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Characterization of an aphid-specific, cysteine-rich protein enriched in salivary glands

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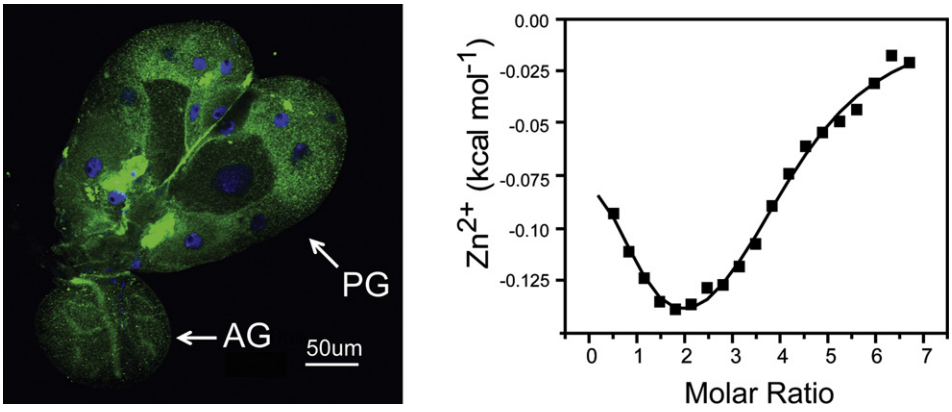
HIGHLIGHTS

- An aphid specific cysteine-rich protein is expressed highly in salivary glands.
- This protein is a monomer globular molecule with a high extent of beta strand.
- This protein is able to bind zinc ions at two binding sites.
- Aphids require more of this protein when feeding on plants.

GRAPHICAL ABSTRACT



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ABSTRACT

Aphids secrete saliva into the phloem during their infestation of plants. Previous studies have identified numerous saliva proteins, but little is known about the characteristics (physical and chemical) and functions of these proteins in aphid–plant interactions. This study characterized an unknown protein (ACYPI39568) that was predicted to be enriched in the salivary glands of pea aphid. This protein belongs to an aphid-specific, cysteine-rich protein family that contains 14 conserved cysteines. ACYPI39568 is a monomeric globular protein with a high beta strand extent. The binding stoichiometric ratios for Zn^{2+} and ACYPI39568 were approximately 3:1 and 1:1 at two binding sites. ACYPI39568 was predominantly expressed in the first instar stage and in the salivary glands. Aphids required more ACYPI39568 when feeding on plants than when feeding on an artificial diet. However, the interference of ACYPI39568 expression did not affect the survival rate of aphids on plants.

1. Introduction

Aphids comprise more than 4700 species worldwide [1]. Polyphagous species, such as *Myzus persicae* (Sulzer) and *Aphis gossypii* Glover, are notorious pests in agriculture, forestry, and horticulture. Unlike other insects with chewing or licking mouthparts, aphids have caused great economic losses by inflicting direct damage and by acting as vectors of phytopathogenic viruses, during which their saliva functions essentially [2,3]. Salivation continues throughout probing and feeding on phloem sap [4]. Many studies have focused on aphid saliva in recent years; however, the function of aphid saliva in the insect's feeding and colonization behavior remains almost unknown.

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Miles reviewed previous enzymatic studies on aphid saliva [2]. Abundant genomic and proteomic data, including the sequenced genome of the pea aphid *Acyrthosiphon pisum*, were used to identify the molecular composition of saliva. The expressed sequence tags (ESTs) from the salivary glands of *Ac. pisum* and *M. persicae* were employed to identify candidate saliva proteins and their abundance [5,6]. Numerous proteins have been identified from the saliva of various aphid species by using mass spectrometry-based proteomic profiling [7,8]. Glucose oxidase, glucose dehydrogenase, NADH dehydrogenase, α-glucosidase, and α-amylase were discovered in the saliva of *M. persicae* [9]. Nine proteins have been identified in the saliva of *Ac. pisum*, of which four were homologs of known proteins: an angiotensin-converting enzyme, an M1 zinc-dependent metalloprotease, a glucose–methanol–choline oxidoreductase, and a regucalcin homolog [10]. Twelve and seven proteins have been identified from the saliva of *Sitobion avenae* and *Metopolophium dirhodum*, respectively [11]. A previous study focused on the Russian wheat aphid *Diuraphis noxia* and found that aphids fed with different substrates secrete watery saliva with qualitative and quantitative differences in soluble proteins [12]. The composition of salivary proteins may be an important determinant of the plant range of different aphid species [13].

The functions of individual saliva proteins in aphid–plant interaction have not been explored adequately. Knockdown of the well-known saliva protein c002 alters the foraging and feeding behavior as well as reduces the survival of pea aphid on fava beans [14,15]. The fecundity of *M. persicae* is enhanced by c002 overexpression in *Nicotiana benthamiana* [6]. Mp10, another candidate saliva protein from *M. persicae*, specifically induces chlorosis and local cell death in *N. benthamiana*; this protein reportedly suppresses pathogen-associated molecular pattern-triggered immunity [6]. However, the physical and chemical characteristics of individual saliva proteins must be elucidated to understand the interaction between saliva proteins and plant counterpart molecules.

Carolan et al. used transcriptomic and proteomic approaches to predict 42 candidate effector proteins enriched in the salivary glands of pea aphid, 33 of which possess unknown functions [5]. Among these unknown proteins, four belong to an undescribed protein family, which is characterized by having 14 conserved cysteine residues [16]. This family contains 13 genes but encodes for 14 proteins because one gene (ACYPI008667) has two isoform transcripts [16]. In this study, this family is designated as aphid cysteine-rich protein family (AphidCRP). The present study aims to explore the physical and chemical characteristics, temporal and spatial expression profiles, and possible involvement in aphid feeding on host plants of the ACYPI39568 gene belonging to the AphidCRP family.

2. Materials and methods

2.1. Insect

The pea aphid *Ac. pisum* was collected in 2010 from peas (*Pisum sativum*) at Yuxi, Yunnan Province, China, and reared on fava beans (*Vicia fabae*) in climate chambers under the following conditions: 21 ± 1 °C, 60% ± 5% relative humidity, and 16 h: 8 h (L:D) photoperiod.

2.2. Protein sequence analysis

A secretory signal peptide was analyzed with the SignalP server (http://www.cbs.dtu.dk/services/SignalP) [17], and the protein molecular weight was calculated at http://web.expasy.org/protparam [18]. Potential O- and N-glycosylation sites were predicted at http://www.cbs.dtu.dk/services/NetOGlyc [19] and http://www.cbs.dtu.dk/services/NetNGlyc [20], respectively. The secondary structure was predicted at http://scratch.proteomics.ics.uci.edu. Protein function was predicted at http://zhanglab.ccmb.med.umich.edu/I-TASSER [21–23]. Confidence score, i.e., EC-score, was used to estimate the quality and functional similarities of predicted models. EC-score > 1.1 indicates functional similarity between the query and template proteins. Both homologous proteins and EST sequences from other species were tracked down with BlastP/tBlastn software at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and then aligned with Bioedit software [24]. A phylogenetic tree was constructed with the neighbor-joining method (complete deletion and Poisson correction model) using MEGA 5 software [25]. Bootstrap analysis (1000 replicates) was applied to evaluate the internal support of the tree topology.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from whole bodies or various tissues of pea aphid using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and then treated with DNase to eliminate DNA contamination. cDNA was reverse-transcribed from 2 μg of total RNA using MMLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.4. Protein expression, purification, and antibody preparation

The full length of ACYPI39568 was cloned from the cDNA library of whole bodies using primers ACYPI39568-F1 and ACYPI39568-R1 (Table 1). ACYPI39568 was inserted into the vector pFastbacI between the EcoRI and XhoI sites using primers ACYPI39568-EcorI and ACYPI39568-XhoI with a hexa histidine-tag at the 3′ terminal (Table 1). The resulting plasmids after sequencing verification were used to generate recombinant baculoviruses with the Bac-to-Bac baculovirus system (Invitrogen, Carlsbad, CA, USA). *Spodoptera frugiperda* Sf9 cells were infested with the recombinant baculovirus at a multiplicity of infection of 3 and then incubated at 28 °C for 72 h. The cell-free medium was loaded on a Ni-NTA agarose column (Qiagen) that was equilibrated with a binding buffer (20 mM Tris–HCl, 50 mM NaCl, 10 mM imidazole, pH 8.0). After washing with a binding buffer containing 20 mM imidazole, the protein was eluted from the column with a binding buffer containing 100 mM imidazole and then dialyzed against a dialysis buffer (20 mM Tris–HCl, pH 8.0) at 4 °C overnight. The dialyzed protein was

Table 1
Primer sequence.

Primer name	Primer (5′ → 3′)	PCR product (bp)
ACYPI39568-F	AATCACAGCAATAATACACATAC	829
ACYPI39568-R	ATAATTTGCATACCACTGTCAC	
ACYPI39568-EcorI	GCGCGAATTCATGTTTATGT	437
ACYPI39568-XhoI	GGCCCTCGAGTTAGTGGTGGTGGTGGTGGTGCTGGCAAGTT	
ACYPI39568-qPCR-F	AGACTCAGGCATTCCCGAAAC	104
ACYPI39568-qPCR-R	AGCCACTGTTGCATTCGTCAC	
L27-qPCR-F	TCGTTACCCTCGGAAAGTC	108
L27-qPCR-R	GTTGGCATAAGGTGGTTGT	
dsACYPI39568-F	AAGGCCGTCTTTGCTTAC	246
dsACYPI39568-R	GAAATTGATTTGTGGGTTC	
dsGFP-F	CACAAGTTCAGCGTGTCCG	420
dsGFP-R	GTTCACCTTGATGCCGTTC	