Vol. 18(31), pp. 978-990, November, 2019
DOI: 10.5897/AJB2018.16860
Article Number: BB697EC62233
ISSN: 1684-5315
Copyright ©2019
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Expression profiling, phylogenetic, and structural analyses of a laccase gene from the red palm weevil, *Rhynchophorus ferrugineus*

Babiker M. A. Abdel-Banat* and Hamadttu A. F. El-Shafie

Date Palm Research Center of Excellence, King Faisal University, Al-Hofuf, Al-Ahsa 31982, Saudi Arabia.

Received 14 May 2019; Accepted 18 September, 2019

Laccases, member of multicopper oxidase (MCO) family enzymes, play crucial roles in insects' cuticle tanning and pigmentation. The purpose of this study was to identify and characterize a laccase gene from the red palm weevil (RPW), *Rhynchophorus ferrugineus*. The isolated RPW laccase gene sequence was 3,389 bp, including a 2,163 bp open reading frame that encodes 720 amino acids. The RPW laccase gene conserved the MCO functional motifs Type-3, Type-1, and Type-2, respectively. Phylogenetic analysis categorized this protein into the functional cluster 2 of insect laccases (*Lac2*). The primary transcripts for *R. ferrugineus* laccase 2 (*RfeLac2*) were highly expressed in the adult's cuticle, elytra, and hindwings, and in the larval cuticles four days before molting. Then, the transcripts were declined drastically in the larval cuticles three days before molting. This implies that the suppression of *RfeLac2* in the larvae occurs earlier than expected. *RfeLac2* transcripts were very low in the gut and adipose tissues of larvae and adults, irrespective to the span to undergo molting. This suggests that *RfeLac2* is not active in the tissues that do not undergo heavy sclerotization. Three-dimensional (3-D) structure modeling of *RfeLac2* predicted eight histidines, one glycine, and one phenylalanine, as copper-binding ligands on the laccase active center. The study finding indicates that the pattern of the RPW *RfeLac2* expression varies from other coleopteran insects, a phenomenon that requires further investigation.

Key words: Cuticle, expression, laccase, phylogeny, Red palm weevil.

INTRODUCTION

Laccases (EC 1.10.3.2) are metalloenzymes that belong to the multicopper oxidase (MCO) family (Ye et al., 2015). These enzymes catalyze the oxidation of various aromatic substrates with simultaneous reduction of molecular oxygen to water. They lack the monooxygenase activity, but they are able to oxidize *ortho*- and *para*-phenols, meanwhile they catalyze the oxidation of polyphenols, diamines, substituted phenols, and aromatic amines (Riva, 2006). A typical laccase active center contains four copper atoms and ten highly conserved histidines (Shi et al., 2017). Laccases are widely distributed in nature and with broad physiological functions depending on both their origin and on their biochemical and structural properties (Cazares-Garcia et al., 2013). They are present in bacteria, plants, fungi, insects, and marine invertebrates. They function in

*Corresponding author. E-mail: babikera@hotmail.com; bahmed@kfu.edu.sa. Tel: +966 13 589 8749. Fax: +966 13 589 7243.

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

pigmentation, lignin synthesis and degradation, iron homeostasis, sporulation, rhizomorph formation, morphogenesis, and immune defense (Dittmer and Kanost, 2010; Shi et al., 2014). This immense functional versatility is partly because laccases possess low substrate specificity and exhibit a broad range of redox potentials (Giardina et al., 2010).

Several laccase isoforms have been identified and characterized in insects and have been suggested to be involved in cuticle sclerotization and pigmentation (Hattori et al., 2005, 2010; Coy et al., 2010; Yatsu and Asano, 2009; Arakane et al., 2005; Dittmer et al., 2004; Futahashi et al., 2011; Yang et al., 2017). There are two main isoforms of insect laccases identified so far. One of them (laccase 2) has been proven to be involved in sclerotization and pigmentation of cuticles of the red flour beetle *Tribolium castaneum TcLac2* and the pine sawyer *Monochamus alternatus MaLac2* by using RNA interference (RNAi) (Arakane et al., 2005; Niu et al., 2008). Laccase 2 of the mosquito *Culex pipiens pallens CppLac2* was found to induce heavy sclerotization of the cuticle, which could reduce insecticide penetration and thus confer insecticide resistance because a higher level of *CppLac2* mRNA was observed in the insecticide-resistant populations (Pan et al., 2009). The other isoform is laccase 1, which was expressed in the midgut, Malpighian tubules, and fat body as well as in the epidermis of the tobacco hornworm *Manduca sexta*. It may function to oxidize toxic compounds ingested by the insect (Dittmer et al., 2004). There are salivary gland laccases identified in the green rice leafhopper *Nephotettix cincticeps* (Uhler) *NcLac1S* and the whitefly *Bemisia tabaci* MED *BtLac1*. These possibly function in the detoxification of plant phenolic compounds and coagulation of the salivary sheath during feeding (Hattori et al., 2010; Yang et al., 2017). Phylogenetic analysis of genes from seven insect species belonging to four orders led to the identification of putative orthologs of MCO1 (Lac1) and MCO2 (Lac2) in all of the insect genomes examined (Gorman et al., 2008). Whereas, MCO3 (Lac3), MCO4 (Lac4) and MCO5 (Lac5) were found only in *Anopheles gambiae* and other species of mosquito, such as *Aedes aegypti*. The genes in this mosquito-specific cluster share a common ancestor with MCO2 (Gorman et al., 2008).

The red palm weevil (RPW), *Rhynchophorus ferrugineus*, is an invasive and globally important quarantine pest of palm trees. The weevil was introduced to Saudi Arabia from Southeast Asia during the 1980s. It subsequently spread to all Middle East countries and has since migrated into Spain and Southern France (Dembilio and Jaques, 2015; Al-Dosary et al., 2016). Food and Agriculture Organization of the United Nations (FAO) has classified the RPW as category-1 pest on date palm in the Middle East (Al-Dosary et al., 2016). The weevil completes its entire larval life cycle within the palm trunk, which renders detection of its early infestation difficult and its control with the conventional methods have evidenced unsuccessful (Faleiro et al., 2012; Hoddle et al., 2013). Looking for possible alternative control methods, thus, synthetic biology approaches were proposed. Such as disruption of pheromone communication machinery that interrupts the weevil's olfaction to find host and mate, and finally interrupting its reproduction leading to population decline (Antony et al., 2016, 2018; Soffan et al., 2016). Additionally, cuticular proteins such as laccases and others also proposed as important targets for disruption since they function in cuticle hardening to protect insects from environmental stress and mechanical damage. Understanding the structure of the functional motifs of the laccase gene from the RPW and elucidation of its phylogenetic relationship to laccases from other insect species will help to formulate tactics to the utilization of these motifs for further studies aiming at the gene disruption. Therefore, the objective of this study was to isolate laccase gene from the RPW, analyze its functional motifs, and to study its expression profile in the tissues of different developmental stages of the RPW.

MATERIALS AND METHODS

RPW rearing and tissue collection

The RPW, at all developmental stages, was reared in the date palm research center facilities as described previously (Abdel-Banat et al., 2018; El-Shafie et al., 2013). For the purpose of egg-laying, male and female adults were fed on sugarcane kept in TATAY storage boxes (51 cm × 38 cm × 26 cm) with perforated lids. The boxes are made of polypropylene and bisphenol A (BPA) free (www.tatay.com). Eggs were removed from the sugarcane with a brush and placed in Petri dishes that contained cotton and moist filter paper and incubated at 28°C until the eggs hatch. First instar larvae were collected daily and reared on pineapples or date palm trunk. Samples of different developmental stages were collected periodically for the integument and other tissues collection. Larvae were dissected by cutting off their heads using a standard stainless steel entomology dissection set. The integument was cut longitudinally to separate the adipose tissues and the guts. The dissected tissues were immediately frozen in liquid nitrogen. Eggs, elytra, forewings, and the adult's body were directly frozen in liquid nitrogen. All samples were stored at -80°C for the subsequent experiments.

BLAST search and sequence alignment

The online Basic Local Alignment Search Tool (BLAST®) was used to search for potential laccase gene sequences in the RPW Transcriptome Shotgun Assembly (TSA) (Wang et al., 2013; Antony et al., 2016). *T. castaneum* and other insect's laccase gene sequences that are available in the NCBI GenBank® were used to search for similar sequences in the RPW TSA dataset. The identified RPW TSA sequences were pools of unannotated sequences with gaps in the sequenced contigs. Multiple sequence alignment was done using MEGA X (Kumar et al., 2018) software in order to locate the highly conserved signature sequences in the amino acids of known insect laccases. Only RPW TSA contigs that show highly conserved sequences of multicopper oxidase, namely

Table 1. Primers used for *RfeLac2* cDNA synthesis and cloning, sequencing, and semi-quantitative RT-PCR analysis.

Primer name	Sequence (5′→3′)	Purpose
RfLac-1	GCCAAATTTTTTCAGCAGCAGCGCGGTAATA	Full cDNA; RT-PCR
RfLac-2	GAAGATGGACGGCATCTACGGCAGCATC	Sequencing
RfLac-3c	GCATCCAATCGGACAGGAGGATGACGTG	RT-PCR; Sequencing
RfLac-4	CACTTATAACAGGCATTTAGTTGCTCCA	Sequencing
RfLac-5c	GGTGCACATACAGTTGGGACCACAGTCG	Sequencing
RfLac-6	CTATCTTTCGGTGCCATCGGTCTCGGTC	Sequencing
RfLac-7c	GGAGACTGGCGTTGACGCGTCCAAGGAT	Sequencing
RfLac-8c	CTCTTTACAATAATAGAACATCGAAGGAGT	Sequencing
RfLac-9c	ATACATAAATTATAATTTTATTTCTATATCCA	Full cDNA; Sequencing

Types-3, -1, and -2 motifs, were used to synthesize the primers (Table 1), which have been used for amplification of the RPW laccase gene.

RNA isolation and first-strand cDNA synthesis

Frozen tissues from individual RPW larva were ground into fine powder in liquid nitrogen using mortar and pestle. Total RNA was isolated from 50 mg larval and adult tissues using RNeasy Plus Universal Mini Kit (QIAGEN) according to the manufacturer's protocol. The total RNA concentration was measured using NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). Elongase™ enzyme mix was obtained from Invitrogen®. Primers used to amplify the full-length cDNA of laccase, to study its expression pattern, and those for sequencing purpose are shown in Table 1. Reverse transcription of RNA to synthesize the first-strand cDNA for laccase was done using RevertAid RT Kit according to the manufacturer's protocol (Thermo Fisher Scientific). Briefly, 0.5 µg total RNA, 100 µM random hexamer primer, 5× reaction buffer, 20 U RiboLock RNase inhibitor, 20 mM dNTP mix, 200 U RevertAid RT, and nuclease-free H_2O were mixed in a total reaction volume of 20 µl. The mixture was incubated at 25°C for 5 min then followed by 60 min at 45°C. The reaction was terminated by heating at 70°C for 5 min. Double-stranded cDNA was amplified in a total volume of 50 µl using 2 µl from the first-strand cDNA reaction as template, pair of gene-specific primers (10 pmol/µl each), 200 µM each dNTP, 1 µl Elongase™ enzyme mix, and 1.8 mM final [Mg^{2+}]. The thermocycling program was as follows: One cycle for initial denaturation at 94°C for 3 min, followed by 35 cycles for denaturation at 94°C for 2 s, annealing at 57°C for 25 s, and extension at 68°C for 6 min. The program was ended with a final extension cycle for 10 min at 68°C. The thermocycler used for cDNA synthesis and the subsequence amplifications was Veriti® Thermal Cycler (96 well) supplied by Applied Biosystems™. Amplified PCR products were electrophoresed on 0.7% agarose D1 (Pronadisa) gel, stained with ethidium bromide, visualized using INGENIUS Syngene Bio Imaging System, and documented using GeneSnap software from Syngene. Then, the cDNA was purified either from the excised gel using QIAquick® Gel extraction kit (QIAGEN) or directly from the PCR products using the DNA Pure Kit (Geneaid®) following the manufacturers' protocols. The recovered cDNA was used for the subsequent PCR amplification, cloning, or direct sequencing.

Gene cloning and sequencing

The PCR-amplified cDNA was cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. Ligation, cloning, and transformation processes were done according to the standard protocols (Sambrook and Russell, 2001). The manipulated plasmids were transformed into *Escherichia coli* strain DH5α. Plasmids maintained by the bacterium were isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) according to the supplier's instructions. Multiple sequencing rounds were done to clarify the dubious and to read long uncovered sequences. Sequencing was performed at Macrogen service facilities (Seoul, South Korea).

Sequence and domain structure analyses

The deduced amino acid sequences of isolated laccase were analyzed and compared with insect laccases using the BLAST algorithm. Prediction of the signal peptide and the cleavage site was performed with the program SignalP server (http://www.cbs.dtu.dk/services/SignalP-3.0/) (Bendtsen et al., 2004). Phyre2, a protein fold recognition server (Kelley et al., 2015), 3DLigandSite server for ligands prediction (Wass et al., 2010), and InterPro, a protein sequence analysis and classification database server (Mitchell et al., 2019) were used to predict the RPW laccase 3-D structural modeling, ligand-binding sites, and the laccase signature domain structure.

Phylogenetic analysis

Multiple sequence alignment of the deduced amino acids of laccases and the phylogenetic analyses were performed using the software MEGA X (Kumar et al., 2018). Sequences were aligned using ClustralW program integral to the MEGA X software and the phylogenetic tree was constructed by the neighbor-joining method. This analysis involved 104 amino acid sequences from 70 insect species belonging to seven orders. More than five trees were constructed using these sequences on the same program. The sequences (with GenBank® accession numbers) used for the analysis were listed in Supplementary Table 1.

Expression profile of laccase in different developmental stages

The expression profiles of the RPW laccase gene at larval and adult developmental stages were analyzed (Abdel-Banat et al., 2018). Tissues of the middle-aged larvae were collected four days to one day before molting. Adult cuticles and gut tissues as well as forewings (elytra) and hindwings were also used for the laccase

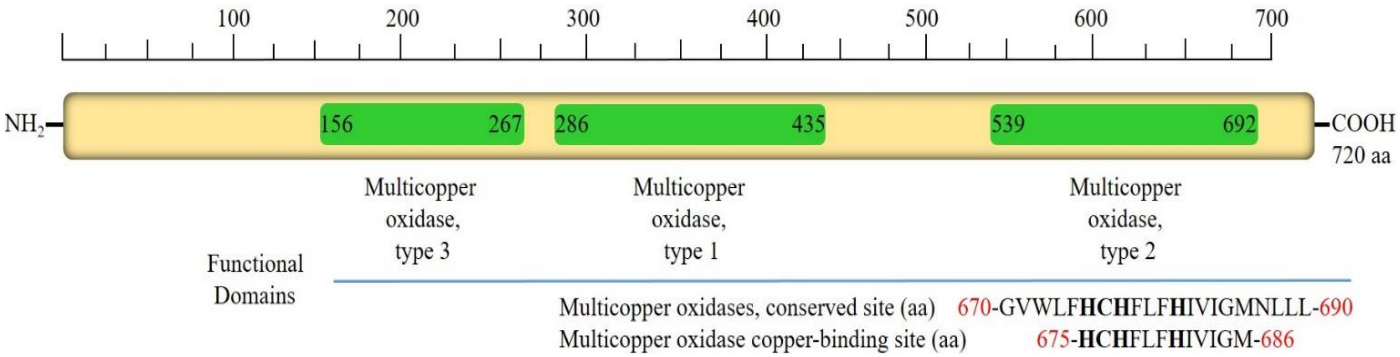

Figure 1. Domain organization of putative RPW *RfeLac2*. The gene encodes 720 amino acids. Functional motifs of MCO Type-3, MCO Type-1, and MCO Type-2 were depicted relative to their positions within the sequence.

expression analysis. Reverse transcription PCR for the laccase gene and the *Rfeβ-actin1* internal reference gene was done in a 25 µl reaction mixtures containing 1 µl template from the first-strand cDNA synthesized from 0.5 µg total RNA, 10 pmol/µl each gene-specific primer, 12.5 µl Master Mix (Biomatik Corporation, Canada), and nuclease-free water. The thermocycler program for RT-PCR was as follows: Initial denaturation cycle at 94°C for 3 min followed by 30 cycles at 94°C for 25 s, 60°C for 25 sec, and 72°C for 2 min, and a final extension cycle at 72°C for 10 min. PCR products were analyzed on 1% agarose gel. Experiments for RT-PCR were replicated at least three times using independent total RNA preparations.

RESULTS

Molecular characterization of *RfeLac2*

A cDNA clone of a laccase gene was isolated from the RPW and the sequence was identified and deposited at the GenBank® database with the accession number (MK655469). The complete mRNA consists of 3,389 base pairs (bp). The open reading frame is 2,163 bp, which encodes a putative protein of 720 amino acids (aa) including a predicted N-terminal signal peptide of 21 aa. Three conserved MCO motifs were found in the putative sequence of *RfeLac2* protein. The amino acids from 156 to 267, from 286 to 435, and from 539 to 692 (Figure 1) exemplify the motifs of MCO Type-3, MCO Type-1, and MCO Type-2, respectively. Within those conserved regions, there are eight histidines (His203, His205, His247, His249, His601, His603, His673, and His675), one glycine (Gly206), one phenylalanine (Phe245), and one cysteine (Cys674) (Figure 2). The sequence $_{111}$-C~C-$_{115}$ at the N-terminal region of *RfeLac2* represents the insect-specific (C-X-R-X-C) sequence commonly found in all identified insect laccases (where X represents any residue). Downstream to the insect-specific sequence is a sequence composed of 24 amino acids ($_{203}$-HWHGIWQKGSQYYDGVPFVTQCPI-$_{226}$) that contain consensus sequences in all laccases (underlined).

A phylogenetic tree was constructed by the neighbor-joining method from 104 amino acid sequences compiled from the GenBank® database to compare with *RfeLac2* (Figure 3). The sequences represent 70 insect species from seven orders (Supplementary Table 1). In general, the phylogenetic tree showed clustering of laccases according to the orders of insects. *RfeLac2* was clustered into the group of insect laccase 2 that includes, for instance, *T. castaneum TcaLac2*, *M. alternatus MalLac2*, *Chrysomela populi CpoLac2*, and *Phaedon cochleariae PcoLac2*. However, the *C. pipiens pallens CppLac2* was clustered in the dipterous laccases clade and the *N. cincticeps NciLac2* was clustered in the hemipterans clade. Whereas group 1 laccases (Lac1) from various insects including *T. castaneum TcaLac1*, *A. gambiae AgaLac1*, *M. sexta MseLac1*, *N. cincticeps NciLac1G* and *NciLac1S*, *Acyrthosiphon pisum ApiLac1*, *B. tabaci BtaLac1*, and *Apis mellifera AmeLac1*, in addition to MCO1 from *Drosophila melanogaster DmeMCO1A*), *Helicoverpa armigera HarMCO1*, and *Bombyx mori BmoMCO1* were clustered together on the phylogenetic tree.

Expression patterns of *RfeLac2*

During and immediately after molting the beetle's exoskeleton and mouthparts are soft and fragile, thus, throughout this period the insect stops feeding. Therefore, adult or larva starts feeding soon after sufficient cuticle sclerotization and the body maintains its physical strength. There is a clear variation in *RfeLac2* transcripts expression pattern in the larval tissues and the adult's body parts (Figure 4). A strong expression level of *RfeLac2* was found in the elytra (forewings), hindwings, and the adult's cuticle, but a weak expression level of the gene was observed in the adult's gut. It is noticeably high *RfeLac2* transcripts in the larval cuticles were observed four days before the start of molting, but very little or hardly detectable transcripts were observed in the gut and fat body of the larvae investigated during the same period. Remarkably, *RfeLac2* transcripts were decreased drastically in all larval tissues three days before the onset

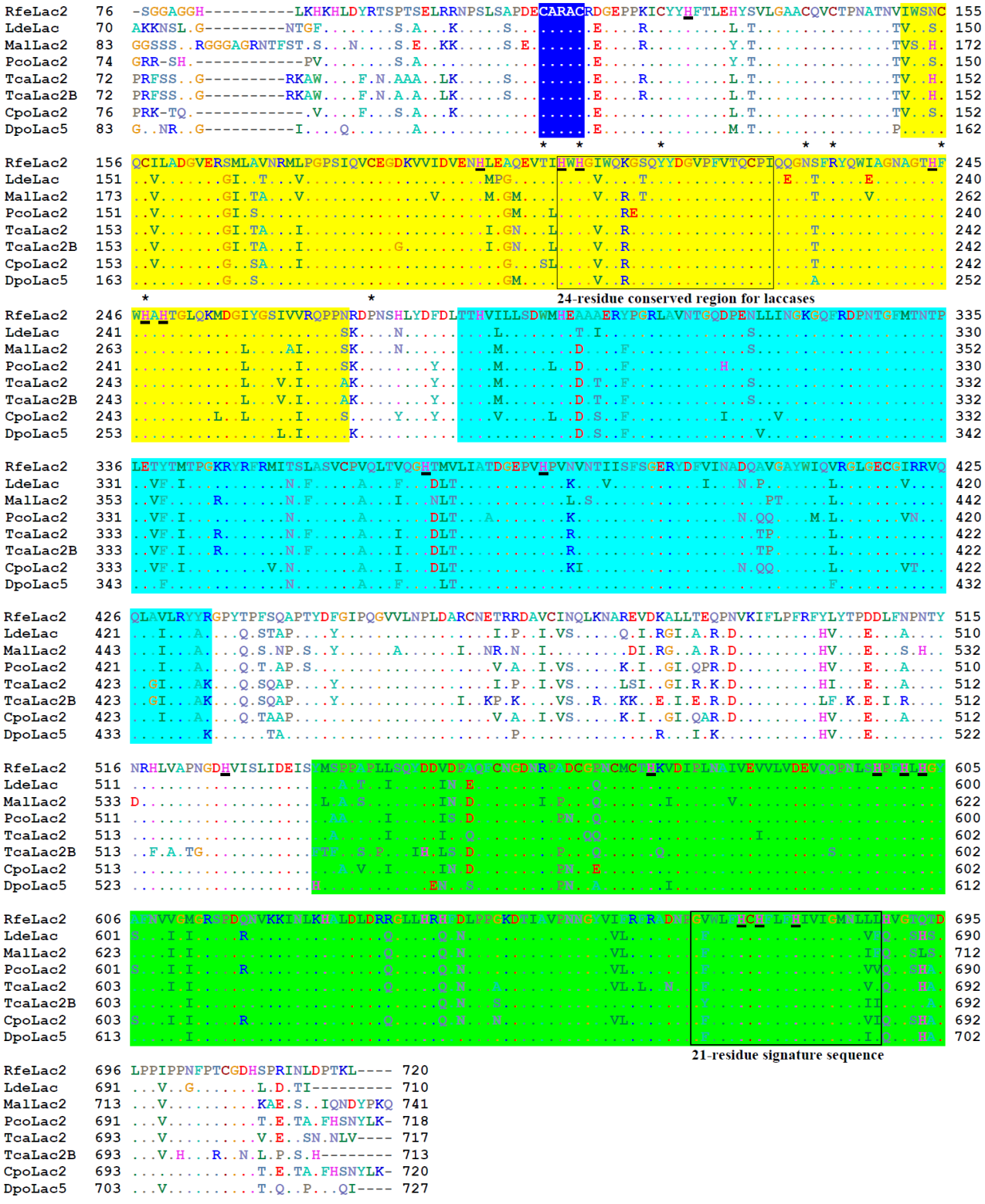

Figure 2. Multiple sequence alignment of the deduced amino acids of laccase 2 genes (*Lac2*). Sequences from eight coleopteran insects were aligned. The insect laccase-specific sequence C-X-R-X-C is highlighted in blue. Asterisks below the sequences indicate cysteine residues conserved at N-terminal in all laccases. MCO Type-3 sequence is highlighted in yellow, Type-1 is highlighted in turquoise, and Type-2 is highlighted in green. The 24-residue conserved region at Type-3 and the 21-residue signature sequence at Type-2 ligand binding sites are boxed. Conserved histidine residues are underlined. *Rfe, Rhynchophorus ferrugineus; Lde, Leptinotarsa decemlineata; Mal, Monochamus alternatus; Pco, Phaedon cochleariae; Tca, Tribolium castaneum; Cpo, Chrysomela populi; Dpo, Dendroctonus ponderosae*.

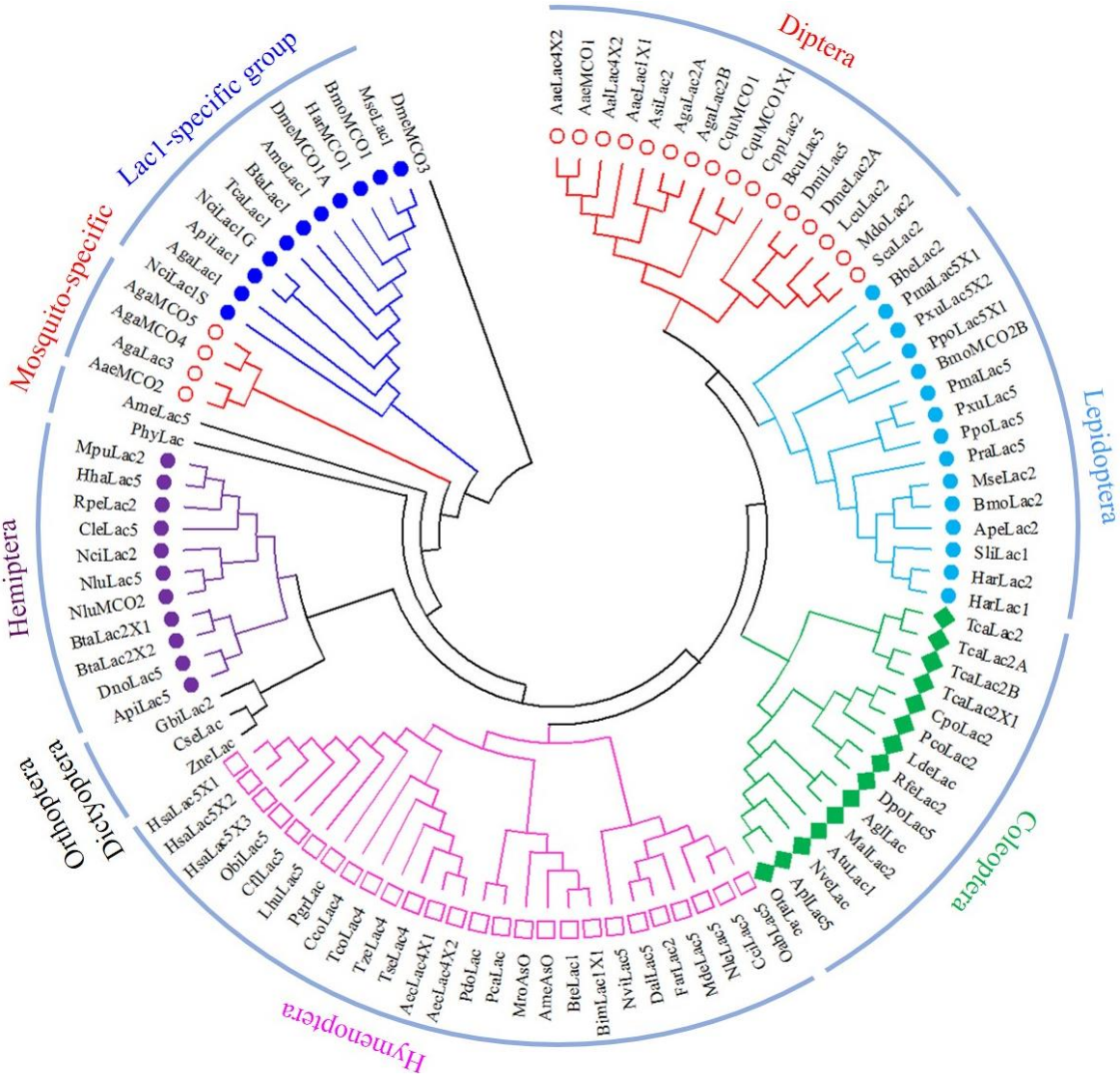

Figure 3. Phylogenetic analysis of insect laccases (Lac) and multicopper oxidases (MCO). The tree was constructed from the deduced amino acid sequences of 104 genes by the Neighbor-Joining method implemented in MEGA X. The evolutionary distances were computed using the Poisson correction method integral to MEGA X software and are in the units of the number of amino acid substitutions per site. The genes that used to construct this tree and their accession numbers are provided in details as Supplementary Table 1.

of molting (Figure 4).

Prediction of *RfeLac2* three-dimensional structure and ligand binding

Three-dimensional (3-D) structure of *RfeLac2* was predicted by the homology modeling approach. Templates used for the prediction were c3ppsD, c3sqrA, c2q9oA, c1zpuE, and c1gycA. Ligands found in the predicted binding site are eight histidine residues, one glycine, and one phenylalanine (Figre 5). The *RfeLac2* Type-3 (residues 156 to 267) and Type-2 (residues from 539 to 692) copper centers were analyzed separately to predict the 3-D structures for these centers. Predicted ligands at Type-3 copper center were four histidine residues, one glycine, and one phenylalanine and those predicted at Type-2 center were four histidine residues (Figure 5). No ligand was predicted for *RfeLac2* Type-1 copper center when analyzed separately (data not shown).

DISCUSSION

In this study, the RPW laccase 2 (*RfeLac2*) cDNA was

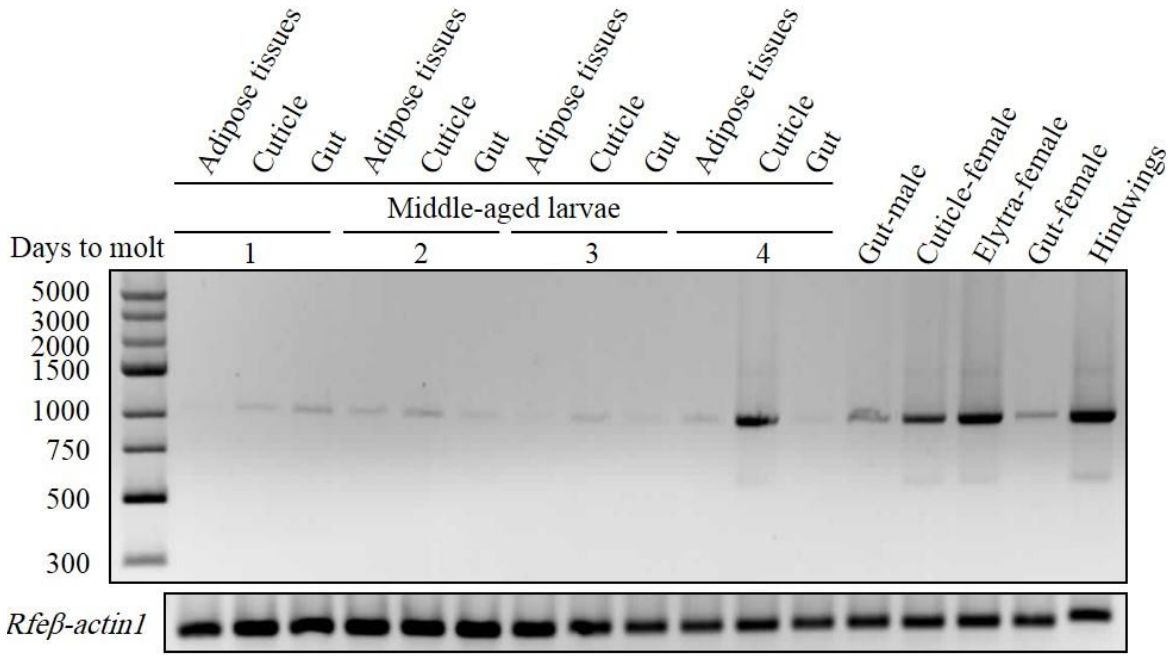

Figure 4. *RfeLac2* expression patterns in the larval and adults tissues. The expression of *RfeLac2* gene was evaluated four- to one-day pre-molting in the adipose tissues, cuticles, and guts of the middle-aged larvae. Likewise, the gene's expression level was investigated in the adult's cuticle, gut, elytra, and hindwings. *Rfeβ-actin1* was used as a reference gene for the RT-PCR.

isolated and its entire sequence was identified. The deduced amino acid sequence of *RfeLac2* shows high identity to those of other insect *laccase 2* genes, particularly to *Lac2* genes of *T. castaneum TcaLac2* (Arakane et al., 2005; Julio et al., 2017), *C. populi CpoLac2* and *P. cochleariae PcoLac2* (Pentzold et al., 2018), *Leptinotarsa decemlineata LdeLac* (Clements et al., 2016), and *M. alternatus MalLac2* (Niu et al., 2008). It also shows high identity to the laccases predicted by automated computational analysis of the genomic sequences of *Dendroctonus ponderosae* and *Anoplophora glabripennis*. The deduced protein from *RfeLac2* contains the MCO conserved regions as described in many insect laccases (Dittmer and Kanost, 2010). The consensus sequence for insect laccases has been defined as HWHG-$(X)_9$-DGVP-$(X)_3$-QCPI, whereas the consensus sequences for fungal and plant laccases have been defined as HWHG-$(X)_9$-DG-$(X)_5$-QCPI and HWHG-$(X)_9$-DGP-$(X)_3$-TQCPI, respectively (Kumar et al., 2003). The first and the last four underlined amino acids of the consensus sequence were common in laccases of insects, fungi, and plants. The sequence ($_{673}$-HCHFLFHIVIGM-$_{684}$) at the C-terminal of *RfeLac2* was conserved in all insect laccases. It is twelve amino acids long representative of Type-2 copper oxidase signature and the same consensus sequence has been defined in fungi as HCH-$(X)_3$-H-$(X)_3$-[A/G]-[L/M] (Kumar et al., 2003). The core of this consensus motif is three histidines and one cysteine.

Phylogenetic analysis of putative insect laccases from 70 species has shown clustering of these proteins according to their respective insect orders with some exceptions. The coleopteran laccase 2 proteins, including *T. castaneum TcaLac2* (Arakane et al., 2005; Jacobs et al., 2015; Julio et al., 2017)*, M. alternatus MalLac2* (Niu et al., 2008)*, Chrysomela populi CpoLac2*, and *Phaedon cochleariae PcoLac2* (Pentzold et al., 2018), were grouped into one clade together with the currently investigated *RefeLac2*. Moreover, the dipterous and hemipterans laccase 2 proteins were grouped into their respective orders despite the fact that many of them have been proven to function in hardening of cuticles, proper morphology, and pigmentation (Pan et al., 2009; Hattori et al., 2010) as do the coleopteran laccase 2. The phylogenetic tree also showed an interesting feature for most functionally characterized insects' laccase 1. This suggests that they probably share common functional properties. The branch of the laccase1-specific group includes laccase 1 and MCO1 proteins from five orders namely Hemiptera, Lepidoptera, Diptera, Hymenoptera, and Coleoptera. This group of enzymes was found to function in the detoxification of secondary plant compounds (Yang et al., 2017) and in iron homeostasis (Dittmer et al., 2004; Lang et al., 2012; Liu et al., 2015). It is notable that the group of MCOs previously described as mosquito-specific (*AgaMCO3-5* and *AaeMCO2*) (Dittmer and Kanost, 2010) was clustered together in a separate branch of the current phylogenetic tree.

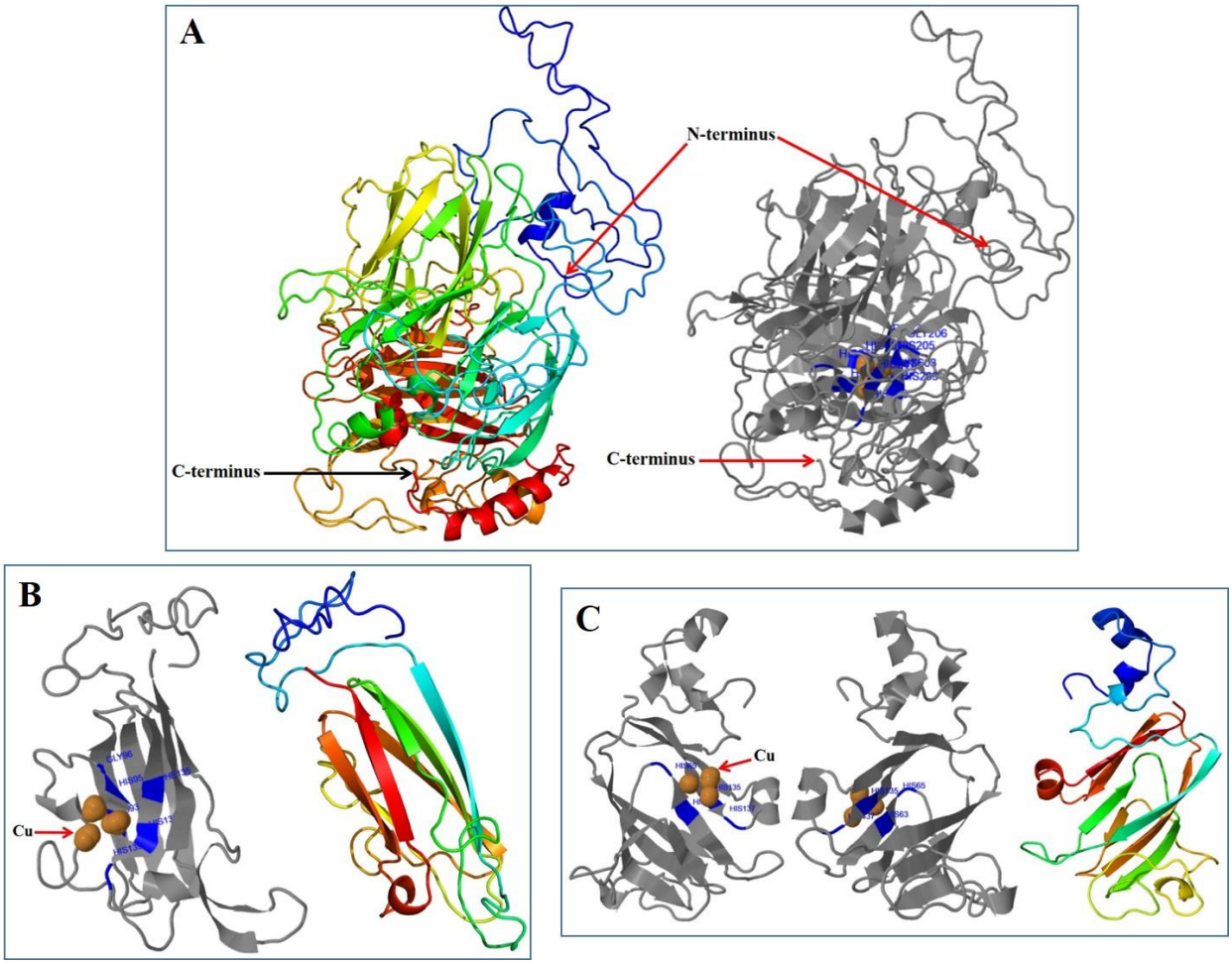

Figure 5. Three-dimensional (3-D) structure of *RfeLac2* and the copper-binding ligands. (**A**) Predicted 3-D structure of *RfeLac2.* Copper appears in the predicted binding site. Ligands in the predicted binding site are eight histidine residues, one glycine, and one phenylalanine (right panel). (**B**) Predicted 3-D structure of multicopper oxidase Type-3 domain of *RfeLac2* (residues 111 to 267) and (**C**) Predicted structure of multicopper oxidase Type-2 domain of *RfeLac2* (residues 539 to 692).

The *RfeLac2* primary transcripts are most abundant in hindwings, elytra, and the cuticle of adult weevil as well as in the cuticle of larvae examined four days before molting. The expression is very low in the adult gut and sharply declined in the cuticle of larvae examined one to three days before molting. Similar expression pattern was observed in the stinkbug, *Riptortus pedestris* (Hemiptera: Alydidae) *RpeLac2* (Futahashi et al., 2011). Thus, the findings from these two studies highlight the expression of *Lac2* peaks days before the larval molting in both species. Contrary to the expression patterns of *RfeLac2* and *RpeLac2*, are the studies, for instance, on *T. castaneum*, *M. sexta*, *B. mori*, and *M. alternatus*, in which the expression of *Lac2* was reported in the epidermis just prior to larval molt (Yatsu and Asano, 2009; Dittmer et al., 2004; Niu et al., 2008). Therefore, the opposite expression patterns of *RfeLac2* and *RpeLac2* relative to the other species, particularly in the larval cuticle, could be attributed to the broad span of the examined samples from the two studies. The expression pattern of the RPW *RfeLac2* suggests that this enzyme promotes the larval cuticle sclerotization, together with other proteins, and its activity gradually diminishes as the larva approach molting in order to facilitate the de-sclerotization of the cuticle making it amenable to the subsequent degrading enzymes.

The *RfeLac2* 3-D structure was predicted on the basis of the topology of the crystal structure of an ascomycete fungal laccases from *Thielavia arenaria* (Kallio et al., 2011) and from *Botrytis aclada* (Osipov et al., 2014), since no crystal structure for insect laccase is available to date. The analysis predicted the basic topology for *RfeLac2*, the copper active centers, and the ligands that bind copper during enzymatic catalysis. Eight histidine residues, a glycine and phenylalanine residues appeared as copper ligands, but the conserved residue cysteine

does not appear as a ligand on the predicted 3-D structure of *RfeLac2*. It was proposed that residues that do not ligate with copper ions were either conserved or semi-conserved to maintain a local 3-D fold (Kumar et al., 2003). This observation might be common to many laccases due to the hidden features of laccases that are not clear by comparison of the amino acid sequences alone or by comparison of the 3-D structures alone (Kumar et al., 2003). It has been reported that Type-1 copper center shows coordination with two histidines, one cysteine, and one methionine as ligands. The Type-2 copper has two histidines and water as ligands. The Type-3 copper coordination with three histidines and a hydroxyl bridge, maintains the strong anti-ferromagnetic coupling between the Type-3 copper atoms (Dwivedi et al., 2011).

Conclusion

On the basis of the results of sequence and phylogenetic analyses, expression profiling, and the 3-D structure modeling, the RPW laccase reported in this study belongs to the group of insect laccase 2. Tissue expression of *RfeLac2* highlights the release of this enzyme earlier than the onset of the RPW larval molting and then suppressed before the beginning of molting process. Further studies on targeting disruption of each functional motif of the *RfeLac2* gene are required for further clarification of the specific role of *RfeLac2* in the RPW during the growth and development.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors acknowledge the financial support provided by the Date Palm Research Center of Excellence (DPRC) through the research grant number DPRC-2(2015).

REFERENCES

Abdel-Banat BMA, El-Shafie HAF, Alhudaib KA, El-Araby WS, Al-Hajhoj MR (2018). Molecular characterization and tissue expression analysis of five genes for chitinase in the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae). African Journal Biotechnology 17:447-457. DOI: https://doi.org/10.5897/AJB2018.16419.

Al-Dosary NMN, Al-Dobai S, Faleiro JR (2016). Review on the management of red palm weevil *Rhynchophorus ferrugineus* Olivier in date palm *Phoenix dactylifera* L. Emirates Journal of Food and Agriculture 28:34-44. DOI: https://doi.org/10.9755/ejfa.2015-10-897.

Antony B, Johny J, Aldosari SA (2018). Silencing the odorant binding protein RferOBP1768 reduces the strong preference of palm weevil for the major aggregation pheromone compound ferrugineol. Frontiers in Physiology 9:252. DOI: https://doi.org/10.3389/fphys.2018.00252.

Antony B, Soffan A, Jakše J, Abdelazim MM, Aldosari SA, Aldawood AS, Pain A (2016). Identification of the genes involved in odorant reception and detection in the palm weevil *Rhynchophorus ferrugineus*, an important quarantine pest, by antennal transcriptome analysis. BMC Genomics 17:69. DOI: https://doi.org/10.1186/s12864-016-2362-6.

Arakane Y, Muthukrishnan S, Beeman RW, Kanost MR, Kramer KJ (2005). Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proceedings of the National Academy of Sciences of the United States of America 102:11337-11342. DOI: https://doi.org/10.1073/pnas.0504982102.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004). Improved prediction of signal peptides: SignalP 3.0. Journal of Molecular Biology 340(4):783-95. DOI: https://doi.org/10.1016/j.jmb.2004.05.028.

Cazares-Garcia S, Vazquez-Garciduenas MS, Vasquez-Marrufo G (2013). Structural and phylogenetic analysis of laccases from *Trichoderma*: A bioinformatic approach. PLoS One 8:e55295. DOI: https://doi.org/10.1371/journal.pone.0055295.

Clements J, Schoville S, Peterson N, Lan Q, Groves RL (2016). Characterizing molecular mechanisms of imidacloprid resistance in select populations of *Leptinotarsa decemlineata* in the Central Sands Region of Wisconsin. PLoS ONE 11:e0147844. DOI: https://doi.org/10.1371/journal.pone.0147844.

Coy MR, Salem TZ, Denton JS, Kovaleva ES, Liu Z, Barber DS, Campbell JH, Davis DC, Buchman GW, Boucias DG, Scharf ME (2010). Phenol-oxidizing laccases from the termite gut. Insect Biochemistry and Molecular Biology 40(10):723-32. DOI: https://doi.org/10.1016/j.ibmb.2010.07.004.

Dembilio Ó, Jaques JA (2015). Biology and management of red palm weevil. In: Sustainable Pest Management in Date Palm: Current Status and Emerging Challenges, Wakil, W., J.R. Faleiro and T.A. Miller, (Eds.). New York, NY; Springer International Publishing pp. 13-36. DOI: https://doi.org/10.1007/978-3-319-24397-9_2.

Dittmer NT, Kanost MR (2010). Insect multicopper oxidases: Diversity, properties, and physiological roles. Insect Biochemistry and Molecular Biology 40:179-188. DOI: https://doi.org/10.1016/j.ibmb.2010.02.006.

Dittmer NT, Suderman RJ, Jiang H, Zhu YC, Gorman MJ, Kramer KJ, Kanost MR (2004). Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae.* Insect Biochemistry and Molecular Biology 34(1):29-41. DOI: https://doi.org/10.1016/j.ibmb.2003.08.003.

Dwivedi UN, Singh P, Pandey VP, Kumar A (2011). Structure-function relationship among bacterial, fungal and plant laccases. Journal of Molecular Catalysis B: Enzymatic 68(2):117-128. DOI: https://doi.org/10.1016/j.molcatb.2010.11.002.

El-Shafie HAF, Faleiro JR, Abo-El-Saad MM, Aleid SM (2013). A meridic diet for laboratory rearing of red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae). Scientific Research and Essays 8(39):1924-1932. DOI: https://doi.org/10.5897/SRE2013.5502.

Faleiro JR, Ben Abdullah A, El-Bellaj M, Al-Ajlan AM, Oihabi A (2012). Threat of red palm weevil, *Rhynchophorus ferrugineus* (Olivier) to date palm plantations in North Africa. Arab Journal of Plant Protection 30(2):274-280. URL: http://www.asplantprotection.org/PDF/.

Futahashi R, Tanaka K, Matsuura Y, Tanahashi M, Kikuchi Y, Fukatsu T (2011). Laccase2 is required for cuticular pigmentation in stinkbugs. Insect Biochemistry and Molecular Biology 41: 191-196. DOI: https://doi.org/10.1016/j.ibmb.2010.12.003.

Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, *et al*. (2010). Laccases: a never-ending story. Cellular and Molecular Life Sciences 67(3):369-85. DOI: https://doi.org/10.1007/s00018-009-0169-1.

Gorman MJ, Dittmer NT, Marshall JL, Kanost MR (2008). Characterization of the multicopper oxidase gene family in *Anopheles gambiae*. Insect Biochemistry and Molecular Biology 38(9):817-24. DOI: https://doi.org/10.1016/j.ibmb.2008.07.001.

Hattori M, Konishi H, Tamura Y, Konno K, Sogawa K (2005). Laccase-type phenoloxidase in salivary glands and watery saliva of the green rice leafhopper. *Nephotettix cincticeps*. Journal of Insect Physiology 51(12):1359-65. DOI: https://doi.org/10.1016/j.jinsphys.2005.08.010.

Hattori M, Tsuchihara K, Noda H, Konishi H, Tamura Y, Shinoda T, Nakamura M, Hasegawa T (2010). Molecular characterization and expression of laccase genes in the salivary glands of the green rice leafhopper, *Nephotettix cincticeps* (Hemiptera: Cicadellidae). Insect Biochemistry and Molecular Biology 40(4):331-338. DOI: https://doi.org/10.1016/j.ibmb.2010.02.009.

Hoddle MS, Al-Abbad AH, El-Shafie HAF, Faleiro JR, Sallam AA, Hoddle CD (2013). Assessing the impact of areawide pheromone trapping, pesticide applications, and eradication of infested date palms for *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) management in Al Ghowaybah, Saudi Arabia. Crop Protection 53:152-160. DOI: https://doi.org/10.1016/j.cropro.2013.07.010.

Jacobs CGC, Braak N, Lamers GEM, van der Zee M (2015). Elucidation of the serosal cuticle machinery in the beetle *Tribolium* by RNA sequencing and functional analysis of Knickkopf1, Retroactive and Laccase2. Insect Biochemistry and Molecular Biology 60:7-12. DOI: https://doi.org/10.1016/j.ibmb.2015.02.014.

Julio AHF, Gigliolli AAS, Cardoso KAK, Drosdoski SD, Kulza RA, Seixas FA, Ruvolo-Takasusuki MC, de Souza CG, Lapenta AS (2017). Multiple resistance to pirimiphos-methyl and bifenthrin in *Tribolium castaneum* involves the activity of lipases, esterases, and laccase2. Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 195:27-43. DOI: https://doi.org/10.1016/j.cbpc.2017.01.011.

Kallio JP, Gasparetti C, Andberg M, Boer H, Koivula A, Kruus K, Rouvinen J, Hakulinen N (2011). Crystal structure of an ascomycete fungal laccase from *Thielavia arenaria* – common structural features of asco-laccases. The FEBS Journal 278(13):2283-2295. DOI: https://doi.org/10.1111/j.1742-4658.2011.08146.x.

Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE (2015). The Phyre2 web portal for protein modeling, prediction and analysis. Nature Protocols 10(6):845-858. DOI: https://doi.org/10.1038/nprot.2015.053.

Kumar S, Phale P, Durani S, Wangikar PP (2003). Combined sequence and structure analysis of the fungal laccase family. Biotechnology and Bioengineering 83(4):386-94 DOI: https://doi.org/10.1002/bit.10681.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018). MEGA X. Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35(6):1547-1549. DOI: https://doi.org/10.1093/molbev/msy096.

Lang M, Braun CL, Kanost MR, Gorman MJ (2012). Multicopper oxidase-1 is a ferroxidase essential for iron homeostasis in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences of the United States of America 109(33):13337-13342. DOI: https://doi.org/10.1073/pnas.1208703109.

Liu X, Sun C, Liu X, Yin X, Wang B, Du M, An S (2015). Multicopper oxidase-1 is required for iron homeostasis in Malpighian tubules of *Helicoverpa armigera*. Scientific Reports 15:14784. DOI: https://doi.org/10.1038/srep14784.

Mitchell AL, Attwood TK, Babbitt PC, Blum M, Bork P et al. (2019). InterPro in 2019: improving coverage, classification and access to protein sequence annotations. Nucleic Acids Research 47 Database issue D351-D360. DOI: https://doi.org/10.1093/nar/gky1100.

Niu BL, Shen WF, Liu Y, Weng H-B, He LH, Mu JJ, Wu ZL, Jiang P, Tao YZ, Meng ZQ (2008). Cloning and RNAi-mediated functional characterization of MaLac2 of the pine sawyer, *Monochamus alternatus*. Insect Molecular Biology 17(3):303-12. DOI: https://doi.org/10.1111/j.1365-2583.2008.00803.x.

Osipov E, Polyakov K, Kittl R, Shleev S, Dorovatovsky P, et al (2014). Effect of the L499M mutation of the ascomycetous *Botrytis aclada* laccase on redox potential and catalytic properties. Acta Crystallographica Section D 70(Pt 11):2913-23 DOI: https://doi.org/10.1107/S1399004714020380.

Pan C, Zhou Y, Mo J (2009). The clone of laccase gene and its potential function in cuticular penetration resistance of *Culex pipiens pallens* to fenvalerate. Pesticide Biochemistry and Physiology 93(3):105-111. DOI: https://doi.org/10.1016/j.pestbp.2008.12.003.

Pentzold S, Grabe V, Ogonkov A, Schmidt L, Boland W, Burse A (2018). Silencing cuticular pigmentation genes enables RNA FISH in intact insect appendages. Journal of Experimental Biology 221(Pt 18). DOI: https://doi.org/10.1242/jeb.185710.

Riva S (2006). Laccases: blue enzymes for green chemistry. Trends in Biotechnology 24(5):219-226. DOI: https://doi.org/10.1016/j.tibtech.2006.03.006.

Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual. 3rd Edn., New York, USA; Cold Spring Harbor Laboratory Press, Cold Spring Harbor. ISBN-13: 978-0879695774.

Shi L, Chan S, Li C, Zhang S (2017). Identification and characterization of a laccase from *Litopenaeus vannamei* involved in anti-bacterial host defense. Fish and Shellfish Immunology 66:1-10. DOI: https://doi.org/10.1016/j.fsi.2017.04.026.

Shi L, Yu H, Dong T, Kong W, Ke M, Ma F, Zhang X (2014). Biochemical and molecular characterization of a novel laccase from selective lignin-degrading white-rot fungus *Echinodontium taxodii* 2538. Process Biochemistry 49(7):1097-1106. DOI: https://doi.org/10.1016/j.procbio.2014.03.028.

Soffan A, Antony B, Abdelazim M, Shukla P, Witjaksono W, Aldosari SA, Aldawood AS (2016). Silencing the olfactory co-receptor rferorco reduces the response to pheromones in the red palm weevil, *Rhynchophorus ferrugineus*. PLoS ONE 11(9):e0162203. DOI: https://doi.org/10.1371/journal.pone.0162203.

Wang L, Zhang XW, Pan LL, Liu WF, Wang DP, Zhang GY, Yin YX, Yin A, Jia SG, Yu XG, Sun GY, Hu SN, Al-Mssallem IS, Yu J (2013). A large-scale gene discovery for the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae). Insect Science 20(6):689-702. DOI: https://doi.org/10.1111/j.1744-7917.2012.01561.x.

Wass MN, Kelley LA, Sternberg MJ (2010). 3DLigandSite: predicting ligand-binding sites using similar structures. Nucleic Acids Research 38(Web Server issue):W469-73. DOI: https://doi.org/10.1093/nar/gkq406.

Yang CH, Guo JY, Chu D, Ding TB, Wei KK, Cheng DF, Wan FH (2017). Secretory laccase 1 in *Bemisia tabaci* MED is involved in whitefly-plant interaction. Scientific Reports 7:3623. DOI: https://doi.org/10.1038/s41598-017-03765-y.

Yatsu J, Asano T (2009). Cuticle laccase of the silkworm, *Bombyx mori*: Purification, gene identification and presence of its inactive precursor in the cuticle. Insect Biochemistry and Molecular Biology 39(4):254-262. DOI: https://doi.org/10.1016/j.ibmb.2008.12.005.

Ye YX, Pan PL, Kang D, Lu JB, Zhang CX (2015). The multicopper oxidase gene family in the brown planthopper, *Nilaparvata lugens*. Insect Biochemistry and Molecular Biology 63:124-132. DOI: https://doi.org/10.1016/j.ibmb.2015.06.010.

Supplementary Table 1. List of laccases (Lacs) multicopper oxidase family enzymes (MCOs) from different insect species and their GenBank® accession numbers

Species	Order	Gene name	GenBank Accession #
Rhynchophorus ferrugineus	**Coleoptera**	Laccase 2 (*RfeLac2*)	MK655469
Tribolium castaneum		Laccase 1 (*TcaLac1*)	AAX84206.1
		Laccase 2 (*TcaLac2*)	NP_001034487.2
		Laccase 2A *(TcaLac2A)*	AAX84202.2
		Laccase 2B (*TcaLac2B*)	AAX84203.2
		Laccase 2 variant X1 (*TcaLac2X1*)	XP_008199220.1
Aethina tumida		Laccase 1-like (*AtuLac1*)	XP_019875844.1
Anoplophora glabripennis		Laccase (*AglLac*)	XP_018575474.1
Nicrophorus vespilloides		Laccase-like (*NveLac*)	XP_017781263.1
Leptinotarsa decemlineata		Laccase (*LdeLac*)	XP_023022290.1
Agrilus planipennis		Laccase 5 (*AplLac5*)	XP_018324480.1
Onthophagus taurus		Laccase (*OtaLac*)	XP_022900258.1
Monochamus alternatus		Laccase 2 (*MalLac2*)	ABU68466.1
Dendroctonus ponderosae		Laccase 5 (*DpoLac5*)	XP_019754547.1
Chrysomela populi		Laccase 2 (*CpoLac2*)	AWK23445.1
Phaedon cochleariae		Laccase 2 (*PcoLac2*)	AWK23446.1
Culex quinquefasciatus	**Diptera**	Multicopper oxidase 1 (*CquMCO1*)	XP_001867157.1
		Multicopper oxidase 1 variant 1 (*CquMCO1X1*)	XP_001861600.1
Culex pipiens pallens		Laccase 2 (*CppLac2*)	ACG63789.1
Aedes aegypti		Laccase 1 variant X1 (*AaeLac1X1*)	XP_021698133.1
		Laccase 4 isoform X4 (*AaeLac4X2*)	XP_021698134.1
		Multicopper oxidase 1 (*AaeMCO1*)	AAY29698.1
		Multicopper oxidase 2 (*AaeMCO2*)	AAY32604.1
Aedes albopictus		Laccase 4 isoform X2 (*AalLac4X2*)	XP_019553181.1
Anopheles sinensis strain LS-WX		Laccase 2 (*AsiLac2*)	ARG47519.1
Musca domestica		Laccase 2 (*MdoLac2*)	XP_005177649.2
Anopheles gambiae		Laccase 1 (*AgaLac1*)	AAN17505.1
		Laccase 2 isoform A (*AgaLac2A*)	AAX49501.1
		Laccase 2 isoform B (*AgaLac2B*)	AAX49502.1
		Laccase 3 (*AgaLac3*)	ABQ95972.2
		Multicopper oxidase 4 (*AgaMCO4*)	ABY84643.1
		Multicopper oxidase 5 (*AgaMCO5*)	ABY84644.1
Stomoxys calcitrans		Laccase 2 (*ScaLac2*)	XP_013106835.1
Lucilia cuprina		Laccase 2 (*LcuLac2*)	XP_023306400.1
Bactrocera (Zeugodacus) cucurbitae		Laccase 5 variant X1 (*BcuLac5X1*)	XP_011177989.1
Drosophila miranda		Laccase 5 (*DmiLac*5)	XP_017149067.1
Drosophila melanogaster		Multicopper oxidase 1 isoform A (DmeMCO1A)	NP_609287.3
		Laccase 2 (DmeLac2A)	NP_724412.1
		Multicopper oxidase 3 (DmeMCO3)	NP_651441.1
Manduca sexta	**Lepidoptera**	Laccase 1 (*MseLac1*)	AAN17506.1
		Laccase 2 (*MseLac2*)	AAN17507.1
Helicoverpa armigera		Laccase 1 (*HarLac1)*	XP_021185007.1
		Laccase 2 (*HarLac2*)	AHA15412.1
		Multicopper oxidase 1 (*HarMCO1*)	KP318028
Pieris rapae		Laccase 5 (*PraLac5*)	XP_022124207.1

Supplementary Table 1. Contd.

Antheraea pernyi		Laccase 2 (*ApeLac2*)	AII19522.1
Papilio machaon		Laccase 5 (*PmaLac5*)	NP_001303942.1
		Laccase 5 variant X1 (*PmaLac5X1*)	XP_014370419.1
Papilio polytes		Laccase 5 (*PpoLac5*)	NP_001298599.1
		Laccase 5 variant X1 (*PpoLac5X1*)	XP_013146294.1
Papilio xuthus		Laccase 5 (*PxuLac5*)	NP_001298899.1
		Laccase 5 variant X2 (*PxuLac5X2*)	XP_013180620.1
Spodoptera litura		Laccase 1 (*SliLac1*)	XP_022819652.1
Biston betularia		Laccase 2 (*BbeLac2*)	AEP43806.1
Bombyx mori		Multicopper 1 (*BmoMCO1*)	DAA06286.1
		Multicopper 2 isoform B (*BmoMCO2B*)	DAA06287.1
		Laccase (*BmoLac2*)	ABU68465.1
Acyrthosiphon pisum	**Hemiptera**	Laccase 1 (*ApiLac1*)	XP_003241886.1
		Laccase 5 (*ApiLac5*)	XP_001950788.1
Nephotettix cincticeps		Laccase 1 isoform G (*NciLac1G*)	BAJ06132.1
		Laccase 1 isoform S (*NciLac1S*)	BAJ06131.1
		Laccase 2 (*NciLac2*)	BAJ06133.1
Bemisia tabaci		Laccase 2 variant X2 (*BtaLac2X2*)	XP_018913180.1
		Laccase 2 variant X1 (*BtaLac2X1*)	XP_018913179.1
		Laccase 1 (*BtaLac1*)	AQY62684.1
Cimex lectularius		Laccase 5 (*CleLac5*)	XP_014240544.1
Nilaparvata lugens		Multicopper oxidase 2 (*NluMCO2*)	AKN21380.1
		Laccase (*NluLac5*)	XP_022184002.1
Riptortus pedestris		Laccase 2 (*RpeLac2*)	BAJ83487.1
Halyomorpha halys		Laccase 5 (*HhaLac5*)	XP_014271851.1
Diuraphis noxia		Laccase 5 (*DnoLac5*)	XP_015374008.1
Megacopta punctatissima		Laccase 2 (*MpuLac2*)	BAJ83488.1
Orussus abietinus	**Hymenoptera**	Laccase 5 (OabLac5)	XP_023290784.1
Cephus cinctus		Laccase-5-like (CciLac5)	XP_015602372.1
Fopius arisanus		Laccase 2 (FarLac2)	XP_011307332.1
Apis mellifera		Laccase 1 (AmeLac1)	XP_026295929.1
		Laccase 5 (AmeLac5)	XP_625189.3
		L- Ascorbate oxidase (AmeAsO)	XP_006562317.1
Diachasma alloeum		Laccase 5 (DalLac5)	XP_015111370.1
Neodiprion lecontei		Laccase 5 (NleLac5)	XP_015522336.1
Megachile rotundata		L-Ascorbate oxidase (MroAsO)	XP_012134606.1
Bombus impatiens		Laccase 1 variant X1 (BimLac1X1)	XP_003490974.1
Bombus terrestris		Laccase 1 (BteLac1)	XP_003399477.1
Harpegnathos saltator	**Hymenoptera**	Laccase 5 variant X1 (*HsaLac5X1*)	XP_011142481.1
		Laccase 5 variant X2 (*HsaLac5X2*)	XP_011142482.1
		Laccase 5 variant X3 (*HsaLac5X3*)	XP_011142483.1
Microplitis demolitor		Laccase 5 (*MdeLac5*)	XP_008557222.1
Cyphomyrmex costatus		Laccase 4 (*CcoLac4*)	XP_018394374.1
Trachymyrmex cornetzi		Laccase 4 (*TcoLac4*)	XP_018363669.1
Polistes canadensis		Laccase (*PcaLac*)	XP_014599609.1
Trachymyrmex zeteki		Laccase 4 (*TzeLac4*)	XP_018302362.1
Acromyrmex echinatior		Laccase 4 variant X1 (*AecLac4X1*)	XP_011062541.1
		Laccase 4 variant X2 (*AecLac4X2*)	XP_011062542.1
Ooceraea biroi		Laccase 5 (*ObiLac5*)	XP_011336181.1

Supplementary Table 1. Contd.

Trachymyrmex septentrionalis		Laccase 4 (*TseLac4*)	XP_018346813.1
Pseudomyrmex gracilis		Laccase (*PgrLac*)	XP_020298349.1
Nasonia vitripennis		Laccase 5 (*NviLac5*)	XP_016843007.1
Polistes dominula		Laccase (*PdoLac*)	XP_015186385.1
Camponotus floridanus		Laccase 5 (*CflLac5*)	XP_011259955.1
Linepithema humile		Laccase (*LhuLac5*)	XP_012216530.1
Pimpla hypochondriaca		Laccase (*PhyLac*)	CAD20461.1
Zootermopsis nevadensis	**Dictyoptera**	Laccase (*ZneLac*)	XP_021934069.1
Cryptotermes secundus		Laccase (*CseLac*)	XP_023707482.1
Gryllus bimaculatus	**Orthoptera**	Laccase 2 (*GbiLac2*)	BAM09185.1