Aphid (*Sitobion yakini*) investigation suggests thin-walled sieve tubes in barley (*Hordeum vulgare*) to be more functional than thick-walled sieve tubes

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Barley, like most other grasses that have been studied, contains two kinds of sieve tube. The first formed are called thinwalled sieve tubes because of their thin wall compared to the late-formed, and are associated with companion cells. The late-formed are thick-walled sieve tubes, which differentiate next to the metaxylem vessels and lack companion cells. Aphid (*Sitobion yakini* (Eastop) feeding was studied using light microscopy to determine if they preferentially feed from thin- or thick-walled sieve tubes in the barley leaf. Penetration of the stylets through the leaf epidermis and mesophyll was largely intercellular, becoming partly intercellular and, partly, intracellular inside the vascular bundle. Sixteen of 19 pairs of stylets (84%), and 293 of 317 (92%) stylet tracks terminated at the thin-walled sieve tubes, suggesting that *Sitobion yakini* feeds preferentially on the thin-walled sieve tubes which seem to be more attractive to the aphid. These thin-walled sieve tubes are thus probably the most functional in terms of phloem loading and transport.

Introduction

Barley and other members of the Poaceae, including wheat, sugarcane, maize and Southern African grasses such as *Themeda triandra*, contain both thin-and thick-walled sieve tubes, at least in the intermediate and small vascular bundles of leaf blades (see Kuo and O'Brien 1974, Walsh 1974, Botha et al. 1982a,b, and references cited therein, Colbert and Evert 1982). The metaphloem, which is formed first, consists of sieve tubes and associated companion cells. The sieve tubes are called thin-walled sieve tubes, because their walls are thin compared to the walls of the other, late-formed, metaphloem sieve tubes, whose elements differentiate adjacent to the metaxylem vessels. These are thick walled and lack companion cells (see Evert et al. 1996 and references cited therein).

Studies of the small and intermediate bundles of the barley leaf by Evert et al. (1996) and subsequently by Botha and Cross (1997) suggest that the sieve tube-companion cell complexes and the thick-walled sieve tubes are virtually isolated symplastically from each other and that the thick walled sieve tubes have no symplasmic contact with companion cells.

The presence of two types of sieve tubes in grass leaves has led to speculation about their possible role in phloem loading and transport of assimilate. Kuo and O'Brien (1974) first suggested that the thick-walled sieve tubes in *Triticum aestivum* (wheat) may be specialized for long distance transport, or serve as temporary storage reservoirs for sugar in excess of what can be transported by the thin-walled sieve tubes. However, a micro-

autoradiographic study of ^{14}C-photosynthate transport in wheat leaves did not support a functional role for the thick-walled sieve tubes, neither in storage nor directly in the transport of photosynthates (Cartwright et al. 1977). Evert et al. (1978) suggested that the thick-walled sieve tubes in *Zea mays* may play a role in retrieving solutes entering the leaf apoplast in the transpiration stream. This suggestion was based on the close spatial association between the thick-walled sieve tubes and the metaxylem vessels, as well as on their possession of plasmalemma tubules, which greatly increase the apo-plast–symplast interface (Evert et al. 1977). Microautoradiographic studies of phloem loading and transport in the leaf of maize (Fritz et al. 1983) showed that the thin-walled sieve tubes are capable of accumulating sucrose and photosynthates from the apoplast, without the companion cells serving as intermediaries, and are primarily involved in the uptake of current photosynthates. Thick-walled sieve tubes also received photosynthates, primarily transferred to them after accumulation by contiguous vascular parenchyma cells, but possibly also through retrieval from the xylem, into which solutes could have leaked from the phloem free space. However, these experiments were based on detached leaves and may not reflect the situation in the whole plant. Fritz and Evert (1983) also suggested that the thick-walled sieve tubes in the maize leaf are not involved in long distance transport, but could quickly pass their contents laterally to thin-walled sieve tubes of the larger bundles.

Accumulation of Prussian blue crystals in the cell walls of both thin-and thick-walled sieve tubes in the leaves of *T. triandra, Z. mays, Saccharum officinarum* and *Bromus unioloides* (Botha et al. 1982a, Evert et al. 1985, Botha and Evert 1986) demonstrated the ease with which water moves from the lumina of the vessels to the phloem apoplast.

Based on these studies on grasses and the findings of Botha and van Bel (1992), it is clear that there is evidence to support a disjunction between the thin-walled sieve-tube companion cell complex and the thick-walled sieve tubes. Even though there is evidence that both thin-and thick-walled sieve tubes are capable of assimilate uptake and transfer (Fritz et al. 1983) and again that the cell walls of thin-and thick-walled sieve tubes are apparently freely permeable to solutes (Botha and Evert 1986 and literature cited), there is no real evidence for a long-term function for the thick-walled sieve tubes in grasses. Clearly, alternative methods need to be explored to determine the role of the thick-walled sieve tubes in plants such as barley.

Feeding aphid colonies have been shown to be very useful for examining potential functionality of specific phloem groups (Evert et al. 1968, 1972, Botha et al. 1975, Botha and Evert 1978). In this study, the presence of stylet tips and the lack of sheath material in association with them in living sieve elements was used as a criterion to identify functional sieve elements (see Evert et al. 1968, Botha et al. 1975, Botha and Evert 1978). Aphids appear to select their feeding sites according to the quantity and quality of the food that is yielded. It has been suggested that high levels of sucrose, amino acids and organic ions, which are characteristic constituents of phloem sap, may provide the stimuli by which phloem-feeding aphids recognize sieve elements (Mittler and Dadd 1965). By inference, the sieve element that is chosen as a feeding site by the aphid should contain more of these substances and can therefore be regarded as being more functional than other sieve tubes.

The feeding habits of the maize aphid (*Rhopalosiphum maidis*) on barley were studied by Evert et al. (1972) using an electron microscope to determine the manner of stylet penetration. This aphid probes preferentially from the adaxial surface of the leaf, penetrating the epidermis largely intercellularly and the vascular bundles mainly intracellularly. Unfortunately Evert et al. (1972) did not determine which sieve tubes (thin-or thick-walled) were penetrated.

Given the earlier work, which suggests no apparent role for the thick-walled sieve tubes, we decided to investigate further, to determine the site of penetration of aphid stylets and the role of the thick-and thin-walled sieve tubes, using colonies of aphids feeding on barley leaves. As aphid feeding has been equated to preference feeding, implying that it is functional (transporting) phloem that is penetrated (Evert et al. 1968, 1972, Botha et al. 1975, Botha and Evert 1978), determination of the site of stylet penetration would indicate if thin or thick-walled sieve tubes are active in phloem transport.

We hypothesize that there may be no functional role for the thick-walled sieve tube under normal (unstressed) conditions in barley. This study focuses on the termination point of the stylets and stylet tracks in order to assess feeding preference. It also sets out to examine the feeding habit and manner of penetration of the barley leaf by the aphid *Sitobion yakini.*

Materials and methods

Plant host material

Colonies of the *Sitobion yakini* Eastop aphid were established on barley plants (14 d after germination) and kept in insect cages in the greenhouse at the Botany Department, Rhodes University, Grahamstown, South Africa. Greenhouse settings were 25°C, 14 h sunlight/day, with high relative humidity. The aphid colonies were transferred to young barley plants every 2 weeks to ensure succulent hosts. Portions of mature leaves to which the aphids remained attached were selected for light microscopic investigations of stylet penetration. The aphids were killed in situ by rapidly exposing them to a 100% acrolein atmosphere to minimize stylet withdrawal. Six segments from six different plants were randomly selected from the pots.

Light microscopy

Leaf segments containing attached aphids were fixed in FAA for 24 h. The segments were then cut into smaller pieces and dehydrated through an alcohol and tertiary butanol series. The material was infiltrated with a number of changes of wax over 3 d, in an embedding oven at 60°C. Blocks were mounted and trimmed and serial sections were cut at 15 µm using a Minot rotary microtome (Leitz Wetzlar, Germany). Sections were stained in safranin and fast green, mounted onto slides with Canada Balsam and dried in an oven at 37°C for 3 weeks. Serial transverse sections were carefully examined for stylets and stylet tracks or sheaths, which are easily preferentially stained. The use of Zeiss Planapochromat oil immersion lenses (magnification X 40,X 100 thick-walled sieve tubes) made it possible to see the tips of the stylets. In all 2000 serial sections were cut from these segments, about 330 sections from each segment.

Data analysis

All data collected were analysed using Statistica 99 software (Sta-soft, incl.). The significant differences between the number of times a sieve tube and/or vascular bundle is visited by the aphid were calculated using a one-way analysis of variance (ANOVA) and Chi square-tests.

Results

Brief description of the leaf segments examined

The leaf of barley has a typical Pooid grass anatomy. The vascular bundles are widely separated by a loosely arranged mesophyll and are surrounded by two sheaths, an inner mestome and an outer parenchymatous bundle sheath. Three orders of longitudinal vascular bundles are present, namely, large (first order), intermediate (second order) and small (third order). Mature, large bundles contain a protoxylem lacuna and two large metaxylem vessels, one on either side of the lacuna. Protophloem and metaphloem are also present, but only metaphloem elements are seen in mature bundles. The intermediate bundles lack protoxylem lacuna as well as the large metaxylem vessels, which are therefore characteristic of the large bundles only. As in other grasses (see Botha et al. 1982, Dannenhoffer et al. 1990), large and intermediate bundles in the barley leaf are associated with girders or strands of hypodermal sclerenchyma. Small bundles are smaller than the intermediate bundles and contain vascular tissue composed of metaxylem and metaphloem only. The metaphloem of all three types of longitudinal bundles contain both thin-and thick-walled sieve tubes. The spatial distribution and separation of thin-and thick-walled sieve tubes (open and filled circles, respectively, Fig. 1) as well as the lack of recognizable companion cells associated with the thick-walled sieve tubes is evident in this intermediate vascular bundle. Based upon this and other electron micrographs the intermediate and small vascular bundles all contained at least one, but not more than two, thick-walled sieve tubes, and between 3 and 5 thin-walled sieve tubes (see Botha and Cross 1997 and literature cited therein, for a further discourse on thick-and thin-walled sieve tubes). On average there is one thick-walled sieve tube in small vascular bundles and two in intermediate vascular bundles.

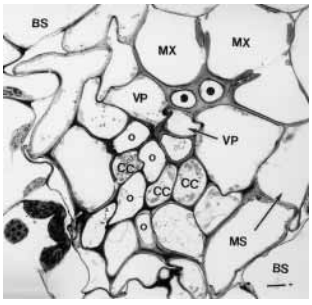


Fig. 1 Transmission electron micrograph (TEM X 1500) showing an intermediate bundle in transection. Two thick-walled sieve tubes (solid dots) occur adjacent to the metaxylem (MX). Thin-walled sieve tubes (open circles) and their associated companion cells (CC) are present. Bundle sheath (BS) surrounds a mestome sheath (MS). Bar =2 µm.

Stylet penetration of the leaf blade

Penetration of the leaf blade by the aphid was determined microscopically by following the pathway of the stylet tracks or sheaths in serial transverse sections of the host tissue. Stylet tracks were examined for point of origin (i.e. from the ad-or abaxial leaf surface) to the point of termination (i.e. in the thin-or thick-walled sieve tubes). The principal data obtained during this study are presented in Table 1.

Statistical analyses of results show a highly significant difference between penetrations of thin-and thick-wall-ed sieve tubes for each plant and for the total of all the plants. The thin-walled sieve tubes (SE, 49 (± 10) are significantly more visited by the aphid than the thick-walled sieve tubes TWSE, 4 (± 4). Almost 90% of the stylet tracks seen, terminated in the thin-walled sieve tubes. In addition, 16 of 19 pairs of stylets encountered were lodged in the thin-walled sieve tubes and only one pair in the thick-walled sieve tubes. The entrance of the stylets through the epidermis and mesophyll was largely intercellular, the pathway apparently becoming intracellular only once inside the vascular bundle (Figs 1D,E).

Commonly, the aphid's stylets approached the veins obliquely, not directly from above or below. Branched stylet tracks were relatively infrequent (see Figs 2A,C). Where branching occurred it was mostly either near or in the bundles themselves. Figure 2F shows the termination of a stylet sheath within a thin-walled sieve tube. Examination of serial sections revealed that the aphid, which formed this sheath first, bypassed the bundle, entering the mesophyll on the upper side of the small vein, as shown by the salivary sheath left behind (unlabelled black arrowheads point to unsuccessful probes). Partly withdrawing its stylet, the aphid then probed downwards reflexing its stylets through approximately 90°, before penetrating the bundle sheath and a thin-walled sieve tube. The aphid first penetrated the bundle sheath cell, a metaxylem vessel, then probed the thick-walled sieve tube and adjacent parenchymatous elements, before penetrating the thin-walled sieve tube below. Note the unoccluded maxillary stylet tips (arrow points to tips of the stylets) which can be seen just protruding into the lumen of the thin-walled sieve tube. The lack of saliva deposition within the sieve tube, and the presence of open stylet tips inside the sieve tube indicates active feeding (Evert et al. 1968, Botha et al. 1975, Prado and Tjallingii 1994) within this small vein.

In all successful probes of the phloem tissue only the tips of the maxillary stylets were inserted into the sieve tubes (see Figs 2A,B,C,F). Figure 2C shows the only successful probe of the thick-walled sieve tubes in which the stylets were still present. In this case, the aphid probed the metaxylem intercellularly, before penetrating the thick-walled sieve tube. This micrograph shows that only the tips of the maxillary stylets (arrow) entered the thick-walled sieve tube. They are free of salivary material, suggesting active feeding before the aphid was killed. Unlike the pair of stylet tips in Figs 2A,C,F which were unoccluded, the stylets in Figs 2D,E were surrounded by salivary material. It is likely that the aphid began to withdraw its stylets during manipulation of the leaf and, having been disturbed, may thus have ejected saliva. It was therefore not possible to determine whether the aphid was feeding. Penetration of several sieve tubes can be seen in the intermediate bundle (Fig. 2E). Two probes apparently unsuccessful can be seen inside the intermediate bundle. The aphid first probed a bundle sheath cell intercellularly (black

arrowheads) and then continued through the central file of thin-walled sieve tubes. Apparently it did not probe the thick-walled sieve tube, but terminated at a thin-walled sieve tube. Figure 2D shows a similar probe, where the aphid first penetrated the bundle sheath cells (apparently intercellularly) and subsequently, a thick-walled sieve tube (white arrowhead) and ultimately, several sieve elements, before terminating in one of these (star points to terminal probe).

The majority of stylet probes terminating in the thin-walled sieve tubes, were initiated from the abaxial surface of the leaf (see Table 1).

Of a total of 317 stylet tracks encountered during the course of this investigation, 142 were associated with the small vascular bundles, and 92 and 83 in the large and intermediate bundles, respectively (see Table 1). Results from One-way anova for the variation between the number of times each vascular bundle (small, intermediate and large) were visited showed significant differences between the vascular bundles. It is important to note that the small bundles in the barley leaf-blade, contain only two or three thin-walled sieve tubes, whilst the intermediate and large bundles contain more. The aphids thus aim at a smaller cross-sectional area of (presumably) functional phloem when they visit the small bundles, compared with the intermediate and large vascular bundles.

Discussion

The data presented here support the hypothesis that *S. yakini* feeds preferentially on the thin-walled sieve tubes in barley. The thin-walled sieve tubes are more attractive to *S. yakini* as a feeding site, in that 94% of stylets and 92% of stylet tracks, were seen to terminate in the thin-walled sieve tubes of the longitudinal veins in mature barley leaves. This observation together with those of Cartwright et al. (1977) and Fritz et al. (1983) on translocation and phloem loading and transport in leaves of wheat and maize (which also contain both thin-and thick-walled sieve tubes), respectively, strongly support the idea that thin-walled sieve tubes play a more important role in phloem loading and transport than the thick-walled sieve tubes.

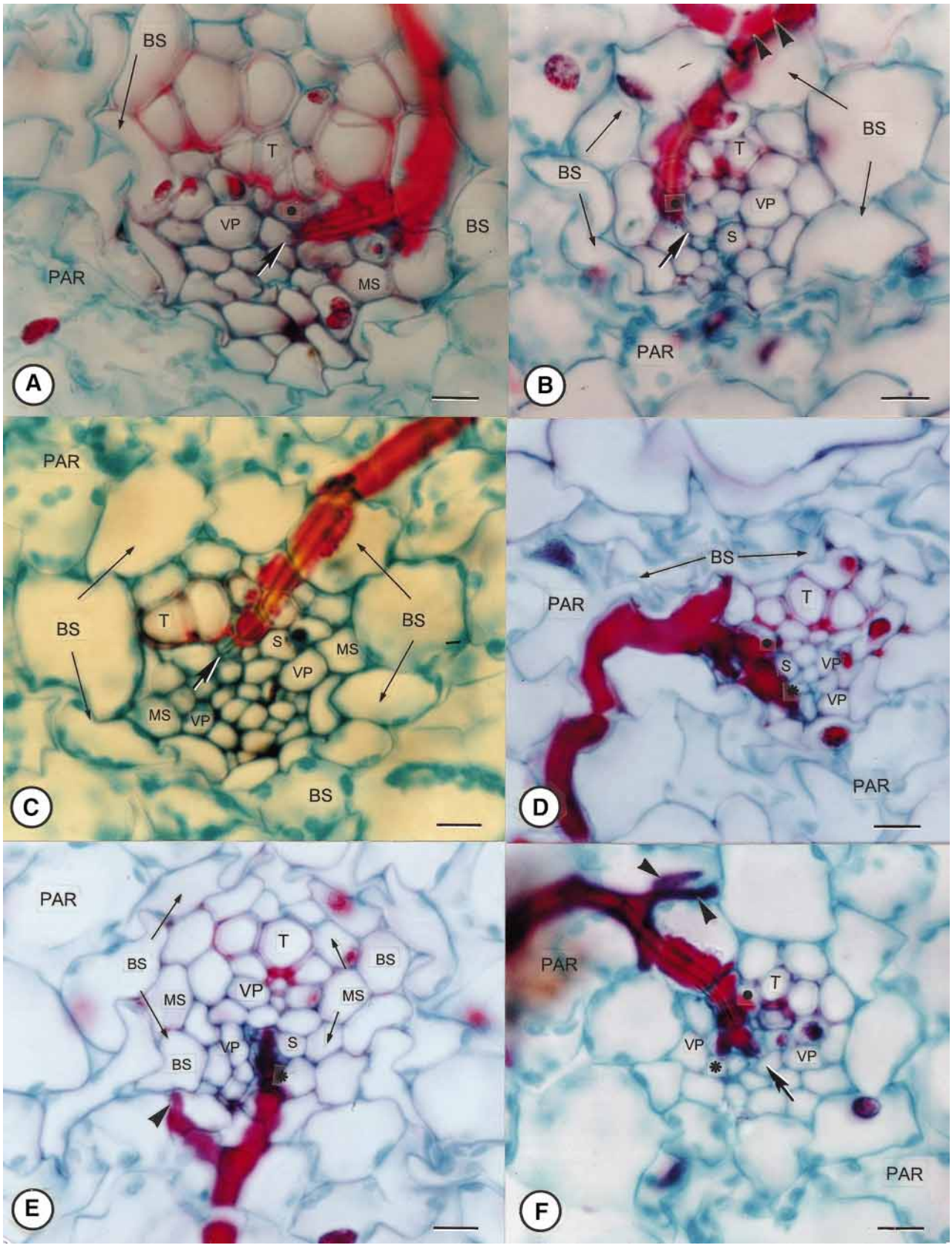
Prado and Tjallingii (1994) reported that during ingestion of phloem sap, the stylet tips of aphids projected beyond the salivary sheath that terminates at the cell wall, as the salivary sheath does not reach the sieve element. Several other authors also demonstrated that the maxillary stylet tips (of other aphids) projected beyond the salivary sheath during penetration (see Evert et al. 1968, Botha et al. 1975, Botha and Mabindisa 1977, Botha et al. 1977, Botha and Evert 1978). In the present study, it has been demonstrated that the maxillary stylet tips of *S. yakini* project beyond the salivary sheath in most cases and are open within the sieve tube being tapped. It would seem then, that the presence of open stylet tips and lack of associated sheath material within the sieve elements, still constitutes sufficient evidence for such sieve elements to be identified as functional.

Table 1. Statistical analysis of the origin of probes at the adaxial and abaxial epidermis, the distribution of stylets and stylet tracks/sheaths within the leaf and the termination of stylet tips and stylet tracks in either thin-or thick-walled sieve tubes, together with the number of times the thin (SE) and thick (TWSE) walled sieve tubes are visited, with the mean standard deviation (Chi -square test level of significance; *** P <0.005) and results from one-way anova for the variation between and within of the number of times a sieve elements (thin-(SE) or thick-walled (TWSE) and the bundles (small, intermediate, large) are visited with the mean standard deviation. The level of significance *** *P* <0.005

Termination of aphid stylets and stylet tracks in different sieve tube types.

Visits as indicated by Stylets	SE	TWSE	Total
adaxial origin	9	1	
abaxial origin	7	0	
Stylet sheaths			
adaxial origin	93	9	
abaxial origin	184	14	
Total visits	293*** (92%)	24 (8%)	317 100%
Distribution over 6 replicates	65, 57, 46, 48, 45, 42	9, 8, 5, 1, 0, 1	
Mean and sd	49*** ± 10	4 ± 4	
Distribution of visits in different bundles			
Small vascular bundle	142	0	142
Intermediate vascular bundle	77	6	83
Large vascular bundle	74	18	92
Mean and sd	98.7 ± 38.4	8 ± 9	
Significance in difference between bundles	***	***	

Figs 2A-F. show penetration of vascular tissue by *Sitobion yakini*. Fig. 2A. Penetration of an intermediate bundle, from the adaxial leaf surface. The aphid's stylet tips are visible (unlabelled arrow) just beneath a thick-walled sieve tube (white arrowhead). T = tracheary element; BS = bundle sheath; MS = mestome sheath; VP = vascular parenchyma; PAR = mesophyll parenchyma. Scale bars = 10 µm. Fig. 2B. Penetration by stylets of a thin-walled sieve tube in a small vascular bundle. The aphid first probed the bundle sheath (unlabelled black arrowheads), mestome sheath, xylem before terminating in a thin-walled sieve tube. The tips are lodged within the sieve tube (unlabelled arrow). Lack of saliva associated with tips, suggests that the aphid was feeding from a functional sieve tube. S = sieve tube. Fig. 2C. Penetration of a thick-walled sieve tube in a small vein. The aphid probed the xylem both intercellularly and intracellularly, before penetrating the thick-walled sieve tube. Stylet tips (unlabelled arrow) terminate within the thick-walled sieve tube. The tips are open and free of salivary material. Fig. 2D,E. Penetration of a small (Fig. 1D) and intermediate (Fig. 1E) leaf vascular bundle. Both penetrations originated from the abaxial side of the leaf. Figure 2D. The aphid penetrated this vascular bundle from the side, recurved its tips, and successively penetrated a thick-walled sieve tube (white arrowhead) and then sequentially probed all remaining thin-walled sieve tubes, terminating at a functional thin-walled sieve tube. Star points to a terminal probe. Lack of salivary material within sieve tube suggests that the aphid fed from this sieve tube. Figure 2(E) shows a branched stylet track. After penetrating the bundle sheath to the left (unlabelled black arrowhead), the aphid pierced successive thin-walled sieve tubes. Fig. 2F. Aphid stylets extending from the abaxial surface of the leaf to a small bundle, and terminating in a thin-walled sieve tube. The aphid first probed a mesophyll cell (unlabelled black arrowheads) then continued downward through bundle sheath and parenchyma cell, before terminating in the thin-walled sieve tube (unlabelled arrow).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Quite significantly, there was a 100% strike for thin-walled sieve tubes in the small vascular bundles, with more than 90% of the probes of intermediate vascular bundles also terminating in thin-walled sieve tubes, and 80% of the 92 probes terminating within the thin-walled sieve tubes in large bundles (see Table 1). Our findings thus support a positive loading role for the small bundles as they were implicated by Altus and Canny (1982) and Evert et al. (1996), with a decreased 'palatability' in respect of the transport veins (intermediate and large veins) in the barley leaf. The closer the stylets are to the site of vein loading, the greater is both the quality and quantity of the food.

Our results also demonstrate that the aphids probed preferentially from the abaxial leaf surface, results that differ from those of earlier studies showing that aphids feed preferentially on the adaxial surface of the barley leaf (see Evert et al. 1972). However, this may simply be due to a difference in aphid species used.

While it has been suggested that aphids and other suctorial insects find their target cells by trial and error (Evert et al. 1968 and literature cited therein, Botha et al. 1975), there is evidence that suggests that the aphids' stylets do not enter the tissue haphazardly, but are directed to their objective with marked precision (Chatters and Schlehuber, 1951, cited in Evert et al. 1968). Based upon our observations, *S. yakini* appears sometimes to locate the phloem by chance (Fig. 2D) and at other times with a degree of precision (Figs 2B,C,E). Precise location appears to be more frequent, as branched tracks, which are suggestive of trial and error, were relatively infrequent. It is possible that multiple probes (interpreted by us as trial and error probes) could result in low stylet pressure within the sieve tubes, leading to decreased flow rate, causing the aphid to withdraw its stylets to look for a new feeding site.

A question arising from the present study is: why do aphids feed almost exclusively on thin-walled sieve tubes? It can be argued that the structure or composition of the walls of the thick-walled sieve tubes makes it difficult for the aphid to penetrate compared with that of the thin-walled sieve tubes. However, it must be borne in mind that aphids have been found to probe the bark of tree (Evert et al. 1968) and penetrate through the xylem both intercellularly and intracellularly over long distances, in search of a more suitable feeding site (i.e. internal phloem) in the stems of *Gomphocarpus physocarpus* (Botha et al. 1975). However, it is more plausible that the thin-walled sieve tubes may contain some substances desirable to the aphid that are either lacking in the thick-walled sieve tubes, or present in smaller amounts. Alternatively, perhaps greater quantities of assimilate are transported in the thin-walled sieve tubes and that the aphids might be drawn to higher sucrose concentration in such actively transporting sieve tubes. With regard to all these possibilities, it must be kept in mind that the greater majority of stylet tips and tracks initiated, bypassed the thick-walled sieve tubes without any evidence of penetration of the thick-walled sieve tubes. Figures 2D,F clearly demonstrate this point. Instead of penetrating a thick-walled sieve tube next to the one the aphid had penetrated (see examples in Figs 2D,F) it redirected its stylets and searched for a thin-walled sieve tube further down. In addition, where the thick-walled sieve tubes were penetrated, the aphid proceeded further on in search of a more suitable feeding site and usually terminated successfully in the thin-wall-ed sieve tubes (Figs 2A,B,D,F). The earlier

formation and quantitative dominance of the thin-walled sieve tubes over thick-walled sieve tubes lends support to the idea that more assimilates are translocated in thin-than in thick-walled sieve tubes.

Whatever the case, it is clear from this study that *S. yakini* prefers to feed on the thin-walled sieve tubes in barley. Whether it is sucrose or some other substance, which is the attractant, remains to be determined.

Conclusion

Based on the preponderance of stylet tips and penetrations or probes of the thin-walled sieve tubes, we conclude that the aphid preferentially fed from the thin-walled sieve tubes, due either to the presence of some desirable compounds or possibly due to higher osmotic potential resulting from higher sucrose concentration in the thin-walled sieve tubes.

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