Aphid salivary proteases are capable of degrading sieve-tube proteins

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Abstract

Sieve tubes serve as transport conduits for photo-assimilates and other resources in angiosperms and are profitable targets for piercing–sucking insects such as aphids. Sieve-tube sap also contains significant amounts of proteins with diverse functions, for example in signalling, metabolism, and defence. The identification of salivary proteases in *Acyrthosiphon pisum* led to the hypothesis that aphids might be able to digest these proteins and by doing so suppress plant defence and access additional nitrogen sources. Here, the scarce knowledge of proteases in aphid saliva is briefly reviewed. In order to provide a better platform for discussion, we conducted a few tests on *in vitro* protease activity and degradation of sieve-tube sap proteins of *Cucurbita maxima* by watery saliva. Inhibition of protein degradation by EDTA indicates the presence of different types of proteases (e.g. metalloproteases) in saliva of *A. pisum*. Proteases in the watery saliva from *Macrosiphum euphorbiae* and *A. pisum* were able to degrade the most abundant phloem protein, which is phloem protein 1. Our results provide support for the breakdown of sieve-element proteins by aphid saliva in order to suppress/neutralize the defence responses of the plant and to make proteins of sieve-tube sap accessible as a nitrogen source, as is discussed in detail. Finally, we discuss whether glycosylation of sieve-element proteins and the presence of protease inhibitors may confer partial protection against the proteolytic activity of aphid saliva.

Key words: Aphid, aphid–plant interaction, aphid saliva, protease, protease inhibitor, sieve-element occlusion, sieve-tube protein.

General functions of saliva in aphid–plant interaction

Aphids are serious agricultural pest insects causing direct damage to crops as phloem feeders and indirect yield losses by virus transmission. Viruses are secreted together with aphid saliva during cell penetration by the stylet (Powell et al., 1995; Martin et al., 1997). Along the stylet track, plant cells are regularly probed (Tjallingii and Hogen Esch, 1993) by ingesting small amounts of cell sap after secretion of watery saliva into penetrated cells, (Martin et al., 1997), which possibly serves for orientation (Hewer et al., 2010, 2011). Stylet movement is accompanied by pulsed secretion of gel saliva (Miles et al., 1964). After secretion, gel saliva hardens and forms a tubular structure, through which the stylet is pushed forward (Kimmins, 1986; Will et al., 2012). After the stylet has pierced the sieve-element plasma membrane, continuous secretion of watery saliva precedes regular intervals of saliva secretion during sap ingestion (Prado and Tjallingii, 1994). During ingestion, saliva may no longer reach the sieve-element lumen, but rather mixes with ingested phloem sap in the tip region of the stylet, the so-called common duct (Uzest et al., 2010).

Abbreviations: EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; O-GlcNAc, O-linked β-N-acetylglucosamine; PP1, phloem protein 1; PP2, phloem protein 2; RFU, relative fluorescence unit; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SEO, sieve-element occlusion

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Watery saliva contains a variety of proteins (Madhusudhan and Miles, 1998; Carolan et al., 2009; Will et al., 2009; Cooper et al., 2011; Rao et al., 2013), some of which are species-specific, while others appear to be universally present, such as the putative sheath protein and glucose-methanol-choline (GMC) oxidoreductase (Rao et al., 2013). In particular, detoxifying enzymes such as peroxidase and phenoloxidase occur in the saliva of diverse aphid species (Madhusudhan and Miles, 1998; Harmel et al., 2008; Cooper et al., 2011).

In addition, saliva of various aphid species contains proteinaceous effectors, which appear species specific and adapted to specific host plants (summarized in Will et al., 2013). RNA interference techniques and in planta expression of effectors indicate a relevance for aphid–plant interaction by facilitating ingestion, but functions on the molecular level have not been identified to date (Pitino et al., 2011; Pitino and Hogenhout, 2013).

Calcium-binding proteins, detected in saliva of the aphid species *Megoura vicina* (Will et al., 2007) and in the saliva and salivary glands of *Acyrthosiphon pisum* (Carolan et al., 2009, 2011), may belong to the group of effectors, as they presumably interfere with calcium-induced sieve-element occlusion (SEO; see Will et al., 2013). Apart from saliva proteins involved in preventing, suppressing, or manipulating host plant defence, two potential proteases of the M1 and M2 family, respectively, have been detected in *A. pisum* saliva and salivary glands by a proteomic approach (Carolan et al., 2009, 2011). In contrast, no structural homologues have been detected in the saliva of other aphid species (Pitino and Hogenhout, 2013). These conglomertes form either protein depositions on the sieve plates after severe metabolic disturbance (Gaufels et al., 2008a) or gelled exudate plugs on the cutting surface of cucurbit vascular bundles (Alosi et al., 1988; Clark et al., 1997; Furch et al., 2010; Zimmermann et al., 2013).

Other abundant proteins in the protein plugs of *C. maxima* are allocated to the SEO protein family (Ernst et al., 2012). Members of the SEO gene family (Rüping et al., 2010; Zielonka et al., 2014) were first described as a group of occlusion proteins arranged as highly organized spindle-like forisome bodies in *Medicago truncatula* (Pélissier et al., 2008). SEO genes encode forisome subunits in legumes (e.g. Müller et al., 2010; Rüping et al., 2010). In the plant species *Arabidopsis thaliana*, *Nicotiana tabacum*, and *C. maxima* (Anstead et al., 2012; Ernst et al., 2012; Jekat et al., 2012; Zielonka et al., 2014) that do not belong to the Fabaceae, SEO genes encode non-forisome proteins, occurring as loosely organized protein depositions or filaments (Müller et al., 2010; Anstead et al., 2012). In addition to the above-mentioned proteins, numerous antioxidant defence-related proteins were found in cucurbit exudates (Alosi et al., 1988; Walz et al., 2002, 2004). We tackled the question of whether cucurbit sieve-element proteins can be degraded by aphid saliva components by conducting some experiments, as reported in the following section.

**Potential target proteins for salivary proteases from aphids**

Proteomes of sieve-tube sap have been analysed, for example for *Brassica napus*, *Ricinus communis*, and *Cucurbita maxima* (Barnes et al., 2004; Giavalisco et al., 2006; Lin et al., 2009). In the exudate of *C. maxima*, ~1100 different proteins have been identified (Lin et al., 2009). Of those, two structural proteins—phloem protein 1 (PP1) and phloem protein 2 (PP2; Read and Northcote, 1983a, b; Thompson and Schulz, 1999; Walz et al., 2002; Atkins et al., 2011)—were the most abundant. PP1 is a 96 kDa filamentous protein with diameters of 7–13 nm. PP2 is a monomeric (25 kDa) or dimeric (46 kDa) lectin that covalently interacts with PP1 (Read and Northcote, 1983a; Alosi et al., 1988). PP1 and PP2 are linked by oxidation in the presence of free oxygen (Alosi et al., 1988) or reactive oxygen species (Gaufels et al., 2008a). These conglomerates form either protein depositions on the sieve plates after severe metabolic disturbance (Gaufels et al., 2008a) or gelled exudate plugs on the cutting surface of cucurbit vascular bundles (Alosi et al., 1988; Clark et al., 1997; Furch et al., 2010; Zimmermann et al., 2013).

**Saliva of *A. pisum* contains different proteases**

Whereas Cherqui and Tjallingii (2000) were unable to identify proteases in aphid saliva by functional approaches, later proteomic studies provided evidence for two different proteases, an M1 zinc metalloprotease and an angiotensin-converting enzyme (M2 metalloprotease), in the saliva of *A. pisum* (Carolan et al., 2009). Metalloproteases have been found in the saliva of other insects such as the phytophagous thrips *Frankliniella occidentalis* (Stafford-Banks et al., 2014) and the blood-feeding tick *Ixodes scapularis* (Decrem et al., 2008). They are believed to counteract protein-mediated defence responses of their hosts (Francischetti et al., 2003; Carolan et al., 2009; Stafford-Banks et al., 2014). No homologues to metalloproteases from *A. pisum* were detected in the saliva of the two cereal aphid species *S. avenae* and *M. dirhodum* (Rao et al., 2013).

To verify the presence of proteases in saliva of *A. pisum*, we used a fluorescence-based functional assay (Voss et al., 1996). Over a period of 2000 s, degradation of fluorescein isothiocyanate (FITC)-labelled albumin led to an increase in fluorescence that increased rapidly to a maximum intensity of ~2300 relative fluorescence units (RFUs) in ~500 s (Fig. 1). In the presence of 5 mM EDTA, fluorescence reached a maximum of ~1000 RFUs after 600 s (Fig. 1). Thus, the increase of fluorescence is strongly reduced in the presence of EDTA, a chelator of divalent cations. The breakdown time profiles demonstrate the presence of proteases in aphid saliva; the EDTA-reduced breakdown of albumin is indicative for involvement of metalloproteases (Parvathy et al., 1997; Windle and Kelleher, 1997) and non-metalloproteases in protein degradation. A similar reduction of protease activity by EDTA, observed for the tick *I. scapularis*, was also attributed to the presence of metalloproteases in its saliva (Francischetti et al., 2003). Non-metalloproteases were not identified in previous studies, but likely candidates are among the high numbers of unidentified proteins in the salivary glands of *A. pisum* (Carolan et al., 2009, 2011).
As for their function, angiotensin-converting enzymes and proteases of the M1 type bind zinc at their active catalytic domain (Lausten et al., 2001; Kim et al., 2003), while calcium regulates their enzymatic activity (Goto et al., 2007). Although both protease types are putatively exopeptidases (Iturrioz et al., 2001; Naqvi et al., 2005), solely cleaving the terminal amino acids from the albumin body, some M1 metalloproteases in *Escherichia coli* and angiotensin-converting enzymes in *Musca domestica* may possess endopeptidase activity as well (Lamango et al., 1996). One may argue that exopeptidases would hardly degrade albumin due to the globular structure of albumin (Voss et al., 1996) which would limit the increase in fluorescence as a consequence of fluorescence quenching. In contrast, albumin degradation by endopeptidases may produce more and larger fragments, leading to lower quenching and, hence, a stronger increase in detected fluorescence. Based on our results, M1 metalloproteases and angiotensin-converting enzyme of *A. pisum* could both be engaged in the degradation of FITC-labelled albumin. Finally, activities of metalloproteases from other classes must be taken into consideration in future research, since EDTA is not a specific inhibitor of M1 and M2 metalloproteases.

**Impact of salivary (metallo)proteases on plant defence and aphid feeding**

Breakdown of sieve-tube sap proteins involved in defence, signalling, and/ or occlusion may lead to suppression of plant defence (Carolan et al., 2009). A similar role has been attributed to metalloproteases in ticks and thrips, where salivary proteases were believed to degrade defence-related proteins in their respective hosts (Francischetti et al., 2003; Stafford-Banks et al., 2014).

SEO proteins in Fabaceae (Péliéssier et al., 2008) and in *A. thaliana* (Anstead et al., 2012), as well as PP1 and PP2 in cucurbits (Walz et al., 2004; Ernst et al., 2012) are potential targets for salivary proteases to prevent food deprivation (Will et al., 2008). The underlying molecular mechanisms of occlusion by SEO proteins as well as PP1 and PP2 seem to be diverse (Read and Northcote, 1983a; Knoblauch et al., 2001; Furch et al., 2009; Jekat et al., 2013). Degradation of occlusion proteins may therefore be an option for generalist feeders to enable ingestion (e.g. *Myzus persicae* and *Macrosiphum euphorbiae*) rather than for specialists such as *A. pisum*. The latter aphid species does not trigger the dispersion of SEO proteins in *Vicia faba* (Walker and Medina Ortega, 2012), presumably by preventing an increase of calcium in the sieve-tube lumen during stylet penetration (Will et al., 2013). In contrast, *M. euphorbiae* induces sieve-tube occlusion in *V. faba* (Medina-Ortega and Walker, 2014).

The question was raised of whether degradation of sieve-tube proteins by salivary proteases enables aphids to access an amino acid source (Carolan et al., 2009) to supplement the limited amount of essential amino acids in sieve-tube sap (Gündüz and Douglas, 2009). During ingestion, saliva mixes with sieve-tube sap inside the aphid’s stylet tip (Tjallingii, 2006). This could speed up degradation of ingested peptides and proteins, and thus facilitate the digestion in the aphid’s stomach as in *A. pisum* (Rabbé et al., 1995).

The breakdown strategy might work best for dicotyledons because high protein levels were reported for the sieve-tube sap of dicotyledonous plants [e.g. *R. communis* 2–5 mg ml⁻¹ (Schobert et al., 1998), *C. maxima* 19–100 mg ml⁻¹ (Richardson et al., 1982; Schobert et al., 1998; Zimmermann et al., 2013]). Monocotyledonous plants possess lower amounts of protein [e.g. barley 0.1–0.4 mg ml⁻¹ (Schobert et al., 1998; Gaupels et al., 2008b) and wheat 1 mg ml⁻¹ (Fisher et al., 1992)].

The functionality of salivary proteases is speculative, because direct interaction between salivary proteins and sieve-tube proteins was only tested once for the aphid species *M. viciae* in combination with forisomes from *V. faba* without a focus on protease activity (Will et al., 2007). To test proteolysis of sieve-tube proteins by salivary proteases, we collected sieve-tube exudate from *C. maxima* (the exudate is easy to collect) and mixed it with concentrated aphid saliva from *A. pisum* (having a known salivary proteome) or *M. euphorbiae* (a natural pest on cucurbits). Sieve-tube protein degradation was followed by polyacrylamide gel electrophoresis.

Analysis of SDS-PAGE by calculation of trace quantity shows that the protein band intensity of the PP2 monomer (PP2mm) did not decrease with time when phloem exudate

![Fig. 1. Detection of protease activity in watery saliva from Acyrthosiphon pisum. Protease activity assays were conducted using fluorescein isothiocyanate (FITC)-labelled albumin (Sigma-Aldrich, St Louis, MO, USA) that is suitable as a substrate for a wide range of proteases. Its globular structure results in low fluorescence of undigested FITC-labelled albumin due to fluorescence quenching (Voss et al., 1996). Saliva samples were collected and processed as described earlier (Will et al., 2007). To identify the presence of metalloproteases (Carolan et al., 2009, 2011), protease activity of aphid saliva was measured in Tris-buffered saline (TBS, pH 7.2) with/without 5 mM EDTA (Francischetti et al., 2003) as a chelating agent for binding divalent cations (e.g. Ca²⁺, Mg²⁺, and Zn²⁺). Each sample contained 20 μl of aphid saliva from ~4000 aphids, 180 μl of buffer, and 5 μg ml⁻¹ FITC-labelled albumin. The blank sample contained aphid diet (Will et al., 2007) instead of aphid saliva. A Infinite® F200 microplate reader (Tecan, Männedorf, Switzerland) was applied for fluorescence detection with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Samples were incubated in black 96-well plates at 25 °C and were shaken for 1 s prior to each measurement. The relative fluorescence intensity was calculated from 20 measurements per well and was amplified ×50. The entire measuring time encompassed 2000 s with intervals of 25 s. Measurements of relative fluorescence units (RFUs) of the blank were subtracted from RFUs of saliva samples.](image-url)
was mixed with saliva from *M. euphorbiae* and *A. pisum*, respectively (Fig. 2A, B), indicating that lectins are difficult to degrade. This interpretation finds support in the fact that artificially fed lectin was found intact in honeydew of *A. pisum* (Rahbé et al., 1995), implying that gut proteases are unable to digest lectins (Rahbé et al., 1995). In contrast to controls with aphid diet (Fig. 2C), the intensity of PP1 bands declined with time in the presence of aphid saliva.

High degradation rates of PP1 occurred with saliva from *M. euphorbiae* (Fig. 2A) as a possible adaptation to its natural host. Less degradation was observed with saliva from *A. pisum* (Fig. 2A), that does not feed on Cucurbitaceae. Degradation of PP1 may prevent formation of protein plugs (Furch et al., 2009) because PP1 and PP2 need to be cross-linked for plug formation (Read and Northcote, 1983a), whereas PP1 is the primary structural protein (Walker and Thaine, 1971; Walker, 1972). Another option is that *M. euphorbiae* degrades the plugs after their formation, which would require a high proteolytic activity. This mode of action concurs with the observation that *M. euphorbiae* induces sieve-tube occlusion in *V. faba*, but resumes ingestion some time after sieve-element penetration (Medina-Ortega and Walker, 2014).

### Are proteins in sieve-tube sap protected from degradation by salivary proteases?

Given the protease activity in saliva of *A. pisum* (Figs 1, 2B) and *M. euphorbiae* (Fig. 2A), sieve-tube proteins seem to be protected from proteolytic degradation because PP1 and PP2 need to be cross-linked for plug formation. The proteolytic activity of aphid saliva suggests that these proteins are not available for degradation by gut proteases. The concentration of saliva proteases may be too low to degrade PP1 and PP2. However, high degradation rates of PP1 observed with saliva from *M. euphorbiae* suggest a possible adaptation to its natural host. This suggests that saliva proteases may not be able to degrade PP1 and PP2.

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**Fig. 2.** Proteolytic degradation of sieve-tube proteins from *Cucurbita maxima* by aphid saliva. To test the proteolytic activity of aphid watery saliva from *M. euphorbiae* (A) and *A. pisum* (B) on sieve-tube proteins of *C. maxima* (21–28 d after germination), 5 μl of saliva from ~4000 aphids per species was mixed with 0.5 μl of sieve-tube sap. Total protein amount per sample was ~4.2 μg for *M. euphorbiae* and 8.5 μg for *A. pisum* (FluoroProfile Protein Quantification Kit, Sigma-Aldrich). Sieve-tube sap was sampled according to a protocol described by Zimmermann et al. (2013). The samples were incubated for 0, 60, and 120 min, respectively, in a Thermomixer Comfort (Eppendorf, Hamburg, Germany) at 37 °C and 1000 rpm (Williams et al., 1994), and were directly frozen in liquid nitrogen and stored subsequently at −80 °C. As a negative control (C), phloem sap was mixed with artificial diet, used for saliva collection (Will et al., 2007). Samples of pure sieve-tube sap (PS) were taken as the reference in all three treatments. One-dimensional SDS-PAGE of the samples was carried out according to Laemmli (1970) by using a 4% stacking gel and a 12% separation gel in a MiniProtean 3 Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA) with Precision Plus Protein Standard-All Blue (Bio-Rad) as a protein size marker. SDS-polyacrylamide gels were stained with colloidal Coomassie Blue (Roti-Blue; Carl-Roth, Karlsruhe, Germany), documented with the Gel Doc XR system (Bio-Rad), and analysed by means of Quantity One 1-D analysis software (Bio-Rad). Three replicates were made for each aphid species (*A. pisum* and *M. euphorbiae*) and the negative control. The trace quantity, the quantity of a band as measured by the area under its intensity profile curve, of PP1 (*), PP2 dimer (PP2dm; **), and PP2 monomer (PP2mm; ***) was calculated. Trace quantity of pure sieve-tube sap samples was defined as 100% and the percentage of trace quantity of further samples was calculated. Units are intensity/mm.
to be prone to degradation as a result of aphid infestation. An intact set of proteins is mandatory for the fulfilment of the entire spectrum of tasks of sieve tubes (e.g. van Bel et al., 2011, 2014), which calls for protection from proteolysis.

Glycosylation could be one of the tools used for protection. Glycosylation is a post-translational modification, responsible for correct folding (Pfěil, 2002) and stabilization of the tertiary structure of many proteins (Wyss et al., 1995). There is evidence in favour of preventive measures against proteolysis (Russe f et al., 2009). O-linked β-N-acetylglucosamine (O-GlcNAc)-glycosylated proteins were detected in the sieve-tube sap of C. maxima by labelling with an anti-O-GlcNAc monoclonal antibody (Taoka et al., 2007). In-gel detection revealed several proteins with low glycosylation labelling and an intensively labelled protein with a molecular weight of ~28 kDa, most probably PP2.

Protease inhibitors may present an alternative or parallel mode of protecting sieve-tube proteins. Protease inhibitors of sieve-tube sap were reported to protect plants against phytopathogenic insects (Christeller et al., 1998; Ryan, 2000; Walz et al., 2004). An appreciable set of different protease inhibitors, such as phloem-serpin-1, aspartic, tryp tic, and chymotryptic protease inhibitors, and cystatin, which all act against different types of proteases, were found in the sieve-tube sap of Cucurbitaceae (Walz et al., 2004). Infestation by the aphid species Schizaphis graminum increased transcripts of several protease inhibitor genes in Sorghum (Zhu-Salzman et al., 2004). Moreover, infestation with M. euphorbiae or M. persicæ increased protease inhibitor-I and -II transcripts in tomato (De Ilarduya et al., 2003). In poplars, Kuntriz trypsin inhibitor 3 expression is increased as a response to aphid colonization (Voelckel et al., 2004). This on-demand expression of protease inhibitors potentially enables a flexible reallocation of resources inside the plant.

In conclusion, plants appear to possess mechanisms to protect sieve-tube proteins from degradation. At the same time, degradation experiments (Fig. 2) show that plant defence mechanisms such as protein glycosylation and protease inhibitor production may fall short for full protection from proteolysis by saliva proteases at an initial state of aphid infestation. Degradation of sieve-element proteins may therefore be a dynamic balancing act between proteolytic attack and defence.

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References


