PROTEOMIC ANALYSIS OF COWPEA APHID APHIS CRACCIVORA KOCH SALIVARY GLAND USING LC-MS/ MS ANALYSIS

S PAVITHRAN1, M MURUGAN1*, M JAYAKANTHAN2, V BALASUBRAMANI1, S HARISS3 and N SENTHIL2

1Department of Agricultural Entomology; 2Department of Plant Molecular Biology and Biotechnology; 3Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore 641003, Tamil Nadu, India
*Email: muruganmarimuthu@tnau.ac.in (corresponding author): ORCID ID 0000-0002-7485-1153

ABSTRACT

The present study identified 151 proteins from the salivary gland of cowpea aphid Aphis craccivora Koch, using LC-MS/MS analysis. These included enzymes mainly involved in the digestion and detoxification of secondary metabolites and proteins related to cell development and molecular function. Enzymes like peroxidase, trehalase, cytochrome P450 monoxygenase, glutathione peroxidase, esterase, peptidase, carboxypeptidase, maltase, and beta-galactosidase were prevalent in the proteome. Additionally, several proteins were assigned to cellular and molecular functions of salivary gland. These proteins may be involved in host-plant interactions. Comprehensively, these results provide a database for elucidating aphid-plant interactions at the molecular level in the future.

Key words: Aphis craccivora, Vigna radiata, salivary gland, protein, LC-MS/MS, digestion, detoxification enzymes, functional annotation, gland dissection, proteomic analysis

Aphids are piercing and sucking type of insects that are adapted to phloem feeding. Over 5000 aphid species have been described, and only 250 species of aphids have economic significance by causing a severe threat to global crop cultivation (Blackman and Eastop, 2000; Dedryver et al., 2010). The feeding mechanism of aphids is very inconspicuous compared to other insects, causing minimal damage. Aphids initiate the probing events on the plant surface with their stylets to reach the phloem via an intercellular pathway (Tjallingii and Esch, 1993; Tjallingii, 2006). Intriguingly, saliva is the first component in the aphid that interacts with the plant at any point of interaction time (Miles, 1999). At the initial probing event, aphids secrete gelling saliva that gets oxidized when released into the plant environment, forming a gelly material that protects the beak and helps lubricate stylets (Morgan et al., 2013). They also possess detoxification enzymes to suppress plant surface defenses. Sheath saliva contains many proteins, phospholipids, and carbohydrates that efficiently modulate plant defenses (Hogenhout and Bos, 2011). Aphids frequently assay the nutritional quality of the host plant by testing the contents of the sieve element, epidermis, and mesophyll cells. At this event, aphids secrete watery saliva, mainly proteins, metabolites, and non-coding RNAs, and may efficiently modulate the host plant's physiology (Naalden et al., 2021). Finally, the aphid stylets penetrate the sieve tube by using navigational cues such as pH and sucrose, which prolong the feeding process if it is a suitable host (Hewer et al., 2010). Throughout the feeding process, from probing to ingestion, aphids continuously salivate within the plant, which helps in successful colonization (Tjallingii, 1995; Will et al., 2007). Plants are sessile organisms that interact with various microorganisms and herbivores, including insect pests (Smith and Clement, 2012). Jones and Dangl (2006) proposed a theoretical zigzag model for describing different levels of plant pathogen immunity; this model can be extended to interactions between plants and insects, where herbivore effectors play a key role in plant resistance and susceptibility to effector-triggered immunity. These effector molecules are a major component of the arms race between herbivores and their host plants.

The C002 gene of the pea aphid is the first salivary effector identified in aphids. When the expression of the C002 gene is suppressed by injection of double-stranded RNA into aphids, these have difficulty reaching the phloem, which leads to greater mortality (Mutti et al., 2006; Mutti et al., 2008). Some of these potential effectors have functions known as pectinases, glucanases, or amylases involved in the degradation of the wall of plant cells. Other potentially secreted proteins have detoxification functions such as metalloproteases, oxidoreductases, peroxidases, or phenol oxidases (Rao et al., 2013; Thorpe et al., 2016; Zhang et al., 2017; Zhang et al., 2023). The cowpea aphid, Aphis craccivora Koch (Hemiptera: Aphididae), is an important legume
pest and poses a severe threat to agriculture causing yield losses of up to 40% and also transmits harmful plant pathogenic viruses, including cowpea aphid-borne mosaic virus and others (Blackman and Eastop, 2000; Boukar et al., 2016). Since, salivary glands are the main component in producing the effectors, studying the salivary proteins and their interactions will give an understanding of aphid-plant interactions. Hence, the current study has undertaken to unravel the proteins present in the salivary gland of A. craccivora through a mass spectrometric approach.

MATERIALS AND METHODS

The stock culture of A. craccivora was maintained in the Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, and aphids were reared on cowpea, Vigna unguiculata (L.), and CO6-susceptible variety under a 14-hr (28± 2°C) day and a 10-hr (20± 2°C) night photoperiod with a relative humidity of 60-70%. The detailed method of salivary gland dissection was followed after Pavithran et al. (2024). Briefly, 500 pairs of adult parthenogenic female salivary glands were isolated under a proteinase-free condition. Immediately after dissection, salivary glands were transferred to 1X phosphate-buffered saline (PBS) on ice. The glands were homogenized in 1X PBS using a micropestle in a tube. Sample preparation was done by following Filter Aided Sample Preparation Method (Huang et al., 2020). The peptide samples were reconstituted in 50 μL of 0.1% formic acid and performed their separation by UPLC over 90 minutes using an Acquity BEH C18 column (75 μm x 150 cm x 1.7 μm). The mobile phase consisted of LC-MS-grade water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient elution started at 53% B and linearly increased to 85% B within 90 minutes. The Xevo-G2-XS system performed mass spectrometric analysis (MS and MS/MS) of the separated peptides in positive ESI mode. Data acquisition parameters after dissection mode include: Collision Energy: 1. Low Energy: Trap = 4 V, Transfer = 4 V; 2. High Energy: Trap Collision Energy Ramp = 15–40 V, Transfer Collision Energy Ramp = OFF, Cone Voltage = 40 V with Continuum Mode: scan time = 0.5 s, mass range = 50–2000 Da in TOF. Leucine encephalin (200 pg/μL) was used for external calibration and performed lock mass acquisition every 30 seconds.

MassLynx v4.0 software was used for data acquisition and subsequent protein identification. Progenesis QI for Proteomics Software V4.0 (Nonlinear Dynamics) identified proteins from the MS/MS spectra. Protein identification was performed with Progenesis QI using the built-in ion accounting algorithm against the Aphididae family of proteins retrieved from Uniprot. The search parameters were: trypsin digestion with two missed cleavages, minimum of two fragment ions/peptide and five/protein, minimum of two peptides/protein, two unique peptides for confident protein identification, fixed modification: Carbamidomethylation of cysteine, variable modifications: oxidation of methionine and N-terminal glutamine converted to glutamic acid (Q-Pyro-E). The identified proteins from the mass spectrometric approach were analyzed for annotation in several databases using OmicsBox (v. 2.0.36). The proteins were initially searched against the Uniprot database to identify similarities with an e value of ≤ 1.0E−3. Subsequently, the proteins were annotated with Gene Ontology using Blast2GO under the GOA Version 2022.08, with an e-value of 1.0E -6. Then, these proteins were searched for domains, sites, families, and repeats in the Interpro database (Blum et al., 2022). Finally, to identify biological function and metabolic pathways, proteins were searched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to retrieve their function (Kanehisa, 2002).

RESULTS AND DISCUSSION

One hundred fifty-one proteins were identified from the raw mass spectra file using the Aphididae database. Furthermore, proteins were searched against various databases for functional annotation. For GO annotation, the proteins were categorized at level 2 distribution (Fig. 1A). In the biological process category, cellular processes, metabolic processes, biological regulation, regulation of biological processes, responses to stimulus, and localization were the most recorded. Under the molecular function category, binding, catalytic activity, and ATP binding activity were the most highly represented categories. Cellular anatomical activity, protein-containing complexes, and virion components were the most expressed cellular components. The majority of salivary proteins belonged to enzymes mainly involved in digestion, detoxification, and regulation of host processes. So, proteins were searched against the Enzyme Code database, which results in seven different databases including oxidoreductase (7 sequences), hydrolase (16 sequences), transferase (17 sequences), ligase (4 sequences), lyase (3 sequences), isomerase (3 sequences), and translocase (2 sequences) enzymes.
Fig. 1. Annotation of salivary proteins with various databases. The x axis represents number of sequences and y axis represents categories. A. The gene ontology classification corresponds to three main categories, BP: Biological process, MF: Molecular function, CC: Cellular component. B. Annotation of proteins based on the enzyme code classes. C. Distribution of proteins in to various biological pathways using the KEGG database.
Furthermore, these proteins were searched for domains, sites, and repeats in the Pfam database. Zinc finger C2H2-type, Glucose-methanol-choline oxidoreductase, N-terminal, Thioredoxin domain, Peptidase S8/ S53 domain, Peptidase M12B, ADAM/repoylpsin, Glucose-methanol-choline oxidoreductase, C-terminal, EGF-like domain, Thioesterase, alcohol dehydrogenase-like, C-terminal, Peptidase S8, pro-domain, Acyl transferase was the most represented domain in the salivary gland proteome. However, in terms of sites, the most proteins fell under Peptidase S8 (Asp, Ser, His-active site), EF-Hand 1, calcium-binding site, low-density lipoprotein (LDL) receptor class A, conserved site, Thioredoxin, conserved site, Glycoside hydrolase family 27/ 36, conserved site, Carboxylesterase type B, Serine proteases, trypsin family, ubiquitin-specific protease, WD40 repeat, conserved site. For KEGG functional annotation, our sequences were grouped into 118 different pathways, including amino sugar and nucleotide sugar metabolism, protein digestion and absorption, glutathione metabolism, salivary secretion, the hippo signaling pathway, drug metabolism (cytochrome P450), the cAMP signaling pathway, and purine and nitrogen metabolism, which are the most represented pathways (Fig. 1C).

Diverse defense mechanisms emerge through the coevolution between aphids and plants; these strategies will continue to develop at an accelerated rate (Walling, 2000; Schatz et al., 2017). The salivary glands secrete proteins that contain an indispensable milieu of effectors modulating aphid-plant interaction (Jones et al., 2022; Wang et al., 2023). A bimodal defense system, consisting of constitutive and induced defenses, is present in plants. When an aphid penetrates a plant, the initial barrier it encounters is the cell wall (Silva-Sanzana et al., 2020; Mafa et al., 2022). Beta-galactosidase, present in the salivary gland proteome of *A. craccivora*, is the principal enzyme accountable for hydrolyzing plant cell wall constituents, particularly hemicellulose polysaccharides. Additionally, surface waxes significantly impede the colonization of aphids by plants (Wójcicka, 2020; Cardona et al., 2023). The proteome of *A. craccivora* includes lipase, which may break down the plant’s defense lipids, making it easier for the aphid stylet to get inside. Insects rapidly eliminate plant defenses by incorporating an additional oral digestion mechanism to acquire more food when plant phloem is enriched with sucrose (Cantón and Bonning, 2020). The presence of alpha mannosidase in the proteome of *A. craccivora* could facilitate the hydrolysis of sucrose into monomers. Aphids ingest a wide variety of plant secondary metabolites while imbibing the sap from the sieve element (Hogenhout and Bos, 2011). This ultimately results in a food intake reduction. Aphids, to avoid plant toxic metabolites, utilize detoxification enzymes, primarily cytochrome P450, esterase, carboxypeptidase, and glutathione peroxidase, in their saliva and these enzymes are known in this study. These detoxification enzymes were commonly reported in insects, including aphids (Bos et al., 2010; Rao et al., 2013; Thorpe et al., 2017; Zhang et al., 2023), hoppers (Hattori et al., 2015; Liu et al., 2016), whiteflies (Su et al., 2012; Huang et al., 2020), thrips (Stafford-Banks et al., 2014), and psyllids (Yu et al., 2021). The trehalase enzyme has been detected in the proteome of cowpea aphids and has also been documented in the salivary glands of numerous insects (Nicholson et al., 2012; Liu et al., 2016; Yu and Killiny, 2018; Zhang et al., 2023).

Trehalose, a non-reducing disaccharide that serves various physiological functions in insects, including growth and reproduction, is hydrolyzed by trehalase into two glucose molecules (Nardelli et al., 2019). Trehalose functions as a signal molecule in *Arabidopsis thaliana* (L.) during herbivore infestation, inducing the expression of the PAD4 gene that generates toxic metabolites (Louis et al., 2012). Trehalase detected in the saliva of green peach aphid, *Myzus persicae* (Sulzer) circumvents this by demonstrating effector activity that degrades plant trehalase, thereby inhibiting PAD4 gene expression (Louis and Shah 2014). Plant phloem, in addition to carbohydrates, contains amino acids that are vital to the survival of aphids. The proteinases identified in this study, which consist of peptidase, peptididase M12B, and furin-like protease, may play a crucial role in the amino acid metabolism-related degradation of plant proteins (Zhang et al., 2017). Plants, being sessile organisms, utilize a highly sensitive surface receptor capable of rapidly detecting herbivore feeding within seconds (Kaloshian and Walling, 2016). This detection results in the generation of reactive oxygen species (ROS). Singlet oxygen, hydrogen peroxide (H₂O₂), and superoxide dismutase compose ROS, which induce hypersensitive responses resulting in cell death (Nalam et al., 2019). Peroxidase and glucose dehydrogenase as oxidoreductive enzymes were also identified in the proteome of *A. craccivora* that may scavenge reactive oxygen species (ROS) in plants, thereby enhancing their virulence. The sieve tube is an integral component of a continuous sap transport conduit; any injury to a single section result in significant sap loss (Van-Bel, 2003).
Phloem is an essential component for the proper functioning of plants. It employs various defense mechanisms to thwart intruders, including the occlusion of sieve tubes by phloem proteins and the influx of calcium ions (Mou et al., 2023). This study's identification of regucalcin suggests that it may reduce occlusion by forming an affinity with calcium ions. The vacuolar sorting-associated protein (VSAP) was identified in this investigation. It was known that VSAP is implicated in vesicular processes such as viral budding and exocytosis (Nicholson and Puterka 2014). By means of its interaction with the transcription factor OsWRKY71, vitellogenin inhibits the production of hydrogen peroxide in rice, functioning as a novel effector in small brown planthopper, *Laodelphax striatellus* (Fallén) (Ji et al., 2021). Vitellogenin was reported in this study which may also participate in plant interactions. In addition to these proteins, this study identified numerous other proteins that were expressed in the salivary proteome of *A. craccivora* and may perform routine molecular and cellular functions. Another class of proteins, yet to be characterized, implicated in plant infestation is present in aphids, which may complicate interactions between aphids and plants. Because these proteins have not been investigated, fundamental research into the molecular mechanisms underlying aphid-plant interactions requires continuous attempts.

**ACKNOWLEDGEMENTS**

Authors thank the Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore for providing facilities and technical support. S. P: Acknowledges the financial assistance provided by Indian Council of Agricultural Research, Agricultural Education Division, New Delhi (F. No. EDN/1/25/2105-Exam Cell) through ICAR Senior Research Fellowship.

**CONFLICT OF INTEREST**

No conflict of interest.

**AUTHOR CONTRIBUTION STATEMENT**

S P: Dissection, sample preparation, analysis and interpretation of data; drafting the manuscript. M M: Advisor for the research work, conceptualization and for drafting the manuscript and reviewing the manuscript. M J: Bioinformatic analysis and reviewing the manuscript. V B: Methodology and reviewing the manuscript. S H: Formal analysis and reviewing the manuscript. N S: Providing facilities and reviewing the manuscript.

**REFERENCES**


(Manuscript Received: January, 2024; Revised: January, 2024; Accepted: January, 2024; Online Published: January, 2024)
Online First in www.entsocindia.org and indianentomology.org Ref. No. e24897