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Potato Aphid Salivary Proteome: Enhanced Salivation Using Resorcinol and Identification of Aphid Phosphoproteins

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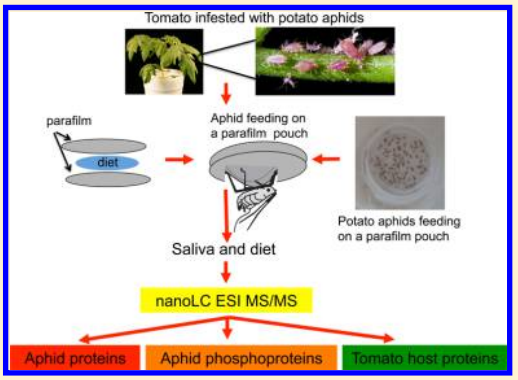
Supporting Information

ABSTRACT: Aphids deliver saliva into plants and acquire plant sap for their nourishment using a specialized mouthpart or stylets. Aphid saliva is of great importance because it contains effectors that are involved in modulating host defense and metabolism. Although profiling aphid salivary glands and identifying secreted proteins have been successfully used, success in direct profiling of aphid saliva have been limited due to scarcity of saliva collected in artificial diets. Here we present the use of a neurostimulant, resorcinol, for inducing aphid salivation. Saliva of potato aphids (*Macrosiphum euphorbiae*), maintained on tomato, was collected in resorcinol diet. Salivary proteins were identified using mass spectrometry and compared with the existing *M. euphorbiae* salivary proteome collected in water. Comparative analysis was also performed with existing salivary proteomes from additional aphid species. Most of the proteins identified in the resorcinol diet were also present in the water diet and represented proteins with a plethora of functions in addition to a large number of unknowns. About half of the salivary proteins were not predicted for secretion or had canonical secretion signal peptides. We also analyzed the phosphorylation states of *M. euphorbiae* salivary proteins and identified three known aphid effectors, Me_WB01635/Mp1, Me10/Mp58, and Me23 that carry phosphorylation marks. In addition to insect proteins, tomato host proteins were also identified in aphid saliva. Our results indicate that aphid saliva is complex and provides a rich resource for functional characterization of effectors.



KEYWORDS: *aphids, effectors, Macrosiphum euphorbiae, phloem proteins, phosphoproteins, resorcinol, saliva*

INTRODUCTION

Aphids (Hemiptera: Aphididae) are sap-sucking insects that cause serious economic losses to crops directly by feeding on plant sap and indirectly by transmitting viruses.[1] Aphids have a worldwide distribution, and while some have a broad host range and are known as generalists, others infest a restricted number of host plants and are referred to as specialists. The potato aphid, *Macrosiphum euphorbiae*, is a generalist that can feed on a large number of plant host species including solanaceous species such as potato and tomato.

Aphids use a specialized mouthpart, or stylet, to feed. After settling on a plant, aphids probe the plant surface using their stylets and penetrate the host tissues to reach the phloem sieve element where they feed. Penetration of host tissues is mainly intercellular, causing minimum tissue damage, although brief intracellular punctures are quite common.[2] In this process, aphids secrete two types of saliva, gelling and liquid.[2] Gelling saliva, also known as sheath saliva, is secreted during stylet penetration of host tissue and solidifies soon after exiting the stylet tip. It forms a continuous sheath enveloping the stylet and remains behind in the host tissues after the stylet is retracted.[3] It is presumed that gelling saliva seals the punctured sites in various cell types and reduces loss of phloem sap due to stylet puncture and therefore plays a role in minimizing aphid-feeding damage. The majority of liquid saliva is secreted into the sieve element lumen; however, liquid saliva is also secreted intercellularly in the stylet path and intracellularly during cell puncture.[2,4]

Aphid salivary components play an important role in modulating plant defense responses. Salivary proteins can suppress or enhance plant defense responses[5−9] and alter aphid performance on plants expressing these proteins.[6,7,10,11] Besides evading or altering host defense responses, aphid feeding also modifies host plant physiology to their advantage.[12,13] Therefore, identification and characterization of aphid salivary components could assist in developing resistance in crops effective against agriculturally important aphid species.

Recently, with the development of genomics and transcriptomic resources and the availability of highly sensitive proteomics technology, the composition of saliva from a number of aphid species has been investigated.[5,14−21] Two main approaches have been used to identify aphid salivary proteins: transcriptome or mass spectrometry (MS) and

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Table 1. Outline for Saliva Source, Peptide Analyses, and Databases Searched in the Present Study

	liquid saliva				gelling saliva[a]	
		water diet			water diet	
organism and database	resorcinol diet	single peptide[a]	2 or more peptides[a]	phosphopeptides	single peptide	2 or more peptides
Macrosiphum euphorbiae (NCBI)	Y	Y	Chaudhary et al.[b]	Y	Y	Chaudhary et al.[b]
Acyrthosiphon pisum (aphidbase)	Y	Y	Y	N	Y	Y
Solanum lycopersicum (Solgenomics)	Y	Y	Y	N	N	N

[a]Peptides detected in water diet reported in Chaudhary et al.[5] were used for these searches. [b]Reported in Chaudhary et al.[5]

protein profiling of aphid salivary gland tissues combined with prediction of secreted proteins or analyzing directly the protein composition of the saliva. Proteins with secretion signals or predicted for secretions were identified from a few aphid species using salivary gland transcriptome and deduced amino acid sequences. Transcriptome analysis of the pea aphid, *Acyrthosiphon pisum*, salivary gland predicted 273 transcript contigs (33%; 273/835 contigs) to encode secreted proteins, while the proteome analysis of the salivary glands identified 156 (17%; 156/925) proteins to have a secretion signal.[17,22] In contrast, proteome analyses of the *A. pisum* saliva identified only 34 secreted proteins.[18,21] Similarly, transcriptome analysis of the green peach aphid, *Myzus persiace*, salivary glands identified 115 transcript contigs (115/5919 redundant sequences) to encode secreted proteins,[6,23] while the proteome analyses of the saliva identified only 42 secreted proteins.[16,21] The low number of detected proteins in the aphid saliva compared with the number of predicted secreted proteins is likely due to scarcity of aphid saliva collected in vitro.

Our *Macrosiphum euphorbiae* salivary gland transcriptome analysis identified 125 proteins predicted to be secreted (27%; 125/460).[10] A total of 105 proteins were identified by MS in the saliva collected from a large number (100 000) of mixed developmental stages of *M. euphorbiae*.[5] Because our analysis involved a much larger number of aphids, the comparatively small number of proteins previously identified in vitro with the other aphid species is likely a consequence of the smaller sample sizes used in these studies that may have resulted in leaving many salivated proteins under the technical detection limit. Alternatively, the low number of proteins identified in these previous studies may reflect that the used aphid species salivate different amounts in vitro or the amount of saliva may vary depending on the composition of the diet used for the collection.[14,15,24]

Only 63 of the 105 *M. euphorbiae* proteins identified in the saliva had secretion signals or were predicted to be secreted, while our transcriptome analysis suggested a larger number of secreted proteins. Thus, there must be a large number of yet undetected proteins in the saliva of this aphid we still have not directly detected.[5]

Salivary proteins from three additional aphid species have been identified using MS. Thirty-four, 32, 12, 7, and 2 proteins have been identified in the saliva of the Russian wheat aphid, *Diuraphis noxia*, the greenbug, *Schizaphis graminum*, two cereal aphids, *Sitobion avenae* and *Metapolophium dirhodum*, and the vetch aphid, *Megoura viciae*, respectively.[19−21,25] The composition of the salivary proteins varied greatly among these aphid species. Variation in the composition of salivary proteins has been detected between different biotypes of the same aphid species and even in the saliva of the same aphid species fed on different diets.[14,19,24−27]

In the present study, we show that the use of a neurostimulant, resorcinol, can enhance aphid salivation in vitro. We present the proteomic and phosphoproteomic analyses of *M. euphorbiae* saliva and identification of insect and host plant proteins in this saliva using high-performance liquid chromatography nanoelectrospray ionization and tandem MS (nanoLC−ESI−MS/MS). Proteomic analysis was performed on saliva collected in water supplemented with resorcinol and compared with salivary proteome identified in saliva collected in water only by Chaudhary et al.[5] The resulting *M. euphorbiae* salivary proteome was compared with predicted secreted proteins from salivary gland proteomes or transcriptomes of other aphid species validating the presence of these proteins in aphid saliva. We also compared the *M. euphorbiae* salivary proteome to salivary proteomes previously reported for other aphid species. An outline of saliva collection and database searches can be found in Table 1.

MATERIALS AND METHODS

Aphid Colony

A colony of the parthenogenetic *M. euphorbiae* was reared on tomato cv. UC82B plants. The colony was maintained in insect cages in a pesticide-free greenhouse at 22−26 °C supplemented with 75 μE light for a 16 h light photoperiod. Plants were fertilized weekly with MiracleGro (18−18−21; Stern's MiracleGro Products).

Saliva Collection

Unless otherwise stated, aphid saliva was collected by feeding mixed developmental stages of the aphid on an artificial diet system as previously described,[26,28] with some modifications. A feeding chamber consisted of a plastic cylinder with one end covered with a double layer of parafilm forming a pouch, containing the diet, while the opposite end was used to introduce the aphids (Supplemental Figure S1A in the Supporting Information (SI)). After aphid introduction, the open end of the cylinder was secured with a piece of cheesecloth and a rubber band. Aphids were given access to feed on the pouch, containing 150 μL of diet prepared in ultrapure autoclaved water, for 16 h under yellow light at 22 °C. All components were either sterilized or treated with alcohol. All material was handled under aseptic conditions in a laminar flow hood. Diet containing saliva was collected under aseptic conditions by gently opening the pouch and collecting the liquid using a pipet. Aphids were not reused for saliva collection.

Three diets were used in this study: 0.4% resorcinol, cocktail of amino acids in 15% sucrose,[26] and a water-only diet; all three diets were at pH 6.8. For the comparative saliva analysis, for each diet type, saliva was collected from 800 aphids by pooling the samples from eight pouches. One hundred age-synchronized fourth instar and 1 day old adult aphids were

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used per pouch. Samples were concentrated using a Vivaspin 500 concentrators with 3 kDa MWCO (GE Healthcare). Samples were mixed with Laemmli buffer and boiled for 5 min before loading on sodium dodecyl sulfate (SDS) 12% polyacrylamide gels. Proteins were visualized by staining the gel with SilverQuest Silver Staining Kit following manufacturer's protocol (Life Technologies, USA). This experiment was repeated twice with similar results. Protein concentration was measured using Bradford assay (Sigma-Aldrich).

For the nanoLC−ESI−MS/MS analysis, saliva was collected from an estimated 28 000 aphids fed on 0.4% resorcinol as previously described. About 150−200 mixed developmental stages of aphids were used per pouch, as previously described above, and all collections were pooled together before processing. For phosphoprotein identification, saliva was collected from aphids fed on only water as earlier described. Saliva was stored at −80 °C until ready for MS.

Saliva Preparation for MS/MS Analysis

Saliva was heated to 94 °C for 5 min to denature the proteins. A final concentration of 25 mM 50 mM Hepes was added to the saliva to adjust the pH to 7.2. Cysteines were reduced and alkylated using 0.5 mM tris(2-carboxyethyl) phosphine (Fisher, AC36383) at 94 °C for 5 min then 1.25 mM iodoacetamide (Fisher, AC12227) at 37 °C in dark for 15 min. Proteins were digested with 20 μg trypsin (Roche, 03 708 969 001) at 37 °C overnight, desalted and concentrated by Sep-Pak tC18 solid phase extraction cartridge (Waters, WAT054925). Eluted peptides were dried in a vacuum concentrator at 4 °C, resuspended in 500 μL of water, and used for MS analysis. A single main MS analysis was performed.

Phosphopeptide enrichment was performed using CeO_2 affinity capture.[29] One mg of trypsin-digested proteins from liquid saliva was used. Twenty μL of 1% (w/v) colloidal CeO_2 (Sigma, 289744) was added to the acidified peptide solution in 3% TFA. After brief vortexing, CeO_2 with captured phosphopeptides was spun down at 1000*g* for 1 min, and the CeO_2 pellet was washed with 1 mL of 1% TFA. Phosphopeptides were eluted by adding 20 μL of eluting buffer (200 mM $(NH_4)_2HPO_4$, 2 M $NH_3{\cdot}H_2O$, 10 mM EDTA, pH 9.5) to the CeO_2 pellet and vortexed briefly. CeO_2 was removed from the phosphopeptide solution by precipitating with 20 μL of 10% formic acid and 100 mM citric acid to a final pH of 3. Sample was centrifuged at 16 100*g* for 1 min, and the supernatant containing phosphopeptides was used for MS analysis.

Nanoliquid Chromatography−MS/MS Analysis

Three salivary protein analytical replicates representing saliva from ∼28 000 aphids were analyzed. Automated 2D nanoflow LC−MS/MS analysis was performed using an LTQ tandem mass spectrometer (Thermo Electron, San Jose, CA) employing automated data-dependent acquisition. An Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) was used to deliver a flow rate of 500 nl min^{-1} to the mass spectrometer through a splitter. Chromatographic separation was accomplished using a three-phase capillary column. Five μm Zorbax SB-C18 (Agilent) packing material was packed into a fused silica capillary tubing (200 μm ID, 360 μm OD, 10 cm long) using an in-house-constructed pressure cell to form the first dimension RP column (RP1). A similar column (200 μm ID, 5 cm long) packed with 5 μm PolySulfethyl (PolyLC) packing material was used as the SCX column. A zero dead volume 1 μm filter (Upchurch, M548) was attached to the exit of each column for column packing and connecting. A fused silica capillary (200 μm i.d., 360 μm o.d., 20 cm long) packed with 5 μm Zorbax SB-C18 (Agilent) packing material was used as the analytical column (RP2). One end of the fused silica tubing was pulled to a sharp tip with the i.d. <1 μm using a laser puller (Sutter P-2000) as the electrospray tip. The peptide mixtures were loaded onto the RP1 column using the same in-house pressure cell. Peptides were first eluted from RP1 column to SCX column using a 0−80% acetonitrile gradient for 150 min. Then peptides were fractionated by the SCX column using a series of 29 step salt gradients (10 mM, 15 mM, 20 mM, 22.5 mM, 25 mM, 27.5 mM, 30 mM, 32.5 mM, 35 mM, 37.5 mM, 40 mM, 42.5 mM, 45 mM, 47.5 mM, 50 mM, 52.5 mM, 55 mM, 57.5 mM, 60 mM, 65 mM, 70 mM, 75 mM, 80 mM, 85 mM, 90 mM, 150 mM, and 1 M ammonium acetate for 20 min), followed by high-resolution reverse-phase separation using an acetonitrile gradient of 0 to 80% for 120 min. For phosphoproteome profiling, peptides were fractionated by the SCX column using a series of 19 step salt gradients (5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 12 mM, 15 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, and 1 M ammonium acetate for 20 min), followed by high-resolution reverse-phase separation using an acetonitrile gradient of 0 to 80% for 120 min.

Spectra were acquired on LTQ linear ion trap tandem mass spectrometers (Thermo Electron, San Jose, CA), employing automated data-dependent acquisition. The mass spectrometer was operated in positive ion mode with a source temperature of 250 °C. The precursor isolation window was 4 Da and the CID collision energy was 35%. As a final fractionation step, gas-phase separation in the ion trap was employed to separate the peptides into three mass classes prior to scanning; the full MS scan range was divided into three smaller scan ranges (300−800, 800−1100, 1100−2000 Da) to improve the dynamic range. Each MS scan was followed by four MS/MS scans of the most intense ions from the parent MS scan. A dynamic exclusion of 1 min was used to improve the duty cycle of MS/MS scans.

Database Searches

Raw data were extracted and searched using Spectrum Mill (Agilent, version B.04.00). MS/MS spectra with a sequence tag length of 1 or less were considered to be poor spectra and discarded. The enzyme parameter was limited to full tryptic peptides with a maximum miscleavage of 1. All other search parameters were set to default settings of Spectrum Mill (carbamidomethylation of cysteines, ±2.5 D for precursor ions, ±0.7 D for fragment ions, and a minimum matched peak intensity (SPI%) of 50%). For nonphosphoproteome data, ox-Met and n-term pyro-Gln were defined as variable modifications for total proteome data. A maximum of 1 modification per peptide was used. For phosphoproteome data, ox-Met, n-term pyro-Gln, and phosphorylation on Ser, Thr, or Tyr were defined as variable modifications. A maximum of two modifications per peptide was used. Phosphorylation sites were localized to a particular amino acid within a peptide using the variable modification localization score in Agilent's Spectrum Mill software.[30] Raw spectra have been deposited at the Mass Spectrometry Interactive Virtual environment (MassIVE) repository, http://massive.ucsd.edu/ProteoSAFe/status.jsp?task=1a2b12b424a9445985e4ac0002f95a93 (proteome ID MSV000078978; data set password: Aphid2014).

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The filtered MS/MS spectra were searched separately against three databases: (1) a predicted *M. euphorbiae* protein database; (2) a predicted *A. pisum* protein database (v2.1) (http://www.aphidbase.com); and (3) a predicted tomato protein database (http://solgenomics.net). The *M. euphorbiae* proteome database was constructed by performing six-frame translation of the *M. euphorbiae* transcripts.[5] For each of the three databases, a 1:1 concatenated forward−reverse database was constructed to calculate the false discovery rate (FDR). The tryptic peptides in the reverse database were compared with the forward database and were shuffled if they matched to any tryptic peptides from the forward database. The details of the three databases are summarized in Supplemental Table S1 in the SI. Cutoff scores (Supplemental Tables S2−S9 in the SI) were dynamically assigned to each data set to maintain low FDR (Supplemental Tables S10−S14 in the SI), which means there were no proteins from the decoy database passing the filtering criteria

Proteins that share common peptides were grouped to address the protein database redundancy issue. The proteins within the same group shared the same set or subset of unique peptides.

All validated spectra from the three database searches were compared against each other. All spectra matched either to the same peptide sequences or peptides with amino acid polymorphisms that the LTQ instrument cannot distinguish (I/L, Q/K).

All single unique peptides with multiple spectra of aphid origin were manually validated (Supplemental Tables S15 and S16 in the SI).

Annotation, Gene Ontology Classification, Signal Peptide Prediction, and Protein Localization

M. euphorbiae transcripts matching the sequenced peptides were annotated by performing reciprocal TBLASTX analyses with pea aphid, a close relative of *M. euphorbiae* with a genome sequence, predicted sequences (aphidbase_2.1_mRNA), and against the NCBI nucleotide (nt/nr) database. The annotated sequences were assigned to different gene ontology (GO) categories using InterProScan or Uniprot.

Amino-acid sequences of putative full-length pea aphid homologues of the *M. euphorbiae* secreted proteins were subjected to de novo signal peptide prediction analysis using SignalP 4.1[31] and TargetP 1.1[32] programs. Hidden Markov model scores higher than 0.45 were considered for SignalP prediction, while TargetP predictions were determined by a predefined set of cutoffs that yielded specificity >0.95 on the TargetP test sets.

Amino-acid sequences of putative full-length pea aphid homologues of the *M. euphorbiae* secreted proteins were subjected to protein subcellular localization prediction using WoLF PSORT.[33]

For the comparative analysis with the secretomes of aphid species, for aphids other than *A. pisum*, the ACYPI numbers were identified using the published Genbank sequences in TBLASTX analysis on NCBI and aphidbase.

RESULTS

Protein Profile of *M. euphorbiae* Liquid Saliva Obtained in Different Diets

Resorcinol is a neurostimulant that has been used successfully to induce esophageal gland secretions in plant parasitic nematodes.[34,35] We tested whether resorcinol can also enhance aphid salivation. But first, in a preliminary experiment we investigated whether *M. euphorbiae* is negatively affected when briefly fed on 0.4% resorcinol diet, the concentration used with plant parasitic nematodes. Our results showed that aphids were able to survive on 0.4% resorcinol for 16 h, and no early mortality was observed compared with water-only diet when moved from the diet onto tomato plants (Supplemental Figure S1B in the SI). To test whether resorcinol can stimulate salivation, we collected liquid saliva in vitro by feeding *M. euphorbiae* on three different diets: 0.4% resorcinol, 15% sucrose plus amino acids[26] or water. About 13.6% more saliva was detected from the diets with 15% sucrose and amino acids (1 μg/800 aphids) compared with water (0.88 μg/800 aphids). Interestingly, 16% more protein was detected in the resorcinol diet (1.16 μg/800 aphids) compared with 15% sucrose and amino acids (Figure 1). The salivary proteins from the three diets, examined on denaturing gels, consistently gave overall similar protein banding patterns (Figure 1). Relatively more intense and higher numbers of protein bands were detectable on denaturing gels with the resorcinol diet compared with the other two diets, suggesting enhanced aphid salivation in the presence of this neurostimulant (Figure 1). Some of the lower molecular weight protein bands that were easily visible with the resorcinol diet were difficult to visualize in the water-only diet. This could be because of lower amount of saliva in the nonresorcinol diets or the possible degradation of proteins in the resorcinol diet. In the former case, these results suggest that

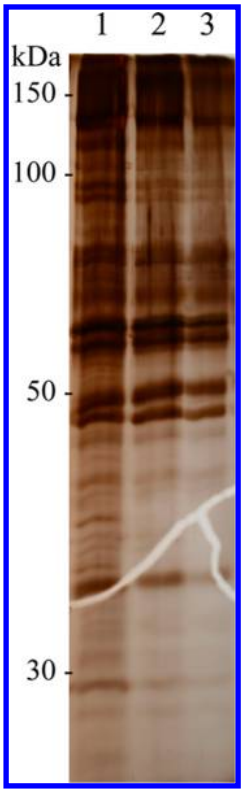

Figure 1. *Macrosiphum euphorbiae* salivary proteins collected in different diets resolved by 1D electrophoresis. Liquid saliva collected 16 h after feeding 800 aphids on 0.4% resorcinol (lane 1; 1.16 μg), cocktail of amino acids in 15% sucrose (lane 2; 1 μg), or water-only (lane 3; 0.88 μg) diets. Saliva samples were separated on 12% acrylamide denaturing gel and silver stained. This experiment was repeated twice with similar results.

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Table 2. *Macrosiphum euphorbiae* Salivary Proteins in Resorcinol Diet

Proteins Identified with Peptides Matching to *M. euphorbiae* Transcript Contigs with Homologues in *Acyrthosiphon pisum*					
M. euphorbiae contig	no. of peptides	*A. pisum* homologue	annotation	SignalP[a]	TargetP[a]
Me_WB00449	27	ACYPI000986	glucose dehydrogenase	Y	Y
Me_WB23639	8				
Me_WB23842	1	ACYPI005582	glucose dehydrogenase	N	Y
Me_WB07812	3				
Me_WB00699	24	ACYPI000288	glucose dehydrogenase [acceptor]-like	Y	Y
Me_WB07325	15	ACYPI000113	RE11240p	Y	Y
Me_WB08982	14	ACYPI30207	peroxidase-like	N	N
Me_WB02834	4	ACYPI26959	peroxidase-like	Y	Y
Me_WB20217	1	ACYPI002439	glutathione peroxidase, (Me23)	N	Y
Me_WB11501	4	ACYPI23752	carbonic anhydrase 7	Y	Y
Me_WB17297	4	ACYPI003917	CG16995	Y	Y
Me_WB17201	2	ACYPI33755	zinc finger protein 853-like (Mp55)	Y	Y
Me_WB11730	2	ACYPI001127	yellow-g	Y	Y
Me_WB16739	21	ACYPI009881	uncharacterized; putative sheath protein	N	Y
Me_WB17099	4				
Me_WB19907	3				
Me_WB19760	3				
Me_WB17822	7	ACYPI003078	phosphatidylinositol-4-phosphate 5-kinase type-1 beta-like	N	Y
Me_WB17524	2				
Me_WB18643	1				
Me_WB02473	1	ACYPI002298	trehalase (Me5)	Y	Y
Me_WB09954	1	ACYPI21663	pancreatic lipase-related protein 1	Y	Y
Me_WB02724	1	ACYPI001551	pancreatic lipase-related protein 2	Y	Y
Me_WB03558	1	ACYPI009980	beta-*N*-acetylglucosaminidase NAG2	Y	Y
Me_WB00118	1	ACYPI008617	effector (C002)	Y	Y
Me_WB05003	1	ACYPI006899	glutathione S-transferase	N	Y
Me_WB12198	1	ACYPI009586	glutathione S-transferase	Y	Y
Me_WB02B10	71	ACYPI000422	apolipophorins	Y	Y
Me_WB20951	27				
Me_WB01635	15	ACYPI006346	effector (Mp1/PInto1)	Y	Y
Me_WB22873	5	ACYPI28420	effector (Mp57)	Y/N[b]	Y/N[b]
Me_WB18513	7				
Me_WB02065	9	ACYPI008224	effector (Me10/Mp58)	Y	Y
Me_WB24410	2	ACYPI000474	heat shock 70 kDa protein cognate 4-like	N	N
Me_WB07124	7	ACYPI006969	beta-actin-like protein 2	N	N
Me_WB27675	1	ACYPI009912	beta-actin-like protein	N	N
Me_WB11597	1	ACYPI008923	elongation factor 2	N	N
Me_WB16709	1	ACYPI000061	ATP synthase subunit beta	N	N
Me_WB17364	1	ACYPI003154	14−3−3 protein theta	N	N
Me_WB18340	1	ACYPI085026	histone H4	N	N
Me_WB01704	1	ACYPI56566	uncharacterized (Me13)	Y	Y
Me_WB18312	14	ACYPI003601	uncharacterized	Y	Y
Me_WB23244	2				
Me_WB05437	33	ACYPI004904	uncharacterized	Y	Y
Me_WB20948	15				
Me_WB23672	4	ACYPI000558	uncharacterized	Y	Y
Me_WB06686	15				
Me_WB18038	2				
Me_WB07137	14	ACYPI000132	uncharacterized	Y	Y
Me_WB02800	8	ACYPI25940	uncharacterized	Y	Y
Me_WB03274	8	ACYPI001887	uncharacterized	Y	Y
Me_WB03846	9	ACYPI45102	uncharacterized	N	Y
Me_WB25797	2				
Me_WB24687	3				
Me_WB17417	1				
Me_WB25041	1				
Me_SG514	2				
Me_WB07850	3	ACYPI066018	uncharacterized	Y	Y
Me_WB21842	7				

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Table 2. continued

Proteins Identified with Peptides Matching to *M. euphorbiae* Transcript Contigs with Homologues in *Acyrthosiphon pisum*					
M. euphorbiae contig	no. of peptides	*A. pisum* homologue	annotation	SignalP[a]	TargetP[a]
Me_WB29764	3	ACYPI36033	uncharacterized	Y	Y
Me_WB17984	6				
Me_WB08177	1	ACYPI003041	uncharacterized	Y	Y
Me_WB06034	4	ACYPI48357	uncharacterized	N	Y
Me_WB26955	5				
Me_WB06556	5	ACYPI069321	uncharacterized	Y	Y
Me_WB01764	5	ACYPI001606	uncharacterized	Y	Y
Me_WB06861	4	ACYPI28320	uncharacterized	Y	Y
Me_WB07163	4	ACYPI21412	uncharacterized (Me20)	Y	Y
Me_WB06653	4	ACYPI53825	uncharacterized (Me17)	Y	Y
Me_WB06871	3	ACYPI37407	uncharacterized	Y	Y
Me_WB03406	3	ACYPI45597	uncharacterized	Y	Y
Me_WB03762	2	ACYPI47142	uncharacterized	Y	Y
Me_WB00462	1	ACYPI065889	uncharacterized	Y	Y
Me_WB12139	2	ACYPI26018	uncharacterized	Y	Y
Me_WB17745	1	ACYPI006269	uncharacterized	Y	Y
Me_WB00766	2	ACYPI000472	uncharacterized	Y	Y
Me_WB00286	1	ACYPI002746	uncharacterized	Y-TM	Y
Me_WB16809	2	ACYPI001152	uncharacterized	Y	Y
Me_WB08862	2	ACYPI28317	uncharacterized	Y	Y
Me_WB00573	1	ACYPI56506	uncharacterized	N	Y
Me_WB09681	1	ACYPI43454	uncharacterized	N	N
Me_WB25811	2				
Me_WB12126	1				
Me_WB10406	3	ACYPI071192	uncharacterized	N	N
Me_WB24535	2	ACYPI53036	uncharacterized	N	N
Me_WB24825	2				
Me_WB11395	2				
Me_WB16795	4	ACYPI25151	uncharacterized	N	N
Me_WB01249	4	ACYPI008945	uncharacterized	N	N
Me_WB18544	2	ACYPI073017	uncharacterized	N	N
Me_WB09034	2	ACYPI083098	uncharacterized	N	N
Me_WB02965	1	ACYPI45290	uncharacterized	N	N
Me_WB05965	2	ACYPI21413	uncharacterized	N	N
Me_WB16604	1	ACYPI30341	uncharacterized	N	N
Me_WB11286	1	ACYPI081323	uncharacterized	N	N
Me_WB04987	2	ACYPI073017	uncharacterized	N	N
Me_WB07390	5	CN582179.1	uncharacterized	N	N
Proteins Identified with Peptides Matching to *M. euphorbiae* Transcript Contigs with No Homologues in *A. pisum*					
M. euphorbiae contig	no. of peptides	*A. pisum* homologue	Annotation	SignalP[a]	TargetP[a]
Me_WB10316	5	NA	NA	N	N
Me_WB13484	3	NA	NA	N	N
Me_WB11404	1	NA	NA	N	N
Proteins Identified with Peptides Matching to *A. pisum* Predicted Proteins with No *M. euphorbiae* Transcript Contigs					
no. of peptides	*A. pisum* IDs	annotation		SignalP[a]	TargetP[a]
1	ACYPI31940	Scm-like with four MBT domains protein		N	N
1	ACYPI005638	ATP synthase subunit alpha		N	N
1	ACYPI001007	tubulin beta-3 chain-like		N	N
2	ACYPI007765	polyubiquitin-A like		N	N
1	ACYPI34081	cell division protein FtsJ		N	N
	ACYPI23689				
1	ACYPI070462	elongation factor 1-alpha 2		N	N
1	ACYPI56064	uncharacterized		N	N
1	ACYPI087669	uncharacterized		N	N

[a]Predicted for secretion by these programs. Y = yes; N = no. [b]ACYPI28420 is not secreted while the *M. persicae* ortholog is.

similar to nematodes[36] the neurostimulant resorcinol may have increased salivation.

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Proteomics Analysis of Saliva Using *M. euphorbiae* Predicted Proteome

To assess the quality of the salivary proteins in the resorcinol diet, we performed MS analysis. Salivary proteins collected from ~28 000 aphids were identified by nanoLC−ESI−MS/MS. High stringency mass spectral quality was used along with very low peptide FDR (Supplemental Table S12 in the SI). Because the genome of *M. euphorbiae* has not been sequenced, the MS spectra were searched against the predicted *M. euphorbiae* proteome derived from its transcriptome, which we previously developed using Illumina sequencing.[5] Seventy three *M. euphorbiae* transcript contigs were found to represent proteins with at least two unique peptides and at least two valid MS/MS spectra each (Table 2; Supplemental Table S17 in the SI). Because the *M. euphorbiae* transcripts are not full-length sequences and could correspond to nonoverlapping regions of the same protein, we used the identified *M. euphorbiae* transcript contigs in BLAST analysis against the *A. pisum* genome and identified their homologues. The 73 transcript contigs corresponded to 52 *A. pisum* genes, while two contigs did not have *A. pisum* homologues (TBLASTX, *e* value < 1×10^{-3}) (Table 2; Supplemental Table S17 in theSI).

Manual validation of proteins with a single unique peptide but multiple valid spectra (Supplemental Table S15 in the SI) identified an additional 29 *M. euphorbiae* contigs corresponding to 22 *A. pisum* genes, while a single contig did not have a *A. pisum* homologue (TBLASTX, *e* value < 1×10^{-3}) (Table 2; Supplemental Table S17 in the SI). Of these 23 proteins, 14 had matching transcripts in the *M. euphorbiae* salivary gland transcriptome.[10]

Previously we reported on *M. euphorbiae* liquid and gelling salivary proteomes, collected in water-only diet, identified with at least two unique peptides.[5] Using these data sets, we manually validated proteins identified with a single unique peptide, with at least 2 valid MS/MS spectra (Supplemental Tables S16 in the SI), and obtained 21 and 41 *M. euphorbiae* contigs in the liquid and gelling saliva, respectively (Supplemental Table S18 in the SI). Six of these were common in both liquid and gelling saliva. Except for three that were unique to *M. euphorbiae*, each *M. euphorbiae* contig corresponded to a single *A. pisum* gene. Of the 21 proteins in the liquid saliva and 41 proteins in the gelling saliva, 3 and 15, respectively, had matching transcripts in the *M. euphorbiae* salivary gland transcriptome.

The majority of the proteins identified with two unique peptides in the liquid saliva in the resorcinol diet (51/54 proteins) were also present in the water-only diet reported by Chaudhary et al.[5] or identified by a single unique peptide in this study. Of the remaining three proteins, one was previously identified in the gelling saliva in water-only diet, while two were not previously detected.[5] Similarly, the majority of the proteins identified with a single unique peptide in the liquid saliva from the resorcinol diet (14/23) were also present in the water-only diet. Of the remaining nine proteins, three were identified in the gelling saliva from the water-only diet, while six were not previously identified.[5] Taken together, salivary proteins identified in the resorcinol diet and water-only diet were of similar composition.

Proteomics Analysis of Saliva Using Pea Aphid Database

To identify additional aphid salivary proteins that could have been missed in the spectra searches against the incomplete *M. euphorbiae* predicted proteome, we also searched the MS spectra from both resorcinol and water-only diets against the *A. pisum* predicted proteome (aphidbase).[37] Proteins with at least two valid MS/MS spectra were reported, and proteins with a single unique peptide but multiple spectra were manually validated (Supplemental Tables S15 and S16 in the SI). Searches against the *A. pisum* database identified an additional eight proteins in the liquid saliva from the resorcinol diet with the majority (7/8) detected by a single unique peptide (Supplemental Table S17 in the SI). Among these, a single peptide matched to two different proteins; therefore, its origin could not be unequivocally confirmed. Taken together, the combined number of aphid proteins in the resorcinol diet identified using *M. euphorbiae* transcriptome and *A. pisum* database searches, was 85 (Figure 2).

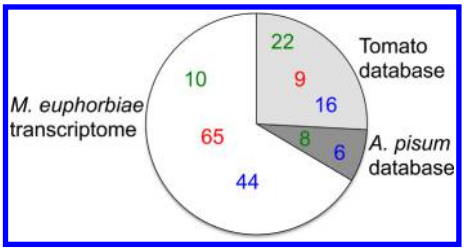

Figure 2. Pie chart representing the number of proteins identified in liquid saliva of *Macrosiphum euphorbiae* in searches against tomato database, *M. euphorbiae* transcriptome and additional proteins identified using *Acyrthosiphum pisum* database. In red are the number of proteins common between water and resorcinol diets, in green are the number of proteins only in resorcinol diet, and in blue are the number of proteins only in water diet. Proteins in the water diet are the combined numbers identified in this work and those reported in Chaudhary et al.[5]

The *A. pisum* predicted proteome was also used to search *M. euphorbiae* salivary peptide spectra, from the water diet, reported by Chaudhary et al.[5] In the liquid saliva, six additional proteins were identified with majority (4/6) also detected by a single unique peptide (Supplemental Table S18 in the SI). In the gelling saliva, 19 additional proteins were detected with also the majority (15/19) identified by a single unique peptide (Supplemental Table S18 in the SI). Four of these proteins were common between the liquid and gelling saliva from the water-only diet.

Figure 2 shows the total number of proteins identified in *M. euphorbiae* liquid saliva collected in resorcinol or water-only diets reported in this work and in Chaudhary et al.[5] It also shows the number of common proteins between the two diet types. Although the majority of the proteins identified in the resorcinol diet using *M. euphorbiae* transcriptome searches were common between the two types of diets, none of the proteins identified searching the *A. pisum* database were common between them (Figure 2).

Protein Annotation and GO Term Assignments

Salivary protein-encoding transcripts were annotated by performing TBLASTX analysis against the aphidbase and NCBI databases. More than half of the salivary proteins from the resorcinol diet (50/85; 59%) were uncharacterized with no known function (Table 2). The remaining represented proteins with a plethora of functions including glucose dehydrogenases, *β*-*N*-acetylglucosaminidase, trehalase, lipases, peroxidases, glutathione S-transferases (GSTs), a heat shock protein, and cytoskeletal proteins.

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Table 3. Comparative Analysis of *Macrosiphum euphorbiae* Secretome with Other Aphid Species

proteins[a]		*M. euphorbiae*[b]	*A. pisum*		*M. persicae*		*D. noxia*		*S. avena*[c]	*M. dirhodum*[c]	*S. graminum*[d]
annotation	ACYPI	saliva	saliva[e]	salivary gland[f]	saliva[g]	salivary gland[h]	saliva[i]	salivary gland[j]	saliva	saliva	saliva
glucose dehydrogenase	000288, 000986, 005582	Y	Y	Y	Y		Y	Y	Y	Y	Y
uncharacterized, putative sheath	009881	Y	Y	Y	Y				Y	Y	
trehalase (Me5)	009351, 002298	Y		Y			Y	Y	Y	Y	
effector C002	008617	Y	Y	Y	Y			Y			
apolipophorin	000422	Y	Y	Y		Y	Y				Y
effector (Mp1/ Pinto1)	006346	Y	Y	Y	Y	Y					
effector (Me10/ Mp58)	008224	Y	Y	Y	Y	Y					
uncharacterized	004904	Y		Y					Y	Y	
uncharacterized	001606	Y		Y		Y				Y	
ribosomal	several	Y	Y		Y						
alanyl aminopeptidase-N	003669	Y	Y				Y				
peroxiredoxin[k]	009090	Y	Y	Y							
uncharacterized	003601	Y		Y	Y	Y					
carbonic anhydrase (Me25)	006300, 23752	Y				Y			Y		Y
peroxidase like	26959, 30207	Y				Y			Y		
uncharacterized	000472	Y		Y	Y						
yellow-like	001127	Y				Y			Y		
uncharacterized	008945	Y			Y						Y
uncharacterized	007406	Y		Y		Y					
uncharacterized	001719	Y		Y		Y					
glutathione peroxidase (Me23)	002439	Y		Y							Y
uncharacterized	001887	Y		Y							Y
zinc finger (Mp55)	33755	Y			Y	Y					
effector (Mp57)	28420	Y			Y	Y					
glutathione S-transferase	009586,006899	Y				Y					
cytochrome oxidase	007006	Y					Y				
beta-actin like	006969, 009912	Y							Y		
uncharacterized	56506	Y			Y						
pancreatic lipase-related	001479, 21663, 001551	Y		Y							
chitinase-like	001365	Y		Y							
kazal-type proteinase inhibitor	37181	Y		Y							
heat shock protein cognate 3	007166	Y		Y							
CG16995	003917	Y		Y							
uncharacterized	001152	Y		Y							
uncharacterized (Me20)	21412	Y		Y							
uncharacterized	006559	Y		Y							
uncharacterized (Me13)	56566	Y		Y							
uncharacterized	000558	Y		Y							
uncharacterized	000490	Y		Y							
uncharacterized	002746	Y		Y							
uncharacterized (Me17)	53825	Y		Y							
uncharacterized	003041	Y		Y							
uncharacterized	47142	Y		Y							
uncharacterized	005249	Y		Y							
uncharacterized	53824	Y		Y							
uncharacterized	37407	Y				Y					
uncharacterized	004796	Y				Y					
uncharacterized	28320	Y				Y					

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Table 3. continued

proteins[a]		*M. euphorbiae*[b]	*A. pisum*		*M. persicae*		*D. noxia*		*S. avena*[c]	*M. dirhodum*[c]	*S. graminum*[d]
annotation	ACYPI	saliva	saliva[e]	salivary gland[f]	saliva[g]	salivary gland[h]	saliva[i]	salivary gland[j]	saliva	saliva	saliva
uncharacterized	009585	Y				Y					
uncharacterized	25151	Y									Y

[a]Includes also glyceraldehyde-3-phosphate dehydrogenase present in *M. euphorbiae* and *Megoura viciae*.[21] [b]This study and Chaudhary et al.[5] [c]Rao et al.[20] [d]Nicholson and Puterak.[25] [e]Carolan et al.[18] and Vandermoten et al.[21] [f]Carolan et al. (based on the presence of a secretion signal).[17] [g]Harmel et al.[16] and Vandermoten et al.[21] [h]Bos et al.[6] and Ramsey et al.[23] (based on the presence of a secretion signal). [i]Nicholson et al.[19] and Cooper et al.[14] [j]Cui et al. (based on the presence of a secretion signal).[38] [k]Present also in *M. viciae*.[21]

Table 4. *Macrosiphum euphorbiae* Salivary Phosphoproteins

M. euphorbiae contig	no. of peptides	*A. pisum* homologue	annotation	liquid saliva in resorcinol	liquid saliva in Chaudhary et al.[a]	predicted to be secreted[b]
Me_WB06686	13	ACYPI000558	uncharacterized	Y	Y	Y
Me_WB23672	1					
Me_WB18038	1					
Me_WB18312	8	ACYPI003601	uncharacterized	Y	Y	Y
Me_WB23244	3					
Me_WB16795	6	ACYPI25151	uncharacterized	Y	Y	N
Me_WB11286	4	ACYPI081323	uncharacterized	Y	Y	N
Me_WB17984	4	ACYPI36033	uncharacterized	Y	Y	Y
Me_WB01635	3	ACYPI006346	effector (Mp1/PInto1)	Y	Y	Y
Me_WB01249	3	ACYPI008945	uncharacterized	Y	Y	N
Me_WB20953	1					
Me_WB03274	3	ACYPI001887	uncharacterized	Y	Y	Y
Me_WB23639	3	ACYPI000986	glucose dehydrogenase	Y	Y	Y
Me_WB22285	1					
Me_WB03846	2	ACYPI45102	uncharacterized	Y	Y	N
Me_WB17417	2					
Me_WB24687	1					
Me_WB20217	2	ACYPI002439	glutathione peroxidase (Me23)	Y	Y	Y
Me_WB02800	2	ACYPI25940	uncharacterized	Y	Y	Y
Me_WB22544	2	ACYPI41445	uncharacterized	N	Y	N
Me_WB24321	1					
Me_WB19760	1	ACYPI009881	uncharacterized, putative sheath protein	Y	Y	Y
Me_WB02065	1	ACYPI008224	effector (Me10/Mp58)	Y	Y	Y
Me_WB12139	1	ACYPI26018	uncharacterized	Y	Y	Y
Me_WB09954	1	ACYPI21663	pancreatic lipase-related 1	Y	Y	N
Me_WB00575	1	ACYPI003340	histone acetyltransferase Gcn5	N	Y	Y
Me_WB17658	1	ACYPI004591	uncharacterized	N	Y	Y
Me_WB09298	1	ACYPI54505	uncharacterized	Y	Y	Y
Me_WB16809	1	ACYPI001152	uncharacterized	Y	Y	Y

[a]Chaudhary et al.[5] [b]Predicted for secretion by either SignalP or TargetP programs. Y= Yes; N = No.

To obtain a functional overview of the proteins, we performed GO term designation using the matching pea aphid ACYPI using InterProScan. GO terms, categorized as molecular function (MF) and biological process (BP), could be assigned for 22 (19/85) and 28% (24/85), respectively (Supplemental Table S17 in the SI).

Annotation of the salivary protein-encoding transcripts in the liquid saliva from the water diet, detected with a single peptide or through *A. pisum* database searches, identified 56% (15/27) of these proteins as uncharacterized. BP could be assigned for 26% (7/27) and MF 30% (8/27) of these transcripts (Supplemental Table S18 in the SI). Proteins in the gelling saliva from the water diet, detected with a single peptide or through *A. pisum* database searches, were 35% (21/60) uncharacterized. BP could be assigned for 57% (34/60) and MF 73% (44/60) of these transcripts. A good number of these annotated proteins were ribosomal (12/60; 20%) or heat-sock (6/60; 10%). The remaining candidates represented proteins like ATP synthases, structural proteins, and cell maintenance proteins (Supplemental Table S18 in the SI).

Secretion Signal Prediction

SignalP and TargetP software programs were used to predict which of the salivary proteins have a secretion signal or are predicted for secretions, respectively. Because not all of the *M. euphorbiae* transcripts are full-length, we used putative protein sequences of their *A. pisum* homologues to predict whether these proteins are secreted. Forty nine percent (42/85) of the proteins found in the *M. euphorbiae* liquid saliva from the resorcinol diet were predicted to have a secretion signal by SignalP 4.1 (Table 2; Supplemental Table S17 in the SI). TargetP identified eight additional secreted proteins that were

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Table 5. Tomato Proteins Identified in *Macrosiphum euphorbiae* Saliva

tomato accession number	no. of peptides	annotation	liquid saliva in resorcinol[a]	liquid saliva in water[a]
Solyc01g007500.2.1	3	photosystem II CP47 chlorophyll apoprotein	Y	N
Solyc01g007330.2.1[b]	3	ribulose bisphosphate carboxylase	Y	Y
Solyc01g007320.2.1[b]	3	ATP synthase subunit beta	Y	N
Solyc11g005670.1.1[b]	3	ubiquitin	Y	N
Solyc06g076090.2.1[b]	4	actin	Y	Y
Solyc06g005910.2.1[b]	2	tubulin beta chain	Y	Y
Solyc04g077020.2.1[b]	1	tubulin alpha-3 chain	N	Y
Solyc06g063330.2.1[b]	2	V-type ATP synthase alpha chain	Y	N
Solyc11g066060.1.1[b]	2	heat shock protein 70	Y	Y
Solyc02g062340.2.1	2	fructose-bisphosphate aldolase	Y	N
Solyc09g009260.2.1[b]	1	fructose-bisphosphate aldolase	Y	Y
Solyc08g062920.2.1[b]	2	Elongation factor EF-2	Y	Y
Solyc01g008950.2.1	2	calmodulin 5/6/7/8-like protein	N	Y
Solyc06g005060.2.1[b]	1	elongation factor 1-alpha	Y	Y
Solyc01g106210.2.1	1	chaperone DnaK	Y	N
Solyc01g028810.2.1	1	chaperonin	Y	N
Solyc05g055310.2.1	1	copper chaperone	Y	N
Solyc02g070790.2.1	1	3-oxoacyl-(acyl-carrier-protein) synthase 2	Y	N
Solyc10g005510.2.1	1	glyceraldehyde-3-phosphate dehydrogenase	Y	N
Solyc03g111010.2.1	1	glyceraldehyde-3-phosphate dehydrogenase	N	Y
Solyc11g007690.1.1[b]	1	pyruvate kinase	Y	N
Solyc10g086010.1.1[b]	1	60S ribosomal protein L4/L1	Y	N
Solyc06g075180.1.1[b]	1	60S ribosomal protein L12//ribosomal protein L11 family	Y	Y
Solyc09g075290.2.1	1	ribosomal protein L18	Y	N
Solyc10g085550.1.1[b]	1	enolase	N	
Solyc10g007690.2.1	1	chlorophyll *a-b* binding protein 8	Y	N
Solyc09g014520.2.1	1	chlorophyll *a-b* binding protein 6A	Y	N
Solyc08g061000.2.1[b]	1	ATP-dependent RNA helicase	Y	N
Solyc05g051260.2.1	1	endo-1,4-beta-xylanase	Y	N
Solyc12g094580.1.1	1	Os03g0133300 protein	Y	N
Solyc06g084090.2.1[b]	1	histone H2A	Y	N
Solyc01g094790.2.1	1	cysteine synthase	Y	N
Solyc08g005710.2.1	1	Ent-copalyl diphosphate synthase	Y	N
Solyc11g011030.1.1	1	Pto-responsive gene 1 protein	Y	N
Solyc12g015880.1.1[b]	1	Heat shock protein 90	N	Y
Solyc01g109660.2.1	1	glycine-rich RNA-binding protein	N	Y
Solyc12g014250.1.1[b]	1	phosphoenolpyruvate carboxylase 1	N	Y
Solyc01g080280.2.1[b]	1	glutamine synthetase	N	Y
Solyc07g066610.2.1[b]	1	phosphoglycerate kinase	N	Y
Solyc01g009180.2.1[b]	1	5-methyltetrahydro pteroyltriglutamate--homocysteine methyltransferase	N	Y
Solyc02g067720.1.1	1	RNA exonuclease 4	N	Y
Solyc10g086190.1.1[b]	1	adenosine kinase	N	Y
Solyc08g021810.1.1	1	transposon Ty1-A Gag-Pol polyprotein	N	Y
Solyc02g027080.1.1	1	*Cc*-nbs-lrr, resistance protein	N	Y
Solyc12g040670.1.1[b]	1	mutator-like transposase	N	Y
Solyc10g006320.2.1	1	vacuolar sorting protein SNF8	N	Y
Solyc10g038080.1.1	1	shikimate dehydrogenase	N	Y

[a]Y= yes; N = no. [b]Matching to multiple members of the gene family.

not predicted by SignalP, bringing the total number of secreted proteins to 50 (50/86; 58%).

About 52% (14/27) of the proteins present in the liquid saliva in the water diet, identified in this study, were predicted to have a secretion signal by SignalP 4.1 (Supplemental Table S18 in the SI). TargetP identified one additional secreted protein that was not predicted by SignalP. This brings the total number of predicted secreted proteins in liquid saliva in water diet to 15 (15/27; 55%). About 18% (11/60) of the proteins present in the gelling saliva in the water diet, identified in this study, were predicted to have secretion signal by SignalP 4.1 (Supplemental Table S18 in the SI). TargetP identified one additional secreted protein that was not predicted by SignalP. This brings the total number of secreted proteins in gelling saliva in water diet to 12 (12/60; 20%).

Comparative Analysis of Aphid Salivary Proteins

A comparative analysis of *M. euphorbiae* salivary proteins identified with nanoLC−ESI−MS/MS in saliva, combined data sets reported in this work and previously,[5] was performed with

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previously published reports of salivary proteins detected by MS analysis from *A. pisum*,[18,21] *M. persicae*,[16,21] *D. noxia*,[14,19] *S. avenae* and *M. dirhodum*,[20] *S. graminum*,[25] and *M. viciae*[21] (Table 3). This comparative analysis also included aphid proteins predicted to be secreted based on salivary gland transcriptome or salivary gland proteome analyses.[6,17,23,38] To simplify this comparison, we grouped together proteins with similar annotations.

Present in seven of the eight aphid species were different types of glucose dehydrogenases. The second most common proteins among these aphids were trehalases, apolipophorin, and an uncharacterized protein referred to as putative sheath protein. Two uncharacterized proteins (ACYPI001606/Mp15 and ACYPI004904), effector C002, and carbonic anhydrases were reported from four aphid species. Thirteen proteins, including glutathione peroxidase, aminopeptidase, peroxiredoxin, effectors (Me10/Mp58 and Mp1/Pinto1), yellow-like, and six uncharacterized were reported from three aphid species. Twenty nine proteins, including effectors (Mp55 and Mp57) and 18 uncharacterized, were reported from only two aphid species. *M. euphorbiae* salivary proteome provided evidence of secretion of 24 proteins that were previously only predicted to be secreted.[6,10,17,23]

Identification of Phosphoproteins

Phosphorylation is a posttranscriptional modification that may affect protein function. To identify phosphorylated proteins in the aphid saliva, saliva was collected from *M. euphorbiae* in water-only diet and phosphopeptides were enriched using cerium oxide prior to nanoLC−ESI−MS/MS analysis. Peptides matching the *M. euphorbiae* database identified 21 phosphoproteins, all of which were previously identified in liquid saliva from water diet.[5] Of these, 14 have secretion signal and one protein was predicted to be secreted (Table 4; Supplemental Table S19 in the SI). Among the phosphoproteins with secretion signals or predicted to be secreted were an uncharacterized protein referred to as putative sheath protein and known aphid effectors, Mp1/PInto1, Me23, and Me10/Mp58, that alter aphid fecundity when expressed in planta.[7,10,11]

Tomato Proteins in Aphid Saliva

Plant phloem is the conduit that transports photoassimilates from source to sink and consists of sieve element and companion cells. Aphids mostly feed on phloem sap using their stylets, which are composed of two canals, the salivary canal and the food canal. The outlet of the salivary canal is at some distance from the stylet tip, providing a chamber where both saliva and ingested plant sap meet. To identify plant host proteins in aphid saliva, we searched spectra from *M. euphorbiae* salivary proteins, collected in resorcinol or water diets, against the tomato database (ITAG2.3) (Supplemental Table S13 in the SI). Proteins, belonging to family members, identified with the same peptides were grouped together. A total of 31 and 25 tomato proteins were identified in the saliva collected in resorcinol and water diets, respectively (Table 5; Supplemental Tables S20 and S21 in the SI). The majority of proteins were identified with a single peptide, suggesting scarcity of these tomato-derived proteins in the saliva. Only nine proteins were common in both saliva collections, suggesting random retention of these proteins in the insect stylet (Table 5; Figure 2). The tomato sap proteins represent proteins with a plethora of functions including metabolism-, transport-, structure-, and defense-related (Supplemental Tables S20 and S21 in the SI).

DISCUSSION

The neurostimulant resorcinol has been used to induce in vitro salivation of plant parasitic nematodes and collect salivary secretions for proteomics profiling of nematode saliva.[34,35,39] Its use in piercing sucking insects was not tested before. At high concentrations, resorcinol has antibacterial properties; however, at low concentrations, this compound does not seem to have adverse effects on nematodes.[40] In a preliminary study, we also did not detect short-term adverse effects on potato aphids, as aphids fed on 0.4% resorcinol for 16 h survived at a similar rate as control aphids fed on water when transferred onto tomato plants. In the present study, we demonstrate that resorcinol can also be used to enhance aphid salivation in vitro. In addition, the composition of the *M. euphorbiae* salivary protein set collected in resorcinol, as demonstrated by the proteins identified by our nanoLC−ESI−MS/MS analysis, is similar to that collected in water, further supporting its use.

The overall protein profile of the saliva collected in resorcinol, evaluated by 1D gel electrophoresis, was similar to that of proteins collected in water or in 15% sucrose supplemented with amino acids. Furthermore, the protein profile of the saliva collected in water-only diet was also similar to that in 15% sucrose and amino acids, indicating that *M. euphorbiae* salivary proteins are not highly affected by the stylet environment. Unlike *M. euphorbiae*, saliva collected from *D. noxia* and *M. viciae* was highly affected by the diet composition, where very low amounts of salivary proteins were collected from non-sucrose-containing diets, making it difficult to compare the protein profiles in these different diets.[14,24] Interestingly, it has been shown that salivary secretions were affected by the pH of the diet more than its composition, as less *D. noxia* saliva was detected in 15% sucrose plus amino acids at pH 5.5 compared with saliva collected in 15% sucrose-only diet at pH 6.8.[14] Because the pH of all of the diets in this study was 6.8, it could be the reason for the reduced variability of the *M. euphorbiae* saliva in the three diets. Alternatively, it is possible that the population of *M. euphorbiae* used in this study has the inherent ability to salivate better in water compared with other aphid species. It is also possible that salivation by generalists, such as *M. euphorbiae*, that can feed on a number of plant families is not affected by the stylets environment.

The MS analysis further supported the 1D denaturing gel data, indicating that more salivary proteins per aphid can be collected when resorcinol is used. Our data suggested that by using about 1/3 number of aphids with the resorcinol diet we could detect over 66% of the proteins that were found with water-only diet, detected by a single peptide reported in this study combined with those reported by Chaudhary et al.[5] Of the 85 proteins in the resorcinol diet, only 10 were not present in the water-only diet with majority of these10 being uncharacterized (Supplementary Table S17 in the SI). Although one of these, a GST, has a secretion signal peptide and is likely delivered into plants through aphid saliva, the remaining resorcinol-induced proteins did not have a secretion signal or were predicted for secretion and might not be commonly present in aphid saliva. Taken together, resorcinol stimulates aphids to salivate more in vitro and can be successfully used to collect aphid saliva.

In the peptide searches against the pea aphid proteome, we identified additional proteins in both water-only and resorcinol diets that were missed in the searches against the *M. euphorbiae* proteome. This is most likely due to incomplete nature of the

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M. euphorbiae transcriptome. Interestingly, in the liquid saliva collected from both diet types, water-only[5] and resorcinol diets (this study), we found only five transcript contigs that were specific to *M. euphorbiae* with no clear homologues in the *A. pisum* genome. This suggests that *A. pisum* has the full repertoire of *M. euphorbiae* salivary proteins. Considering that *A. pisum* and *M. euphorbiae* have very different host range, with *A. pisum* being more specialized feeding mainly on leguminous plants while *M. euphorbiae* is the generalist with a wide host range, this overall similarity of putative salivary proteins in these two aphid species was unexpected. However, the low number of *M. euphorbiae* specific salivary proteins could be an underestimate because of the incomplete nature of the *M. euphorbiae* transcriptome. Alternatively, the variation in the sequences encoding the salivary proteins or their expression could be the host range determinant factors.

Only about half of the proteins identified in the *M. euphorbiae* liquid saliva had secretion signal or were predicted for secretion. Similar proteins not predicted for secretion have also been reported from saliva of other aphid species.[16,19−21] These salivary proteins could be from cells other than the salivary glands. It is unlikely that these proteins originate from the stylets as the aphid maxillary stylets, which form both the salivary and food canals, are made of chitin, and have no cells.[41−43] Recently, it has been shown that proteins from the aphid primary endosymbiont, *Buchnera aphidicola*, were also present in aphid saliva,[5,21] indicating movement of proteins from the insect hemocoel through the salivary gland and into the salivary secretory canal. Both the principal and accessory salivary glands in aphids possess extensive microvilli directed toward the salivary ducts, suggesting the passage of proteins from the hemocoel into the gland cells.[44] It has been suggested that in aphids, in the absence of an excretory system, such as the Malpighian tubules, salivary glands may also function as an auxiliary excretory system.[44,45] A possible source of these proteins of aphid origin is the cells of the outer membrane of the bacteriome that houses the *Buchnera* bacteriocyte. The disintegration of the bacteriome with age may release its content into the hemocoel.[46,47] Interestingly, although the proteins in the saliva with no secretion signal or prediction for secretion have a plethora of functions, those involved in cellular core functions and expected to be abundant, such as actin and ribosomal proteins, are not well-represented among these, indicating some selectivity in movement of these proteins through the salivary glands.

Most of the published work describing aphid salivary proteome composition did not distinguish between proteins with secretion signal or predicted for secretion and those without secretion function. Therefore, for the comparative analysis of salivary proteomes among aphids, we used the entire repertoire of proteins identified in aphid saliva irrespective of a secretion signal or prediction for secretion. Glucose dehydrogenases were detected in saliva from all seven aphid species, including *M. euphorbiae*, with salivary proteome analysis (Table 3). Three different secreted members of this enzyme were detected in the *M. euphorbiae* saliva; one of these was among the salivary phosphoproteins (ACYPI000986) and two, including this phosphoprotein, are under positive evolutionary selection, suggesting a role in aphid virulence.[17] Glucose dehydrogenases are oxidoreductases involved in insect immune responses[48] and possibly involved in detoxification and suppression of plant defense responses.[17,19] Another enzyme involved in sugar metabolism and plant immunity present in aphid saliva of four aphids is trehalase. Previously, trehalase was found only in the saliva of aphids feeding on cereals, *D. noxia*, *S. avenae*, and *M. dirhodum* and thought to be limited to aphids feeding on monocots.[19,20] The presence of trehalase in *M. euphorbiae* saliva suggests a broader role for this enzyme beyond aphids feeding on monocots. Aphid trehalase may hydrolyze trehalose that has been associated with plant defense against aphids.[49] However, the role of aphid trehalase in modulating plant immunity against aphids remains unclear because transient expression of *M. euphorbiae* trehalase (Me5) in *N. benthamiana* did not result in altered aphid performance phenotype.[10]

An additional oxidoreductase, peroxiredoxin, was also identified in *M. euphorbiae* saliva. Peroxiredoxin has been also detected in the saliva of both *M. vicia* and of *A. pisum*.[21] Peroxiredoxin may suppress defense responses by interfering with the oxidative burst, a hallmark of immunity in plants, by reducing hydrogen peroxide. *M. euphorbiae* feeding is known to induce oxidative burst in tomato. Thus, the presence of peroxiredoxin in aphid saliva may modulate this response.[50] We also detected in *M. euphorbiae* saliva glutathione peroxidase, also known as the *M. euphorbiae* effector Me23, which when transiently expressed in *N. benthamiana* resulted in enhanced aphid performance, suggesting a role for peroxidases in modulating plant defense to enhance aphid virulence similar to a microbial pathogen.[10,51] Although peroxidase activity has been reported for saliva from several aphid species[26] and a peroxidase was identified in the saliva of *S. avenae*,[20] glutathione peroxidase has been reported from the saliva of only *S. graminum*[25] and *M. euphorbiae*.

Another possible defense modulator present in the saliva of *M. euphorbiae*, *A. pisum*, *D. noxia*, and *S. graminum* is the lipid-binding protein apolipophorin.[19,21,25] Apolipophorins are involved in lipoprotein metabolism and lipid transport and in insect immunity against microbial pathogens.[52] Lipids and fatty acids are plant defense signal molecules, and secreted apolipophorin in aphid saliva may bind to them and directly interfere with plant immune responses.[53] A pancreatic lipase-like protein was also detected only in *M. euphorbiae* saliva, which was previously predicted to be secreted from *A. pisum*.[17] Lipases break down lipids and fatty acids, and their breakdown products trigger defense responses in plants. Lipase activity has been detected in oral secretions of a grasshopper, and this activity was responsible for the induction of jasmonic-acid (JA) hormone-mediated defense responses.[54] It is likely, therefore, that aphid secreted lipases also trigger plant JA-induced defense responses.

Two GSTs, with secretion signal or prediction for secretion, were detected in *M. euphorbiae* saliva. One of these (ACYPI009586) was predicted to be secreted from *M. persicae*,[23] and GST activity has been reported from a number of aphid species and partially correlated with host range.[55,56] This family of detoxifying enzyme conjugates endogenously induced or xenobiotic compounds to reduced glutathione (GSH) to target them for degradation.[57,58] GSTs in insects, including aphids, have been implicated in resistance to insecticides or allelochemicals.[56,59,60] In addition, mammalian GSTs have GSH-dependent peroxidase activity against products of oxidation such as hydrogen peroxide.[61] The presence of GSTs in aphid saliva suggests detoxification of allelochemicals happens directly inside the plant host before ingestion. In addition, GSTs in the saliva could contribute to

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detoxification in the insect gut because saliva is ingested by aphids.

Aminopeptidase N-like proteins, known for their role in protein processing including protein turnover and catabolism of bioactive peptides, were detected in *M. euphorbiae* saliva as well as saliva of other aphid species, suggesting their ubiquitous presence in aphid saliva (Table 3).[62] The presence of aminopeptidases in aphid saliva further implicates a role for saliva in the degradation of host proteins and detoxification including conjugated glutathione.[58]

Yellow-like proteins and carbonic anhydrase were also only detected in the saliva of the cereal aphid *S. avenae* and were predicted to be secreted from the *M. persicae* salivary gland transcriptome.[20,23] Yellow-like proteins, present mostly in insects, bacteria, and fungi, are multifunctional and diverse gene family members.[63−67] Similar to *M. persicae*, the member (ACYP001127) detected in *M. euphorbiae* saliva has a secretion signal; however, the member detected in *S. avenae* (ACYP001857) does not.[20] It has been postulated that yellow-like proteins could be involved in modulating plant immunity by interfering with phenol oxidase−related defense, similar to their role in insect immunity and melanization.[20] Carbonic anhydrases, on the other hand, are zinc-containing metalloenzymes that reversibly catalyze the conversion of carbon dioxide to bicarbonate and in this process maintain pH homeostasis. Although it is speculated that plant tissue pH guides the aphid stylets to the phloem element, how carbonic anhydrases can assist in the process remains unclear. Another uncharacterized protein ACYPI1606 present in *M. euphorbiae* saliva was detected in the saliva of the cereal aphid *M. dirhodum* and was predicted to be secreted from pea aphid and *M. persicae*, further demonstrating the similarity of salivary proteins from aphids feeding on different host ranges.

Several known aphid effectors were common among these aphid species (C002, Mp1, Mp55, Mp57, Me10/Mp58). C002 is the first characterized aphid effector that alters aphid performance when expressed in planta in a species-specific and plant-specific manner.[6,11] Silencing *C002* affects aphid fecundity and longevity,[11,22,68] indicating the importance of this effector in plant−aphid interactions. The *M. persicae* effectors Mp55 and Mp57 also affect *M. persicae* fecundity when expressed in *Nicotiana tabacum* or *Arabidopsis*. Mp55 enhances aphid fecundity by suppressing plant defense and reducing accumulation of 4-methoxyindol-3-ylmethylglucosinolate, hydrogen peroxide, and callose.[7] Similar to *C002*, silencing *Mp55* also reduces aphid fecundity. Because the molecular functions of these two effectors are unknown, it is not clear how these effectors are able to alter plant defenses. In contrast with C002 and Mp55, in planta expression of Mp57 reduces aphid fecundity likely by inducing plant defenses.[7] Two additional effectors, Mp1 and Mp58/Me10, were also among the identified effectors in the *M. euphorbiae* saliva. These two effectors are among the salivary phosphoproteins and are discussed later.

Among the *M. euphorbiae* proteins with potential role in virulence previously not detected in aphid saliva are a chitinase-like (Cht1), pancreatic lipase-related proteins, and a Kazal-like proteinase inhibitor (Table 2). Nine chitinase-like genes have been identified in *A. pisum* genome.[69] Sequence analysis indicated that Cht1 belongs to insect chitinase-related family, imaginal disc growth factors (IDGFs), lacking chitinase activity with growth promoting functions.[69,70] It is unclear what role IDGF can play in aphid−plant interactions and its contribution to aphid virulence. Considering that the majority of chitinase-like proteins, not originating from the salivary glands, including Cht1, are secreted and have secretion signal peptides, Cht1 could be originating from cells other than the salivary glands. Three pancreatic-lipase like proteins were detected in *M. euphorbiae* saliva, and their presence suggests that aphids degrade plant lipids. Lipids are an important constituent of prokaryotic and eukaryotic cells and are precursors of signaling molecules including plant defense signaling.[71] Plant phloem sap contains lipids and plant lipid-based signaling participates in aphid defense. *Arabidopsis* lipase1 (*MPL1*) is induced by *M. persicae* feeding and is a positive regulator of defense against aphids. Alternatively, aphid-secreted lipases could function as a virulence factor to promote colonization, as has been shown with fungal pathogens, by interfering with plant epicuticular waxes to initiate infection.[71−74] Several secreted Kazal-like proteinase inhibitors have been identified from plant pathogenic oomycetes *Phytophthora* species.[75] Two of these effectors (EPI1 and EPI10) have been shown to target subtilisin serine proteases to enhance *Phytophthora* virulence.[75] It is therefore likely that *M. euphorbiae*-secreted Kazal-like protein also contributes to aphid virulence.

Phosphatidylinositol-4-phosphate 5-kinase (PIP5K) was also identified only in *M. euphorbiae* saliva. In plants, PIP5K phosphorylates phosphatidylinositol-4-phosphate to produce phosphatidylinositol-4,5-bisphosphate as a precursor of two second messengers, inositol-1,4,5-triphosphate and diacylglycerol, and as a regulator of many cellular proteins involved in signal transduction, stress, and cytoskeletal organization.[76] It is conceivable that aphid PIPK5 may function in modulating these responses in plants to its advantage.

Another enzyme that was detected in *M. euphorbiae* saliva and might be involved in enhancing aphid virulence is a maltase. Starch is one of the major products of photosynthesis, and accumulation of starch has been associated with aphid defense.[49,77] Starch is degraded to sustain growth, and maltose is one of its major degradation products.[78] Because maltases break down maltose to glucose, aphid salivary maltases could facilitate degradation of starch biproducts to counteract starch-based plant defense.

An uncharacterized protein (ACYPI009881), thought to be a principle component of the salivary sheath, has been identified in liquid saliva from almost all aphids with salivary proteome MS analysis (Table 3). This protein is also among the phosphorylated salivary proteins identified in this study. When the *M. euphorbiae* sheath and liquid saliva proteome were profiled with nanoLC−ESI−MS/MS,[5] this protein was identified in both liquid and sheath saliva, albeit with only a few peptides in the gelling saliva compared with a very larger number of peptides in the liquid saliva. Several additional proteins were detected in both saliva types, suggesting either cross contamination between the two saliva types or that both salivas partially contain redundant proteins.; however, the detection of far larger number of matching peptides to this protein in the liquid saliva combined with identification of this protein in liquid saliva from different aphid species suggests that this protein is part of the liquid saliva.

Several additional uncharacterized proteins were also common among the different aphid species. The presence and absence of these proteins in the saliva of different aphid species may reflect the diverse host range they feed on. However, it is possible that more proteins are common in the saliva of distinct aphid species but were not detected due to

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different experimental approaches used and variation in aphid salivation mentioned above. Moreover, our work identified a large number of uncharacterized proteins that were only previously predicted to be secreted based on salivary gland transcriptome or proteome analyses. These unknowns provide a treasure chest of putative aphid effectors to explore to better understand what makes aphids such a successful plant pest.

Phosphorylated Effectors

Our work represents the first report for aphid salivary phosphoproteins and identifies three known aphid effectors, Me_WB01635/Mp1/Pinto1, Me10/Mp58, and Me23, among these phosphorylated salivary proteins.[6,7,10,11] Me_WB01635 encodes a putative full length mature, excluding the secretion signal peptide, CDS, of 123 amino acids. The Serine residues S(106) and S(111) are phosphorylated in Me_WB01635 (Supplemental Figure S2 in the SI). The S(106) corresponding residue is replaced with isoleucine in the *M. persicae* homologue Mp1 (I104; excluding the signal peptide), while the S(111) corresponding residue is conserved (S108; excluding the signal peptide). In contrast, in *A. pisum* (ACYPI006346), the S106 corresponding residue (S106; excluding the signal peptide) is conserved while the S(111) corresponding residue is replaced with threonine (T110; excluding the signal peptide). The role of Me_WB01635 in planta has not been evaluated. There is no consensus for the role Mp1in *M. persicae* virulence, which seems to vary even when expressed in the same plant. Expression of Mp1 in *Arabidopsis* resulted in either no effect[7] or enhanced aphid fecundity,[11] while expression in *N. benthamiana*[6] or *N. tabacum* had no effect on aphid performance.[7] In addition, silencing *Mp1* in *M. persicae* does not result in an aphid performance phenotype.[11] Taken together, Mp1 seems not to contribute considerably to aphid virulence. It remains to be seen whether the *M. euphorbiae* phosphorylated homologue (Me_WB01635) has a virulence function.

The second phosphorylated aphid effectors is Me10 (Me_WB02065)/Mp58. The full length mature, excluding the secretion signal peptide, Me10 CDS, is 127 amino acids long. Two serine residues, S(107) and S(120), are phosphorylated in Me10 (Supplemental Figure 3 in the SI). The S(107) corresponding residue is replaced with alanine in the *M. persicae* homologue Mp58 (A107; excluding the signal peptide), while the S(120) corresponding residue is conserved (S120; excluding the signal peptide). In contrast, in *A. pisum* (ACYPI008224), the S107 corresponding residue is missing because there is a three amino acid deletion in this region of the protein (Supplemental Figure 3 in the SI),[10] while the S(120) corresponding residue is conserved (S117; excluding the signal peptide). In tomato and *N. benthamiana*, expression of Me10 resulted in enhanced aphid fecundity, while expression of Mp58 in *N. tabacum* or *Arabidopsis* resulted in a decrease in aphid fecundity, indicating differences in recognition of these two homologues by different plant species. It is intriguing to speculate that this difference is due to the variation in these phosphorylation sites.

The third identified phosphorylated *M. euphorbiae* effector is Me23. Me23 encodes a glutathione peroxidase and has been shown to enhance aphid fecundity when expressed in *N. benthamiana* but not in tomato, indicating that this aphid effector acts in a plant species-specific manner. Because no Me23 homologue has been detected in saliva from other aphid species, it is unclear whether the Me23 phosphorylation sites are conserved among aphids and could affect plant−aphid interactions.

Tomato Proteins in Aphid Saliva

Because aphids mostly feed on the phloem sap, the tomato proteins identified in the aphid saliva are likely representative of phloem sap proteins. Indeed, the majority of the tomato proteins identified in the aphid saliva have been previously reported in the phloem sap (Supplemental Tables S20 and S21 in the SI). Therefore, aphid saliva could be another possible source for identification of phloem sap proteins. These plant proteins were likely acquired by aphids during feeding and were either stuck to the stylet wall or mixed with the saliva in the stylet chamber, where both feeding and salivary canals meet.[42]

CONCLUSIONS

Aphid saliva contains a large number and diverse repertoire of proteins and is far more complex than previously thought. The novel aphid proteins need to be further characterized to identify their unique roles in aphid feeding and successes in host colonization as well as in altering host defenses. The presence of proteins with no secretion signal peptides or prediction for secretion suggests movement of proteins from other organs into the salivary gland and salivary canal and requires re-evaluation of movement of proteins through the salivary glands. In addition, the presence of secretion signal peptide or sequences predicted for secretion cannot be used as the only criterion to determine whether a protein is originating from the salivary gland. A possible criterion for this purpose is enrichment of transcripts encoding these proteins in the salivary glands. In the absence of genetic transformation system for aphids, the ultimate proof for secretion of these unexpected proteins into the plant host is to detect them in planta using antibodies. Aphid-specific proteins or those sufficiently divergent from plant proteins, with high abundance in aphid saliva, could be targeted for these experiments.

ASSOCIATED CONTENT

Supporting Information

Supplemental Figure S1. Saliva collection setup and aphid survival on resorcinol diet. Supplemental Figures S2 and S3. Phosphorylated effectors amino acid alignments. Supplemental Tables S1−S14. Summary of searched databases and filtering criteria used. Supplemental Tables S15 and S16. Validation of single peptide spectra. Supplemental Tables S17 and S18. List aphid proteins in resorcinol or water diets. Supplemental Table S19. Salivary phosphoproteins. Supplemental Tables S20 and S21. Tomato proteins in saliva in resorcinol or water diets. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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