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INTERGENERIC DISTRIBUTION AND IMMUNOLOCALIZATION OF A PUTATIVE ODORANT-BINDING PROTEIN IN TRUE BUGS (HEMIPTERA, HETEROPTERA)

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Summary

Lygus antennal protein (LAP) is an olfactory-related protein of the tarnished plant bug *Lygus lineolaris* (Hemiptera, Heteroptera: Miridae), a hemimetabolous insect. In previous work, a polyclonal antiserum was generated against the N-terminal sequence of LAP; LAP immunoreactivity was strongest in antennae of adult males, but was also present in antennae of adult females and of nymphs. In the current study, LAP immunoreactivity was examined to determine the species specificity and the tissue and cellular localization of LAP expression. Western blot analysis indicated that LAP immunoreactivity was present in the antennae of the male congeners *L. lineolaris* and *L. hesperous*, but was not detectable in male antennae of the more distant relatives *Podisus maculiventris* or *Nezara viridula* (Hemiptera, Heteroptera: Pentatomidae). Western blot analysis further confirmed that LAP expression was restricted to antennal tissue. Histological analyses showed that LAP expression within the antennae was specifically associated with chemosensory sensilla on the antenna. Within the sensilla, LAP immunoreactivity was distributed throughout the extracellular lumen and was concentrated in dense granules within the cytoplasm of sensillar support cells. LAP immunoreactivity was restricted to a subset of antennal chemosensory sensilla, specifically the multiporous olfactory sensilla. These findings suggest that LAP has an important olfactory function in *Lygus* sp., possibly related to that of odorant-binding proteins (OBP) found in other insect orders. If so, LAP would be the first OBP-like protein characterized outside the Endopterygota.

Key words: olfaction, odorant-binding protein, insect, immunogold labeling, microwave fixation, sensilla, incomplete metamorphosis, exopterygote, Miridae, *Lygus lineolaris*.

Introduction

Olfaction plays a role of major importance in the life of insects. Odorous pheromones are used to communicate information related to sexual receptivity, alarm responses and tracks to food sources as well as to maintain developmental hierarchies in social insects (Birch, 1974). Non-pheromonal odorants are used to locate appropriate host organisms for feeding (Bernays and Chapman, 1994; Bernays, 1995). Molecular mechanisms underlying insect olfaction have been studied primarily in adult insects showing holometabolous development (i.e. distinctly different larval and adult forms), such as Lepidoptera (moths) and Diptera (flies) (Vogt, 1995; Carlson, 1996; Pelosi, 1996; Steinbrecht, 1996; Breer, 1997). Development in such animals involves complete metamorphosis in which the adult olfactory system entirely replaces the larval olfactory system. In contrast, in hemimetabolous insects, such as Orthoptera (e.g. grasshoppers and cockroaches) and Heteroptera (true bugs), the adult form is an elaboration of the larval form; the larval olfactory system is maintained but additional olfactory sensilla are added to accommodate adult requirements such as sexual communication.

Lygus lineolaris (Hemiptera, Heteroptera: Miridae) is a polyphagous hemimetabolous insect broadly distributed through North America; it is a serious pest damaging important crops including tomatoes, soybeans and cotton (Snodgrass *et al.* 1984; Young, 1986). It is also a small and easily reared insect that has proved readily amenable to analysis of olfactory function. The nymphal antennae of *L. lineolaris* are typical of hemimetabolous insects in being complex and resembling the adult antenna (Chapman, 1982; Dickens *et al.* 1995; Chinta *et al.* 1997). Fifth (last)-instar nymphal and adult antennae are approximately 4 mm long and are composed of four segments all of which have numerous sensilla; male and female nymphal antennae have approximately 650 sensilla while adult antennae

have approximately 2000 sensilla (Dickens *et al.*. 1995; Chinta *et al.* 1997). While several recognizable types of sensillum are present on both nymphs and adults, there is little sexual dimorphism among them, but there is an increase in the number of sensilla from nymph to adult, especially on segments 2 and 3. The most dramatic increase in sensilla from nymph to adult is in the total number of porous basiconic and trichoid sensilla and the addition of an adult-specific subclass of basiconic sensillum. While there is little information about olfactory organization in earlier nymphal stages, it would be characteristic to observe a gradual increase in the numbers of sensilla from one instar to the next (Chapman, 1982).

Recently, an antennal-specific protein (LAP, lygus antennal protein, approximate molecular mass 17 kDa) was partially purified from the adult male *L. lineolaris* (Dickens *et al.* 1995). The N-terminal amino acid sequence was obtained, and a polyclonal antiserum was generated against a peptide synthesized from this sequence. Western blot analysis suggested that LAP was uniquely expressed in antennal tissue (Dickens and Callahan, 1996). LAP immunoreactivity was most abundant in the antennae of adult males; LAP was also observed at lower levels in the antennae of adult females and fifth-instar nymphs.

LAP was proposed to be a putative odorant-binding protein (OBP) on the basis of its small size, relative abundance and specific localization to antennal tissue (Dickens *et al.* 1995; Dickens and Callahan, 1996). OBPs were originally identified in Lepidoptera as small, water-soluble and antennal-specific proteins (Vogt and Riddiford, 1981; Gyorgyi *et al.* 1988; Raming *et al.* 1989, 1990; Vogt, 1995; Pelosi, 1996; Steinbrecht, 1996). OBPs are extracellular proteins expressed in sensillar support cells and secreted into the aqueous fluid in the sensillar lumen surrounding the olfactory neurons. OBPs are thought to function by solubilizing and transporting lipophilic odorants through this lumen to the receptors in the membranes of olfactory neurons (Vogt *et al.* 1985; Vogt, 1995; Pelosi, 1996; Steinbrecht, 1996). Three classes of lepidopteran OBP have been identified, two associating with plant-odor-sensitive sensilla (general odorant-binding proteins GOBP1 and GOBP2) and one associating with sex-pheromone-sensitive sensilla (pheromone-binding proteins, PBPs) (Vogt *et al.* 1991*b*); several different OBP-like proteins have also been identified in dipteran insects (McKenna *et al.* 1994; Pikielny *et al.* 1994; Ozaki *et al.* 1995). In Lepidoptera, PBPs are typically found in high concentrations in male antennae, corresponding to the role male antennae play in detecting sex pheromone (Vogt *et al.* 1991*b*). The observation that LAP expression was most abundant in adult male *L. lineolaris* contributed to the suggestion that LAP is an OBP, possibly involved in sex pheromone detection (Dickens *et al.* 1995; Dickens and Callahan, 1996).

In the current study, the distribution of LAP immunoreactivity was examined using the polyclonal antiserum previously prepared against synthetic LAP N terminus. The purpose of this study was to establish a baseline for LAP expression in the adult male against which we can evaluate the developmental changes in LAP expression from nymph to adult as well as between male and female adults. LAP represents a useful molecular genetic marker for identifying specific olfactory pathways, targeting sensilla for electrophysiological studies and investigating olfactory development in hemimetabolous insects.

Materials and methods

Animals

Lygus lineolaris (Palisot de Beauvois) fifth-instar nymphs and adults were obtained from a laboratory colony annually infused with feral insects and maintained at the United States Department of Agriculture – Agricultural Research Service, Southern Insect Management Laboratory, Stoneville, MS, USA (Snodgrass and McWilliams, 1992). Fifth-instar nymphs were sexed on the basis of their abdominal sexual characters, and both adults and nymphs were sexed and separated into paper containers. Insects were fed green beans and held at 25 °C and a photoperiod of 14 h:10 h L:D until use, usually within 1–3 days. Adult *L. hesperous* were obtained from a laboratory colony at the USDA–ARS, Cotton Research Laboratory, Shafter, CA, USA. *Podisus maculiventris* and *Nezara viridula* were supplied from colonies maintained at the USDA–ARS, Insect Chemical Ecology Laboratory, Beltsville, MD, USA.

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Body parts, including legs, heads, wings, abdomens, thoraces, probosci and antennae, were excised from the insects, collected in micro-tissue grinders (Wheaton 357848) and stored at −70 °C until use within a few days. Tissues were homogenized on ice following the addition of 30 µl of sample buffer (50 mmol l^{-1} Tris–HCl, pH 6.8; 10 % glycerol). Homogenates were centrifuged (4 °C) for 2 min at 12 000 ***g***. The resulting supernatants (soluble protein fractions) were collected and stored at −70 °C. Samples of soluble protein fractions were mixed with an equal volume of 2× SDS sample buffer (125 mmol l^{-1} Tris–HCl, pH 6.8; 20 % glycerol, 4 % SDS, 10 % mercaptoethanol) and denatured by heating at 95 °C for 5 min prior to PAGE. Relative protein concentrations in the different sample types were estimated from 1 µl samples using a dye-binding method described by Marder *et al.* (1986) and then equalized prior to electrophoresis by dilution with 1× SDS sample buffer. Typically, the protein concentrations of the soluble protein fractions were approximately 0.1 mg ml^{-1}.

SDS–PAGE (Laemmli, 1970) was performed using a mini-gel apparatus (Bio-Rad) with a 4.5 % acrylamide stacking gel and a 15 % acrylamide resolving gel. Electrophoresis was at room temperature with constant 50 V for 1 h followed by 150 V until completion. Duplicate gels were run for each analysis; one gel was silver-stained (after Dickens *et al.* 1995) to visualize total protein, while the other gel was used for immunoblotting (after Dickens and Callahan, 1996).

Immunocytochemistry

For light microscopy, fresh antennae were opened at both ends and fixed in 2 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS-T, 10 mmol l^{-1} Na_2HPO_4, 150 mmol l^{-1} NaCl, pH 7.0, containing 0.1 % Tween-20) for 1 h at 20 °C. Tissue was rinsed twice in PBS-T, then treated with 20 % dimethyl sulfoxide (DMSO) in methanol overnight at 4 °C (or stored at −20 °C). For further processing, tissue was washed in PBS, dehydrated through a graded series of ethanol concentrations and toluene, and incubated in melted paraffin (Periplast) for 2 h before embedding in plastic molds. Sections (8–10 μm) were cut, and ribbons of sections were transferred to water droplets on glass slides coated with albumin (EM Diagnostic) before drying overnight on a slide warmer at 37 °C. Slides were de-waxed with toluene and rehydrated through a graded series of ethanol concentrations to PBS. Sections were reacted in primary antiserum (LAP, 1:500 dilution) in 3 % non-fat dry milk (NFDM) in PBS overnight at 4 °C. Following repeated washing in NFDM-PBS, sections were reacted with secondary antibody [goat anti rabbit IgG (H+L) horseradish peroxidase conjugate (ICN), 1:100 dilution with NFDM-PBS] for 2 h at 20 °C. Following four washes in NFDM-PBS, sections were stained by addition of DAB substrate (Sigma FAST DAB tablets). Finally, sections were washed with PBS and mounted with Aqua Mount Medium (Lerner Laboratories) under coverslips. Sections were photographed using either normal bright-field or differential interference contrast optics.

For electron microscopy, two methods of chemical fixation were used and compared in this study. In one method, antennae were plunge-frozen and freeze-substituted according to the procedure described by Steinbrecht *et al.* (1992). A second method using microwave-enhanced fixation was employed to improve preservation of antennal ultrastructure. In this procedure, the antennae were cut from the insect with a razor blade under a drop of fixative consisting of 4 % paraformaldehyde and 2 % glutaraldehyde in 0.1 mol l^{-1} phosphate buffer, pH 7.4. Next, the antennae were transferred to vials containing 4 ml of the fixative and placed in a microwave (500 W) oven, together with a beaker containing 300 ml of water, which served as a heat sink. The microwave was operated on high for 25 s. Immediately following microwave fixation, the vials containing the antennae were removed from the oven, the samples were rinsed three times in phosphate buffer, dehydrated in an ethanol series and embedded in LR White resin as described above. This microwave procedure gave superior results and all the figures presented here derive from tissue prepared using this procedure. Sections 90 nm thick, which were taken with a Diatome diamond knife on a Riechert/AO Ultracut microtome, were mounted on 400 mesh nickel grids. The grids containing sections were placed into a droplet of blocking buffer containing 0.05 mol l^{-1} Tris-buffered saline (TBS) pH 7.4, 0.5 % bovine serum albumin and 3.3 % normal goat serum for 20 min, incubated overnight at 4 °C in the primary antibody diluted in TBS at 1:100–1:1000 (or overnight in the pre-immune serum control at similar concentrations). The grids were then rinsed three times in TBS for 10 min each and incubated for 1 h with 10 nm colloidal gold goat anti-rabbit immunoglobulin complex (IgG, 10 nm, BioCell Research Laboratories, Cardiff, UK), then rinsed three times in TBS and three times in distilled water for 10 min each. The grids were stained in aqueous 4 % uranyl acetate for 10 min. Sections were observed in a Hitachi H-500H transmission electron microscope operating at 75 kV.

Results

Species- and tissue-specificity of LAP immunoreactivity

The immunoreactivity of LAP antiserum was examined against antennal extracts of four species: *L. lineolaris* and its congener *L. hesperous* (Miridae), and two more distantly related heteropterous species (Pentatomidae), the predaceous bug *Podisus maculiventris* and the plant feeder *Nezara viridula* (Fig. 1). LAP antiserum recognized single protein bands of similar apparent molecular masses in both *L. lineolaris* and *L. hesperous.* However, no immunoreactivity was observed in antennal extracts of *P. maculiventris* or *N. viridula*. The immunoreactive band was observed in male and female antennae of both *Lygus* species, although immunoreactivity was at a comparatively low level in female antennal extracts. While an immunoreactive band was clearly visible in *L. hesperous* females, LAP immunoreactivity was barely detectable in *L. lineolaris* females.

The immunoreactivity of the LAP antiserum was examined against homogenates of a variety of tissues isolated from adult male *L. lineolaris*, including hemolymph, head, wing, leg, abdomen, thorax, proboscis and antenna (Fig. 2). A single strongly immunoreactive band was observed only in antennal extracts. The apparent molecular mass of this protein was consistent with that of previously characterized LAP (Dickens and Callahan, 1996).

Immunocytochemical localization of LAP expression in antennae of adult male L. lineolaris

Immunocytochemical analysis of thick antennal sections (10 μm) using LAP antiserum revealed focal staining associated with chemosensory sensilla (Fig. 3). In longitudinal sections (e.g. Fig. 3A), staining could be seen in epithelia on opposite sides of the antenna; staining appeared in discrete patches, the edges of which were diffusely defined. A grazing section of the antenna (Fig. 3B) visualized these stained patches in an orientation parallel to the antennal surface; the patches appeared irregular at their edges as if limited by a highly folded membrane.

To determine the distribution of immunoreactivity within individual sensilla, thin sections were examined using electron microscopy (Figs 4, 5). Within sensilla, immunogold particles were observed almost exclusively over the extracellular sensillar lymph, with only an occasional grain over the

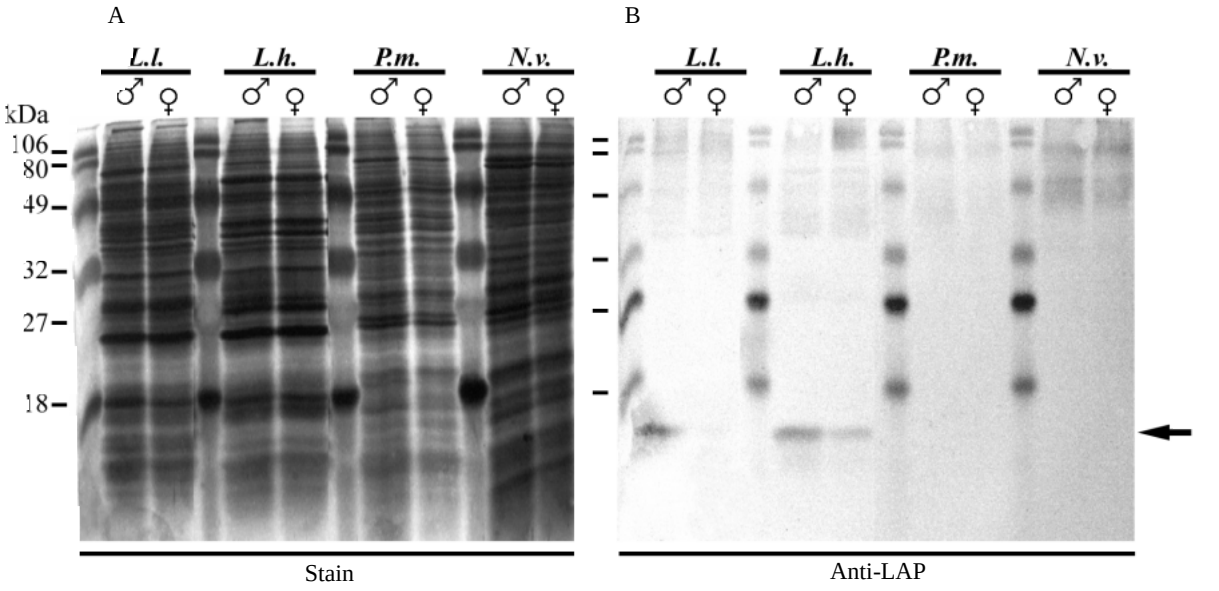

Fig. 1. Intergeneric distribution of LAP among some true bugs. (A) SDS–PAGE of soluble protein fractions of antennae of male and female Heteroptera: congeneric Miridae, *Lygus lineolaris* (*L.l.*) and *L. hesperous* (*L.h.*), and two Pentatomidae, *Podisus maculiventris* (*P.m.*) and *Nezara viridula* (*N.v.*). Proteins were visualized by silver staining. (B) Immunoblot of a duplicate SDS–PAGE gel after reaction with LAP antiserum and visualization with anti-rabbit IgG alkaline phosphatase. The arrow identifies immunoreactive LAP migrating at approximately 15 kDa and detectable only in congeneric *Lygus* species. Reaction with preimmune serum was negative for LAP (Dickens and Callahan, 1996).

dendritic cytoplasm or sensillar cuticle (Figs 4A,B,D, 5B). Immunogold particles were often observed either in close proximity to or in apparent contact with dendritic cell membranes (Fig. 4A,B). Furthermore, the number of particles appeared proportionately greater at higher antiserum concentrations (1/1000, compare Fig. 4A with 1/200, Fig. 4B). Immunogold particles were abundant in the lymph cavities beneath the sensilla (Fig. 5A,B); these cavities are lined by support cells having highly folded apical cell membranes. Immunogold particles were also observed within the cytoplasm of the support cells neighboring the sensillar lymph cavity. These particles were diffuse in the cytoplasm but appeared more highly concentrated within electron-dense structures near nuclei (Fig. 5C). Comparable labeling was not observed in

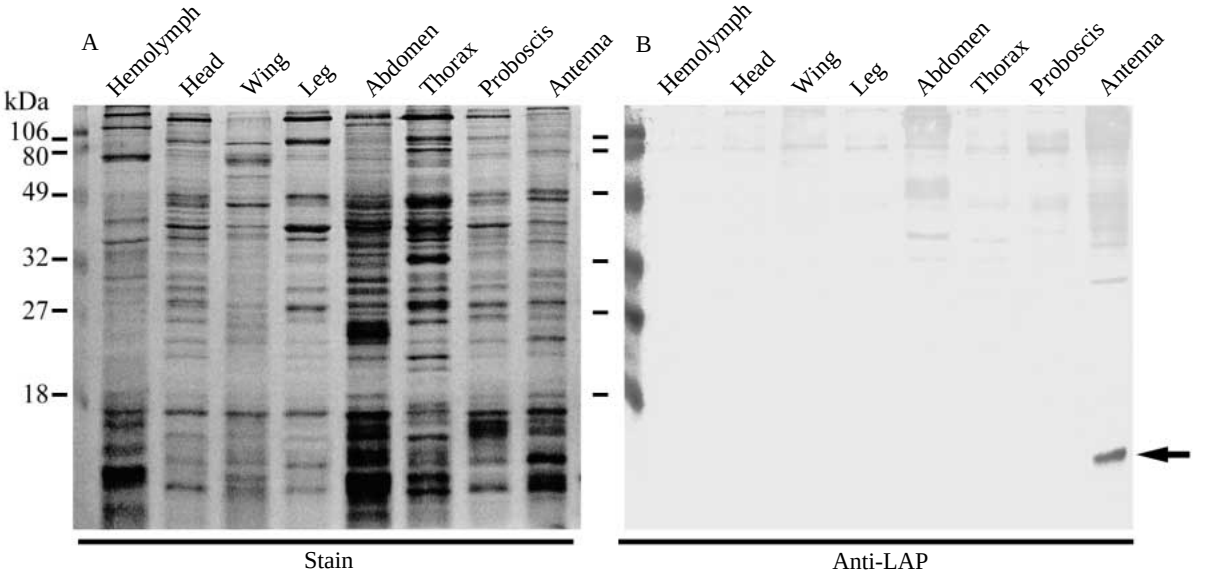

Fig. 2. Tissue distribution of LAP in *Lygus lineolaris*. (A) SDS–PAGE of soluble protein fractions of hemolymph, head, wing, leg, abdomen, thorax, proboscis and antenna of adult male *Lygus lineolaris*. Proteins were visualized by silver staining. (B) Immunoblot of duplicate SDS–PAGE gel after reaction with LAP antiserum and visualization with anti-rabbit IgG alkaline phosphatase. The arrow indicates the presence of a LAP band only in antennae.

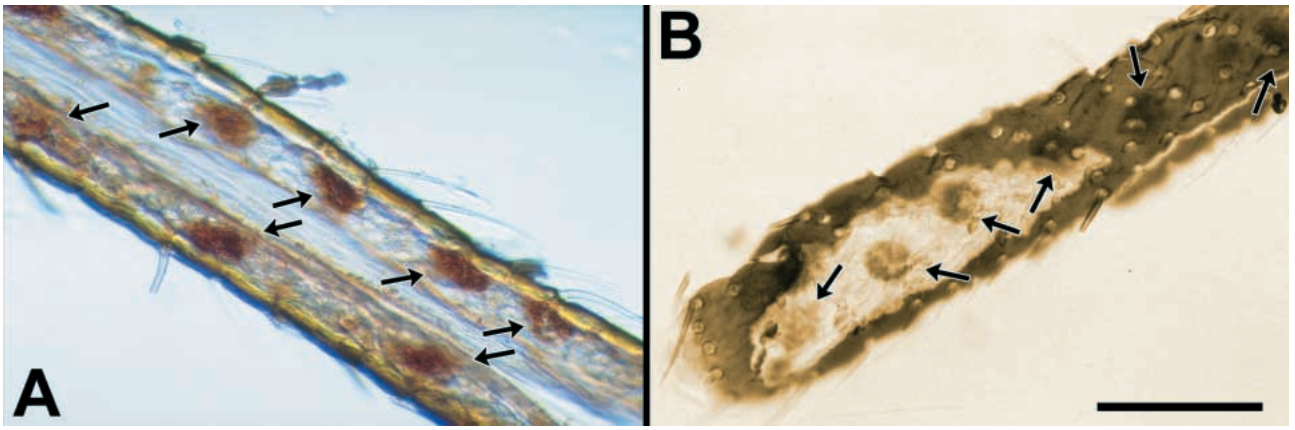

Fig. 3. Immunocytochemical localization of LAP in *Lygus lineolaris* antennae. (A) Immunostaining in an adult female antenna is focal, co-localizing with chemosensory sensilla (arrows). (B) Immunostaining of a grazing longitudinal section reveals focal staining in oblique orientation in the sensory epithelium; arrows point to stained structures visible throughout the antennal cuticle. Scale bar, 61 μm (A), 70 μm (B).

Table 1. *Characteristics and types of sensilla on antennae of adult* Lygus lineolaris *and their relative immunoreactivity to LAP antiserum in the current study*

Sensillar type	Characteristics	Present in nymphs	Role	Sexual dimorphism (number in males/ number in females)	Immunogold particles*
Type 1	ST†, only on distal region of terminal segment, multiporous, 3 neurons, 40 μm length	Yes	Olfaction	1.0 (16M/16F)	NA
Type 2	SC, only on basal 3 segments, uniporous, 1 neuron in sheath, 40 μm length	Yes	Taste	1.0 (34M/35F)	–
Type 3	SC, on all 4 segments, uniporous, 2 chambers in cross section, 2 neurons, 60 μm length	Yes, but greatly reduced numbers	Taste	1.2 (825M/678F)	–
Type 4	ST, on distal 3 segments, multiporous, 3 neurons, 50 μm length	Yes, but greatly reduced numbers	Olfaction	1.2 (874M/746F)	+++
Type 5	SB, only on segment 2, multiporous, 2 neurons, 34 μm length	No	Olfaction	1.4 (123M/89F)	+++
Type 6	SB, only on distal 3 segments, fluted (multiporous), radial channels, 5 neurons, 12 μm length	Yes, but greatly reduced numbers	Olfaction	1.4 (235M/166F)	+

Data for *L. lineolaris* are taken from Chinta *et al*. (1997).

*LAP antiserum concentration 1/1000: –, fewer than 10 immunogold particles within outer wall of sensillum per cross section ; +, 10–80 immunogold particles within the outer wall of the sensillum per cross section; +++, more than 140 immunogold particles within the outer wall of the sensillum per cross section.

†ST, sensilla trichodea; SC, sensilla chaetica; SB, sensilla basiconica (Chinta *et al*., 1997).

NA, not available.

M, male; F, female.

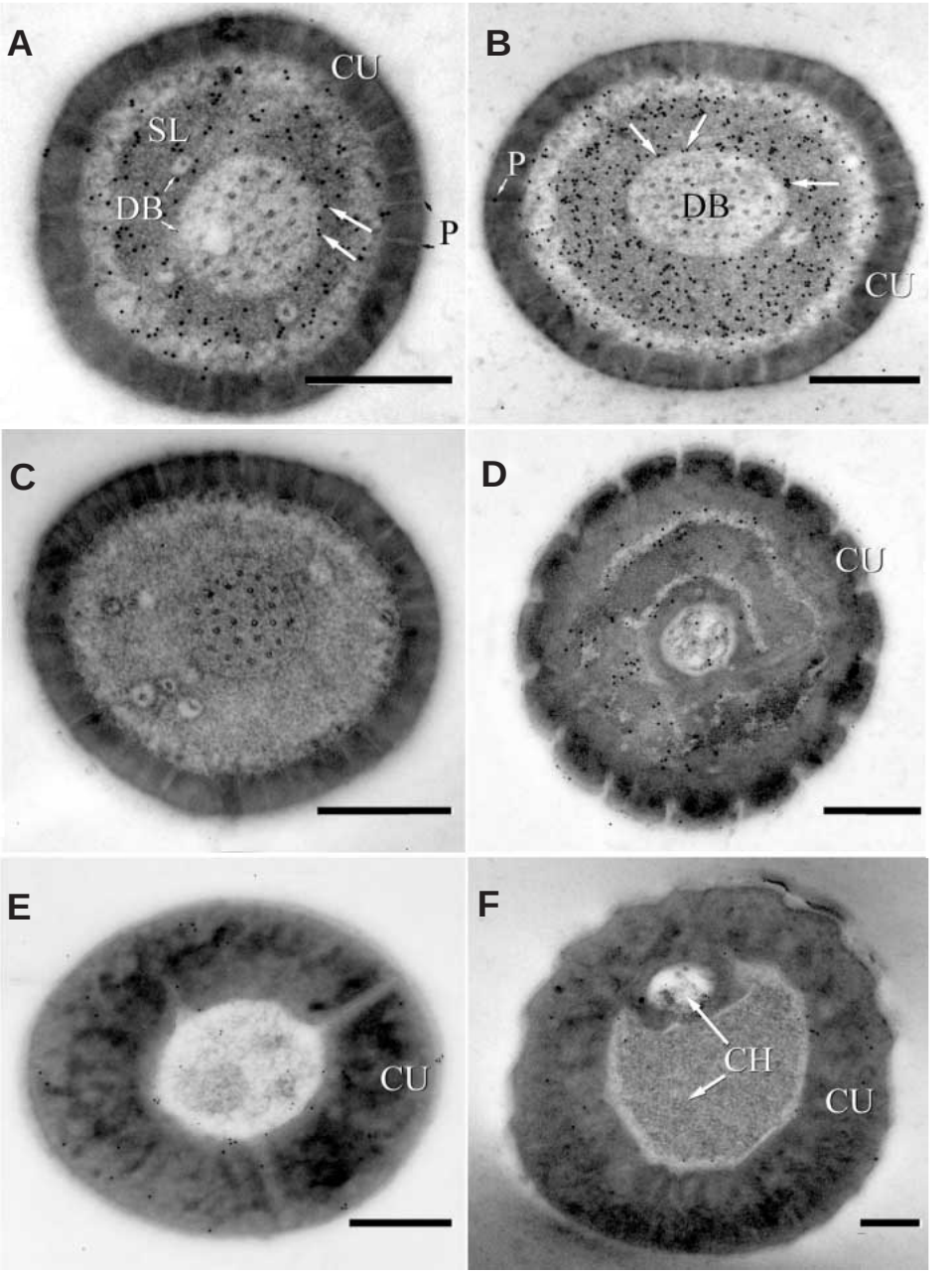

Fig. 4. Immunocytochemical localization of LAP within various sensillar types on adult male antennae of *Lygus lineolaris*. Immunogold labeling of LAP in cross sections of individual sensilla reveals that LAP is associated with the sensillar lymph (SL). The density of immunogold particles in SL of type 4 sensilla increased proportionally with antiserum concentration (1/1000, A *versus* 1/200, B). Arrows indicate particles in close association with membranes of dendritic branches (DB) (A,B). At the higher antiserum concentration (B), label is also seen in the pores (P) of the cuticle (CU). The specificity for LAP detection in type 4 sensilla was confirmed by the absence of gold particles in sections treated with pre-immune serum (1/1000, C). Cross section of a type 6 sensillum also shows significant labeling of LAP at 1/1000 antiserum dilution (D). Only occasional gold labeling is present in type 2 (E) and type 3 (F) sensilla. The type 3 sensillum has two chambers (CH). Scale bars, 0.5 μm.

other antennal tissues. Sections reacted with preimmune serum showed no significant immunogold labeling (Fig. 4C).

LAP antiserum reacted only with a subset of antennal chemosensory sensilla, specifically those involved in olfaction (Figs 4, 5; Table 1). At least six types of chemosensory sensilla have been identified on the antenna of adult male *L. lineolaris* (Table 1; Chinta *et al.* 1997). Four types (1, 4, 5, 6) are multiporous, suggesting a role in the detection of volatile odorants; two types (2, 3) are uniporous, suggesting a role in contact chemoreception or taste. The surface structure and ultrastructure of types 1 and 4 sensilla are nearly identical; the major difference being that type 1 sensilla are somewhat curved and restricted in location to the distal end of the terminal antennal segment (Table 1). Sections of type 1 sensilla were not obtained owing to their low numbers (16 in adults, 11 in nymphs). At the electron microscopical level, types 2, 3, 4 and 6 sensilla were readily identifiable by cross-sectional morphology and antennal location (Table 1; Chinta *et al.* 1997). Immunogold labeling was clearly visible in type 4 (Fig. 4A,B) and type 6 sensilla (Fig. 4D); labeling was consistently at lower densities in the type 6 sensilla. Significant labeling was never observed in the non-olfactory types 2 and 3 sensilla (Fig. 4E,F). Labeling was indicated in type 5 sensilla by inference. Type 5 sensilla are restricted to the second antennal segment, co-mingling with types 2, 3, 4 and 6 sensilla. Types 5 and 4 sensilla are similar in cross-section; however, three neurons associate with type 4 sensilla while only two neurons associate with types 5 and 3 sensilla

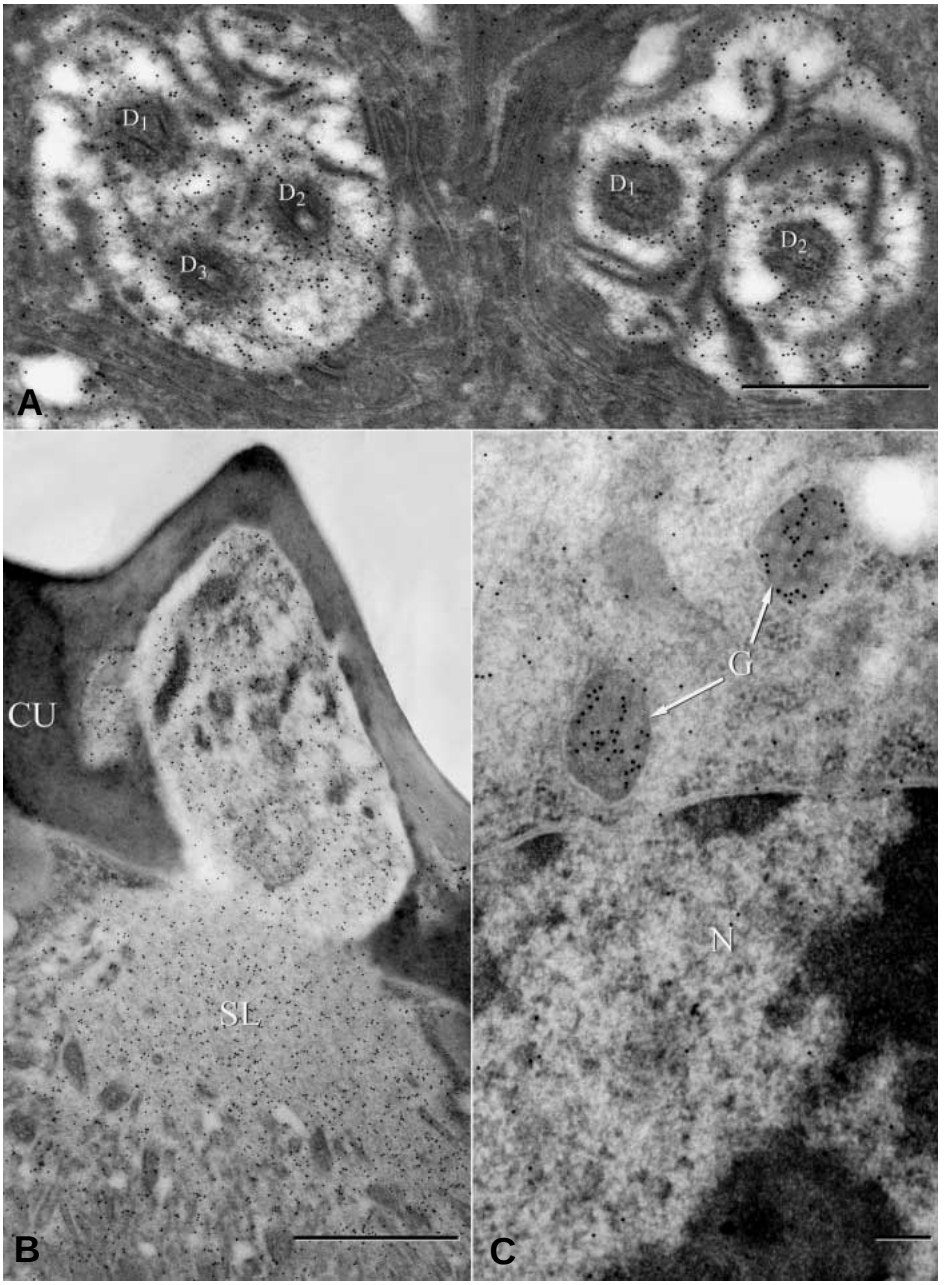

Fig. 5. Relative immunogold labeling of LAP in sensillar lymph, dendrites and supporting cells of sensilla of *Lygus lineolaris*. (A) Cross section of inner dendritic segments of two adjacent sensilla, one with two dendrites, D_1 and D_2 (type 5 sensillum), the other with three dendrites, D_1, D_2 and D_3 (type 4 sensillum), on the second antennal segment of an *L. lineolaris* male. Note that anti-LAP label is present within the sensillar lymph of both sensilla but is sparse in the area of the dendrites. (B) Longitudinal section of a sensillum showing uniform distribution of LAP within the lumen beneath the sensillum through the cuticle (CU) into the base of the sensillum. (C) LAP occurs in the cytoplasm of supporting cells but is concentrated in electron-dense granules (G) near the nucleus (N). SL, sensillar lymph. Scale bars, 0.5 μm.

(type 2 sensilla contain one neuron and type 6 contain five neurons). We observed immunogold labeling of second-segment sensilla containing both two and three neurons. Since the readily identifiable type 3 sensilla showed no labeling (Fig. 4F), staining within the two-neuron sensilla implicated type 5 sensilla as immunoreactive.

Discussion

Using an antiserum generated previously (Dickens and Callahan, 1996), the current study has shown that LAP immunoreactivity appeared to be restricted to the genus *Lygus* and was not detectable in two other members of the heteropteran order. Within *L. lineolaris* adult males, LAP immunoreactivity was restricted to the antenna. In the adult male antenna, LAP immunoreactivity was associated only with chemosensory sensilla. Within sensilla, LAP immunoreactivity was distributed throughout the lumen, identifying LAP as an abundant extracellular protein. LAP immunoreactivity was further observed in dense granules within the sensillar support cells as might be expected of a secreted protein. Finally, LAP immunoreactivity was associated only with the multiporous olfactory sensilla; LAP did not associate with the uniporous contact or taste sensilla. All of these observations suggest that the expression of LAP is linked to the function of the olfactory sensilla, that is, to the detection of behaviorally important environmental signals.

These observations further support the speculation that LAP

may be a hemipteran OBP. In general, OBPs are small (approximately 15 kDa), water-soluble antennal-specific proteins expressed by sensillar support cells and secreted into the sensillar lumen, where they are present at high concentration. Several classes of OBP have been identified in both Lepidoptera and Diptera, and members of each class are only expressed in subsets of olfactory sensilla (Vogt and Riddiford, 1981; Breer *et al.* 1990; Vogt *et al.* 1991*a*,*b*; Krieger *et al.* 1993; McKenna *et al.* 1994; Pikielny *et al.* 1994; Vogt, 1995; Pelosi and Maida, 1995; Nagnan-Le Meillour *et al.* 1996; Breer, 1997). Immunocytochemical studies using OBP antisera in moths and *Drosophila* revealed spatial patterns of OBP antigenicity virtually identical to that of LAP antigenicity in *L. lineolaris*, both within the lumen of the sensilla and within dense granules in the cytoplasm of the support cells (Laue *et al.* 1994; Steinbrecht *et al.* 1992, 1995; Steinbrecht, 1996; Hekmat-Scafe *et al.* 1997). Although antisera to a moth pheromone-binding protein do not cross-react with proteins in *L. lineolaris* antennae (Dickens *et al.* 1995), the properties of LAP are entirely consistent with observations regarding the OBPs in moths (Laue *et al.* 1994; Steinbrecht *et al.* 1992, 1995; Steinbrecht, 1996) and suggest that LAP may have an OBP-like function.

The differential immunoreactivity of the LAP antiserum with the respective heteropteran species is also consistent with observations of OBP immunoreactivity in Lepidoptera, where antisera generated against the OBPs of one species recognize non-identical homologs in other species (Steinbrecht *et al.* 1992; Maida *et al.* 1993; Dickens *et al.* 1995; Steinbrecht, 1996). For those lepidopteran OBPs involved in pheromone detection, variations in protein sequence and cross-immunoreactivity between different species have been proposed to correlate with structural differences among the sex pheromones of the same species (Vogt *et al.* 1991*b*; Steinbrecht, 1996). *Lygus* species represent the heteropteran infraorder Cimicomorpha, while both *Nezara viridula* and *Podisus maculiventris* are of the heteropteran infraorder Pentatomorpha (Scuh, 1986). The phylogenic distances between these species may correlate with similar differences in the compositions and structures of their sex pheromones. If LAP is involved in sex-pheromone detection, the differences in LAP immunoreactivity may similarly correlate with differences in the sex pheromones of these heteropteran species. However, LAP function is presumably not restricted to pheromone processing, since LAP expression associates, albeit disproportionately, with sensilla of both sexes as well as with those of non-reproductive nymphs (Dickens and Callahan, 1996). Furthermore, LAP immunoreactivity associates with multiple classes of olfactory sensilla, some of which are presumably tuned to plant volatiles rather than to sex pheromones.

OBP-like proteins have been identified in a relatively narrow range of insects belonging to the Lepidoptera, Diptera and Coleoptera (Vogt, 1995; Steinbrecht, 1996; Pelosi, 1996; W. Leal, personal communication). All are relatively advanced evolutionarily, being phylogenetically classified in the division Neoptera within the subdivision Endopterygota (=Holometabola). Heteropterans, such as *L. lineolaris*, are classified with the hemipteroid assemblage (=Acercaria, Paraneoptera), a group viewed as entirely separate from the Endopterygota (Hennig, 1981; Boudreaux, 1979; Kristensen, 1991). If LAP represents an OBP-like protein, it would be the first such protein identified outside the Endopterygota; the gene duplication(s) which gave rise to this family of proteins would predate the founding of these phylogenetic groups. Indeed, recent and as yet incomplete analysis of the entire LAP sequence indicates that LAP is related to the OBP-like proteins identified in *Drosophila melanogaster* and in the moth *Heliothis* sp. (R. G. Vogt, F. E. Callahan and J. C. Dickens, unpublished observation; McKenna *et al.* 1994; Pikielny *et al.* 1994; Krieger *et al.* 1993). The evolution of OBPs has been suggested to be an adaptation for the terrestrialization of olfaction, accommodating a switch from water-soluble aquatic odorants to lipophilic airborne volatile odorants (Vogt, 1987, 1995). If this is true, one would predict that the OBPs are widespread, having duplicated and diverged many times in parallel with the establishment of new species. In this context, the OBPs provide a relatively simple genetic trail that follows the evolution of the olfactory systems of insect species as these species diverged to exploit new olfactory resources. Information obtained from the LAP sequence should thus prove valuable in following this trail.

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