MOLECULAR STUDIES OF THE SALIVARY GLANDS OF THE PEA APHID, *ACYRTHOSIPHON PISUM* (HARRIS)

by

NAVDEEP S. MUTTI

M. S., Punjab Agricultural University, India, 1998

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

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Manhattan, Kansas

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ABSTRACT

Salivary secretions are a key component of aphid-plant interactions. Aphids' salivary proteins interact with plant tissues, gaining access to phloem sap and eliciting responses which may benefit the insect. In an effort to isolate and identify key components in salivary secretions, we created a salivary gland cDNA library. Several thousand randomly selected cDNA clones were sequenced. We grouped these sequences into 1769 sets of essentially identical sequences, or clusters. About 22% of the clusters matched clearly to (non-aphid) proteins of known function. Among our cDNAs, we have identified putative oxido-reductases and hydrolases that may be involved in the insect's attack on plant tissue. C002 represents an abundant transcript among the genes expressed in the salivary glands. This cDNA encodes a novel protein that fails to match to proteins outside of aphids and is of unknown function. *In situ* hybridization and immunohistochemistry localized C002 in the same sub-set of cells within the principal salivary gland. C002 protein was detected in fava beans that were exposed to aphids, verifying that C002 protein is a secreted protein. Injection of siC002-RNA caused depletion of C002 transcript levels dramatically over a 3 day period after injection. With a lag of 1 – 2 days, the siC002-RNA injected insects died, on average 8 days before the death of control insects injected with siRNA for green fluorescent protein. It appears, therefore, that siRNA injections of adults will be a useful tool in studying the roles of individual transcripts in aphid salivary glands.

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Salivary secretions are a key component of aphid-plant interactions. Aphids' salivary proteins interact with plant tissues, gaining access to phloem sap and eliciting responses which may benefit the insect. In an effort to isolate and identify key components in salivary secretions, we created a salivary gland cDNA library. Several thousand randomly selected cDNA clones were sequenced. We grouped these sequences into 1769 sets of essentially identical sequences, or clusters. About 22% of the clusters matched clearly to (non-aphid) proteins of known function. Among our cDNAs, we have identified putative oxido-reductases and hydrolases that may be involved in the insect's attack on plant tissue. C002 represents an abundant transcript among the genes expressed in the salivary glands. This cDNA encodes a novel protein that fails to match to proteins outside of aphids and is of unknown function. *In situ* hybridization and immunohistochemistry localized C002 in the same sub-set of cells within the principal salivary gland. C002 protein was detected in fava beans that were exposed to aphids, verifying that C002 protein is a secreted protein. Injection of siC002-RNA caused depletion of C002 transcript levels dramatically over a 3 day period after injection. With a lag of 1 – 2 days, the siC002-RNA injected insects died, on average 8 days before the death of control insects injected with siRNA for green fluorescent protein. It appears, therefore, that siRNA injections of adults will be a useful tool in studying the roles of individual transcripts in aphid salivary glands.

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DEDICATION

Dedicated to my parents, Dr. Darshan Singh and Mrs. Narinder Kaur, for their constant support and encouragement.

CHAPTER 1

Chapter 1: Review of Literature

Aphid Taxonomy

Approximately, 4000 species of aphids have been described (Dixon, 1998). Of these, 250 species are considered pest species (Blackman and Eastop, 2000). Species belonging to tribe Macrosiphini include important agricultural pests, such as the green peach aphid (*Myzus persicae*), the Russian wheat aphid (*Diuraphis noxia*) and the pea aphid (*A. pisum*) (von Dohlen and Moran, 2000; Martinez-Torres et al., 2001; Ortiz-Rivas et al., 2004). Detailed classification of important aphid species is described in Table 1. The pea aphid, *Acyrthosiphon pisum* (Harris), is a member of superfamily Aphidoidae and family Aphididae, within the order Hemiptera (Sorensen, 1995; von Dohlen and Moran, 1995).

Diagnostic morphological features of aphids are shown in Fig. 1. (A) The base of the proboscis lies between and behind the fore coxae; (B) the antennae have two short thick basal segments and a thinner flagellum; (C) there is an ocular tubercle made up of three lenses (a triommatidium) situated behind each compound eye; (D) there are two tarsal segments; (E) the wings have only one prominent longitudinal vein; and (F) there is a pair of siphunculi on the dorsum of the fifth abdominal segment (Heie, 1980; Dixon, 1998).

It is estimated from fossil evidence that Aphidoidae appeared 280 million year ago, in the Carboniferous era (Dixon, 1998). Reproduction by means of unfertilized eggs

(parthogenesis) may have appeared in the late carboniferous or early Permian, over 200 million years ago (Heie, 1967). Viviparity, and other characteristics like shape and venation of their wings and the structure of their proboscis and legs, appeared by the Jurassic (146 million years ago), whereas the cauda and siphunculi appeared later, in the Cretaceous (65 million years ago) (Shaposhnikov, 1977).

Aphids have a soft cuticle; wings, if present, are membranous. Winged aphids are known as alatae and wingless aphids as apterae. They have both sexual (which produce fertilized eggs that overwinter) and parthenogenetic reproduction. Short developmental time and ability of adult females to reproduce several nymphs per day enable aphids to achieve very high rates of increase. Aphids produce the phenotype they require to suit the environmental circumstances they encounter. These kinds of environmentally induced discrete variants are called polyphenisms. If aphids develop on a plant that is crowded with many other aphids, they may develop with wings and fly to a new host plant (Blackman, 1987; Braendle et al., 2005). The mechanisms that allow aphids to switch between alternative morphs have remained obscure. It is believed that well-known insect hormones (like juvenile hormone and ecdysone hormone) regulate these switches (Hardie, 1980; Nijhout, 1999).

Aphid Feeding

Many aphids have a narrow host range. For example, the mustard aphid, *Lipaphis erysimi* feeds only on cruciferous plants. The pea aphid, *A. pisum*, feeds on leguminous host plants, including peas and alfalfa (Blackman and Eastop, 2000). Some aphids have a

broader host range and are classified as polyphagous (Dixon, 1975; Blackman and Eastop, 2000). For example, the host range of the greenbug, *Schizaphis graminum*, includes 70 graminaceous species (monocots) including sorghum and wheat (Michels 1986). The green peach aphid, *Myzus persicae*, has a wider host range covering more than 100 families of plants (Baker, 1982; Cabreray Poch et al., 1998) and is considered as the most polyphagous of all aphids, and can cause significant crop losses (Blackman and Eastop, 1984).

Aphids feed on phloem sap, which they obtain from sieve elements using their stylets. Stylets are needle-like and are formed by the mandibles and maxillae. Mandibular stylets tightly enclose the maxillary pair. The maxillary stylets are always firmly interlocked and appear as a single structure enclosing two minute canals. The food canal is larger (0.7 µm) than salivary canal (0.3 µm) (Ponsen, 1987). The average diameter for an aphid stylet bundle (consisting of 2 maxillary and 2 mandibular stylets) is 4-5 µm. The stylet size varies in relation to species, instar and morph. In general, tree-dwelling aphids, *Adelges spp.* (family Adelgidae, order Hemiptera) and *Eriosoma spp*. (family Aphididae, order Hemiptera) have longer stylet bundles and penetrate inter- or intracellularly to phloem. Herbaceous-dwelling species penetrate intercellulary to the phloem (Pollard, 1973). The stylets penetrate either through the middle lamella, or between the plasmalemma and cell wall (Tjallingii, 1988; Tjallingii and Hogen Esch, 1993). Intercellular penetration is thought to be less deleterious to the plant than intracellular penetration. In the latter process, cells are damaged that may trigger host-plant defenses responses (Walling, 2000).

Phloem sap seems to be an unbalanced diet, with high ratios of sugars:amino acids, non-essential: essential amino acids and K^+:Na^+ ions and low lipid levels (Marschner et al., 1996; Girousse et al., 1996; Douglas, 2003). Carbohydrates, especially sugars, are dominant compounds in the phloem sap. Sucrose accounts for more than 95% of the phloem sugars in many plants, with concentrations ranging between 0.5 M – 1.5 M (Winter et al., 1992). Nitrogen is mainly present in phloem sap as free amino acids (Sandstrom and Pettersson, 1994; Telang et al., 1999). The phloem sap of *Vicia faba* is dominated by two amino acids, asparagine (72%) and glycine (10%). In addition, all the essential amino acids were also detected in the phloem but are present at low concentration (8.2%) (Douglas, 2006).

Aphid antennae bear many sensilla which are used in chemoreception and the perception of the leaf surface (Bromley and Anderson, 1982). Aphids probe the surface of plant with tip of their proboscis. The tactile receptors on the tip of the proboscis respond to contact and surface texture and enable aphids to detect the contours of veins, their preferred feeding site (Tjallingii, 1978). They then probe into the plant with their stylets. Periods of stylet movement and salivation (initially resulting in formation of stylet sheaths) alternate with each other and with periods of suction until the destination, usually phloem, is reached (Prado and Tjallingii, 1994).

Salivary Glands

The salivary glands of species within the suborder Sternorrhyncha (aphids, whiteflies, coccids and psyllids) are labial glands and lie mainly in the anterior region of the thorax. Aphids' salivary glands are paired and consist of two principal glands and two accessory glands. The principal salivary gland is a symmetrical and bi-lobed organ. The duct of the accessory gland unites with the duct of the principal gland to form principal ducts, which further unite to form a common salivary duct that discharges into the salivary canal (Fig. 2) (Ponsen, 1972). The principal salivary gland in *M. persicae* contains 8 different cell-types (Ponsen, 1972). On the other hand, Weidemann (1968) described 9 cell-types in the principal salivary gland in *M. persicae*. According to Ponsen (1972), there are 6 cells of type 5. Weidemann (1968) classified these 6 cells into two different cell types called A and F. Correspondence of cell-types between Ponsen's and Weidemann's nomenclature is shown in Table 2. The accessory salivary gland is composed of few cells (4 cells in *A. pisum*).

Weidemann (1968) has described cell types in the salivary glands of *M. persicae*, and his classification is discussed in detail here. Each lobe is composed of 6 cover cells (or *Deckzellen*) and 15 main cells (or *Hauptzellen*) (Fig. 3). The so called "cover cells" are differentiated in two cell types called H and I based on nuclei staining. Cover cells with irregularly shaped nuclei are called H cells and there are five H cells in each lobe. In addition a cell with a round and translucent nucleus called the I cell is also present in each lobe.

The main cell (or *Hauptzellen*) region is comprised of seven cell types (Fig. 3). Along the caudal external border of each lobe, there are four cells in two planes designated the A cells. Their nuclei are round and contain a dense (thick) chromatin structure similar to that found in the B cells. There are two B cells. The adjacent C cells of irregular shape and with nuclei twice the size of B cell and are found only once in each lobe.

On the innermost edge of the glands, there are two D cells; their nuclei are slightly smaller in size than those of A cells. Above D cells are two E cells; their nuclei are small and typically weakly stained. The F cells are found adjacent to the E cells. They are easily mistaken for A cells because they are similar in appearance and have nuclei of approximately same size. G cells have the most prominent nuclei of the salivary gland bordering the cover cells and can be distinguished from the nuclei of the C cell, by their loose structure as well as their almost perfectly round shape. There are 2 G cells in each lobe.

The fact that these cells are morphologically different suggests that they may have differentiated to serve different functions. A bit of evidence for this is provided by immunodetection of prominent salivary proteins (identified by Baumann and Baumann, 1995, namely 66, 69 and 154 kDa) in the posterior part of the principal salivary gland in greenbug (Cherqui and Tjallingii, 2000). Presumably, aphids' salivary secretion is a mix of the products from one or more cell types.

The ultra-structure of the cells of the principal salivary gland show the presence of a well developed rough endoplasmic reticulum and also the presence of secretory granules, suggesting that they are involved in the synthesis of salivary proteins (Moericke and Wohlfarth-Bottermann, 1960 and 1963; Wohlfarth-Bottermann and Moericke, 1960). On the other hand, function of the accessory gland is largely unknown; but it is involved in virus transmission, based on presence of potato leaf roll virus particles as seen by electron microscopy studies (Glidow et al., 2000).

Like those in aphids, *Drosophila* embryos salivary glands consist of two major cell types: secretory cells and duct cells. Secretory cells are columnar epithelial cells that synthesize and secrete high levels of proteins. At the onset of metamorphosis they also secrete the glue to paste the pupae to the substrate. The high production level of glue proteins is achieved by genome amplification. The chromosomes of the salivary gland nuclei undergo endoreplication (DNA replication without division) and become giant polytene chromosomes (Andrew, 1998). The duct cells are cuboidal epithelial cells that form the simple tubes connecting the secretory cells and form a common duct that discharges into salivary canal.

The initial specification of salivary cells in *Drosophila* occurs within a two-dimensional sheet of cells, the ectoderm, with no known induction from underlying layers. The salivary gland primordium is bilaterally symmetric and consists of approximately 100 cells on either side of the ventral midline (Andrew, 1998; Campos-Ortega and Hartenstein, 1997; Andrew et al., 2000). Salivary glands arise from two ventral

ectodermal plates, in the region of the presumptive posterior head (Panzer et al., 1992; Andrew et al., 2000). Salivary glands of *Drosophila* differentiate without further cell division and increase in size simply by increasing the volume of individual cells (Andrew et al., 2000). Salivary gland development begins at 4.5 h of development and finishing by 10 h of development. This initial specification, which is complete by embryonic stage 10 (about 5.5 h of development), occurs only within a specific region of the anterioposterior axis: parasegment two. In contrast little is known about the embryonic development of the salivary glands of aphids besides the anatomical studies by Ponsen (1972) and Weidemann (1968) on the salivary gland.

Aphid Saliva and Salivary Proteins

Electric penetration graph (EPG) studies have shown four phases of salivary secretion during penetration of host plants by aphids: (1) intercellular sheath secretion, (2) intracellular salivation into cells along the stylet path, (3) initial phloem salivation (into sieve elements); and (4) phloem feeding salivation (Tjallingii, 1988; Tjallingii, 1990; Prado and Tjallingii, 1994; Cherqui and Tjallingii, 2000). Thus, there is ample opportunity for salivary secretions to elicit plant responses, block wound responses or detoxify phytochemicals. Aphid saliva holds the potential to better understand the co-evolution of insect-host interactions (Miles, 1998; Miles, 1999).

Aphids inject a variety of physiologically and biochemically active substances into host plants to facilitate feeding (Miles, 1968; Miles, 1999). Aphid saliva is a mix of ions, amino acids, hemolymph (pumped from myoepithelioid cell) (Ponsen, 1972) and salivary

proteins / enzymes secreted from principal and accessory salivary gland (Miles, 1998; Miles, 1999). The proteins of aphid saliva are of two types, structural and enzymatic. The structural proteins provide a tube-like sheath (Miles 1999; Cherqui and Tjallingi 2000) and are probably few in number, corresponding to major bands on gels with estimated molecular masses of 154 kDa and 66/69 kDa (Baumann and Baumann, 1995). Polyclonal antibodies against these proteins stained sheaths (Cherqui and Tjallingi 2000). Enzymatic assays have been carried out on diluted saliva of aphids (Adams and McAllan, 1956 and 1958; Madhusudhan et al., 1994; Miles, 1999; Cherqui and Tjallingii, 2000). The secreted salivary enzymes fall into two broad categories: hydrolases (pectinases, cellulases, oligosaccharases) and oxidation / reduction enzymes (phenol oxidase (E.C. 1.14.18.1) and peroxidases) (Campbell and Dreyer, 1985; Miles amd Peng, 1989; Miles 1999). The roles of these enzymes during aphid attack on plants are not well understood.

Aphid saliva is believed to perform multiple functions; including creation of the stylet sheath, assisting the penetrations of substrate for food (by the action of pectinases, cellulases, β-glucosidases etc), digesting nutrients (polysaccharases), detoxification of phenolic glycosides ingested during feeding by the action of polyphenol oxidases or peroxidases (oxidation-reduction enzymes) and suppression of host defenses or elicitation of host responses (Miles, 1972; Miles, 1987; Urbanska et al., 1998; Miles, 1999). The salivary sheath is formed as stylets penetrate plant tissue and are left behind as a solid structure of salivary origin after aphid feeding in host plants and on parafilm when feeding on artificial diet (Miles, 1959; Miles 1964a). The sheath material that encases the

stylets is at least partly proteinaceous and begins to gel immediately after it leaves the tips of the stylets (Miles, 1965; Miles, 1990; Miles and Harrewijn, 1991).

Aphid saliva may play a role in the ability of aphids to counter resistance factors in plants, since some species or biotypes of aphids can feed on plants that are resistant to other species or biotypes (Miles, 1999). Additionally, aphid saliva may enhance the bulk flow of solutes across the sieve plates of phloem, and components of saliva may diffuse from one sieve tube and affect the physiology of phloem transport (Miles, 1965; Prado and Tjallingii, 1997; Miles, 1999) and/or block wound response in sieve elements.

Plants cope with a wide variety of physical and chemical, abiotic and biotic stresses. Sieve elements are sensitive to injury; they immediately react to damage in which P-proteins gel in response to the change in the redox condition of the cell (Alosi et al., 1988; Wil and van Bel, 2006). P-proteins, PP1 (96 kDa) and PP2 (48 kDa) have 16 and 6 cysteine residues respectively. Droplets of phloem exudates may form gel due to oxidation of sulfhydryls of the cysteine residues of the P-proteins leading to the formation of intermolecular disulfide bonds (Read and Northcote, 1983, Alosi et al., 1988; Knoblauch et al., 2001). Puncturing of the sieve element of fava beans by a glass microelectrode (with diameter of ~0.1 μm) evokes plugging of its sieve plate within minutes (Knoblauch and van Bel, 1998); whereas an aphid is not only able to puncture but also suck sap from a sieve element for hours and even days (Tjallingii, 1995).

The "redox hypothesis" proposed by Miles and Oertli (1993) states that the oxidative processes in healthy plants are subject to control by reducing systems of the plant such as antioxidants like glutathione and ascorbic acid, and the aphid salivary enzymes serve to change the natural redox equilibrium in the plant to the aphid's advantage. Plants respond to damage by sucking insects by mobilizing and oxidizing phenolic compounds especially monomeric *o*-quinones or phenolic compounds, which are deterrent to insects. Miles and Oertli (1993) proposed that the effective defense by the plant requires oxidation of phenolics at a controlled rate that maintains a deterrent titer of the monomers and at the same time allows controlled oxidation of monomeric quinones and phenols to form polymers and phenol-protein conjugates, which are non-toxic, but serve to seal off damaged cells. Aphid salivary oxidases on the other hand may act by enhancing oxidation in the affected tissue, thereby decreasing concentrations of monomeric phenols and quinones, which may be toxic to the aphid (as electrophilic o-quinones can be alkylated by cellular nucleophiles leading to the formation of reactive oxygen species or isomerization of quinones can lead to quinone methides, which could cause cellular damage) (Miles, 1964b; Miles and Oertli, 1993).

Injected saliva may play a crucial role in the prevention of the plant's wound responses but it may also act as an elicitor of a plant's reaction, resulting in damage during a later stage of the infestation. Greenbugs cause necrotic spots and red spots on wheat and sorghum, respectively (Ma et al., 1990; Girma et al., 1992). Wheat infested by Russian wheat aphid exhibits white streaks (Deol et al., 2001). Some species of aphids can cause chlorosis and necrosis on the growing tip of their host plants whereas other species can

form galls and stunt the growth of fruits (Miles, 1999). These symptoms are attributed to aphid saliva but it is also possible that symptoms are due to the hypersensitive reaction by the host plant.

Recently, researchers have employed a functional genomics approach in order to identify proteins by sequencing of randomly selected clones from various cDNA libraries (whole body, head, gut or salivary glands). A list of the expressed sequence tag (EST) projects undertaken within the order Hemiptera is shown in Table 3. A large scale sequencing of 40,904 ESTs from the pea aphid was carried out (Sabater-Muñoz et al., 2006) leading to 12,082 unique transcript. About 59% (7,146 sequences) showed no match to any protein of known function. Among the 4,936 annotated sequences, 4,080 and 3,977 has a significant match in *D. melanogaster* and *Anopheles gambiae* respectively (Sabater-Muñoz et al., 2006). A similar approach using ESTs to study of the regulation of reproductive modes in aphids was carried out in the cereal aphid, *Rhopalosiphum padi*. The majority of the ESTs sequenced were without matches or encoded hypothetical proteins (56%) followed by housekeeping polypeptides (38%) (Tagu et al., 2004). The likely reason of for such a high proportion of unknown sequences can be either the sequences are too short or may correspond to 5' or 3' untranslated regions. It is also possible that these partial sequences may correspond to a non-conserved domain of a polypepetide, and a longer sequence will allow better identification.

Most of our knowledge of salivary proteins in insects comes from blood feeding insects. Large scale sequencing of ESTs from salivary gland cDNA libraries have led to the

identification of proteins expressed in salivary glands that may play a vital role in blood feeding (Francischetti et al., 2002; Valenzuela et al., 2003; Calvo et al., 2004). The secreted proteins identified from sequencing of salivary glands of mosquitoes contains α-glucosidases and α-amylases that initiate the digestion of carbohydrates present in dietary sugar sources and also other enzymes and peptides involved in blood feeding and ingestion, such as anticoagulants, vasodilators, and platelet aggregation inhibitors (Stark and James, 1996). Some mosquito salivary proteins are immunogens that elicit allergic reactions in the vertebrate hosts (Peng et al., 1995; Peng and Simons, 1997).

Plant Resistance and Defense Response to Aphids

Plant-herbivore relationships are the product of long evolutionary struggles between host and predator (Schoonhoven et al., 1998). Plant conditions can affect probing behavior of aphids, due to change in plant properties, chemical contents of the sap, and/or physiological changes induced by aphid saliva (Hays et al., 1999; Harborne, 1988; Karban and Baldwin, 1997; Prado and Tjallingii, 1997; Ponder et al., 2001; Pegadaraju et al., 2005). Aphid feeding induces changes in plant metabolism and gene expression (Moran and Thompson, 2001; Walling, 2000; Moran et al., 2002).

Plant defenses against insect herbivores can be divided into “static” or constitutive defenses and “active” or induced defenses (Kessler and Baldwin, 2002). A constitutive defense can be a physical barrier, as in lignification or resin production, or an allelochemical that reduces growth and development, or can be a biochemical signal perceived by the herbivore, as in deterrents of feeding or egg deposition, or can act as a

toxin (Harborne, 1988; Bennett and Wallsgrove, 1994). On the other hand, an active or induced mechanism results in the synthesis of proteins, which could act as toxins, or have potential to disrupt pest metabolism (Ryan, 1978). Active defenses normally involve systemic induction. The systemic response may result in the production of defensive proteins (Lamb and Dixon, 1997; Durner et al., 1998; Walling, 2000; Kessler and Baldwin, 2002).

Coordination of these pathways is complex, since the wound and defense response pathways communicate at several levels (Kessler and Baldwin, 2002). First, wound-induced and salicylic acid-activated, mitogen-activated protein kinases appear to coordinate activity of these pathways (Seo et al., 1995; Romeis et al., 1999; Kumar and Klessig, 2000; Petersen et al., 2000). Second, salicylic acid interferes with jasmonic acid biosynthesis, blocking expression of wound-response genes (Pena-Cortes et al., 1993; Dempsey et al., 1999). Third, in *Arabidopsis* the jasmonic acid/ethylene- and salicylic acid-dependent defense pathways appear to converge at regulatory junctions that involve the NPR1, SSI1, and CPR6 gene products (Clarke et al., 1998; Shah et al., 1999; Staswick et al., 1998; Pegadaraju et al., 2005). Thus, plants appear to perceive phloem-feeding herbivores (such as aphids) similar to pathogens and can activate the salicylic acid and jasmonic acid/ethylene signaling pathways (Moran and Thompson, 2001). On the other hand, chewing insects and cell content feeders activate a wound-signaling pathway mediated by jasmonic acid and ethylene (Walling, 2000; Kaloshian and Walling, 2005).

Aphid feeding induces defense response leading to the expression of specific genes. Transcript profiling using cDNA microarrays containing 240 genes from tobacco, *Nicotiana attenuata* revealed that aphid attack (*Myzus nicotianae*) upregulated the expression of defense related and proteinase inhibitor genes but down regulated the expression of photosynthesis regulated genes (Voelckel et al., 2004). Zhu-Salzman and co-workers (2004) used cDNA microarrays with 672 cDNA fragments from sorghum, observed that *S. graminum* elicited a strong induction of salicyclic acid regulated pathogenesis related genes and a weak induction of jasmonic acid pathway regulated genes. Heidel and Baldwin (2004) used oligo-microarrays with 789 genes from tobacco observed that *M. nicotianae* elicited few responses, with up-regulation of genes involved in nitrogen assimilation and transport but it did not alter the expression of threonine deaminase, jasmonic acid methyl transferase and proteinase inhibitor genes (jasmonic acid pathway genes). Moran and co-workers (2002) used cDNA microarrays with 105 ESTs from *Arabidopsis* revealed that aphid attack (*M. persicae* and *Brevicoryne brassicae*) upregulated genes involved in oxidative stress pathway, pathogenesis related proteins and tryptophan biosynthesis. A detailed list of microarray studies done on aphid-plant interactions is provided in Table 4. In addition to the upregulation of various genes upon aphid feeding, there was also down regulation of genes involved in oxidative stress pathway like superoxide dismutase and peroxidase and signaling pathway genes like alpha-dioxygenase and endo-transglycosylase (Moran et al., 2002). Voelckel and co-workers (2004) also observed down regulation of germin and light-harvesting protein. Many photosynthetic pathway genes like RUBISCO, a protein in photosystem II and plastidic aldolase were down regulated upon aphid attack (Heidel and Baldwin, 2004).

Similarly, Bede and co-workers (2006) found that *Spodoptera exigua* salivary factors (possibly glucose oxidase) can act to suppress genes involved in plant defense pathway.

Plant resistance to insect herbivores in some instances can be mediated via constitutive gene effects. For example the tomato gene, *Mi* 1.2 encodes a 1,257 amino acid residues cytoplasmic protein that is a member of leucine zipper, nucleotide-binding, leucine-rich repeat family of R genes. The gene confers multiple resistance to a biotype of potato aphid, *Macrosiphon esculentum* and three species of root-knot nematodes (*Melodogyne arenaria, M. incognita, M. jaranica* and two biotypes of whitefly, *Bemisia tabaci* (Milligan et al., 1998; Rossi et al., 1998; Nombela et al., 2003). In melon, another gene, Vat (virus aphid transmission), encodes a protein with 1,473 amino acid residues and is member of the coiled-coils, nucleotide binding, leucine-rich repeat family of R gene (Dogimont et al., 2003). This gene confers resistance in melon to the cotton melon aphid, *Aphis gossypii* and also to the transmission of certain non-persistent viruses by this aphid (Chen et al., 1997; Martin et al., 1997).

Economic Importance of Aphids

Aphids are among the most important insect pests of temperate agriculture and cause significant losses to U.S. agriculture and also worldwide (Blackman and Eastop, 2000). They damage crops by transmitting pathogenic viruses, depleting photoassimilates, covering plants with honeydew, and altering normal plant physiology (Blackman and Eastop, 2000). Total world insecticide market is worth about $6 billion dollars, of which

$2 billion dollars are spent for the control of sucking pests (Robert Lind, Syngenta; personal communication).

Aphids as Carriers of Viruses

Accessory salivary glands of aphids are important in virus transmission (Glidow et al., 2000). Virus particles are observed in the lumen of the salivary duct. Aphids transmit viruses by one of two general processes (Kennedy et al., 1962). *Non-persistent* viruses are concentrated in the epidermis of the plant, and aphids acquire the virus when they probe the surface of infected plants. Aphids can acquire these viruses with a single probe, within seconds, and also can subsequently transmit it to a healthy plant within seconds. However, non-persistent viruses are retained by the aphid for only a short period – usually only an hour or two. After that point the aphid no longer can transmit the virus unless it feeds on another infected plant (Gray and Gildow, 2003; Reavy and Mayo, 2002). Because of the rapid acquisition and transmission of the non-persistent viruses, insecticides have little or no effect on reducing spread by aphids. Examples of non-persistent viruses spread by aphids include potato virus Y and alfalfa mosaic virus. Potato aphid and green peach aphid are highly efficient vectors of non-persistent viruses, other aphid species can also transmit these viruses.

Persistent viruses are concentrated in the phloem, and aphids acquire the virus only after feeding on the phloem for a while. This process takes a minimum of 30 minutes after probing a plant and often considerably longer. Once an aphid has acquired a persistent virus, the virus moves internally in the insect and eventually migrates to the accessory

salivary gland (Ponsen, 1972). Completion of this circulation within the insect can take days after feeding on an infected plant. However, once the virus begins to appear in the salivary glands the aphid will transmit it for the remainder of its life. Insecticides can be somewhat more effective in reducing spread of persistent viruses than non-persistent viruses, particularly if the insecticide rapidly incapacitates the aphid vector. Examples of persistent viruses spread by aphids include potato leafroll virus and beet western yellows virus (Gray and Gildow, 2003). Aphids do not transmit the mechanically transmitted viruses like potato virus X.

The pea aphid is an important vector of viral diseases of legumes (Zitter and Provvidenti, 1984). Peas are susceptible to a large number of aphid- transmitted viruses. Pea enation mosaic virus infects legumes in the temperate regions of the world. In addition to pea, pea enation mosaic virus also infects broadbean, sweet pea, and alfalfa. The virus is spread in nature most efficiently by the pea aphid and to a lesser extent by the green peach aphid. The virus is transmitted in a persistent (circulative) manner. Pea leafroll mosaic virus, red clover vein mosaic virus, clover yellow vein virus and bean yellow mosaic virus are also transmitted by pea aphid but in non-persistent manner (Zitter and Provvidenti, 1984).

Symbionts of Aphids

As mentioned earlier, phloem sap provides aphids an unbalanced diet. Aphids overcome this imbalance partly through the nutritional contribution from their symbiotic micro-organisms. Neither an aphid nor its symbionts can fix atmospheric nitrogen (Douglas,

1998; Dixon, 1998). Therefore, the aphid has to ingest the necessary amount of nitrogen for protein synthesis from the phloem sap. Thus, the symbiont can improve aphid nutrition only by “correcting” the composition of ingested amino acids in the phloem sap, using its broader biosynthetic capabilities (Wilkinson and Ishikawa, 1999). Aphids feeding on different plants appear to vary depending on their symbionts for their overall essential amino acid synthesis, due to the large variation in proportion of essential amino acids in phloem sap from different plant species (Sandstrom and Moran, 1999). Generally methionine and leucine are always present in low concentration in the phloem sap, suggesting a higher dependence on the symbiont for the synthesis of these amino acids.

The term symbiosis was first introduced by Anton de Bary in 1879 as “the permanent association between two or more specifically distinct organisms, at least during a part of the life cycle.” Symbiosis is only when both partners benefit from the association. It is estimated that at least 15-20% of all insects live in symbiotic relationships with bacteria (Buchner, 1965). Symbiotic relationship between insects and bacteria could be the key factor in the evolutionary success of insects (Moran and Baumann, 2000). Insect endosymbionts live inside specialized host cells called bacteriocytes, in the body cavity of insects (Douglas, 1989). Endosymbionts cannot be cultured outside of host and host needs the bacteria for normal growth and reproduction (Gil et al., 2002). Bacteriocyte-associated endosymbionts are vertically transmitted from mother to the offspring through developing egg or embryo (Buchner 1965; Houk and Griffiths, 1980).

The mutualism between aphids and their primary (obligate) bacterial endosymbiont *Buchnera aphidicola* is well characterized (Munson et al., 1991; Wilkinson et al., 2001; Douglas, 2006). *Buchnera* lives only within specialized aphid cells called bacteriocytes and can synthesize essential amino acids and can supplement nutrients present at low concentration in the phloem sap (Douglas, 1998). Removal of *Buchnera* with antibiotics severely debilitates aphid performance and fecundity (Prosser and Douglas, 1991). The sequencing of three *B. aphidicola* genomes revealed the presence of genes coding for the biosynthesis of essential nutrients (especially amino acids) that are lacking in the aphids' diet (Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003).

B. aphidicola is believed to complement an aphid's diet by synthesizing vitamins, sterols and certain amino acids (Douglas, 2003; Douglas, 2006). In particular, in *M. persicae*, the symbionts incorporate inorganic sulphate into the methionine and cysteine (Douglas, 1988). Symbionts synthesize tryptophan in *A. pisum* and *S. graminum* (Douglas and Prosser, 1992; Munson and Baumann, 1993). The gene (*trp*EG) responsible for tryptophan biosynthesis in *S. graminum* is present in multiple copies in *Buchnera* (Moran et al., 2003).

In addition to the primary symbiont, some aphids harbor other intercellular symbionts. They are called secondary (facultative) symbionts. It is likely that they have been acquired independently many times in various aphid species beacuse they are not confined to a particular group of aphids. The pea aphid can lack secondary symbionts or contain various combinations of at least five kinds of secondary symbionts: three γ-3

proteobacteria designated as the R, T and U types, a *Rickettsia* and a *Spiroplasma* (Sandstrom et al., 2001, Chen et al., 1996; Fukatsu et al., 2001). Endosymbionts are vertically transmitted (from mother to daughter), and the infection status of a particular parthenogenetic aphid lineage is stable in the laboratory (Sandstorm et al., 2001). Within hosts, secondary symbionts are found in and near bacteriocytes, sporadically in other cell types, and free in the hemolymph (Oliver et al., 2003). A vertically transmitted symbiont, or one with low levels of horizontal transmission, will be lost from a population if carrying it imposes a cost on the host, so it must have some beneficial effects of carrying secondary symbionts. Secondary symbiont infection with γ-proteobacterium called pea aphid U-type symbiont plays a vital role in the host plant specialization of pea aphid, thereby improving growth and reproduction of the pea aphid on non host white clover (Tsuchida et al., 2004). Pea aphid U-type symbiont also plays role in providing resistance to pea aphid against major fungal pathogen *Pandora neoaphidis* (Scarborough et al., 2005).

RNA Interference in Insects

The term RNA interference or “RNAi” was coined by Fire and coworkers to describe the observation that gene expression can be blocked by double-stranded RNA (dsRNA) in *Caenorhabditis elegans* (Fire et al., 1998). RNAi occurs posttranscriptionally and involves mRNA degradation by complementary siRNAs, small (21-23 nucleotide) double-stranded RNAs thus can act as specific determinants for down-regulation of gene expression. Therefore, siRNA provides a valuable reagent for inactivation of gene expression. The most important feature of the mechanism of RNAi is the processing of

long dsRNA into duplexes of 21-23 nucleotide RNAs (Zamore et al., 2000). RNAi has become an important tool for down-regulating specific gene expression in many species.

RNAi appears to be related to the posttranscriptional gene silencing mechanism of cosuppression in plants (Cogoni and Macino, 1999; Fagard et al., 2000). Cosuppression is the ability of some transgenes to silence both themselves and homologous chromosomal loci simultaneously. The initiator molecule for cosuppression is believed to be aberrant RNA, possibly dsRNA, and some components of the RNAi machinery are required for posttranscriptional silencing by cosuppression (Catalantto et al., 2000; Ketting and Plasterk, 2000; Dernburg et al., 2000).

Dicer, a cytosolic ribonuclease III, digests long double-stranded RNA into oligonucleotides of length 21-23-nucleotide units (Elbashir et al., 2001; Hamilton and Baulcombe, 1999). The two strands of the siRNA are generated but the antisense strand, relative to the mRNA target, exhibits greater silencing efficiency if it has a relatively thermodynamically unstable 5' end (Martinez et al., 2002). Recent evidence suggests that binding of RNA-induced silencing complex to siRNA is coordinated with Dicer cleavage. Moreover, the loss of Dicer 2 in *Drosophila melanogaster* also results in loss of RNAi activity mediated by siRNA (Kim et al., 2005). The RNA-induced silencing complex contains the Argonaute 2 catalytic subunit that binds siRNA and mediates mRNA target recognition and inactivation (Yan et al., 2003). The success of RNAi, hinges on the affinity of siRNA molecule for its target mRNA (Miyagishi and Taira, 2005). The

regulatory targets of siRNAs are usually very similar in sequence to the target gene (Hammond et al., 2000; Allen et al., 2005).

RNAi has been successfully used in arthropods. Injections of dsRNA or siRNA in post-embryonic stages have been used successfully in: the honeybee, *Apis mellifera* (Beye et al., 2002; Amdam et al., 2003); the giant silkmoth, *Hyalophora cecropia* (Bettencourt et al., 2002); the fall armyworm, *Spodoptera litura* (Rajagopal et al., 2002); the silkmoth, *Bombyx mori* (Uhlirova et al., 2003); the malarial mosquito, *Anopheles gambiae* (Osta et al., 2004); the yellow fever mosquito, *Aedes aegypti* (Attardo et al., 2003); the tobacco hornworm, *Manduca sexta* (Levin et al., 2005); and the red flour beetle (*Tribolium castaneum* (Tomoyasu et al., 2005). Apparently injected dsRNA and /or siRNA can move from the hemolymph into various tissues or organs, and can lead to target mRNA degradation.

Specific Objectives

In my research I have undertaken a functional genomics approach to identify components of aphid saliva. Identification of secreted proteins from the salivary glands is essential in understanding the interaction between aphid and its host plant. We have chosen to do this work with pea aphid, *A. pisum*, because of its large size (compared with other aphid species), thus making dissections of salivary glands relatively easy and also it is a model aphid species and is chosen for genome sequencing (http://www.hgsc.bcm.tmc.edu/projects/aphid/).

a) To build a salivary gland cDNA library and sequence several thousand randomly selected clones and analyze ESTs.

b) To clone and characterize C002 an abundant cDNA in our library.

c) To examine the effect of C002 transcript levels on survival and fecundity.

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Figure 1. Diagnostic morphological features of an aphid.

(A) The base of the proboscis lies between and behind the fore coxae; (B) the antennae have two short thick basal segments and a thinner flagellum; (C) there is an ocular tubercle made up of three lenses (a triommatidium) situated behind each compound eye; (D) there are two tarsal segments; (E) the wings have only one prominent longitudinal vein; and (F) there is a pair of siphunculi on the dorsum of the fifth abdominal segment. (Used with permission) (Heie, 1980).

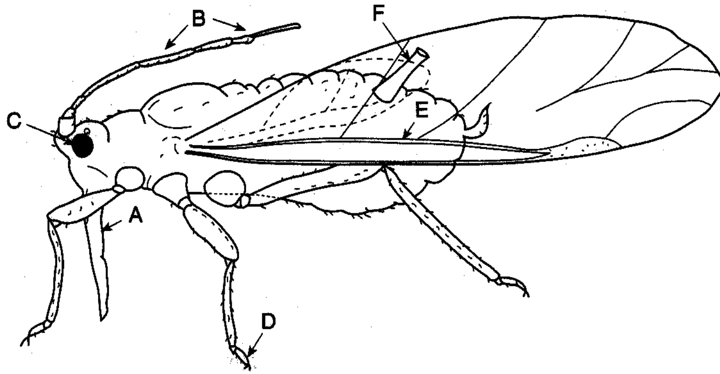
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Figure 2. Anatomy of the salivary gland of the green peach aphid, *M. persicae*.

(A) Graphical representation of the salivary gland from 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 represents *Deckzellen* (dz) and cell types 3-8 represents *Hauptzellen* (h). (B) Transverse section of the common salivary duct (csd). (C) Transverse section of the middle region of the principal 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).

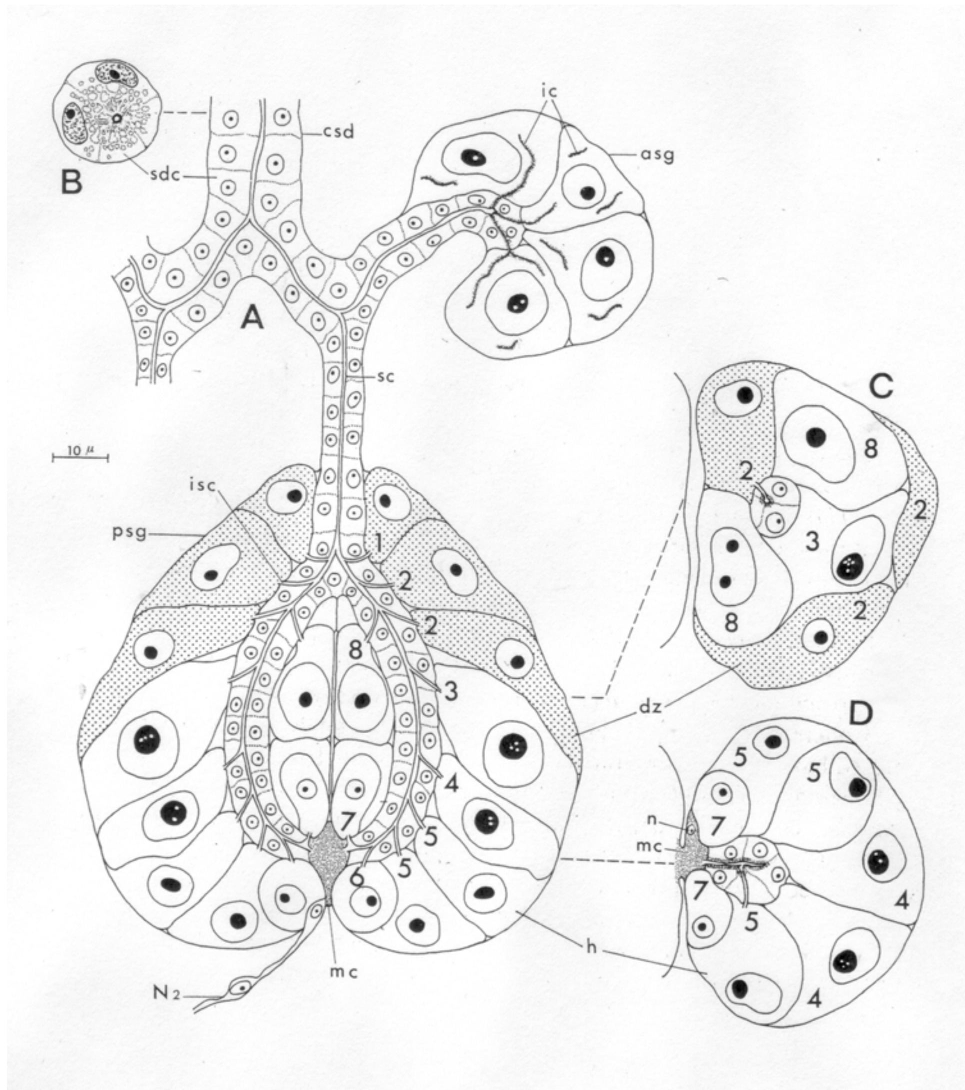
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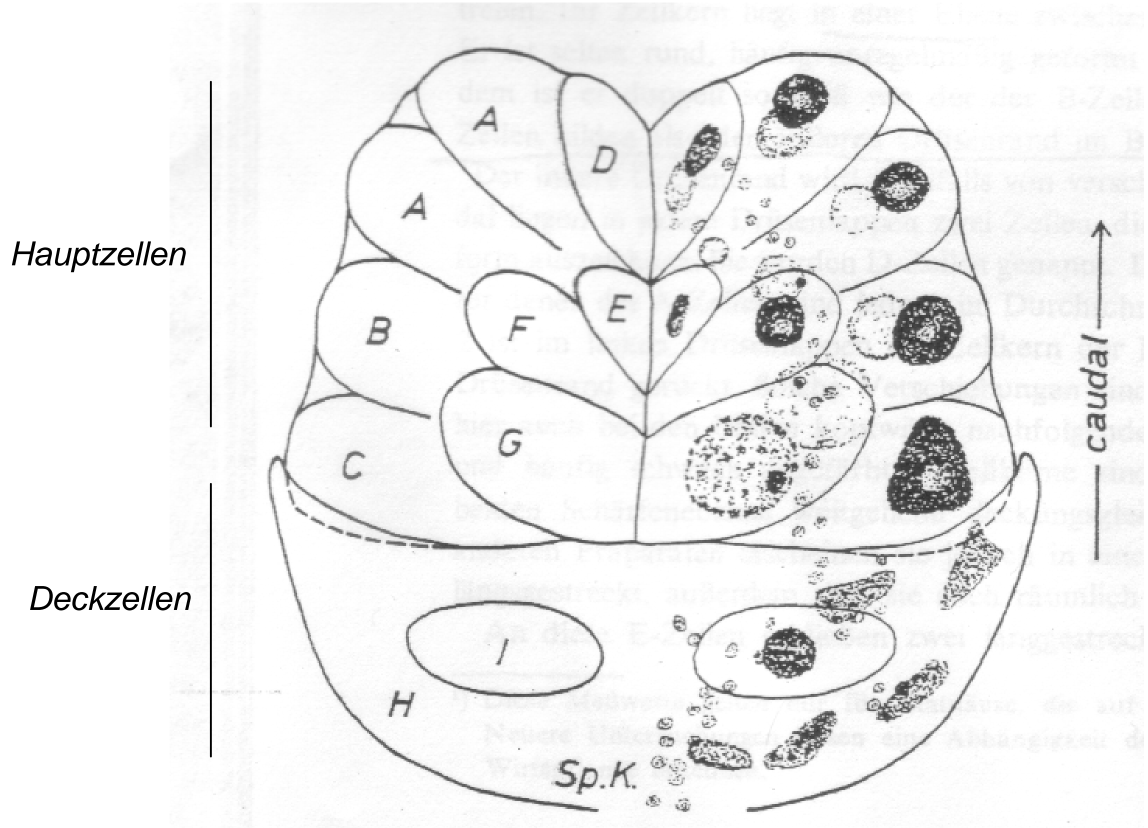
Figure 3. Feulgen stained principal salivary gland of *M. persicae*.

Cell types H and I represent *Deckzellen* and cell types A, B, C, D, E, F, and G represent *Hauptzellen* (Used with permission) (Weidemann, 1968).



Hauptzellen
Deckzellen
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A
D
B
F
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H
I
Sp.K.
caudal

Table 1. Classification of selected aphid species.

Common name	Scientific name	Tribe	Reference
Pea aphid	*Acyrthopisum pisum*	Macrosiphini	von Dohlen and Moran, 2000 ; Martinez-torres et al., 2001; Ortiz-Rivas et al., 2004
Russian wheat aphid	*Diuraphis noxia*	Macrosiphini	Heimpel et al., 2004
Green peach aphid	*Myzus persicae*	Macrosiphini	Martinez-torres et al., 2001 ; Ortiz-Rivas et al., 2004
Greenbug	*Schizaphis graminum*	Aphidini	von Dohlen and Moran, 2000; Martinez-torres et al., 2001; Ortiz-Rivas et al., 2004
Soybean aphid	*Aphis glycines*	Aphidini	Heimpel et al., 2004

Note: I have classified aphids under order Hemiptera instead of Homoptera, which is the deeper clade than Homoptera. Since the members of the Homoptera and Hemiptera are mixed on the same phylogenetic tree, it is convenient to place them all under Hemiptera (Sorensen, 1995; von Dohlen and Moran, 1995).

Table 2. Classification of cell types of salivary glands of *M. persicae*.

<table>
<tr><th rowspan="2"></th><th colspan="2">Ponsen, 1972</th><th colspan="2">Weidemann, 1968</th></tr>
<tr><th>Cell types</th><th>No. of Cells</th><th>Cell types</th><th>No. of Cells</th></tr>
<tr><td rowspan="2">Hauptzellen</td><td>1</td><td>1</td><td>I</td><td>1</td></tr>
<tr><td>2</td><td>5</td><td>H</td><td>5</td></tr>
<tr><td rowspan="7">Deckzellen</td><td>3</td><td>1</td><td>C</td><td>1</td></tr>
<tr><td>4</td><td>2</td><td>B</td><td>2</td></tr>
<tr><td rowspan="2">5</td><td rowspan="2">6</td><td>A</td><td>4</td></tr>
<tr><td>F</td><td>2</td></tr>
<tr><td>6</td><td>2</td><td>D</td><td>2</td></tr>
<tr><td>7</td><td>2</td><td>E</td><td>2</td></tr>
<tr><td>8</td><td>2</td><td>G</td><td>2</td></tr>
</table>

Table 3. EST projects within Hemiptera.

Insect	Tissue	No. of EST	Reference
A. pisum	Head	20,988	Sabater-Muñoz et al., 2006
	Whole body	6,668	
	Whole insect	11,092	
	Digestive tract	9,307	
	Parthenogenetic embryo	5,442	
	Antennae	10,096	
	Salivary gland	4,517	Unpublished
Toxoptera citricida	Whole insect	4,304	Hunter et al., 2003
M. persicae	Whole body	6,996	Unpublished
R. padi	Whole body	459	Tagu et al., 2004
Homalodisca coagulata	Whole body	4,529	Unpublished
Rhodnius prolixis	Salivary gland	44(252)*	Ribeiro et al., 2004

* 252 ESTs were sequenced by Ribeiro et al. (2004) but only 44 ESTs were deposited at NCBI.

Table 4. Plant genes up-regulated in response to aphid feeding based on microarray analysis.

Insect	Plant	Gene up-regulated	Reference
S. graminum	Sorghum	**Salicyclic acid pathway** β-1,3-glucanase; Chitinase; Thaumatin-like protein; Wound-induced PI; PR10. **Jasmonic acid pathway** Lipoxygenase; Bowman-Birk protease inhibitor; Dhurrinase. **Signal transduction** Defense related protein. LRR-containing glycoprotein. **Active oxygen related** Glutathione-S-transferase, Lactoyglutathione lyase. **Secondary metabolites** Methyltransferase; Flavanone 3-hydroxylase. **Abiotic stress** Aldehyde oxidase; Drought, salt, low temperature responsive protein. **Cell maintenance** Nitrite reductase.	Zhu-Salzman et al., 2004
M. nicotianae	Tobacco	Nitrogen-uptake and metabolism genes Nitrate transpoter; Feroxin-dependent glutamate synthase; Glutamine synthetase.	Heidel and Baldwin, 2004
M. persicae	*Arabidopsis*	Pathogenesis related protein, Anthranilate synthase beta subunit, Glutathione-S-transferase; ACC oxidase.	Moran et al., 2002
B. brassicae	*Arabidopsis*	β-1,3-glucanase; defensin; PR-1; Sugar transpoter gene.	Moran et al., 2002
M. nicotianae	Tobacco	Trypsin protease inhibitor; Lipoxygenase; Xyloglucan-endotransglycosylase; Glutamate synthase.	Voelckel et al., 2004

* Only prominent differences are listed in the table.

CHAPTER 2

Chapter 2: An EST library from Salivary Glands of the Pea Aphid, *Acyrthosiphon pisum*.

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Key words: pea aphid, salivary glands.

Abbreviations: EST, expressed sequence tag; NCBI, National Center for Biotechnology Information. PCR, polymerase chain reaction; PBS, phosphate buffered saline; ORF, open reading frame

Abstract

Several thousand ESTs in a salivary gland cDNA library of the pea aphid, *Acyrthosiphon pisum* have been sequenced. A cluster analysis based on sequence similarity grouped 5,098 sequences into 1,769 clusters. A majority of the clusters (about 78% or 1,392 clusters) did not match to any sequence, only about 22% (or 377 clusters) were assigned putative functions based on BLASTX against the UniProt database. These 377 clusters encode proteins with putative secretion signals, housekeeping genes or hypothetical proteins. Among secreted proteins, we found 14 clusters predicted to code for proteases, 11 clusters predicted to code for oxidoreductases, and 8 clusters predicted to code for other hydrolases. Among the housekeeping cDNAs, we found clusters coding for heat shock proteins, cytochrome oxidases, transcription factors, polymerases, calcium or metal binding proteins, ATPases and enzymes of electron transport and DNA binding proteins.

Introduction

Aphid saliva holds the potential to better understand the co-evolution of insect-host interactions (Miles, 1999). Aphid saliva is believed to perform multiple functions, including the creation of the stylet sheath, assisting the penetration of substrate for food (by the action of pectinases, cellulases, β-glucosidases, etc.), digesting nutrients (polysaccharases and proteases), detoxification of phenolic glycosides ingested during feeding by the action of polyphenol oxidases or peroxidases (oxidation-reduction enzymes), and the suppression of host defenses or the elicitation of host responses (Miles, 1972; Miles, 1987; Urbanska et al., 1998; Miles, 1999). The fact that aphid saliva is only available in very small quantities makes the direct study of salivary components difficult (Miles, 1965; Madhusudhan et al., 1994; Miles and Harrewijn, 1991).

In an attempt to reveal the complexity of the pea aphid, *Acyrthosiphon pisum* (Harris) salivary glands, a high-throughput approach designed to identify a large number of cDNAs in the salivary glands has been employed in the present work. ESTs have become an effective means of gene discovery. In the past, similar approaches have been very successful in salivary glands of blood-sucking bug, *Rhodnius prolixus* or mosquito, *Anopheles stephens* (Valenzuela et al., 2003; Ribeiro et al., 2004), particularly when the cDNA libraries have been prepared from tissues with high activity for the respective enzymes.

We have chosen to work with the pea aphid, *A. pisum*, because of its large size (compared with other aphid species), thus making dissections of salivary glands less

difficult. Generation of a set of pea aphid salivary cDNAs, along with the availability of the pea aphid genome later this year (http://www.hgsc.bcm.tmc.edu/projects/aphid/), will provide an indispensable tool for the systematic analysis of proteins/enzymes that may play roles in the aphid-plant interactions. Here we describe the annotation of 1,726 clusters representing 5,098 mRNA sequences. Remarkably, only about 22% of our cDNA sequences match to sequences of known functions.

Material and Methods

Plants and Aphids

Aphids were originally collected from alfalfa plants in the summer of 1999 by Dr. Marina Caillaud at Cornell University. Thereafter, the aphids were reared at KSU on fava beans (*Vicia fabae*) grown in pots (10 cm diameter) at room temperature under high intensity sodium lights with a L:D of 16:8. Salivary glands from adult aphids were dissected and separated from the brain tissue overlaying the glands. Dissected glands were transferred to 50µl of PBS (137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) for 50 paired glands and washed 3 times in PBS to remove hemocytes. Salivary glands were kept at –75°C until needed.

Phagmid cDNA Library Construction

Total RNA was isolated from 250 salivary glands using the Micro RNA isolation kit from Stratagene, La Jolla, CA. The PCR-based cDNA library was made following instructions with the SMART cDNA library construction kit (Clontech, Palo Alto, CA). Total RNA

was reverse transcribed using PowerScript reverse transcriptase and CDS III primer provided in the kit (Clontech, Palo Alto, CA). Second strand synthesis was performed through the PCR-based protocol using SMART III and CDS III primers from Clontech. Double strand synthesis was followed by proteinase K digestion. Double strand cDNA was ligated into a Lambda TriplEx2 vector (Clontech, Palo Alto, CA), and the resulting ligation reaction was packaged using Gigapack Gold III from Stratagene following the manufacturer's specifications.

Sequencing, Sequence Processing, and Annotation

Sequencing of the cDNA clones was done either at the Kansas State University sequencing facility or at Genoscope, Evry cedex, France (http://www.cns.fr/). Raw EST data were analyzed using the Lucy program (Chou and Holmes, 2001) for sequence quality and vector sequence removal. Then ESTs were clustered into groups of nearly identical sequences using the CAP3 software tool using default settings (Huang and Madan, 1999). The non-redundant set of clusters (contigs and singletons) was searched against the UniProt reference database (Bairoch et al., 2005) using the BLASTX program downloaded from NCBI. A query sequence was annotated using the best hit in UniProt with E value threshold set to 1e-5. The functional annotation included text description as well as gene ontology terms of the matched reference sequence (Camon et al., 2003). cDNAs were translated and analyzed for signal peptide and cleavage information using SignalP 3.0 Server (Nelson et al., 1997) using default parameters (http://www.cbs.dtu.dk/services/SignalP/).

Results

Salivary Transcript Catalog for *A. pisum*

We have sequenced 5,098 ESTs from the salivary gland cDNA library. A cluster analysis based on sequence similarity grouped 5,098 EST sequences into 1,769 clusters. Cluster refers to both contigs (containing 2 or more ESTs) and singletons. Putative functions corresponding to these salivary gland ESTs collection was assigned by comparing these ESTs with UniProt database using BLASTX with E value threshold set to 1e-5. Among the 1,769 clusters, 1392 (78%) showed no sequence similarity with any other protein sequences and only 377 clusters (22%) were assigned putative functions.

Classification of Salivary Transcripts of *A. pisum*

Classification of 377 clusters with significant matches to sequences in UniProt database is provided in Table 5. 72 clusters correspond either to hypothetical proteins or to proteins that were not annotated. There are several clusters corresponding to ribosomal proteins (42), ATPase/ATP binding proteins (27), polymerases (20), cytochromes (16), DNA binding proteins (15), proteases (14), metal binding proteins (14), mitochondrial proteins (14), oxidoreductases (11), transcription factors (10), protein kinases (9), heat shock proteins (9) and other hydrolases (8). We also found several clusters encoding putative secreted proteins including several oxidoreductases, several proteases and several carbohydrases. Among other secreted proteins identified included glutathione-S-transferase, apolipophorin precursor protein, carbonic anhydrase, 11 kDa salivary protein,

odorant binding protein, armet like protein. There is a group of 13 clusters which were previously assigned under structural component of cell wall, cell membrane and nucleus, now listed listed as a part of Table 6 under group X for miscellaneous proteins.

We also found two clusters corresponding to viral sequences, and one cluster corresponding to a plant sequence. The two viral clusters are non-overlapping fragments of a 89.2 kDa capsid protein from an aphid-infecting virus, aphid lethal paralysis virus (van Munster et al., 2002). This virus belongs to the recently recognized family Dicistroviridae (Mayo, 2002). The plant cluster corresponds to a phospholipase C from *Arabidopsis thaliana* and is probably the result of contamination from the host plant. It was not found in the latest sequencing of 4,517 ESTs.

Further analysis using TBLASTX against NR database at NCBI of 72 clusters corresponding to hypothetical or unannotated proteins is shown in Table 6. Signal P analysis performed on homologs from other species identified 8 clusters encoding secreted proteins. We also found three clusters encoding proteins from a pea aphid secondary endosymbiont (*Candidatus hamiltonella*). In total putative functions were assigned to 57 clusters (not just looking at the top match but also looking other matches with significant e value). Only 15 clusters (out of 72) were not assigned any putative function and encoded either a hypothetical protein or protein of unknown function.

The clusters with putative functions were classified based on the number of ESTs in a contig (Table 7). Contig 90 contains 109 ESTs and is an aphid-infecting virus, aphid

lethal paralysis virus. Contig 33 represents a cluster with 57 EST sequences and is cytochrome oxidase subunit I. Other clusters in Table 7 include ribosomal protein (ribosomal protein S15A, contig 94 with18 EST sequences) and hypothetical protein (contig 97 with 40 EST sequences). We also found clusters representing glucose dehydrogenase (14 ESTs), angiotensin-converting enzyme (15 ESTs), glutathione peroxidase (10 ESTs), apolipophorin (8 ESTs) and superoxide dismutase (6 ESTs).

Many clusters (1,392) showed no sequence similarity with any other sequences. Most of the clusters either have short ORF's or entirely lack ORF (Table 8). There are 21 clusters which, despite having an ORF of 200 or more amino acids residues, showed no similarity to any other sequence. The presence of ORF drops with number of ESTs represented in a cluster. Most of the clusters with three ESTs have no ORF or very short ORF. Clusters with two or one EST sequence are not listed in Table 8. This high number of unmatched clusters might reflect the limited sequence quality delivered by single-pass sequencing (for example, too short sequences, wrong base identified leading to frame shift errors). Among these clusters of unknown function, an abundant cluster (contig 32) representing 46 EST sequences referred to as C002 was further characterized (Chapter 3) and also used to develop RNAi as a tool to study gene expression in pea aphid (Chapter 4).

Proteases and Other Hydrolases in the Salivary Glands of *A. pisum*

Annotation of clusters revealed the presence of 14 clusters representing putative proteases and 5 clusters representing other putative hydrolases (Table 9). Among proteases we have identified subtilisin related protease, a member angiotensin-converting

enzyme family, peptidase M1, cathepsin L, cathepsin B, signal peptidase, endoprotease FURIN and ubiquitin specific protease. SignalP analysis done on homologs from other species (as our pea aphid salivary sequences are not full length) identified in BLAST analysis at NCBI shows that some encode proteins with a putative signal peptide. Therefore based on SignalP analysis, 8 clusters out of 14 clsuter encoding putative proteases represent putative secreted proteases. Among other hydrolases we have identified putative S-adenosyl homocysteine hydrolase, prolyl 4-hydroxylase alpha subunit, alpha-glucosidase, trehalase, carbon-nitrogen hydrolase and phosphoesterase. Based on SignalP analysis described above, 3 clusters represent putative secreted other hydrolases.

Oxidoreducatases in the Salivary Glands of *A. pisum*

Annotation of clusters also revealed the presence of 11 clusters representing putative oxidoreductases (Table 10). These clusters represent putative glucose dehydrogenase, glutathione peroxidase, aldehyde dehyrogenase, thioredoxin peroxidase, phosphoglycerate dehydrogenase, peroxiredoxin-like protein, dimethylaniline monooxygenase, superoxide dismutase and peroxidase. SignalP analysis done on the homologs (as our pea aphid salivary sequences are not full length) identified in BLAST analysis at NCBI, shows that some encode a putative secreted protein and has a putative signal peptide. Therefore, based on SignalP analysis, 5 clusters represent putative secreted oxidoreductases.

Discussion

To our knowledge, this is the first attempt to create a catalog of the cDNAs from the salivary glands of the pea aphid, *A. pisum* or any other aphid species. The majority of the clusters (78%) could not have their putative function annotated. The most likely reason for this lack of similarity is that some of the sequences are too short or may represent 5'- or 3'-untranslated regions. Second it is possible that these partial sequences correspond to a non-conserved domain of polypeptide: a longer sequence should allow a better identification of these clusters. Finally, this might reflect limitations of single pass sequencing (too short sequences, wrong base calling leading to frame shift errors). For the clusters represented under hypothetical proteins or unannotated proteins (75 clusters), it is possible that some of these proteins without matches correspond to aphid specific proteins of cellular functions not yet elucidated. Genes involved in environmental adaptation evolve quickly and might correspond to unannotated sequences (Domazet-Loso and Tautz, 2003). As aphids are highly sensitive to environmental changes, it is possible that these clusters may correspond to rapidly evolving sequences.

The largest proportion of functionally annotated sequences falls into following categories: ribosomal proteins represented in 42 clusters, there are several clusters corresponding to ATPase/ATP binding protein (27), polymerases (20), cytochromes (17), DNA binding proteins (15), proteases (14), metal binding proteins (14), mitochondrial proteins (14), oxidoreductases (11), transcription factors (10), protein kinases (9) heat shock proteins (9) and other hydrolases (8). We also found several clusters encoding putative secreted proteins including several oxidoreductases, several proteases and

several carbohydrases. Among other secreted proteins identified included glutathione-S-transferase, apolipophorin precursor protein, carbonic anhydrase, 11 kDa salivary protein, odorant binding protein, armet like protein.

A large scale sequencing of 40,904 ESTs from the pea aphid was carried out (Sabater-Muñoz et al., 2006) leading to 12,082 unique transcripts. About 59% (7,146 sequences) showed no match to any protein of known function. Among the 4,936 annotated sequences, 4,080 and 3,977 has a significant match in *D. melanogaster* and *Anopheles gambiae* respectively (Sabater-Munoz et al., 2006). A similar approach using ESTs to study of the regulation of reproductive modes in aphids was carried out in the cereal aphid, *Rhopalosiphum padi*. The majority of the ESTs sequenced were without matches or encoded hypothetical proteins (56%) followed by housekeeping polypeptides (38%) (Tagu et al., 2004).

The “redox hypothesis” proposed by Miles and Oertli (1993) states that the oxidative processes in healthy plants are subject to control by reducing systems of the plant such as antioxidants like glutathione and ascorbic acid, and that the aphid salivary enzymes serve to change the natural redox equilibrium in the plant to the aphid’s advantage. Plants respond to damage by sucking insects by mobilizing and oxidizing phenolic compounds especially monomeric *o*-quinones or phenolic compounds, which are deterrent to insects (Miles and Oertli 1993; Harmatha and Nawrot 2002). Several putative oxidoreductases were identified in our study (Table 9). Five of them encode enzymes with putative

secretion signals; these salivary oxidases can act by enhancing oxidation, thereby decreasing the concentration of monomeric phenols and quinones.

In *Helicoverpa zea*, glucose oxidase present in saliva suppresses jasmonic acid related plant defense, presumably by the production of hydrogen peroxide (Musser et al., 2002). Glucose oxidase and glucose dehyrogenase are also present in our salivary gland library. They may play a similar role in suppressing jasmonic acid mediated plant defense, as jasmonic acid regulated pathway genes are up-regulated in sorghum upon greenbug feeding (Zhu-Salzman et al., 2004).

In addition, glycosyl hydrolases present in the salivary gland hydrolyse glycosidic bonds in carbohydrates. Alpha-amylase hydrolyses α-1,4 linkages in starch to maltose, which is then hydrolysed to glucose by an α-glucosidase. Amylase activity has been detected in the salivary glands of other phytophagous heteropterans (Zeng and Cohen, 2000; Boyd, 2003). Thus, it is possible that aphids are capable of partially digesting starches before ingestion. In addition to serving as energy and carbon sources, sugars function as messengers in signal transduction (Rolland et al., 2002). Sucrose and trehalose are two sugars that are involved in signal transduction in plants. Trehalose is also the predominant hemolymph sugar in insects (Becker et al., 1996). Trehalose can be hydrolysed into two glucose molecules by trehalase. Putative secreted trehalase is present in the salivary glands of the pea aphid and may play a crucial role in breakdown of plant trehalose, thereby disrupting signal transduction in plant and thus can aphid feed continuously.

Recently in the social aphid, *Tuberaphis styraci*, cathepsin B protein was preferentially expressed in soldiers and was localized in the midgut of soldiers. It is injected (probably from gut) into the body of prey during attack (Kutsukake et al., 2004), although it is possible that enzyme is also synthesized in salivary glands. We have also identified a putative secreted protein encoding cathepsin B. In bird cherry-oat aphid, *Rhopalosiphum padi*, there were changes in the activity of glutathione-S-transferase and glutathione reductase, enzymes involved in detoxification of plant allelochemicals (Laskowska et al., 1999). In most cases, the activities of these enzymes depend upon the composition of aphid's diet, when fed on cereals, glutathione-S-transferase activity further increased and glutathione reductase activity decreased (Laskowska et al., 1999). We have identified putative secreted glutathione peroxidase and glutathione-S-transferase in the salivary glands of pea aphid. It is possible that these enzymes are involved in the detoxification of plant allelochemicals and thus may play a vital role in aphid feeding.

In addition, one of our cluster is apparently a homolog of an 11 kDa salivary protein identified in our studies is also present in the salivary glands of sand fly, *Lutzomyia longipalpis* (Oliveira et al., 2006; Valenzuela et al., 2004). In the sand fly, 11 kDa protein encodes a novel protein. Its function has not been elucidated (Oliveira et al., 2006). We have also identified a putative carbonic anhydrase; carbonic anhydarse has been previously reported in the salivary glands of the cockroach, *Periplaneta americana* (Just and Walz, 1994). It functions by maintaining pH homeostasis in various tissues by catalyzing the hydration of CO_2 and dehydration of bicarbonate (Kivela et al., 1999). It is

possible that the putative secreted carbonic anhydarse in aphid saliva may regulate pH of the sieve element.

We have also identified 12 full-length putative secreted cDNAs encoding proteins with unknown functions. An abundant cluster, C002 was further characterized and RNAi studies show that C002 is important for survival and reproduction of the pea aphid (see Chapter 3 and 4). It is possible that these putative secreted proteins of unknown function may play similar important function in pea aphid performance on the host plant.

Proteins including enzymes, identified in our study with putative secretion signals may constitute aphid saliva. Together these enzymes may play roles in aphid-plant interaction. This information derived from the large sequencing of ESTs from the salivary glands of pea aphid, *A. pisum* along with the avaliability of the pea aphid genome later this year will provide an indispensable tool to study the molecular basis of aphid-plant interactions.

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Table 5. Classifications of 377 clusters based on BLASTX against UniProt database.

Identification	No. of Clusters
Hypothetical / Unannotated proteins	72
Ribosomal proteins	42
ATPase complex/ATP binding proteins	27
Polymerases (DNA / RNA)	20
Cytochromes	16
DNA binding proteins	15
Proteases	14
Metal ion binding proteins	14
Mitochondrial proteins / enzymes of electron transport	14
Group X	13
Oxidoreductases	11
Whole genome shotgun sequence (*Tetraodon nigroviridis*)	11
Transcription factors	10
Protein kinases	9
Heat shock proteins	9
Enzymes of carbohydrate metabolism	9
Calcium binding proteins	9
Other hydrolases	8
Allatotropin	6
Amino acid transporters / enzymes nitrogen of metabolism	7
Enzymes of lipid metabolism	4
Signal tranducers	3
Pherophorin-C2 proteins (extension-like proteins)	3
Actin binding proteins	3
Enzymes of chitin metabolism	3
Aquaporin	2
Cuticle proteins (aphids and *Bombyx mori*)	2
Ubiquitin protein ligase	2
Apoptosis related proteins	2
Viral proteins	2
Plant enzyme	1
Armet like protein	1
Integrin	1
Apolipophorin precursor protein	1
Carbonic anhydrase	1
Super cysteine rich protein	1
Luciferin regenerating enzyme	1
11 kDa salivary protein (sand fly)	1
Anthranilate synthase	1
Senescence associated protein	1

Ornithine decarboxylase inhibitor	1
Odorant binding protein	1
Glutathione-S-transferase	1
S-adenosyl-L-methionine decarboxylases	1

Table 6. Classification of 72 clusters encoding "hypothetical proteins."

Cluster	Secreted	Putative function	Species	Other insect species
Contig 5	Yes	Putative secreted salivary protein	*Ixodes scapularis*	Yes
Contig 10	No	mitochondrial phosphate carrier protein	*Drosophila melanogaster*	Yes
Contig 11	No	Der1-like domain family member 1	*Bombyx mori*	Yes
Contig 18		No matches	-	No
Contig 31	Possible cleavage b/w 22 & 23	Hypothetical protein	*Ixodes scapularis*	Yes
Contig 37	Yes	Hypothetical protein	*Apis mellifera*	Yes
Contig 39	No	integral membrane protein 2A	*Gallus gallus*	Yes
Contig 55	No	Hypothetical protein	*Tribolium castaneum*	Yes
Contig 65	No	Hypothetical protein	*Anopheles gambiae*	Yes
Contig 97	No	18S ribosomal RNA	*A. pisum*	Yes
Contig 108	No	Ubiquitin Associated domain (Peptidase C19)	*Apis mellifera*	Yes
Contig 130	No	translation factor and RNA binding protein	*Drosophila melanogaster*	No
Contig 145	No	Hypothetical protein	*Tribolium castaneum*	No
Contig 337	No	Tetratricopeptide repeat domain	*Anopheles gambiae*	Yes
Contig 363	No	No matches	-	No
Contig 431	No	No matches	-	No
Contig 461	No	DNA-directed RNA-polymerase II subunit	*Anopheles gambiae*	Yes
Contig 483	No	DNA repair protein	*Anopheles gambiae*	Yes
A3_A09_t7_065	Possible cleavage b/w 33 & 34	FAR-17a/AIG1-like protein	*Drosophila melanogaster*	Yes
A3_C02_t7_006	No	*Drosophila* yakuba	Drosophila melanogaster	Yes
A3_C09_t7_066	No	Low density lipoprotein receptor	*Drosophila melanogaster*	Yes
A3_G03_t7_020	No	Ribosomal protein S29	*Apis mellifera*	Yes

AphidB1_A12_t7_085	No	Adaptin N terminal region	*Anopheles gambiae*	Yes
AphidB1_C07_t7_050	Yes	Unknown function	*Tribolium castaneum*	Yes
AphidB1_D08_t7_062	No	Cytochrome c oxidase	*Bos Taurus*	No
AphidB1_F01_t7_011	Yes	Aldehyde dehydrogenase	*Homo sapiens*	Yes
AphidB1_H02_t7_016	No	Protein kinase C	*Anopheles gambiae*	Yes
PlateI_D10	No	Cytochrome b	*Anopheles gambiae*	Yes
PlateI_F09	No	E3 ubiquitin ligase	*Apis mellifera*	Yes
PlateI_G01	No	ADP-ribosylation factor	*Homo sapiens*	Only in *Anopheles*
PlateKEP_C05	No	hydroxypyruvate isomerase	*Homo sapiens*	Yes
PlateKEP_G09	No	TatD DNase domain	*Apis mellifera*	Yes
PlateKEP_A07	No	Bladder cancer-related protein BC10	*Anopheles gambiae*	Yes
PlateKEP_C06	Yes	Odorant binding protein	*Drosophila melanogaster*	Yes
PlateKEP_A05	Possible cleavage b/w 17 & 18	Eukaryotic protein of unknown function	*Apis mellifera*	Yes
PlateKEP_B04	No	Protein of unknown function	*Drosophila melanogaster*	Yes
PlateKEP_E09	No	Ezrin/radixin/moesin family	*Anopheles gambiae*	Yes
ID0AAH1DD03ZM1	No	Sybindin-like family	*Tribolium castaneum*	Yes
ID0AAH10CA01ZM1	No	ribosome-associated membrane protein	*Bombyx mori*	Yes
ID0AAH11DD09ZM1	No	aphid secondary symbionts (*Candidatus hamiltonella*)	*A. pisum*	No
ID0AAH11DG05ZM1	No	DnaA from secondary symbiont	*Glossina morsitans*	No
ID0AAH12AE10ZM1		Leucine-rich repeat (LRR) protein	*Anopheles gambiae*	Yes
ID0AAH13CD06ZM2	No	No matches	-	No
ID0AAH14AA08ZM1	No	Unknown function	*Mus musculus*	No
ID0AAH14BA05ZM1	No	No matches	-	No
ID0AAH14BC12ZM1	No	peroxisomal biogenesis factor	*Homo sapiens*	Yes
ID0AAH14DE12ZM1	No	Enhancer-of-zeste, (Trithorax) domain	*Apis mellifera*	Yes
ID0AAH14DH11ZM1	No	aphid secondary	*A. pisum*	No

		symbionts (*Candidatus hamiltonella*)		
ID0AAH15CC04ZM1	No	Cupin metalloenzyme superfamily	*Drosophila melanogaster*	Yes
ID0AAH15DA03ZM1	No	prefoldin beta subunit	*Bombyx mori*	Yes
ID0AAH15DB03ZM1	No	Bacteriophage lysis protein	aphid secondary symbionts (*Candidatus hamiltonella*)	No
ID0AAH15DE02ZM1	No	18S Ribosomal protein	*A.pisum*	Yes
ID0AAH15DG12ZM1	No	Unknown function	*Drosophila melanogaster*	Yes
ID0AAH2BH08ZM1	No	Acid phosphatase	*Drosophila melanogaster*	Yes
ID0AAH2CB01ZM1	No	Predicted GTPase	*Anopheles gambiae*	Yes
ID0AAH2CD04ZM1	No	Uncharacterized conserved protein (Function unknown)	*Anopheles gambiae*	Yes
ID0AAH2CG02ZM1	No	Putative transcriptional repressor	*Anopheles gambiae*	Yes
ID0AAH3DG05ZM1	No	Unknown function	*Anopheles gambiae*	No
ID0AAH4BE12ZM1	No	Eukaryotic initiation factor	*Apis mellifera*	Yes
ID0AAH5BB03ZM1	No	Spectrin repeats, found in several proteins involved in cytoskeletal structure	*Apis mellifera*	Yes
ID0AAH5CD01ZM1	No	Unknown function	*Anopheles gambiae*	Yes
ID0AAH6BF03ZM1	No	ubiquitin processing protease	*Bos taurus*	No
ID0AAH6BH08ZM1	No	Protein of unknown function	*Anopheles gambiae*	Yes
ID0AAH6CF02ZM1	No	Cloning vector	-	No
ID0AAH6CF09ZM1	No	kettin-like protein	*Helicoverpa armigera*	Yes
ID0AAH6CF10ZM1	No	Protein of unknown function	*Anopheles gambiae*	Yes
ID0AAH6DC03ZM1	No	TBC1 domain family	*Anopheles gambiae*	Yes
ID0AAH7BG10ZM1	No	Protein of unknown function	*Anopheles gambiae*	Yes
ID0AAH8AF04ZM1	No	putative replication-associated protein	*Glossina morsitans*	No
ID0AAH8AG07ZM1	No	RING3 protein	*Apis mellifera*	Yes

ID0AAH9AA10ZM1	No	unknown function	*Anopheles gambiae*	Yes
ID0AAH9CG08ZM1	No	nuclear prelamin A recognition factor	*Homo sapiens*	No
Group X: Miscellaneous clusters				
Contig332	Yes	unknown function	*Aedes aegypti*	Yes
AphidB1_D04_t7_030	Yes	Rnp24-prov protein	*Apis mellifera*	Yes
PlateKEP_D10	No	Actin	*A. pisum*	Yes
PlateKEP_D11	Possible cleavageb/w pos. 37 and 38	Amino acid permease	*Anopheles gambiae*	Yes
PlateKEP_H01	No	Sugar transporter	*Anopheles gambiae*	Yes
ID0AAH1CD04ZM1	No	No matches	-	-
ID0AAH1CG07ZM1	No	No matches	-	-
ID0AAH10AB05ZM1	No	No matches	-	-
ID0AAH11AH05ZM1	Yes	hypothetical protein	*Buchnera aphidicola*	-
ID0AAH13AH01ZM2	Yes	seven transmembrane receptor	*Drosophila melanogaster*	Yes
ID0AAH2CE10ZM1	No	No matches	-	-
ID0AAH3DB05ZM1	Yes	emp24/gp25L/p24 family	*Anopheles gambiae*	Yes
ID0AAH7BG10ZM1	No	TMEM9 domain family	*Anopheles gambiae*	Yes

Table 7. Clusters with three or more sequences of known function.

Cluster	Length	Functional annotation using UniProt (BLASTX)	# ESTs
Contig 90	2014	similar to Q8B107_9VIRU (Q8B107) Capsid protein (Fragment) (Eval: 0.0, Coding: 67%); GO:0005198 structural molecule activity [MF]; GO:0019028 viral capsid [CC]	**109**
Contig 33	1638	similar to Q699N8_SCHGA (Q699N8) Cytochrome oxidase subunit I (Eval: e-171, Coding: 91%); GO:0004129 cytochrome-c oxidase activity [MF]; GO:0005739 mitochondrion [CC]; GO:0006118 electron transport [BP]; GO:0016020 membrane [CC]	57
Contig 405	1638	similar to Q8B594_9VIRU (Q8B594) Nonstructural polyprotein (Eval: 0.0, Coding: 26%); GO:0003723 RNA binding [MF]; GO:0003724 RNA helicase activity [MF]; GO:0003968 RNA-directed RNA polymerase activity [MF]; GO:0006350 transcription [BP]; GO:0019079 viral genome replication [BP]	43
Contig 97	486	similar to Q7RN92_PLAYO (Q7RN92) Hypothetical protein (Eval: 1e-05, Coding: 39%)	40
Contig 18	1174	similar to Q5TVN3_ANOGA (Q5TVN3) ENSANGP00000027660 (Fragment) (Eval: 5e-11, Coding: 23%)	35
Contig 377	853	similar to Q699N4_SCHGA (Q699N4) Cytochrome oxidase subunit III (Eval: 3e-58, Coding: 91%); GO:0004129 cytochrome-c oxidase activity [MF]; GO:0005739 mitochondrion [CC]; GO:0006118 electron transport [BP]; GO:0016020 membrane [CC]	33
Contig 329	920	similar to Q9B7Q6_ACYPI (Q9B7Q6) ATP synthase A chain subunit 6 (Eval: 8e-36, Coding: 100%); GO:0005739 mitochondrion [CC]; GO:0015992 proton transport [BP]; GO:0016020 membrane [CC]; GO:0016469 proton-transporting two-sector ATPase complex [CC]; GO:0016820 hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances [MF]	20
Contig 94	593	similar to Q56FF3_9HYME (Q56FF3) Ribosomal protein S15A (Eval: 3e-63, Coding: 100%); GO:0003735 structural constituent of ribosome [MF]; GO:0005622 intracellular [CC]; GO:0005840 ribosome [CC]; GO:0006412 protein biosynthesis [BP]; GO:0030529 ribonucleoprotein complex [CC]	18
Contig 194	717	similar to Q2QKX4_9HEMI (Q2QKX4) Cytochrome c oxidase subunit II (Eval: 1e-74, Coding: 97%); GO:0005739 mitochondrion [CC]	16
Contig 138	215	similar to Q5WPT4_LUTLO (Q5WPT4) 71 kDa salivary protein (Eval: 3e-16, Coding: 11%); GO:0004246 peptidyl-dipeptidase A activity [MF]; GO:0006508 proteolysis [BP]; GO:0016020 membrane [CC]	15

Contig 5	1029	similar to Q9VQA7_DROME (Q9VQA7) CG16995-PA (Eval: 4e-26, Coding: 100%); GO:0005576 extracellular region [CC]	14
Contig 54	1647	similar to Q6WMV9_DROEU (Q6WMV9) Glucose dehydrogenase (Fragment) (Eval: 2e-53, Coding: 89%); GO:0006118 electron transport [BP]; GO:0016491 oxidoreductase activity [MF]; GO:0016614 oxidoreductase activity, acting on CH-OH group of donors [MF]; GO:0050660 FAD binding [MF]	14
Contig 4	1511	similar to Q7QFX9_ANOGA (Q7QFX9) ENSANGP00000015052 (Fragment) (Eval: 3e-49, Coding: 64%); GO:0006066 alcohol metabolism [BP]; GO:0006118 electron transport [BP]; GO:0016491 oxidoreductase activity [MF]; GO:0016614 oxidoreductase activity, acting on CH-OH group of donors [MF]; GO:0050660 FAD binding [MF]	13
Contig 223	1431	similar to Q3HTK5_CHLRE (Q3HTK5) Pherophorin-C2 protein precursor (Eval: 1e-08, Coding: 4%); GO:0005199 structural constituent of cell wall [MF]	13
Contig 40	775	similar to Q9LML5_ARATH (Q9LML5) F10K1.6 protein (Eval: 3e-84, Coding: 30%); GO:0016788 hydrolase activity, acting on ester bonds [MF]	11
Contig 331	1123	similar to Q9FEV2_ORYSA (Q9FEV2) Putative phospholipid hydroperoxide glutathione peroxidase (EC 1.11.1.9) (Eval: 7e-27, Coding: 100%); GO:0004601 peroxidase activity [MF]; GO:0004602 glutathione peroxidase activity [MF]; GO:0004602 glutathione peroxidase activity [MF]; GO:0006979 response to oxidative stress [BP]; GO:0016491 oxidoreductase activity [MF]	10
Contig 257	583	similar to Q699N4_SCHGA (Q699N4) Cytochrome oxidase subunit III (Eval: 1e-16, Coding: 44%); GO:0004129 cytochrome-c oxidase activity [MF]; GO:0005739 mitochondrion [CC]; GO:0006118 electron transport [BP]; GO:0016020 membrane [CC]	10
Contig 50	1724	similar to APLP_LOCMI (Q9U943) Apolipophorins precursor [Contains: Apolipophorin-2 (Apolipophorin II) (apoLp-2); Apolipophorin-1 (Apolipophorin I) (apoLp-1)] (Eval: 8e-61, Coding: 16%); GO:0005319 lipid transporter activity [MF]; GO:0006810 transport [BP]; GO:0006869 lipid transport [BP]; GO:0006869 lipid transport [BP]; GO:0008289 lipid binding [MF]; GO:0016055 Wnt receptor signaling pathway [BP]	8
Contig 131	698	similar to Q699M8_SCHGA (Q699M8) Cytochrome b (Eval: 3e-61, Coding: 61%); GO:0005506 iron ion binding [MF]; GO:0005739 mitochondrion [CC]; GO:0005746 mitochondrial electron transport chain [CC]; GO:0006118 electron transport [BP]; GO:0006118 electron transport [BP]; GO:0006810	8

		transport [BP]; GO:0016020 membrane [CC]; GO:0016020 membrane [CC]; GO:0016021 integral to membrane [CC]; GO:0016491 oxidoreductase activity [MF]; GO:0046872 metal ion binding [MF]	
Contig 67	1314	similar to Q7QGY7_ANOGA (Q7QGY7) ENSANGP00000012700 (Fragment) (Eval: 3e-79, Coding: 98%); GO:0005509 calcium ion binding [MF]; GO:0005509 calcium ion binding [MF]	7
Contig 106	381	similar to Q7Q0B4_ANOGA (Q7Q0B4) ENSANGP00000011194 (Fragment) (Eval: 7e-22, Coding: 7%); GO:0000166 nucleotide binding [MF]; GO:0004672 protein kinase activity [MF]; GO:0004674 protein serine/threonine kinase activity [MF]; GO:0004674 protein serine/threonine kinase activity [MF]; GO:0005524 ATP binding [MF]; GO:0005524 ATP binding [MF]; GO:0006468 protein amino acid phosphorylation [BP]; GO:0016301 kinase activity [MF]; GO:0016740 transferase activity [MF]	7
Contig 68	897	similar to Q7Z8K5_9APHY (Q7Z8K5) Manganese superoxide dismutase (Fragment) (Eval: 1e-05, Coding: 14%); GO:0004784 superoxide dismutase activity [MF]; GO:0006801 superoxide metabolism [BP]; GO:0016491 oxidoreductase activity [MF]; GO:0046872 metal ion binding [MF]	6
Contig 167	462	similar to Q8ITC5_AEQIR (Q8ITC5) Ribosomal protein L30 (Eval: 3e-43, Coding: 100%); GO:0003735 structural constituent of ribosome [MF]; GO:0005622 intracellular [CC]; GO:0005840 ribosome [CC]; GO:0006412 protein biosynthesis [BP]	7
Contig 108	640	similar to Q9VSC5_DROME (Q9VSC5) CG8209-PA (GM09977p) (Eval: 5e-18, Coding: 22%)	5
Contig 130	434	similar to Q803P1_BRARE (Q803P1) Zgc:55443 (Eval: 4e-08, Coding: 28%)	5
Contig 173	555	similar to Q4RV41_TETNG (Q4RV41) Chromosome 15 SCAF14992, whole genome shotgun sequence. (Fragment) (Eval: 2e-08, Coding: 100%)	4
Contig 260	627	similar to Q4LB03_BIPLU (Q4LB03) Ribosomal protein S24e (Eval: 1e-49, Coding: 100%); GO:0003735 structural constituent of ribosome [MF]; GO:0005622 intracellular [CC]; GO:0005840 ribosome [CC]; GO:0006412 protein biosynthesis [BP]	4
Contig 394	591	similar to Q7Q7P4_ANOGA (Q7Q7P4) ENSANGP00000021586 (Eval: 6e-14, Coding: 97%); GO:0015986 ATP synthesis coupled proton transport [BP]; GO:0016469 proton-transporting two-sector ATPase complex [CC]; GO:0046933 hydrogen-transporting ATP synthase activity, rotational mechanism [MF]; GO:0046961 hydrogen-transporting ATPase activity, rotational mechanism [MF]	4

Contig 409	281	similar to PSF1_HUMAN (Q14691) DNA replication complex GINS protein PSF1 (Eval: 6e-09, Coding: 27%); GO:0005634 nucleus [CC]; GO:0005634 nucleus [CC]; GO:0005737 cytoplasm [CC]; GO:0006260 DNA replication [BP]	4
Contig 478	178	similar to Q8B594_9VIRU (Q8B594) Nonstructural polyprotein (Eval: 6e-16, Coding: 2%); GO:0003723 RNA binding [MF]; GO:0003724 RNA helicase activity [MF]; GO:0003968 RNA-directed RNA polymerase activity [MF]; GO:0006350 transcription [BP]; GO:0019079 viral genome replication [BP]	4

Table 8. Clusters with more than 2 sequences with unknown function.

Cluster	Length	Secreted	# ESTs	ORF
Contig 14	1385		236	-
Contig 7	2236		134	690
Contig 78	610	Full-length (secreted) cleavable site (19-20)	74	141
Contig 181	719		72	-
Contig 164	921	May be secreted, possible cleavable site (27-28)	49	154
Contig 32	**1054**	**C002,** Full-length (secreted) cleavable site (23-24)	**46**	213
Contig 140	1041		46	232
Contig 9	1535	Full-length (secreted) cleavable site (27-28)	35	227
Contig 34	1413	Full-length (secreted) cleavable site (28-29)	35	132
Contig 6	1452		34	294
Contig 86	2948		34	441
Contig 356	762		26	-
Contig 62	1627		21	402
Contig 498	876	Full-length (secreted) cleavable site (23-24)	20	208
Contig 122	1151	Full-length (secreted) cleavable site (19-20)	19	141
Contig 283	635		19	128
Contig 20	1178		17	267
Contig 42	1481		16	492
Contig 475	950		16	247
Contig 462	821		16	64
Contig 81	716		15	-
Contig 235	1038		14	-
Contig 422	360		14	-
Contig 38	1319		13	271
Contig 358	1302	Full-length (secreted) cleavable site (25-26)	13	263
Contig 15	813		13	155
Contig 258	577		13	78
Contig 216	488		13	-
Contig 159	952		12	178
Contig 339	865		12	179
Contig 300	445		11	148
Contig 160	251		11	69
Contig 73	138		11	-
Contig 154	1865		10	-
Contig 3	1336		10	344
Contig 12	936		10	-
Contig 470	731		10	-
Contig 36	673		10	109
Contig 171	623		10	67
Contig 85	422		10	139
Contig 349	417		10	-
Contig 510	660		9	219
Contig 275	645	Full-length (no signal peptide)	9	107
Contig 308	565		9	-
Contig 334	543		9	-
Contig 186	189		9	-

Contig 240	155		9	51
Contig 52	1044	Full-length (secreted) cleavable site (24-25)	8	228
Contig 371	993		8	-
Contig 211	601		8	-
Contig 193	320		8	-
Contig 444	172		8	57
Contig 399	757		7	122
Contig 176	682		7	-
Contig 184	633		7	-
Contig 357	622		7	-
Contig 278	441		7	-
Contig 445	247		7	82
Contig 133	209		7	-
Contig 69	954	Full-length (secreted) cleavable site (22-23)	6	249
Contig 265	831		6	202
Contig 212	749		6	152
Contig 178	741		6	-
Contig 429	638		6	109
Contig 16	619		6	-
Contig 515	613		6	-
Contig 336	549		6	182
Contig 203	492		6	163
Contig 183	387		6	-
Contig 198	339		6	-
Contig 205	260		6	-
Contig 424	242		6	-
Contig 163	1217		5	-
Contig 446	836		5	180
Contig 61	700		5	-
Contig 200	665	Full-length (secreted) cleavable site (26-27)	5	156
Contig 125	642		5	180
Contig 436	551		5	-
Contig 366	516		5	-
Contig 353	492	Secreted, cleavable site (26-27)	5	141
Contig 241	490		5	-
Contig 303	418		5	-
Contig 208	404		5	-
Contig 438	343		5	114
Contig 248	250		5	-
Contig 229	246		5	82
Contig 413	241		5	-
Contig 157	236		5	-
Contig 128	168		5	-
Contig 418	154		5	-
Contig 43	1074		4	253
Contig 149	934		4	200
Contig 479	864		4	92
Contig 22	811		4	82
Contig 2	813		4	212
Contig 96	745		4	-
Contig 82	740		4	-
Contig 53	714		4	155

Contig 296	671		4	-
Contig 17	666		4	143
Contig 344	661		4	-
Contig 213	654		4	-
Contig 262	619		4	-
Contig 319	608		4	199
Contig 44	605		4	201
Contig 217	467		4	123
Contig 417	424		4	-
Contig 323	422		4	-
Contig 134	408		4	-
Contig 111	379		4	-
Contig 136	377		4	78
Contig 247	358		4	-
Contig 218	356		4	-
Contig 129	354		4	-
Contig 448	353		4	-
Contig 351	342		4	-
Contig 118	320		4	-
Contig 375	274		4	-
Contig 249	261		4	-
Contig 391	231		4	-
Contig 443	218		4	-
Contig 365	207		4	-
Contig 282	206		4	-
Contig 161	196		4	-
Contig 511	195		4	-
Contig 324	192		4	-
Contig 414	179		4	-
Contig 397	176		4	-
Contig 280	169		4	-
Contig 166	160		4	-
Contig 110	151		4	-
Contig 529	1099		3	-
Contig 306	901		3	-
Contig 251	823		3	-
Contig 19	756		3	-
Contig 520	719		3	-
Contig 255	707		3	-
Contig 335	693		3	-
Contig 307	684		3	-
Contig 408	676		3	-
Contig 148	672		3	-
Contig 121	668		3	-
Contig 13	665		3	137
Contig 383	656		3	-
Contig 153	653		3	-
Contig 124	640	Secreted, cleavable site (23-24)	3	124
Contig 180	638		3	-
Contig 256	617		3	154
Contig 254	610		3	-
Contig 269	584		3	-

Contig 284	578		3	119
Contig 204	561		3	-
Contig 263	552		3	-
Contig 372	544		3	-
Contig 458	509		3	108
Contig 192	505		3	-
Contig 139	501		3	-
Contig 210	488		3	-
Contig 76	482		3	-
Contig 132	472		3	-
Contig 385	468		3	-
Contig 355	463		3	-
Contig 126	454		3	-
Contig 233	440		3	-
Contig 309	426		3	-
Contig 158	416		3	-
Contig 177	414		3	109
Contig 84	409		3	69
Contig 273	407		3	-
Contig 103	399		3	-
Contig 271	395		3	-
Contig 150	386		3	-
Contig 250	372		3	-
Contig 313	334		3	-
Contig 419	333		3	-
Contig 295	320		3	-
Contig 361	313		3	-
Contig 352	311		3	-
Contig 435	311		3	-
Contig 185	304		3	-
Contig 314	298		3	-
Contig 514	294		3	-
Contig 496	290		3	-
Contig 406	281		3	-
Contig 318	278		3	-
Contig 484	270		3	-
Contig 195	245		3	-
Contig 197	232		3	-
Contig 364	229		3	-
Contig 322	224		3	-
Contig 362	223		3	-
Contig 302	215		3	-
Contig 343	210		3	-
Contig 467	209		3	-
Contig 503	199		3	-
Contig 398	192		3	-
Contig 387	190		3	-
Contig 77	168		3	-
Contig 79	168		3	-
Contig 127	155		3	-
Contig 370	145		3	-

Table 9. Functional annotation of proteases and other hydrolases.

Cluster[a]	Functional annotation[b]	Organism[c]	Secreted[d]	ORF[e]
Proteases				
Contig 46	Subtilisin- related protease	*Apis mellifera*	-	140
Contig 57	Angiotensin-converting enzyme	*Bombyx mori*	Yes	150
Contig 209	Peptidase M1	*Drosophila melanogaster*	Yes	63
Contig 360	Angiotensin-converting enzyme	*Apis mellifera*	Yes	147
PlateA3_G10	Cathepsin L	*Apis gossypii*	Yes	130
PlateKEP_G10	Cathepsin B	*Myzus persicae*	Yes	150
PlateKEP_A11	NTPase	*Drosophila melanogaster*	-	149
PlateKEP_B07	Proteasome subunit	*Drosophila melanogaster*	-	180
ID0AAH12DB08ZM1	Ubiquitin specific protease	*Candida albicans*	-	-
ID0AAH15AH08ZM1	Signal peptidase	*Gallus gallus*	-	-
ID0AAH15CF12ZM1	Angiotensin-converting enzyme	*Locusta migratoria*	Yes	56
ID0AAH3DD05ZM1	Endoprotease FURIN	*Drosophila melanogaster*	Yes	118
ID0AAH4BD04ZM1	Ubiquitin specific protease	*Danio rerio*	-	-
ID0AAH7AD10ZM1	Signalosome complex	*Danio rerio*	-	177
Other hydrolases				
Contig 40	Phosphoesterase (hydrolase)	*Arabidopsis thialiana*	Yes	165
Contig 266	Carbon-nitrogen hydrolase	*Anopheles gambiae*	-	120
Contig 398	Prolyl-4-hydroxylase	*Drosophila melanogaster*	-	113
PlateKEP_A06	Fructose 1-6, bisphosphatase	*Drosophila melanogaster*	-	147
PlateKEP_H08	Alpha-glucosidase	*Apis mellifera*	Yes	100
AphidB1_G05	Trehalase	*Apis mellifera*	Yes	167
ID0AAH11DE05ZM1	Prolyl 4-hydroxylase alpha subunit	*Apis mellifera*	-	159
ID0AAH9BB06ZM1	S-adenosyl homocysteine hydrolase	*Apis mellifera*	-	129

[a] Cluster (comprising contig and singlets) in pea aphid salivary database.

[b] Functional annotation based to top hit at NR database at NCBI.

[c] Top match at NR database using TBLASTX.

[d] SignalP analysis using full-length homolog of pea aphid cluster.

[e] ORF represent number of amino acid residues in pea aphid cluster.

Table 10. Functional annotation of oxidoreductases.

Cluster[a]	**Functional annotation**[b]	**Organism**[c]	**Secreted**[d]	**ORF**[e]
Contig 40	Glucose dehydrogenase	*Apis mellifera*	Yes	503
Contig 54	Glucose dehydrogenase	*Anopheles gambiae*	Yes	375
Contig 331	Glutathione peroxidase	*Aedes aegypti*	Yes	259
AphidB1_F01	Aldehyde dehyrogenase	*Apis mellifera*	-	167
PlateI_D06	Thioredoxin peroxidase	*Toxoptera citricida*	-	254
PlateKEP_C04	Phosphoglycerate dehydrogenase	*Mus musculus*	-	-
PlateKEP_D04	Peroxiredoxin-like protein	*Aedes aegypti*	-	202
PlateKEP_G03	Dimethylaniline monooxygenase	*Aedes aegypti*	-	171
PlateKEP_B09	Superoxide dismutase	*Gryllotalpa orientalis*	-	94
ID0AAH2DE03ZM1	Peroxidase	*Aedes aegypti*	Yes	219
ID0AAH6CC11ZM1	Peroxidase	*Aedes aegypti*	Yes	140

[a] Cluster (comprising contig and singlets) in pea aphid salivary database.

[b] Functional annotation based to top hit at NR database at NCBI.

[c] Top match at NR database using TBLASTX.

[d] SignalP analysis using full-length homolog of pea aphid cluster.

[e] ORF represent number of amino acid residues in pea aphid cluster.

CHAPTER 3

Chapter 3: A Novel Transcript and Protein from the Salivary Glands of Pea Aphid, *Acyrthosiphon pisum*.

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Key words: pea aphid, salivary glands.

Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; EST, expressed sequence tag; NCBI, National Center for Biotechnology Information; PBS, phosphate buffered saline; DIG, digoxigenin; LB, luria broth; IPTG, isopropyl-β-D thiogalactoside; SDS, Sodium dodecyl sulfate.

Abstract

Salivary secretions are a key component of aphid-plant interactions. Aphid salivary proteins interact with plant tissues, enabling aphids to gain access to phloem sap and possibly eliciting responses which may benefit the insect. In an effort to isolate and identify key components in salivary secretions, we created a salivary gland cDNA library. Several thousand randomly selected cDNA clones were sequenced as described in Chapter 2. The transcript corresponding to an abundant cDNA (called C002), was characterized. Based on *in situ* hybridization and immunohistochemistry, transcript as well as protein was localized to the same sub-set of cells within principal salivary glands. The encoded protein fails to match any protein of known function and any sequence outside of the family Aphididae. The protein encodes a secreted protein and is injected into the host plant during aphid feeding. RNAi directed toward the C002 transcript had no affect on growth and development on liquid diet, though, as shown in the next chapter it causes lethality when aphids are placed on fava bean leaves.

Introduction

Aphid saliva plays a major role in the interaction of aphids and host plants (Miles, 1999). Although, aphid-plant interactions have been studied extensively, not much is known at the molecular level. The availability of aphid saliva in small quantities makes the direct study of salivary components difficult (Miles, 1965; Madhusudhan et al., 1994; Miles and Harrewijn, 1991). The proteins of aphid saliva are of two types, structural and enzymatic. The structural proteins provide a tube-like sheath (Miles 1999; Cherqui and Tjallingi 2000) and are probably few in number corresponding to major bands on gels, with estimated molecular masses of 154 kDa and 66/69 kDa (Cherqui and Tjallingi 2000). Limited N-terminal sequence information is available on these bands (Baumann and Baumann 1995). The secreted salivary enzymes fall into two broad categories: hydrolases (pectinases, cellulases, oligosaccharases) and oxidation/reduction enzymes (phenol oxidase (E.C. 1.14.18.1) and peroxidases) (Miles 1999). The role of these enzymes during aphid attack on plants is not well-understood.

Aphid saliva is believed to perform multiple functions; including creation of the stylet sheath, assisting the penetration of substrate for food (by the action of pectinases, cellulases, β-glucosidases etc), digesting nutrients (polysaccharases), detoxification of phenolic glycosides ingested during feeding by the action of polyphenol oxidases or peroxidases (oxidation-reduction enzymes), and suppression of host defenses or elicitation of host responses (Miles, 1972; Miles, 1987; Urbanska et al., 1998; Miles, 1999). Aphid saliva may also play a role in the ability of aphids to counter resistance

factors in plants, since some species or biotypes of aphids can feed on plants that are resistant to other species or biotypes (Miles, 1999).

We have undertaken a functional genomics approach to identify components of aphid saliva. We have chosen to do this work with pea aphid, *A. pisum*, because of its large size (compared with other aphid species), thus making dissections of salivary glands less difficult. In this paper, we report cloning of an abundant cDNA (C002) from a cDNA library of pea aphid salivary glands. The cDNA encodes a protein that fails to match to proteins outside of aphids, is of an unknown function, but appears to be vital to pea aphid feeding on plant tissue.

Material and Methods

Plants and Aphids

Aphids were originally collected from alfalfa plants in the summer of 1999 by Dr. Marina Caillaud at Cornell University. Thereafter, the aphids were reared at KSU on fava beans (*Vicia fabae*) grown in pots (10 cm diameter) at room temperature under high intensity sodium lights with a L:D of 16:8. Salivary glands from adult aphids were dissected and separated from the brain tissue overlaying the glands. Dissected glands were transferred to 50µl of PBS (137mM NaCl, 2,7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4; pH 7.4) for 50 paired glands and washed 3 times in PBS to get rid of hemocytes. Salivary glands were kept at –75°C until needed.

Phagmid cDNA Library Construction and Sequencing

Total RNA was isolated from 250 salivary glands using the Micro RNA isolation kit from Stratagene, La Jolla, CA. The PCR-based cDNA library was made following instructions with the SMART cDNA library construction kit (Clontech, Palo Alto, CA). Total RNA was reverse transcribed using PowerScript reverse transcriptase and CDS III primer provided in the kit. Second strand synthesis was performed through the PCR-based protocol using SMART III and CDS III primers from Clontech. Double strand synthesis was followed by proteinase K digestion. Double strand cDNA was ligated into a Lambda TriplEx2 vector (Clontech, Palo Alto, CA), and the resulting ligation reaction was packaged using Gigapack Gold III from Stratagene following the manufacturer's specifications.

Sequencing of the cDNA clones was done either at the Kansas State University sequencing facility or at Genoscope, France. Vector stripped sequences were blasted against the Gen Bank non redundant protein database from the National Center for Biotechnology Information using TBLASTX. cDNAs were analyzed for signal peptide and cleavage information using SignalP 3.0 Server (Nelson et al., 1997) using default parameters (http://www.cbs.dtu.dk/services/SignalP/).

RNA Isolation and RT-PCR

Total RNA was isolated from salivary glands, guts or adult aphids using TRI reagent (Molecular Research Center, Inc. Cincinnati, OH) following the procedure provided by the manufacturer. For RT-PCR, total RNA was treated with DNaseI (Ambion, Austin,

TX)) following standard instructions. AMV reverse transcriptase was used along with oligoDT primers to synthesize single-stranded cDNA following procedure from Promega technical Bulletin no. 099. PCR was done using 5' -- CCA GTG CGA TAG CGA TAA TTT ACA AC -- 3' and 5' -- CAC CTC TCT TAT GAT GAA CGC CAA C -- 3' for C002 forward and reverse primers, respectively, giving a final product of 397 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 reverse, primers, respectively, giving a final product of 231 base pairs.

RNA Isolation and Northern Blotting

Total RNA was extracted from adult aphids using TRI reagent (Molecular Research Center, Inc. Cincinnati, OH). Total RNA (10 µg) was separated on a 1.2% agarose gel containing formaldehyde and blotted on to GeneScreen membrane (Perkin Elmer, Beltsville, MD). The RNA was fixed onto the membrane using UV-light. [^{32}P]-labeled RNA probes generated using a random labeling kit from Stratagene (La Jolla, CA). Hybridization was carried out overnight at 42^{0}C in a hybridization bottle containing a 15 ml hybridization solution (10% dextran sulphate/1% SDS/1 m NaCl, pH 8.0). After hybridization, the membranes were washed twice with 2X SSC at room temperature for 30 min, followed by 2 washes with 2X SSC plus 1% SDS at 65^{0}C for 30 min, and finally 2 washes with 0.1X SSC plus 1% SDS at room temperature for 30 min. The membranes were then exposed to Kodak SR-5 X-ray film.

Genomic DNA Isolation and Southern Blotting

DNA was extracted from adult aphids following the procedure of Bender et al. (1983). Southern blotting was carried out according to the protocol of Shen et al (2003) with minor modifications. The genomic DNA (10 μg) was digested with various restriction enzymes, separated by agarose gel electrophoresis, and then transferred and cross-linked to a GeneScreen membrane (Perkin Elmer, Beltsville, MD). The membrane was treated with hybridization solution (25 mM phosphate buffer (pH 7.0), 5X SSC, 5X Denhardt's solution, 50 μg/ml salmon sperm DNA, 50 % formamide) at 42° C for 2 h. The cDNA was [^{32}P]-labeled using random labeling kit from Stratagene (La Jolla, CA) and, after boiling for 5 min, was added immediately to the hybridization solution. The membrane was hybridized for 15 h at 42° C. Following hybridization the membrane was washed twice for 10 min in 2X SSC and 0.1 % SDS at room temperature and once for 30 min in 0.1X SSC and 0.5 % SDS at 37° C. Finally, the membrane was washed in 0.1X SSC and 0.5 % SDS at 68° C for 30 min and then was exposed to Kodak SR-5 X-ray film.

In situ Hybridization

Whole mount *in situ* hybridization of salivary glands of the pea aphid was done with RNA digoxigenin-labeled probe. Salivary gland were dissected in PBS and fixed in 4% paraformaldehyde in PBS for 1 hr at room temperature. All experimental procedures were performed in a humidified chamber. After fixing, salivary glands were washed 3 times at 10 min interval in PBST (1X PBS and 0.6% Triton-X) at room temperature. Thereafter tissues were sequentially dehydrated for 2 min with 50%, 75%, and 100% ethanol, respectively. Again, salivary glands were sequentially rehydrated for 2 min with

100%, 75%, and 50% ethanol. Prehybridization was performed without probe in hybridization solution (5XSSC, 2% blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS, and 50% formamide) at 60^0C for 1 h. Salivary glands were hybridized with digoxigenin-labeled single-strand sense or antisense RNA probes (100ng/ml) in hybridization solution at 60^0C for 16-18 h. Sense probe was used as negative control. The DIG-labeled RNA probes were prepared with the DIG RNA Labeling Kit (Roche Molecular Biochemicals). A 397-bp fragment of clone C002 was amplified by PCR and subcloned into a pGEM-T Easy vector and was used as a template to generate RNA probes. Right insertion was confirmed by *Eco*R1 digestion and insertion direction was determined by PCR. This plasmid was linearized with *Spe*I and *Apa*I and transcribed with SP6 RNA polymerase and T7 RNA polymerase to generate antisense and sense probes, respectively. After hybridization, glands were washed with hybridization solution at 55^0C for 2-4 h followed by 3 washes at 10 min interval with PBST. Salivary glands were then incubated with alkaline phosphatase conjugated anti-DIG antibody (1:300 dilution in PBST) for over night at 4^0C. The salivary glands were then washed in PBST, followed by 3 washes in detection buffer (Roche Molecular Biochemicals). Finally the salivary glands were stained by adding nitroblue tetrazolium salt/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP, 1:50 dilution in detection buffer). Staining was monitored in a dissection microscope. Color reaction was stopped by repeated washes with PBS and mounted in 100% glycerol. Photographs were taken using digital camera attached to the compound microscope (Nikon Eclipse E800).

Expression of Recombinant Protein in *E. coli* for Antibody Preparation

A cDNA encoding a full-length C002 was amplified by PCR. We used a forward primer 5'--TAG CTG TAG CCA TGG AAG TTA GAT GCG--3' containing an *Nco*I site and a reverse primer 5'--GTA TGG ACA AGC TTA TTA AAA ACG TCG--3' containing a *Hind*III site. The resulting DNA encoded residues 1-196 of the mature protein. The PCR product (632 bp) was ligated into a pGEM-T easy vector and used to transform *E. coli* strain JM109. LB/ampicillin/IPTG/X-Gal (100 µg/mL ampicillin, 0.5mM IPTG, 80 µg/mL X-Gal) plates were used to grow transformed bacteria. Resulting white colonies were selected for DNA sequencing. The PCR product was excised from the vector by digestion with *Nco*I and *Hind*III and purified by low melting point agarose gel electrophoresis, ligated into vector H_6pQE_{60} (Lee et al., 1994), and then used to transform *E.coli* strain JM109. JM109 cells were spread on an LB agar plate containing ampicillin. Correct insertion for C002 was confirmed sequencing. A single colony from the plate was used to inoculate 3 mL 2xYT medium containing 100 µg/mL ampicillin. Culture was shaken at 300 rpm, 37°C overnight. The 3 mL overnight culture was then used to inoculate 200 mL 2 x YT medium with ampicillin and still incubated at 300 rpm, 37°C until A_{600} was ~ 0.7. Recombinant protein expression was then induced by adding IPTG to 1 mM final concentration, and culture was incubated for another five hours. Bacteria were harvested by centrifuging at 5,000 rpm, 20 min at 4°C and then resuspended in 4 mL lysis buffer (8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris.Cl, pH 8.0). A 5 µL sample was reserved for western blot analysis. The bacteria were incubated on ice for 30 min with Triton X-100 at a final concentration of 2% and lysozyme to a final concentration of 1mg/mL and then sonicated on ice (6x10 sec bursts at 200 Watts, with 1 min interval

between bursts). The lysates were centrifuged at 12,000 rpm for 20 min at 4°C. Supernatants and pellets were saved and 5 µl of each sample was used for western blot analysis.

Recombinant protein expressed from a total of 2 l. medium was purified under denaturing conditions by affinity chromatography with nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Valencia, CA). The protein was concentrated with an YM-10 centricon (Millipore, Bedford, MA) to 400 µL, mixed with 2 x SDS loading buffer, separated by electrophoresis in a 12.5% acrylamide gel (ISC Bioexpress, Kaysville, UT) and stained with 0.025% Commassie blue R-250 in water. Then the protein band (approximately 600 µg) was cut out and sliced into pieces for injection into rabbit to generate antisera (Cocalico Biologicals, Reamstown, PA).

Western Blotting

Polyclonal rabbit antibodies were purified by immobilizing recombinant protein (C002) to matrix composed of cross-linked 4% beaded agarose activated to form aldehyde functional groups using AminoLink Plus Immobilization Kit (Pierce, Rockford, IL) following manufacturer's instructions. For western blot, approximately 500 hundred pea aphids were placed on fresh fava bean plants and all the aphids were removed after 24 h. 1.5 g of plants tissue was homogenized in PBS on liquid nitrogen. Frequent freeze-thaw cycles were used for complete extraction, and the extract was centrifuged at 12,000 xg for 5 min. About 2 ml of supernatant was concentrated to 200 µl using YM3 microcon centrifugal filter devices (Millipore, Bedford, MA) and were also filtered through YM50

microcon centrifugal filter devices (Millipore, Bedford, MA) to get rid of contaminating large protein. 6X SDS-PAGE sample buffer was added to concentrate and subjected to SDS-PAGE on 4-20% gradient gels (ISC Bioexpress, Kaysville, UT) and then transferred onto PVDF membrane. Non-specific protein binding sites were blocked with 5% instant non-fat dry milk (BestChoice®) and membranes were incubated with purified polyclonal antibody (1:200) overnight followed by extensive washing for 3 h with frequent changes of 1xPBST. The antigen-antibody complexes were visualized with horse radish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL) at a dilution of 1:15,000 and detected with SuperSignal West Femto maximum sensitivity substrate kit (Pierce, Rockford, IL) on X-ray film.

Immunohistochemistry

Pea aphid salivary glands were dissected in 1X PBS and washed three times in PBST (137mM NaCl, 2.7mM KCl, 10mM Na_2HPo_4, 1.8mM KH_2Po_4, 0.1% Triton-X100; pH 7.4). Thereafter, salivary glands were fixed in Bouin (71% saturated picric acid, 24% formaldehyde (37-40%) and 5% glacial acetic acid) for 10 min at room temperature in a humified chamber. Salivary glands were washed extensively with PBST and incubated with primary antibody (raised in rabbit against recombinant C002 expressed in *E. coli*) at 1:100 dilution overnight at 4^oC. Following morning salivary glands were washed 3 times at 15 min interval with PBST and were blocked with 5% normal goat serum in PBST for one hour and then washed 3 times at 15 min interval with PBST and followed by incubation with secondary antibody Cy-3 conjugated goat anti-rabbit (Jackson Immuno

Research Laboratories, West Grove, PA) at 1:500 dilution overnight at 4°C. Salivary glands were washed extensively with PBST at 15 min interval. Nuclei staining was done using TO-PRO-3 (Molecular probes, Invitrogen, CA) at 5µm conc. for 30 min in dark at room temperature. Glands were washed extensively with PBST and mounted on mounting media (Gel/Mount™; Biomeda corp., Foster city, CA) on a glass slide. Photographs were taken using Nikon Zeiss LSM 5 Pascal (Laser Scanning Confocal Microscope).

Preparation of dsRNA, siRNA and siRNA Injections

The detailed procedure is described Chapter 4. Injected aphids were placed on artificial aphid diet (Mittler and Dadd, 1965).

Results

The C002 Transcript and Protein

C002 is an abundant cDNA in our salivary gland cDNA library from the pea aphid. The C002 cDNA and its predicted amino acid sequence are shown in Fig. 4a. The predicted protein contains 219 amino acid residues. The N-terminal sequence of the protein was predicted by SignalP to be a signal peptide for an extracellular protein, (http://www.cbs.dtu.dk/services/SignalP/), with cleavage predicted between residues 23 and 24. The predicted mass of the mature protein is 21.8 kDa. There are no potential O-glycosylation sites (http://www.cbs.dtu.dk/services/NetOGlyc/) or N-glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/).

Alignment of all 46 cDNA sequences reveals two forms of the transcript differing at 4 positions resulting in 4 amino-acid substitutions as shown in Fig. 4b. There are 22 cDNA clones representing the C002a isoform and 24 cDNA clones representing the C002b isoform. These can be either variants or alleles of the same gene, or can be two separate genes. The encoded protein fails to match anything of known function and to any other sequence outside of the family Aphididae. BLAST search against EST-database aligns the pea aphid, C002 with its homologs from other aphid species. ClustalW alignment of the pea aphid C002, with the sequences from brown citrus aphid, *Toxoptera citricida*, cotton aphid, *Aphis gossypii*, green peach aphid, *Myzus persicae* and Russian wheat aphid, *Diuraphis noxia* is shown in Fig. 5a. The signal cleavage site in all three proteins is conserved. Brown citrus aphid, cotton aphid and green peach aphid sequences are not full-length. The pea aphid, C002 and brown citrus aphid sequences are 63.8% identical and the pea aphid C002 and cotton aphid sequences are 58.8 % identical. The pea aphid, C002 and green peach aphid sequence are 71.5% identical. The pea aphid, C002 and Russian wheat aphid sequences are 53.8% identical. The highest level of sequence similarity pea aphid C002 is to green peach aphid sequences, as reflected in the phylogram in Figure 5b.

In addition BLAST searches using three different short conserved regions taken from the alignments of all aphid C002 sequences had a top match to a hypothetical protein. This approach also failed to match a conserved region of C002 protein to a putative conserved domain in other known proteins.

Expression of C002 in the Salivary Gland and Gut

To determine the transcript levels in salivary gland and gut of the pea aphid, RT-PCR was conducted. As shown in Fig. 6a, the levels of C002 after 35 cycles, is significantly higher in the salivary gland than gut of the pea aphid. Equal amounts of RNA were loaded as shown as L27, which serves as internal RNA control. Transcript from salivary glands can be seen on an agarose gel only after 20 cycles of PCR whereas it takes 28 cycles of PCR for band to appear in gut RT-PCR. The C002 transcript is 256 times more abundant in salivary glands than in guts of the pea aphid.

RT-PCR on RNA isolated from various aphid species; greenbug (*Schizaphis graminum*), green peach aphid (*M. persicae*), Russian wheat aphid (*D. noxia*), and soybean aphid (*Aphis glycine*) using the primers from the pea aphid C002, amplified the fragment of 397bp in all the species as was expected for the pea aphid C002 transcript (Fig. 6b).

Northern and Southern Blot Analyses

Northern blot analysis of total pea aphid RNA using full-length C002 probe revealed a single band of 1126 bases (Fig. 7a). In Southern analysis using multiple enzymes that don’t cut within entire length of C002 clone, we observed a single band, consistent with one gene (or locus) for pea aphid C002 (Fig. 7b Lanes 1, 2, 3 and 4). The size of the band seen in *Eco*RV digest is about 1.2 kb (Lane 3, Fig. 7b) is almost same as the transcript size of C002 based on northern blot (Fig. 7a). The possibility that there may be two genes is eliminated. Thus, two isoforms C002a and C002b are alleles of the same gene.

In situ Hybridization

The pea aphid salivary glands were probed with digoxygenin-labeled C002 RNA (anti-sense) revealed that the C002 transcript occurred in the principal salivary glands (Fig 8a and b). There was no hybridization to the accessory salivary glands. Within principal salivary glands only a sub-set of cells were positively stained for C002 transcript. The negative control (with a sense probe) showed no hybridization to RNA within salivary glands (Fig. 8c and d).

Immunohistochemistry

Recombinant C002 was expressed in *E. coli* and was purified using Ni-NTA resin under denaturing conditions. This purified protein (as a gel band) was used to raise antibodies in rabbit. Rabbit polyclonal antibodies were used to localize C002 protein in the salivary glands of pea aphid (Fig. 9a). No staining of the accessory salivary gland was observed. This is consistent with the *in situ* hybridization results shown in Fig. 8. Nuclei staining with TO-PRO-3 followed by analysis of different z-sections taken by confocal microscope show 10 cells in each principal lobe (5 on each side, as the principal salivary gland is symmetrical) stained positive for C002 protein. Under higher magnification, C002 protein appeared to be located in secretory vesicles (Fig. 9b). Pre-immune serum was used as a negative control and showed no significant labeling in the salivary glands (Fig. 9c).

Western Blotting

In odder to verify that C002 is indeed a secreted protein, western blot analysis using purified C002 antibodies was done. Approximately five hundred pea aphids were placed on fresh fava bean plants and after 24 h of feeding, aphids were removed from plants. Leaf tissue was extracted and western blot was done to probe for C002 protein using purified polyclonal rabbit antibodies. C002 protein was detected in the plants that were exposed to aphids and was not detected in plants not exposed to aphids (Fig. 10a, lane 2 versus lane 3). Recombinant protein is shown in Lane 1 (Fig. 10a). This verifies the fact that C002 protein is a secreted protein. It is part of pea aphid saliva and is injected into the host-plant during feeding. Further as positive control, C002 protein was also detected from protein extracts from 5 pea aphid heads and 5 salivary glands (Fig. 10a, lanes 4 and 5 respectively). In addition there was an additional band of about 75 kDa detected in all lanes (expect the salivary gland extract), this band was also detected in negative control with pre-immune rabbit serum in plant extracts with and without aphid feeding (Fig. 10b). Nothing was detected on X-ray film in a western blot with 2^0 antibody only.

Effects of RNAi on Feeding on Artificial Diet

We injected siC002-RNA and the injected pea aphids were placed onto artificial diet (Mittler and Dadd, 1965) and allowed to feed across stretched parafilm membrane. siC002-RNA injected aphids had comparable survival when compared to siGFP-RNA injected aphids (Fig. 11). Over 70% of siC002-RNA injected aphids survived to day 7. After day 7, aphids still alive were removed from diet and placed onto fava bean leaves in

agar. Over 70% of siC002-RNA injected aphids died within 2 days on leaves compared to less that 25% for siGFP-RNA injected aphids (Fig. 11).

Discussion

Physiological and biochemical studies have established the importance of salivary secretions in the attack of aphids on plant tissue (Miles, 1999). Although aphid-plant interactions have been studied extensively, not much is known at the molecular level. The availability of aphid saliva in small quantities makes the direct study of salivary components difficult (Miles, 1965; Madhusudhan et al., 1994; Miles and Harrewijn, 1991).

In this work, a functional genomics approach was undertaken to identify components of aphid saliva. Several thousand randomly selected cDNA clones from a pea aphid salivary gland cDNA library were sequenced. cDNA were arranged into clusters of identical sequences. A cluster representing 46 clones (C002) was characterized. The encoded protein does not match any protein of known function. The encoded protein has a mass of 21.8 kDa and has a predicted secretion signal peptide, suggesting that it is a secreted protein.

The gene encoding C002 is apparently a single copy in the pea aphid genome based on southern blot analysis (Fig. 7b). Thus the two forms of C002 are two different alleles of the same gene. This heterozygosity could reflect the heterogeneity of the aphid colony used for library construction. Alternatively, this could also reflect a permanent

heterozygosity in individuals of this colony. Considering the fact that the ratio for the two alleles is 1 to 1 (22:24 respectively) and there has been no sexual reproduction in the colony, it is more likely that the two forms coexist in individual aphids rather than an equal distribution of alleles within the population. Also if one allele had a fitness advantage over a second allele, then the second allele would have been lost in the population. Thus it is highly likely that both alleles are present in individual aphids and thus the alleles appear to coexist with stable equilibrium. This would be due to balancing selection, where the heterozygote has the highest fitness. This is very different than from the ultimate result of random genetic drift, which would be fixation of one allele and the loss of all others, which would happen unless there is constant input of alleles into the population by processes such as mutation or migration. These can be ruled out, as our colony has been maintained for 7 years from initial population collected, there has been no migration into the colony, and 7 years is too short a time to accumulate mutations. Thus it appears that the polymorphism is actively maintained by a balancing selection (Graur and Li, 2000).

C002 was first identified in a positive screen for pectin methylesterase. In order to test for the activity, a pectin methylesterase assay using purified soluble recombinant protein was performed using procedure from Shen et al., 1999. However, no activity was observed and we conclude that C002 is not a pectin methylesterease.

C002 transcript and protein were localized in the same sub-set of 10 cells within one lobe of the principal salivary gland by both *in situ* hybridization and immunohistochemistry.

C002 protein can be detected in Cherqui and Tjallingii (2000) localized three salivary proteins identified by Baumann and Baumann (1995), (namely 66, 69 and 154 kDa proteins) within principal salivary glands and the pattern of staining observed is clearly different from the pattern of staining observed with C002 protein in the principal salivary gland. Thus, it seems reasonable to speculate that morphologically different cell types identified by Weidemann (1968) and Ponsen (1972), may perform different functions and at any given time (or stage of feeding). In other words aphids' saliva can be mix of the products from one or more cell types of principal salivary gland.

C002 protein was detected in fava bean plants that were exposed to aphids (Fig. 10a). This verifies directly that C002 is a secreted protein. It is part of pea aphid saliva and is injected into the host-plant during feeding. Being in aphid saliva, it is postulated that it may play a role in aphid-plant interactions. RNAi experiment verifies importance of the protein, as it is not needed to feed on articial diet but it is necessary for survival on host plants.

C002 transcript is also present in other aphid species. But the absence of a homolog of C002 outside of aphids, in spite of the presence of huge amount of sequence information in various database (Non-redundant-NCBI, EST-database and UniProt Knowledgebase database at ExPASy) and also with the availability of genome sequences of many insects (*Drosophila melanogaster*, *Anopheles gambiae*, *Tribolium castaneum, Bombyx mori* and *Apis mellifera*), suggests that C002 might be specific to Aphididae or phloem feeding Hemiptera.

C002 could have any of many functions in aphid-plant interactions. It is apparently not a digestive enzyme for any component of artificial diet, as it is not required to feed on such a diet. It is possible that C002 protein is a putative hydrolase and assists an aphid's penetration of the leaf tissue or helps divert plant responses. It could be an inhibitor that functions to inhibit potential toxic plant products (may be by conjugation with C002) or C002 protein could function to avoid a potential response from phloem (callose formation) as the siC002-RNA injected aphids are unable to feed continuously and tend to move around a good deal, whereas uninjected aphids or aphids injected with siGFP-RNA stay quite still on the underside of the fava bean leaves, or it could be part of the mechanism of preventing "plugging" of the sieve element once stylets has entered phloem. Further experiments are needed to elucidate the role of C002 in aphid-plant interactions. Using electrical penetration graph methods on the siC002-RNA injected aphids, we hope to understand the effect of C002 transcript knockdown on detailed aspects of the insects' feeding behavior.

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Kansas State University Museum of Entomological and Prairie Arthropod Research (KSU-MEPAR).

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Figure 4. Pea aphid, *A. pisum* C002 sequence.

a) Nucleotide and deduced amino acid sequence of pea aphid, C002 cDNA clone. N-terminal signal peptide by SignalP is shown in bold. Arrow indicates a signal-peptide cleavage site. b) Alignment of two alleles/variants of C002. The two alleles differ at four positions, which are shown in bold.

a)

GGCCGGGGGTTCAAACAAATATCTCGTCGTGTATCCAGTGCGATAGCGATAATTTACAAC 60

ATGGGAAGTTACAAATTATACGTAGCCGTCATGGCAATAGCCATAGCTGTAGTACAGGAA 120

M G S Y K L Y V A V M A I A I A V V Q E

GTTAGATGCGATTGGTCTGCCGCTGAACCGTACGATGAGCAGGAAGAAGCGTCTGTCGAA 180

V R C ↑ D W S A A E P Y D E Q E E A S V E

TTACCGATGGAGCACCGTCAGTGCGATGAATACAAATCGAAGATCTGGGACAAAGCATTT 240

L P M E H R Q C D E Y K S K I W D K A F

AGCAACCAGGAGGCTATGCAGCTGATGGAACTAACGTTTAATACAGGTAAGGAATTAGGC 300

S N Q E A M Q L M E L T F N T G K E L G

TCCCACGAAGTGTGCTCGGACACGACGCGGGCCATTTTTAACTTCGTCGATGTGATGGCC 360

S H E V C S D T T R A I F N F V D V M A

ACCAACCAGAACGCCCATTACTCGCTGGGTATGATGAACAAGATGTTGGCGTTCATCATA 420

T N Q N A H Y S L G M M N K M L A F I I

AGAGAGGTGGACACGACGTCCAACAAATTCAAAGAGACGAAGGAGGTTTTCGAACGCATC 480

R E V D T T S N K F K E T K E V F E R I

GCGAAAACTCCAGAGATCCGAGACTATATCAAGCACACGACCGCCCGGACCGTCGACTTG 540

A K T P E I R D Y I K H T T A R T V D L

CTCAAAGAGCCCGTGATTAGAGGCCGACTGTTCAAAGTGGTGAAAGCCTTCGAGGGTCTG 600

L K E P V I R G R L F K V V K A F E G L

ATAAAACCGTCCGAAAACGAGGAATTGGTCAAGCAGAGGCTTAAGAGGATAACCAATGCT 660

I K P S E N E E L V K Q R L K R I T N A

CCCGCCAAGATGGCTATGGGAGCCATAAATAAGTTTGGAAGTTTCCTTCGACGTTTTTA 720

P A K M A M G A I N K F G S F L R R F *

ATAAGCGCGTCCATACAGACTAGTGATATATTATATATATATACTTATAA 770

b)

```
C002a     MGSYKLYVAVMAIAIAVVQEVRCDWSAAEPYDEQEEASVELPMEHRQCDEYKSKIWDKAF 60
C002b     MGSYKLYVAVMAIAIAVVQEVRCDWSAAEPYDEQEEASFELPMEHRQCDEYKSKIWDKAF 60
          **************************************.*********************

C002a     SNQEAMQLMELTFNTGKELGSHEVCSDTTRAIFNFVDVMATNQNAHYSLGMMNKMLAFII 120
C002b     SNQEAMQLMEITFNTGKELGSNEVCSDTTRAIFNFVDVMATNQNAHYSLGMMNKMLAFII 120
          **********:**********:**************************************

C002a     REVDTTSNKFKETKEVFERIAKTPEIRDYIKHTTARTVDLLKEPVIRGRLFKVVKAFEGL 180
C002b     REVDTTSNKFKETKEVFERITKTPEIRDYIKHTTARTVDLLKEPVIRGRLFKVVKAFEGL 180
          ********************:***************************************

C002a     IKPSENEELVKQRLKRITNAPAKMAMGAINKFGSFLRRF 219
C002b     IKPSENEELVKQRLKRITNAPAKMAMGAINKFGSFLRRF 219
          ***************************************
```

Figure 5. Multiple sequence alignments.

a) Alignment of C002 amino acid sequences from pea aphid, *A. pisum*, with sequences of cotton aphid, *A. gossypii*, brown citrus aphid, *T. citricida*, green peach aphid, *M. persicae* and Russian wheat aphid, *D. noxia*. The sequences were aligned using ClustalW version 1.82 and followed by BOXSHADE server design.

http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html

b) Phylogram of C002 sequences corresponding to the conserved regions in the alignment. The phylogram was created starting with a multiple alignment created with ClustalW version 1.82.

a)

```
A.gossypii      1 MGRYQLYVAVMAISSLAVIQKASCAGGPNAYNTTEQYI----------------ESKDGL
T.citricida     1 MGRYQLYVAVVAISSLAVIQKASCAGGSNAYPSTEQYD----------------ESKDKL
D.noxia         1 MGSYKLYLAVIAIACIAAVQEASCSDEQVQYDDGEEVIGLEKEQEEVSEPDMEEKEEPES
A.pisum         1 MGSYKLYVAVMAIAIAVVQEVRCDWSAAEPYDEQEEAS-------------------FEL
M.persicae      1 ------------------------------------------------------------


A.gossypii     45 EMEHHQCDEYKSKIWNKAFSNPAAMQLVDVVLKTAKEMGTDNVCSDTIRVLSNFIDVMAT
T.citricida    45 EMEHHQCDEYKSKIWNKAFSYPAAMQLMDVIFETAKEMGTNDVCSDTIRVLSNFIDVMAT
D.noxia        61 EMEYHQCEEYKSKIWDNAFSNKDAMDMMKLTFITAEKMGSDAVCTDTARAFINFIEVMAT
A.pisum        42 PMEHRQCDEYKSKIWDKAFSNQEAMQLMEITFNTGKELGSNEVCSDTTRAIFNFVDVMAT
M.persicae      1 ----------------------------------AKELGSNEVCSDTTRALFNFVDVMAT


A.gossypii    105 NQNSHYSVGMLAKMLAFIAREADMTSDKFRDTKEVFDRIVQNADIRDYIRNTASRVVDLL
T.citricida   105 NQNSHYVVGMLGKMLAFIAREVDTTSDKFRETTEVFERIAKNADIRDYIRH---------
D.noxia       121 NSNSQYTRSMFKKLVAFIVRELNTTSDNFRETSEVFERIWTTPEIRDFIRDSVTRTNNVL
A.pisum       102 NQNAHYSLGMMNKMLAFIIREVDTTSNKFKETKEVFERITKTPEIRDYIKHTTARTVDLL
M.persicae     27 SPYAHFSLGMFNKMVAFILREVDTTSDKFKETKQVVDRISKTPEIRDYIRNSAAKTVDLL


A.gossypii    165 KLPVMRN---------------------------------------------------
T.citricida       ----------------------------------------------------------
D.noxia       181 KEPRMRSRLFKVIEAAMDLMSKSKDGESMKQKFKGMYRAPTKMARXAMDKVGNFFRKL
A.pisum       162 KEPVIRGRLFKVVKAFEGLIKPSENEELVKQRLKRITNAPAKMAMGAINKFGSFLRRF
M.persicae     87 KEPKIRARLFRVMKAFESLIKPNENEALVKQKIKGLTNAPVKLAKGAMKTVGRLFRHF
```

b)

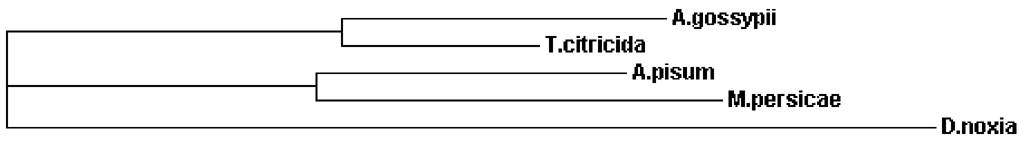
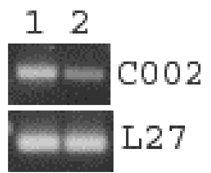


Figure 6. Expression of C002 in salivary glands and gut of pea aphid.

a) RT-PCR of the C002 transcript from pea aphid, salivary glands (Lane 1) and guts (Lane 2). PCR products after 35 cycles. b) RT-PCR of the C002 transcript from various aphid species using primer pair from the pea aphid clone. Pea aphid, *A. pisum* (Lane 1), greenbug, *S. graminum* (Lane 2), green peach aphid, *M. persicae* (Lane 3), Russian wheat aphid, *D. noxia* (Lane 4), soybean aphid, *A. glycine* (Lane 5).

a)



b)

Figure 7. Northern and Southern blot analysis of C002 transcript and gene.

a) Northern blot analysis of the pea aphid C002 transcript. b) Southern blot analysis of the pea aphid C002. Genomic DNA was extracted from adult aphids. The cDNA shown in Figure 1 was radiolabelled and used as the probe. Lane 1, *Xba*I; Lane 2, *Nco*I; Lane 3 *Eco*RV and Lane 4 *Eco*RI.

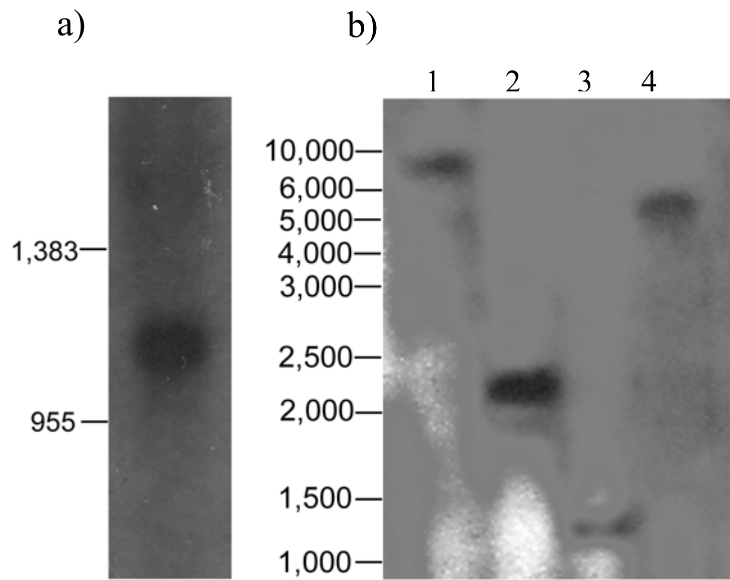

a)
b)
1,383
955
1
2
3
4
10,000
6,000
5,000
4,000
3,000
2,500
2,000
1,500
1,000

Figure 8. Detection of C002 mRNA in the salivary glands by *in situ* hybridization.

Paraformaldehyde fixed salivary glands were treated with a digoxigenin (DIG)-labeled RNA, antisense (A and B) and control, using sense probe (C and D) followed by incubation with an anti-DIG antibody.

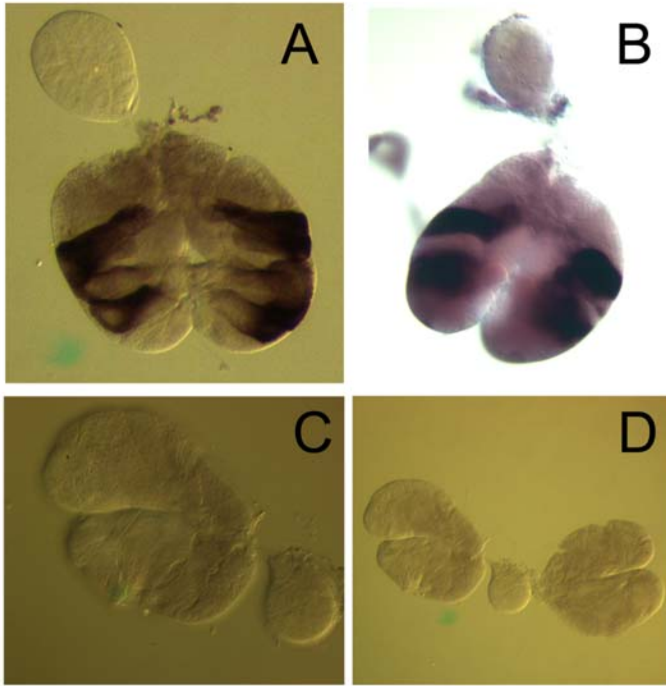

A
B
C
D

Figure 9. Immunohistochemical localization of C002 protein.

Localization of C002 protein within principal salivary gland of the pea aphid. a) TO-PRO-3 staining of nuclei and Cy-3 antibody staining of C002 protein of principal and accessory salivary glands (at 20X magnification). b) Cy-3 antibody staining of C002 protein of principal salivary glands under higher magnification (40X) showing C002 protein in secretory vesicles in four positively stained cells. (c) Negative control using pre-immune rabbit serum instead of polyclonal C002 antibody.

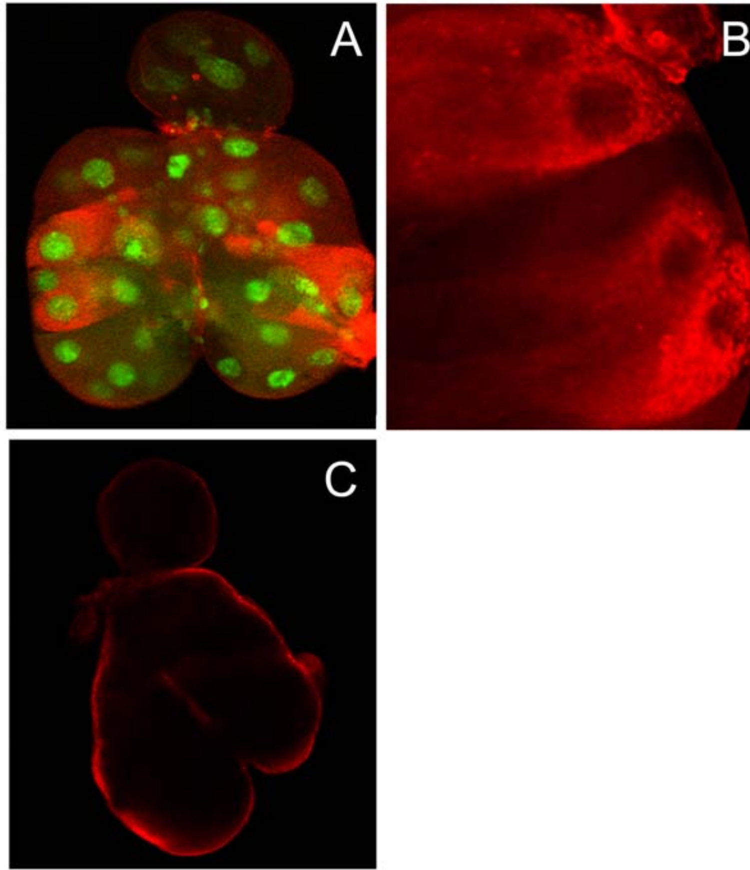
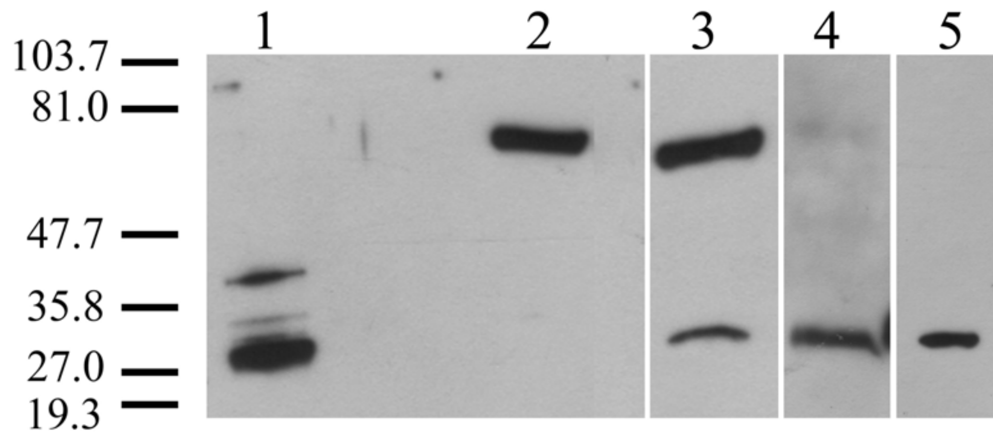

A
B
C

Figure 10. Detection of C002 protein using purified C002 antibodies.

a) Western blot indicating that C002 is injected into the fava bean plants during feeding. Lane 1. Recombinant C002 protein. Lane 2. Fava bean plant without aphid feeding, which serves as negative control. Lane 3. Fava bean plant extract after pea aphid feeding. Lane 4. Protein extract from 5 aphid heads. Lane 5. Protein extract from 5 salivary glands. b) Negative control using pre-immune rabbit serum instead of polyclonal C002 antibody. Lanes 1 and 2. Fava bean plant extract without aphid feeding. Lane 3. Fava bean plant extract after aphid feeding.

a)



b)

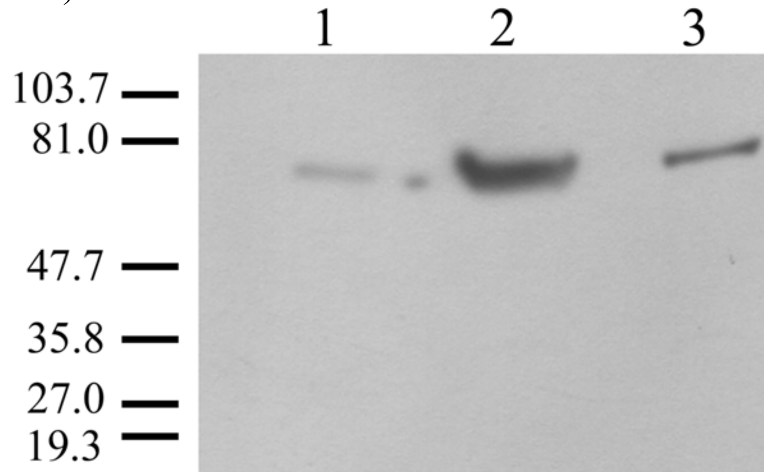
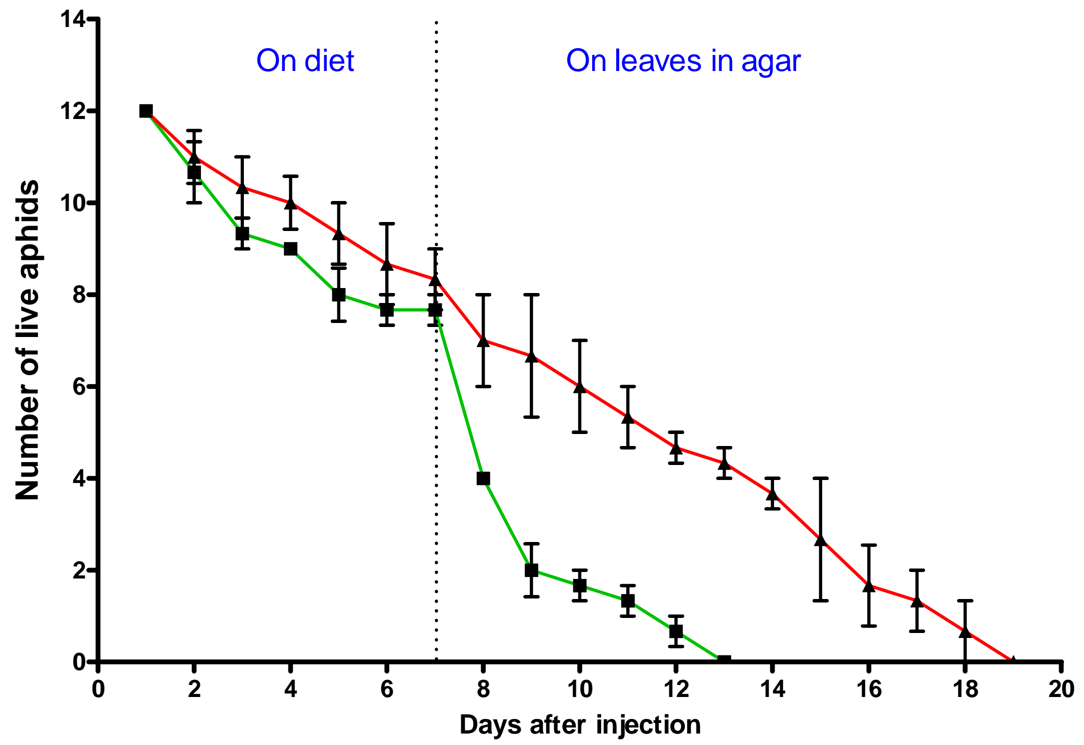


Figure 11. Survival of siRNA injected aphids on artificial diet.

siRNA injected aphids kept on aphid diet for 7 days after injection, thereafter, they were moved to fava bean leaves in Petri plates on agar. Green line: injections with siC002-RNA; Red line: injections with siGFP-RNA (control). Data from 3 independent experiments are shown; there were 12 aphids in each experiment. The bars depict standard errors.


On diet
On leaves in agar
Number of live aphids
Days after injection
0
2
4
6
8
10
12
14
16
18
20

CHAPTER 4

Chapter 4: RNAi Knockdown of a Salivary Transcript Leading to Lethality in the Pea Aphid, *Acyrthosiphon pisum*.

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Key words: pea aphid, salivary gland, RNA interference, RNAi, small interfering RNA, siRNA

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; dsRNA, double-stranded RNA; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; EST, expressed sequence tag; GFP, green fluorescent protein; NCBI, National Center for Biotechnology Information

Abstract

Injection of siRNA (small interfering RNA) into parthenogenetic adult pea aphids (*Acyrthosiphon pisum*) is shown here to lead to depletion of a target salivary-gland transcript. The siRNA was generated from double stranded RNA that covered most of the open reading frame of the transcript, which we have called C002. The C002 transcript level decreases dramatically over a 3-day period after injection of siRNA. With a lag of 1 to 2 days, the siC002-RNA injected insects died, on average 8 days before the death of control insects injected with siRNA for green fluorescent protein. It appears, therefore, that siRNA injections into adults will be a useful tool in studying the roles of individual transcripts in aphid salivary glands, and our results suggests that siC002-RNA injections can be a useful positive control in such studies.

Introduction

Double-stranded RNA (dsRNA), when injected into or ingested by an organism or introduced into cells in culture, can specifically lower the level of the transcript of a target gene. This method, initially documented in *C. elegans* and named RNA interference, or RNAi, has become a very powerful tool to examine the role of individual genes (Fire et al. 1998; Zamore et al. 2000). Among insects, injections of interfering RNAi in post-embryonic stages have been used successfully in the honeybee, *Apis mellifera* (Amdam et al. 2003), the moths *Hyalophora cecropia* (Bettencourt et al. 2002), *Spodoptera litura* (Rajagopal et al. 2002), *Bombyx mori* (Uhlirova et al. 2003) and *Manduca sexta* (Levin et al. 2005), the mosquitoes, *Anopheles gambiae* (Osta et al. 2004) and *Aedes aegypti* (Attardo et al. 2003), the fruit fly, *Drosophila melanogaster* (Goto et al. 2003), a grasshopper, *Schistocerca americana* (Dong and Friedrich 2005), the red flour beetle *Tribolium castaneum* (Arkane et al. 2005a and 2005b, Tomoyasu et al. 2005,) and a termite, *Reticulitermes flavipes* (Zhou et al. 2006).). Evidently, in some insect species, injected interfering RNA can move from the hemolymph into tissues or organs, where it then exerts its transcript-lowering effect, presumably by promoting degradation of the target mRNA. Extending this method to an aphid, and in particular, to the aphid salivary gland, is the objective of the work reported here.

Relatively long dsRNA is often used as an interfering RNA, but in two cases listed above (Levin et al. 2005 and Zhou et al. 2006) small interfering RNA (siRNA) was injected instead. siRNAs of 21-base pairs are highly effective in eliciting RNAi (Elbashir et al.

2001) and can be produced *in vitro* using the enzyme Dicer, a form of RNase III and the enzyme believed to produce siRNA *in vivo* (Bernstein et al. 2001).

Saliva is very important in the interaction between aphids and host plants. Proteins, including enzymes, of aphid saliva have been postulated to play several roles, including the formation of a sheath around the stylets, the creation of an extracellular path by the stylet, overcoming plant defense and possibly stimulating plant defense in non-host plants (Miles 1999; Cherqui and Tjallingii 2000). As transcripts of potential interest are found in cDNA libraries of aphid salivary glands (for instance, among the roughly 4500 pea aphid salivary expressed sequence tags (ESTs) recently deposited at NCBI as accession numbers DV747494 through DV752010), a method will be needed to examine the importance of transcripts of individual genes, and RNAi is a potentially powerful approach for doing so.

The most abundant cDNA from a salivary gland cDNA library prepared from the peaaphid, *Acrythosiphon pisum* (Reeck et al., unpublished observations), was selected as the target transcript for this study. This cDNA was arbitrarily designated C002 (i.e., Cluster 2). This transcript was also found, infrequently, among whole-body and whole-insect pea aphid ESTs, where there are 7 occurrences in approximately 17,800 such ESTs deposited at the National Center for Biotechnology Information (Sabeter-Munoz et al. 2006). As a point of comparison, ESTs for cytochrome oxidase subunit-1, a widely distributed protein, occur over 160 times among the whole-body and whole-insect pea aphid ESTs. The translated nucleotide sequence of C002 does not match other sequences

except in aphids, where there are matches to translated ESTs from *Aphis gossypii* and *Toxoptera citricida*. The entire open reading frame of the C002 transcript can be found at accession number CN763138. The encoded pea aphid protein includes a predicted signal peptide, and the protein is of entirely unknown function.

To test the efficacy of RNAi in aphids, siRNA coding for C002 was injected into adult aphids. It was found that injection of siRNA leads to knockdown of the C002 transcript level in salivary glands and results in a greatly reduced lifespan of the injected insects. These results provide the basis for the use of this technique in studies of other salivary transcripts and, possibly, transcripts in other organs.

Materials and Methods

Plants and Aphids

Aphids were originally collected from alfalfa plants in the summer of 1999 by Dr. Marina Caillaud at Cornell University. Thereafter, the aphids were reared at KSU on fava beans (*Vicia fabae*) grown in pots (10 cm diameter) at room temperature under high intensity sodium lights with a L:D of 16:8. For the RNAi experiments, even-aged cohorts were established by collecting nymphs from young parthenogenetic females over a 24-h period. Cohorts formed in this way were maintained on plants for 7 days and then used for siRNA injections.

Preparation of dsRNA and siRNA

PCR primers with T7 promoter sequences were used to prepare double-stranded RNA (Tomoyasu et al. 2005). For C002 RNA, the primers had the following sequences: 5'--TAA TAC GAC TCA CTA TAG GGA AGT TA--3' and 5'--TAA TAC GAC TCA CTA TAG GGA AAC TT--3' (forward and reverse, respectively). The two primers cover a region that extends from position 5 to position 637 in the open reading frame that, in its entirety, is 660 bases. Primers for green fluorescent protein RNA that were used in controls had the following sequences: 5'--TAA TAC GAC TCA CTA TAG GGC GAT GC--3' and 5'--TAA TAC GAC TCA CTA TAG GGC GGA CT -- 3' (forward and reverse, respectively). These cover a region of 520 bases in the open reading frame for the green fluorescent protein.

PCR products were gel purified using Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). dsRNA was then made using Megascript RNAi Kit (Ambion, Austin, TX) following the manufacturer's protocol. dsRNA was purified using phenol:choloform extraction. siRNA was generated from dsRNA using the Dicer siRNA Generation Kit T5200002 (Genetherapy Systems) and purified using siRNA purification columns of Genlantis. Products of Dicer digestion were checked for size (21 – 23 base pairs) on 15% acrylamide gels.

siRNA Injections

Glass needles (outer diameter of 1.0 mm, inner diameter of 0.50 mm; Sutter Instruments, Navato, CA) were made using a micropipette puller (Model P-87, Sutter Instruments) at

settings of: heat, 355; velocity, 50; time, 150. A PMI-200 pressure microinjector (Dagan) was used for siRNA injections. Aphids were held on their dorsa over a small hole in piece of plastic tubing (5 mm inner diameter) that was blocked at one end, held in place on a flat surface and connected at the other end to a small vacuum pump (Pro-Craft, Grobet USA, Carlstadt, NJ). Aphids were injected at the suture joining the ventral mesothorax and metathorax, at an angle of about 45 degrees, aimed toward the head of the aphid. We estimate that 5 nl of siRNA (10μg/μl) was injected into each aphid.

Leaves were cut from healthy, intact fava beans and put into a sterilized 2% agar (Fisher Scientific) supplemented with 0.1% Miracle Grow fertilizer and 0.03% methyl 4-hydroxybenzoate (Sigma-Aldrich, St. Louis, MO) as a fungicide, essentially as described on the David Stern website (http://www.princeton.edu/%7Edstern/PlatesProtocol.htm) for *Medicago* leaves. About 7 ml of medium was placed in a Petri plate (100 x 15 mm) and one leaf was inserted into the agar as it cooled. One injected aphid was placed on each leaf. The plates were placed under GE Utility Shoplite with F48/25 watt/UTSL fluorescent lights, with 16:8 L:D, at a temperature of 23° C. Plates were checked several times a day for dead aphids, which were identified by lack of movement, being off the leaves and, after several hours, darkened coloration.

Examining Transcript Levels by RT-PCR

Total RNA was isolated from individual pea aphid heads using TRI reagent (Molecular Research Center, Inc. Cincinnati, OH) following the procedure provided by the manufacturer. RNA was treated with DNase I (Ambion, Austin, TX) following the

company's instructions. AMV reverse transcriptase was used with oligo-dT primers to synthesize single-stranded cDNA following the procedure in Technical Bulletin 099 of Promega. PCR was done using 5'--CCA GTG CGA TAG CGA TAA TTT ACA AC--3' and 5'--CAC CTC TCT TAT GAT GAA CGC CAA C--3' for C002 forward and reverse primers, respectively, giving a final product of 397 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 reverse, primers, respectively, giving a final product of 231 base pairs. The sequence of pea aphid L27 has accession number CN584974. Both primer pairs were used in each PCR, with L27 serving as an internal control. PCR was performed for one cycle at 95° C for 2 min followed by 26 cycles of: 95° C for 30 s, 54° C for 30 s and 72° C for 35 s. Primers were used at 0.3 μM and PCR master mix from Promega (Madison, WI) was used in a final volume of 50 μl. PCR products were separated on 1% agarose gels prepared in 40 mM Tris-acetate (pH 8.3) and 1 mM EDTA. Ethidium bromide was added to a final concentration of 0.7 μg/ml before allowing the agarose to solidify. The gels were photographed under ultraviolet light and band intensities were obtained using SigmaScan's Pro5 image measurement software.

Results and Discussion

Injection of siC002-RNA into adult parthenogenetic pea aphids led to greatly reduced life-span, as shown in Figure 1. Aphids injected with siC002-RNA died well before control aphids injected with green fluorescent protein si-RNA. Half of the aphids injected with siC002-RNA had died at 3 days after injection whereas 11 days was required for

death of half of the aphids injected with control siRNA. The survival of uninjected aphids was similar to that of aphids injected with control siRNA, indicating that the small injection wound was tolerated by the aphids and that injection of control siRNA and buffer components not have a toxic effect.

RT-PCR was used to assess C002 transcript levels in RNA extracted from heads. The signal obtained by RT-PCR using head RNA as template is from transcripts in salivary glands, since using RNA from heads from which salivary glands had been removed by injected aphids are shown in Fig. 2. Using the transcript for ribosomal protein L27 as internal control, we found that transcript levels from C002 dropped dramatically within 3 days after injection with siC002-RNA. On the other hand, in control siRNA injected insects, C002 transcript levels, normalized to L27 transcript levels, did not change significantly.

Semiquantitative data from such measurements are plotted in Figure 3 (open triangles), where, for purposes of comparison of the timing, the decline in the number of live insects (from Fig. 1) is again shown. We note that the measurements of C002 transcript levels may somewhat overestimate the levels of the transcript and thus underestimate the timing of the knockdown, since in examining transcript levels only insects that were alive at each time point were used. Insects that had already died might well have had extensive knockdown of the C002 transcript.

These data demonstrate that siRNA injection into adult pea aphids can lower the transcript level of a target gene, C002, expressed in the salivary gland. The injections have a profound effect on lifespan, lowering the time to 50% survival from 11 days in control injections to about 3 days for injection of siC002-RNA. Several questions remain unanswered. Will the same procedure work for other salivary transcripts? There are no data at this time regarding this point, but we will soon undertake siRNA studies on other salivary transcripts. Will the same procedure work for lowering transcript levels in other organs? Again, there are no data on this point. Judging from results in other insects, it seems likely that gut (Rajagapol et al. 2002, Osta et al. 2004, Arakane et al. 2005), fat body (Attardo et al. 2003, Amdam et al. 2003) and hemocytes (Levin et al. 2005) would be sensitive to the effects of injected interfering RNA. Does siRNA enter aphid embryos developing within the injected adult female? We have not studied this systematically, but, informally, we have noticed that a significant fraction of nymphs from adults injected with siC002 RNA do die prematurely. This suggests the possibility that siRNA does indeed enter at least some embryos.

Unanswered by these experiments is the function of C002. We had no way of anticipating the profound effect of knockdown of the C002 transcript and in light of that effect, we will be investigating the function of C002 in ongoing experiments. One interesting piece of information that we have at this point is that siC002-RNA-injected aphids exhibit a peculiar behavior. Whereas uninjected aphids or aphids injected with siGFP-RNA stay quite still on the underside of the fava bean leaves, the siC002-RNA-injected aphids move around a good deal and do not stay confined to the underside of the leaves. Using

electrical penetration graph methods we hope to understand the effect of C002 transcript knockdown on detailed aspects of the insects' feeding behavior.

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Figure 12. Survival of pea aphids after injection of siRNA.

The graph shows the number of surviving aphids at daily intervals after injection. Green line and open triangles: injections with siC002-RNA. Red line and closed triangles: injections with siGFP-RNA (control). Blue line and open squares: uninjected insects. For the siRNA-injected insects, the data points are averages from three experiments, each of which began with 12 insects in the experimental and control groups. The bars depict standard errors.

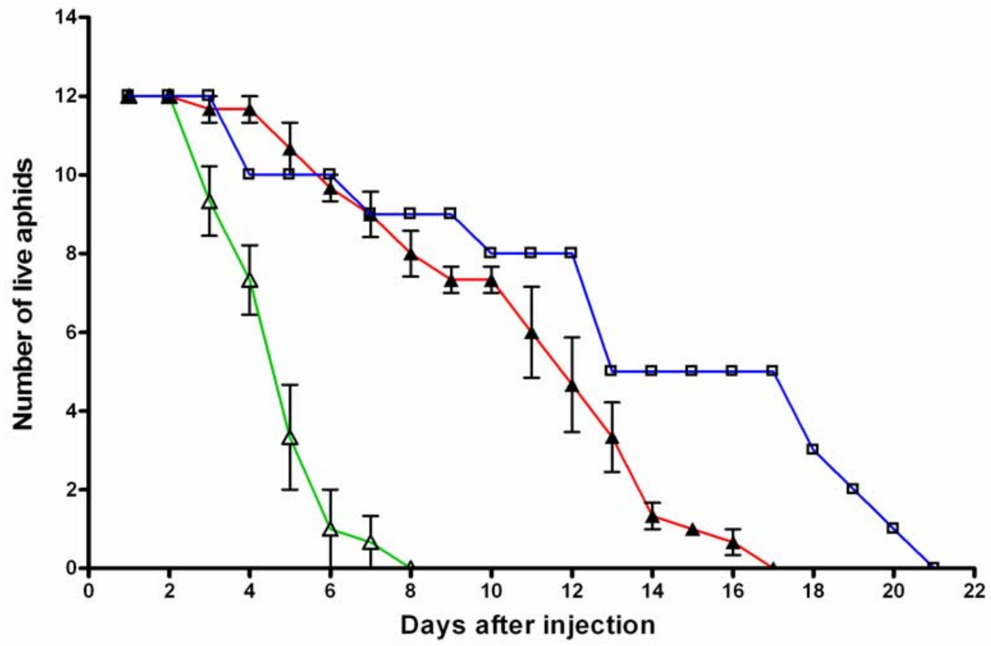

Number of live aphids
Days after injection
0
2
4
6
8
10
12
14
16
18
20
22
0
2
4
6
8
10
12
14

Figure 13. Knockdown of the C002 transcript after siRNA injections.

Insects were injected either with siC002-RNA or siGFP-RNA. RNA from heads of injected insects was used in RT-PCR in which two primer-pairs were included, for C002 itself and for the transcript encoding ribosomal protein L27. The L27 PCR product serves as an internal control. The results shown (agarose gels after ethidium bromide staining) are of individual aphids at time points from 1 to 5 days after injection. Panel A: PCR products from reactions (26 cycles) with head RNA from siC002-RNA-injected insects. Panel B: PCR products from reactions (26 cycles) with head RNA from siGFP-RNA-injected insects. Panel C: PCR products from reactions (26 cycles) carried out on RNA extracted from heads from which salivary glands had been removed. Analysis of samples from 4 separate insects are shown.

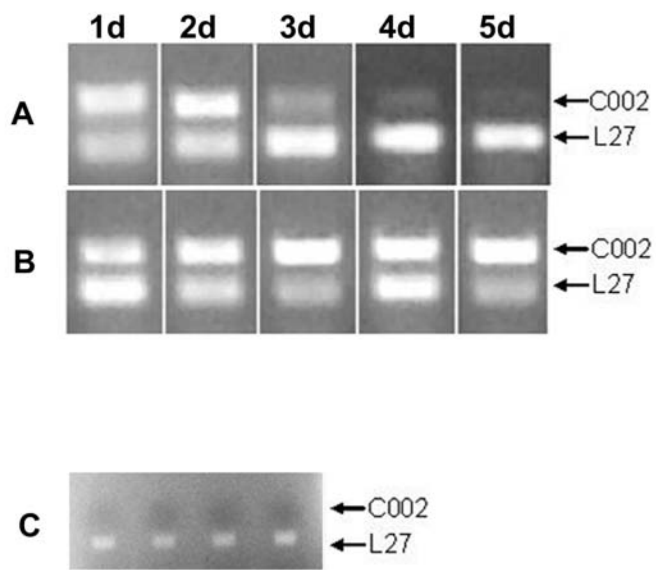
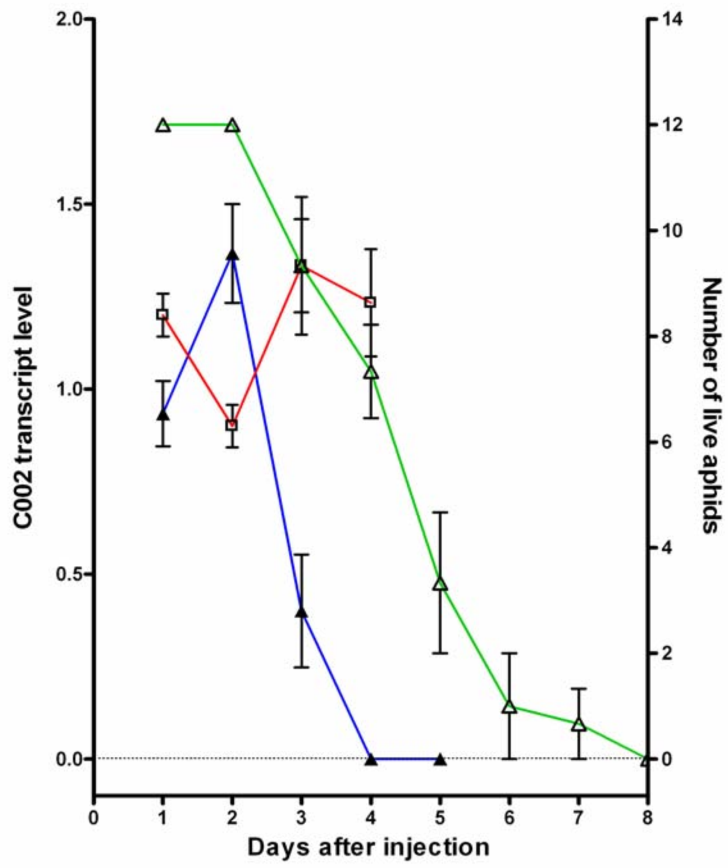

1d
2d
3d
4d
5d
A
B
C
C002
L27
C002
L27
C002
L27

Figure 14. Timing of knockdown of C002 transcript after siRNA injection.

Data from RT-PCR analysis of C002 transcript levels (normalized against L27 transcript levels) are plotted over a several day period after injection with either siC002-RNA (blue line, closed triangles) or siGFP-RNA (red line, open squares). Data are averages of normalized intensities from several individual insects at each time point and bars depict standard errors. The green line (open triangles) shows the survival of siC002-RNA-injected insects from Figure 1.


C002 transcript level
Number of live aphids
Days after injection
2.0
1.5
1.0
0.5
0.0
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8

Appendix 1

Supplemental Information to Chapter 4

Injection of siC002-RNA into adult parthenogenetic pea aphids led to greatly reduced life-span, as shown in Fig. 15. Aphids injected with siC002-RNA died well before aphids injected with siGFP-RNA (as a control). Results from 3 experiments (each with 12 replications) at different time intervals (Feb 03, Feb 13 and Feb 18) and 3 experiments with aged cohorts (March 20, May 21 and June 13) also with 12 replication for experimental group (siC002-RNA injections) and control group (siGFP-RNA injections) are shown in Fig. 15. Half of the aphids injected with siC002-RNA had died at about 3 days after injection whereas 11 days was required for death of half of the aphids injected with siGFP-RNA. The survival of uninjected aphids was similar to that of aphids injected with siGFP-RNA, indicating that the wound created was tolerated by the aphids and that injection of control siRNA and buffer components not have toxic effects.

RT-PCR data from individual aphid heads injected with siC002-RNA is shown in Fig. 16a and with siGFP-RNA injections in Figure 16b. There was no effect on the transcript level within 48 h of injection but the transcript levels decrease rapidly thereafter to almost undetectable levels at 120 h after siC002-RNA injections (Fig. 16a). On the other hand siGFP-RNA injections had no effect on transcript levels of C002 (Fig. 16b).

Suppression of C002 transcript had a drastic effect on the reproduction of siC002-RNA injected aphids, when compared with siGFP-RNA injected aphids. Different experiments conducted over time and with different experimental set-up are shown in Fig. 17(a, b, c and d). Aphids injected with dsC002-RNA had on an average of 1.5 – 2.0 nymphs / day of life, whereas, dsGFP-RNA injected aphids has 3.5 – 4.5 nymphs / day of life (Fig. 17a). RT-PCR on dsC002-RNA injected aphids did not show reduction in transcript levels. Therefore, injections were done using siRNA instead of dsRNA and RT-PCR analysis on siC002-RNA injected aphids showed the reduction in the transcript level. Injections done at various time points (Nov 27, Dec 06, Dec 22, Jan 09 and Jan 26) show a dramatic decrease in the reproduction of siC002-RNA injected aphids. Aphids injected with siC002-RNA had on an average 0.5 – 1.5 nymphs / day of life, when compared to 4.0 – 5.0 nymphs / day of life, in case of siGFP-RNA injected aphids (Fig. 17b). For these experiments, data on the survival was not thoroughly recorded, as the experiment was terminated, when all siC002-RNA injected aphids were dead.

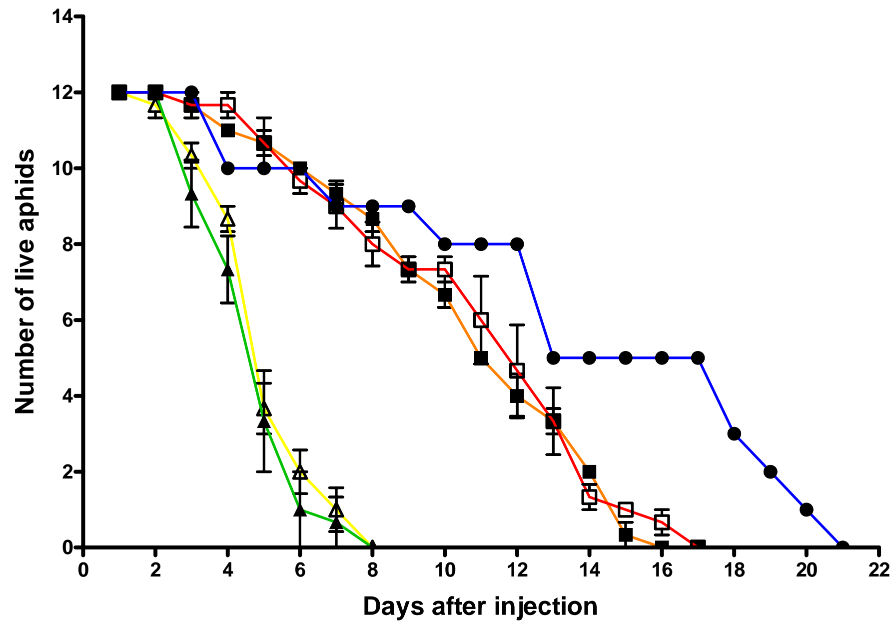
Reproduction data from 3 different experiments done on Feb 03, Feb 13 and Feb 18, and the corresponding survival data on these injected aphids is part of Fig. 15 (yellow and orange line for siC002-RNA and siGFP-RNA injected aphids respectively) is shown in Fig. 17c. siC002-RNA injected aphids had an average of 1.3 - 1.8 nymphs / day of life, whereas siGFP-RNA injected aphids had 3.9 – 4.6 nymphs / day of life (Fig. 17c). Finally 3 different experiments with aged cohorts are shown in Fig. 17d. All the injected aphids were of same age i.e. 7-8 days old. siC002-RNA injected aphids had 0.7 – 1.1

nymphs / day of life when compared to 4.4 – 4.7 nymphs / day of life in case of siGFP-RNA injected aphids (Fig. 17d).

Behavior of the siC002-RNA injected aphids was considerably different from siGFP-RNA injected aphids. siC002-RNA injected aphids did tend to move around (in petri-plate, on agar and on the upper-side of the fava bean leaves). This behavior was evident within two hours of injection in siC002-RNA injected aphids, whereas, in contrast the siGFP-RNA injected aphids, settled down within an hour of injection and thereafter stayed on the underside of the leaves. This behavioral change may explain the decrease in reproduction observed in case of siC002-RNA injected aphids. It is therefore hypothesized that the less spent on feeding on leaves led to the decrease in the survival and reproduction of a siC002-RNA injected aphids.

Figure 15. Survival of siRNA injected pea aphids.

The graph shows the number of surviving aphids at daily intervals after injection. Yellow line and open triangles: injections with siC002-RNA done on Feb 03, Feb 13 and Feb 18. Green line and closed triangles: injections with siC002-RNA done on March 20, May 21 and June 13. Orange line and closed squares: injections with siGFP-RNA (control) on Feb 03, Feb 13 and Feb 18. Red line and open squares: injections with siGFP-RNA (control) done on March 20, May 21 and June 13. Blue line and closed circles: uninjected insects. For each siRNA-injection experiment, the data points are averages from three experiments, each of which began with 12 insects in the experimental and control groups. The bars depict standard deviations.

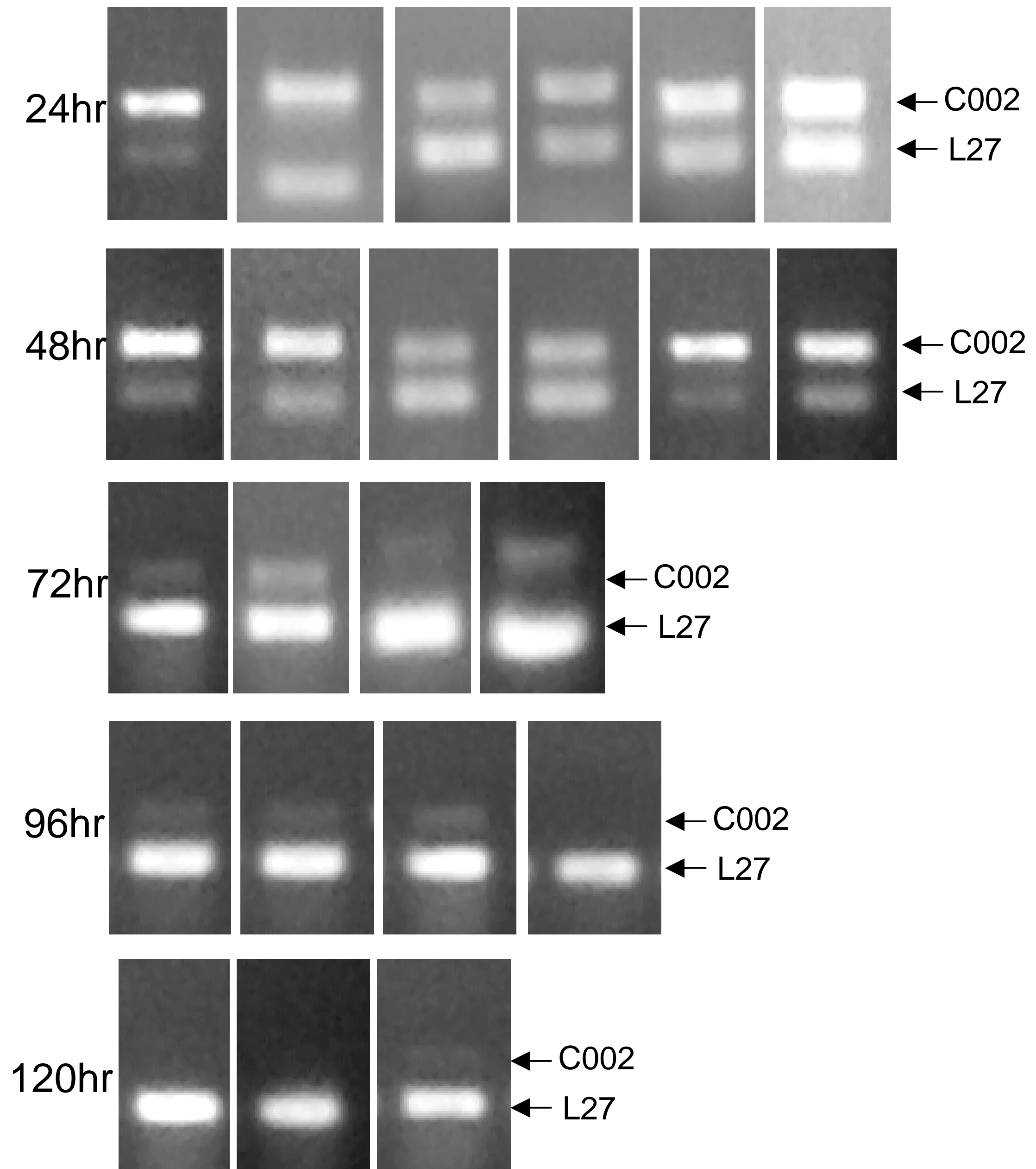


Number of live aphids
Days after injection
0
2
4
6
8
10
12
14
16
18
20
22

Figure 16. Knockdown of the C002 transcript after siRNA injections.

Insects were injected either with a) siC002-RNA b) siGFP-RNA. RNA from heads of injected insects was used in RT-PCR in which two primer-pairs were included, for C002 itself and for the transcript encoding ribosomal protein L27. The L27 PCR product serves as an internal control. The results shown are of individual aphids at time points from 24hours to 120 hours after injection. PCR products from reactions (26 cycles) with head RNA either from siC002-RNA-injected insects (a) or from siGFP-RNA-injected insects (b) are shown after agarose gel electrophoresis and ethidium bromide staining.

a)



b)

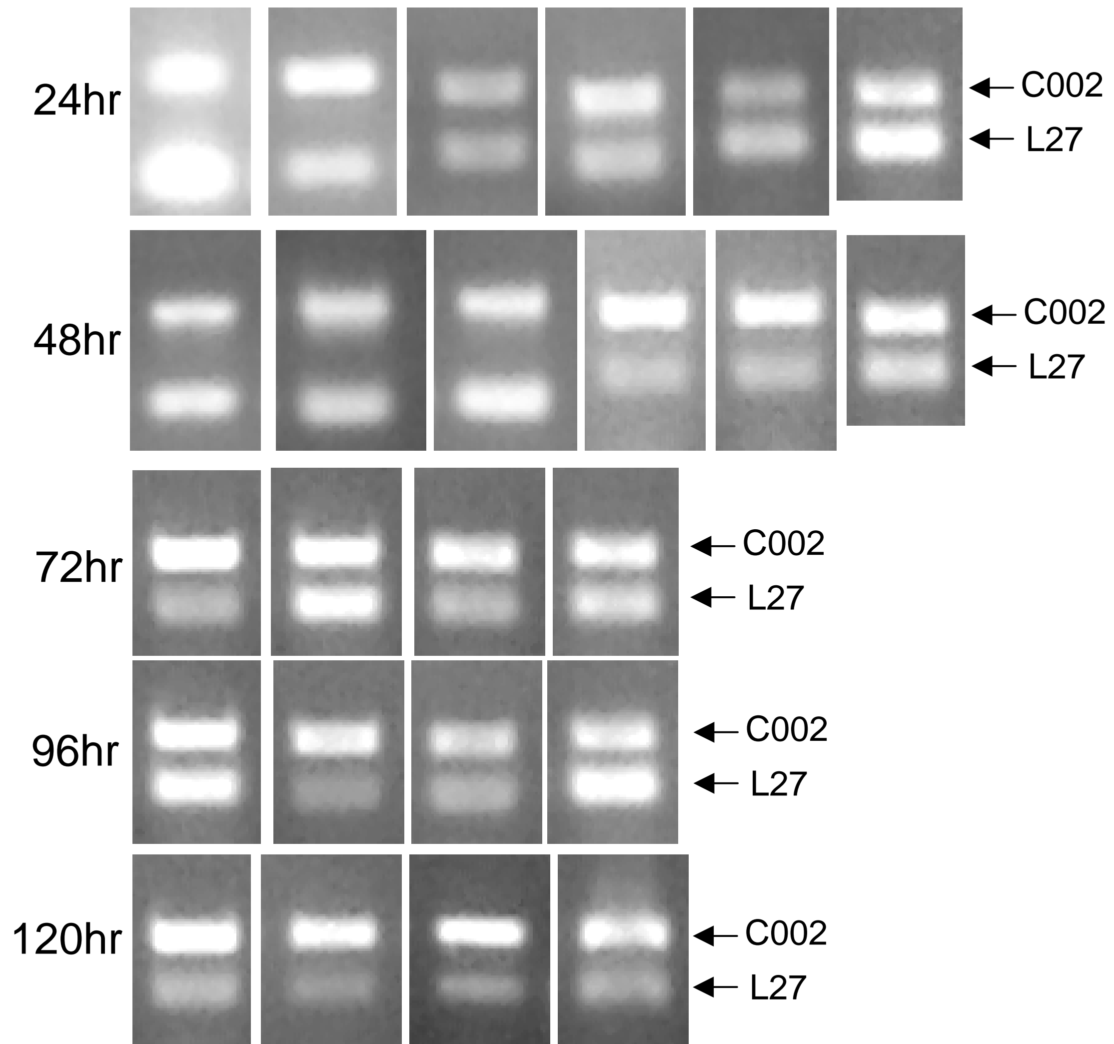
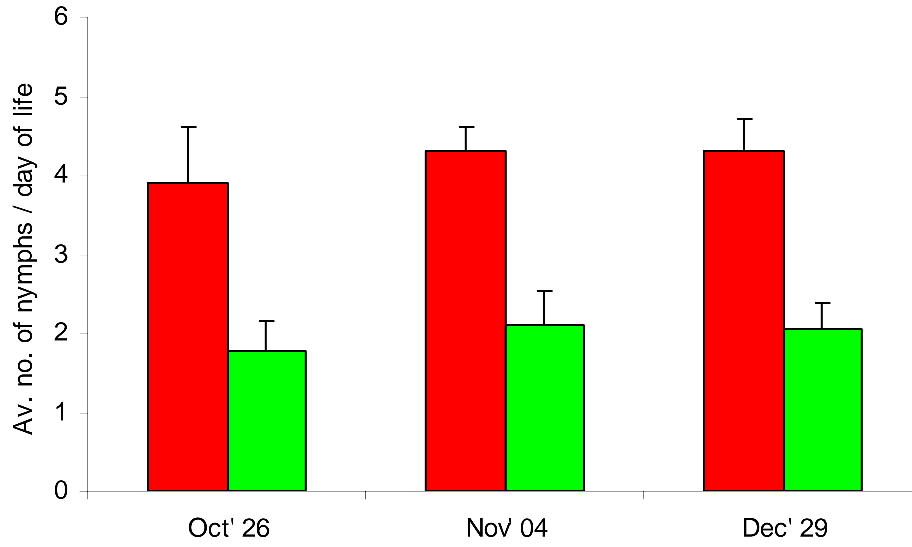


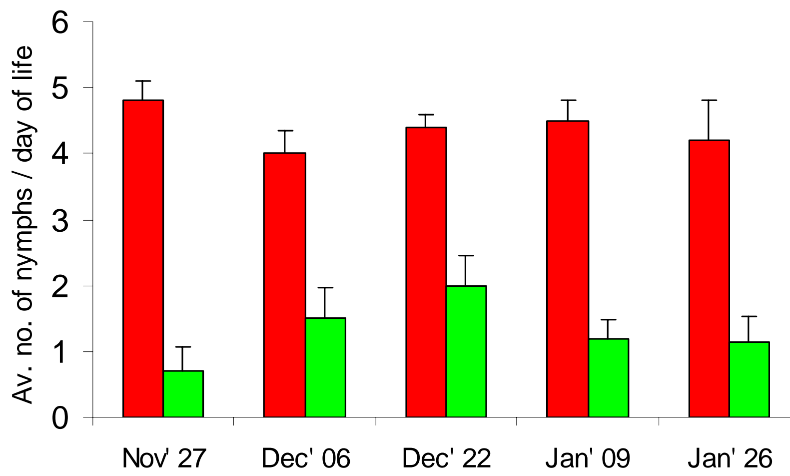
Figure 17. Reproduction of pea aphids after siRNA injections.

a) Reproduction of dsRNA injected aphids (3 experiments), red bar: dsGFP-RNA injections; green bars: dsC002-RNA injections. b) Reproduction of siRNA injected aphids (3 experiments), red bar: siGFP-RNA injections; green bars: siC002-RNA injections. c) Reproduction of siRNA injected aphids (3 experiments for these survival data is shown in Fig. 1), red bar: siGFP-RNA injections; green bars: siC002-RNA injections. d) Reproduction of siRNA injected aphids (3 experiments with aged cohorts and for these survival data is also shown in Fig. 1), red bar: siGFP-RNA injections; green bars: siC002-RNA injections. Each experiment began with 12 insects in the experimental and control groups. The bars depict standard deviations.

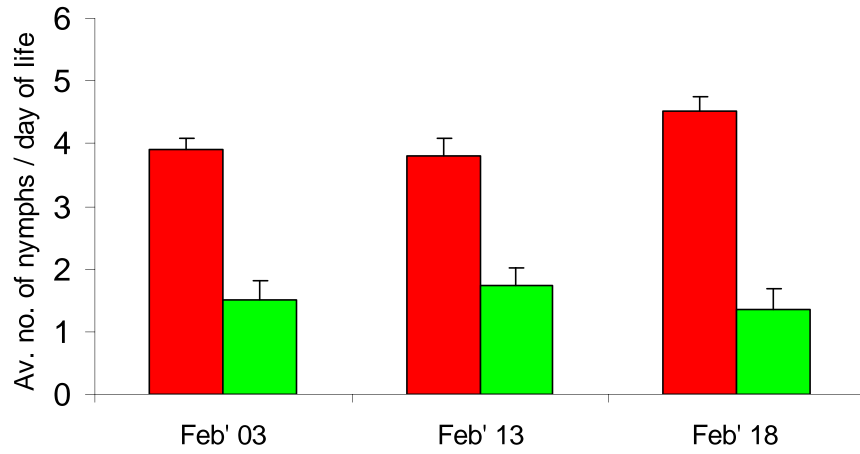
a)



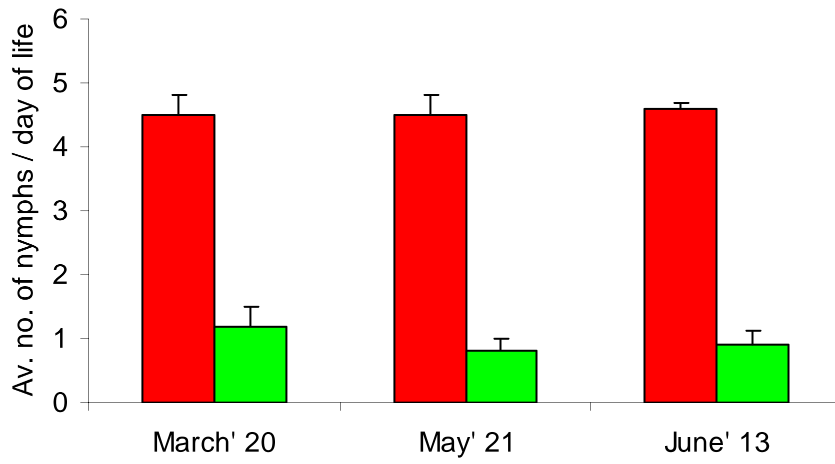
b)



c)



d)



SUMMARY

The pea aphid, *Acyrthosiphon pisum* (Harris), is a member of superfamily Aphidoidae and family Aphididae, within the order Hemiptera. Aphid saliva plays a major role in the interaction of aphids and host plants (Miles, 1999). The availability of aphid saliva in small quantities makes the direct study of salivary components difficult (Miles, 1965; Madhusudhan et al., 1994; Miles and Harrewijn, 1991). The proteins of aphid saliva are of two types, structural and enzymatic. The structural proteins provide a tube-like sheath (Miles 1999; Cherqui and Tjallingi 2000). The secreted salivary enzymes fall into two broad categories: hydrolases (pectinases, cellulases, oligosaccharases) and oxidation/reduction enzymes (phenol oxidase (E.C. 1.14.18.1) and peroxidases) (Miles 1999). The role of these enzymes during aphid attack on plants is not well-understood.

Aphid saliva is believed to perform multiple functions; including creation of the stylet sheath, assisting the penetrations of substrate for food (by the action of pectinases, cellulases, β-glucosidases etc), digesting nutrients (polysaccharases), detoxification of phenolic glycosides ingested during feeding by the action of polyphenol oxidases or peroxidases (oxidation-reduction enzymes) and suppression of host defenses or elicitation of host responses (Miles, 1972; Miles, 1987; Urbanska et al., 1998; Miles, 1999). Aphid saliva may also play a role in the ability of aphids to counter resistance factors in plants, since some species or biotypes of aphids can feed on plants that are resistant to other species or biotypes (Miles, 1999).

We have undertaken a functional genomics approach to identify components of aphid saliva. 5,098 randomly selected cDNA clones were sequenced. We grouped these sequences into 1769 sets of essentially identical sequences, or clusters. Putative functions corresponding to these salivary gland ESTs collection was done by comparing these ESTs with UniProt database using BLASTX with E value threshold set to 1e-5. Among the 1,769 clusters, 1392 (78%) showed no homology with any other protein sequences and only 377 cluster (22%) were assigned putative functions. Among our cDNAs, we have identified putative oxido-reductases and hydrolases that may be involved in the insect's attack on plant tissue. C002 represents an abundant transcript among the genes expressed in the salivary glands and was further characterized and also used to develop RNAi as a technique in aphids. This cDNA encodes a putative secreted protein that fails to match to proteins outside of aphids and is of unknown function. *In situ* hybridization and immunohistochemistry localized C002 in the same sub-set of cells within the principal salivary gland. C002 protein is detected in fava beans that were exposed to aphids, verifying that C002 protein is a secreted protein. Injection of siC002-RNA causes depletion of C002 transcript levels dramatically over a 3 day period after injection. With a lag of 1 - 2 days, the siC002-RNA injected aphids died, on average 8 days before the death of control aphids injected with siGFP-RNA. siC002-RNA-injected aphids exhibit an peculiar behavior, they tend to move around a good deal and do not stay confined to the underside of the leaves, whereas uninjected aphids or aphids injected with siGFP-RNA stay quite still on the underside of the fava bean leaves. It appears, therefore, that siRNA injections of adults will be a useful tool in studying the roles of individual transcripts in aphid salivary glands.