesh (2 × 2 mm) stapled to the tree to prevent beetle escape and encourage mining activity. All assays were realized in laboratory at room temperature, and once all insects were introduced simultaneously into the logs, these were placed in the dark. Five unfed pre-emerged males or females were used as controls for the relative expression calculations. After time had elapsed, the insects were removed from the tree logs and dissected in phosphate buffered solutions (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4, 2 mM KH2PO4) under a stereomicroscope. Three biological replicates were performed.

Total RNA Isolation and cDNA Synthesis

The insects’ guts were separated from the fat body and the Malpighian tubules and sectioned into foregut, midgut, and hindgut. Five midguts pooled from each sex and experimental condition (fed solitary or paired) were placed into 1.5-ml vials with 200 µl TRI Reagent solution (Invitrogen Corp., Carlsbad, CA) and macerated using sterile pestles. Later, every vial was filled with TRI Reagent to complete 1 ml and frozen in liquid N2. Tissues were kept at −80°C until RNA was extracted using the RiboPure Isolation of High-Quality Total RNA Kit following the manufacturer’s protocol (Ambion Inc the RNA Company, Austin, TX). RNA integrity and non-DNA contamination were verified on 1% denatured gels of agarose UltraPure (Invitrogen Corp., Austin, TX). RNA isolation and cDNA synthesis were verified on 1% denatured gels of agarose UltraPure (Invitrogen Corp., Austin, TX). RNA and cDNA were stored at −20°C until cDNA synthesis.

Two micrograms of each sample of total RNA were reverse-transcribed using High Capacity RNA to cDNA Kit (Applied Biosystems, United States), following the manufacturer’s protocol. The cDNA obtained was stored at −20°C until the quantitative real-time polymerase chain reaction experiments (RT-qPCR) were performed.

RT-qPCR Assays

All experimental procedures related to qPCR were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Supp. Table S1)
In particular, the CYP6DJ2 gene expression was statistically significant among times and sexes (P < 0.001); whereas this gene was always expressed in the first hours, its expression pattern was different between females and males, being higher in solitary (120,298-fold) and paired (1,837-fold) females than males in both conditions (Fig. 1A); decreased expression was observed at 8 h in males and down-regulation in females in both conditions.

Significant differences were found in the CYP6BW5 gene expression pattern among times and conditions (P < 0.001) but not between sexes; while the expression level was high during the first 4 h, the expression fell down at 6, 8, or 12 h and increased again at 8, 12, or 18 h, respectively; the major expression was observed in solitary females at 12 h (1,926-fold) followed by paired females at 18 h (735-fold) (Fig. 1B).

In the case of the CYP6DG1 gene, significant differences were observed in the expression pattern between times and conditions (P < 0.001) but not between sexes; gene expression was high during the first 6 h, but it was down-regulated at 8 and 18 h, with a slight increase at 12 h. The highest expression level was reached by solitary males (777-fold) after 1 h of feeding (Fig. 1C).

Significant differences in the CYP9Z18 gene expression pattern were found among times, sexes, and conditions; the highest expression was observed in solitary males (4,424-fold); a down expression at 8 h and a slight up-regulation at 12 and 18 h were observed at both sexes and conditions, which was not greater than those recorded in early hours, except for solitary females (Fig. 1D). Finally, significant differences were observed in the CYP9Z20 gene expression among times and sexes, despite its expression being variable but continuous through time (Fig. 1E).

Discussion

In this study, we examined the expression of five CYP genes—three of the family 6 (CYP6BW5, CYP6DG1, and CYP6DJ2) and two of the family 9 (CYP9Z18 and CYP9Z20)—presumably involved in the detoxification of xenobiotics in D. rhizophagus and/or other metabolic pathways for the biosynthesis of pheromonal components, hormones, or compounds related to reproduction as suggested in other Dendroctonus species (Pitt et al. 2014; Tittiger and Blomquist 2016, 2017; Nadeau et al. 2017).

Our findings showed that the CYP genes analyzed in D. rhizophagus are induced rapidly, as soon as the beetles get into the entrance hole and come into contact with the phloem, suggesting that only a short time of feeding on phloem and/or the immediate exposure to constitutive oleoresin are enough to induce an important overexpression in these genes.

Previous studies have also documented differential expression of some CYP genes of families 4, 6, and 9 in stimulated or fed bark beetles; however, no one has evaluated these genes before 8 h in Dendroctonus species (Huber et al. 2007, Cano-Ramirez et al. 2013, López et al. 2013, Dai et al. 2015, Obregón-Molina et al. 2015). Nevertheless, our results and those previously reported for CYP6BW5, CYP6DG1, CYP6DJ2, CYP9Z18, and CYP9Z20 genes in stimulated and fed D. rhizophagus insects beyond 8 h (Cano-Ramirez et al. 2013, Obregón-Molina et al. 2015), jointly suggest that the expression of these CYPs is a dynamic process with up- and down-regulations in response to the colonization process and probably to mating and oviposition (Obregón-Molina et al. 2015).

In particular, our data highlight the early expression of CYP6BW5, CYP9Z18, and CYP9Z20 genes that is maintained across time in both sexes. The up- and down-regulation recorded
Fig. 1. Relative expression of CYP6DJ2, CYP6BW5, CYP6DG1, CYP9Z18, and CYP9Z20 genes (mean ± SE) in midgut from male and female solitary and paired of D. rhizophagus during the early hours of drilling and settling into the tree’s bark. Values > 0 on logarithmic y-axis indicate overexpression. CYP expression was normalized with the CYP4G55 reference gene. Different letters indicate a significant difference in the gene’s expression between times for each condition (SM, PM, SF, PF). The 2⁻ΔΔCT and SE values were transformed at log₂ for plotting (SM = solitary male; PM = paired male; SF = solitary female; PF = paired female).
could be the response to the variation in the concentration of terpenes during the tree drilling, followed perhaps by the production of induced oleoresin by the host tree as a result of the advance of the insect into the gallery (Leufven and Birgersson 1987, Wallin and Raffa1999, Zhao et al. 2011, Keefover-Ring et al. 2016). A high expression of these genes has also been observed in the gut and antennae of female and male of both D. rhizophagus (Cano-Ramírez et al. 2013) and D. valens (López et al. 2013), after exposing them to single host monoterpenes. The expression patterns of these genes are not sex-specific, suggesting that they have a similar function in males and females (Feyereisen 1999, 2011; Huber et al. 2007; Schuler 2011; Cano-Ramírez et al. 2013; López et al. 2013; Robert et al. 2013; Dai et al. 2015; Obregón-Molina et al. 2015).

While no one has demonstrated the direct involvement of gut cytochromes of Dendroctonus species in terpene metabolism, the CYPs expression observed in this study in D. rhizophagus during the first hours of bark drilling, suggests that these enzymes are involved in the hydroxylation of terpenes. Nevertheless, the differences in the general expression pattern of CYPs, and its independence with respect to the insect condition (i.e., sex, pairing status), point to a similar function of these genes with different levels of performance and specificity during early colonization.

The differential expression of CYP6DG1 and CYP6DJ2 genes and their drop at different times during tree drilling are indicative of their differential performance and probable participation in other metabolic functions. In particular, CYP6DJ2 transcript levels were higher in solitary and paired females than males throughout the different times assayed, suggesting that this gene could be involved in other sex-specific roles in the endogenous metabolism. This CYP pattern is consistent with that reported by Obregón-Molina et al. (2015), who registered an elevated number of transcripts in females at 8 and 24 h, as well as through different colony stages in the field. In addition, expression patterns in which the induction of a gene is paused in one sex while in the other continues, have been reported in other bark beetles (Sandstrom et al. 2006, Huber et al. 2007), suggesting different functions in each sex or a change of the metabolic pathway in which they are involved (Pitt et al. 2014). In fact, a proteomic analysis carried out in D. ponderosa adults fed during 24 h revealed not only the accumulation of chaperone proteins, a cytochrome P450 from CYP 6 family (CYP6DE1), and a glutathione S-transferase enzyme in both sexes, but these researchers found significant changes in the enzymes required for vitellogenesis in females, which discloses a trade-off between colonization and reproduction (Pitt et al. 2014). Recently, the functional characterization of CYP6DE1 demonstrated that it produces trans-verbolen as the major product of (−)-α-pinene and (+)-α-pinene hydroxylation. Also, it can use other substrates such as (−)-β-pinene, (+)-β-pinene, and (+)-3-carene, which are the most abundant compounds of pine oleoresin (Chiu et al. 2019).

Other studies have also demonstrated the performance and specificity of CYPs’ bark beetles. For example, in Ips species (Curculionidae: Scolytinae), the expression profiles of CYP9T1 and CYP9T2 in fed insects showed up-regulation of these genes at 4 h only in males, in females, they were induced at 8 (CYP9T1) and 32 h (CYP9T1 and CYP9T2) (Sandstrom et al. 2006, 2008). In addition, CYP9T2 and its orthologous CYP9T3 were able to hydroxylate several terpenes (myrcene, α-pinene, 3-carene, and limonene), but showed greater specificity for myrcene (Song et al. 2013), a compound that is hydroxylated by these cytochromes to produce de novo pheromones, ipsenol, and ipsdienol, through the mevalonate pathway.

In addition, comparative transcriptomic studies carried out with both fed and unfed D. ponderosae adults suggest that α-pinene is hydroxylated to trans-verbolen—an oxygenated monoterpene aggregation pheromone in this species—probably by multiple CYP enzymes (CYP6DJ1, CYP6DJ2, CYP349B2, and CYP4BD4) (Nadeau et al. 2017). In the case of D. rhizophagus, the early expression of CYPs could be associated with α-pinene hydroxylation to oxygenated-monoterpenes, among which trans-verbolen stands out as a suspected component of the sex pheromone of this species (Cano-Ramírez et al. 2012). In the case of Dendroctonus species, it has been reported that pheromone production in D. frontalis, D. mesoamericanus (Niño-Domínguez et al. 2015), and D. rufipennis (Isitt et al. 2018) is more copious around 24 h after feeding on host phloem. Hence, the metabolic activity previous to this time is probably dedicated to the detoxification process. Moreover, the colonization progress requires different types of pheromone components for aggregation or anti-aggregation, thus diverse enzymes are needed at each colonization stage, and such transition may have an effect on the gene expression levels of the involved genes (Sullivan et al. 2016). Nevertheless, biochemical studies of heterologous expression should demonstrate the degree of specificity of these CYP on terpenes in D. rhizophagus, as well as their capacity to hydroxylate these compounds to trans-verbolen.

In summary, our findings provide strong evidence of the rapid induction of CYP genes in D. rhizophagus gut, immediately after the insects are exposed to the host terpenes as they initiate the drilling into the bark. In the expression patterns obtained, there are some visible variations, which could be related to the changes in the concentration of terpenes as colonization progresses, or to metabolic shifts from the primary need of detoxification to other metabolic requirements including the biosynthesis of hormones, pheromone components, or reproduction-related compounds. Ongoing molecular approaches will characterize the specific functional role of CYPs in different metabolic processes.

### Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.
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