An immuno-suppressive aphid saliva protein is delivered into the cytosol of plant mesophyll cells during feeding.

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Abstract

Herbivore selection of plant hosts and plant responses to insect colonization have been subjects of intense investigations. A growing body of evidence suggests that for successful colonization to occur, (effector/virulence) proteins in insect saliva must modulate plant defense responses to the benefit of the insect. A range of insect saliva proteins that modulate plant defense responses have been identified, but there is no direct evidence that these proteins are delivered into specific plant tissues and enter plant cells. Aphids and other sap-sucking insects of the order Hemiptera use their specialized mouthparts (stylets) to probe plant mesophyll cells, until they reach the phloem cells for long-term feeding. Here we show by immunogold-labeling of ultrathin sections of aphid feeding sites that an immuno-suppressive aphid effector localizes in the cytoplasm of mesophyll cells near aphid stylets, but not in cells further away from aphid feeding sites. In contrast, another aphid effector protein localizes in the sheaths composed of gelling saliva that surround the aphid stylets. Thus, insects

26 deliver effectors directly into plant tissue. Moreover, different aphid effectors locate
27 extracellularly in the sheath saliva or are introduced into the cytoplasm of plant cells.
28
29 **Introduction:**
30
31 Insect herbivores likely modulate a range of plant processes in order to successfully
32 colonize their host plants. A growing body of evidence suggests that the saliva of
33 herbivorous insects contains virulence factors (effectors) that interfere with host plant
34 defences and facilitate colonisation (Musser et al. 2002; Will et al. 2007; Mutti et al
35 2008; Bos et al. 2010; Stuart et al. 2012; Atamian et al. 2013; Pitino and Hogenhout
36 2013; Chaudhary et al., 2014; Elzinga et al. 2014; Guo et al. 2014; Rodriguez et al.
37 2014; Naessens et al. 2015; Wang et al. 2015) Sap-sucking insects of the order
38 Hemiptera, such as aphids, whiteflies, leafhoppers, psyllids and planthoppers, are
39 stealthy feeders. These insects possess piercing-sucking mouthparts, consisting of a
40 pair of stylets, which navigate to vascular elements for long-term feeding.
41 Hemipterans cause direct feeding damage to plants and also transmit the majority of
42 plant viruses and several plant pathogenic bacteria (Hogenhout et al 2008; Guerrieri
43 and Digilio 2008; Orlovskis et al. 2015). The green peach aphid (GPA) *Myzus*
44 *persicae* alone transmits over a 100 plant viruses.
45
46 The feeding behaviour of aphids has been studied extensively. While navigating to the
47 phloem, in the pathway phase, aphid stylets probe various plant cells of the mesophyll
48 (Tjalingi 2006). During a probe the aphid stylets penetrate cells, deposit saliva inside,
49 and acquire cell contents (Martín et al. 1997). The pathway phase continues until the
50 aphid reaches a phloem sieve-element cell, where they remain to feed often for many

51 hours. The phloem feeding phase starts with release of saliva followed by extended
52 periods of phloem sap acquisition that are occasionally interrupted with short
53 salivation periods. Whereas it has been shown that this feeding behaviour is
54 responsible for the transmission of a diversity of viruses by aphids, the biological
55 significance of this behaviour to the aphids themselves is not fully understood.
56
57 Aphids produce different types of saliva: 'gelling' saliva that forms a protective
58 sheath around the stylets, and 'watery' saliva that is thought to be injected into plant
59 cells during the pathway and phloem feeding phases (Tjallingii and Esch 1993;
60 Martín et al. 1997). A number of proteins are detected in aphid saliva (Ramsey et al.
61 2007; Harmel et al. 2008; Carolan et al. 2009; Cooper et a. 2010; Nicholson et al.
62 2012, 2014; Rao et al. 2013; Vandermoten et al. 2014; Chaudhary et al. 2015). Aphid
63 saliva proteins have also been detected in lysate of plant tissues previously exposed
64 to aphids (Mutti et al. 2008; Naessens et al. 2015). Some components of aphid saliva
65 trigger plant defence responses that are characteristic of pattern-triggered immunity
66 (PTI), including reactive oxygen species (ROS) bursts and callose deposition, that
67 require the plant membrane-associated receptor-like kinase (RLK)
68 BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE (BAK1)
69 (Chaudhary et al. 2014; Prince et al. 2014). BAK1 is a key regulator of several cell
70 membrane-localized pattern recognition receptors (PRRs), which mediate the first
71 step of the plant defence response via the recognition of pathogen-associated
72 molecular patterns, such as flg22 (Chinchilla et al. 2007; Chaudhary et al. 2014).
73
74 Aphid saliva contains effectors that suppress plant defence responses (Will et al.
75 2007; Mutti et al 2008; Bos et al. 2010; Atamian et al. 2013; Pitino and Hogenhout

76 2013; Chaudhary et al., 2014; Elzinga et al. 2014; Guo et al. 2014; Rodriguez et al.
77 2014; Naessens et al. 2015; Wang et al. 2015). Mp10 is one of a number of candidate
78 effector proteins that have been identified and was found to suppress the plant ROS
79 burst in response to flg22 (Bos et al. 2010; Rodriguez et al. 2014). Mp10 and another
80 candidate effector protein expressed in GPA salivary glands, MpOS-D1, belong to the
81 chemosensory protein (CSP) family (Bos et al. 2010; Zhou et al. 2010). Other aphid
82 effector candidates, such as MpPIntO1 and MpC002, promote aphid colonization
83 (Mutti et al 2008; Pitino and Hogenhout 2013; Pitino et al. 2011; Coleman et al. 2015)
84 and are found in aphid saliva and/or in extracts of aphid-exposed leaves (Mutti et al
85 2008; Harmel et al. 2008). However, evidence that insects deliver effectors directly
86 into plant cells is currently lacking.
87
88 How pathogens and pests deliver effectors to the appropriate site of action has been a
89 major research topic of many research groups (Dodds and Rathjen 2010). Gram-
90 negative bacterial pathogens deliver their effectors via specialized mechanisms, such
91 as type III secretion systems (Gopalan et al. 1998). For fungi and oomycetes it was
92 discovered that effectors require conserved sequences, such as an RXLR motif, at
93 their N-termini for entering the plant host, but the mechanisms underlying their
94 delivery is not fully understood (Petre and Kamoun 2014). It is as yet unclear how,
95 where and when aphids deposit their effectors in the plant; they may do so via the
96 'gelling' saliva to embed the proteins within the sheaths surrounding the stylets in the
97 apoplastic space of plant tissue, or via 'watery' saliva to introduce them into plant
98 cells. In addition, aphids may secrete different effectors during the pathway and
99 phloem feeding phases.
100

101 Here we studied ultra-thin sections of plant tissues containing tracks of aphid stylets
102 and used antisera raised to the aphid effectors Mp10, MpOS-D1, MpC002 and
103 MpPIntO1 for immuno-gold labelling of the sections. Antisera of Mp10, but not of
104 MpOS-D1, labelled the cytosol and chloroplasts of plant mesophyll cells adjacent to
105 aphid stylet tracks, whereas antisera to MpPIntO1 and MpC002 labelled the aphid
106 stylet sheaths. These data indicate that aphid effectors are delivered into the cytosol of
107 plant cells during probing in the pathway phase, whereas other effectors are
108 embedded within the sheaths that surround the stylets in the apoplastic space of
109 mesophyll tissue.
110
111 **Results**
112
113 **Candidate effector proteins are detected in total protein extracts of aphid-**
114 **exposed leaves**
115
116 Affinity purified antisera were raised against recombinant GPA candidate effectors
117 and examined for specificity and background levels in aphid and plant extracts by
118 protein immuno-blotting. The antisera to Mp10, MpOSD1, MpPIntO1 and MpC002
119 detected bands of the predicted sizes in total protein extracted from whole aphids
120 (Fig. 1). Antisera to the CSPs Mp10 and MpOS-D1 detected Mp10 and MpOS-D1,
121 respectively, down to the ng level (Supplementary Fig. S1), indicating that the
122 antisera to these proteins are specific and sensitive. The antisera to all four effectors
123 detected proteins of the predicted sizes in extracts of *A. thaliana* leaves previously
124 exposed to GPA (Fig.1), but not in unexposed leaves. These bands were not detected
125 in blots probed with corresponding pre-immune sera (Supplementary Fig. S2). The

126 Mp10 and MpOS-D1 antibodies and pre-immune sera reacted with some non-specific
127 bands of different sizes in the aphid-infested and uninfested plant tissues, but the
128 bands corresponding to the Mp10 and MpOS-D1 proteins were only detected in
129 infested tissues and not with the pre-immune sera (Fig. 1, Supplementary Fig. S2).
130 Thus, GPA delivers all four effectors into plant tissues during feeding.
131
132 **The aphid effector protein Mp10 localizes to the cytoplasm and chloroplasts of**
133 **mesophyll cells adjacent to aphid feeding sites**
134
135 To investigate if GPA delivers effectors in or near feeding sites, semi-thin sections of
136 GPA-infested leaf samples were stained with toluidine blue to localize GPA stylets
137 tracks (Fig. 2A). Ultra-thin sections of the same sample were then used for immuno-
138 gold-labelling (IGL) with the Mp10 antiserum (Fig. 2B-D). Controls included the
139 GPA-infested *A. thaliana* leaf samples sections incubated with pre-immune sera and
140 sections of uninfested *A. thaliana* leaf samples incubated with antisera (Fig, 3, 4 and
141 Supplementary Fig. S2).
142
143 IGL with Mp10 antiserum (dilutions 1:10 and 1:100) showed high labelling density in
144 cytoplasm and chloroplasts of mesophyll cells adjacent to the aphid stylets tracks in
145 two independently prepared GPA-infested leaf samples (Fig. 2B-D, 3A-B), whereas
146 only low density background labelling was observed with pre-immune sera (dilution
147 1:50) in the sections of aphid-infested leaves (Fig. 3C) or with Mp10 antisera
148 (dilution 1:10) in sections of uninfested control samples (Fig. 3D). Thus, despite some
149 aspecific labelling of plant proteins on immuno-blots (Fig. 1), the labelling of plant
150 tissue adjacent to the aphid feeding site is specific. Quantification of the labelling

151 seen on micrographs revealed consistently more labelling with Mp10 antisera of the
152 cytosol and chloroplasts with Mp10 antisera in mesophyll cells adjacent to aphid
153 feeding sites compared to the two control treatments (Fig. 3G, Supplementary Fig.
154 S3). Labelling of mesophyll cells distal to the site of feeding was not detected (Fig.
155 3E; Supplementary Fig. S3). Chloroplasts are known to be prone to high levels of
156 non-specific background labelling in IGL experiments, however we do not see similar
157 levels of labelling of plastids in the uninfested or pre-immune controls, or of plastids
158 distal to the site of feeding. Therefore, these data suggest that Mp10 localizes in the
159 cytosol and chloroplasts of mesophyll cells adjacent to the stylets tracks.
160
161 Occasionally we found that cells adjacent to the stylets' track contained unusual
162 features (Fig. 3 A, C). The cytoplasm of these cells showed reduced granularity and a
163 higher number of vesicle-like structures that may be endoplasmic reticulum (ER)-
164 derived. These were not seen in all aphid-infested samples (e.g. Fig. 3 B), but may
165 represent a response of the cell to aphid probing.
166
167 Because GPA feeds from the phloem sieve cells, we quantified Mp10 antisera
168 labelling of the vascular bundles closest to the aphid feeding site (Fig. 2 A; Fig. 3F).
169 We did not detect specific labelling of any cell type in the vascular tissue in
170 experimental compared to the control samples (Supplementary Fig. S2B) suggesting
171 that Mp10 is not delivered into the vasculature or is not present at high enough
172 concentrations for detection in these tissues.
173 We also investigated labelling with antisera to MpOS-D1, a family member of Mp10,
174 in and near stylets tracks. We did not detect specific labelling of mesophyll or
175 vascular tissues with MpOS-D1 antisera in or near GPA feeding sites compared to

176 controls (Supplemental Fig. S2C, D). While the Mp-OSD1 antisera detected
177 denatured MpOS-D1 protein on immuno-blots (Fig. 1), it is possible that these
178 antisera do not detect this effector in resin sections. Alternatively, GPA may not
179 deliver Mp-OSD1 or delivers this effector at low concentrations or to different
180 locations inside plant tissues compared to Mp10.
181
182 **Aphid effector MpPIntO1 localizes to the sheaths surrounding aphid stylets at**
183 **feeding sites.**
184
185 To compare localizations of aphid effectors, we also conducted IGL with antisera to
186 MpPIntO1 and MpC002 on ultra-thin sections of tissues containing aphid stylets
187 tracks. We observed very strong and specific labelling of the stylets sheaths by the
188 MpPIntO1 antisera (Fig. 4B; Suppl. Fig. 2G), but not by the corresponding pre-
189 immune serum (Fig. 4C; Suppl. Fig. 2G).The MpC002 antisera also labelled the
190 sheaths more strongly than other tissues in the plant and this labelling was stronger
191 than detected with the corresponding pre-immune sera (Fig. 4D-E; Suppl. Fig. 2E).
192 However, the background labelling of uninfested plant tissue by the MpC002 antisera
193 was much higher than for MpPIntO1, suggesting that anti-MpC002 may be less
194 specific (Suppl. Fig. 2E-H). These data suggest that MpPIntO1, and possibly also
195 MpC002, are found in the sheaths that surround the stylets during feeding.
196
197 **Discussion**
198
199 In this study, we generated specific antisera to the GPA proteins Mp10, MpOS-D1,
200 MpC002 and MpPIntO1, which were previously shown to have activities

201 characteristic of effector proteins (Mutti et al 2008; Bos et al. 2010; Pitino et al. 2011;
202 Pitino and Hogenhout 2013), and used these antisera in immuno-gold labelling
203 experiments to examine if GPA secretes effector proteins into plants during feeding.
204 We found that the Mp10 antisera specifically labelled the cytoplasm and chloroplasts
205 of mesophyll cells adjacent to the aphid stylet tracks. No such labelling was observed
206 in cells further removed from the stylets tracks. In addition, immuno-gold labelling
207 experiments with the antisera to the three other effectors did not label these
208 compartments. Finally, we observed no or much reduced labelling densities in the
209 pre-immune sera and uninfested controls. Taken together, these experiments provide
210 strong evidence for Mp10 delivery by GPA into mesophyll cells, where Mp10 resides
211 in the cytosol and the chloroplasts.
212
213 We previously found that Mp10 suppresses the flg22-induced ROS burst (Bos et al.
214 2010) which is dependent on BAK1 (Chinchilla et al. 2007). The ROS burst seen in
215 response to aphid elicitors is also dependent on BAK1 (Chaudhary et al. 2014; Prince
216 et al. 2014). BAK1 is a key regulator of several plasma membrane-localized pattern
217 recognition receptors (PRRs), which sense potential invaders early on and trigger
218 downstream immune signalling. Thus, given that we found Mp10 in the cytoplasm of
219 mesophyll cells adjacent to stylets tracks, this effector may suppress the ROS burst
220 induced by aphids shortly after initial probing by the aphid in the pathway phase. The
221 additional detection of Mp10 in chloroplasts is of interest because chloroplasts are a
222 major sources of ROS and have a critical role in PTI (Shapiguzov et al. 2012; Nomura
223 et al. 2012; Caplan et al. 2015).
224

225 In addition to the suppression of PTI, heterologous expression of Mp10 in plants
226 elicits an effector-triggered immunity (ETI)-type response, leading to chlorosis and
227 activation of JA- and SA- signalling pathways (Bos et al. 2010; Rodriguez et al.
228 2014). However, in these expression experiments Mp10 is present at high levels in
229 many cells, whereas our data indicate that Mp10 is delivered in only some cells near
230 the aphid stylets in the presence of other effectors (such as MpPIntO1). Hence, it
231 remains to be investigated how Mp10 modulates cell defense responses during aphid
232 feeding.
233
234 Our IGL data show different localizations of MpPIntO1 and MpC002 compared to
235 Mp10. MpPIntO1 and MpC002 are abundant proteins in aphid saliva and can be
236 readily detected by protein immuno-blotting in artificial diets fed upon by aphids and
237 extracts of aphid-exposed leaves (Mutti et al. 2008; Harmel et al. 2008). Our IGL data
238 provide evidence that MpPIntO1, and possibly also MpC002, are present in the stylets
239 sheaths. We did not detect MpPIntO1 inside plant mesophyll and vascular cells
240 indicating that aphids do not deliver this effector inside these cells or else they are
241 present at or below detection-level concentrations. Our data are in agreement with
242 previous findings showing that MpPIntO1 is abundant in the gelling saliva, which
243 generate the sheaths (Harmel et al. 2008). We previously found that aphids reproduce
244 better on transgenic *A. thaliana* which produce MpPIntO1 and MpC002 under control
245 of 35S promoters (Pitino and Hogenhout 2013), though the effect of MpPIntO1 on
246 aphid performance is variable (Pitino and Hogenhout 2013; Elzinga et al. 2014). In
247 addition, RNA interference (RNAi)-mediated knock down of MpC002 in aphids
248 reduced aphid performance on plants, whereas MpPIntO1-RNAi aphids were not
249 affected (Pitino et al. 2011). Several pathogens deliver effectors in the apoplastic

250 space to modulate defence responses (Tian et al. 2004, 2007; De Wit 2016). Our IGL
251 data open up the possibility that the aphid MpPIntO1 and MpC002 effectors act in the
252 plant apoplast from within sheath saliva.
253
254 In some cases, changes in subcellular structures occurs in response to aphid feeding
255 (Fig. 3, A, C). It is known that pathogen attack can induce dramatic reorganisations of
256 subcellular structures, including organelles (Hardham et al. 2008; Ben Khaled et al.
257 2015). Aphids can also cause significant tissue damage and disruption of plant tissue
258 during feeding (Saheed et al. 2007). Moreover, probing of cells by aphid stylets
259 induces a rapid subcellular relocalization of vesicle-associated *Cauliflower mosaic*
260 *virus* (CaMV) particles that is essential for the acquisition and transmission of this
261 virus by aphids (Martiniere et al. 2013). The increase in abundance of vesicular
262 structures we observed might be associated with the activation of plant defence
263 responses, such as the delivery of defence compounds via vesicles to deter attack.
264 Alternatively, aphid effectors may modulate plant cell processes, such as the
265 reprogramming of vesicle trafficking, as has been shown to occur for pathogens (Ben
266 Khaled et al. 2015; Bozkurt et al. 2015).
267
268 **Materials and methods**
269
270 **Antibody generation**
271
272 Coding sequences corresponding to the predicted mature effector proteins (minus
273 predicted secretory signal peptide) were expressed in *Escherichia coli* as *N*-terminal
274 6Xhis tag fusions (Mp10, MpPIntO1 and MpOSD1) or as a *N*-terminal dC, and 6XHis

275 tag fusion (MpC002), purified and checked for purity by protein immuno-blotting
276 using anti-His-tag antibody (BacPower and FoldArt technologies, Genscript). Proteins
277 were injected into chicken (Mp10) or rabbit (MpOSD1, MpPIntO1, and MpC002) by
278 Genscript. Specific antisera were affinity purified using immobilized recombinant
279 protein. Aliquots of pre-immune serum were collected from the animals before
280 injection.
281
282 **Western blotting**
283
284 *Arabidopsis thaliana* Col-0 plants were exposed to GPA (*Myzus persicae* clone O) for
285 24h (1000 aphids per 3-week old plant), aphids were carefully removed, and the plant
286 rosette was rinsed in distilled water to remove any remaining aphid material from the
287 surface. Total protein was extracted from plant and aphids in sodium dodecyl sulfate
288 (SDS) buffer (5 µl per mg plant tissue, or 200 µl per mg aphid tissue), and 10 µl was
289 size-separated by SDS-polyacrylamide gel electrophoresis and blotted onto
290 nitrocellulose membrane using standard methods. Blots were probed overnight with
291 1:100 or 1:1000 antibody dilutions (or with pre-immune sera at double the
292 concentrations), and a secondary antibody-horseradish peroxidase-conjugate.
293
294 **Immuno-localisation**
295
296 Samples were harvested from aphid-infested (or uninfested) *A. thaliana* plants and
297 immediately fixed in 4% formaldehyde / 0.5% glutaraldehyde in phosphate-buffered
298 saline (PBS) at 4 degrees overnight. The samples were then embedded in LR White
299 resin (Agar Scientific) using the progressive lowering of temperature (PLT) method

300 using the Leica EM AFS2 (Automatic Freeze Substitution) machine (Caillaud et al.
301 2014). Sections were prepared on a Leica UC6 ultramicrotome. Sections of 0.5 µm
302 were stained with toluidine blue for light microscopy (Nikon Microphot-SA) and
303 ultrathin sections of approximately 90 nm, were picked up on to pyroxylin- and
304 carbon-coated gold grids (Agar Scientific) for immuno-labelling.
305
306 The ultrathin sections were immuno-gold labelled according to the following
307 protocol: 50mM glycine/PBS for 15 mins; Aurion blocking buffer (Aurion, The
308 Netherlands for 30 mins; 0.1% acetylated bovine serum albumin (BSA-C) (Aurion,
309 The Netherlands) in PBS for 2 X 5 mins; primary antibody of choice at 1:10 to 1:100
310 dilutions in 0.1% BSA-C/PBS for 90 mins; 0.1% BSA-C/PBS for 6 X 5 mins;
311 secondary antibody conjugated to 10nm gold particles (1/40 dilution in 0.1% BSA-
312 C/PBS) for 90 mins; 0.1% BSA-C/PBS for 6 X 5 mins; PBS for 2 X 5 mins; and
313 water for 2 X 3 mins.
314
315 Grids were viewed in a FEI Tecnai 20 transmission electron microscope (FEI UK Ltd,
316 Cambridge, UK) at 200 kV and imaged using an AMT XR60 digital camera (Deben,
317 Bury St Edmunds, UK) to record TIF files. Images were analysed using ImageJ. The
318 areas and the numbers of gold particles detected in each subcellular compartment
319 were measured across a series of images across each sample. The density across
320 different compartments in infested/uninfested samples were analysed by general
321 linear model (GLM, Genstat). Where differences in labelling intensity were detected
322 between aphid-infested and uninfested samples, replicate sections were probed with
323 the corresponding pre-immune serum, at a higher concentration that the antibody was
324 used at, to demonstrate that labelling was specific.

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Figure legends

Figure 1. Aphid salivary proteins are detected in extracts of aphid-infested host plant tissue.

Total protein extracts from whole aphids (GPA) (lane 8) and *A. thaliana* rosettes uninfested (lanes 1-2, two different plants) or infested with GPA (lanes 3-5, three different plants) were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antisera raised against the aphid proteins Mp10, MpOS-D1, MpPIntO1 and MpC002 as shown at the left of the blots. The bottom panel shows an amido-black stained loading control. Arrows at right point to bands of predicted sizes for the effectors, as indicated. Arrowheads indicate non-specific bands detected by antibodies.

Figure 2. Mp10 protein is detected in the cytoplasm of mesophyll cells near an aphid stylets track.

(A): Semi-thin section (0.5 µm) from an aphid-infested *A. thaliana* leaf stained with toluidine blue. Aphid stylets tracks (ST) are visible between mesophyll cells (Mc) and a vascular bundle (VB). (B-D): Immuno-gold labeling of ultra-thin sections (90 nm)

350 of the tissue shown in (A) with anti-Mp10 (1:10 dilution) revealed a high density of
351 gold particles (arrows in D) in the cytoplasm (Cy) of mesophyll cells (Mc). No
352 obvious labeling was observed of aphid stylets (St), cell wall (CW), intercellular
353 space (IS), mesophyll cell vacuole (V) and inside vesicles (Ve). Images shown in B to
354 D are sequentially higher magnifications of the same area of a cell adjacent to the
355 stylets track. Scale bars, 500 nm.
356
357 **Figure 3. Mp10 detection is specific and restricted to the cells adjacent to stylets**
358 **tracks.**
359 Ultra-thin sections from *A. thaliana* leaves infested with GPA, and labeled with anti-
360 Mp10 (1:100 dilution) show labelling (examples indicated with arrows) in the cytosol
361 "Cy" (A-B, G) and plastid "P" (B, G) of cells adjacent to the stylets track, but not
362 when labelled with pre-immune serum (1:50) (C, G). Samples from uninfested plants
363 are not labelled with anti-Mp10 above background levels (D, G). Labeling with anti-
364 Mp10 was not detected in mesophyll cells distal to the feeding site (E), or an area of
365 the vascular bundle closest to the feeding site in GPA-infested tissues (F).
366 Quantification of labelling density (G) based on measurements of compartment area
367 and gold particle count across multiple images of different areas from the samples.
368 Values show mean (+/- SEM) density (infested, antiMp10 (at 1:100 dilution) n=13;
369 infested, pre-immune (1:50) n=4, uninfested anti-Mp10 (1:100) n=5). Asterisks
370 indicate significant differences in labelling both between infested vs. uninfested
371 samples, and between anti-Mp10 and pre-immune labelled samples for that
372 compartment (p<0.01, GLM). Abbreviations: "IS" intercellular space; "Cy"
373 cytoplasm; "V" vacuole; "CW" cell wall; "Ve" vesicles; "P" plastid; "U" unidentified
374 compartment. In (F): "CC" is a phloem companion cell, and "SE" are phloem sieve

375 elements. Insets (A, C, D and F) are magnified regions of the image, all scale bars are
376 500 nm.
377
378 **Figure 4. MpPIntO1 and MpC002 antisera label the stylets sheaths**
379 Ultra-thin sections from aphid-infested samples showing the gelling saliva that forms
380 the stylets sheath ("ST"), immuno-gold labelled with anti-MpPIntO1 (A-B at 1:100
381 dilution) or with the corresponding pre-immune serum (C, 1:50); or with anti-
382 MpC002 (D, 1:100) or the corresponding pre-immune serum (E, 1:50). Examples of
383 gold-labelling, where present, are indicated with arrows. Compartments are indicated
384 as: "ST" stylet track; "IS" intercellular space; "Cy" cytoplasm; "V" vacuole; "CW"
385 cell wall; "Ve" vesicles; "P" plastid; "M" mitochondria. Scale bars all indicate 500
386 nm. Quantification of labelling density can be found in Supplemental Fig. 3.
387
388 **Supplementary Figure 1. Mp10 and MpOSD1 antisera are specific and do not**
389 **cross react.**
390 Immuno-blots of pure recombinant Mp10 and MpOSD1 proteins probed with antisera
391 raised against the proteins show the sensitivity and specificity of these antisera. (A)
392 Replicate blots of a range of quantity of Mp10 protein (1-25ng) and 25ng OSD1
393 protein probed with (from top) decreasing concentration of the anti-Mp10 serum
394 (from 1:500 to 1:10,000) or with corresponding pre-immune serum (at 1:1000
395 dilution). (B) Replicate blots of a range of quantities of OSD1 protein (1-25ng) and
396 25ng Mp10 protein probed with (from top) decreasing concentration of the anti-OSD1
397 serum (from 1:500 to 1:10,000) or with corresponding pre-immune serum (at 1:1000
398 dilution).
399

400 **Supplementary Figure 2. Immuno-blots of aphid-exposed plant tissue probed**
401 **with antisera raised against candidate effector proteins and also with**
402 **corresponding pre-immune serum.**
403 Immuno-blots of total protein extracts from Arabidopsis rosettes; uninfested (lanes 1-
404 2) or infested with GPA (lanes 3-5), and extract from GPA aphids (lane 9), were
405 probed with antisera raised against the aphid salivary proteins (as shown in Fig. 1),
406 and with the corresponding pre-immune sera (PI); (from the top) Mp10, Mp10-PI,
407 OSD1, OSD1-PI, MpPIntO1, MpPIntO1-PI, MpC002, and MpC002-PI. The bottom
408 panel shows loading of the plant extract samples by amido-black staining of the
409 membrane. Arrowheads indicate bands of the expected sizes detected in aphid-
410 exposed plants and aphids, but not unexposed plants. Arrows indicate presumably
411 non-specific bands of other sizes. Pre-immune sera were used at higher concentrations
412 than all corresponding antibodies to ensure that specific antibody-labelled bands were
413 not also detected by pre-immune serum.
414
415 **Supplementary Figure 3. Quantification of immuno-gold labelling over different**
416 **cellular compartments and tissues.** Graphs show the density of gold particles in
417 different subcellular compartments (mean +/- SEM (n= from 3 to 13)) for (left) cells
418 adjacent to the aphid stylet track (or equivalent mesophyll cells from uninfested
419 tissues), and (right) cells in the vascular bundle. Samples were probed with affinity-
420 purified antibody (all at 1:100 dilution) raised against Mp10 (A-B); MpOSD1 (C-D);
421 MpC002 (E-F) or MpPIntO1 (G-H), or (where indicated) with the corresponding pre-
422 immune serum (all at 1:50 dilution). Asterisks indicate significant difference in
423 labelling both between infested and uninfested samples, and between antibody and
424 pre-immune labelled samples for that compartment ($p < 0.01$, GLM). Plus signs

425 indicate significantly higher labelling of stylet track relative to other compartments in
426 infested tissue (for which no uninfested control plants could be tested) and for which
427 the signal was significantly higher than for infested samples probed with the
428 corresponding pre-immune serum ($p < 0.01$, GLM). "ND": Not determined.
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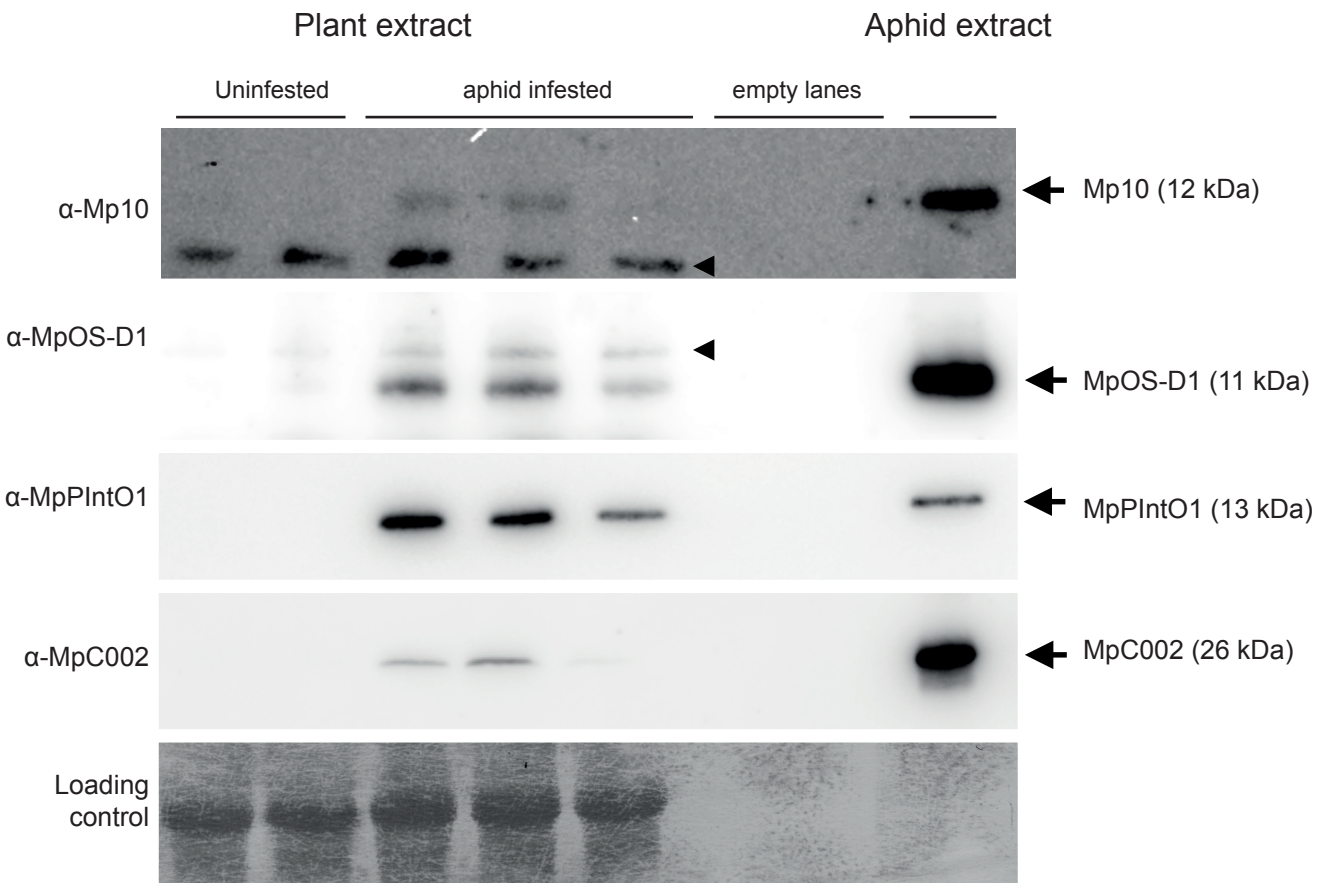


Figure 1. Aphid salivary proteins are detected in extracts of aphid-infested host plant tissue.
Total protein extracts from whole aphids (GPA) (lane 8) and *A. thaliana* rosettes uninfested (lanes 1-2, two different plants) or infested with GPA (lanes 3-5, three different plants) were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antisera raised against the aphid proteins Mp10, MpOS-D1, MpPIntO1 and MpC002 as shown at the left of the blots. The bottom panel shows an amido-black stained loading control. Arrows at right point to bands of predicted sizes for the effectors, as indicated. Arrowheads indicate non-specific bands detected by antibodies.

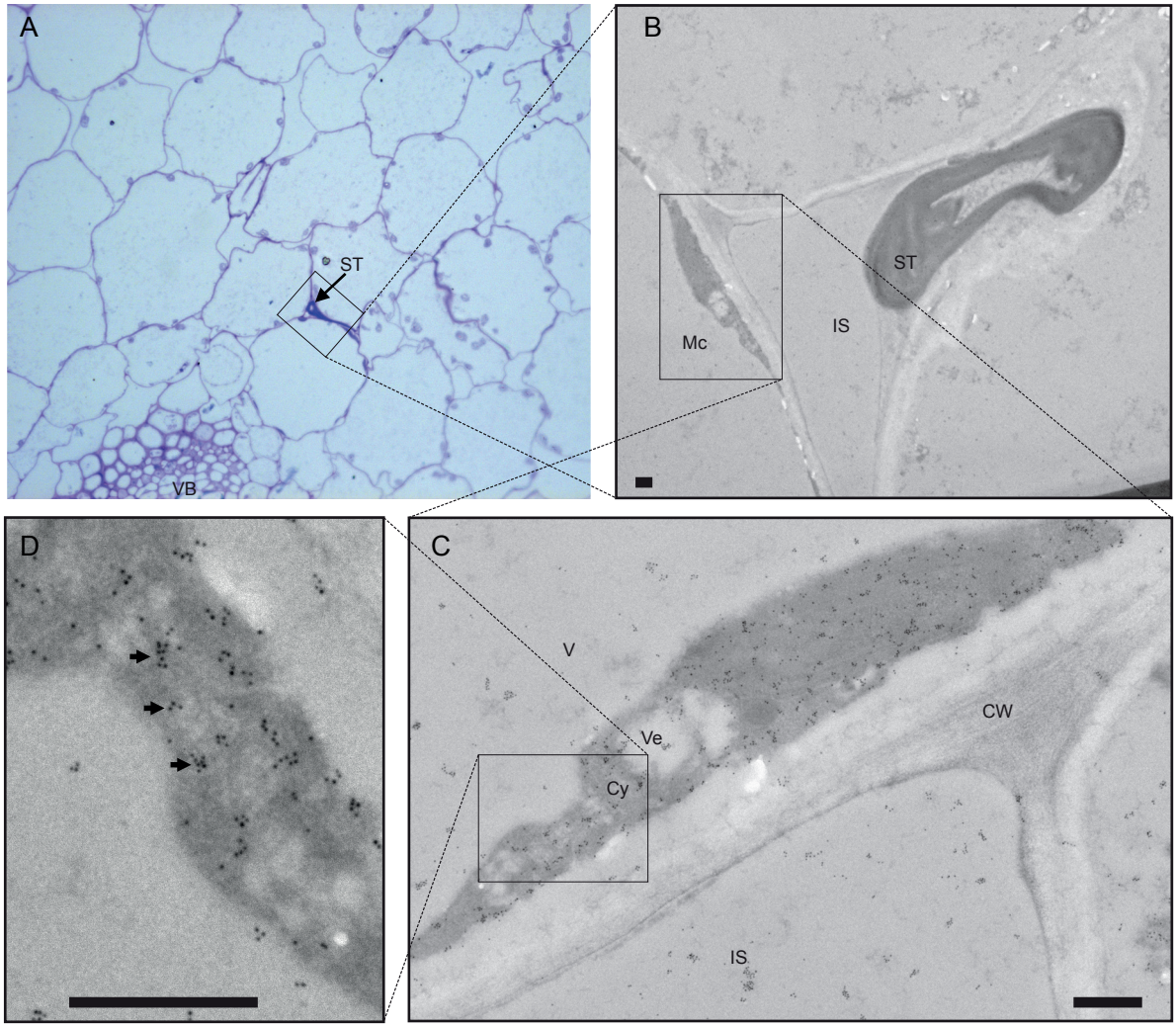


Figure 2. Mp10 protein is detected in the cytoplasm of mesophyll cells near an aphid stylets track.
(A): Semi-thin section (0.5 µm) from an aphid-infested *A. thaliana* leaf stained with toluidine blue. Aphid stylets tracks (ST) are visible between mesophyll cells (Mc) and a vascular bundle (VB). (B-D): Immuno-gold labeling of ultra-thin sections (90 nm) of the tissue shown in (A) with anti-Mp10 (1:10 dilution) revealed a high density of gold particles (arrows in D) in the cytoplasm (Cy) of mesophyll cells (Mc). No obvious labeling was observed of aphid stylets (St), cell wall (CW), intercellular space (IS), mesophyll cell vacuole (V) and inside vesicles (Ve). Images shown in B to D are sequentially higher magnifications of the same area of a cell adjacent to the stylets track. Scale bars, 500 nm.

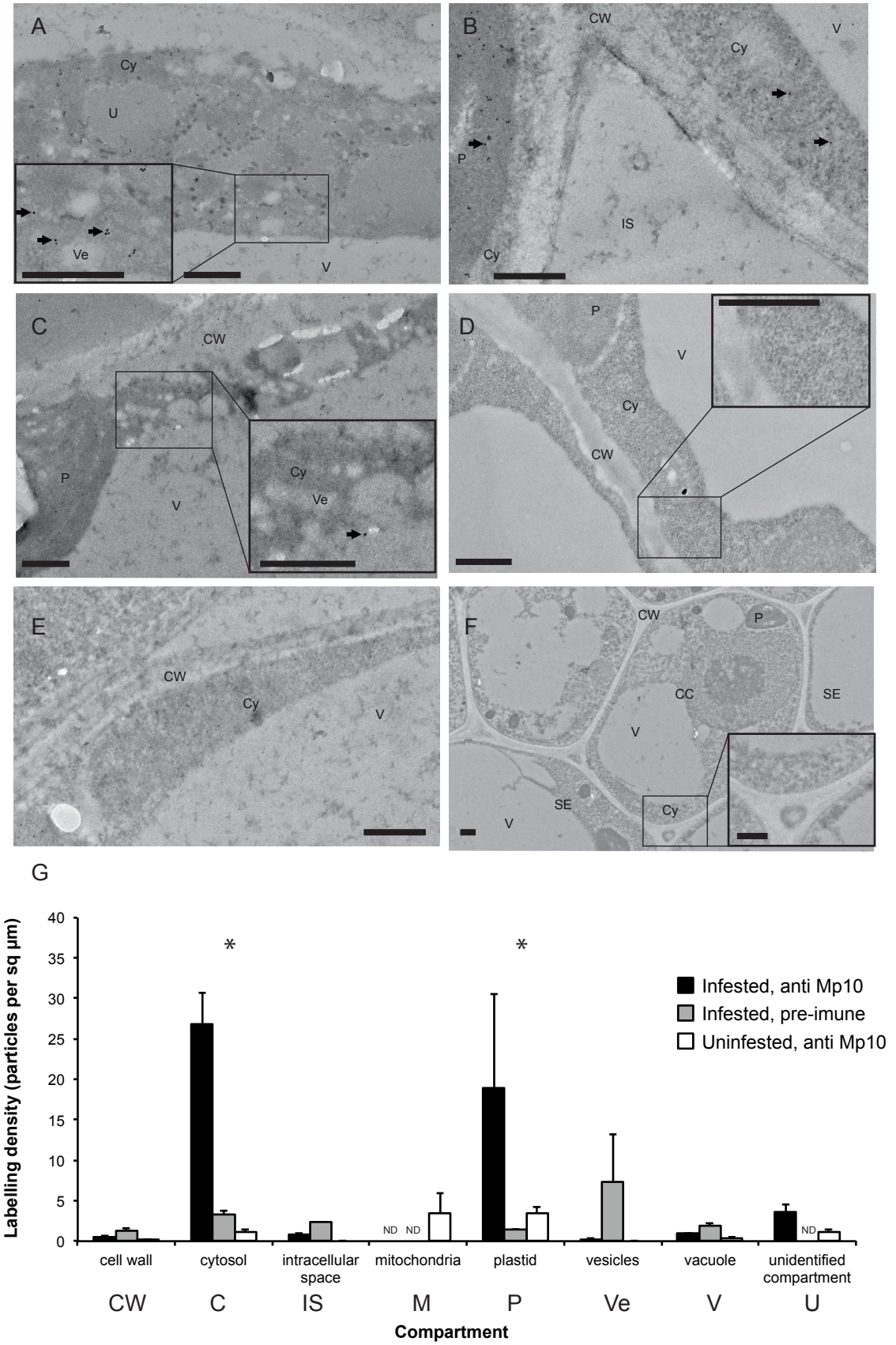


Figure 3. Mp10 detection is specific and restricted to the cells adjacent to stylets tracks.
Ultra-thin sections from *A. thaliana* leaves infested with GPA, and labeled with anti-Mp10 (1:100 dilution) show labelling (examples indicated with arrows) in the cytosol "Cy" (A-B, G) and plastid "P" (B, G) of cells adjacent to the stylets track, but not when labelled with pre-immune serum (1:50) (C, G). Samples from uninfested plants are not labelled with anti-Mp10 above background levels (D, G). Labeling with anti-Mp10 was not detected in mesophyll cells distal to the feeding site (E), or an area of the vascular bundle closest to the feeding site in GPA-infested tissues (F).
Quantification of labelling density (G) based on measurements of compartment area and gold particle count across multiple images of different areas from the samples. Values show mean (+/- SEM) density (infested, antiMp10 (at 1:100 dilution) n=13; infested, pre-immune (1:50) n=4, uninfested anti-Mp10 (1:100) n=5). Asterisks indicate significant differences in labelling both between infested vs. uninfested samples, and between anti-Mp10 and pre-immune labelled samples for that compartment ($p<0.01$, GLM). Abbreviations: "IS" intercellular space; "Cy" cytoplasm; "V" vacuole; "CW" cell wall; "Ve" vesicles; "P" plastid; "U" unidentified compartment. In (F): "CC" is a phloem companion cell, and "SE" are phloem sieve elements. Insets (A, C, D and F) are magnified regions of the image, all scale bars are 500 nm.

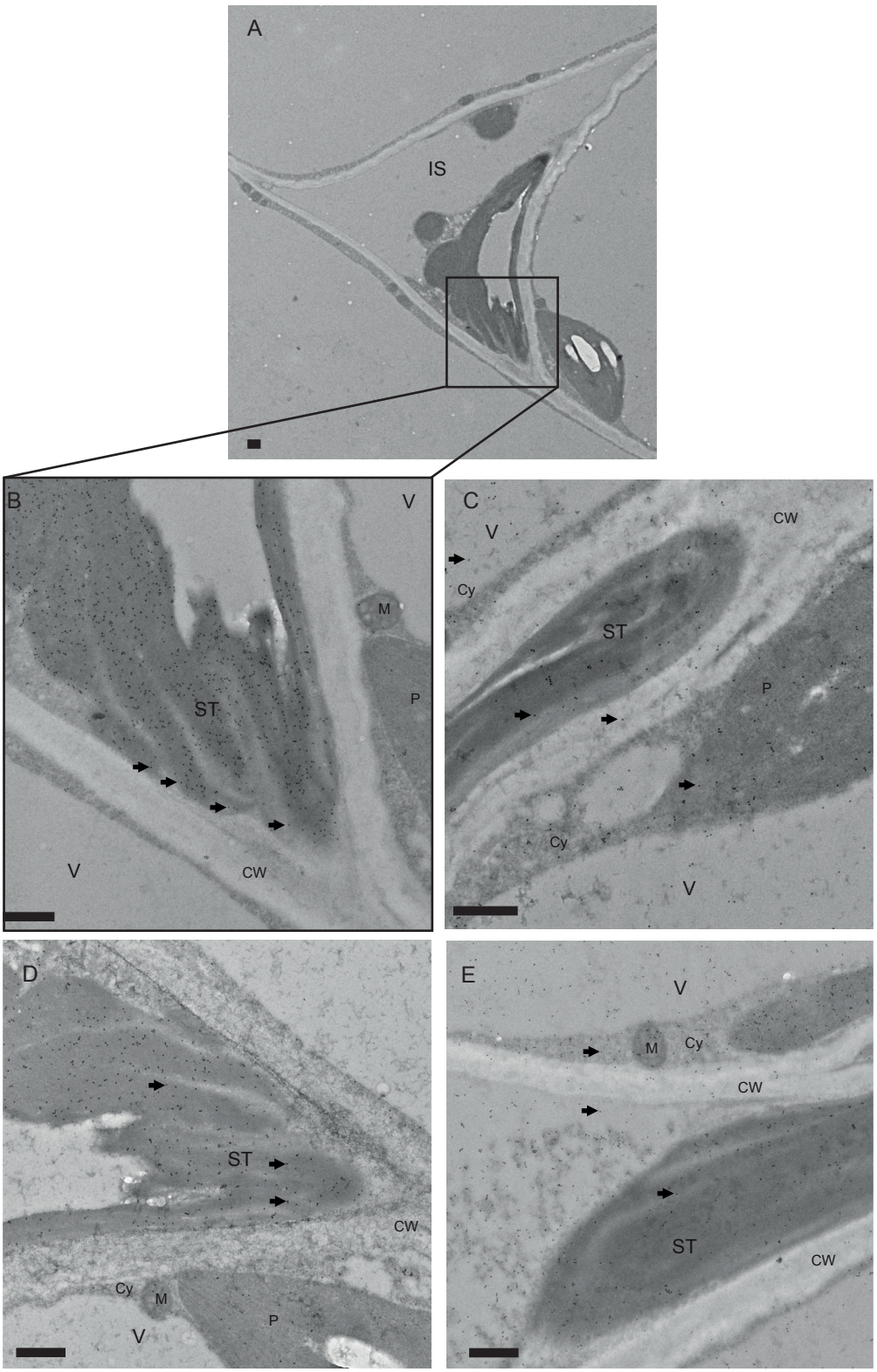
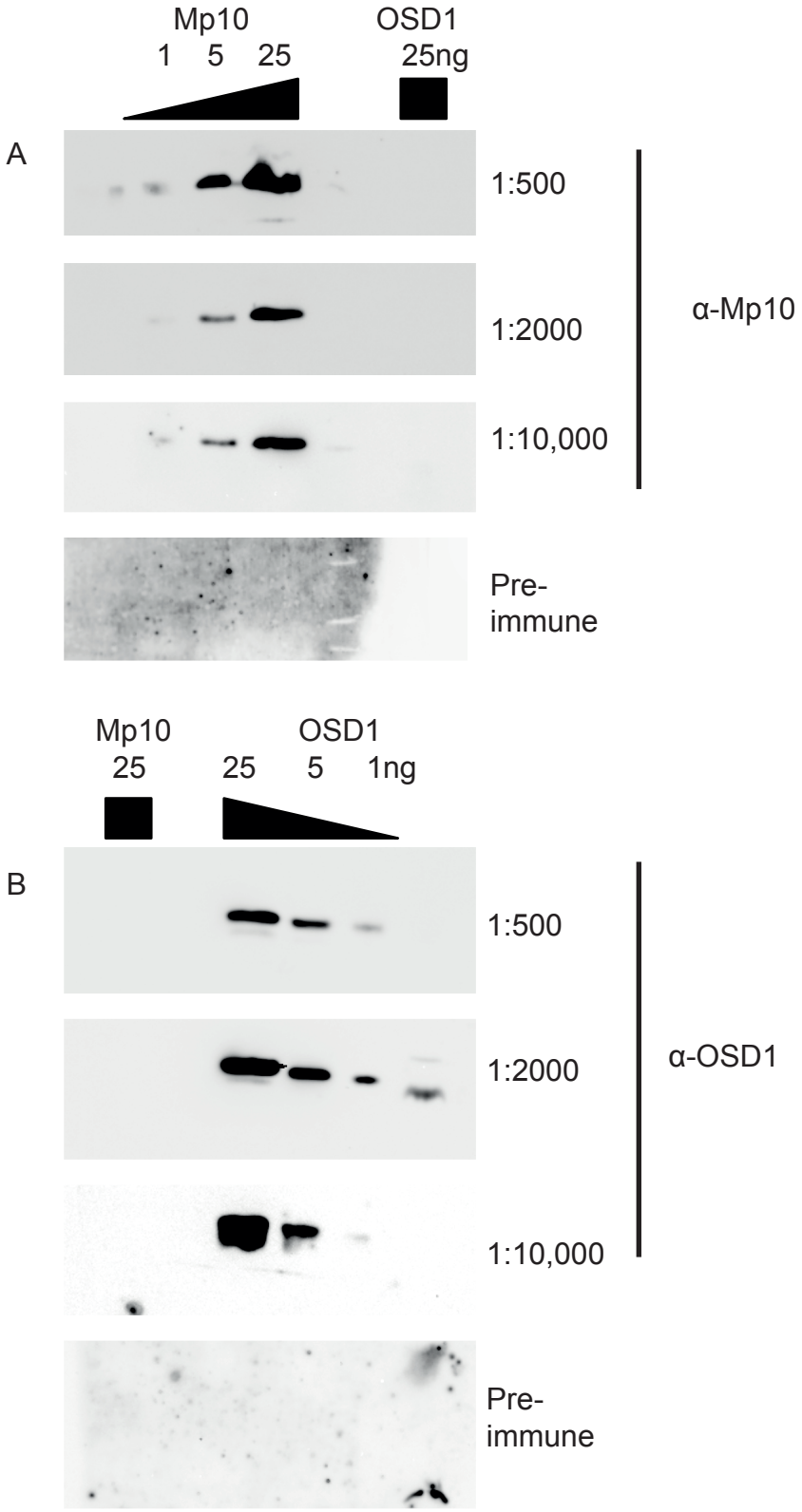
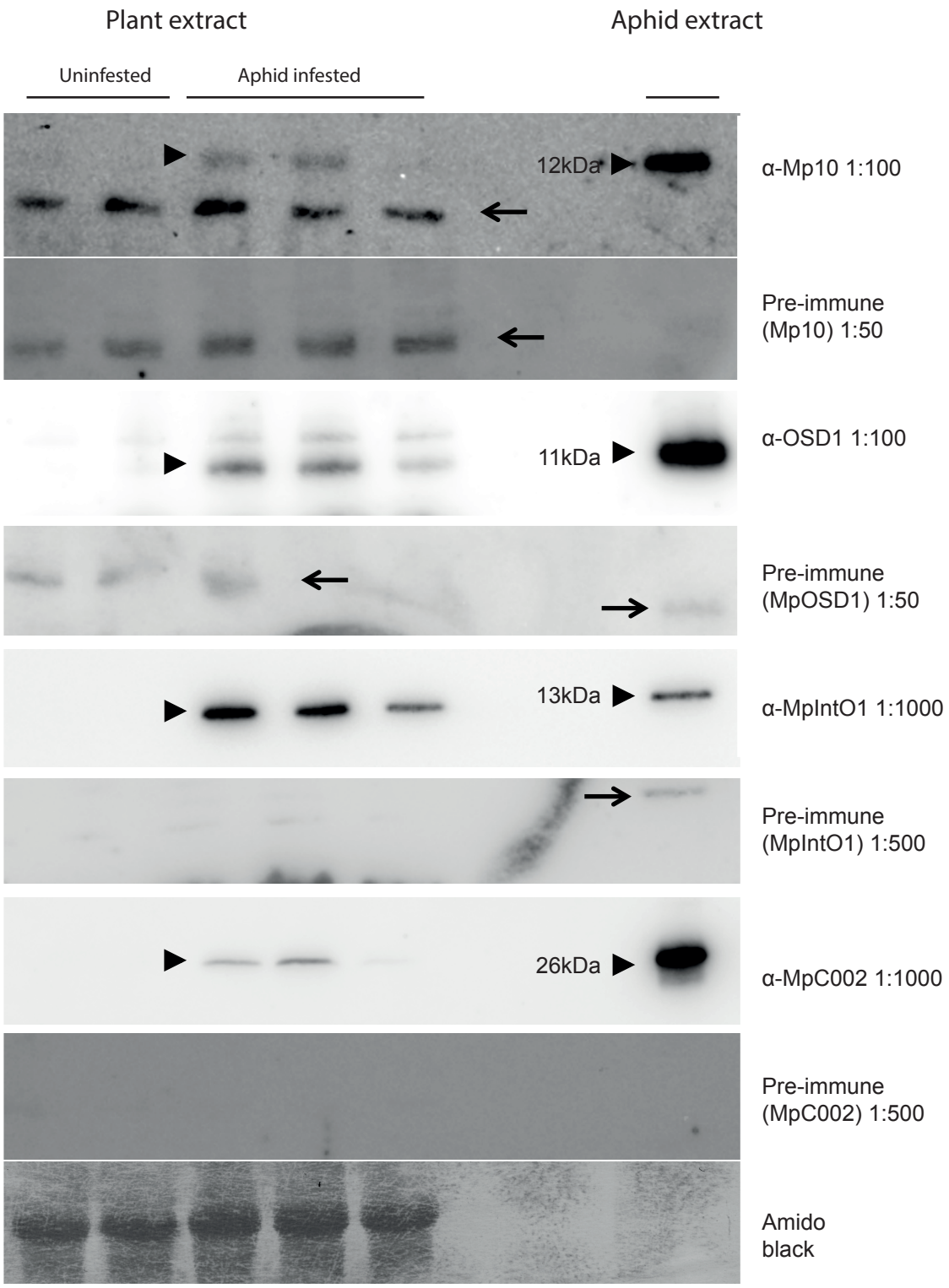


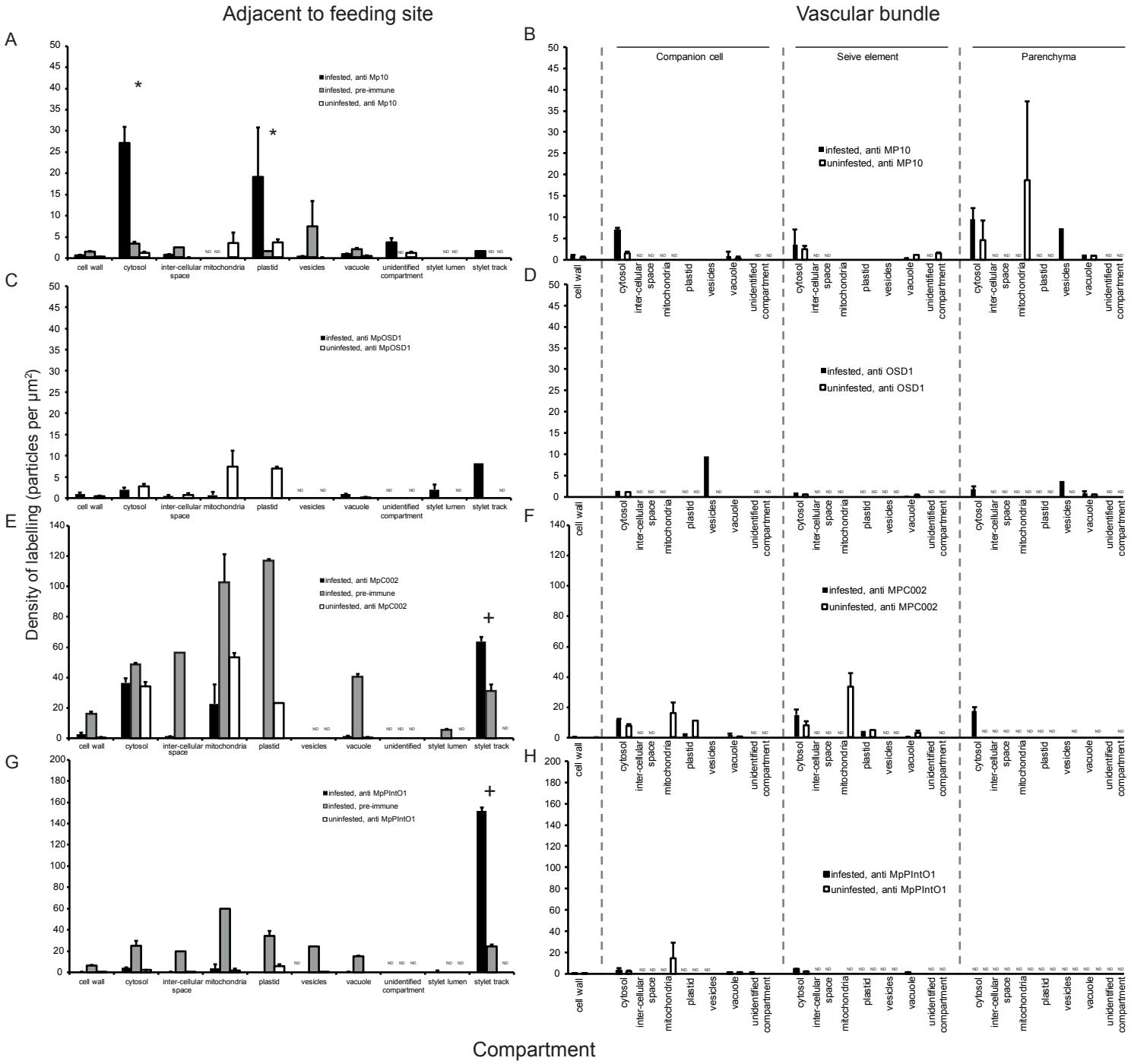
Figure 4. MpPIntO1 and MpC002 antisera label the stylets sheaths
Ultra-thin sections from aphid-infested samples showing the gelling saliva that forms the stylets sheath ("ST"), immuno-gold labelled with anti-MpPIntO1 (A-B at 1:100 dilution) or with the corresponding pre-immune serum (C, 1:50); or with anti-MpC002 (D, 1:100) or the corresponding pre-immune serum (E, 1:50). Examples of gold-labelling, where present, are indicated with arrows. Compartments are indicated as: "ST" stylet track; "IS" intercellular space; "Cy" cytoplasm; "V" vacuole; "CW" cell wall; "Ve" vesicles; "P" plastid; "M" mitochondria. Scale bars all indicate 500 nm. Quantification of labelling density can be found in Supplemental Fig. 3.



Supplementary Figure 1. Mp10 and MpOSD1 antisera are specific and do not cross react. Immuno-blots of pure recombinant Mp10 and MpOSD1 proteins probed with antisera raised against the proteins show the sensitivity and specificity of these antisera. (A) Replicate blots of a range of quantity of Mp10 protein (1-25ng) and 25ng OSD1 protein probed with (from top) decreasing concentration of the anti-Mp10 serum (from 1:500 to 1:10,000) or with corresponding pre-immune serum (at 1:1000 dilution). (B) Replicate blots of a range of quantities of OSD1 protein (1-25ng) and 25ng Mp10 protein probed with (from top) decreasing concentration of the anti-OSD1 serum (from 1:500 to 1:10,000) or with corresponding pre-immune serum (at 1:1000 dilution).



Supplementary Figure 2. Immuno-blots of aphid-exposed plant tissue probed with antisera raised against candidate effector proteins and also with corresponding pre-immune serum. Immuno-blots of total protein extracts from Arabidopsis rosettes; uninfested (lanes 1-2) or infested with GPA (lanes 3-5), and extract from GPA aphids (lane 9), were probed with antisera raised against the aphid salivary proteins (as shown in Fig. 1), and with the corresponding pre-immune sera (PI); (from the top) Mp10, Mp10-PI, OSD1, OSD1-PI, MpPIntO1, MpPIntO1-PI, MpC002, and MpC002-PI. The bottom panel shows loading of the plant extract samples by amido-black staining of the membrane. Arrowheads indicate bands of the expected sizes detected in aphid-exposed plants and aphids, but not unexposed plants. Arrows indicate presumably non-specific bands of other sizes. Pre-immune sera were used at higher concentrations than all corresponding antibodies to ensure that specific antibody-labelled bands were not also detected by pre-immune serum.



Supplementary Figure 3. Quantification of immuno-gold labelling over different cellular compartments and tissues. Graphs show the density of gold particles in different subcellular compartments (mean +/- SEM (n= from 3 to 13)) for (left) cells adjacent to the aphid stylet track (or equivalent mesophyll cells from uninfested tissues), and (right) cells in the vascular bundle. Samples were probed with affinity-purified antibody (all at 1:100 dilution) raised against Mp10 (A-B); MpOSD1 (C-D); MpC002 (E-F) or MpPIntO1 (G-H), or (where indicated) with the corresponding pre-immune serum (all at 1:50 dilution). Asterisks indicate significant difference in labelling both between infested and uninfested samples, and between antibody and pre-immune labelled samples for that compartment ($p < 0.01$, GLM). Plus signs indicate significantly higher labelling of stylet track relative to other compartments in infested tissue (for which no uninfested control plants could be tested) and for which the signal was significantly higher than for infested samples probed with the corresponding pre-immune serum ($p < 0.01$, GLM). "ND": Not determined.