

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, Krokene 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).

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 (Fäldt 2000, Keeling et al. 2006). The monoterpenes, 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 (Berenbaum 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 monoterpenes, 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-emerged insects exposed to different monoterpene 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 monoterpene 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 (Cibrián-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 monoterpene 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 (Armendáriz-Toledano 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 μ l 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) and visualized in UV transilluminator (Alpha Innotech Corporation). RNA concentration and purity (A_{260}/A_{280} ratio) were quantified in a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was kept at -80°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)

(Bustin et al. 2009). Primers and High specificity TaqMan MGB probes (Applied Biosystems) previously reported for the *CYP6* (*CYP6BW5-AIRR8ZM*, *CYP6DG1-AIS07DF*, and *CYP6DJ2-AIRR867*) and *CYP9* (*CYP9Z18-AIT9SJN* and *CYP9Z20-AIQJATE*) genes were used in the qPCR assays (Supp. Table S2) (Cano-Ramirez et al. 2013).

PCRs were carried out in a Step One Real-Time PCR System (Applied Biosystems) under the following conditions: each reaction contained custom TaqMan primers and probes with a final concentration of 900 nM of each primer and 250 nM TaqMan probe, TaqMan Universal Master Mix II, no UNG (Applied Biosystems) 1X, and 5 µl of diluted cDNA sample in a final volume of 20 µl. The manufacturer's standard amplification conditions were used: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 60 s. The results were normalized with *CYP4G55* (AIS065U) as reference gene (Cano-Ramirez et al. 2013). Three technical replicates were performed for each biological replicate. To assess the specificity of the primers, amplicons were analyzed by electrophoresis on 1.5% agarose gels. Calibration curves for the reference and target genes were constructed with five cDNA dilutions, starting from the initial concentration with dilution factor of 1:5 to obtain the amplification efficiencies. The RT-qPCR efficiency and validation of both genes were assessed using a linear regression analysis from the average values obtained in three replicates of the quantification cycles (C_T). The slopes obtained were -3.58 (*CYP6DG1*), -3.56 (*CYP6DJ2*), -3.35 (*CYP9Z20*), -3.57 (*CYP9Z18*), -3.61 (*CYP6BW5*), and -3.62 (*CYP4G55*) with R^2 from 0.98 to 0.99.

Statistical Analyses

Relative expression values of all genes were determined using the C_T ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen 2001). The unfed insects were used as control and the *CYP4G55* gene was used for the data normalization. The $2^{-\Delta\Delta C_T}$ values were transformed to \log_2 for graphing and statistical analyses. To evaluate the significant differences in the expression, a three-way analysis of variance (ANOVA) was performed for each gene with factors sex, feeding time (1, 2, 4, 6, 8, 12, and 18 h), and beetle condition (solitary and paired). When ANOVA was significant, Tukey's post hoc comparisons were performed. All statistical analyses were performed with SigmaStat v3.5 software (Systat Software, Inc., San Jose, CA).

Results

Relative gene expressions (*CYP6BW5*, *CYP6DG1*, *CYP6DJ2*, *CYP9Z18*, and *CYP9Z20*) by exposure time (1, 2, 4, 6, 8, 12, and 18 h), sex (male or female), and experimental condition (solitary or paired) (Fig. 1), and the ANOVA results are reported (Table 1).

General trends in the expression were obtained and described as follows: 1) all *CYP* genes are rapid and continuously induced from 1 to 6 or 8 h independently to the sex and condition; 2) this rapid induction occurs in both sexes regardless of solitary or paired condition; 3) the expression of *CYP6BW5*, *CYP6DG1*, *CYP6DJ2*, and *CYP9Z18* genes decreases in most treatments at 8 h; 4) the *CYP6DJ2* and *CYP6DG1* genes are up-regulated at 12 h and down-regulated at 18 h regardless of sex and condition, in contrast to the *CYP6BW5* and *CYP9Z18* genes that are up-regulated at 12 and 18 h; 5) the relative expression in *CYP9Z20* is up-regulated independently of time, sex, or condition; and 6) for all genes, significant differences between the feeding times were found, as well as feeding time versus condition (solitary and paired) and feeding times versus sex.

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

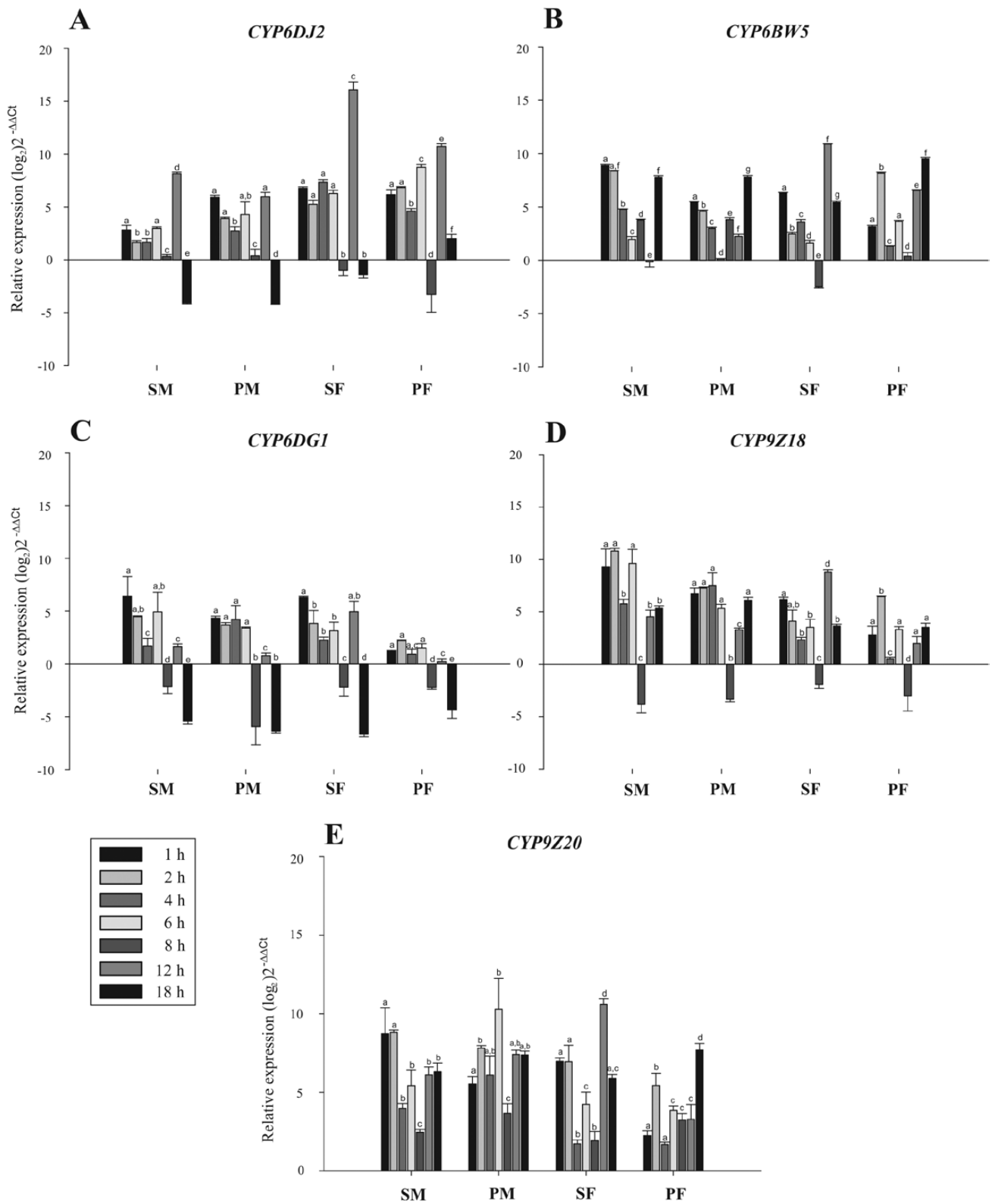


Fig. 1. Relative expression of *CYP6DJ2*, *CYP6BW5*, *CYP6DG1*, *CYP9Z18*, and *CYP9Z20* genes (mean \pm SE) in midgut from male and female solitary and paired of *D. rhizophagus* during the early hours of drilling and settling into the tree's bark. Values > 0 on logarithmic y -axis indicate overexpression. *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 $2^{-\Delta\Delta Ct}$ and SE values were transformed at \log_2 for plotting (SM = solitary male; PM = paired male; SF = solitary female; PF = paired female).

Table 1. Three-way ANOVA results of the relative expression of five *CYP* genes in males and females of *D. rhizophagus* during the early hours of drilling and settling into the tree's bark and, under two different conditions (solitary or paired)

Gene/interaction	df	<i>CYP6DJ2</i>	<i>CYP6BW5</i>	<i>CYP6DG1</i>	<i>CYP9Z18</i>	<i>CYP9Z20</i>
Sex (S)	1	248.161*	4.122	1.147	76.368*	39.906*
Condition (C)	1	0.628	15.496*	21.078*	28.162*	1.393
Feeding time (T)	6	260.775*	744.312*	90.635*	91.505*	23.516*
S*C	1	11.342*	217.031*	1.183	0.430	19.984*
S*T	6	29.083*	539.434*	3.512**	14.008*	4.956*
C*T	6	17.564*	131.299*	3.929*	5.704*	11.098*
S*C*T	6	7.387*	214.095*	3.941*	8.307*	5.273*

F values were statistically significant at **P* < 0.001 and ***P* < 0.05.

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 (Leufvén and Birgersson 1987, Wallin and Raffa 1999, 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 colonization 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. ponderosae* 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*-verbenol 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 (*CYP9T2*) 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*-verbenol—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*-verbenol 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*-verbenol.

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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