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Forisomes defend *V. faba* against generalist aphids

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## Original Article

## Faba bean forisomes can function in defence against generalist aphids

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## ABSTRACT

Phloem sieve elements have shut-off mechanisms that prevent loss of nutrient-rich phloem sap when the phloem is damaged. Some phloem proteins such as the proteins that form forisomes in legume sieve elements are one such mechanism and in response to damage, they instantly form occlusions that stop the flow of sap. It has long been hypothesized that one function of phloem proteins is defence against phloem sap-feeding insects such as aphids. This study provides the first experimental evidence that aphid feeding can induce phloem protein occlusion and that the aphid-induced occlusions inhibit phloem sap ingestion. The great majority of phloem penetrations in *Vicia faba* by the generalist aphids *Myzus persicae* and *Macrosiphum euphorbiae* triggered forisome occlusion and the aphids eventually withdrew their stylets without ingesting phloem sap. This contrasts starkly with a previous study on the legume-specialist aphid, *Acyrtosiphon pisum*, where penetration of faba bean sieve elements did not trigger forisome occlusion and the aphids readily ingested phloem sap. Next, forisome occlusion was demonstrated to be the cause of failed phloem ingestion attempts by *M. persicae*: when occlusion was inhibited by the calcium channel blocker lanthanum, *M. persicae* readily ingested faba bean phloem sap.

**Key-words:** sieve element, phloem occlusion, P-protein, plant defense, host plant resistance, calcium signaling, stylet penetration, feeding behavior, EPG, insect-plant-interactions.

## INTRODUCTION

Phloem is the plant transport system that translocates nutrients throughout the plant. Specialized elongated phloem cells, called sieve elements, are connected symplastically end to end to form sieve tubes which are the living conduits for transporting phloem sap over long distances in the plant (Schulz 1998). Phloem sap is transported by an osmotically generated turgor pressure gradient where turgor pressure is high in 'source tissue' where solutes are loaded into the sieve elements and low in 'sink tissue', such as developing plant parts, where solutes are unloaded from sieve elements (Knoblauch & Oparka 2012; De Schepper *et al.* 2013).

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Because sieve tubes are a pressurized transport system, any breach in a sieve tube could result in considerable loss of sap. Consequently, plants have evolved mechanisms, referred to as sieve element occlusion (SEO), to seal or plug damaged sieve elements to prevent sap loss (Eschrich 1975; van Bel 2006). Two well-studied mechanisms of SEO are deposition of callose ( $\beta$ -1,3-glucans) in the sieve pores (the pores that connect adjacent sieve elements in a sieve tube) and plugging by 'P-proteins' (Eschrich 1975; van Bel 2006). Callose plugs require *de novo* synthesis and thus take several minutes to fully block a sieve tube whereas P-proteins are constitutively present in sieve elements and, in response to damage, can rapidly (<1 s) transform from a physical state that does not impede the flow of sap to a physical state that plugs the sieve element (Knoblauch *et al.* 2001; Furch *et al.* 2007; Xie *et al.* 2011). A general model for prevention of loss of phloem sap because of injury involves a two-step process: rapid development of a proteinaceous plug seals the sieve plates which is then followed by a slower deposition of callose which eventually chokes off the sieve pores (van Bel 2006). However, the universality of P-proteins effectively sealing sieve plates has recently been questioned (Knoblauch *et al.* 2014). While there is strong experimental evidence that P-proteins of legumes can form effective plugs that stop the flow of sap (Knoblauch *et al.* 2014) and there is a correlation between formation of P-protein plugs and stoppage of sap flow in cucurbits (Furch *et al.* 2010), P-proteins of Arabidopsis readily pass through the sieve plates without stopping the flow of sap (Froelich *et al.* 2011).

Sieve element occlusion by P-protein has been best studied in papilionoid legumes, especially faba bean *Vicia faba* L., where P-proteins form discrete bodies called forisomes, usually one forisome per sieve element (Peters *et al.* 2006). In undisturbed sieve elements, forisomes are in a compact spindle-shaped configuration (referred to as the low-volume state) that provides little or no interference with sap flow in the sieve element (Fig. 2d; Peters *et al.* 2006). In response to damage, forisomes instantaneously swell to a high-volume state that occludes the sieve element lumen and stops the flow of sap. Sieve element occlusion by callose and forisomes are both triggered by an influx of calcium into the sieve element (Kauss 1987; Knoblauch *et al.* 2001) and is reversible: callose plugs can be broken down by  $\beta$ -1,3-glucanase (Levy *et al.* 2007) and forisome plugs dissipate after several minutes as the forisomes revert back to their compact,

low-volume state (Knoblauch *et al.* 2001; Furch *et al.* 2007, 2009; Medina-Ortega & Walker 2013).

It has been hypothesized many times over the past two decades that one function of P-protein and callose plugs is to deter phloem sap-feeding insects such as aphids and whiteflies (Cole 1994; Gironse & Bournoville 1994; Caillaud *et al.* 1995a,b; Caillaud & Niemeyer 1996; Klingler *et al.* 1998, 2005; Sauge *et al.* 1998; Garzo *et al.* 2002; Cardoza *et al.* 2005; Tjallingii 2006; Jiang & Walker 2007; Zhu *et al.* 2011; Lightle *et al.* 2012). Many of these studies compared the feeding behaviour of aphids on susceptible and resistant plants and found that penetration of a sieve element on a susceptible plant is usually followed by a short (ca. 30–60 s) bout of salivation, which is subsequently followed by a long bout of sap ingestion; whereas on resistant plants, sieve element penetration is often followed by very long periods of salivation and the stylets are frequently withdrawn from the sieve element without engaging in sap ingestion or are withdrawn after only a short bout of sap ingestion. It was hypothesized in these studies that sieve element penetration on resistant plants triggers phloem occlusion, preventing the insects from making a successful transition from salivation to phloem sap ingestion.

Recently, Walker & Medina-Ortega (2012) demonstrated that penetration of faba bean sieve elements by pea aphid, *Acyrtosiphon pisum* (Harris), does not trigger P-protein occlusion and following sieve element penetration, the aphid engages in short bouts of salivation followed by long bouts of sap ingestion. Pea aphid is a legume-specialist and faba bean is a favoured host, so this study represents a system of a specialist aphid feeding on a highly susceptible host. More recently, we observed that when the generalist aphids *Myzus persicae* (Sulzer) and *Macrosiphum euphorbiae* (Thomas) penetrated faba bean sieve elements, they exhibited similar behaviour as described earlier for many aphid species feeding on resistant plants: very long periods of salivation and frequent stylet withdrawn from the sieve element without engaging in sap ingestion. Faba bean has been the model plant for studying sieve element occlusion by P-proteins; consequently, this provided an opportunity to test for the first time the long-held hypothesis that aphid stylet penetration of a sieve element can trigger sieve element occlusion and prevent the aphid from ingesting phloem sap.

## MATERIALS AND METHODS

### Plants and insects

Faba bean (*V. faba* L. cv. Windsor) and sugar beet (*Beta vulgaris* L.) plants were grown in a greenhouse in pots under natural light conditions and used at the age of 20–25 and 60–75 d, respectively. Green peach aphids, *My. persicae* (Sulzer), were maintained on sugar beet, and potato aphids, *Ma. euphorbiae* (Thomas), were maintained on faba bean plants grown in a greenhouse. Apterous adult aphids and large late-instar nymphs were used in the experiments.

### Aphid performance on faba bean and sugar beet plants

Green peach aphid performance was compared with sugar beet, a preferred host, and faba bean. Four plants of each species were infested with a total of 10 adult aphids; two aphids per leaf on the first five leaves from top to bottom. Plants were spatially arranged in a completely randomized design in a greenhouse under natural light conditions. Twelve days after infestation, the number of nymphs and adult aphids per plant were counted. Total number of aphids was compared with sugar beet and faba bean using the Kruskal–Wallis test.

### Electrical penetration graph (EPG) recordings

Aphid feeding behaviour was monitored using a Giga 4 or Giga 8 DC-EPG (EPG Systems, Wageningen, The Netherlands) using standard methods as described previously (Walker & Medina-Ortega 2012; Medina-Ortega & Walker 2013). EPG recordings on both faba bean and sugar beet were made from aphids feeding on young, mature leaves intact on the plant. During EPG recording, the aphids fed on the abaxial side of the leaves, their normal feeding site. Faba bean leaves normally are oriented horizontally which makes it difficult to initially place the wired aphids on the abaxial side of the leaf without them falling off. Consequently, a device described previously (Walker & Medina-Ortega 2012) was used so that the wired aphids could be placed on the leaf with the abaxial side facing up, and once they secured a grip on the leaf, the leaf could be turned over to the normal position of abaxial side down. EPGs were recorded with the leaves oriented in their normal position. Sugar beet leaves are more erect, so this device was not needed to facilitate the wired aphids settling on the abaxial leaf surface.

### Cryofixation, staining and examination of leaf tissue

To determine the state of forisomes [low volume (non-occluding) or high volume (occluding)] when aphids penetrate a faba bean sieve element and initiate 'phloem phase' (Pettersson *et al.* 2007), aphid feeding was monitored by EPG, and within a few minutes after the aphid reached phloem phase, the aphid and leaf were instantaneously cryofixed with 95% ethanol chilled with liquid nitrogen to a temperature of about  $-120^{\circ}\text{C}$ , as described previously (Walker & Medina-Ortega 2012). Following cryofixation, samples were freeze-substituted, stained and examined by confocal laser-scanning microscope (CLSM) as described previously (Walker & Medina-Ortega 2012).

### Aphid feeding behaviour on faba bean and sugar beet

Feeding behaviour of *My. persicae* on faba bean and sugar beet was recorded by EPG. Each replicate was recorded for 8.5 h and used a different aphid and different plant. There

**Table 1.** *Myzus persicae* feeding behaviour on sugar beet versus faba bean [8.5 h electrical penetration graph (EPG) recordings] and on faba bean infiltrated with saline plus lanthanum (La<sup>3+</sup>) versus saline without La<sup>3+</sup> (4 h EPG recordings)

EPG calculated parameters	Sugar beet	Faba bean	Prob	With La <sup>3+</sup>	Without La <sup>3+</sup>	Prob
Proportion of aphids that reached phloem phase (PP)	1.00	1.00	1.0000	1.00	1.00	1.0000
Proportion of aphids that reached phloem sap ingestion (E2)	1.00	0.53	0.0106	1.00	0.47	<0.0001
Proportion of aphids that reached E2 during the first PP	1.00	0.05	<0.0001	0.69	0.16	<0.0001
Proportion of PP that reached E2 <sup>a</sup>	0.98 ± 0.02	0.07 ± 0.02	<0.0001	0.80 ± 0.06	0.15 ± 0.05	<0.0001
Number of PPs per aphid	1.8 ± 0.5	9.0 ± 1.1	<0.0001	2.7 ± 0.4	5.5 ± 0.7	0.0002
Number of 'failed phloem phases' (PP with no E2) prior to first 'successful phloem phase' (PP with an E2) <sup>b</sup>	0	6.4 ± 1.1	<0.0001	0.4 ± 0.1	3.7 ± 0.6	<0.0001
Time (min) from start of recording to first PP	155 ± 37	91 ± 16	0.0852	41.4 ± 8.9	42.6 ± 6.9	0.2553
Time (min) from start of recording to first successful PP <sup>c</sup>	155 ± 37	338 ± 44	0.0089	63 ± 11	174 ± 20	<0.0001
Time (min) to first successful PP from start of its probe <sup>d,e</sup>	31.4 ± 4.8	66 ± 16	0.1927	23.6 ± 5.0 (26)	48.3 ± 15.2 (9)	0.1360
Average time (min) to PP from start of probes that produced PP <sup>f</sup>	30.4 ± 4.5	16.7 ± 1.7	0.0017	15.4 ± 1.9	15.9 ± 1.5	0.3888
Total time (min) in E2	194 ± 44 (10) <sup>g</sup>	95 ± 29	0.0303	135 ± 14	25 ± 10	<0.0001
Average duration (min) of each bout of E2 <sup>e,h</sup>	159 ± 44	158 ± 40 (10)	0.5974	95 ± 13 (26)	42 ± 17 (9)	0.0224
Total time (min) in active phloem watery salivation (E1)	1.2 ± 0.3 (10) <sup>g</sup>	20.9 ± 4.4	<0.0001	3.0 ± 0.8	14.0 ± 2.8	<0.0001
Average duration (s) of each bout of E1 <sup>h</sup>	41.4 ± 5.2	134 ± 23	0.0003	70 ± 23	173 ± 44	0.0007
Average duration (s) of each bout of E1 followed by E2 <sup>e,h</sup>	41.4 ± 5.2	645 ± 263 (10)	0.0017	75 ± 32	427 ± 239 (9)	0.0062
Average duration (s) of each bout of E1 not followed by E2 <sup>h</sup>	82 (1)	98 ± 14	NA <sup>i</sup>	129 ± 39 (9)	155 ± 29	0.4312

Sample sizes:  $n = 19$  for faba bean,  $n = 11$  for sugar beet,  $n = 26$  for saline with La<sup>3+</sup> and  $n = 19$  for saline without La<sup>3+</sup> (unless otherwise specified in parentheses). In the first three rows, probability levels (Prob) are from Fisher's exact test (two-tailed). Data in the remaining rows were given as mean ± SEM with probability levels from the Kruskal–Wallis test.

<sup>a</sup>Proportions were first calculated for each aphid and then the Kruskal–Wallis test was used to compare these proportions between sugar beet and faba bean or between 'with La<sup>3+</sup>' and 'without La<sup>3+</sup>'.

<sup>b</sup>If an aphid did not produce E2, then the total number of PPs produced during the recording was entered as the total number of 'failed phloem phases' prior to first PP with an E2.

<sup>c</sup>If the aphid did not reach E2 during the recording, then the duration of the recording was entered as the time to first PP with E2 from start of recording.

<sup>d</sup>'Its probe' is the probe that produced the first E2.

<sup>e</sup>If the aphid had no successful PPs (i.e. no E2) during the recording, then this variable was entered as missing data.

<sup>f</sup>For each aphid, the average time from start of probe to the first PP in that probe was calculated from all probes that produced PP; the Kruskal–Wallis test was then used to compare these averages between sugar beet and faba bean or between 'with La<sup>3+</sup>' and 'without La<sup>3+</sup>'.

<sup>g</sup>One aphid on sugar beet had only an 8 h recording rather than 8.5 h; consequently, data from this aphid were omitted from the calculation of these variables because total time would be affected; all variables other than 'total time' variables were unaffected by this difference.

<sup>h</sup>For each aphid, the average bout duration was calculated for all bouts produced by the aphid; the Kruskal–Wallis test was then used to compare these averages between sugar beet and faba bean or between 'with La<sup>3+</sup>' and 'without La<sup>3+</sup>'.

<sup>i</sup>Not applicable; only one aphid on sugar beet had one single event of E1 without E2; consequently, a statistical comparison was not calculated.

were 19 replicates for *My. persicae* feeding on faba bean and 11 replicates on sugar beet. Variables calculated from the recordings and statistical tests used to analyse them are given in Table 1. Statistical analyses were conducted using JMP software (SAS Institute, Cary, NC, USA). EPGs also were recorded from *Ma. euphorbiae* feeding on faba bean. Recordings were 8 h in duration and there were 17 replicates, each with a different aphid and plant. The same variables listed in Table 1 were calculated.

### Forisome response to feeding by *My. persicae* and *Ma. euphorbiae*

EPGs were recorded from *My. persicae* and *Ma. euphorbiae* feeding on secondary (lateral) veins of faba bean. Aphids were kept confined to the lateral veins as described in Walker & Medina-Ortega (2012). Aphids and leaves were cryofixed soon after the aphids initiated phloem phase (9–146 s for *My. persicae* and 16–487 s for *Ma. euphorbiae*)

and were prepared for CLSM to determine the state of the forisomes in the penetrated sieve elements and nearby sieve elements as described earlier. The distance that the forisome response (transformation to the high-volume state) extended away from the aphid's stylets was measured with an Olympus BX50wi compound microscope with a 60× water immersion DIC lens and ocular micrometer (Olympus Corporation, Shinjuku, Tokyo, Japan). The direction of flow within faba bean phloem is indicated by the location of the forisomes within the sieve elements: they are usually located at the downstream end of the sieve element (Peters *et al.* 2006). Therefore, measurements were made both upstream and downstream from the stylets to the point where no more high-volume forisomes were observed. Twenty-six and 14 usable replicates were obtained for *My. persicae* and *Ma. euphorbiae*, respectively, each replicate with a different aphid and different plant.

### Aphid feeding on faba bean when forisome occlusion is prevented

EPGs were used to record *My. persicae* feeding on faba bean leaves where forisome occlusion was inhibited by lanthanum, a calcium channel blocker (Furch *et al.* 2009). Young mature leaves, still attached to the plant, were infiltrated with either physiological saline (10 mM KCl, 5 mM NaCl, 10 mM CaCl<sub>2</sub>) or 'lanthanum (La<sup>3+</sup>) saline' (10 mM KCl, 5 mM NaCl, 2 mM LaCl<sub>3</sub>) using a hypodermic syringe. Leaves were injected from the adaxial side. After about an hour, the water-soaked appearance of the leaves had mostly dissipated and a second injection was then applied from the adaxial side. About an hour later when the water-soaked appearance of the leaves had again mostly dissipated, EPG recordings were made from aphids confined to a secondary (lateral) vein. Recordings were 4 h in duration and there were 19 and 26 replicates for aphids feeding on physiological saline and La<sup>3+</sup> saline-treated leaves, respectively. Each replicate used a different aphid and different plant. Variables calculated from the recordings and statistical tests used to analyse them are shown in Table 1.

To verify that the lanthanum treatment had the intended effect on forisomes, a total of five and six additional samples of aphids feeding on leaves treated with physiological saline and La<sup>3+</sup> saline, respectively, were cryofixed within 1.3 min after the aphids initiated phloem phase. Leaf tissue was then processed as described previously to determine the state of the forisomes.

## RESULTS

### Faba bean is a poor host for *My. persicae*

Twelve days after infesting each plant with 10 adult aphids, aphid numbers increased to 118 ± 10 (±SEM) aphids per plant on sugar beet, a preferred host, compared with only 22.5 ± 8.7 on faba bean ( $P = 0.0304$ , Kruskal–Wallis test).

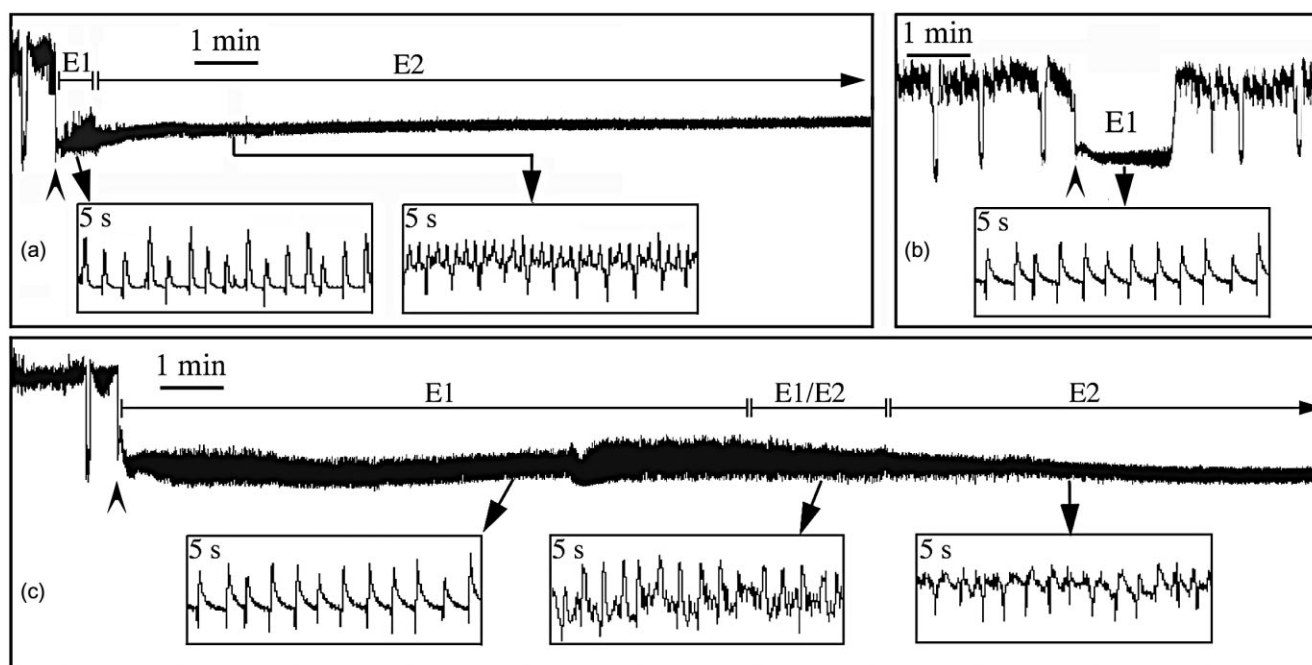
### *My. persicae* feeding behaviour on faba bean and sugar beet

'Phloem phase' is characterized by two stereotypic behaviours that occur with the stylet tips in a sieve element: secretion of watery saliva into the sieve element (represented by EPG waveform E1) and ingestion of phloem sap with concurrent salivation (represented by EPG waveform E2). Phloem phase always begins with waveform E1 which may or may not be followed by waveform E2 (Pettersson *et al.* 2007). Herein, 'successful phloem phase' is defined as a phloem phase where the aphid makes a successful transition from sieve element salivation (E1) to phloem sap ingestion (E2) and 'failed phloem phase' is defined as a phloem phase that terminates in E1 without ingesting phloem sap (i.e. E1 only; no E2). Examples of EPGs from typically successful and failed phloem phases of *My. persicae* feeding on faba bean and a successful phloem phase of *My. persicae* feeding on sugar beet are shown in Fig. 1. Results of the EPG study are tabulated in Table 1 and summarized in the succeeding text. All aphids on both plant species reached phloem phase during the 8.5 h EPG recording; however, those on faba bean were much less successful at achieving phloem sap ingestion (E2) during phloem phase. All aphids on sugar beet engaged in phloem sap ingestion, but only 10 out of 19 aphids did so on faba bean ( $P = 0.0106$ , Fisher's exact test). Aphids on faba bean engaged in many more phloem phases than aphids on sugar beet ( $9.0 \pm 1.1$  versus  $1.8 \pm 0.5$ , mean ± SEM;  $P < 0.0001$ , Kruskal–Wallis test), but far fewer of these phloem phases successfully made the transition from salivation into the sieve element (E1) to phloem sap ingestion (E2) (7% versus 98%;  $P < 0.0001$ , Kruskal–Wallis test). Only one out of 19 aphids on faba bean successfully engaged in phloem sap ingestion during their first phloem phase, whereas all aphids on sugar beet did so during their first phloem phase ( $P < 0.0001$ , Fisher's exact test). On average, aphids on faba bean made  $6.4 \pm 1.1$  failed phloem phases before their first successful phloem phase.

Aphids on faba bean spent much more total time engaged in sieve element salivation (E1) than aphids on sugar beet ( $20.9 \pm 4.4$  versus  $1.2 \pm 0.3$  min, respectively;  $P < 0.0001$ , Kruskal–Wallis test) and the average duration of individual bouts of sieve element salivation was over three times longer on faba bean than on sugar beet ( $134 \pm 23$  versus  $41 \pm 5$  s;  $P = 0.0003$ , Kruskal–Wallis test). Considering only bouts of sieve element salivation that were followed by phloem sap ingestion, the average duration of individual bouts of sieve element salivation was over 15 times longer on faba bean than on sugar beet ( $645 \pm 263$  versus  $41 \pm 5$  s, respectively;  $P = 0.0017$ , Kruskal–Wallis test). There was only one bout of sieve element salivation that was not followed by phloem sap ingestion on sugar beet, so a statistical comparison with faba bean was not possible for that variable.

While the data clearly indicate that *My. persicae* has difficulty initiating phloem sap ingestion on faba bean, once they initiate ingestion, faba bean and sugar beet phloem sap seems equally acceptable. The average duration of bouts of phloem sap ingestion was almost identical between faba bean and





**Figure 1.** Electrical penetration graph (EPG) recordings of *Myzus persicae* during phloem phase on sugar beet and faba bean. Y axis is voltage; X axis is time from left to right. The larger EPG displays in (a), (b) and (c) are all at the same time scale (1 min scale is given in each figure). Boxed insets show details of 5 s segments at the points indicated in the larger EPG displays. Phloem phase begins with a sharp drop in voltage (arrow heads). Phloem phase waveforms E1 (salivation into sieve element), E2 (phloem sap ingestion) and overlapping E1/E2 transitions are labelled. (a) Example of a typical phloem phase on sugar beet: E1 is brief (ca. 37 s) followed by a very short (ca. 3 s) E1/E2 transition (not labelled) and a long E2 which lasted ca. 2 h. (b) Example of a typical 'failed' phloem phase (phloem phase that does not produce E2) on faba bean: phloem phase consists entirely of E1 which lasted ca. 95 s before stylet withdrawal from the sieve element (sharp voltage increase). (c) Example of a typical 'successful' phloem phase on faba bean: E1 was very long (ca. 595 s) followed by a long E1/E2 transition (ca. 130 s) and finally by E2 which lasted ca. 2.5 h.

sugar beet ( $158 \pm 40$  versus  $159 \pm 44$  min, respectively). Furthermore, aphids had no difficulty reaching the phloem on faba bean. All 19 aphids did so on faba bean and the time it took to reach phloem phase was actually much shorter on faba bean than on sugar beet. Shorter times to first phloem phase indicate easier access to the phloem, and two variables that measure this were much shorter on faba bean than on sugar beet: time to first phloem phase from the beginning of the recording ( $91 \pm 16$  versus  $155 \pm 37$  min;  $P = 0.0852$ , Kruskal–Wallis test) and average time from the beginning of a probe to the first phloem phase in that probe ( $16.7 \pm 1.7$  versus  $30.4 \pm 4.5$  min;  $P = 0.0017$ , Kruskal–Wallis test). However, the time it took to reach the first successful phloem phase was much longer on faba bean than on sugar beet ( $338 \pm 44$  versus  $155 \pm 37$  s;  $P = 0.0089$ , Kruskal–Wallis test) primarily because of a large number of 'failed' phloem phases (no E2) prior to the first successful phloem phase.

### *Ma. euphorbiae* feeding behaviour on faba bean

The same variables calculated for *My. persicae* were tabulated for *Ma. euphorbiae* in Table 2, and the most relevant are summarized in the succeeding text. Similar to *My. persicae*, *Ma. euphorbiae* feeding on faba bean also had (1) a low success rate (41%) ingesting phloem sap during the 8 h rec-

ording; (2) a low proportion (6%) of aphids that achieved phloem sap ingestion during their first phloem phase; (3) a low proportion (20%) of phloem phases that were successful; and (4) very long durations of E1 for both failed and successful phloem phases ( $421 \pm 184$  and  $1366 \pm 468$  s, respectively).

### Difficulty ingesting phloem sap is associated with sieve element occlusion

The state of the forisome in the sieve element penetrated by the aphids' stylets as well as forisomes in nearby sieve elements was determined.

Out of 26 samples cryofixed between 9 and 146 s ( $31 \pm 26$  s; mean  $\pm$  SD) from the beginning of *My. persicae* phloem phase, forisome expansion to the high-volume state was triggered in 22 samples. The most striking observation was that forisomes in most or all sieve elements in the vicinity of the stylets were in the high-volume state, even in sieve elements that were not in direct contact with the stylets and were not part of the same sieve tube as sieve elements in contact with the stylets (Fig. 2a). High-volume forisomes were observed as far as  $408 \pm 199$  microns upstream and  $409 \pm 147$  microns downstream (mean  $\pm$  SD;  $n = 22$ ) from the stylet tips.

Of the 22 samples where forisomes were in the high-volume state, the stylet tips were in a sieve element in 14

**Table 2.** *Macrosiphum euphorbiae* feeding behaviour on faba bean [8 h electrical penetration graph (EPG) recordings]

EPG calculated parameters	Faba bean
Proportion of aphids that reached phloem phase (PP)	0.82 (14 out of 17)
Proportion of aphids that reached phloem sap ingestion (E2)	0.41 (7 out of 17)
Proportion of aphids that reached E2 during the first PP	0.06 (1 out of 17)
Proportion of PP that reached E2 <sup>a</sup>	0.20 ± 0.08 (14)
Number of PPs per aphid	2.8 ± 0.5
Number of 'failed phloem phases' (PP with no E2) prior to first 'successful phloem phase' (PP with an E2) <sup>a,b</sup>	2.9 ± 0.5 (14)
Time (min) from start of recording to first PP <sup>c</sup>	222 ± 39
Time (min) from start of recording to first successful PP <sup>d</sup>	413 ± 24
Time (min) to first successful PP from the start of its probe <sup>a,e,f</sup>	138 ± 30 (7)
Average time (min) to PP from start of probes that produced PP <sup>a,g</sup>	53 ± 8 (14)
Total time (min) in E2	55 ± 21
Average duration (min) of each bout of E2 <sup>e,h</sup>	134 ± 32 (7)
Total time (min) in active phloem watery salivation (E1)	21 ± 5
Average duration (s) of each bout of E1 <sup>a,h</sup>	492 ± 113 (14)
Average duration (s) of each bout of E1 followed by E2 <sup>e,h</sup>	1366 ± 468 (7)
Average duration (sec) of each bout of E1 not followed by E2 <sup>a,h,i</sup>	421 ± 184 (13)

In the first three rows, proportions are given in decimal format and ratio. Other data were given as mean ± SEM ( $n = 17$ , unless otherwise specified in parentheses).

<sup>a</sup>If the aphid had no PP, then this variable was entered as missing data.

<sup>b</sup>If an aphid produced PP, but did not produce E2, then the total number of PPs produced during the 8 h recording was entered as the total number of 'failed phloem phases' prior to first PP with an E2.

<sup>c</sup>If the aphid did not reach PP, then the duration of the recording (8 h) was entered as the time to first PP from beginning of recording.

<sup>d</sup>If the aphid did not reach E2, then the duration of the recording (8 h) was entered as the time to first successful PP from beginning of recording.

<sup>e</sup>If the aphid had no successful PPs (i.e. no E2) during the recording, then this variable was entered as missing data.

<sup>f</sup>'Its probe' is the probe that produced the first E2.

<sup>g</sup>For each aphid, the average time from beginning of probe to the first PP in that probe was calculated from all probes that produced PP; the average of these averages is presented.

<sup>h</sup>For each aphid, the average bout duration was calculated for all bouts produced by the aphid; the average of these averages is presented.

<sup>i</sup>If all bouts of E1 were followed by E2, then this variable was entered as missing data.

samples and in a companion cell in five samples. In three samples, we were unable to identify which cell was penetrated. Out of the 14 samples with stylets inside a sieve element, the forisome in the penetrated sieve element was unambiguously identified in nine samples, and it was in the high-volume state in all nine samples, as were the forisomes

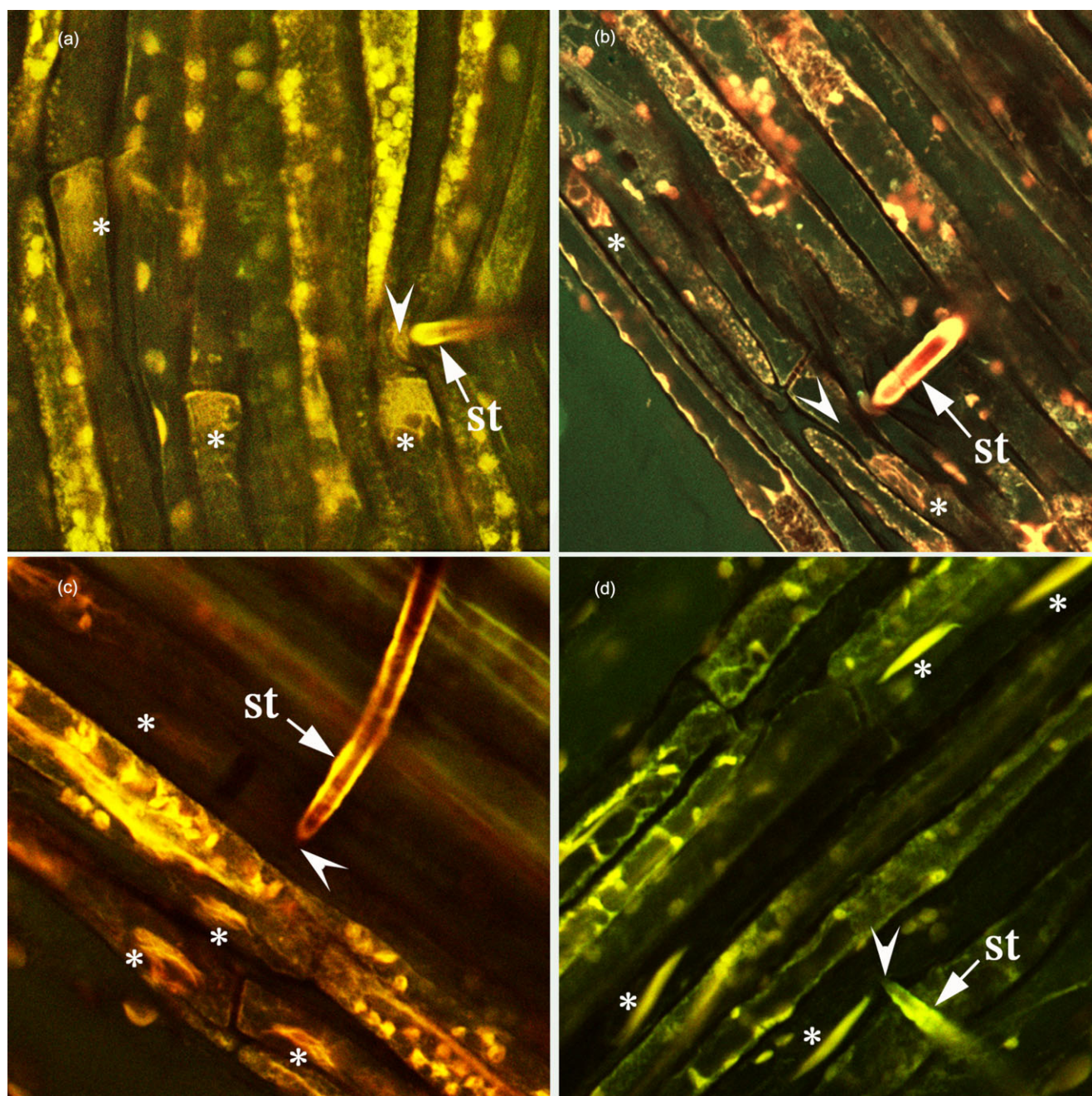
in most or all of the nearby sieve elements. In the five samples where we could not unambiguously identify the forisome in the penetrated sieve element and in the five samples where the stylet tips were in a companion cell, forisomes in all or most of the sieve elements in the vicinity of the stylet tips were in the high-volume state. There were only four out of 26 samples with forisomes in a low-volume state. We were able to identify unambiguously the forisome in the penetrated sieve element in three of these samples, and in each case, it as well as forisomes in nearby sieve elements were in a low-volume state.

Results for *Ma. euphorbiae* were similar to those for *My. persicae* (Fig. 2b). Out of 14 samples cryofixed during E1, between 16 and 487 s ( $92 \pm 126$ ; mean ± SD) from the beginning of phloem phase, forisome occlusion was triggered in 12 samples. Forisome occlusion was triggered not only in the penetrated sieve element (nine samples where the forisome in the penetrated sieve element could be confidently identified), but also in nearby upstream and downstream sieve elements. In three of the 12 samples, we could not determine with confidence whether the stylet tips were penetrating a sieve element or companion cell, but all forisomes near the stylet tips were in the high-volume state. In two of the 14 samples, only low-volume forisomes were observed. For one of these samples, we could not unambiguously identify the specific penetrated sieve element, but all forisomes near the stylet tips were in a low-volume state.

### Sieve element occlusion is a mechanism of aphid resistance

When forisome occlusion was prevented by the calcium channel blocker lanthanum (Furch *et al.* 2009), *My. persicae* readily ingested phloem sap from faba bean (Table 1). During the 4 h recording period, all aphids reached phloem phase on leaves treated with lanthanum saline ( $n = 26$ ) and control saline ( $n = 19$ ). However, only nine out of 19 aphids successfully ingested phloem sap on the control treatment whereas all 26 aphids on the lanthanum treatment successfully ingested phloem sap ( $P < 0.0001$ , Fisher's exact test). Aphids in the control treatment engaged in twice as many phloem phases as aphids in the lanthanum treatment ( $5.5 \pm 0.7$  versus  $2.7 \pm 0.4$ ; mean ± SEM;  $P = 0.0002$ , Kruskal–Wallis test), but far fewer of the phloem phases in the control treatment made a successful transition from sieve element salivation to phloem sap ingestion (15% versus 80%;  $P < 0.0001$ , Kruskal–Wallis test). Only three out of 19 aphids (16%) successfully engaged in phloem sap ingestion during their first phloem phase on control saline-treated leaves, whereas 18 out of 26 (69%) aphids on lanthanum saline-treated leaves did so during their first phloem phase ( $P < 0.0001$ , Fisher's exact test). On average, aphids on control saline-treated leaves made  $3.7 \pm 0.6$  failed phloem phases before their first successful phloem phase in contrast to only  $0.4 \pm 0.1$  on lanthanum saline-treated leaves ( $P < 0.0001$ , Kruskal–Wallis test). Additionally, the average duration of individual bouts of phloem sap ingestion was





**Figure 2.** Confocal laser-scanning microscope (CLSM) micrographs of faba bean phloem penetrated by aphid stylets (st) shortly after initiation of phloem phase. Asterisks mark forisomes in a high-volume state (a, b, c) and in a low-volume state (d). Arrow heads mark location of stylet tips, which in (a) and (b) are in a slightly different focal plane than the micrograph (focal plane of micrographs was optimized for a clear view of forisomes near the stylet tips). (a) *Myzus persicae* stylets cryofixed during E1, 146 s after beginning of phloem phase. Note the presence of high-volume forisomes in sieve elements that are not in contact with stylets nor in the same sieve tube as the sieve element penetrated by the stylets (the two forisomes on the left side of the micrograph). (b) *Macrosiphum euphorbiae* stylets cryofixed during E1, 28 s after beginning of phloem phase. The forisome in the penetrated sieve element (near arrow head) is in a high-volume state as well as a forisome in a sieve element that is not in contact with stylets nor in the same sieve tube as the sieve element penetrated by the stylets (upper left in micrograph). (c) *My. persicae* stylets in saline-without-lanthanum-treated phloem cryofixed during E1, 14 s after beginning of phloem phase. Forisome in the penetrated sieve element is in a high-volume state (out of view in micrograph) as well as the forisomes in nearby sieve elements that are not in contact with stylets nor in the same sieve tube as the sieve element penetrated by the stylets (three forisomes at bottom of micrograph). (d) *My. persicae* stylets in saline-lanthanum-treated phloem cryofixed 57 s after beginning of phloem phase. This aphid had already transitioned to E2 after 30 s of E1 when cryofixed. Note that the forisome in the penetrated sieve element (next to the stylet tips) is in a high-volume state as well as forisomes in nearby sieve elements.



over twice as long in the lanthanum treatment than in the control ( $95 \pm 13$  versus  $42 \pm 17$  min, respectively;  $P = 0.0224$ , Kruskal–Wallis test).

Aphids in the lanthanum treatment spent much less total time engaged in sieve element salivation (E1) than aphids in the control ( $3.0 \pm 0.8$  versus  $14.0 \pm 2.8$  min, respectively;  $P < 0.0001$ , Kruskal–Wallis test) and the average duration of individual bouts of sieve element salivation was less than half as long in the lanthanum treatment than in the control ( $70 \pm 23$  versus  $173 \pm 44$  s, respectively;  $P = 0.0007$ , Kruskal–Wallis test). Considering only bouts of sieve element salivation that were followed by phloem sap ingestion, the average duration of individual bouts of sieve element salivation was less than one-fifth as long in the lanthanum treatment than in the control ( $75 \pm 32$  versus  $427 \pm 239$  s, respectively;  $P = 0.0062$ , Kruskal–Wallis test). For bouts of sieve element salivation that were not followed by phloem sap ingestion, their average duration was similar in the lanthanum and control treatments ( $129 \pm 39$  versus  $155 \pm 29$  s, respectively;  $P = 0.4312$ , Kruskal–Wallis test).

To confirm that the  $\text{La}^{3+}$  treatment suppressed forisome occlusion of the sieve elements when the aphid entered phloem phase, five samples were taken from  $\text{La}^{3+}$ -treated leaves cryofixed between 34–79 s after the aphid initiated phloem phase. In two of these samples, the aphid was still in the salivation (E1) stage of phloem phase and in three samples, the aphid had already made the transition to ingestion (E2) after 26–30 s of salivation. In all five samples, the forisome in the penetrated sieve element was in the low-volume, non-occluding state even though in some samples the stylet tips were in close proximity and appeared to be in contact with the forisome; forisomes in nearby sieve elements were also in the low-volume, non-occluding state, verifying that our method of delivering lanthanum to the leaf was effective at suppressing forisome occlusion (Fig. 2d). Eight samples were taken from control saline-treated leaves cryofixed between 14–49 s after the aphid initiated phloem phase. All were still in the salivation (E1) stage of phloem phase. In six of eight samples, the forisome in the penetrated sieve element was in the high-volume state as well as forisomes seen in the nearby sieve elements (Fig. 2c). In two samples, the forisome in the penetrated sieve element was in the low-volume state and forisomes in nearby sieve elements were a mix of low and high volume.

## DISCUSSION

Results of the present study demonstrate for the first time, a long-held hypothesis that plants can respond to aphid penetration of sieve elements by P-protein occlusion and that the aphid-induced response inhibits phloem sap ingestion. This plant defence response is aphid species-specific. In a previous study using the same methodology as the present study, penetration of faba bean sieve elements by the legume-specialist aphid, *A. pisum*, did not trigger sieve element occlusion (Walker & Medina-Ortega 2012) and *A. pisum* typically engages in only short bouts of sieve element salivation (<60 s) before transitioning to prolonged periods of sap

ingestion. In contrast, in this study, penetration of faba bean sieve elements by the generalist aphids, *My. persicae* and *Ma. euphorbiae*, normally triggered sieve element occlusion and the aphids generally engaged in very long periods of sieve element salivation and usually did not transition to sap ingestion behaviour. Results of lanthanum experiment clearly demonstrate that forisome occlusion is not just correlated with inhibition of phloem sap ingestion, but that it is the cause of the observed inhibition. In the absence of sieve element occlusion, *My. persicae* readily ingests phloem sap from faba bean; thus sieve element occlusion can be a defence against phloem-feeding insects.

Behaviour of *My. persicae* and *Ma. euphorbiae* on faba bean is similar to that observed in many studies on aphid feeding behaviour on resistant plants: very long periods of salivation and/or frequent stylet withdrawn from the sieve element without engaging in sap ingestion (Cole 1994; Caillaud *et al.* 1995b; Klingler *et al.* 1998; Sauge *et al.* 1998; Garzo *et al.* 2002; Tjallingii 2006; Lightle *et al.* 2012). This study supports the hypothesis generated in these earlier studies that very long periods of E1 salivation following sieve element penetration and frequent stylet withdrawn from the sieve element without engaging in sap ingestion are indicative of inhibition of phloem sap ingestion by sieve element occlusion. This study also supports the hypothesis proposed in these studies and many others that sieve element occlusion can provide at least some degree of aphid resistance.

Faba bean is not ‘completely resistant’ *per se* to the strains of aphids used in this study; both aphid species can survive and reproduce on faba bean; however, at least in the case of *My. persicae*, it does not perform as well on faba bean as it does on a more favourable host such as sugar beet. The EPG data also indicate that the plants are not completely resistant: despite numerous failed attempts to ingest phloem sap from faba bean, about half the green peach aphids managed to successfully ingest phloem sap during the 8.5 h recording period and presumably more would have if the recording period was longer. This leads to the question: if sieve element occlusion is less than 100% effective at preventing sap ingestion, can it contribute to resistance? Aphid ecology may provide insights on this question. Although not exceptionally fecund by insect standards, aphids are classic r-strategists and their high reproductive rate is largely a function of parthenogenic reproduction and very short generation time, which is a consequence of live birth and fast development. Thus, their populations are able to grow faster than their many natural enemies can regulate. Difficulty in establishing phloem sap ingestion may reduce the assimilation of nutrients per day and reduce the rapid development rate that aphids depend on to maintain their high population growth rates. The median time it took the green peach aphid to reach phloem sap ingestion was three times longer on faba bean than on sugar beet (6.7 versus 2.2 h) which would be expected to result in a lower rate of nutrient assimilation. Furthermore, alate aphids, which are responsible for initial colonization of plants, may abandon the plant after multiple unsuccessful attempts to ingest phloem sap, thus reducing the number of infested plants in a field.

Aphids produce a proteinaceous salivary sheath that surrounds their stylets over the full length of their penetration in the plant tissue (Miles 1999). The salivary sheath, which readily stains with the fluorochromes used in this study, remains in the plant after stylets are withdrawn, and consequently, everywhere in the plant tissue where the stylets penetrated is easily detectable by the presence of salivary sheaths in the micrographs. As a result, it is clear that forisome occlusion occurred not only in the sieve element penetrated by *My. persicae* and *Ma. euphorbiae*, but also in sieve elements that were not penetrated, were not touched by the stylet pathway and were not likely to be part of the same sieve tube as the penetrated sieve element (i.e. sieve elements that are laterally separated from the penetrated sieve element) (e.g. Fig. 2a–c). For *My. persicae*, forisome occlusion was observed in most sieve elements within  $408 \pm 199$  microns upstream and  $409 \pm 147$  microns downstream ( $n = 22$ ) from the stylet tips, which is approximately the length of two sieve elements in either direction. Results were similar for *Ma. euphorbiae*. Upstream and downstream forisome occlusion is significant because, as Knoblauch *et al.* (2014) point out, if P-protein occlusion occurred only in the penetrated sieve element, it would clog the downstream sieve plate, but the sieve element would still be connected to upstream sieve elements and thus the aphid would not be cut off from the flow of nutrients from the source organs in the plant. The observed occlusion of sieve elements both upstream and downstream from the penetrated sieve element isolate the sieve element penetrated by *My. persicae*'s and *Ma. euphorbiae*'s stylets and cut off their access to the translocation stream.

The forisome response in non-penetrated sieve elements shows that the sieve element does not have to be penetrated to respond, so what is the elicitor that triggers the forisome response to *My. persicae* and *Ma. euphorbiae*? A direct effect of saliva secreted into the penetrated sieve element is an unlikely elicitor to generate the observed pattern of forisome occlusion in nearby sieve elements because it would be expected to have only downstream effects in sieve elements connected to the same sieve tubes as the penetrated sieve element. Forisome occlusion in upstream sieve elements and in sieve elements not part of the same sieve tube as the penetrated sieve element make this an unlikely explanation. We propose two hypotheses for what triggers forisomes to occlude in sieve elements in the general vicinity of the penetrated sieve element.

**Hypothesis 1:** Moreno *et al.* (2011) demonstrated that during stylet penetration, aphids secrete watery saliva into the apoplast (intercellular space that includes plant cell walls). The apoplast is not part of the translocation stream; therefore apoplastically secreted saliva would diffuse from the stylets throughout the general vicinity of the stylet pathway, both upstream and downstream relative to the flow of sap in the sieve tubes and consequently would account for the observed pattern of forisome occlusion. Hypothesis 1 postulates that apoplastically secreted saliva diffuses away from the

stylet pathway and activates  $\text{Ca}^{2+}$  channels in nearby sieve elements, triggering forisome occlusion.

**Hypothesis 2:** Variation potentials are a type of electropotential wave in the phloem that can be triggered by damage to the phloem and spread a short distance from their initiation site and activate voltage-gated calcium channels that trigger forisome occlusion (van Bel *et al.* 2014). Hypothesis 2 postulates that physical penetration of the sieve element or salivation into the penetrated sieve element triggers a variation potential that is propagated a short distance in all directions from the penetrated sieve element and triggers forisome occlusion in sieve elements within the distance.

For the phloem phases where *My. persicae* and *Ma. euphorbiae* were successful at engaging in ingestion (E2), ingestion was preceded by exceptionally long periods of E1 salivation on faba bean (>10 min for *My. persicae* on faba bean compared with 41 s on sugar beet – Table 1 and >22 min for *Ma. euphorbiae* on faba bean – Table 2). This leads to the question of whether or not prolonged E1 salivation eventually reversed the forisome plug. Will *et al.* (2007) found *in vitro* evidence that aphid saliva collected and concentrated from thousands of aphids can reverse forisomes from a high-volume, occluding state to a low-volume state; however, Medina-Ortega & Walker (2013) found that in the natural *in vivo* situation of a single pea aphid salivating into a single sieve element, there was no evidence that aphid saliva can reverse forisome occlusion. The same lack of an *in vivo* effect was also found for the vetch aphid, *Megoura vicia* Buckton, the aphid species used in the Will *et al.* study (unpublished data). Forisome occlusion is transient (Knoblauch *et al.* 2001; Furch *et al.* 2007, 2009; Medina-Ortega & Walker 2013); so lacking *in vivo* evidence that salivation reverses forisome plugs, the eventual engagement in sap ingestion after 10 min of salivation seems likely because of the forisomes spontaneously reverting back to a non-plugging state, same as they eventually do in the absence of aphid saliva.

Why does stylet penetration by the generalist aphids, *My. persicae* and *Ma. euphorbiae* (this study), trigger forisome occlusion while stylet penetration by the legume-specialist, *A. pisum*, (Walker & Medina-Ortega 2012) does not? This has important practical implications for the potential of breeding aphid resistance in crops based on phloem occlusion defences. Medina-Ortega & Walker (2013) found no evidence that *A. pisum* saliva can reverse forisome occlusion *in vivo*, so the focus is on triggering, not reversing forisome occlusion. We propose three hypotheses that address this question: (1) *My. persicae* and *Ma. euphorbiae* saliva have an elicitor that is detected by *V. faba* and triggers forisome occlusion while *A. pisum* saliva lacks such an elicitor; (2) forisome occlusion is a general 'default' response to sieve element penetration by aphids and *A. pisum* saliva has an effector that suppresses the specific *V. faba* response (calcium channel gating?) while *My. persicae* and

*Ma. euphorbiae* saliva lack such an effector; (3) penetration of a sieve element by stylet tips of *My. persicae* and *Ma. euphorbiae* results in leakage of sap and subsequent turgor loss triggering a variation potential and forisome occlusion; while in contrast, *A. pisum* stylets are sealed more efficiently preventing leakage at the penetration site (Will & van Bel 2006).

There currently is great interest in potential elicitors and effectors in aphid saliva and how they may effect plant response to aphid feeding and determine a plant's resistance or susceptibility to aphids (recently reviewed by Rodriguez & Bos 2013). Most of these studies have examined the impact of salivary components on plant defence pathways without any clear understanding of the ultimate mechanism of the pathways acting directly on the aphid. The present study identifies a specific mechanism of plant defence that acts directly on the aphid, is readily measurable and is differentially elicited or suppressed by different aphid species. Consequently, sieve element occlusion would be a fruitful subject for the study of how salivary elicitors and effectors affect a direct mechanism of plant defence against aphids.

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