Insect Molecular Biology (2008) **17**(2), 165–174

Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*

N. Harmel*, E. Létocart†, A. Cherqui†, P. Giordanengo†, G. Mazzucchelli‡, F. Guillonneau‡, E. De Pauw‡, E. Haubruge* and F. Francis*

**Gembloux Agricultural University, Functional and Evolutionary Entomology, Gembloux, Belgium;*
†*Université de Picardie Jules Verne, Biologie des Plantes et Controle des Insectes Ravageurs, Amiens, France; and*
‡*University of Liège, Mass Spectrometry Laboratory, Sart Tilman, Liège, Belgium*

Abstract

The role of insect saliva in the first contact between an insect and a plant is crucial during feeding. Some elicitors, particularly in insect regurgitants, have been identified as inducing plant defence reactions. Here, we focused on the salivary proteome of the green peach aphid, *Myzus persicae*. Proteins were either directly in-solution digested or were separated by 2D SDS-PAGE before trypsin digestion. Resulting peptides were then identified by mass spectrometry coupled with database investigations. A homemade database was constituted of expressed sequence tags from the pea aphid *Acyrtosiphon pisum* and *M. persicae*. The databases were used to identify proteins related to *M. persicae* with a nonsequenced genome. This procedure enabled us to discover glucose oxidase, glucose dehydrogenase, NADH dehydrogenase, α-glucosidase and α-amylase in *M. persicae* saliva. The presence of these enzymes is discussed in terms of plant–aphid interactions.

Keywords: 2D gel, plant-aphid relation, elicitor, glucose oxydase.

Introduction

Among the 4000 species of aphids that have been described, 250 are considered pest species and are responsible for direct (phloem uptake) and indirect (virus transmission) damage (Blackman & Eastop, 2000). The green peach aphid, *Myzus persicae*, is able to transmit more than 100 viral diseases to more than 400 host plants and the prevalence of this aphid is significant in agronomic terms (Quaglia *et al.*, 1993). The damaged area created by a phloem-feeding insect is not equivalent to that created by a chewing insect and the plant defence responses also differ: attacks from phloem-feeding aphids elicit weaker responses in contrast with tissue-feeding Lepidoptera larvae and mesophyl-sucking insects (Voelckel *et al.*, 2004; De Vos *et al.*, 2005). This is because of insect feeding behaviour: whereas chewing insects, such as caterpillars and beetles, tear tissues with mandibles and other feeding apparatus, the aphid stylet penetrates the plant between the epidermal and parenchymal cells to reach the phloem sieves, thereby inflicting minimal wounding to the plant (Miles, 1999).

The signal responsible for the activation of plant defences is not only mechanical but also chemical through the action of particular molecules, commonly called elicitors. Elicitors have been discovered in the saliva of some chewing insects: glucose oxidase (GOX) from the saliva of *Helicoverpa zea* (Eichenseer *et al.*, 1999), β-glucosidase from *Pieris brassicae* (Mattiaci *et al.*, 1995), volicitin from *Spodoptera exigua* (Alborn *et al.*, 1997) and caeliferin from *Schistocerca americana* (Alborn *et al.*, 2007). Both β-glucosidase and volicitin have been found to induce the release of volatile organic compounds, which are attractive to the natural enemies of the attacking insects. By contrast, it has been suggested that GOX is advantageous for the insect that produces it. The enzyme increases the caterpillar's ability to survive through the suppression of nicotine, an inducible anti-herbivore defence in tobacco plants (Eichenseer *et al.*, 1999) and decreases transcript levels of genes encoding for terpenoid biosynthesis (Bede *et al.*, 2006). No aphid elicitor has been discovered to date but some cues show that this matrix contains plant defence modulating compounds.

The volatile response (terpene emission in particular) of *Solanum tuberosum* infested by *Myzus persicae* aphids has been found to be significantly different from that of healthy or mechanically picked plants and has also been found to influence the foraging and reproductive behaviour

Received 25 September 2007; accepted after revision 7 January 2008. Correspondence: Frédéric Francis, Gembloux Agricultural University, Functional and Evolutionary Entomology, Passage des Déportés 2, 5030 Gembloux, Belgium. Tel.: +32 81 622283; fax: +32 81 622312; e-mail: francis.f@fsagx.ac.be

of *Episyrphus balteatus* (Harmel *et al.*, 2007). In Harmel *et al.*'s experiment, differences in volatile emissions between mechanically picked and infested leaves revealed that aphid damage was not equivalent to mechanical damage. Aphids have been found to elicit plant cell wall remodelling: genes encoding cell wall-modifying enzymes have been found to be up-regulated in aphid infested plants (Voelckel *et al.*, 2004; Divol *et al.*, 2005; Qubbaj *et al.*, 2005). These structural modifications are thought to deter phloem feeding herbivores by strengthening barriers to insect probing within the host tissues.

Thompson & Goggin (2006) reviewed different studies using transcription analysis of gene expression and showed that plant phloem feeders were able to change greatly the physiology of their host plants, including photosynthetic activity, source-sink relations and secondary metabolism. Plant responses to aphids are regulated by salicylate, jasmonate and ethylene signalling pathways. Furthermore, aphids ingest phloem sap without eliciting the normal occlusion response to injury in the sieve tubes. These occlusion mechanisms are calcium-triggered. Will *et al.* (2007) demonstrated that aphid saliva has the ability to prevent sieve tube plugging by molecular interactions between salivary proteins and calcium. The involvement of aphid saliva certainly seems to be preponderant in these phenomena.

Moreover, the electrical penetration graph (EPG) technique allows the electrical monitoring of plant penetration by aphids with piercing mouthparts and the recording of signal waveforms reflecting different insect activities (mechanical stylet work, saliva secretion and sap ingestion) (Tjallingii, 2006). Four periods of salivary secretion have been shown by EPG; one period of gelling salivation and three periods of watery salivation: (1) intercellular sheath salivation that envelops the stylet; (2) intracellular salivation into cells along the stylet path; (3) initial phloem salivation; and (4) phloem feeding salivation (Cherqui & Tjallingii, 2000).

The proteins contained in aphid saliva are of two types: structural and enzymatic. The structural proteins provide a tube-like sheath (Cherqui & Tjallingi, 2000) and correspond to major bands on gels with estimated molecular masses of 154 and 66/69 kDa (Baumann & Baumann, 1995). The secreted salivary enzymes have been found to be hydrolases (pectinases) and oxidation/reduction enzymes (phenol oxidase and peroxidases) (Miles, 1999; Cherqui & Tjallingii, 2000).

Injected saliva may play a crucial role in the prevention of the plant's wound responses but it may also play the role of elicitor of a plant's reaction. The role of these enzymes is not clear. Only enzymatic activity has been detected in aphid saliva. Proteins have never been identified. This is the reason why we investigated the *M. persicae* saliva proteome. We were searching for potential plant defence elicitor(s) in accordance with the approach that has been developed with mosquito saliva. This approach has enabled the identification of, among others, anticlotting, antiplatelet and vasodilator substances that modulate the host immune response and inhibit blood coagulation (Valenzuela *et al.*, 2002; Montgomery *et al.*, 2004). In the present study, different methodologies were used in order to study the aphid saliva proteome: in-solution digestion was performed on aphid salivary proteins, as this is the most sensitive method for protein identification. As a result of the lack of quantification of this technique, spots excised from 2D PAGE were in-gel-digested before ESI MS-MS (nano-electrospray ion trap mass spectrometer) identification.

Results & discussion

A multi-approach experiment based on both in-solution and in-gel (after 2D gel electrophoresis) protein digestion associated to complementary mass spectrometry techniques (LC (liquid chromatography) and Maldi-Tof) was performed in order to investigate the saliva proteome of an aphid species. More than 20 spots were visualized on 2D gels of aphid saliva. Nine proteins were identified with a known function in other insects, while others were related to expressed sequence tag (EST) aphid sequences (Table 2). In the in-solution digestion approach, more than 200 peptides were generated; 71 were identified as a match with known sequences in existing databases (Table 1).

Digested *M. persicae* salivary proteins were submitted to three databases: the NCBI database, the annotated EST database related to the pea aphid *A. pisum* and the peach aphid *M. persicae* database (Table 1). Because the function of many of these cDNAs remains unknown, only sequences presenting a significant Mascot score (> 30) were blasted.

Different kinds of cDNA libraries were used to generate ESTs. Among them, some were related to salivary glands or from aphid head RNAs. The majority of peptides from in-solution digestions matched with these databases (Table 1). These results confirmed that the identified peptides are found in the head and, in particular, in the salivary glands. These specific databases were therefore very useful for identifying salivary proteins collected in the aphid's artificial diet and provided more data in peptide identification than in previous proteomic works on aphids (Francis *et al.*, 2006).

The *M. persicae* database revealed three in-solution digested peptides (Table 1A) matching with the sequence from an EST of *M. persicae* named EC389056. After a BLAST procedure, this nucleotidic sequence was found to match with two insect enzymes: glucose dehydrogenase (GLD) and GOX. This match was confirmed with the *A. pisum* EST database: two common peptides were found to match with an EST, presenting a 95.1% similarity with EC389056 (data not shown). Another peptide was identified that matched with an EST of an *M. persicae* sequence and it also matched with GLD and GOX. This EST, named EE262240 (Table 1A), was quite different from EC389056.

Table 1. In-solution digested salivary peptides resulting from the annotated expressed sequence tags (ESTs) of the *Myzus persicae* aphid research. (A) Peptides and associated EST where BLAST searches led to known function proteins with a significant E-value. (B) Peptides only associated with EST-specific aphid databases

	Organism	Acc no. BLAST	Mw*	pI†	E-value	Peptides	Mascot score	EST GENBANK accession no.	Cov‡
Glucose dehydrogenase	*Aedes aegypti*	EAT44638	61993	6.41	7e-27	K.LEDIDLDGCAK.Y K.YMVSTTSSTAGSCR.M K.DAVVDSELNVIGISNLRAVGR.S	121	EC389056	18%
			68893	9.27	1e-31	K.AYLSPIFGR.E K.IQPDSTTGFGIEGNMK.I	80	EE262240	8%
Glucose oxidase	*Apis mellifera*	NP_001011574	67938	6.48	9e-24	K.LEDIDLDGCAK.Y K.YMVSTTSSTAGSCR.M K.DAVVDSELNVIGISNLRAVGR.S	121	EC389056	18%
					1e-16	K.AYLSPIFGR.E K.IQPDSTTGFGIEGNMK.I	80	EE262240	8%
Alpha-amylase	*Aedes aegypti*	EAT48298	70055	5.36	2e-61	KVDQSIMSQYQDQ	33	EE571055	5%
Alpha-glucosidase	*Apis mellifera*	NP_001035349	66567	5.84	1e-60				
Hydroxyacyl dehydrogenase	*Aedes aegypti*	EAT38824	32115	6.63	1e-56	IILELLNNA	31	DW010534	5%

Aphid EST database references	Peptides	Mascot score	EST GENBANK accession no.	Cov‡
Myzus persicae, line G006, whole aphid library cDNA	K.VYQVYAYTR.D R.EVISHHVILK.T R.DRIPSLDTMK.S R.GYNMITSELQETR.S K.SITQSLLYSGYNPNHMIK.I	445	EC387934	21%
	K.VYEDIER.S R.EVISHHVILK. T R.GYNMITSELQETR.S K.SIVSDCSSSSSTSKSSSTTTTMK.S	341	EC388457	15%
	R.DNIVEDMTK.A K.AGMPDVSSTNR.G K.TGMPDVSSTNR.G K.RDIIVEAMTK.T	72	EE263445	15%
Myzus persicae, tobacco lineage, aphid salivary gland library cDNA	R.YMVLER.G K.WNFNTR.Y R.GYNMITSK.V R.CGINPNYMIK.I K.EVPLVYSYTR.D R.GYNMITSKVQQTR.S K.TIDDLYTFDESYFK.S R.GISIGNDSPITELPSICPK.D	312	EE572212	30%
	R.CGINPNYMIK.I K.EVPLVYSYTR.D K.TIDDLYTFDESYFK.S R.GISIGNDSPITELPSICPK.D K.TGLFCNSYPCTNNQLIFK.D	281	EE571947	27%
	R.YLCQFM.- K.SMSPTVAQPVVA.- K.ATNEFEPTINYQTSDPQK.V	96	EE572100	11%
	R.YMVLER.G K.WNFNTR.Y K.GLDNITIR.K R.GYNMITSK.V R.GYNMITSKVQQTR.S	86	EE264749	11%
	R.HSLIQFK.T R.TTFTEFK.K -.IGFLIVSSK.- K.ALNYFENK.L K.QPGLTLEITEK.K R.SAYNYYSLYNK.Q	79	EE264598	21%
	K.IKLPCXK.K K.TVTEDIIERL.-	39	EE264595	3%
	K.YMAFDMMVK.G K.FCADDSEALYQK.G	77	EE264560	10%
Myzus persicae, tobacco lineage, aphid head library cDNA clone	K.IWNDAFSNPK.A K.ELGSNEVCSDTTR.A	90	EC389283	10%
	-.NHIITX.- R.GNPNLLPQQK.S R.AMALAQLMNMQNR.F K.SSTTQGDQVYAPSMPQFSME GNR.Q	37	EC389929	17%
Myzus persicae, tobacco lineage, whole aphid library cDNA	K.ECVCDGPCYSCVVSAGLDK.S	65	DW013464	9%
	K.QSLGMVGSFSDSSAR.G	53	DW011294	7%
	K.NLQEIENNTVK.Q	47	EC389211	6%
	R.YLGEMEKDGQK.C	40	DW012626	17%
Myzus persicae, line F001, PLRV infected, whole aphid library cDNA	K.SQLVFNIFIVLLYLCTLVSLLT AAEIGETSCR.Y K.IVPLIANK.I R.LLTIEEAIR.M R.NGYYLNSNTR.N	59	EE571076	9%

Cov, coverage per cent
*Molecular weight.
†Isoelectric point.
‡Percentage of sequence coverage of identified peptides related to the corresponding sequence in database.

Table 2. In-gel digested 2D SDS-PAGE salivary peptides resulting from the annotated expressed sequence tags (ESTs) of the *Myzus persicae* aphid research. (A) Peptides and associated EST where BLAST searches led to known function proteins with a significant E-value. (B) Peptides only associated with EST-specific aphid databases

	Organism	Acc no. BLAST	Mw*	pI†	E-value	Spot	Peptides	Mascot score	EST GENBANK accession no.	Cov‡
Solid saliva related to Fig. 3A										
Ran-binding protein	*Aedes aegypti*	EAT38186	293536	6.25	9e-38	7	K.MIIISC.- K.CMSSAR.L	47	EE261552	2%
						1	MIIISC	32		
NADH dehydrogenase	*Aedes aegypti*	EAT43733	18100	9.69	1e-37	2, 4, 5, 11	AHIIL	31	EE264957	3%
Retinol dehydrogenase 13	*Tribolium castaneum*	XP_973517	34004	8.65	4e-67	2, 8, 11	CAVLERTGAK	34	EE572101	4%
Transcriptional intermediary factor 2	*Tribolium castaneum*	XP_967666	164515	6.12	7e-13	6	R.NKMLASLLAK.D R.HCGWARHWCSFR.K	39	EE262675	3%
Protein arginine methyltransferase 5 isoform a isoform 1	*Apis mellifera*	XP_394141	71106	7.47	2e-60	7	R.VEISAIEK.N R.VTIINEDMR.L K.ILSSITSIKER.Y	47	EE264648	5%
AMP dependent coa ligase	*Aedes aegypti*	EAT33078	42583	6.24	6e-49	2	TCEIEG	36	EE571292	2%
ATM protein	*Drosophila melanogaster*	AAR89513	137555	8.42	2e-34	3	NFSKVL	33	DW013327	3%
Soluble saliva related to Fig. 3B										
NADH dehydrogenase	*Aedes aegypti*	EAT43733	18100	9.69	1e-37	2′, 7′	AHIIL	32	EE264957	3%
Retinol dehydrogenase 13	*Tribolium castaneum*	XP_973517	34004	8.65	4e-67	1′, 3′	CAVLERTGAK	34	EE572101	4%

	Spot	Peptides	Mascot score	EST GENBANK accession no.	Cov‡
Solid saliva related to Fig. 3A					
Myzus persicae, tobacco lineage, whole aphid library cDNA	1	R.HSLIQFK.T, K.ALNYFENK.L K.QPGLTLEITEK.K R.SAYNYYSLYNK.Q	160	DW011417	15%
	3	R.HSLIQFK.T R.TTFTEFK.K K.ALNYFENK.L K.QPGLTLEITEK.K R.SAYNYYSLYNK.Q	192	DW011417	18%
	4	R.HSLIQFK.T K.ALNYFENK.L R.TTFTEFKK.S R.SAYNYYSLYNK.Q	166	DW011417	14%
	8	K.SMSPTVAQPVVA.- K.ATNEFEPTINYQTSDPQK.V	93	DW010315	14%
	11	R.HSLIQFK.T K.ALNYFENK.L	70	DW011417	6%
Myzus persicae, tobacco lineage, a phid salivary gland library cDNA	5	-.ILKASVFK.K -.CAVLERTGAK.T	40	EE572069	6%
Myzus persicae, line G006, whole aphid library cDNA	7	-.CAVLERTGAK.T K.CMSSAR	37	EC388700	3%
Myzus persicae, tobacco lineage, aphid head library cDNA	5	-.TTRPHSR.N R.HSLIQFK.T K.ALNYFENK.L K.NLSINECIIVLK.I R.SAYNYYSLYNK.Q	126	EC389308	9%
Soluble saliva related to Fig. 3B					
Myzus persicae, tobacco lineage, whole aphid library cDNA	1′	R.SAYNYYSLYNK.Q K.TLINYDTNIPVTSLDDDXPIDRAIL.-	73	DW011417	15%
	2′	K.VDYSAVER.A K.SMSPTVAQPVVA.	86	DW010315	9%
	3′	K.VDYSAVER.A -.LYSLFDPLK.V K.SMSPTVAQPVVA.- K.DALDDMHENILK.S	130	EC389290	11%
	6′	R.HSLIQFK.T K.ALNYFENK.L K.SYNIARSMGXSSSNDVTGMLVS.-	39	DW011417	6%

Cov, coverage per cent
*Molecular weight.
†Isoelectric point.
‡Percentage of sequence coverage of identified peptides related to the corresponding sequence in database.

We detected GOX activity in aphid saliva samples containing 25 μg of total proteins. We used the same samples heated at 100 °C for 30 min as a negative control and *Apergillus niger* pure GOX as a positive one. GOX activity in aphid saliva was found to be 0.312 ± 0.001 U/mg soluble protein and was in the same range as the labial GOX activities of several caterpillar species, between 3.260 ± 0.028 U/mg soluble protein per pair of fourth instar *S. exigua* labial salivary glands (Merkx-Jacques & Bede, 2005) and 0.160 ± 0.070 U/mg soluble protein per pair of *H. zea* labial salivary glands (Eichenseer *et al*., 1999).

GLD (EC 1.1.1.47) is already known to have an immunological role in insects (Cox-Foster & Stehr, 1994). This was the first enzyme demonstrated in insects to be essential in killing foreign invaders during cellular immune defence or encapsulation. The enzyme has been found to be present in an inactive form in both plasma and specific blood cells. Shortly after invasion of an abiotic or a fungal invader, the enzyme is activated and localized onto the target. GOX (EC 1.1.3.4) has been identified in the hypopharyngeal glands of the worker honeybee *Apis mellifera* (Ohashi *et al*., 1999). This enzyme is needed to convert glucose to gluconic acid and hydrogen peroxide. The gluconic acid keeps the honey acidic and, together with hydrogen peroxide, has an antiseptic action. Moreover, GOX has been reported to inhibit soybean lipoxygenase activity and GOX treatment of tobacco foliage has been found to inhibit JA (jasmonate acid) production (Bi & Felton, unpublished observations). *H. zea* GOX has been shown to inhibit wound-inducible nicotine production in tobacco, *Nicotiana tabacum* (Musser *et al*., 2005). Similar results have been obtained with caterpillars with a cauterized spinneret or with their labial salivary gland surgically removed (Musser *et al*., 2006). It has been established that aphids evade the plant wounding response and activate inefficient defence gene expression, but the mechanism used is unclear (Zhu-Salzman *et al*., 2005). The presence of GOX in *M. persicae* saliva could explain the weak induction of the wounding response and the JA-regulated genes among aphid infested plants. Indeed, H_2O_2 is known to be a potent inactivator of lipoxygenase (Sporn & Peters-Golden, 1998). The role of this GOX needs to be confirmed by further experiments involving protein purification.

One in-solution digested peptide included in *M. persicae* EST EE571055 led to a match with α-glucosidase or α-amylase (Table 1A). Glucosidases are glycoside hydrolase enzymes. Alpha-glucosidases (EC 3.2.1.3) catalyse the hydrolysis of terminal 1,4-linked alpha-D-glucose residues successively from no-reducing ends of the chains with the release of beta-D-glucose. Alpha-glucosidases are widely distributed in microorganisms, plants, mammals and insects (Nishimoto *et al*., 2001). Alpha-glucosidase has been identified as a digestive enzyme in the *A. pisum* midgut (Cristofoletti *et al*., 2003). It is membrane-bound and catalyses *in vitro* transglycosylations in the presence of an excess of the substrate sucrose. Sucrose is present in large amounts in plant phloem sap and has been shown to be used as a power supply, whereas amino acids present in low amounts have been shown to be used for the production of structural compounds (Rhodes *et al*., 1996). Whereas α-glucosidase acts upon α bonds, β-glucosidase cleaves β linkages. Beta-glucosidase has been found in *P. brassicae* regurgitant as an elicitor of cabbage volatiles, which are attractive to the parasitic wasp *Cotesia glomerata* (Mattiaci *et al*., 1995). Alpha-amylase (EC 3.2.1.1) catalyses the endohydrolysis of 1,4-alpha-D-glucosidic linkages in oligosaccharides and polysaccharides. In a study by Ohashi *et al*. (1999), amylase of the honeybee hypopharyngeal gland was thought to be needed to convert plant starch (found in nectar) into glucose, which is then converted into gluconic acid by GOX. Alpha-amylase, as well as α-glucosidase, has been detected in mosquito salivary glands (Effio *et al*., 2003).

NADH dehydrogenase (EC 1.6.5.3) has been found in both in-solution (not shown in Table 1A because of a 28 Mowse score) and in-gel digestions (spots 2, 4, 5 and 11 in Fig. 2A and Table 2A; spots 2′ and 7′ in Fig. 2B and Table 2A). It has also been identified using the *A. pisum* EST database in both in-solution and in-gel digestions (data not shown). This enzyme belongs to complex 1 of the mitochondrial electron transfer chain and catalyses the transfer of electrons from NADH to coenzyme Q. The complex also translocates protons across the inner membrane, leading to an increase in the electrochemical potential used to produce ATP (Fernie *et al*., 2004). We compared the complete mitochondrial genome (15 721 bases) of *Schizaphis graminum* (Thao *et al*., 2004) with the EST of *M. persicae*, blasting with NADH dehydrogenase enzyme. The nucleotide alignment showed that the EST side was similar to a part of the sequence of the *S. graminum* mitochondrial genome (BLAST result not shown).

In the process of identifying the salivary proteins from *M. persicae*, the use of specific EST aphid databases led to the lengthening of the peptide sequences obtained from mass spectrometry. From sequences of only a few amino acids, sequences of hundreds of corresponding nucleotides were obtained using the EST genomic databases after translation and adaptation to mass spectrometry data requirements. It is important to note that the identification of many aphid salivary proteins was possible only because of the use of the specific aphid EST databases. The efficiency of matching the data depended on the aphid part used to build the EST database. Indeed, many of our positive searches were related to more specific parts of aphids, either the head or the salivary glands. This was to be expected, as working on salivary proteins would logically lead to protein identification relating to these parts of the body. Only the Swiss-Prot Database (http://www.expasy.org/sprot) was not adapted to match efficiently proteins for a nonsequenced genome organism such as *M. persicae*.

Several peptides resulting either from the in-solution or the in-gel digestions still failed to present significant Mascot scores or BLAST results. A minority of ESTs presented significant similarities with known insect proteins. It is not surprising to have obtained only a few identified proteins: the large-scale sequencing of 40 904 ESTs from *A. pisum* had led to 10 082 transcripts, among which 59% showed no match to any protein of known function (Sabater-Munoz *et al*., 2006). One reason for the large percentage of unknown proteins may be a factor involved in EST sequencing. Many of these sequences may be 3′ untranslated regions of the transcripts, which are much less conserved than translated regions.

In conclusion, this study constituted a first investigation of an aphid saliva proteome and revealed that data resulting from complementary approaches were needed to allow the identification of some interesting salivary proteins. Among these, we identified a GOX whose enzymatic activity was confirmed in *M. persicae* aphid saliva. In-solution digestion was better adapted than in-gel digestion for the study of aphid saliva proteins, notably because of the gelling consistency of the collected saliva, which might cause some inadequate interference during the first dimension migration in the 2D electrophoresis. It is for this reason that proteins identified after in-solution digestion were not necessarily identified after in-gel digestion. Finally, the importance of the use of an appropriate database (in this case consisting of annotated ESTs of *M. persicae*) was demonstrated when investigating protein identification from non-sequenced genome organisms. It is important to note that all the peptides and ESTs presented in this paper referred first to aphid sequences. Some of these interesting results will be enhanced when a complete aphid genome, that of *A. pisum*, becomes available. Similar studies have already been carried out with mosquito saliva: salivary transcriptome and proteome were combined in order to identify candidate proteins implied in host defence manipulation (Valenzuela *et al*., 2002, 2003; Ribeiro *et al*., 2004).

Numerous papers have dealt with aphid enzymatic activity. Some have established a hypothetical link between saliva and its effect on plants but few have been able to confirm it. We only partially understand why aphids manipulate plant defences. Thus, further experiments are required in order to study the effect of aphid saliva on plant defences.

Experimental procedures

Plants and insects

Broad beans (*Vicia faba* L.) were planted in a mixture of perlite and vermiculite (v:v, 50 : 50) in 12 cm diameter plastic containers and were grown in a controlled environment room at 20 ± 2 °C temperature with a 16/8 h photoperiod. Mass rearing of *M. persicae* Sultzer was performed on bean plants. The aphids ('Gembloux' clones collected in 1997 from beans in a crop field) had been reared on broad bean plants in the laboratory for several years.

Saliva collection

Aphid saliva was collected after 2 days of the artificial diet system (Fig. 1), as previously described by Cherqui & Tjallingii (2000). Up to 30 *M. persicae* adults were transferred from the plants to a feeding chamber, a standing cylinder (PVC tube), 27 mm in diameter and 40 mm high, covered on top with a double layer of Parafilm (Brand GMBH, Wertheim, Germany) with 120 μl of a 15% (w/v) sucrose diet contained in a 'sachet'. The aphids remained in the chamber for 48 h in an incubator at 20 °C, illuminated from above with a constant yellow light. The saliva collected after the incubation of the aphids on the diet could be distinguished as two fractions. Soluble saliva was contained in the artificial diet after the incubation of the aphids and proteins were concentrated by conventional TCA (trichloroacetic acid) protocol. Solid saliva, which consists of a gelling substance secreted to minimize the contact between the aphid stylet and plant cells (Cherqui & Tjallingii, 2000), was collected during the rinsing of each lower Parafilm membrane with a 1% (v/v) Triton solution. Proteins were concentrated by centrifugation (15 000 *g*; 15 min; 4 °C). The technique used allowed us to collect only saliva fluids. The aphid stylet is in fact the only organ able to penetrate the lower Parafilm layer and the salivary canal is the only stylet through which a fluid can be poured into an external media such as plant phloem or an artificial diet (Miles, 1999).

In-solution digestion

Digestion. Salivary proteins (soluble and solid) collected from about 4000 aphids were resuspended in 20 μl of 50 mM NH_4HCO_3, pH 8.0. Cysteines were reduced with 1 μl of a 200 mM DTT (1,4-Dithiothreitol) solution in 100 mM NH_4HCO_3 for 10 min at 50 °C followed by alkylation for 45 min with 0.8 μl of a 50 mM iodoacetamide solution in 100 mM NH_4HCO_3 at room temperature in the dark. Alkylation was halted by neutralizing the remaining iodoacetamide through the addition of 4 μl of a 200 mM DTT in 100 mM NH_4HCO_3 at room temperature for 45 min. Digestion was performed overnight with 0.1 μg of trypsin in water. The resulting peptides were dried in a vacuum centrifuge. This step was performed in two replicates.

Protein identification. Peptide separation by reversed-phase liquid chromatography was performed on an Ultimate LC system (LC Packings, Amsterdam, The Netherlands) complete with Famos autosampler and Switchos II microcolumn switching device for sample clean-up and preconcentration. Each kind of sample (30 μl each) was loaded in duplicate at a flow rate of 200 nL/min on a micro-precolumn cartridge (300 μm i.d. × 5 mm, packed with 5 μm C18 10 nm PepMap). After 5 min, the precolumn was connected to the separating nano-column (75 μm i.d. × 15 cm, packed with C18 PepMap100, 3 μm, 10 nm) and the gradient started. The elution gradient varied from 0 to 30% in buffer B for 30 min. Buffer A consisted of 0.1% formic acid in acetonitrile/water 2 : 98 (vol/vol) and buffer B of 0.1% formic acid in acetonitrile/water 20 : 80 (vol/vol). The outlet of the LC system was directly connected to the nano electrospray source of an Esquire HCT ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass data acquisition was performed in the mass range of 50 to 1700 m/z using the Standard-Enhanced mode (8100 m/z per sec). For each mass scan, a data-dependent scheme picked the four most intense doubly or triply charged ions to be selectively isolated and fragmented in the trap and the resulting fragments were mass analysed using the Ultra Scan mode (50–3000 m/z at 26 000 m/z per sec).

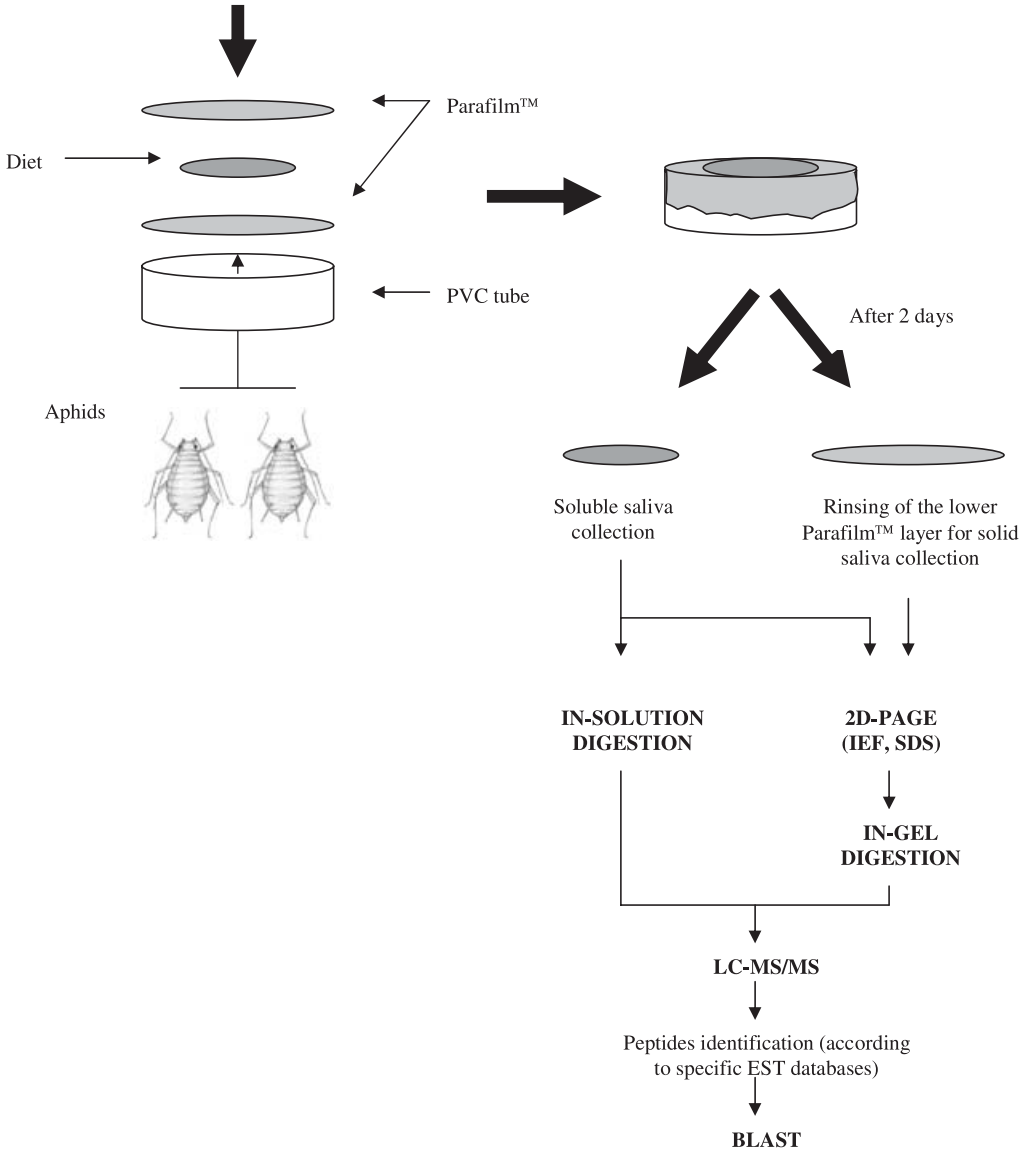

Figure 1. System used for aphid saliva collection. Aphids were transferred from plants to PVC tubes covered on top by a double layer of Parafilm with a 15% sucrose diet in between a 'sachet'. The saliva collected after 2 days of incubation of aphids on the diet can be distinguished as two fractions. Soluble saliva was contained in the artificial diet after incubation of aphids and proteins were concentrated by conventional TCA protocol. Solid saliva was collected by rinsing each lower Parafilm membrane with a 1% Triton solution and proteins were concentrated by centrifugation.

Strategies for protein separation and identification. Soluble and solid fractions of aphid saliva were collected. Whereas solid saliva proteins were separated by 2D-PAGE, soluble saliva proteins were in-solution and in-gel digested. Resulting peptides were analysed by LC MS-MS with the NCBI nonredundant protein database and two other specific databases: one constituted from the annotated expressed sequence tags (ESTs) of the whole pea aphid *Acyrtosiphon pisum* and the other one from annotated ESTs of *A. pisum* salivary glands. The enlarged sequences were submitted to the BLAST procedure to identify peptide functions.

Denaturing polyacrylamide gel electrophoresis (SDS/PAGE)

Analytical 2D gel electrophoresis. The protein extracts were used in two replicates to rehydrate 7 cm IPG (Immobilized pH gradient) strips (pI 3–10 NL from Amersham) for 12 h at 20 °C and at a constant voltage of 50 V. Isoelectric focusing (IEF) was carried out at 250 V for 200 Vh, 500 V for 500 Vh, 1000 V for 1000 Vh and 8000 V for 60 000 Vh at 20 °C and a maximum current setting of 50 μA/strip in an isoelectric focusing unit from Bio-Rad (Hercules, CA, USA). Following IEF, the IPG strips were equilibrated for 15 min in 375 mM Tris (pH 8.8) containing 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS and 130 mM DTT and then for a further 15 min in the same buffer except for the fact that DTT was replaced with 135 mM iodoacetamide. The IPG strips were then sealed with 0.5% agarose in SDS running buffer at the top of slab gels polymerized from 12% (w/v) acrylamide and 0.1% N,N′-methylenebisacrylamide. The 2D electrophoresis was performed at 20 °C in a Slab system (Bio-Rad) at 100 V/gel for 2 h. Because of the low number of purified proteins, the gels were silver stained with a silver-stain compatible MS protocol (Shevchenko *et al.*, 1996). Each gel was scanned and

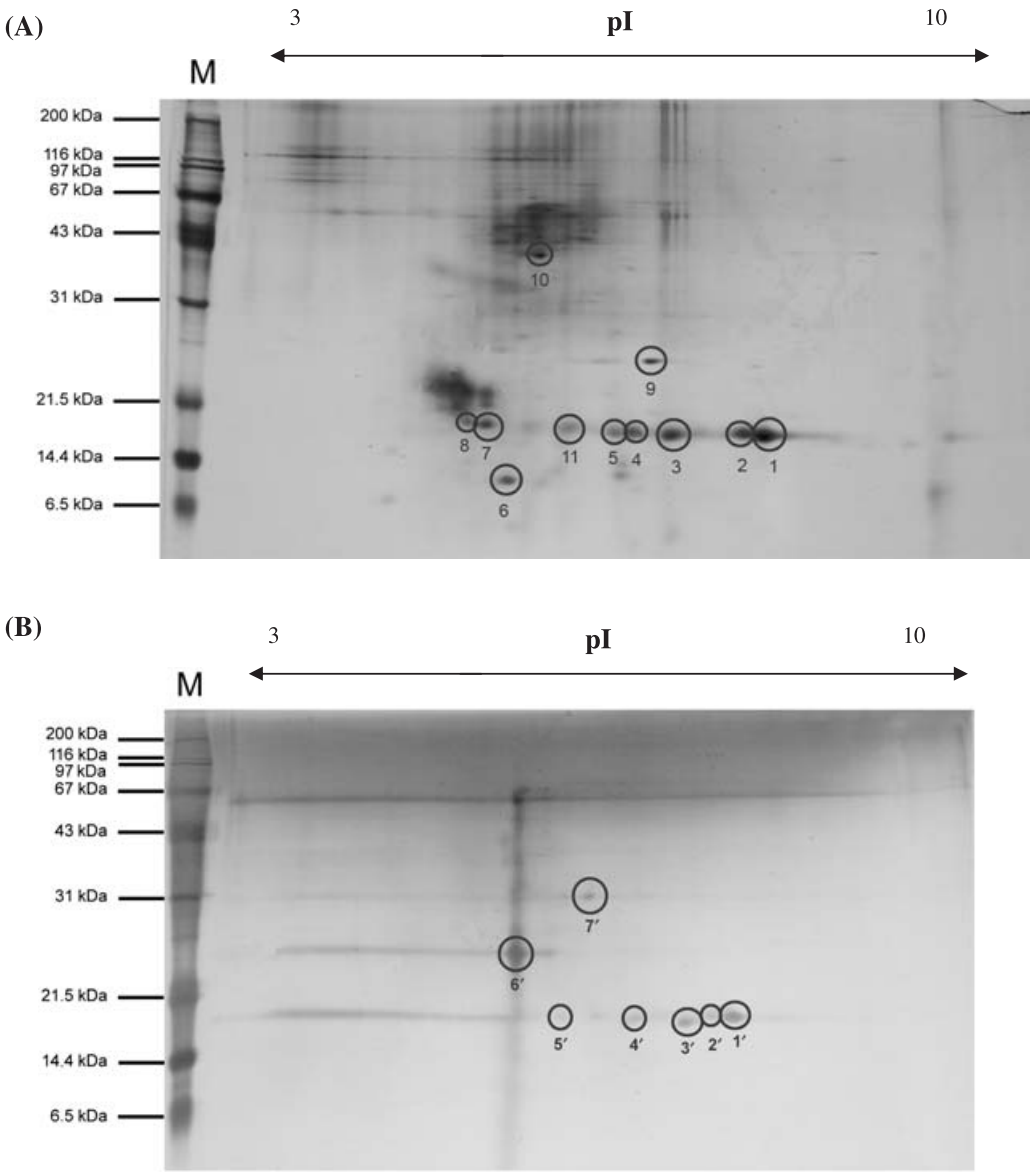

Figure 2. (A) 2D-PAGE of *Myzus persicae* solid saliva proteins. Proteins were separated on a 15% acrylamide gel before being silver stained. (B) 2D-PAGE of *M. persicae* soluble saliva proteins. Proteins were separated on a 15% acrylamide gel before being silver stained.

analysed with Image Master platinum 5.0 software (Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's instructions. Each experiment was repeated in duplicate.

Protein identification after polyacrylamide electrophoresis

Protein spots were manually excised from the gel. Excised gel plugs were first destained (Gharahdaghi *et al.*, 1999) with a 30 mM potassium ferricyanide, 100 mM sodium thiosulphate solution. Gel fragments were then washed three times with water and cysteines were reduced with a 10 mM DTT solution for 45 min at 56 °C followed by alkylation with 50 mM iodoacetamide at room temperature in the dark. Digestion was performed overnight with 12.5 ng of trypsin in 100 mM ammonium carbonate buffer, pH 8.4. The resulting peptides were extracted with 0.1% formic acid in acetonitrile, dried in a vacuum centrifuge and resuspended in 30 µl 0.1% formic acid in water. Peptides were identified in LC MS-MS, as previously described for in-solution digestion.

Peptide analysis

Raw data were analysed in duplicate for each kind of sample and formatted (using Data Analysis software, Bruker Daltonics) for subsequent protein identification against the NCBI nonredundant protein database (last search performed with 8 October 2007 updated database version) and two specific databases constituted from the annotated ESTs of the whole pea aphid *A. pisum* (Sabater-Munoz *et al.*, 2006) and from the annotated ESTs of *M. persicae* (Ramsey *et al.*, 2005; Figueroa *et al.*, 2007) after the last update on 6 October 2007. The numbers of sequences were 28 937 and

76 465 for the peach and pea aphid database, respectively. The MS/MS ions search algorithm on the Mascot search engine was used (www.matrixscience.com). The mass tolerances of precursor and sequence ions were set at 0.5 and 0.3 Da, respectively, and carbamidomethylation of cysteines and methionine oxidation was set as fixed and variable modifications, respectively.

BLAST procedure

EST sequences (obtained from one or some digested peptides) were submitted to a BLAST procedure (BLASTX, NCBI). We only blasted cDNA sequences presenting a significant Mascot score (≥ 30). The resulting protein was characterized by a name, a source, a molecular weight (Mw) and an isoelectric point (pI). Mw and pI were calculated with the Expasy compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html). In order to calculate the coverage per cent of a peptide, the EST sequence was translated into a protein sequence using the Expasy Translate tool (http://www.expasy.org/tools/dna.html). Only BLAST results with a significant E-value ($E < 10^{-5}$) are presented.

All these data are presented in Tables 1 and 2 for in-solution and in-gel digested 2D SDS-PAGE salivary peptides, respectively. Each table is divided into two parts. Part A refers to peptides associated to both an aphid EST and a known protein after the BLAST search in another organism, and Part B refers to peptides associated with EST sequences in aphid databases without any significant homology to known proteins in public databases. The particular aphid EST database reference is shown (aphid species and insect part). The procedure from aphid rearing to protein function identification is represented in Fig. 1.

Assay of glucose oxidase activity

Glucose oxidase activity was assayed using the method of Ohashi *et al*. (1999). The reaction mixture was 800 µl sodium-phosphate buffer 0.1 M, pH 6.1, containing 1.5 M glucose as the substrate and 0.08 mg *o*-dianisidine. In order to determine the amount of H_2O_2 liberated from the glucose, 0.04 ng peroxidase and 10 µl of the test sample was added to the reaction mixture. The solution was incubated for 60 min at 37 °C and then the reaction was halted by adding 10 µl of 1 M HCl; the absorbance at 400 nm (oxidized *o*-dianisidine) was measured using a spectrophotometer.

Acknowledgements

Nicolas Harmel was financially supported by a PhD grant from the Fonds pour la formation à la Recherche dans l'Industrie et l'Agriculture (F.R.I.A.), Belgium. The authors would like to thank the 'Fond National pour la Recherche scientifique (FNRS)' from Belgium for the funding of a proteomic project (FRFC number 2.4.561.06). We would also like to thank Denis Tagu from UMR INRA-Agrocampus BiO3P (INRA Rennes, France) for providing us with the annotated ESTs used for our researches.

References

Alborn, H.T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H. and Tumlinson, J.H. (1997) An elicitor of plant volatiles from beet armyworm oral secretion. *Science* **276**: 945–949.

Alborn, H.T., Hansen, T.V., Jones, T.H., Bennett, D.C., Tumlinson, J.H., Schmelz, E.A. *et al*. (2007) Disulfooxy fatty acids from the American bird grasshopper *Schistocerca americana*, elicitors of plant volatiles. *Proc Natl Acad Sci USA* **104**: 12976–12981.

Baumann, L. and Baumann, P. (1995) Soluble salivary proteins secreted by *Schizaphis graminum*. *Entomol Exp Appl* **7**: 56–60.

Bede, J.C., Musser, R.O., Felton, G.W. and Korth, K.L. (2006) Caterpillar herbivory and salivary enzymes decrease transcript levels of *Medicago truncatula* genes encoding early enzymes in terpenoid biosynthesis. *Plant Mol Biol* **60**: 519–531.

Blackman, R.L. and Eastop, V.F. (2000) *Aphids on the World's Crops: An Identification and Information Guide*, 2nd edn. John Wiley, Chichester, UK.

Cherqui, A. and Tjallingii, W.F. (2000) Salivary proteins of aphids, a pilot study on identification, separation and immunolocalisation. *J Insect Physiol* **46**: 1177–1186.

Cox-Foster, D.L. and Stehr, J.E. (1994) Induction and localization of FAD–glucose dehydrogenase (GLD) during encapsulation of abiotic implants in *Manduca sexta* larvae. *J Insect Physiol* **40**: 235–249.

Cristofoletti, P.T., Ribeiro, A.F., Deraison, C., Rahbé, Y. and Terra, W.R. (2003) Midgut adaptation and digestive enzyme distribution in a phloem feeding insect, the pea aphid *Acyrthosiphon pisum*. *J Insect Physiol* **49**: 11–24.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A.V., Pozo, M.J., Mueller, M.J. *et al*. (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol Plant Microbe Interact* **18**: 923–937.

Divol, F., Vilaine, F., Thibivilliers, S., Amselem, J., Palauqui, J.C., Kusiak, C. *et al*. (2005) Systemic response to aphid infestation by *Myzus persicae* in the phloem of *Apium graveolens*. *Plant Mol Biol* **57**: 517–541.

Effio, P.C., Folgueras-Flatschart, A.V., Montor, W.R., Pernasetti, F.M., Pueyo, M.T. and Sogayar, M.C. (2003) Expression of functional *Anopheles merus* α-amylase in the baculovirus/*Spodoptera frugiperda* system. *Insect Mol Biol* **12**: 415–425.

Eichenseer, H., Mathews, M.C., Bi, J.L., Murphy, J.B. and Felton, G.W. (1999) Salivary glucose oxidase: multifunctional roles for *Helicoverpa zea*? *Arch Insect Biochem Physiol* **42**: 99–109.

Fernie, A.R., Carrari, F. and Sweetlove, L.J. (2004) Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Curr Opin Plant Biol* **7**: 254–261.

Figueroa, C.C., Prunier-Leterme, N., Rispe, C., Sepúlveda, F., Fuentes-Contreras, E, Sabater-Muňoz, B. *et al*. (2007) Annotated expressed sequence tags and xenobiotic detoxification in the aphid *Myzus persicae* (Sulzer). *Insect Sci* **14**: 29–45.

Francis, F., Gerkens, P., Harmel, N., Mazzucchelli, G., De Pauw, E. and Haubruge, E. (2006) Proteomics in *Myzus persicae*: effect of aphid host plant switch. *Insect Biochem Mol Biol* **36**: 219–227.

Gharahdaghi, F., Weinberg, C.R., Meagher, D.A., Imai, B.S. and Mische, S.M. (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20**: 601–605.

Harmel, N., Almohamad, R., Fauconnier, M.L., Verheggen, F., Marlier, M., Haubruge, E. *et al*. (2007) Role of terpenes from aphid infested potato as indirect defence on *Episyrphus balteatus behaviour*. *Insect Sci* **14**: 57–66.

Mattiaci, L., Dicke, M. and Posthumus, M.A. (1995) β-glucosidase: an elicitor of herbivore-induced plant odor that attracts hostsearching parasitic wasps. *Proc Natl Acad Sci USA* **92**: 2036–2040.

Merkx-Jacques, M. and Bede, J.C. (2005) Influence of diet on the larval beet armyworm, *Spodoptera exigua*, glucose oxidase activity. *J Insect Sci* **5**: 48.

Miles, P.W. (1999) Aphid saliva. *Biol Rev Camb Philos Soc* **74**: 41–85.

Montgomery, R.R., Lusitani, D., de Boisfleury Chevance, A. and Malawista, S.E. (2004) Tick saliva reduces adherence and area of human neutrophils. *Infect Immun* **72**: 2989–2994.

Musser, R.O., Cipollini, D.F., Hum-Musser, S.M., Williams, S.A., Brown, J.K. and Felton, G.W. (2005) Evidence that the caterpillar salivary enzyme glucose oxidase provides herbivore offense in solanaceous plants. *Arch Insect Biochem* **58**: 128–137.

Musser, R.O., Farmer, E., Peiffer, M., Williams, S.A. and Felton, G.W. (2006) Ablation of caterpillar labial salivary glands: technique for determining the role of saliva in insect-plant interactions. *J Chem Ecol* **32**: 981–992.

Nishimoto, M., Kubota, M., Tsuji, M., Mori, H., Kimura, A., Matsui, H., *et al*. (2001) Purification and substrate specificity of honeybee, *Apis mellifera* L., α-glucosidase III. *Biosci Biotechnol Biochem* **65**: 1610–1616.

Ohashi, K., Natori, S. and Kubo, T. (1999) Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.). *Eur J Biochem* **265**: 127–133.

Quaglia, F., Rossi, E., Petacchi, R. and Taylor, C.E. (1993) Observations on an infestation by green peach aphids (Homoptera: Aphididae) on greenhouse tomatoes in Italy. *J Econ Entomol* **86**: 1019–1025.

Qubbaj, T., Reineke, A. and Zebitz, C.P.W. (2005) Molecular interactions between rosy apple aphids, *Dysaphis plantaginea*, and resistant and susceptible cultivars of its primary host *Malus domestica*. *Entomol Exp Appl* **115**, 145–152.

Ramsey, J., De Vos, M., Wilson, A., Moran, N., Gray, S., Sun, Q. *et al*. (2005) Microarray analysis of agriculturally relevant gene expression in the peach-potato aphid, *Myzus persicae*. *CSREES/USDA 2005* 35604–15446.

Rhodes, J., Croghan, P. and Dixon, A. (1996) Uptake, excretion and respiration of saccharose and amino acids by the pea aphid *Acyrthosiphon pisum. J Exp Bot* **1999**: 1269–1276.

Ribeiro, J.M.C., Charlab, R., Pham, V.M., Garfield, M. and Valenzuela, J.G. (2004) An insight into the salivary transcriptome and proteome of the adult female mosquito *Culex pipiens quinquefasciatus*. *Insect Biochem Mol Biol* **34**; 543–563.

Sabater-Munoz, B., Legeai, F., Rispe, C., Bonhomme, J., Dearden, P., Dossat, C. *et al*. (2006) Large-scale gene discovery in the pea aphid *Acyrthosiphon pisum* (Hemiptera). *Genome Biol* **7**: R21.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric of proteins from silver stained polyacrylamide gels. *Anal Chem* **68**, 850–858.

Sporn, P.H. and Peters-Golden, M.J. (1998) Hydrogen peroxide inhibits alveolar macrophage 5-lipoxygenase metabolism in association with depletion of ATP. *Biol Chem* **263**: 14776–14783.

Thao, M.L., Baumann, L. and Baumann, P. (2004) Organization of the mitochondrial genomes of whiteflies, aphids, and psyllids (Hemiptera, Sternorrhyncha). *Evol Biol* **4**: 1–13.

Thompson, G.A. and Goggin, F.L. (2006) Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *J Exp Bot* **57**: 755–766.

Tjallingii, W.F. (2006) Salivary secretions by aphids interacting with proteins of phloem wound responses. *J Exp Bot* **57**: 739–745.

Valenzuela, J.G., Pham, V.M., Garfield M.K., Francischetti, I.M.B. and Ribeiro J.M.C. (2002) Towards a description of the sialome of the adult female mosquito *Aedes aegypti*. *Insect Biochem Molec* **32**: 1101–1122.

Valenzuela, J.G., Francischetti, I.M.B., My Pham, V., Garfield, M.K. and Ribeiro, J.M.C. (2003) Exploring the salivary gland transcriptome and proteome of the *Anopheles stephensi* mosquito. *Insect Biochem Mol Biol* **33**: 717–732.

Voelckel, C., Weisser, W.W. and Baldwin, I.T. (2004) An analysis of plant-aphid interactions by different microarray hybridization strategies. *Mol Ecol* **13**: 3187–3195.

Will, T., Tjallingii, W.F., Thonnessen, A. and van Bel, A.J.E. (2007) Molecular sabotage of plant defense by aphid saliva. *Proc Natl Acad Sci USA* **104**: 10536–10541.

Zhu-Salzman, K., Bi, J.L. and Liu, T.X. (2005) Molecular strategies of plant defense and insect counter-defense. *Insect Sci* **12**: 3–15.