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SAAG-4 is a Novel Mosquito Salivary Protein that Programs Host CD4⁺ T Cells to Express IL-4¹

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Abstract

Mosquitoes represent the most important vector for transmitting pathogens that cause human disease. Central to pathogen transmission is the ability to divert the host immune system away from Th1 and towards Th2 responsiveness. Identification of the mosquito factor(s) critical for programming Th2 responsiveness should therefore lead to strategies to neutralize their function and thus prevent disease transmission. In the current study, we used a TCR transgenic adoptive transfer system to screen gene products present in the saliva of the mosquito *Aedes aegypti* for their ability to program CD4 T cells to express the signature Th2 cytokine IL-4. The clone SAAG-4 encodes a secreted protein with a predicted size of 20 kDa whose function has previously been uncharacterized. Notably, SAAG-4 reduced host CD4 T cell expression of the signature Th1 cytokine IFN- γ while simultaneously increasing expression of IL-4. SAAG-4 is therefore the first identified mosquito factor that can program Th2 effector CD4 T cell differentiation.

Keywords

Rodent; Th1/Th2 cells; Cytokines; Mosquitoes; Cell activation

INTRODUCTION

Diseases transmitted by blood feeding arthropods are global public health concerns (1). Female mosquitoes are the most important vectors of infectious agents to humans (2) and there has been a significant increase in the past few decades of newly recognized mosquito-borne arboviruses of medical importance (3). *Aedes aegypti* is a mosquito with anthropophagic feeding preference of vast medical importance in the tropics and subtropics (4). Diseases transmitted by *Ae. aegypti* include dengue and yellow fever, two resurging arbovirus infections (5,6) and chikungunya, an epidemic arbovirosis on the Indian subcontinent and Indian Ocean region (7).

Mosquito modulation of host haemostasis and immune defenses results in increased host arbovirus infection (8). This process can be studied in mice, where mosquito saliva can

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potentiate infection of arboviruses such as Cache-Valley virus (9) and West Nile virus (10). *Ae. aegypti* saliva is known to contain multiple factors that can effect various components of the host immune response (11). Factor Xa inhibitor (12) may inhibit complement activation and leukocyte migration to the bite site (8), apyrase inhibits platelet aggregation (13,14), sialokinin is a vasodilator (15), a nucleosidase potentially inhibits mast cell degranulation (16), D7 proteins scavenge host amines to prevent platelet aggregation, vasoconstriction and pain (17) and other factors inhibit TNF- α release from activated mast cells (18). Importantly, it is thought that *Ae. Aegypti* facilitates arbovirus transmission and infection by inhibiting host expression of type I interferons, down-regulating expression of Th1 cytokines and up-regulating Th2 cytokines (19,20,21,22). One factor, sialokinin, when injected subcutaneously into mice suppressed expression of the Th1 cytokines IL-2 and IFN- γ while up-regulating the Th2 cytokines IL-4 and IL-10 in ConA-stimulated splenocyte cultures at four days post-injection (21).

Analysis of the *Ae. aegypti* salivary gland transcriptome revealed numerous genes that are preferentially expressed in females that encode putatively secreted proteins with no established function (23). In this report, we used an in vivo TCR transgenic adoptive transfer system (24) to identify a novel *Ae. aegypti* salivary gland protein, SAAG-4, which programs CD4⁺ T cells to produce IL-4.

MATERIALS AND METHODS

Mice, adoptive transfer and flow cytometry

6.5 TCR transgenic mice that express a clonotypic TCR specific for an I-E^d- restricted epitope deriving from haemagglutinin (HA) of influenza virus PR8 (¹¹⁰SFERFEIFPKE¹²⁰) expressed on CD4⁺ T cells (25) were maintained on a BALB/c Thy1.1 background. Pooled spleen and lymph nodes processed from 6.5 TCR transgenic mice were depleted of CD8⁺ cells using magnetic beads, and naïve Thy1.1⁺ clonotypic CD4 cells were labeled with CFSE prior to adoptive transfer into Thy1.2⁺ BALB/c recipients that had been exposed to HA antigen, mosquito bites, salivary gland extract (SGE) or DNA vaccination, and subsequent functional analyses were performed as previously described (24,26,27). For CFSE (5–6-carboxyfluorescein diacetate succinimidyl ester)-labeling experiments, clonotypic CD4 T cells were labeled using the previously reported method (24). Briefly, CD8 T cell depleted cell suspensions from 6.5 TCR transgenic mice were resuspended in RPMI medium (Life Technologies) supplemented with 10% FBS (HyClone), 0.1 mM 2-ME, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1x nonessential amino acids and penicillin/streptomycin solutions (Sigma, St. Louis, MO) at a concentration of 1×10^7 cells/ml and incubated with 1 μ M CFSE (Molecular Probes) for 10 minutes at 37°C and then washed three times in Hanks' buffer before adoptive transfer of 1.0×10^6 clonotypic CD4 cells. In brief, adoptive transfer recipients were injected with a bolus of soluble HA peptide (200 μ g) i.d. at the site of mosquito feeding or injection of SGE or DNA. Four days post-transfer, adoptively transferred clonotypic CD4 cells (identified as CD4⁺Thy1.1⁺) were recovered from the draining brachial and axillary lymph nodes, and the ability of the divided (CFSE-diluted) clonotypic CD4 cells to express intracellular cytokines was measured by FACS following in vitro restimulation with 100 μ g/ml HA peptide (to coat APC contained within the lymph node preparations) plus 5 μ g/ml Brefeldin A (Sigma-Aldrich) for 5 h. All quantitative data are expressed as the mean $\pm$ SEM. Protocols used in this study were approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee.

Mosquitoes and mosquito feeding

Pathogen-free *Ae. aegypti* (Liverpool, Black eye stain) were a gift from Dr. Stephen Higgs (Department of Pathology, University of Texas Medical Branch, Galveston, TX) and were maintained as previously described (28). Briefly, adult *Ae. aegypti* were maintained at 27°C, with relative humidity of 70–75% under a 12 hour-light/12 hour-dark photoperiod and fed on a solution of 10% sucrose in water. Mosquito eggs were maintained on semi-wet filter-paper in a humidified chamber. Eggs were placed into a plastic pan with water of approximately 1-inch depth with a small amount of food (1:1:1 powdered laboratory rodent diet, lactalbumin and brewers yeast) added. Under these conditions, larvae developed into the pupae stage in six to seven days. Pupae were removed, sex determined, and transferred into a small cup of water placed in a rearing cage for eclosion. Emerged adults were allowed access to sucrose solution. Females were starved for 12–24 h prior to blood feeding.

Mosquito salivary gland extract preparation and injection into mice

Adult *Ae. aegypti* females were fed on 10% sucrose for 4–5 days prior to collection of salivary glands. Mosquitoes were immobilized by chilling on a snap frozen tray, surface sterilized with 70% ethanol, salivary glands were removed and placed together into a minimal volume of 0.15 M Dulbecco's phosphate buffered saline, pH 7.2 (1xPBS, Gibco) held on ice. Pooled salivary glands were sonicated at 55 kHz for 1 min while held in an ice water bath followed by centrifugation at $14,000 \times g$ for 20 min at 4°C. Supernatant was collected and used as salivary gland extract (SGE). Protein concentration was determined by bicinchoninic acid assay (29). SGE was divided into 10 µl aliquots and stored at –80°C, and subsequently frozen and thawed only once. A 10 µl aliquot is equivalent to 10 pairs of salivary glands and 10 µg protein.

Fur was clipped on the shoulder region of anesthetized (ketamine/xylazine) Thy1.2⁺ BALB/c adoptive transfer recipient mice, which were then positioned on the feeding cage so that a 1 cm diameter area was exposed to the bites of 5 or 10 *Ae. aegypti* on days –1 and +1 respective to adoptive transfer of CFSE-labeled clonotypic CD4 T cells. Control mice were treated in a similar manner but did not receive mosquito bites.

Real-time RT-PCR to measure differential cytokine gene expression at mosquito bite sites

Three to four adult female *Ae. aegypti* were exposed to mouse ear for 10 min. Punch biopsies (2 mm) were then obtained from ear bite sites at 1, 3, 6, 12 and 24 h post-feeding, stored in RNeasy Lysis Buffer (Qiagen) and Trizol reagent (Invitrogen) was used to extract total RNA. Genomic DNA contamination was eliminated by DNase treatment, total RNA was measured using a µQuant spectrophotometer (Bio-Tek Instruments) and RNA quality was analyzed by denaturing gel electrophoresis. First strand cDNA was synthesized from 1 µg total RNA using a Retroscript 1st Strand cDNA synthesis kit (Ambion) and subsequently used as template for real-time RT-PCR analysis. The real-time PCR primers were as follows: GAPDH: TCACTGGCATGGCCTTCC and TCTCCAGGCGGCACGT; IL-2: TGAGCAGGATGGAGAATTACAGG and GTCCAAGTTCATCTTCTAGGCAC; IL-4: TCATCGGCATTTTGAACGAG and CGGAGATGGATGTGCCAAA; IL-10: ACAGCCGGGAAGACAATAACTG and CCGCAGCTCTAGGAGCATG; IL-12 p40: TCAGTGTCTCTGCCAGGAGG and CAGTTCAATGGGCAGGGTCT; IFN-γ: TCAGCTGATCCTTTGGACCC and TCTCAGAGCTAGGCCGCAG; TNF-α: CAAAATTTCGAGTGACAAGCCTG and GAGATCCATGCCGTTGGC. Real-time RT-PCR amplifications were performed using RT²Real-Time™ SYBR Green/Fluorescein PCR master mix (Superarray) in an iCycler (BioRad). Typically, PCR was performed by heating to 95°C for 10 min to heat-activate the HotStart Taq DNA Polymerase followed by 40 cycles of 15 sec at 94°C then 60 sec at 60°C. All reactions were performed in triplicate. GAPDH mRNA was used as a normalizing standard, and RNA from mosquito non-exposed

ear biopsies were considered as naïve and assigned an arbitrary value of 1.0. Changes in mosquito bite-induced cytokine gene expression were calculated as the ratio between mosquito bite and naïve samples divided by the number of feeding mosquitoes.

Construction and inoculation of DNA vaccine plasmids

Selected salivary gland cDNAs (Table I) were PCR amplified and cloned into the DNA vaccine vector pUMVC1 (Aldevron), and endotoxin-free vaccine plasmids were prepared using GenElute HP Select Plasmid Gigaprep Kits (Sigma). To analyze the influence of mosquito salivary gland proteins on cytokine gene expression in skin, 10 µl of vaccine plasmids (1 µg/µl) were injected i.d. into each mouse ear, and at 3 and 6 h post-injection 4 mm punch biopsies from both ears were pooled for RNA extraction. Real-time RT-PCR analysis of cytokine gene expression was performed as described above. To analyze the influence of mosquito salivary gland proteins on the function of host CD4 cells, 10 µg of the indicated plasmids were injected i.d. 24 and 6 h prior to clonotypic CD4 cell adoptive transfer.

Statistical analysis

Statistical analysis were performed with Graph Pad 4.0 Prism software (GraphPad Software, Inc). One way nonparametric ANOVA was performed followed by Tukey post- test with $P \leq 0.05$ considered statistically significant.

RESULTS

Mosquito bites program CD4+ T cells to express IL-4

Our TCR transgenic adoptive transfer system to monitor the functional response of naïve CD4 cells encountering cognate antigen proved a useful approach to assess how mosquitoes influence host CD4 T cell function. As previously observed for tick infestation (27), adoptively transferred CFSE-labeled naïve TCR transgenic clonotypic HA-specific CD4 T cells were induced to undergo significant proliferation in response to a bolus of soluble HA peptide as indicated by the dilution of CFSE fluorescent (Fig. 1A). Only very low levels of the signature Th1 cytokine IFN- γ and negligible amounts of the signature Th2 cytokine IL-4 were produced following in vitro restimulation with HA peptide-pulsed APC (Fig. 1B), consistent with the lack of pathogen-associated molecular patterns (PAMPs) or other adjuvant activities in the soluble HA peptide preparation. Proliferation increased when 10 mosquitoes were allowed to feed at the site of HA peptide injection at days -1 and +1 relative to adoptive transfer and peptide administration ($P < 0.01$), although proliferation was not affected when 5 mosquitoes fed. Mice receiving a second series of mosquito bites (two exposures separated by 14 days) did not develop increased clonotypic CD4 cell proliferation (Fig. 1, A and C). Notably, mosquito bites programmed the clonotypic CD4 cells to express IL-4 ($P < 0.001$), and the percentage of IL-4-expressing clonotypic cells decreased slightly during the second infestation (Fig. 1, B and D).

Mosquito salivary gland extract programs CD4 T cell IL-4 expression

The potential of *Ae. aegypti* salivary gland extract (SGE) to influence clonotypic CD4 cell function was tested by i.d. injecting 5 or 10 µg SGE (corresponds to 5 or 10 pairs of salivary glands, respectively) at the site of soluble HA peptide administration at the time of clonotypic CD4 cells adoptive transfer. Both concentrations of SGE increased clonotypic CD4 cell proliferation relative to controls ($P < 0.01$) (Fig. 2, A and B). Additionally, SGE increased the percentage of clonotypic CD4 cells expressing IL-4 in a dose-dependent manner when injecting 10 µg ($P < 0.001$) or 5 µg ($P < 0.05$) of SGE (Fig. 2, C and D). In fact, the percentage of clonotypic CD4 cells expressing IL-4 in SGE injected mice (Fig. 2D) was

comparable to that observed following mosquito bites (Fig.1D), suggesting that the IL-4 programming activity is contained within mosquito saliva.

Cytokine responses at mosquito bite sites

Mosquitoes generally down-regulate Th1 while up-regulating Th2 cytokines in bulk splenic cultures (8,21,22). To characterize the effect of mosquitoes on cytokine expression in cells at the bite site, cytokine gene expression was measured by real-time RT-PCR in skin biopsies taken throughout the initial 24 h post-feeding (Fig. 3) with GAPDH serving as an internal control. Notably, IL-4 expression was up-regulated 684-fold at 3 h ($P<0.001$) and 91-fold at 6 h. Expression of IL-2, IL-10, IL-12 p40, IFN- γ and TNF- α were not significantly affected throughout the 24 h time course.

IL-4 programming ability of P5F9/SAAG-4

The contribution of individual mosquito saliva components to host cytokine polarization is poorly understood. We therefore randomly selected from a 24 h fed *Ae. aegypti* salivary gland cDNA library (23) five genes that are preferentially expressed in females and predicted to encode secreted proteins (Ag5, 34kDa, P1A3, P1E4, P5F9, refer to Table II) to be cloned individually into a DNA vaccine vector, and then tested their ability to modulate cytokine gene expression in skin following intradermal injection. GAPDH was used as an internal control gene. Additional considerations in selection of these five genes were the absence of known or predicted functions and our hypothesis that they might be important in blood feeding. In screening this initial set of five genes, we observed that clone P5F9 enhanced IL-4 expression 17-fold ($P<0.001$), while the remaining clones failed to increase IL-4 expression above baseline (Fig. 4A). In addition to increasing IL-4 expression, P5F9 increased IL-10 expression ($P<0.01$) and down-regulated expression of IL-12 p40 ($P<0.001$), IFN- γ ($P<0.001$), and TNF- α ($P<0.001$), (Fig. 4B). P5F9 has a predicted molecular weight of 19.6 kDa and a pI of 4.7, and sequence analysis reveals homology to other clones from the same library that all include sequences associated with allergen activity (23). Given P5F9's activity, we renamed it Salivary *Ae. aegypti* IL-4-Inducing Protein (SAAG-4).

To test whether SAAG-4 can program host CD4 cells to express IL-4, the SAAG-4 DNA vector was i.d. injected into clonotypic CD4 cell adoptive transfer recipients. Injection of both the empty DNA vector and vector expressing the 34kDa clone (which did not induce IL-4 in the skin, Fig. 4A) did not alter clonotypic CD4 cell IL-4 expression (Fig. 5, C and D); however, they both increased CFSE-dilution ($P<0.01$ for both clones) (Fig. 5, A and B) and IFN- γ expression ($P<0.001$) (Fig. 5, C and E). These increases likely resulted from the Th1 adjuvant activity of non-methylated DNA sequences in the plasmid vector (30,31). Nevertheless, SAAG-4 reduced CFSE-dilution and IFN- γ expression back to control baseline levels ($P>0.05$) while simultaneously increasing IL-4 expression ($P<0.001$). Thus, SAAG-4 skews host CD4 cells away from Th1 and towards Th2 responsiveness.

DISCUSSION

Blood feeding arthropods modulate host hemostasis and immune defenses to facilitate unimpeded feeding (8,32,33,34). This is an obvious strategy for ticks that can feed for up to two weeks, but less obvious for the mosquito that feeds for only minutes. Nevertheless, the numbers of mosquitoes obtaining a blood meal can potentially be high over prolonged periods of time (35). Mosquito suppression of host immune defenses could thus be beneficial for the population of mosquitoes feeding over time by reducing the likelihood that host immune responses would develop to salivary proteins essential for successful blood

feeding. In fact, mosquitoes can disregulate murine immune responsiveness for up to a week (21).

Mosquitoes tend to skew host immune responses away from a Th1 phenotype (marked by IFN- γ expression) and towards a Th2 phenotype (marked by IL-4 expression) (19,20,21,22). Up to now this phenomenon has only been demonstrated by restimulating bulk spleen cultures prepared from mosquito-infested animals with antigen-non-specific stimuli, while our current study is the first demonstration that mosquitoes can program Th2 differentiation of CD4⁺ helper T cells specific for a bite-associated antigen. This skewing of host immunity towards Th2 responsiveness in turn facilitates the transmission of a number of arboviruses (e.g., Cache-Valley and West Nile viruses) that would otherwise be neutralized by Th1 cytokines (9,19,20,21,22). Thus, this modulation creates a favorable environment for pathogen transmission (36). Identifying the critical components in mosquito saliva responsible for Th2 skewing will therefore provide avenues for their neutralization and thus strategies to block the transmission of these pathogens that cause infectious disease in a significant fraction of the human population. Furthermore, such efforts could potentially reduce mosquito-induced Th2-mediated allergic responses (37).

It has recently been shown that chitin from the nematode *Nippostrongylus brasiliensis* elicits IL-4 expression in eosinophils and basophils (38). In the current study, salivary gland extract from female mosquitoes was able to program host CD4 T cells to express IL-4, indicating that mosquito mouthpart chitin is not required for this Th2-skewing activity. Rather, we found by randomly analyzing a cDNA library generated from 24 hour fed female *Ae. aegypti* mosquitoes that the clone SAAG-4 (which encodes a gene product of previously unknown function) elicited IL-4 expression in skin when introduced intradermally. Nevertheless, this rapid production of IL-4 by innate immune cells in the skin might facilitate the CD4 cell Th2 differentiation that is also observed in the draining lymph nodes. A previous study has shown that the mosquito salivary gland vasodilatory molecule, sialokinin, can induce Th2 skewing as measured in concanavalin-A-stimulated batch splenocyte cultures (21), although SAAG-4 is the first mosquito protein shown to program Th2 differentiation in CD4 cells specific for a bite-associated antigen.

SAAG-4 is a new addition to the limited number of identified molecules capable of driving Th2 responsiveness (39). Previously identified members include sialokinin (21), nematode-derived chitin (38) as well as the synthetic lipopeptide Pam3cys, *Toxoplasma gondii* stimulated lipoxins, soluble egg antigen of *Schistosoma mansoni*, *Porphyromonas gingivalis* LPS and *Candida albicans* (38,40,41,42,43,44). Identification of SAAG-4 as a novel Th2-inducing factor thus not only provides a new model to study Th2 responses as they develop under physiologically and medically relevant conditions, but also raises the exciting prospect of developing strategies to neutralize its activity to prevent mosquito-transmitted infectious diseases.

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Figure 1.

Mosquito bites program CD4 cells responding to associated soluble antigen to develop the capacity to express IL-4. Adoptively transferred Thy1.1⁺ CFSE-labeled clonotypic HA-specific CD4 cells were recovered 4 days following adoptive transfer into Thy1.2⁺ recipients treated i.d. with soluble HA peptide and either spot fed at the site of peptide injection with 2 waves of 5 or 10 *Ae. aegypti* mosquitoes on day -1 and +1 (1° infestation), 2 waves of 5 or 10 mosquitoes given at days -15, -13, -1 and +1 (2° infestation) or non-infested (control or C). A, Representative CFSE-dilution histograms with percentage of CFSE-diluted clonotypic CD4 cells shown. B, Representative IFN- γ vs IL-4 intracellular staining plots corresponding to the CFSE-diluted clonotypic CD4 cells in panel A. Quantitative analysis (mean $\pm$ SEM) of % CFSE-diluted clonotypic CD4 cells (C) and % of divided clonotypic cells expressing IL-4 (D). N=5 per group and asterisks indicate P<0.05 compared to the control.

Figure 2.

The CD4 cell IL-4 programming activity is contained within mosquito salivary gland extract (SGE). Adoptively transferred CFSE-labeled clonotypic CD4 cells were recovered 4 days following adoptive transfer into recipients treated i.d. with soluble HA peptide and pre-treated 1 h earlier with or without 5 or 10 μ g SGE. Representative plots (A) and quantitative analysis (B) of clonotypic CD4 cell proliferation, and representative plots (C) and quantitative analysis (D) of intracellular IFN- γ vs IL-4 expression are presented as in Fig. 1. $N=5$ per group and asterisks indicate $P < 0.05$ compared to the control.

Figure 3.

Mosquitoes induce IL-4 expression at the skin bite site. Three to four *Ae. aegypti* were allowed to feed on mouse ear and total RNA was extracted from biopsies at the indicated times. Real-time RT-PCR was performed to measure expression of the indicated cytokine mRNAs. GAPDH mRNA was used as a normalizing standard. RNA extracted from ears of mice not exposed to mosquitoes were considered as naïve and assigned an arbitrary value of 1.0, and changes in mosquito-induced cytokine gene expression are expressed as the ratio between mosquito and naïve samples divided by the number of feeding mosquitoes. The asterisk denotes a statistically significant difference between the means of naïve and experimental groups ($P < 0.05$). $N = 3$ per group.

Figure 4.

The *Ae. aegypti* gene P5F9/SAAG-4 induces IL-4 expression in skin. Plasmids expressing the salivary gland proteins Ag5, 34kDa, P1A3, P1E4 and P5F9/SAAG-4 (10 μ g) were injected into mouse ears. A, At 3 and 6 h post-injection, RNA samples were prepared and analyzed for IL-4 expression as in Fig. 3 except that samples treated with empty vector were considered as naïve. B, Expression of IL-2, IL-4, IL-10, IL-12 p40, IFN- γ and TNF- α were performed and analyzed in biopsies of skin injected with the P5F9/SAAG-4 vector. GAPDH mRNA was used as a normalizing standard. The asterisk denotes a statistically significant difference between the means of naïve and experimental groups ($P < 0.05$). $N = 3$ per group.

Figure 5.

SAAG-4 programs host CD4 cells to express IL-4. Adoptive transfer recipients were treated with 10 mosquitoes or the indicated plasmids (10 μ g). Representative plots (A) and quantitative analysis (B) of clonotypic CD4 cell proliferation, and representative plots (C) and quantitative analysis (D and E) of intracellular IFN- γ vs IL-4 expression are presented as in previous figures. $N=8$ per group and asterisks indicate $P < 0.05$ compared to the control.

Table I

Ae. aegypti salivary gland clones used in DNA vaccination experiments

Salivary gland gene ID	NCBI accession number	Putative match	Expression (Ribeiro et al. 2007)	Primers for cloning into pUMVC1 vector (5' – 3')
34 kDa	AF466595	34 kDa secreted protein	EF ^a	F: GCGGTCGACCTCGCAATGGAAACATCTCTT R: GCGAGATCTTCAACCCCTATCATTCCATGACAC
Ag5	AF466601	Antigen 5 family member	MF ^b	F: GCGCTGCAGCAGGTGACAATGAAGTCT R: GCGAGATCTACTCGCTCAGCCCGTATT
P1A3	AF466592	8.3 kDa secreted protein	F ^c	F: GCGCTGCAGAAAATGAAACTGATTGTCGC R: GCGAGATCTGCTCTAACGTCCTTCGCTGGTGG
P1E4	AY951940	30 kDa secreted protein	F	F: GCGGTCGACACCATGGGAAAATATTGC R: GCGAGATCTTTAACAGTTGGTCGTCTTG
P5F9	AF466608	30 kDa allergen-like protein	F	F: GCGCTGCAGGGCGTTCGCTACATTATG R: GCGAGATCTTCATGATCTATTGGAAGCAAC

^a Expression enriched in females.^b Expressed in both males and females.^c Expressed specifically in females.