LACCASE-1 IN THE PEA APHID, *ACYRTHOSIPHON PISUM* (HARRIS)

by

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ABSTRACT

Laccases belong to the "multicopper oxidase" family of proteins, and can oxidize *o*-diphenols and *p*-diphenols in the presence of molecular oxygen. Laccases have been well characterized in wood-rotting fungi where they appear to play a role in lignin degradation, morphogenesis, and stress defense. More recently, laccase-2 has been found to play a role in the insect cuticle sclerotization and tanning. In addition, it has been hypothesized that laccase-1 may be involved in the oxidation of toxic phenolic compounds ingested by insects during feeding. A laccase-type phenoloxidase has been identified in the pea aphid, *Acyrthosiphon pisum* (Harris) using a combination of substrates that react with laccase. Within the pea aphid, laccase-1 transcript was found to be localized within the gut and the salivary glands. Finally, the specific regions where laccase-1 was present in the salivary gland was visualized using immunohistochemistry.

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Acknowledgements

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CHAPTER 1 - INTRODUCTION

Aphids are a diverse group of plant-feeding insects that belong to the family Aphididae of the order Hemiptera (Blackman and Eastop 2000). They are predominantly found in temperate climate zones such as North America, Europe, and Central and East Asia (Blackman and Eastop 2000). Out of the approximately 4000 species of aphids that have been described, over 250 feed on various agricultural and horticultural crops throughout the world (Blackman and Eastop 2000). Most species of aphids do not cause physical damage to their host plants, although some aphids cause necrosis on the plant at the site of feeding, resulting in the formation of galls (Blackman and Eastop 2000). Some species of aphids also cause indirect damage to plants by acting as a vector of several viruses (Miles 1999, Blackman and Eastop 2000, Stacey and Fellowes 2002).

Aphids are considered major pests and understanding their molecular biology, interactions with host plants and natural enemies is of immediate economic interest (Blackman and Eastop 2000, Stacey and Fellowes 2002). In the absence of viral transmission, it has generally been assumed that aphid saliva is the causative agent of damage to plant tissues (Miles 1999).

Phloem feeders such as aphids have been shown to elicit a different plant response compared to chewing insects (Voelckel et al. 2004). For instance, it has been shown that after *Macrosiphium euphorbiae/Myzus persicae* attack, lipogenase and the pathogenesis-related protein P4 (PR1) was strongly elicited but proteinase inhibitor (PI) II was not. On the other hand, an attack by *Helicoverpa zea* induced the opposite response (Voelckel et al. 2004). It was found that attack by *M. persicae* induced the transcriptional signatures of salicylic acid signaling

(apoplastic β-1,3,-glucanase, PR-1), and an increase in phenylalanine ammonia lyase (PAL1) and monosaccharide symporter mRNAs, which suggests an imbalance of phenolics and sugars at the wound site as a result of sequestration of phenolics in saliva and/or stylet sheaths (Voelckel et al. 2004). Thus, knowledge of the biochemical nature and physiological function of aphid saliva (and, by extension, the salivary gland) could aid in the genetic engineering of plants that are resistant to aphids (Miles 1999).

Morphology of aphid salivary glands

Aphid salivary glands are paired, and the right and left gland each consist of a large bi-lobed principal salivary gland and a smaller spherical accessory gland. The salivary ducts of right and left glands join to form a common duct leading to the salivary canal (Weidemann 1968, Ponsen 1972). The principal salivary gland is innervated while the accessory gland not (Tjallingii 2006). The principal salivary gland is thought to be made up of two major components: the *Deckzellen* (or "cover cells") and the *Hauptzellen* (or "main cells") (Fig. 1). The *Deckzellen* are situated at the anterior region of salivary gland, while the *Hauptzellen* and situated in the posterior region. Comparatively, the *Hauptzellen* are more opaque, and thus the two regions are clearly distinguishable. This difference between the *Deckzellen* and *Hauptzellen* has been described in several aphid species (Ponsen 1972). According to Ponsen (1972), each lobe of the principal salivary gland of *Myzus persicae* contains 6 *Deckzellen* and 15 *Hauptzellen*. These cells can be further subdivided into different cell types, according to the shape of the cell, the type of cytoplasm, as well as the size of the nucleus and nucleolus. By Ponsen's (1972) classification, the *Deckzellen* can be broken down into 2 cell types (types 1, 2), while the *Hauptzellen* can be broken down into 6 different types (types 3 – 8). On the other hand, Weidemann (1968)

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described 2 different cell types in the *Deckzellen* (types H, I) and 7 different cells types in the *Hauptzellen* (types A – G).

It is worth noting that the cells in the principal salivary glands are extremely large when compared to the duct cells. Using Ponsen's (1972) measurements of *M. persicae* salivary gland cells, the estimated volume of the duct cells was only approximately $30\mu m^3$. In comparison, the smallest cells in the principal salivary gland (types 1 and 6) each had a rough volume of $750\mu m^3$. The largest cell type (type 3) had a rough volume of $2400\mu m^3$.

The fact that principal salivary glands cells vary morphologically suggests that each type may have specialized functions. Some evidence supporting this comes from Miles (1964) who stained the salivary glands of the aphid species *Macrosiphum euphorbiae* and *Aphis nerii* with phenolase substrates (DOPA and catechol) and found that the phenol oxidase activity was localized within a subset of cells in the principal salivary gland. Using antibodies against *Schizaphis graminum* salivary proteins SP154 and SP66/69 (numbers correspond to molecular size of proteins detected in aphid diet and sheaths), Cherqui and Tjallingii (2000) visualized the locations of the antisera using immunohistochemistry and found that these proteins were localized in only the posterior parts of the principal salivary gland. In addition, Sogawa (1968) found that phenolase activity in salivary glands of the plant leafhopper species *Nephotettix cincticeps* and *Inazuma dorsalis* was also localized in a specific subset of cells. This suggests that the specialization of secretory cells in the salivary glands is widely found throughout the order Hemiptera.

Composition of aphid saliva

Two types of aphid saliva have been postulated, although there is no experimental evidence that draws a clear distinction between the two in the sense of their composition. First,

there is the "gelling saliva" which is postulated to consist of a viscous mixture of stylet sheath precursors and rapidly solidifies in the presence of air or aqueous media, and "watery saliva," which is secreted into plant tissues during feeding (Tjallingii 2006, Miles 1999). Salivary enzymes have been categorized into phenoloxidases, pectinases, peroxidases, and glucosidases (Cherqui and Tjallingii 2000, Ma et al. 1990, Baumann and Baumann 1995, Miles 1999). It has been suggested that phenoloxidases have a detoxifying role in plant tissues while glucosidases hydrolyze phenolic glycosides and other oligosaccharides upon ingestion from the phloem (Miles 1999). Pectinases have been previously detected from salivary secretions in the greenbug and have been postulated to aid the insect in its feeding by breaking down pectin (Ma et al. 1990). The activity and even occurrence of these enzymes differ widely among aphid species. For instance, peroxidase activity has been detected in the salivary sheaths of *Acyrthosiphon pisum* and *Myzus persicae*, but not *Schizaphis graminum* (Cherqui and Tjallingii 2000). Miles and Oertli (1993) proposed a model of interaction between aphids and their host plants called the "redox hypothesis." This hypothesis will be described in detail below.

Aphid feeding behavior

After an aphid has chosen a suitable host plant for feeding, it uses its stylet to probe beyond the epidermis into mesophyll and parenchyma tissues (Tjallingii 2006, Powell et al. 2006). Aphid stylets are thin, needle-like appendages formed by the mandibles and maxilla. The mandibular stylets enclose the maxillary pair, which are always interlocked and appear as a single structure enclosing two minute canals. The food canal (0.7 µm in diameter) is larger than the salivary canal (0.3 µm in diameter) (Ponsen 1987). The aphid stylet penetrates the host plant cells intracellularly *en route* to the phloem and gelling saliva is continually secreted as the aphid probes deeper into the plant tissue, forming a sheath around the stylet (Miles 1993, Tjallingii

2006). This salivary sheath remains in the plant even after the stylet has been withdrawn (Tjallingii 2006). The aphid stylet eventually reaches the vascular bundle and penetrates a phloem sieve element. The aphid then begins to secrete saliva into the plant cell. The saliva is believed to inhibit defensive phloem-sealing and other defense mechanisms and allow for sustainable sap extraction (Tjallingii 2006, Powell et al. 2006). Research on aphid salivation and feeding behavior has been done mainly though electrical penetration graph (EPG) studies (Tjallingii 2006). Once the stylet tip has penetrated the phloem, the aphid secretes saliva into the sieve element for about 1 minute. The period is known as the E1 phase (Tjallingii 2006). Following the E1 phase, the aphid begins to ingest phloem sap while continuing salivation. It is believed that during this phase, the saliva does not reach the plant; rather, it is sucked into the food canal along with the phloem sap by hydrostatic pressure in the sieve elements. This period is known as the E2 phase (Tjallingii 2006). During feeding, the E1 phase always preceeds E2. The aphid may also return to the E1 after E2, or alternate between the 2 phases depending on the sieve element (Tjallingii 2006).

The redox hypothesis in aphid-plant interactions

The presence of oxidases, such as catechol oxidase and peroxidase, in the saliva of piercing-sucking insects presents a paradox because similar enzymes are found in their host plants where they play a defensive role against herbivorous insects (Miles and Oertli 1993, Miles 1999). In plants, phenolic substrates are kept in a reduced form by antioxidants such as glutathione and ascorbic acid (Miles and Oertli 1993). As part of the plant wound response mechanism these monomeric phenolics are oxidized into quinones in the presence of phenol oxidases (Miles and Oertli 1993, Miles 1999). Along with monomeric phenols, quinones are believed to be toxic to insects because of their potential to copolymerize with proteins (Miles and

Oertli 1993). If phenolic compounds are oxidized rapidly in the absence of proteins, they can form phenolic polymers that are non-toxic and insoluble (Peng and Miles, 1988). The phenol oxidases found in aphid saliva have been shown to convert toxic monomeric phenols into non-toxic polymers in artificial diets (Peng and Miles 1988, Miles 1999). When aphids ingest plant sap, the phenolics are presumably no longer kept in a reduced state by antioxidants and are free to react with proteins in the aphid (Miles 1999). Therefore, it has been proposed that phenol oxidases in aphid saliva help oxidize potentially toxic phenolic compounds into nontoxic polymers before it reaches the midgut. This hypothesis is known as the "redox hypothesis" (Miles and Oertli 1993, Miles 1999). I will discuss it further in my conclusions.

Laccases

Laccases (EC 1.10.3.2) are polyphenol oxidases that belong to the "multicopper oxidase" family of proteins (Baldrian 2006, Hoegger et al. 2006, Claus 2003). Other enzymes that belong to the same family include ferrioxidases, ascorbate oxidase, and ceruloplasmin (Hoegger et al. 2006). Laccases have a broad substrate range, and so it is difficult to classify them according to their reducing substrates, which vary from one laccase to another and overlap with the substrate range of another oxidoreductase, tyrosinase (EC 1.14.18.1) (Baldrian 2006, Claus 2003). In general, tyrosinase catalyzes the hydroxylation of monophenols and the oxidation of *o*-diphenols but not *p*-diphenols, whereas laccase catalyzes the oxidation of both types of diphenols, but not the hydroxylation of monophenols (Thomas 1989). In addition, laccase also oxidizes aminophenols, methoxyphenols, diamines and anilines (Hakulinen et al. 2002, Thomas 1989). Both laccase and tyrosinase transfer electrons from a substrate to atmospheric oxygen, while another oxidoreductase, peroxidase, requires the presence of hydrogen peroxide to transfer

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electrons (Claus 2003). Typically, for catalytic activity laccases (like other multi-copper oxidases) have at least 4 copper ions per active protein unit. Three different types of copper ions have been identified, differing in light absorbance, and electron-paramagnetic behavior (Claus 2003). In a redox reaction, electrons from the substrate are accepted in the mononuclear center (type 1 copper atom), and are transferred to the trinuclear cluster (one type 2 and two type 3 copper atoms), which acts as the dioxygen binding site and reduces the molecular oxygen upon receipt of the four electrons (Hoegger et al. 2006).

Laccases have been particularly well characterized in wood-rotting fungi where they appear to play a major role in lignin degradation. Fungal laccases have also been associated with morphogenesis, fungal plant-pathogen/host interaction and stress defense. (Baldrian 2006, Hoegger et al. 2006). In plants, it has been suggested that laccases are involved in radical-based mechanisms of lignin polymer formation (Baldrian 2006). The existence of laccases in prokaryotes is still somewhat controversial at this point, although laccase-like enzymes have been found in both gram negative and gram positive bacteria (Baldrian 2006, Claus 2003). Laccase has also been identified as a virulent factor in the human pathogen, *Cryptococcus neoformans* (Williamson 1997).

Because laccases are able to oxidize a wide range of substrates, they have received substantial commercial interest in the areas of paper pulp bleaching, detoxification of textile dyes, removal of phenolic compounds from wine, use as biosensors, and detoxification of pesticides and polycyclic aromatic hydrocarbons (Baldrian 2006, Dittmer et al. 2004). As the most abundant ligninolytic enzyme in soil, laccases have also attracted the interest of ecologists interested in the role of laccase in the ecosystem. It has been found that a significant decrease of laccases and peroxidases in forest soils resulted in elevated nitrogen doses, along with a

simultaneous increase in the litter layer. On the other hand, an increase of phenolic compounds in forest soil after burning resulted in an increase in laccase activity (Baldrian 2006).

Insect laccases

More recently, the functional roles of laccases in insects have been investigated. A feature that is unique to insect laccases (relative to fungal and plant laccases) is a longer amino-terminal sequence characterized by a region with conserved cysteine, aromatic and charged residues (Dittmer et al. 2004). In insects, several forms of laccases have been identified. "Laccase-1" and "laccase-2" have been identified in *Manducca sexta* and *Tribolium castaneum* Arakane et al. 2005, Dittmer et al. 2004)*.* In *Anopheles gambiae,* 5 different forms of laccases have been identified (Dittmer et al. 2004). Alternative splicing in the laccase-2 gene gives rise to two different isoforms, laccase-2A and laccase-2B (Arakane et al. 2005) (Fig. 2). In *Tribolium castaneum*, the genomic sequence encoding the C-terminus of the laccase-2 gene consists of 2 sets of alternative exons with three exons in each set, which results in the production of two different laccase-2 transcripts. These transcripts encode for proteins of 717 and 712 residues, with 74% identity in the alternate C-terminal regions (Arakane et al. 2005). Laccase-2A and 2B isoforms have also been documented *Drosophila melanogaster* and *Anopheles gambiae* (Arakane et al. 2005).

Phylogenetic analysis of insect laccases (based on an alignment from a region of approximately 280 amino acid residues from the amino-terminal region of these insect laccases again plant and fungi laccases) revealed that laccase-1 across several insect species clustered together, while laccase-2 across species clustered together (Dittmer 2004) (Fig. 3, 4). The sequence similarity between laccase-1 and laccase-2 in insects varies considerably, with

identities as high as 80% in some pairs, to less than 40% in other pairs. For instances, in *Manduca sexta*, laccase-1 and laccase-2 only have 36% sequence identity (Dittmer et al. 2004).

Expression of laccases differs from organ to organ. In *Manduca sexta*, it has been found that laccase-2 mRNA was more abundantly expressed the epidermis in a controlled manner, while laccase-1 mRNA was highly expressed in both the epidermis, midgut and Malpighian tubules. It has been suggested that the primary role of laccase 2 is the oxidation of catechols for protein cross-linking during sclerotization, while the suggested role for laccase-1 is the oxidation of toxic compounds ingested by the insect during feeding (Dittmer et al. 2004). These hypothesizes have been reinforced by work done by Arakane et al. (2005) and Hattori et al. (2005).

Arakane et al. (2005) recently discovered recently that laccase-2 was the phenoloxidase gene responsible for beetle cuticle tanning, and not tyrosinase or laccase-1. When tyrosinase or laccase-1 transcript was knocked down via RNA interference (RNAi) in larval *Tribolium castaneum*, the insect could still successfully sclerotize and molt into adults. However, when laccase-2 was knocked down, cuticle tanning could not be completed and resulted in death of the insect.

Hattori et al. (2005) claimed finding laccase in the salivary glands of the green rice leafhopper, *Nephotettix cincticeps.* Using a combination of various substrates for laccase, it was determined that laccase was localized within the "V" cells of the posterior lobe of the salivary gland of the insect. They postulated that salivary laccase from the green rice leafhopper leads to the rapid oxidization of toxic monolignols, resulting in the formation of nontoxic polymers that allows the insect to feed successfully (Hattori et al. 2005).

Work described in this study

The research presented in this dissertation was intended to verify the presence of laccase-1 in the salivary glands and guts of the pea aphid, *Acyrthosiphon pisum,* using a variety of molecular techniques. Once it was established that laccase-1 is present in the salivary glands, the location of the enzyme within the glands was visualized with substrate staining and immunohistochemistry.

CHAPTER 2 - MATERIALS

Chemicals

Agarose, Enzyme grade (high melting): Fisher Scientific

2,2'-Amino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS): Sigma

Coomassie Brilliant Blue R: Sigma Chemical Company

Cy-3 conjugated goat anti-rabbit secondary antibodies: Jackson Immuno Research

3,4'-Dihydroxy-L-phenylanaline (L-DOPA): Sigma

DEPC Treated Water, EDTA-free: Ambion

DNase I/DNase I buffer: Ambion

Ethidium Bromide: Sigma Chemical Company

Filter Paper 3MM: Whatman

Gel/Mount^{TM} Mounting Media: Biomeda corp

Immobilon® Transfer Membrane: Millipore

β-mercaptoethanol: Sigma Chemical Company

Methanol: Fisher Scientific

PCR Master Mix: Promega

Prestained SDS-PAGE standards, Low Range (Control: 300001357): Bio-Rad

Sodium dodecyl sulfate (SDS), electrophoresis grade: Fisher Scientific

SuperSignal West Femto maximum sensitivity substrate kit: Pierce

TRI Reagent (T 9424): Molecular Research Center

Tris-HCl-EDTA-Acetate (TAE) buffer was constituted to a stock solution of 25M concentration.

Buffer salts were all obtained from Fisher.

Insects

A colony of pea aphids, *Acyrthosiphon pisum* (Harris) was established in the summer of 1999 from insects collected from alfalfa plants by Dr. Marina Calliaud (Cornell University) and is currently maintained by Dr. John Reese in the Department of Entomology, Kansas State University on pots of broad beans, *Vicia fabia*, exposed under a mixture of high-pressured sodium and fluorescent lamps at room temperature.

CHAPTER 3 - METHODS

Salivary gland dissections

Salivary glands from adult pea aphids were dissected on sterile glass slides in phosphate buffered saline (0.1 M, pH 7.0) under a dissecting microscope at 40X magnification. First, a cut was made just posterior to the prothorax using a clean razor blade. The head was laid on a slide so that the mouth parts were facing upwards. A needle (0.2 mm in diameter) was inserted into the middle-top portion of the head, between the eyes and above the labrum. A second needle was inserted into the head, right next to where the first needle. Using the second needle, the head was torn apart in a semicircular downwards motion. If the head was not torn into two parts at this step, a second tear was made. This time, starting from the bottom, the needles were inserted between the first pair of legs and torn upwards in a semicircular motion. At least one of the salivary glands would have been exposed at this point, most likely attached to the other salivary gland and the brain. Once at this step, the salivary glands were gently excised from the surrounding head fragments (Fig. 6).

Substrate Staining

After individual salivary glands were dissected out, they were gently picked up with a pair of forceps and placed into small wells (sterile microfudge tube caps glued onto glass slides) containing substrates for various oxidoreductases. In the presence of hydrogen peroxide, 2,2'-amino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) stains for peroxidase activity. In the absence of hydrogen peroxide, ABTS is a specific substrate for laccase (Collins et al. 1998). On the other hand, 3,4'-dihydroxy-L-phenylanaline (L-DOPA) is a substrate for both laccase and tyrosinase. For the purposes of staining the salivary glands, a 5

mM concentration of each substrate was used. Hydrogen peroxide (0.03%) was used along with ABTS when staining for peroxidase activity. ABTS was buffered at pH 5.0, using 0.1 M sodium acetate buffer. L-DOPA was buffered at pH 7.0, using 0.1 M potassium phosphate buffer.

Western blot analysis

Purified *Anopheles gambiae* laccase-1 polyclonal antibodies were obtained from Maureen Gorman in the Department of Biochemistry, Kansas State University. The antigen was a recombinant protein that consisted of residues 351 - 820 from *A. gambiae* laccase-1 (Dittmer et al. 2004), starting with the sequence DHDLSE and ending with VLDESQ.

Twenty pairs of salivary glands, 5 guts, or 10 heads (without salivary glands) were collected in three separate microfudge tubes with 30 µl of 2X SDS sample buffer with β-mercaptoethanol. The samples were incubated for 5 min in boiling water, and then loaded in a precast 4-20% gradient mini gel (BioRad) and run on constant voltage of 140V for approximately 1 h. The separated proteins on the gel were transferred onto a PVDF membrane using a transfer cell. Non-specific protein binding site was blocked with 5% instant non-fat dry milk (BestChoice®) in 1X PBST for 1 h and the membrane was then incubated with purified polyclonal antibody (against *A. gambiae* laccase-1) at 1:100 dilution overnight. The next morning, the membrane was washed with 1X PBST 3 times, at 5 min per wash. The membranes were then incubated with secondary antibodies, horse radish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL), at 1:5000 dilution in 1X PBST for 3 h. The membrane was again washed 3 times and the antigen-antibody complexes were visualized with the SuperSignal West Femto maximum sensitivity substrate kit (Pierce, Rockford, IL) and exposed to X-ray film. Typical exposure times ranged from 10 s to 30 s.

Immunohistochemisty

Salivary glands were dissected in PBS and washed three times in PBST (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 0.1% Triton X-100; pH 7.4).Thereafter, salivary glands were fixed in Bouin (71% saturated picric acid, 24% formaldehyde (37-40% v/w), and 5% glacial acetic acid) for 8 min at room temperature in a humidified chamber. Salivary glands were washed extensively with PBST and incubated with primary antibody (*A. gambiae* laccase-1) at 1:100 dilution in PBST overnight at 4^0C. Salivary glands were then washed 3 times at 15 min intervals with PBST and were blocked with 5% normal goat serum in PBST for one hour, and then washed 3 more times at 15 min intervals with PBST and followed by incubation with secondary antibody Cy-3 conjugated goat anti-rabbit at 1:500 dilution in PBST overnight at 4^0C. Following incubation, the salivary glands were washed extensively with PBST throughout the entire day, and then mounted on Gel/Mount^{TM} mounting media on sterile glass slides. Photographs were taken using Nikon Zeiss LSM 5 Pascal (Laser Scanning Confocal Microscope).

RNA extraction

Twenty pairs of salivary glands, 5 guts, and 10 heads (with the salivary glands removed) were dissected and placed into different microfudge tubes containing 300µl of TRI reagent. These tubes were placed in ice during the dissection to keep the RNA samples from degrading and were then transferred in dry ice during transport. The sample was allowed to stand in the solution at room temperature for 5 min. A volume of 0.2 ml chloroform per ml of TRI Reagent was added to the sample, covered tightly and vortexed for 15 s. The homogenized sample was incubated in the TRI Reagent-chloroform solution for 2 – 15 min at room temperature. The aqueous phase was then transferred to a fresh tube and 0.5 ml of isopropanol per ml of TRI

Reagent (used in the original volume) was added and mixed. The sample was incubated for 5 –
10 min at room temperature and centrifuged at 12,000x g for 10 min at 4ºC. A pellet was
precipitated out on the bottom and side of the tube. The supernatant was removed and the RNA
pellet was washed with 75% ethanol with the equal volume of the original volume of TRI
reagent .

The sample was then vortexed and centrifuged at 7,500x g for 5 min at 4ºC. Ethanol was
decanted and the pellet was air-dried for 5-10 min and redissolved in 10 µl DEPC-treated water.
The dissolved RNA pellet was immediately treated with DNase I (Ambion) by adding 1/10th
volume of 10X DNAse I buffer plus 1 µl DNase. The sample was then incubated for 25 min at
37ºC. After the incubation period, 5 µl of DNAse inactivation reagent was added to the mixture
and was incubated for 2 min at room temperature. It was then centrifuged at 10,000x g for 1 min
to pellet the inactivation reagent. The supernatant was then transferred to a clean Eppendorf tube.
To assess the quality of the RNA sample and also to calculate for its concentration A_{260}/A_{280} was
measured. The RNA sample was stored at -20°C for later use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Full length pea aphid laccase-1 clone (Fig. 5) was obtained by Dr. Navdeep Mutti and
Matthew Hermann from the Department of Biochemistry, Kansas State University. From large
scale sequencing of whole body cDNA libraries, partial sequence data on laccase-1 was
obtained. Two clones represent the N-terminal (CN760195), and the C-terminal (CV836585).
This was followed by PCR with "F4" forward primers and "5RP" reverse primers. Their
sequences were 5'- CAG TTC GTT CCG GTA CGT GTA – 3' and 5' – ACA CAA ATG GCG
TCA GTC CTT – 3' respectively. Next, a nested PCR was done using "F2" and "6RP" forward
and reverse primers with the sequences 5' – CGA TCA CAG ACA GCA TCC AA – 3' and 5' –

CGA TCA CAG ACA GCA TCC AA – 3" respectively. The product was cloned into *E. coli* using the TOPO TA® cloning kit (Invitrogen) according to the manufacturer's instructions and sequenced at the Kansas State University DNA sequencing facility, Department of Plant Pathology.

To transcribe poly(A)+ mRNA from the extracted total RNA in the salivary glands and guts, AMV Reverse Transcriptase was carried out with oligoDT primers to synthesize single-stranded cDNA following procedure from Promega technical bulletin no. 099. Primers were designed using GeneFisher at http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/gf_submit?mode=STARTUP&qid=na&sample=dna . Once the primers were selected they were synthesized commercially from Integrated DNA Technologies, Inc (Coralville, IA).

PCR was done using 5' – AGT CTG CCG GGA CTT CCA – 3' and 5' – CCG GAC ACT GTG TCA CGT AC – 3' for lac1 forward and reverse primers respectively, giving a final product of 160 base pairs, and using 5' – CCG AAA AGC TGT CAT AAT GAA GAC C – 3' and 5' – GGT GAA ACC TTG TCT ACT GTT ACA TCT TG – 3' for ribosomal protein L27 forward and reserve primers respectively, giving a final product of 231 base pairs. All of the PCR products were run on a 1% agarose gel with a 100kb DNA ladder. For two-tube PCR, 10 µl of ssDNA sample was used with 25 µl of Promega Master Mix, and 2.5 µl of each forward and reverse primers and the volume was brought to 50 µl. Initial denaturation was done at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 50 – 55°C for 30 s, and 72°C for 30 s , and a final extension step of 72° C for 5 min.

DNA Electrophoresis

To identify the PCR product, 1% w/v agarose gel with 2.5 µl ethidium bromide (0.5µg /ml) was used. Only 8 - 10 µl of PCR product was analyzed per lane. Gels were run at a constant

100V voltage while submerged in 1 x TAE buffer. For visualization, the agarose gel was exposed to UV light and results were recorded using Kodak DX200 camea. Photos were then cropped and labeled using Adobe Photoshop 7.0 software.

CHAPTER 4 - RESULTS

Substrate staining with ABTS

2,2'-Amino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) is a specific substrate for laccase in the absence of hydrogen peroxide, and a substrate for peroxidase in presence of hydrogen peroxide (Lonergan et al. 1997). In its reduced form, ABTS in solution has a very faint green color. When oxidized, it turns dark green. ABTS was used to detect laccase activity. Pea aphid salivary glands were dissected and immersed in 5 mM ABTS solution buffered at pH 5.0. The first hint of staining (green color) occurred after approximately 1 hour. Staining occurred in the *Deckzellen* and some parts of the *Hauptzellen*, and at approximately the same rate. Within 1.5 h the staining pattern was more or less established, and the entire principal salivary gland, with the exception of the accessory gland and, apparently, cell type 3 (according to Ponsen's classification scheme), was stained green. Interestingly, after the gland was stained green, a deep purple-red color started to develop gradually. Within 6 h, the initial green color was entirely replaced by purple-red. As far as I am aware, this observation has never been documented in scientific literature and may be a further oxidation step of the initial oxidation product of ABTS by an unknown enzyme (Fig. 7).

Substrate staining with ABTS plus H_2O_2

Salivary glands were stained in 5 mM ABTS with hydrogen peroxide (0.03%) to detect the presence of peroxidase activity. Compared to substrate staining in ABTS without hydrogen peroxide, staining with ABTS in the presence of hydrogen peroxide proceeded much quicker. Within approximately 30 sec, a faint green staining pattern was visible in parts of the *Hauptzellen*. Within about 5 min, the staining reached its highest intensity, and appeared to stain

cell types 2, 4 and 8 (according to Ponsen's classification scheme). This staining pattern was markedly different from ABTS staining that lacked hydrogen peroxide. Within 10 min of staining, the green color started to decline and was entirely replaced by purple-red color by the 30 min (Fig. 8).

Substrate staining with L-DOPA

3,4'-Dihydroxy-L-phenylanaline (L-DOPA) is a substrate both tyrosinase and laccase (Haavik 1997). When oxidized by either of the enzymes, it turns from colorless to black. Salivary glands were dissected and immersed in 5 mM DOPA buffered at pH 7.0. At the 2 hour mark, staining was clearly visible in the *Deckzellen* region of the salivary gland. The strongest staining, however, occurred at the type 2 and type 4 cells sandwiching the type 3 cells (which were conspicuously unstained). The staining pattern darkened over time and within 18 h, the entire salivary gland, with the exception of the accessory gland and type 3 cells, was stained (Fig. 9).

Immunohistochemistry

In the samples where anti-*A.-gambia-* laccase-1 primary antibodies were used, the *Deckzellen* was the most fluorescent region of the gland, indicating a relatively high content of laccase-1. Parts of the *Hauptzellen* were also fluorescent, but at a lower intensity compared to the *Deckzellen*. The cells that were fluorescent in the *Hauptzellen* seemed to correspond to cell types 4 and 5 (according to Ponsen's classification scheme). For the control, primary antibodies were left out of the procedure to omit the possibility of random binding by the secondary antibodies. In all of the control samples, no fluorescence was detected (Fig. 10, 11).

Western blot analysis

Western blot analysis was used to determine whether antiserum to *A. gambiae* laccase-1 detects an aphid protein of a size consistent with that expected of a laccase. Three samples were used in western analysis: (1) 20 pairs of salivary glands, (2) 5 guts, (3) 10 heads with salivary glands excised. *A. gambiae* laccase-1 primary antibodies were used to detect the presence of laccase-1 in the samples. Sample (3) was the control, and used to verify that in the head, no other organ contained laccase-1 other than the salivary glands. For samples (1) and (2), two bands were observed at approximately 72 kDa and 88 kDa. The expected size of the band based on the number of amino acid residues is 68 kDa. No bands were observed in sample (3) (Fig. 12). The 72 kDa band is close to the expected protein size of 68 kDa, which suggests that it is laccase-1 with no post-translational modifications.

RT-PCR

RT-PCR was used to verify the presence of laccase-1 transcript within the pea aphid. As with the western blot analysis, three samples were used in RT-PCR: (1) 20 pairs of salivary glands (2) 5 guts (3) 10 heads with salivary glands excised. Two sets of primers were used: laccase-1 (expected product size 160 bp) to detect laccase transcript, and L27 (expected product size 231 bp) primers (control) to detect L27, a constitutively expressed ribosomal protein. At 30 PCR cycles using laccase-1 primers, a band of approximately 160 bp was observed samples (1) and (2), but not in sample (3). Using L27 primers, bands of approximately 230 bp were observed in all 3 samples (Fig. 13).

Figure 1. Anatomy of the salivary gland of the green peach aphid, *M. persicae*.

(A) Visual representation of the salivary gland from the transverse section of a five day old *M. persicae* showing the principal salivary gland (psg), the accessory salivary gland (asg), the salivary canal (sc) and the common salivary duct (csd). Each lobe of the principal gland is composed of 8 cell types. Cell types 1 and 2 form the *Deckzellen* (dz), while cell types 3-8 form the *Hauptzellen* (h). (B) Transverse section of the common salivary duct (csd). (C) Transverse section of the middle region of the principal salivary gland. (D) Transverse section of the posterior region of the principal gland. sdc: salivary duct cell; n: nucleus; mc: myoepitheloid cell; isc: intercellular secretory canaliculum; ic: intracellular canaliculi; N2: branch of medial dorsal nerve.

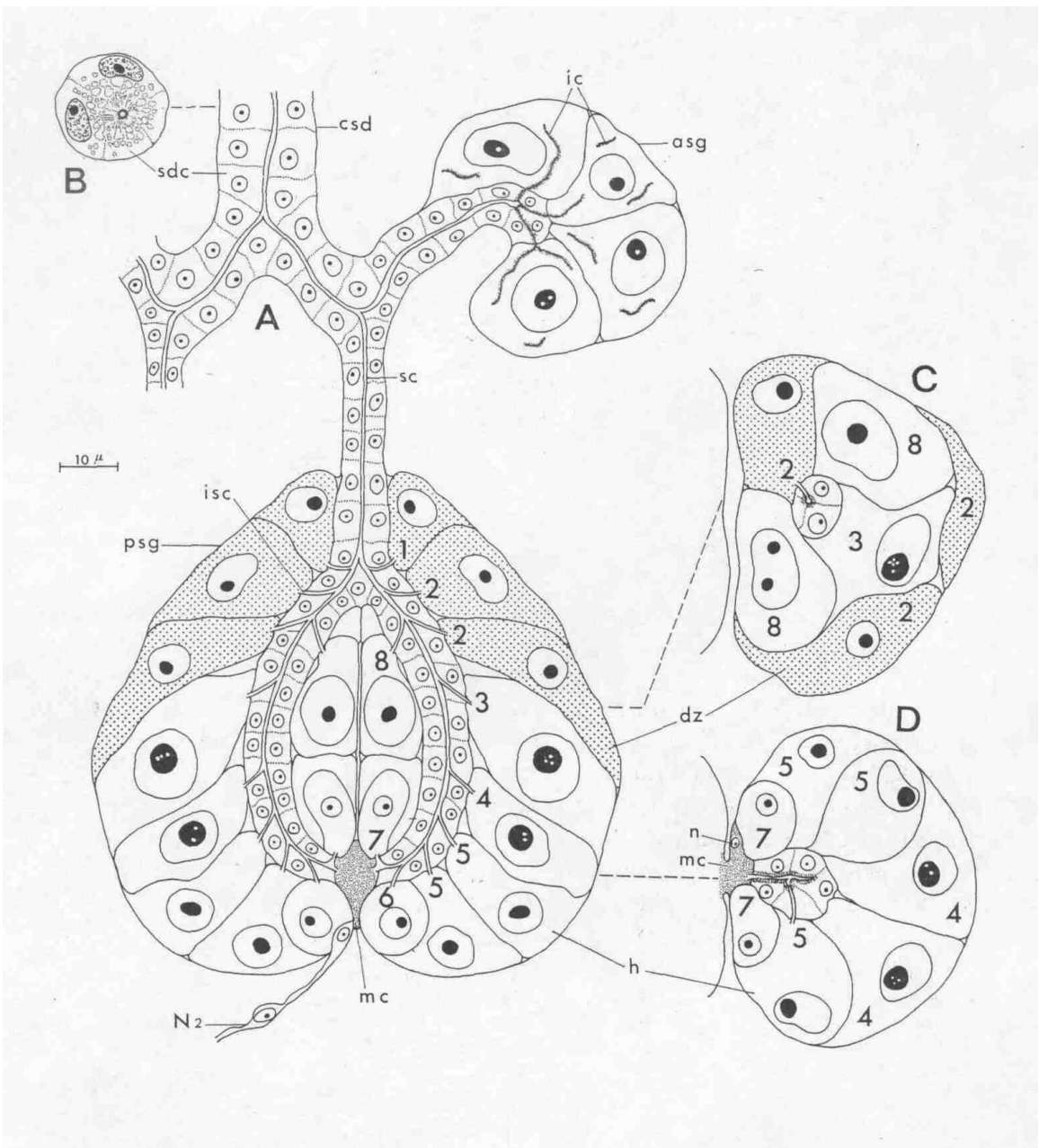(Used with permission) (Ponsen, 1987)

Figure 2. Alignment of full-length insect laccases.

Residues highlighted in yellow were the conserved N-terminal regions used to generate the phylogram in Figure 3. Residues highlighted in green were the C-terminal regions used to generated the phylogram in Figure 4. The numbers 1, 2 and 3, above the residues highlighted in red indicate amino acid residues involved in coordinating the T1, T2, and T3 copper centers.

```
Tribolium_Lac_2A                         MDGTQRYLLIATAALFLFFDLCHGVRAPG--AKKKVG----------- 35
Tribolium_Lac_2B                         MDGTQRYLLIATAALFLFFDLCHGVRAPG--AKKKVG----------- 35
Manduca_lac2                             MGCSGRYCLLT-LFLCLVTELALGVRVVP--KRKKEAI---------- 35
Anopheles_Lac2A                          MAIDWRNRVLSLGILLALAVAADGVRVQQHTSRRFKDE---------- 38
Anopheles_Lac2B                          MAIDWRNRVLSLGILLALAVAADGVRVQQHTSRRFKDE---------- 38
Pea_aphid                                    MRSQCTTTVVLFYYCCCTIAV--------------------- 21
Tribolium_lac1                           MKK-------ITLFMIIIC----------FERNLS------------ 18
Manduca_lac1                             MHRGSWRHIVCSILLVIISTHGINSQTESTAEDNES----------- 36
Anopheles_lac1       MAVRNSTLTAGRHRLPALVLVTATILPILSLMVPIGHSQSVITDCDTSKCQPLSNISEVS 60
                                                            :

Tribolium_Lac_2A     ------------------------------------------------------------
Tribolium_Lac_2B     ------------------------------------------------------------
Manduca_lac2         ------------------------------------------------------------
Anopheles_Lac2A      ------------------------------------------------------------
Anopheles_Lac2B      ------------------------------------------------------------
Pea_aphid            ------------------------------------------------------------
Tribolium_lac1       ------------------------------------------------------------
Manduca_lac1         ------------------------------------------------------------
Anopheles_lac1       LEPGQRIRRELDPCCEILRLYCDTSACPPLIEFCDAERTIRPKNIAGTCCTLQRCDNFCE 120


Tribolium_Lac_2A     -----PIDQSAAAASWHD------FDNSDFFQSEHAVIQTHP----------------- 66
Tribolium_Lac_2B     -----PIDQSAAAASWHD------FDNSDFFQSEHAVIQTHP----------------- 66
Manduca_lac2         ---NVPDDQSTSASWWQAGTATPFRESSNSFSSTHGLVQTHPTA---------DDPFGS 82
Anopheles_Lac2A      ---SFGHDQTPAGSWWSS---HLTEPPSNFYQATHGLLQTHPS-----------VPSLK 80
Anopheles_Lac2B      ---SFGHDQTPAGSWWSS---HLTEPPSNFYQATHGLLQTHPS-----------VPSLK 80
Pea_aphid            ----------------------------------------------------------
Tribolium_lac1       ---------YKVMYNGNN--NVTDLVEYVLLNED------------------------ 41
Manduca_lac1         ---------TTVGLNSENTESVSANLEDVSLDNDQ------------------QSSVQIK 69
Anopheles_lac1       VYANGEVTTRSVGEKWFNMVNETTCMNYECLRNDANETFINSIGIQCNTTCPEGFEAQLS 180


Tribolium_Lac_2A     -------SIG---------------GGPRFS-------SGVGRKAWKHLDFRNSATAELL 97
Tribolium_Lac_2B     -------SIG---------------GGPRFS-------SGVGRKAWKHLDFRNSATAELL 97
Manduca_lac2         SFGGIGSTIGPSSNPYGHSGSGPLSGGVRNNPLPSIARSANGKLSLKHLDFTSSATAELR 142
Anopheles_Lac2A      PVAGAPAAPGPSALPLSSRKSPTVSSAAALNSGFPSIANPNPRSPFRHLDFSTSATAELR 140
Anopheles_Lac2B      PVAGAPAAPGPSALPLSSRKSPTVSSAAALNSGFPSIANPNPRSPFRHLDFSTSATAELR 140
Pea_aphid            ---------------------------------------------LRLTGAVKPRHE 33
Tribolium_lac1       ------------------------------------------------------------
Manduca_lac1         KAEGLESASRVIMPVKPNISRNDVSYASKENVQRHPVEELDEELAKQILSKYAMKKSNIR 129
Anopheles_lac1       EQHCCPQCVQSQCKFNDQFYREGQSWASPDGCIVYRCVKENGFLSISSSRKQCPAVGDCP 240


Tribolium_Lac_2A     KNPSLS----------------------------------SPDECARACREGEPPR 119
Tribolium_Lac_2B     KNPSLS----------------------------------SPDECARACREGEPPR 119
Manduca_lac2         RNPALS----------------------------------APDECARACRENEPPR 164
Anopheles_Lac2A      RNPSLS----------------------------------APDECARACREGEPPR 162
Anopheles_Lac2B      RNPSLS----------------------------------APDECARACREGEPPR 162
Pea_aphid            RGQDYN----------------------------------AVHPCQRECRAGEPPK 55
Tribolium_lac1       ----------------------------------------NPCARKCVKDSVPM 55
Manduca_lac1         AHVRYD--------------------------EVTGELVGGAHPCERECKEGEPPM 159
Anopheles_lac1       DQHIVERDCCRVCNYTEAQMAPGLTTASPVEPEEGVDFYEELSYDNHPCKRACTLGRKPE 300
                                                               . * * *  .  *

Tribolium_Lac_2A     ICYYHFTLELYTVLGAACQVCTPNATNTVWSHCQCVLADGVERGILTANRMIPGPSIQVC 179
Tribolium_Lac_2B     ICYYHFTLELYTVLGAACQVCTPNATNTVWSHCQCVLADGVERGILTANRMIPGPSIQVC 179
Manduca_lac2         ICYYHFTLELYTVMGAACQVCAPNATNVVWSHCQCVLADGVERGILTANRMLPGPSIQAC 224
Anopheles_Lac2A      ICYYHFTVEYYTVLGAACQVCTPNATNTVWSHCQCVLADGVERGILTVNRMIPGPSIQVC 222
Anopheles_Lac2B      ICYYHFTVEYYTVLGAACQVCTPNATNTVWSHCQCVLADGVERGILTVNRMIPGPSIQVC 222
Pea_aphid            TCEYRFKVEWYYTMSKACYDCPYNITDCYRP--DCVPADGVAKPIIVINRSLPGPSIQVC 113
Tribolium_lac1       TCRYTFLLEWYHTLSKACYDCPYNTQDCYRE--DCIPGDGNKRSIIVVNRKMPGPSVEVC 113
Manduca_lac1         VCYYHFNLEWYQTMSKACYNCPFNETDCSRP--DCIPADGMNRALSVVNRKMPGPAIEVC 217
Anopheles_lac1       TCYYRFRLEWYRTLSKACYNCPYNATDCERP--HCITGDGVRRNVAVINRMMPGPAIEVC 358
                      * * * :* *  .:. ** *. *  :        .*: .**  : :  . ** :***:::.*
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                              2 3
Tribolium_Lac_2A   EGDKVVIDVENHIEGNEVTLHWHGVWQRGSQYYDGVPFVTQCPIQQGNTFRYQWIAGNAG 239
Tribolium_Lac_2B   EGDKVVIDVENHIEGNEVTLHWHGVWQRGSQYYDGVPFVTQCPIQQGNTFRYQWIAGNAG 239
Manduca_lac2       ENDKVVIDVENHMEGMEVTIHWHGIWQRGSQYYDGVPFVTQCPIQQGNTFRYQWQG-NAG 283
Anopheles_Lac2A    ENDRVVIDVENHMEGMELTIHWHGIWQRGTQYYDGVPFVTQCPIQQGNTFRYQWTG-NAG 281
Anopheles_Lac2B    ENDRVVIDVENHMEGMELTIHWHGIWQRGTQYYDGVPFVTQCPIQQGNTFRYQWTG-NAG 281
Pea_aphid          LGDTVMVDVENAMMEESTSVHWHGHHQRNSPYMDGVPYVTQCPVPPHSSFRYVYLADNEG 173
Tribolium_lac1     LGDEVIIDVVNHLSSDSTTIHWHGHHQKNSPYMDGVPFVTQCPIHPGMTFRYHFNVHNSG 173
Manduca_lac1       QDDRIIVDVENDLMTEGTTVHWHGQHQRGTPYMDGTPYVTQCPILPETTFRYQFTARHSG 277
Anopheles_lac1     ENDIIVVDVENHLMGESTTIHWHGLHQRRTPYMDGVPHVSQCPISPGTTFRYTFRADNPG 418
                    .* ::::** * :    ::**** *: : * **.*.*:***:   :*** :  : *

                      3 3
Tribolium_Lac_2A   THFWHAHTGLQKMDGLYGSVVIRQPPAKDPNSHLYDYDLTTHVMLLSDWMHEDATERFPG 299
Tribolium_Lac_2B   THFWHAHTGLQKMDGLYGSVVIRQPPAKDPNSHLYDYDLTTHVMLLSDWMHEDATERFPG 299
Manduca_lac2       THFWHAHTGLQKLDGLYGSIVVRQPPSKDPNSHLYDYDLTTHVMLISDWLHDDAAERYPG 343
Anopheles_Lac2A    THFWHAHTGLQKLDGLYGSIVVRQPPSRDPNSHLYDFDLTTHIMLVSDWLHEDAAERYPG 341
Anopheles_Lac2B    THFWHAHTGLQKLDGLYGSIVVRQPPSRDPNSHLYDFDLTTHIMLVSDWLHEDAAERYPG 341
Pea_aphid          THFWHAHSGLQKIDGIYGSIVVRQPPSQDPNSHLYDYDLTTHVVLLSDWLHENGMERFPG 233
Tribolium_lac1     THFWHSHSGFQRSDGTFGPFIVRVPEEDNPHAKLYDYDLSSHVITILDWTKEDGTDKFMS 233
Manduca_lac1       THFWHSHSGMQRADGAAGAFIIRKPKSQEPYESLYDYDRSDHVMIVTDWIHQLAVGMFTD 337
Anopheles_lac1     THFWHSHTGMQRGDGAFGALIIR--KDNDIQELLYDHDLSEHVITVQDWGHEQGVSLFAS 476
                   *****:*:*:*: ** *..::*    :   ***.* : *:: : ** ::.   : .

Tribolium_Lac_2A   RLAVNTGQDPESLLINGKGQ----FRDPN------------------------------ 324
Tribolium_Lac_2B   RLAVNTGQDPESLLINGKGQ----FRDPN------------------------------ 324
Manduca_lac2       RLAVNTGQDPESVLINGKGQ----FRDPN------------------------------ 368
Anopheles_Lac2A    RLAVNTGQDPESLLINGKGQ----FRDPN------------------------------ 366
Anopheles_Lac2B    RLAVNTGQDPESLLINGKGQ----FRDPN------------------------------ 366
Pea_aphid          RLAANTGQDPESLLINGKGQ----FTDPN------------------------------ 258
Tribolium_lac1     HIHNDGDNKPDTILVNGFGR-FKHFVGAD------------------------------ 261
Manduca_lac1       HHHSSGDNKPPTLLINGVGR-FKIFNNDT------------------------------ 365
Anopheles_lac1     HHHSTGDNKPPNLLINGRGKYFQRFAKTTPLTTTTTSTEEPALEPETIMAVEPESTTLME 536
                   :     .:.* .:*:** *:    *

Tribolium_Lac_2A   -------------------------------------------------------TGFMT 329
Tribolium_Lac_2B   -------------------------------------------------------TGFMT 329
Manduca_lac2       -------------------------------------------------------TGFMT 373
Anopheles_Lac2A    -------------------------------------------------------TGFMT 371
Anopheles_Lac2B    -------------------------------------------------------TGFMT 371
Pea_aphid          -------------------------------------------------------TGFSI 263
Tribolium_lac1     --------------------------------------------------------NSTV 265
Manduca_lac1       --------------------------------------------------------EKPV 369
Anopheles_lac1     ELPTTTVPITDAITPDDTELLQASSNTNLKTVLRAEEVRHRTKRQSRTVNFNAIVVPESK 596

Tribolium_Lac_2A   NTPLEVFTITPGRRYRFRMINSFASVCPAQLTIQGHDLTLIATDGEPVHPVRVN-TIISF 388
Tribolium_Lac_2B   NTPLEVFTITPGRRYRFRMINSFASVCPAQLTIQGHDLTLIATDGEPVHPVRVN-TIISF 388
Manduca_lac2       NTPLETFTITAGRRYRFRMINAFASVCPAQVTFEGHNLTVIATDGEPVQPVQVN-TIISF 432
Anopheles_Lac2A    NTPLEIFTITPGRRYRFRMINAFASVCPAQVTIEGHALTVIATDGEPVHPAQVN-TIISF 430
Anopheles_Lac2B    NTPLEIFTITPGRRYRFRMINAFASVCPAQVTIEGHALTVIATDGEPVHPVQVN-TIISF 430
Pea_aphid          NTPLETFTITPGRRYRFRMINALASVCPAQITIQGHPLVLIATDGEPIQPVVVN-TIISF 322
Tribolium_lac1     FVPTARFTVEQGYRYRFRVINAGFLNCPIEVSIDNHTLSVISTDGSDFNATEVD-SLVTY 324
Manduca_lac1       YMKAARFNVEQGYRYRFRVINAEFLNCPIEMSVDGHNITVIASDGYDLEPITAT-SLVTY 428
Anopheles_lac1     HIPLKVFHVDKGRRYRFRLINAEFLNCPVELSIENHNLTVIASDGFGIQPLEDLGSFVSY 656
                    * :   * *****:**:    ** :::.:.* : :*::** ...    :::::

Tribolium_Lac_2A   SGERYDFVINADQTPGAYWIQLRGLGECGI--RRVQQLGILRYAKGPYQPSQAPPTYDYG 446
Tribolium_Lac_2B   SGERYDFVINADQTPGAYWIQLRGLGECGI--RRVQQLGILRYAKGPYQPSQAPPTYDYG 446
Manduca_lac2       SGERYDFVIEANNIPGAYWIQVRGLGECGI--KRAQQLGILRYARGPYQPSLPAPTYDIG 490
Anopheles_Lac2A    SGERYDFVITADQPVGAYWIQLRGLGECGI--KRAQQLAILRYARGPYQPASPPPTYDVG 488
Anopheles_Lac2B    SGERYDFVITADQPVGAYWIQLRGLGECGI--KRAQQLAILRYARGPYQPASPPPTYDVG 488
Pea_aphid          SGERYDFIINAEQPVGAYWIQVRGLGECGN--RRVQQLAILRYARGPYQPKSKAPTYDVG 380
Tribolium_lac1     AGERFDFIVTADQPQDVYWMHFRGLMDCDERFTRAYQVAVLEYKGTQTNYPSYEPTYDNS 384
Manduca_lac1       AGERYDFILDANNEIDNYWIRFRGLMDCDEIYTRAKQVAVLHYEGAMDLEPPGDPSWFEL 488
Anopheles_lac1     AGERFDFIVKANQPIGNYLIRFRGLMDCDERFTSAYQFAVLRYRGAPTDTEYESWPPYDY 716
                   :***:**:: *:: . * ::.*** :*.      . *..:*.*                .
```

```
Tribolium_Lac_2A    IPQGVVLNPLDARCNEIRPDAICVSQLKNALSIDKGILREKPDVKIFLPFRFHIYTPEDL  506
Tribolium_Lac_2B    IPQGVVLNPLDAICNKPRKDAVCVSQLRNAKKVDEAILEERPDVKIFLPFRFLFYKPEDI  506
Manduca_lac2        IPQGVVMNPLDARCNILRNDAICVSQLKNAKHIDPAILQERPDIKIFLPFRFFVYGPETL  550
Anopheles_Lac2A     LPQGVVMNPLDAQCNVQRDDAICVSQLKNAKEIDRALLQDKPDVKIFLPFRFYLYRPEEL  548
Anopheles_Lac2B     LPQGVVMNPLDAVCNVPRPDAVCVSNLRNAKKADKAVLSERPDVKIFLPFRFYFYRVEEL  548
Pea_aphid           LPQGVVMNPLDAVCDRPRTDAICVNQLKNAKVVDKGLLQERPDVKIFLPFKFLFYRPDEL  440
Tribolium_lac1      RREGKQLNPLNKGT-EADSSFVTLPQLHSLDEWDDT-LKEKADFQYYVSYDFYKMNHPVY  442
Manduca_lac1        HNEGLQLNALNKG--EEENETISVAEMRSLAGYDDS-LKEIADYQFYIAYDFYAKNNSHF  545
Anopheles_lac1      EAPGVQLNSLNRGP-GAEN-VITIAETSALDQEDLLLLRNETDYKFYVYYDFYGKDNPHF  774
                        *   :*.*:           : : :        *     *  : .*  ::  : *

Tribolium_Lac_2A    FAPNTYNRHLVAPNG-DHVISLIDEISYMAPPAPLISQYDDIDPQQFCNGDNRPAD--CQ  563
Tribolium_Lac_2B    FRPNTYNRFLAATGG-DHVISLIDEISFTFPPSPPLSQIHDLSPDQFCNGDNRPPD--CG  563
Manduca_lac2        FQPNTYNRYLVAPSG-DHVISLIDEISYMSPPAPLLSQYDDINPEQFCNGDNRPAN--CG  607
Anopheles_Lac2A     FQPNTYNRFLVAPTG-DHVISLIDEISYLSAPAPLLSQYDDINPEQFCNGDNRPAD--CG  605
Anopheles_Lac2B     FTPNTYNKFLVAPGG-DHLISLIDEISYVSPPSPMLSQINDIPPEQFCNGDNRPPD--CG  605
Pea_aphid           FQPHQYNKYLVAPGGGDHVISLVDEISYTSPGSPMISQIDDIPPELFCNGDNKPAN--CG  498
Tribolium_lac1      HKDPHYGFHNVTNTTLQNLTPQLNYISMKLQSFPLLSQRHQIDAKMFCNESSVSN--CEN  500
Manduca_lac1        HRSPYYGYYQVPEQVNRLYTPQLNHISMKMPTSPLLITRPSPEN--FCNASSIDEG-CKE  602
Anopheles_lac1      HVPSLYGFQQVVNNTNRLYTPQLNHISMRMPPVPFLPGKDVLDESQFCNETSVRDRNCRQ  834
                    .      *.   .            . :: **     * :        ***   .

                                                     1 2 3
Tribolium_Lac_2A    QNCMCTHKVDIPLNAIVEIVLVDEVQQPNLSHPFHLHGYAFNVIGIGRSPDQNVKKINLK  623
Tribolium_Lac_2B    QNCMCTHQVDIPLNAIVEVVLVDEVQSPNLSHPFHLHGYAFNVVGIGRSPDQNVKKINLK  623
Manduca_lac2        QNCMCTHKVDIPLNAVVEIVLVDEVQITNLSHPFHLHGYAYNVIGIGRSPDQNVKKINLK  667
Anopheles_Lac2A     ANCMCTHKVDIPLNAIVEVVLVDEVQQPNLSHPFHLHGYAYNVVGIGRSPDSNVKKINLK  665
Anopheles_Lac2B     PNCMCTHKVDIPLNAIVEVVLVDEVQQENLSHPFHLHGHAFHVIGMGRSPDSTVKKINLR  665
Pea_aphid           RNCMCSHKVDIPRHAVVEVVLVDEVQQPNLSHPFHLHGYSFNVIGMGRSPDKNVKKINLK  558
Tribolium_lac1      EYCECTHVVNIPLGTVVEMVLIDKGYAYDANHPFHLHGHSFRVVAMERVG----SHVNVS  556
Manduca_lac1        GYCECPHVLSVKLNAIVEVIIVDEGVTFDANHPFHLHGHAFRVVGLRRLN----RTTTIE  658
Anopheles_lac1      EFCECSHVLQIPLHATVEMVMIDEGFTFDANHPFHLHGHAFRVVGMDRVS----RNTTIE  890
                      *  * *.* :.:    :  **:::*:     : .*******:::.*:.: *        .:

                                                                  313       1
Tribolium_Lac_2A    HALDLDRQGLLHRQFN---LPPAKDTIAVPNNGYVVLRLRANNPGFWLFHCHFLFHIVIG  680
Tribolium_Lac_2B    HALDLDRRGLLHRQFN---LPPSKDTIAVPNNGYVIFRFRADNPGYWLFHCHFLFHIVIG  680
Manduca_lac2        HALDLDRRGLLERHLKQGDLPPAKDTIAVPNSGYVILRFRATNPGFWLLHCHFLFHIVIG  727
Anopheles_Lac2A     HALDLDRRGLLHRQYN---LPPLKDTIAVPNNGYVVLRFRADNPGYWLFHCHFLFHIVIG  722
Anopheles_Lac2B     HTLDLDRRGLLNRQFN---LPPLKDTIAVPNNGYVVLRFRADNPGYWLFHCHFQFHIVIG  722
Pea_aphid           HALDLDRRGLLDRHFN---LPPLKDTIAVPNNGYVVFRFRADNPGYWLFHCHFLFHIVIG  615
Tribolium_lac1      EILKMDQNGQIKRNLVD---APLKDTVTVPDGGFTIIRFKATNPGYWLFHCHIEFHVEVG  613
Manduca_lac1        EIKAFDEAGLLKRNLKN---APIKDTVTVPDGGYTVIRFKADNPGYWLFHCHIEFHVEVG  715
Anopheles_lac1      DIRRMDEEGRLPRRLKR---APIKDTVTIPDGGYTIIRFIANNPGYWLFHCHIEFHAEIG  947
                    .    :*. *  :  *.       .* ***:::*:.*:.::*: * ***:**:***: **   :*

                    1
Tribolium_Lac_2A    MNLVLQVGTHADLPPVPPNFPTCGDHVPEINSNPNLV                        717
Tribolium_Lac_2B    MNLIIHVGTQLIYRPFS-HFPRCGNHLPPISLH                            712
Manduca_lac2        MSLVLQVGTQADLPPVPPGFPTCGDHLPPIPLH                            760
Anopheles_Lac2A     MNLILQVGTHADLPPVPPNFPTCGDHLPPIN                             753
Anopheles_Lac2B     MNLVVHIGTHADLPPVPPNFPRCGNHIPPIKYN                           755
Pea_aphid           MNLVLHVGTHADLPPVPETSPRCGDFLPPVSVH                           648
Tribolium_lac1      MALVFKIGEDYEMPPVPKDFPQCGDYVPSGNSTVDCDDVGTFGAI-LKKLLPKVYEDYCP  672
Manduca_lac1        MALVFKVGEHKDMAPLPRDFPTCGNYMPDDMSLQTEKPKEENPVISISQWWPVVYVNNTI  775
Anopheles_lac1      MSLVLKVGDSSEMLPAPANFPTCYDFKPKLGQLGSGGARHG                   988
                    * *:.::*         *  .   * * :. *

Tribolium_Lac_2A
Tribolium_Lac_2B
Manduca_lac2
Anopheles_Lac2A
Anopheles_Lac2B
Pea_aphid
Tribolium_lac1      TSNSGSVRMSHLGTLVPLILFMLWG      697
Manduca_lac1        SSAT-SVSVSGFLILCSVWILKINVDT 801
Anopheles_lac1      HSLTSSLLVVVLIVVSLQRLL        1009
```

27

Figure 3. Phylogram of conserved laccase N-terminal regions.

Phylogram was generated using ClustalW using maximum parsimony. The branch lengths are proportional to the amount of inferred evolutionary change.
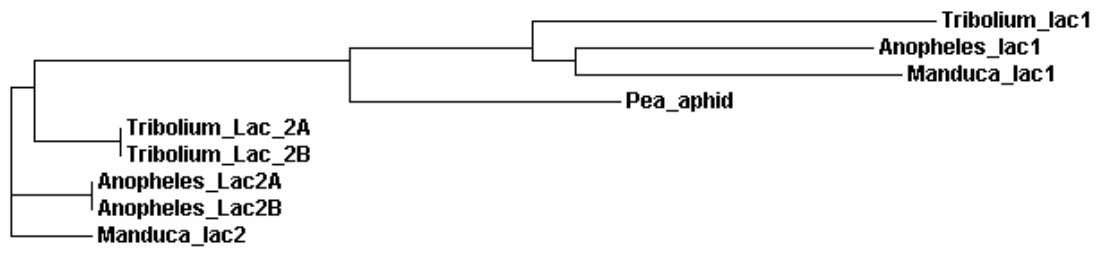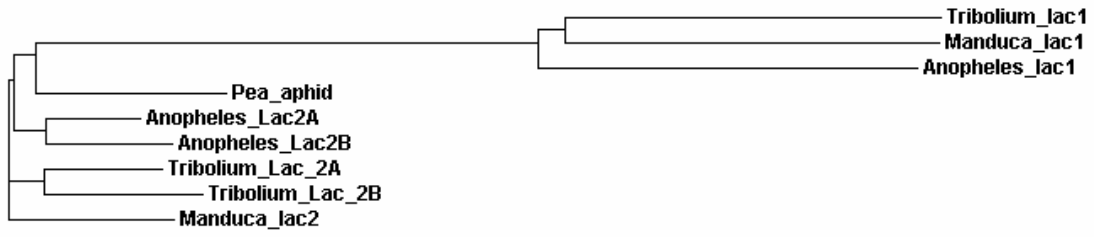
Figure 4. Phylogram of conserved laccase C-terminal regions.

Phylogram was generated using ClustalW using maximum parsimony. The branch lengths are proportional to the amount of inferred evolutionary change.

Figure 5. Full length pea aphid laccase-1 sequence.

Sequence was obtain from Dr. Navdeep Mutti and Matthew Heerman, Department of Biochemistry, Kansas State University. The red arrow indicates the cleavage site of the putative signal peptide predicted by SignalP analysis. Amino acid residues that are colored blue indicate possible N-glycosylation sites as predicted by NetNGlyC analysis. Residues with red highlighting indicate the amino acids involved in coordinating the copper centers of the enzyme. Residues highlighted in yellow indicate conserved cysteine residues (with respect to *T. castaneum, M. sexta, and A. gambiae* laccases.)

Pea Aphid Laccase-1

```
atgagatcacagtgcaccaccaccgtcgtgctgttctactactgctgctgtacgatcgcc   60
 M  R  S  Q  C  T  T  T  V  V  L  F  Y  Y  C  C  C  T  I  A
gtgctgcgcttgacgggcgccgtgaagccgcgacacgaacgcggacaggactacaacgcc  120
 V  L  R  L  T  G  A  V  K  P  R  H  E  R  G  Q  D  Y  N  A
gtccatccttgccagagagaatgtcgcgcgggcgaaccgcccaaaacgtgcgagtaccgt  180
 V  H  P  C  Q  R  E  C  R  A  G  E  P  P  K  T  C  E  Y  R
ttcaaagtggaatggtactacacaatgagcaaggcgtgctacgactgtccgtacaacatt  240
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```

```
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 H  V  G  T  H  A  D  L  P  P  V  P  E  T  S  P  R  C  G  D
tttttacctccggtcagtgtacactgatatcacgttcataacacgtcagttcaaaactaa 1980
 F  L  P  P  V  S  V  H  *
```

34

Figure 6. Dissection technique used to excise salivary glands.

(1) Two dissecting pins are inserted between the eyes and the middle-top portion of the head, between the eyes and above the labrum, the top portion of the head is torn in a semicircular downwards motion. (2) A second tear is made starting from the bottom, the needles are inserted between the first pair of legs. (3) A salivary glands is exposed in the right fragment of the head. (4) Both salivary glands are attached to the brain. (5) The brain has been removed, leaving the salivary glands attached with a duct. (6) A single dissected salivary gland is shown.

3

Exposed salivary gland

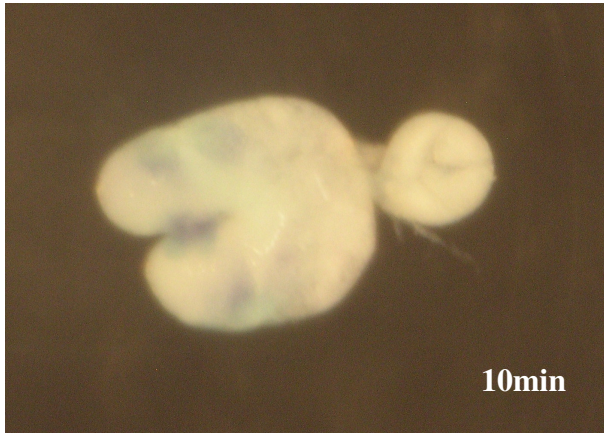40x Zoom

4

Brain

salivary gland

salivary gland

40x Zoom
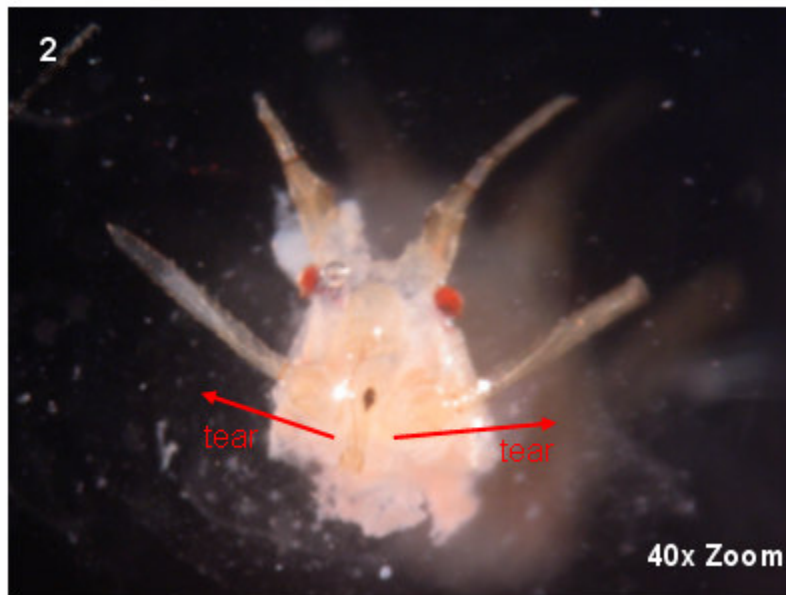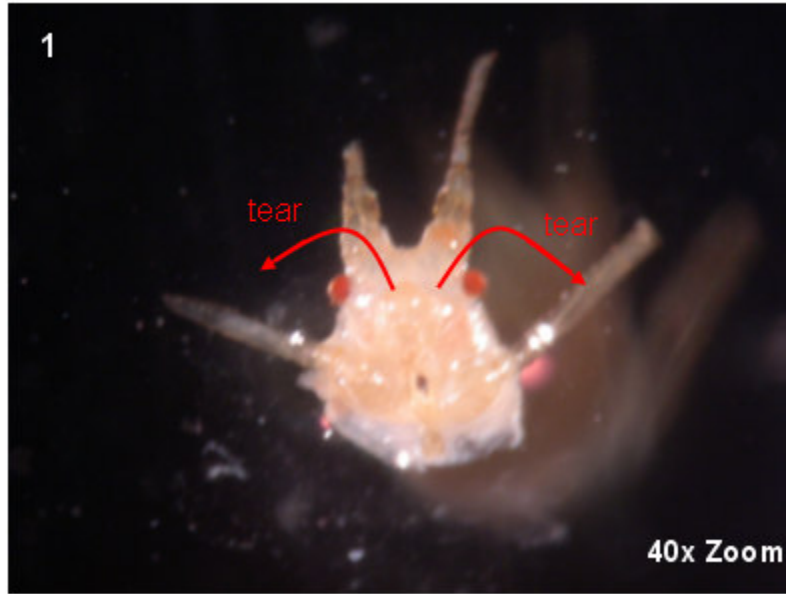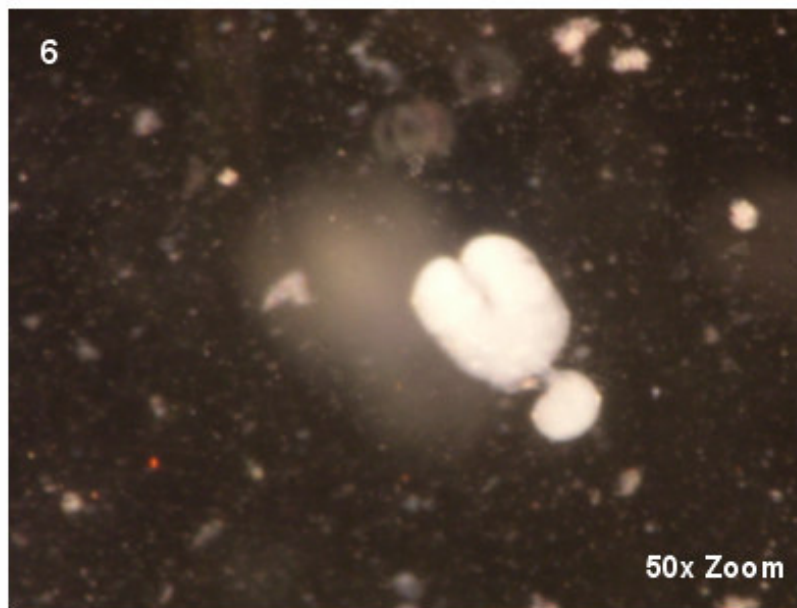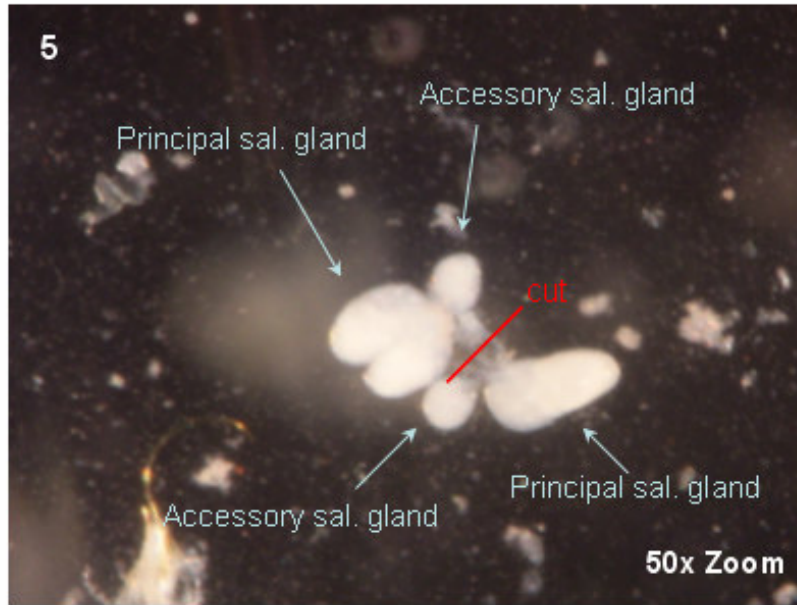
Figure 7. ABTS staining of dissected salivary glands.

Images of salivary glands exposed to 5 mM ABTS dissolved in 0.1 M acetate buffer, pH 5.0 and incubated with substrate from 0 to 6 h. Pictures were taken under 50X magnification

0 min

1 h

1h 30min

2h
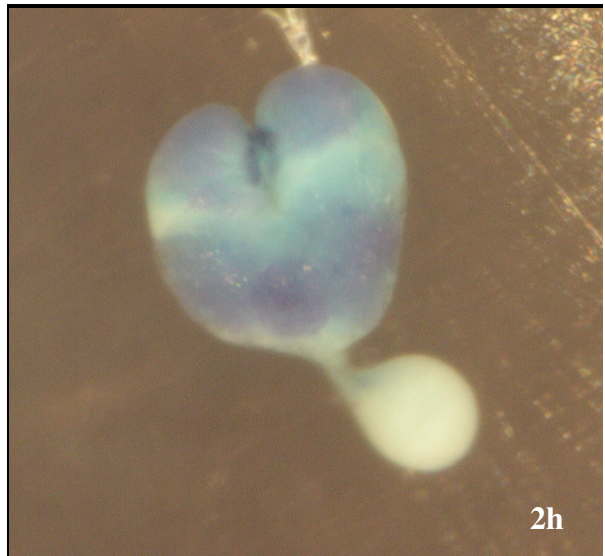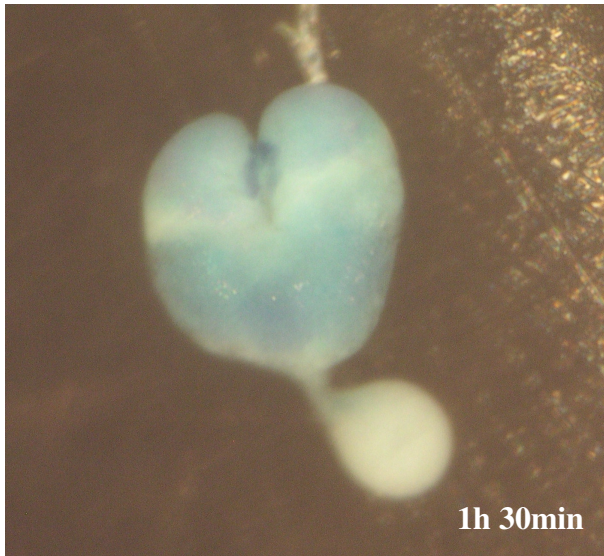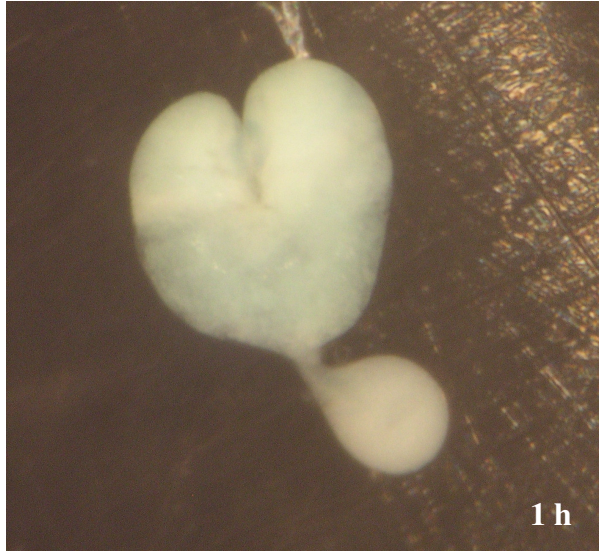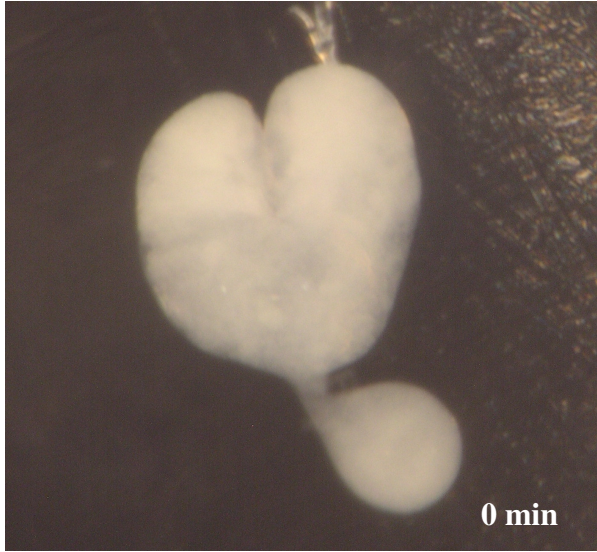
Figure 8. ABTS staining of salivary glands in the presence of H_2O_2.

Images of salivary glands exposed to 5 mM ABTS and 0.3% H_2O_2 dissolved in 0.1 M acetate buffer, pH 5.0 and incubated for up to 30 min. Pictures were taken under 50X magnification
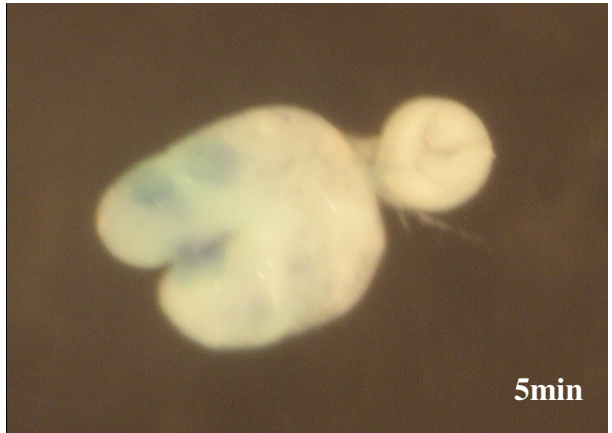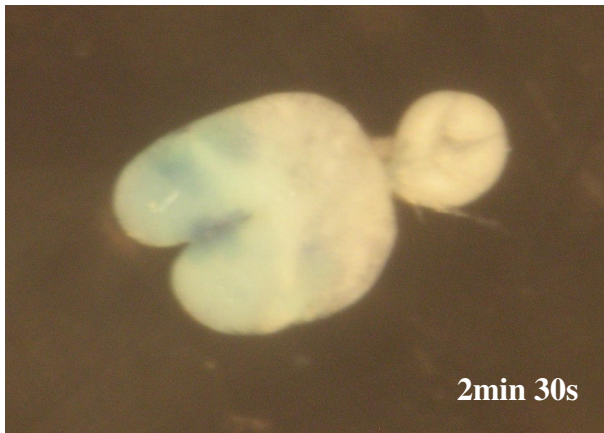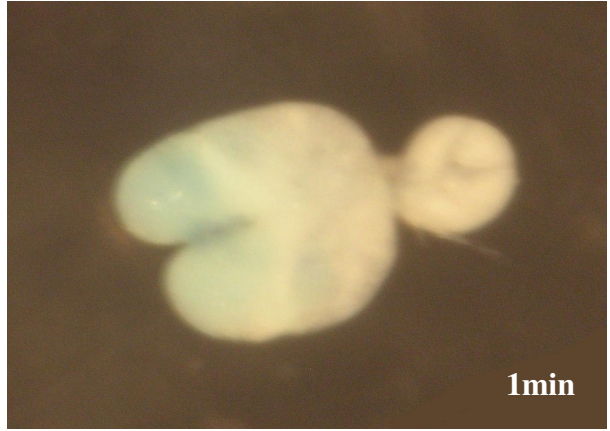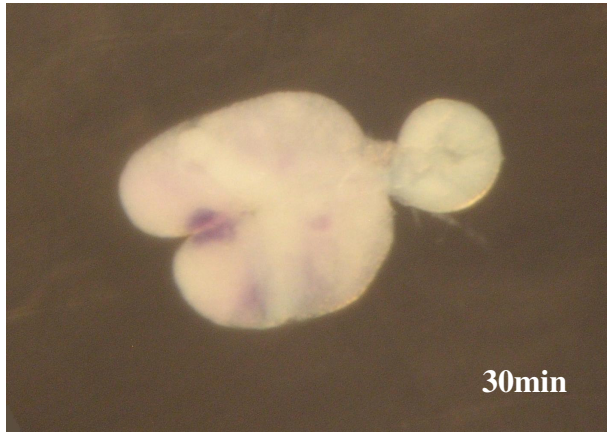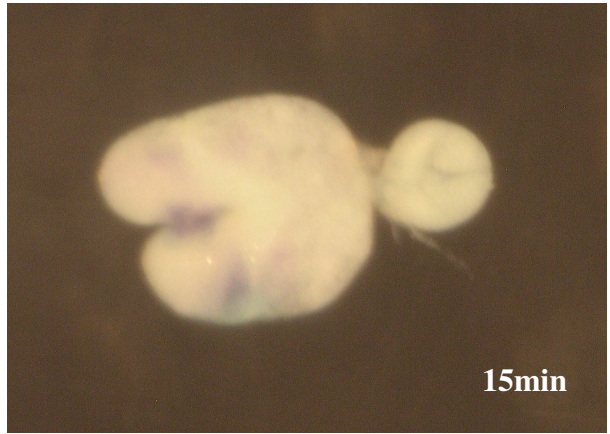
Figure 9. L-DOPA staining of dissected salivary glands.

Images of salivary glands exposed to 5 mM L-DOPA dissolved in 0.1 M phosphate buffer, pH 7.0 and incubated from 0 to 16 h. Pictures were taken under 50X magnification.
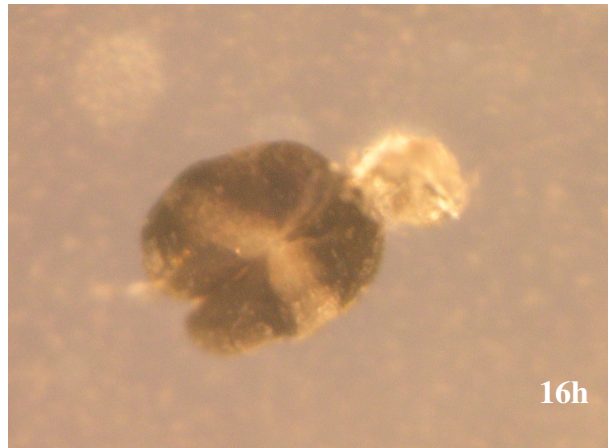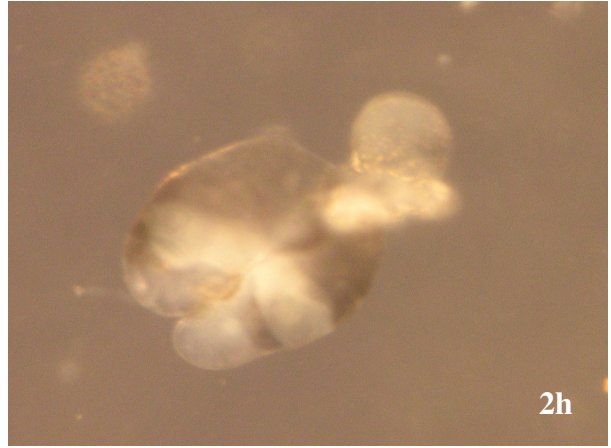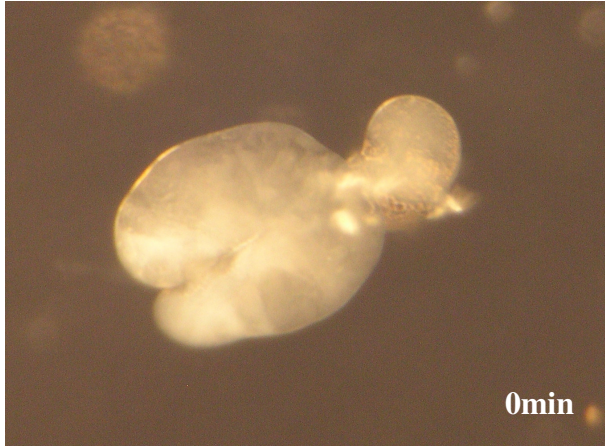
Figure 10. Immunohistochemistry.

Immunohistochemistry using purified polyclonal anti-laccase-1 (*A. gambiae)* antibodies. Two replicates are shown.
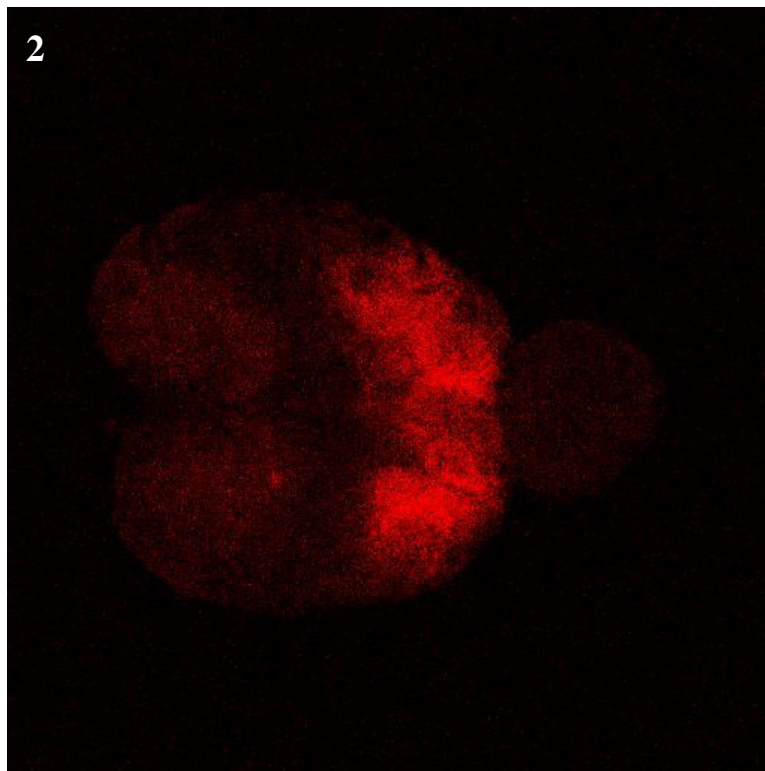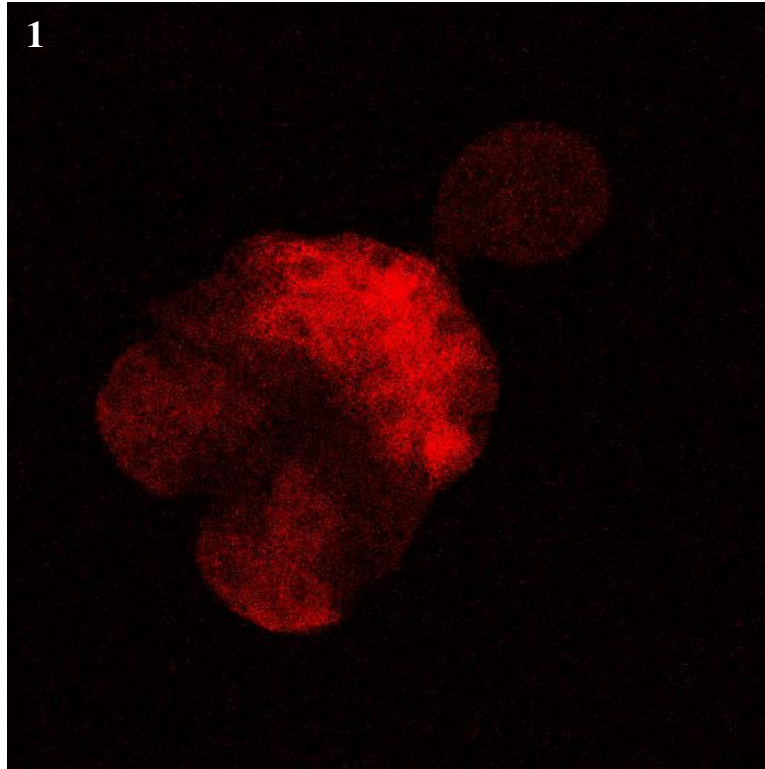
Figure 11. Control for anti-laccase immunohistochemistry

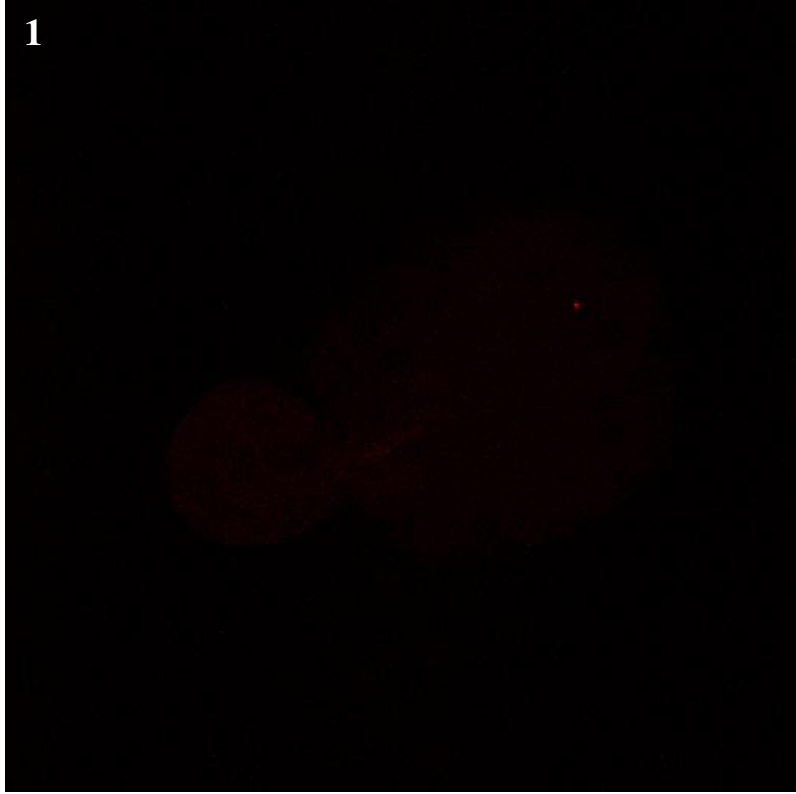Immunohistochemistry control with primary antibodies omitted.

Figure 12. Western blot analysis detects laccase protein in salivary glands and gut. Lane 1: 20 pairs of salivary glands. Lane 2: 5 guts. Lane 3: 10 heads with salivary glands excised. Two bands were observed in lanes (1) and (2), at approximately 72 kDa and 88 kDa (calculated from a standard curve of log molecular weight vs. distance). No bands were observed in lane (3).
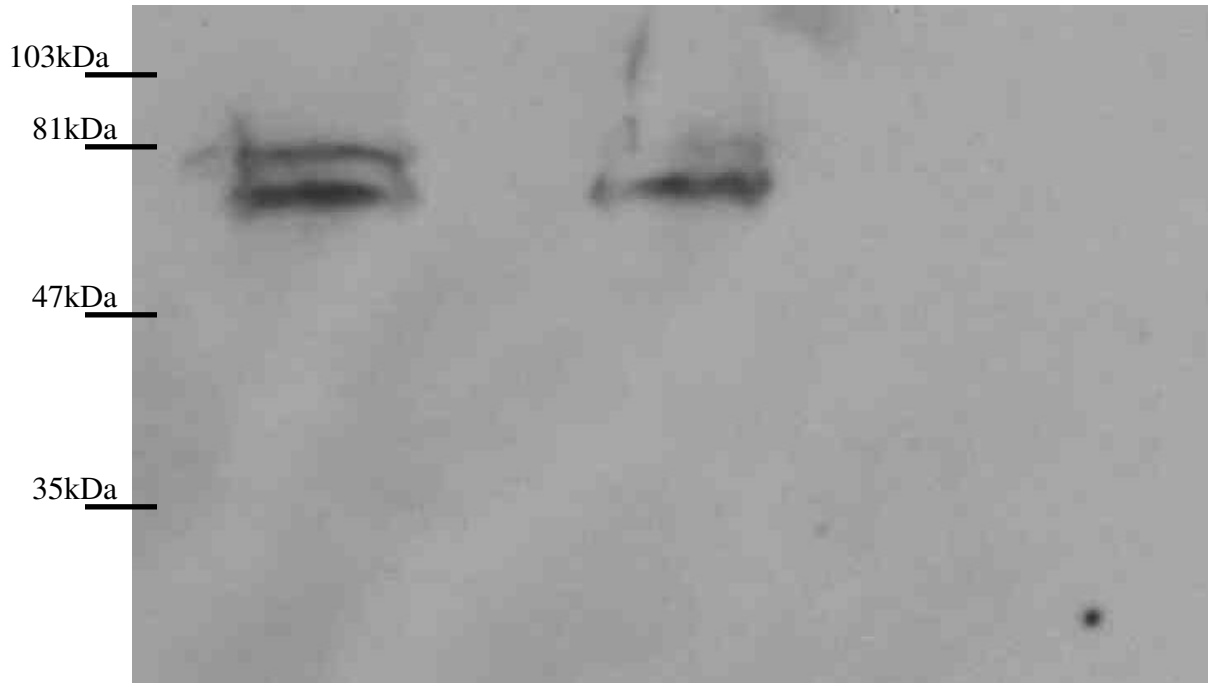
Figure 13. Detection of laccase mRNA by RT-PCR.

RT-PCR using laccase-1 and L27 primers, 30 PCR cycles. Lane 1: 20 pairs of salivary glands. Lane 2: 5 guts. Lane 3: 10 heads with salivary glands excised. For the laccase-1 primer set, bands of approximately 160 bp was observed in lane (1) and (2), but not lane (3) at 30 PCR cycles. For the L27 primer set, a band of approximately 230 kDa was observed for all 3 lanes at 30 PCR cycles.

Laccase-1 Primers
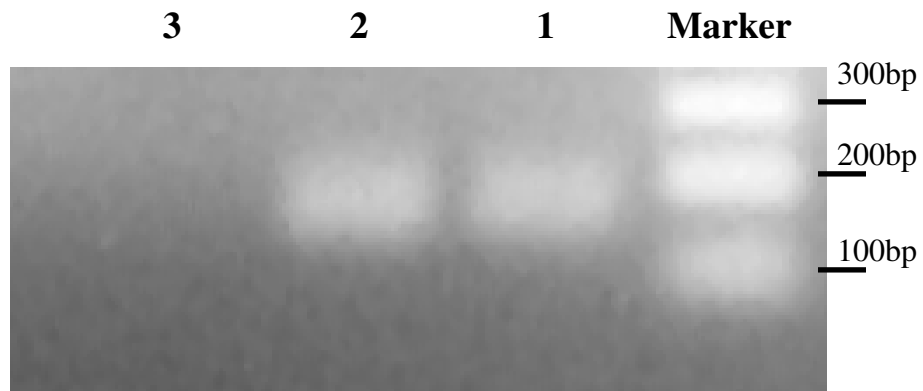
L27 Primers
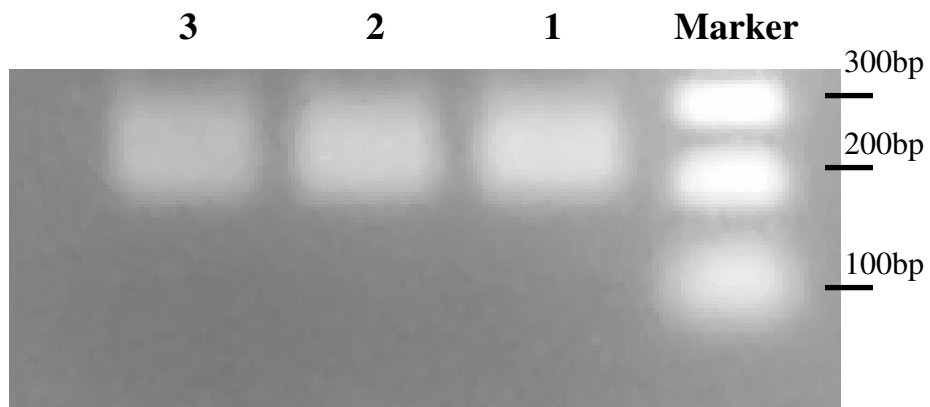
CHAPTER 5 - DISCUSSION

Several forms of experimental evidence indicate that a laccase-type phenol oxidase has been identified in the principal salivary gland and gut of the pea aphid, *Acyrthosiphon pisum.* ABTS, a specific substrate for laccase in the absence of hydrogen peroxide (Hattori et al. 2005), was readily oxidized in certain parts of the salivary gland. Another substrate for laccase, L-DOPA, also tested positive. Both substrates were oxidized in the *Deckzellen*, and, at a slower rate, the posterior regions of the *Hauptzellen* probably corresponding to cell types 4, 5, 6 and 7 in Ponsen's (1972) classification. Further confirmation of the presence of laccase was provided by immunohistochemistry using antibodies against *A. gambiae* laccase-1. In these experiments the regions of the cells that fluoresced were the same regions of the salivary gland that stained positive for the two aforementioned substrates. For the immunohistochemistry control samples, primary antibodies were omitted to negate the possibility of the secondary antibodies binding to specific sites on the salivary gland in the absence of primary antibodies.

When the glands were stained with ABTS in the presence of hydrogen peroxide, a different subset of cells was stained. This result would seem to support the hypothesis that it is indeed laccase that is oxidizing ABTS (in the absence of hydrogen peroxide), and not other peroxidase. Between the two techniques used to visualize the location of laccase within the salivary gland, the immunohistochemical evidence technique was arguably more persuasive because of the specificity of the probe (antibodies against *A. gambiae* laccase-1). Since the glands that were simply stained with substrates were not fixed with fixatives or exposed to detergents, the issue of how readily the substrates diffuse into the membranes may be crucial. One might note that the ABTS staining pattern looked less defined compared to the image produced via immunohistochemistry. Due to specific antibody-antigen binding, the

immunohistochemical technique provided a much clearer representation of laccase localization in the principal gland. Another piece of information that was somewhat lacking with substrate staining was the relative concentration of protein found in various parts of the gland. With immunohistochemistry, it was clear that laccase was most readily found in the *Deckzellen*, where the cells had been lit up intensely, while the posterior regions of the *Hauptzellen* lit up faintly, indicating a lesser concentration. Cell type 3 (the largest cells in the salivary gland) did not fluoresce. Both experimental procedures indicated laccase presence in the essentially same sections of the principal gland.

A secretion signal was predicted using SignalP analysis (Technical University of Denmark), suggesting that laccase-1 may be a secreted protein. Given that the *A. pisum* laccase-1 sequence is 621 residues long (after removing the signal peptide), we can estimate the size of the mature protein to be roughly 68kDa. Western blot analysis revealed 2 bands in the salivary gland sample, as well as the gut sample at approximately 65kDa and 75kDa. These bands, however, were conspicuously missing in the sample which contained the aphid heads with the salivary glands excised. This result suggests that in the head, laccase-1 protein is found only in the salivary glands. Two potential N-glycosylation sites were detected using NetNGlyC (Technical University of Denmark). If laccase-1 is indeed a glycosylated protein that could explain the doublet bands seen on the Western blot. The higher band could be the glycosylated form of the protein, while the lower band is the unglycosylated form. Another possibility, however, is that both bands are glycosylated forms of the proteins. Further experimentation will be required to verify this hypothesis.

RT-PCR revealed that laccase-1 transcript was only expressed in the salivary glands and guts, but not in heads with the salivary glands excised. From this, I conclude that laccase-1

transcript is present in the salivary gland (and nowhere else in the head) and gut. One way to explore laccase-1 expression further would be to obtain thin sections of whole aphids, and do *in-situ* hybridization using probes specific for laccase-1 transcript.

Laccase-1 in relation to the redox hypothesis

Although it has long been known that phenol oxidases are present in the salivary glands of aphids, their functions remain obscure. One approach that we can take when considering the role of laccases in aphid salivary glands is to put it in context of the redox hypothesis (Miles 1993).

The redox hypothesis has been described in the introduction. The crux of the hypothesis is that monomeric plant phenolics are readily oxidizable to quinones that condense with proteins, and are therefore toxic to insects. In this scheme, the initial oxidation of monomeric hydroquinones expresses their toxicity. However, further oxidation considerably decreases it. Hydroquinones may also react with other phenolic compounds to form oligomers that are less toxic and may even be phagostimulants to aphids (Miles 1993).

The redox hypothesis is not without its flaws. The underlying assumption that phloem sap contains a low concentration of protein has been shown to be erroneous. In fact, recent studies demonstrated that in some higher plants, the protein concentration in phloem sap can accumulate to relatively high levels (Kehr 2006). This undermines the scenario where salivary phenol oxidases are hypothesized to be able to rapidly oxidize toxic phenolic compounds to form non toxic polymers in the absence of proteins because, evidently, such conditions are unlikely to exist in nature.

However, the redox hypothesis is still plausible, even in light of high protein concentration in phloem sap. The effect of releasing phenolic compounds in response to insect

feeding, presumably, is that the toxic monomeric quinones and phenols will react and damage internal insect proteins, and thus deter them from feeding. Therefore, as long as the toxic monomers do not reach the internal organs of the aphid, it is safe from its undesired effects. When an aphid releases phenol oxidases into the phloem sap, it may damage plant proteins by reacting them with phenols and quinones. Yet, the aphid itself would not be exposed to the damaging effects of these polymers, and salivation could continue until much of the toxic monomers have been depleted. This could be a partial explanation for continuous salivation of the E1 phase during aphid feeding. An interesting experiment, then, would be to see if the E1 salivation period is longer on aphids feeding on a whole plant, compared to aphids feeding on a single leaf (planted in agarose). It would seem reasonable that a single leaf should have less of the toxic phenols and quinones compared to a whole plant, and as such should take the aphid less time to deplete them before starting the E2 phase of feeding.

The observation that oxygen is depleted in galls formed by some sucking insects due to "enhanced oxidative activities" (Miles 1992) may also be relevant to the role of laccase in the redox hypothesis. Laccase and tyrosinase oxidize substrates by transferring electrons to molecular oxygen, thereby depleting oxygen and forming water in the process. It may be possible, then, that laccase is one of the causative agents of the exhaustion of the oxygen supply in gall formations by aphids and other sucking insects. Tyrosinase is another attractive possibility, but its presence in the salivary gland of *A. pisum* has not yet been confirmed.

The molecular evolution of laccase

Although it is still unclear how laccases evolved as a whole, the fact that laccases have a striking level of conservation with other copper-containing oxidases at the active site level suggests that they are ancient enzymes from an evolutionary point of view (Nakamura and Go

2005, Valderrama et al. 2003). Like other proteins in the multi-copper oxidase family, laccase is believed to have evolved from a single-domain cupredoxin-fold protein family, which includes plastocyanin, azurin, pseudoazurin, rusticyanin, and amicyanin (Nakamura and Go, 2005). Multiplication of cupredoxin domains followed by modification, such as creation of interdomain copper-binding sites and substrate binding sites ultimately led to the formation of contemporary laccases, ascorbate oxidases, ceruloplasmins, and other multicopper oxidases (Nakamura and Go, 2005).

An intriguing but, as of yet, unanswered question is how laccase gene came to exist in insects. One possibility is that of a horizontal gene transfer from an ancient bacterium or fungus, which conferred a primitive laccase-like protein that evolved into its current form today. The fact that laccase-2 has been shown to be crucial in cuticle tanning (Arakane et al. 2005) suggests that laccases should, at least, be as old as ancient insects. This would mean the gene transfer would have taken place before the first "insects" had exoskeletons.

Conclusions

To my knowledge, *Acyrthosiphon pisum* laccase is only the second laccase to be found in the salivary gland of an insect, with the first being discovered by Hattori et al. (2005) in the green rice leafhopper, *Nephotettix cincticeps* (a hemipteran species). Hattori and his colleagues determined that it was laccase, and not other phenol oxidases, using a combination of laccase substrates and inhibitors. Molecular approaches such as RT-PCR, immunohistochemistry and western blot analysis were entirely lacking. In light of this weakness, I would argue that my evidence for the presence of laccase in *A. pisum* is stronger than theirs.

Although the presence of laccase-1 has been established in certain hemiptera species, its exact physiological function remains obscure. To date, there have been no studies done on the

impact of phloem sap proteins on insects, or insect feeding on phloem sap protein composition (Kehr 2006). What is known is that at the whole plant level, phloem-feeding insects such as aphids induce local responses that include the activation of the salicylic acid and jasmonic acid-dependent pathways, as well as upregulation of genes involved in oxidative stress, calcium-dependent signaling, and pathogenesis-related responses such as lipoxygenase, chitinases, peroxidases, and other pathogenesis-related proteins (Kehr 2006).

Laccase could be just one enzyme in a cocktail of phenol oxidases that an aphid utilizes to bypass plant defense. On the other hand, it is also possible that it is a crucial, non-redundant enzyme required for specific metabolic processes. The next logical step would be to do a transcript knockdown of laccase using RNAi and observe the effects on aphid feeding behavior through visual inspection as well as electrical penetration graph. One could also analyze the expression of genes that encode proteins known to be involved in the plant defense mechanisms on plants that have been fed on by normal and laccase-knockdown aphids.

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