Reduction in transport in wheat (*Triticum aestivum*) is caused by sustained phloem feeding by the Russian wheat aphid (*Diuraphis noxia*)

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The Russian wheat aphid (*Diuraphis noxia*) feeds preferentially from the phloem of longitudinal veins of nonresistant wheat leaves. The xenobiotic, 5,6-CFDA was applied to exposed leaf blade mesophyll cells in control and aphid-infested plants. In control plants, the fluorophore moved approximately 5–6cm from the point of application of 5,6-CFDA within 3h of application. The fluorochrome was transported in the sieve tube companion cell complex, including those in the numerous interconnecting transverse veins. Leaf blades on which the Russian wheat aphid had been feeding demonstrated a marked decrease in 5,6-CF transport. Aphids feeding on the leaves formed local sinks and redirected the fluorophore (and presumably associated assimilate) to the aphids' gut, with little longitudinal translocation of 5,6-CF below inserted stylets or aphid feeding areas. Aniline blue staining revealed massive deposits of wound and reaction callose caused by the aphids, with callose associated with the sieve plates, pore-plasmodesma between the companion cells and their associated sieve tubes, as well as with plasmodesmal aggregates in parenchymatous elements within the vascular bundles. Leaves that had been colonised by aphids but from which the aphids were removed, showed extensive wound callose deposits, which persisted for up to 48h after removal of aphid colonies, suggesting that the damage caused by aphid feeding is a long-term, non-transient event in non-resistant plants.

Introduction

The investigation of cell damage caused by the aphid, *D. noxia*, to wheat leaves is limited to laboratory experiments which have shown that changes occurred in the cell organisation within wheat tissue treated with aphid extracts (Fouché *et al.* 1984, Hewitt *et al.* 1984, Kruger and Hewitt 1984, Van der Westhuizen *et al.* 2002). There is limited information available on the physiological consequences of *D. noxia* feeding.

A recent paper by Van der Westhuizen *et al.* (2002) reported on the localisation of β-1,3 glucanase after Russian wheat aphid infestation in resistant and non-resistant wheat lines. The enzyme's substrate, β-1,3 glucan, is reported to accumulate at sites of attempted pathogen penetration (Benhamou 1992). According to Van der Westhuizen *et al.* (2002) the damage caused by the Russian wheat aphid results in the accumulation of β-1,3-glucanases where tissues are affected most by feeding aphids. Furthermore, β-1,3-glucanase accumulation was greater in resistant plants, indicating a possible role for this enzyme in the resistance mechanism against Russian wheat aphids. Clearly this, and other Pathogenesis Related (PR) proteins are related to aphid infestation. Forslund *et al.* (2000) reported that chitinase and β-1,3-glucanase activity are induced by the bird-cherry–oat aphid, *Rhopalosiphum padi* infestation, and that PR protein activity appears to be more strongly induced in resistant lines than in susceptible lines.

The vascular bundles in grasses contain two distinct sieve tube types. The first is described as thin-walled, and the sieve tube members are associated with companion cells. These are the first to mature and in the literature are also referred to as early metaphloem (Evert *et al*. 1978, Botha and Evert 1988). In the second, one, or sometimes two, sieve tubes are found in close proximity to the metaxylem. These lack companion cells, and have thickened walls.

Ni and Quisenburry (1997) and Girma *et al.* (1992) reported that the feeding site of *D. noxia* is the phloem, but did not provide much information on the feeding habit of the aphid, or which sieve tube type was preferred. More recently, Matsiliza and Botha (2002) showed that there appears to be no real role for the thick-walled sieve tubes in barley, as aphids preferentially feed from the thin-walled sieve tubes.

Phloem cells, like other parenchymatous elements, are susceptible to damage caused by osmotic shock (Botha and Cross 2000), which triggers the synthesis of β-1,3 glucan (callose) within a relatively short time period. Callose is easily visualised using aniline blue and a conventional epifluorescence microscope under blue light.

Given that there are reported differences in feeding resistance (and assumedly, differences in the level of damage caused by the RWA), and that in resistant cultivars the aphid apparently probes more but feeds

less, it was felt that a detailed examination of the translocation pathway in uninfested and RWA-infested wheat leaves of a non-resistant cultivar (where the aphid would probe less but feed more), would provide valuable information about the feeding pattern of the aphid, as well as detailed information about the damage caused by the RWA to the phloem.

Despite papers that have appeared recently which deal with susceptibility and resistance to aphids by cereals (Forslund *et al.* 2000, Van der Westhuizen *et al.* 2002), several important questions pertaining to the probing and feeding behaviour of *D. noxia* remain unanswered. For example, can cell damage caused by feeding aphids be visualised *in vivo*? What effect does feeding have on the normal functioning of the phloem itself? Can the transport of assimilate in aphid-infested and control plants be visualised? Do feeding aphids establish themselves as local assimilate sinks? How soon does the aphid affect the physiological capacity of the translocating phloem? Does long-term feeding result in long-term damage to the phloem? What are the effects of sustained feeding on phloem-associated parenchymatic elements? These questions are of significance in understanding the effects of sustained feeding by *D. noxia* on lines of wheat which are susceptible and resistant to the aphid.

The present work focuses on the effects of aphid feeding on phloem translocation using a symplasmically-transported fluorophore, 5,6-CF, to probe transport capacity reduction attributable to aphid feeding in a susceptible wheat line. Damage to sieve tubes, their associated companion cells and vascular parenchyma cells was investigated using aniline blue, a dye that specifically highlights wound callose deposition on sieve plate pores, pore plasmodesmal units between the sieve tube members and their companion cells, as well as that associated with plasmodesmata between companion cells and associated parenchymatic elements.

Materials and Methods

Plant host material

Wheat seeds (*Triticum aestivum* L. cv. Adamtas) were pre-germinated in petri dishes and thereafter sown in potting soil in 17cm diameter pots. The plants were colonised two weeks after germination, with the aphid, *Diuraphis noxia* (Mordvilko). Aphid-colonised plants were kept in insect cages either under controlled conditions (Conviron S10H, Controlled Environments Limited, Winnipeg, Manitoba, Canada) at 28.8°C and RH 50% for a 14h photoperiod, or in the greenhouse (at 30°C). Aphid colonies were transferred to young wheat plants every two weeks, to ensure succulent hosts. Plants with suitably established aphid colonies were selected for further study. The second visible leaf of both aphid-infested and control wheat plants were used were used in all translocation experiments.

Fluorescence microscopy

Aniline blue treatment

Leaf strips (approximately 3cm long) of both infected and control wheat were cut out from whole leaves. The abaxial surface was scraped gently in places, under MES buffer (2-[morpholino]ethanesulfonic acid hydrate, Sigma Aldrich Johannesburg, South Africa, number M-8250, pH 7.2; see Knoblauch and Van Bel (1998) for details of buffer composition). Small windows were scraped into the epidermis using a sharp single-edged razorblade, to expose the underlying mesophyll and vascular tissues. Aniline blue (0.05%, w/v in MES) was applied drop-wise to the exposed area. After 5–15min, leaf strips were examined for callose fluorescence using a Zeiss Standard Junior 18 microscope, fitted with a 50W HBO UV lamp (Zeiss, Johannesburg, South Africa). Callose was visualised using a Chroma (number 1103) violet filter set (BP 425-440, LP 475, FT 460nm, Chroma technology, Battlebro, USA). Callose-related fluorescence was digitally imaged using an Olympus DP-10 camera (Wirsam Scientific, Johannesburg, South Africa) and downloaded as tagged information format images at 1 440 pixels per inch resolution.

Preparation of 5,6-Carboxyfluorescein Diacetate (5,6-CFDA)

Stock 5,6-CFDA was made up as follows: 1ml of dimethyl sulphoxide (DMSO) was added to 100mg of 5,6-CFDA (number C-8166, Sigma Aldrich Chemical Corp. Johannesburg, South Africa). Thereafter, 1–2µl of the stock was added to polypropylene vials containing 1ml distilled water. Stock and working strength 5,6-CFDA were kept foil-wrapped and stored at –4°C until needed.

Observation of leaf tissue

The tissue was prepared by gently scraping small 'windows', under MES buffer. 5,6-CFDA was added directly to the exposed mesophyll cells, and the regions were covered with coverslips to prevent dehydration of the mesophyll tissues.

To observe the transport characteristics of the fluorochrome, leaf segments were excised, gently scraped with a single-edge razor blade under MES buffer to expose small 'windows' into the mesophyll below the point of application of 5,6-CFDA. Leaf strips were mounted under coverslips and immediately viewed using a Zeiss epifluorescence microscope using a GFP filter set with an additional 10-C Topaz emission filter (Chroma Technology, Battlebro, USA) to minimise the autofluorescence normally associated with lignin.

Results

Transport of 5,6-CFDA in control leaf tissue

Three hours after application, 5,6-CF transport was easily distinguished by its bright yellow-green fluorescence. In total, 30 source leaves were examined after allowing 5,6-CF transport using the application technique described previously. In most cases, the fluorescence front was observed up to 6cm from the point of 5,6-CFDA application. 5,6-CF trafficking was observed after gently scraping the lower epidermis with a single-edge razor blade.

The fluorochrome was observed in all vein size classes, but it was more intense in the small and intermediate veins. In cases where fluorescence was observed in larger veins, transverse veins connected to those larger veins showed evidence of lateral transfer. Figures 1 and 2 show 5,6-CF within a transverse vein interconnecting an intermediate (right) and small vein (left). It is not possible to determine if the dye is associated either with vascular parenchyma elements, or with the single sieve tube found in all cross veins of the wheat leaf.

Co-staining with aniline blue allowed easier identification of sieve tube members, sieve plates, lateral sieve area pores and plasmodesmata, as these all showed evidence of callose formation. The vascular parenchyma cell in Figure 2 appears to be associated with the fluorochrome, but not the sieve tube. Figure 3 shows 5,6-CF in the vascular parenchyma cells and their associated sieve tube members.

Transport of 5,6-CF in aphid-infested leaf tissue

Movement of 5,6-CF was examined 3h after application. Figures 4–11 illustrate aspects of the effect of the aphid's feeding habit on long-term translocation. Little fluorochrome was evident, and even after 4h the fluorochrome moved only approximately 2cm from the point of application, suggesting a slower uptake/transport rate. As in the controls, most of the fluorochrome observed was in the smaller veins.

Figures 4 and 5 show several stylets and or tracks (some with stylets still lodged) within longitudinal veins. Several of these stylet tracks contain 5,6-CF, suggesting that the tracks were still open and that the aphids had been ingesting phloem sap, prior to examination. In Figure 5, two stylets remain lodged in the small vein. In many cases where stylet tracks or aphid stylets were visible, the fluorochrome front seemed to terminate at the point where the stylet track terminated at the sieve tube wall or at the point of stylet insertion, with no evidence of 5,6-CF beyond this point. That aphids ingested 5,6-CF along with phloem sap is shown in Figure 6 in which the fluorochrome is visible in the aphid's gut. Figure 7 shows bright fluorescence associated with salivary sheath material that had been ejected into a small vein; the disturbed aphid withdrew its stylets, ejecting salivary material. A mixture of salivary sheath material with 5,6-CF-containing phloem sap moved acropetally as well as basipetally. Where disturbance was evident, little if any evidence of 5,6-CF movement was encountered below the point of stylet withdrawal, suggesting that the sieve tubes were blocked during withdrawal. Figure 8 shows the penetration of a transverse vein. The stylets are lodged at the junction between the transverse vein and a longitudinal vein. No fluorochrome is visible in this cross vein below the probed sieve tube, suggesting that the local sink formed by the aphid was strong enough to redirect assimilate (and 5,6-CF) towards its gut.

Cell damage caused by D. noxia *feeding*

Callose deposition in control leaf tissue

Twenty leaf strips were examined for the presence of wound callose. Figures 9–10 illustrate aspects of

control and wound damage responses attributable to aphid feeding. Callose formation was induced in control plants, simply by partial damage caused by even the gentlest scraping. Sieve plate plugging and the associated callose, as well as that in lateral sieve areas and pore-plasmodesmal units, was evident after 10–15min.

Figure 9 shows the distribution of callose-associated fluorescence in a longitudinal and transverse vein, in which bright fluorescence is associated with sieve plates and poreplasmodesmal units. Other than the sieve plates, very little callose is evident along the longitudinal vein. Figure 10 shows a transverse vein at higher magnification, in which some callose is associated with sieve plates and pore-plas-modesmal units.

Callose deposition in infested leaf tissue

Figures 11–13 show examples of the distribution of callose after prolonged aphid feeding. Most callose deposition was found in the longitudinal veins, and was primarily phloem-associated. Figure 11 shows a stylet track terminating just above a cross vein. The track itself is extremely callosed near the phloem. Figure 12 shows the distribution of wound callose in a transverse vein. The wound callose occurs in a file of sieve tube members. There is a region of massive callose deposition (lower left), as well as a punctate distribution over pore-plasmodesmal units and sieve plates further along up this single file of sieve tube members.

Aphids cause severe cellular damage to the transport components of the phloem. Figure 13 shows a region within phloem tissue 24h after removal of a feeding aphid colony. The wound response is extensive. By 36h, callose was still evident, but reaction with aniline blue was not as intense, suggesting a slow recovery or reversal of the wounding. However, callose was still evident two to three days after removal of the feeding aphids.

Discussion

This study demonstrates that 5,6-CF taken up by the mesophyll cells of source leaves of wheat, moves towards the phloem tissue and follows a classical source to sink pathway to the companion cell/sieve tube complex. Aphids preferentially feed from thin-walled sieve tubes in grasses (Matsiliza and Botha 2002). This finding is supported by the fact that 5,6-CF was often seen in the gut of feeding aphids. Co-local-isation of 5,6-CF with aniline blue, identified 5,6-CF as being in the sieve tubes of cross veins in the wheat leaf.

Where aphids had infested and fed from wheat leaves, little longitudinal or transverse 5,6-CF trafficking was seen, which confirmed that the aphids probably caused massive cellular damage, effectively re-routing assimilates (containing the fluorochrome 5,6-CF) to themselves. This resulted in the formation of strong local secondary sinks, thus ensuring a continuous supply of diverted nutrients. Alteration of the transport pattern is evidenced by the large areas of wound related callose seen in all infested material examined (Figures 10–14), which must lead to a decline in the availability of assimilates either loaded and transported, in source leaves, or unloaded, in sinks.

The results reported here are supported by previous studies. For example, aphids have been reported to grow faster in communities than in isolation (Dixon and Wratten 1971). Cagampang *et al.* (1974) showed that the rate of upward sap transport for rice plants infested by *Nilaparvata lugens* was only 60% of that of their healthy counterparts. Further evidence for a reduction of assimilate trafficking is found in the paper by Hicks *et al.* (1984), who reported that the quantity of labelled assimilates in leaf blades above petioles that had been girdled by two nymphs of the three-cornered alfalfa hopper, (a grazer) was twice that found below the girdle. It is possible, that collectively, they establish a sink of sufficient magnitude to the detriment of the plant. Nielson *et al*. (1990), concluded that the acropetal movement of photosynthates in alfalfa was seriously disrupted by feeding of the potato leafhopper and showed that the rate of photosynthate transport to the tips of exposed stems was reduced by about 60% of that calculated for control plants. Girma *et al.* (1993) showed that plant height, shoot weight, and number of spikes were significantly reduced as a result of the feeding of *D. noxia*.

It is well-known that callose together with P-protein serves to seal the sieve plate pores of injured sieve tubes, thereby preventing the loss of assimilates (Eschrich 1975, Sjölund 1997). Plugging of sieve plate pores with P-protein appears to be an almost instantaneous reaction due to the release of pressure in the active sieve tube (Knoblauch and Van Bel 1998). In contrast, the rate of callose deposition is variable, and relatively slow — usually taking 5–10min or more to develop. Evert *et al.* (1968) presented evidence of blockage of sieve tubes by tylosoids in plants that had been punctured by aphids, and suggested this to be caused by loss of turgor induced by feeding aphids.

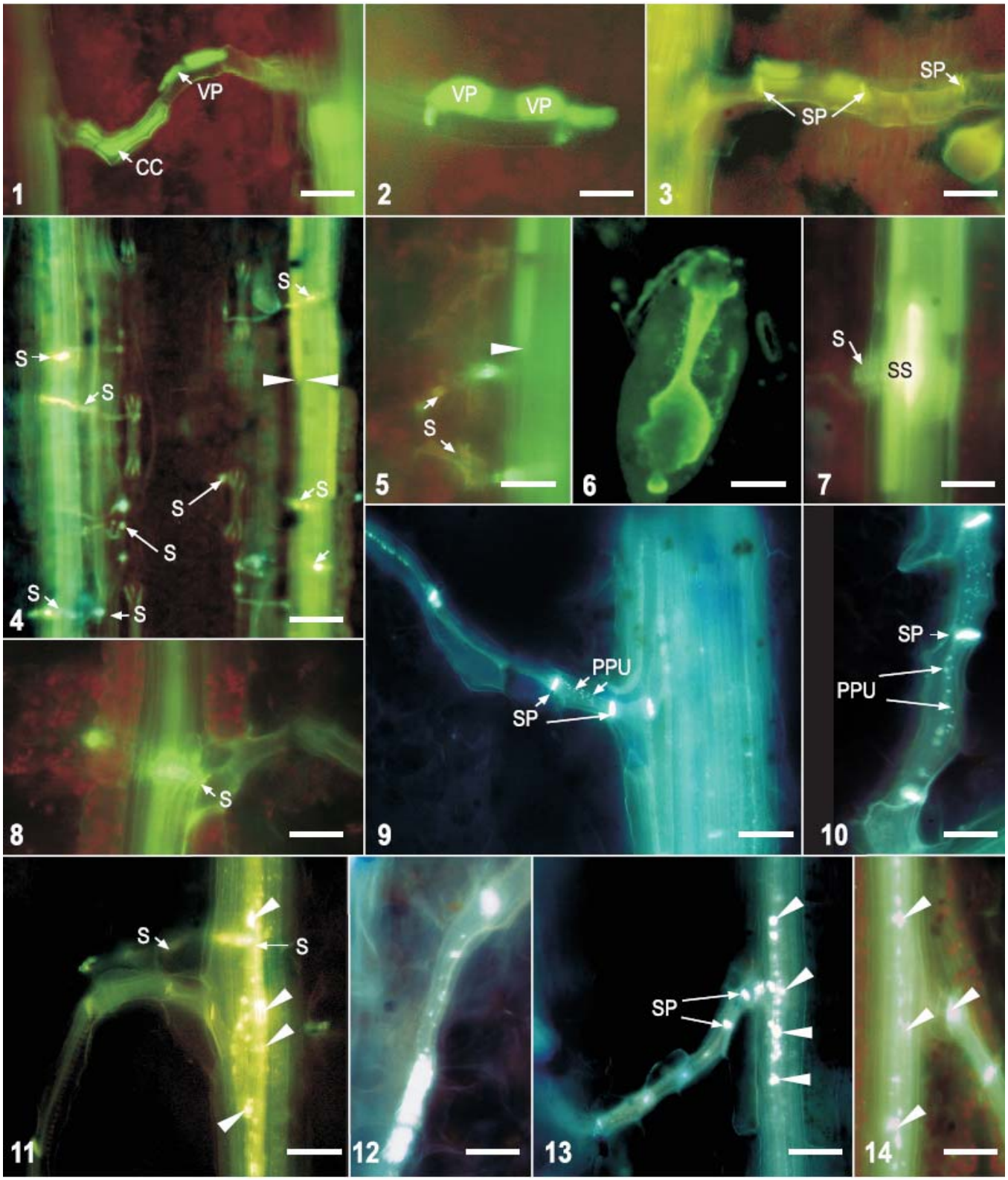

Figure 1: See opposite page for legend
CC = companion cell, SP = sieve plate, S = stylet track/stylet sheath/stylet, Darts = fluorochrome front, SS = salivary sheath material, PPU = pore-plasmodesmal units
Bars represent 10µm in 1–3, 7, 8, 10–12 and 14; 20µm in 4, 5, 9; and 13 and 250µm in 6

Figures 1–14: Details of the distribution of 5,6-CF within veins in a source leaf. **1**: A transverse vein interconnecting an intermediate (right) and small vein (left). It is impossible to determine whether the dye is associated with vascular parenchyma elements or with the single sieve tube visible in these sections. **2**: A cross vein in which 5,6-CF is mostly associated with parenchyma cells. **3**: Three sieve plates in a transverse vein, with some evidence of 5,6-CF in the companion cell, as well as in the sieve tube itself. **4**: Several stylets and/or stylet tracks lodged in longitudinal veins. 5,6-CF occurs in the stylet tracks, which is evidence that the aphids had ingested 5,6-CF-containing phloem sap, prior to examination. **5**: Stylets of two *D. noxia* aphids lodged in a small vein. **6**: An example of an aphid containing 5,6-CF in its gut. **7:** Bright fluorescence associated with salivary sheath material that was deposited in a small vein after feeding. The aphid apparently had withdrawn its stylets, leaving behind a mixture of the salivary sheath and 5,6-CF-containing phloem sap, which appears to have moved acropetally as well as basipetally. **8**: Penetration near the junction with a transverse vein. The stylets are lodged at the junction between the transverse vein and a longitudinal vein. Note that no fluorochrome is visible in this cross vein, behind the stylet insertion region. **9**: Distribution of callose-associ-ated fluorescence in a longitudinal and transverse vein, where bright fluorescence is associated with sieve plates and pore-plasmodesmal units (PPUs). Other than the sieve plates, there is very little callose evident along the longitudinal vein. **10**: Detail of a transverse vein in which callose occurs in association with sieve plates and pore-plasmodesmal units. The PPUs appear as bright punctate spots in the walls of the sieve tube members. **11**: 5,6-CF trafficking and callose stained with aniline blue. A stylet track terminates just above a cross vein. This track is itself very callosed near the phloem, as is the vascular strand within this intermediate vascular bundle. Darts point to large callose deposits.
12: Distribution of wound callose in a transverse vein. The vein contains a single sieve tube. An extensive region of wound callose deposition is visible in the lower-most sieve tube member. Note aggregates associated with the sieve plates as well as large areas associated with the PPU. **13**: Wound callose deposits 24h after removal of feeding aphids. Darts = large wound callose formations in phloem and associated parenchyma tissue. **14**: Wound callose deposits 36h after removal of feeding aphids. Some evidence for a slow reversal of the wounding effect is evidenced by the decrease in fluorescence activity. Darts = large wound callose formations in phloem and associated parenchyma tissue

Our results using a RWA-susceptible cultivar show massive localised callose deposition in response to aphid feeding, whereby the wounding led to constriction of the phloem pathway. Blockage of sieve plate pores and pore plasmodesmal units and the other symplasmic connections between sieve tube members and associated vascular bundle cells, can all be seen quite clearly in Figures 4–5, 7–14. Callose deposition at sieve plates, pore-plasmodesmal units and at plasmodesmal aggregates show a typical wounding response. Callose deposition would effect a decrease in the rate of transport across from the vascular parenchyma through the companion cell-sieve tube complexes, below the wound site. The report by Nielson *et al*. (1990) of a nearly 200% over-accumulation of translocate at the exposed sites in leafhopper-injured stems, was interpreted as being due to phloem blockage which resulted in a permanently diminished assimilate transport rate, as recovery was not observed even after four days. Although not aphids, the leafhopper data (Nielson *et al.* 1990) lend good support to our observation of slow post aphid feeding damage recovery.

Using the phloem mobile fluorophore 5,6-CF confirms that aphids form local sinks once their stylets have penetrated functional phloem. Hill (1962) demonstrated that if the drain of assimilates is sufficiently strong and localised, the plant reacts to it in some respects as if it were a bud. The aphid not only ingests products of photosynthesis, but also competes directly with other parts of the plants which are denied assimilates as a result.

Van der Westhuizen *et al.* (2002) suggest that the RWA apparently causes a significant increase in a pathogenesis-related protein, β-1,3-glucanase, which appears to accumulate selectively in infested resistant cultivars but not in infested non-resistant, or control wheat cultivars. The enzyme's substrate is callose (β-1,3-glucan). The presence of the PR-protein β-1,3-glucanase in RWA-infested wheat leaves, suggests that callose is being actively removed from the probed tissues. If this is so, then the question that remains to be answered is why?

In susceptible lines such as Adamtas, callose accumulation is evident as a result of aphid feeding. Our data support the report by Evert *et al.* (1968), who suggested that aphids could cause turgor loss when puncturing parenchymatic cells and elements of the phloem. Our data unequivocally demonstrate that this in turn appears to trigger callose synthesis. At first glance, impeding callose formation in resistant lines (or limiting it) makes little sense mechanistically, as greater turgor loss would result. However, there is very good evidence for the stimulation of PR protein synthesis. Van der Westhuizen *et al.* (2002), as well as a number of other authors (see Forslund *et al.* 2000, and literature cited for other examples), report significant increases of β-1,3-glu-canase synthesis, which is apparently stimulated in resistant lines in response to the aphid's feeding. The role of the glucanase must therefore be to prevent, or at least regulate callose synthesis. Inhibition of callose synthesis suggests a causal relationship between resistance and susceptibility in which callose synthesis may be specifically inhibited, preventing callose synthesis and thus, the inevitable cessation of transport that follows this damage.

In summary, feeding by *D. noxia* results in redirection of the assimilate flow through the formation of local sinks and also causes massive, possibly long-term, damage to cells and tissues, due to feeding-related pressure loss, through enhanced callose deposition in the damaged functional phloem in non-resistant wheat The resultant reduction of carbohydrate translocation capacity of the phloem must adversely affect plant development.

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