Resistance or tolerance: An examination of aphid (*Sitobion yakini)* phloem feeding on Betta and Betta-Dn wheat (*Triticum aestivum*)

L.R. de Wet [1] and C.E.J. Botha[1,2]

[1.] Department of Botany, Rhodes University, Lucas Avenue Grahamstown 6140.

[2] Corresponding Author T.Botha@ru.ac.za

Summary

Engineering pest resistance into crops is important. However, the mechanisms of resistance are not clearly understood. In this study, we examined the effects of aphid feeding on Russian wheat aphid-resistant and -susceptible cultivars of wheat (*Triticum aestivum* L.); Betta-Dn and Betta, respectively, by the grass aphid, *Sitobion yakini* (Eastop). These cultivars were grown with or without aphid colonies. In each case, we examined the plants specifically for the formation of wound callose associated with the phloem, using aniline blue and fluorescence microscopy. We observed that aphid feeding stimulated the formation of wound callose in the susceptible cultivar, but that callose was comparatively reduced in the resistant cultivar of wheat. In a separate series of experiments, the xenobiotic, 5, 6- carboxyfluorescein diacetate was applied to attached sink leaves, distal to feeding aphids. When leaf segments were examined four hours after application, little evidence of phloem transport of the fluorescent cleavage product, 5, 6-carboxyfluorescein (5, 6-CF), was evident below known aphid-probed sieve tubes. Low levels or absence of 5, 6-CF indicates that either the aphids have successfully redirected sap to themselves, or that the phloem is no longer functional. In contrast, 5, 6-CF transport was evident below sites of aphid probing in Betta-Dn, suggesting that the phloem was still capable of long-distance transport. In addition, callose deposition was reduced in Betta-Dn leaf phloem and it is surmised that transport was not as affected by aphid feeding in the resistant cultivar. This indicates that the 'resistant' wheat cultivar may in fact be tolerant to aphid feeding by successfully overcoming the nutrient drain that feeding aphids imposed on the phloem transport system.

Keywords

Aphid feeding, 5, 6-carboxyfluorescein transport, callose formation, resistance, tolerance.

Introduction

Cultivars of wheat resistant to aphid infestation have been developed over the years, but the precise function of this resistance remains largely unknown. It has been argued that susceptible and resistant wheat cultivars react differently at the genomic level, and, consequently, at the cellular level, through changed cellular function. Since genetically modified plants have been shown to successfully retain pest resistance over several generations (Mohan Babu *et al.* 2003), the development of transgenic plants with enhanced pest resistance would greatly benefit agricultural industries, particularly those involved in the production of food crops.

Aphids are destructive agricultural pests**,** but little is known about the long-term effects of phloem-feeding by these insects on the cells of plants and thus the overall plant response (Moran & Thompson 2001). Aphids probe thin-walled sieve tubes in grasses (Matsiliza & Botha 2002) and eject saliva into cells that they have probed, before commencing feeding (Evert *et al* 1973). The saliva is thought to be responsible for the onset of plant responses (Moran & Thompson 2001, Kimmins & Tjallingii 1985). Additionally, aphids are also capable of themselves becoming 'sinks' to which all or most of the plant sap is diverted, rather than to the plant sinks (Girousse *et al* 2003). Aphids are able to feed at a rapid rate, consuming up to 10^5 times the volume of sap in one cell every hour (Kennedy &

Fosbrooke 1971). Aphids are able to do extensive damage to the plant and are certainly capable of eliciting wound responses, specifically callose formation.

Signals for plant responses enter plant cells within aphid saliva, resulting in the production of pathogen-resistance proteins such as glucanases (Moran & Thompson 2001). A study by van der Westhuizen *et al.* (2002) shows that ?-1,3-glucanases are produced in abundance in wheat cultivars resistant to the Russian wheat aphid but these same glucanases are not produced in susceptible cultivars. This response may well be involved in the formation and deposition of callose in the phloem as a defence against aphid attack (Krishnaveni *et al* 1999), or may be an active defence against aphid salivary toxins. Glucanases are usually produced in response to infection by fungal hyphae and may be produced as a misidentification of the pest. Thus, glucanases may serve no function in combating aphid infestation, but are rather a defence against pathogens that may be introduced into the plant by the aphid saliva (van der Westhuizen *et al.* 2002; Krishnaveni *et al* 1999). Callose is the major substance produced by plant cells in response to wounding (including aphid feeding). It follows then that the resistant gene or gene system must affect callose production and deposition in some way. This could involve signals that elicit callose response, or expression of genes that produce the enzyme systems responsible for the production of callose itself. The changes in deposition of callose must inevitably result in direct and indirect effects on the phloem transport capacity of the plant.

Deposition of callose in plasmodesmata is regarded as a regulatory response to wounding thereby slowing down the transport between phloem cells and thus curbing the possible detrimental effects of wounding on plant health. Callose, a ?-1, 3-glucan (Botha and Matsiliza, 2004, Radford *et al.* 1997) is produced largely in the sieve elements of phloem (Currier 1957). This may be in response to elevated calcium ion levels utilizing a mechanism involving calmodulin (Botha & Cross 2001). Wounding results in the rapid formation and deposition of callose (Botha and Matsiliza, 2004, McNairn & Currier 1968, Currier 1957). In addition, callose has an integral effect on the regulation of plasmodesmatal pore size (Botha & Cross 2001), where it is deposited in the neck region of the plasmodesmata and may block them completely (Radford *et al* 1997) altering the size exclusion limit of the plasmodesmata, thereby primarily reducing sap loss from the phloem, but it may regulate transportation of viruses. We believe that in resistant plants, callose is not produced as a wound response, resulting in the continued flow of the phloem sap, the maintenance of the aphid population, but more importantly, the survival of the plant which does not die as a consequence of aphid feeding. In contrast, callose production in susceptible plants leads to the death of the plant as the flow of the sap in the phloem ceases.

The aim of this study was to further explore the earlier report by Botha and Matsiliza (2004) on the effects of aphid feeding on a non-resistant cultivar (Adamtas), but using newly available cultivars, on callose production and deposition.

The two main questions posed were: first to determine the extent of callose deposition in the two cultivars – one non-resistant, the other stated to be resistant to aphid infestation; and second to determine the possible effects of long-term aphid feeding on functional phloem transport in resistant and non-resistant plants

Materials and Methods

Colonies of the grass aphid, *Sitobion yakini*, were kept on wheat (*Tricium aestivum* L.) plants in a controlled environment chamber (Conviron; Controlled Environments Limited, Winnipeg, Manitoba Canada; Analytical Scientific Instruments CC South Africa) until the plants were used experimentally. Wheat plants were replaced every week to ensure succulent hosts. Two chambers were used, one with infested plants and another with uninfested plants. The chambers were set at 25^{o}C, 70% humidity with a 16-h photoperiod. Two different cultivars were used: Betta, susceptible to the Russian wheat aphid (*Diuraphis noxia*), and its resistant counterpart, Betta-Dn.

For all experiments at least 10 different wheat plants were used onto which aphid colonies were introduced to four exposed leaves. Experiments involved the use of both aniline blue and 5, 6-CFDA to visualize the effects of aphids. Uninfested plants of each of the cultivars were used as control.

Leaves with aphids present and those with no aphids were scraped on the abaxial surface of the leaf to remove the cuticle and epidermis, thereby exposing the mesophyll and shortening the pathway to the phloem. Scraping was carried out in MES (morpholinoethanesulfonic acid, pH 7.2) buffer. The scraped leaves were then stained

with a few drops of aniline blue (0.05% w/v) in MES buffer and left for 5 min to enable staining. Aniline blue stains callose, which appears blue under white light, but fluoresces blue-green under UV light with the appropriate filter set. The specimens were then viewed under UV light, using an Olympus BX61 wide-field florescence microscope (Olympus Tokyo Japan, Wirsam Scientific, Johannesburg, South Africa).

5, 6-Carboxyfluorescein diacetate (5, 6-CFDA) solution was prepared by adding 100mg of the compound to 1ml dimethyl sulphoxide . A volume of 1-2?l of the stock solution were pipetted into polypropylene centrifuge tubes and diluted to 1ml with distilled water. Tubes were covered with aluminium foil to avoid cleavage of the 5, 6-CFDA by light, frozen at -5^{o}C and defrosted when used. A small 'window' was scraped with a needle on the lower surface of leaves and 5, 6-CFDA was applied to this area. After about 4 h, the leaf was removed from the plant and the lower surface scraped as in the aniline blue experiments. The non-polar 5, 6-CFDA is thereby introduced into damaged cells and can move across membranes (Botha 2005). In the diacetate form it does not fluoresce. Once the dye reaches healthy cells (by moving across membranes) the diacetate is cleaved from the molecule and it becomes 5, 6-carboxyfluorescein (5, 6-CF), a polar molecule. The 5, 6-CF cannot move across membranes and is contained within the symplasmic transport system where it is transported largely within the phloem (Botha 2005).

MES buffer was used for both aniline blue and 5, 6-CF treatments. Specimens were double stained, that is; they were first put through the 5, 6-CF procedure and thereafter the leaves were scraped and dyed with aniline blue. Specimens were mounted on glass

slides and viewed using an Olympus BX-61 wide-field fluorescence microscope. Aniline blue slides were viewed using the UMWU2 filter cube (with excitation wavelengths of 425-440nm and an emission of 475nm). 5, 6-CF samples were viewed using the U-YFP filter cube (with an excitation of 513nm and an emission of 527nm). Images were saved in a database using the program analySIS (Soft imaging system GmHb, version 3.0, Germany 2001). Images were imported as bitmaps to Corel Draw 12 (Corel 2003) for presentation.

Results

Betta leaves that were aphid-free generally showed little evidence of callose formation (Fig. 1a). These results demonstrate that damage caused by scraping was effectively minimised, provided MES buffer was used to bathe the 'windows' opened up into the mesophyll and the subtending vascular tissues. As wound callose may develop after about 1h, we examined all aniline blue-treated leaf material as soon as possible so as to minimise long-term effects that could affect the results. Figure 1a shows part of a leaf from the susceptible Betta cultivar stained in aniline blue. There is very little callose-associated fluorescence. Small punctate spots (not shown) were usually found in sieve elements and associated parenchyma elements. This micrograph shows several intermediate leaf blade bundles (IV) and indicates also the position of a cross vein (CV). Figure 1b shows typical and massive callose deposition associated with sieve plates (unlabelled arrow) and with lateral sieve area pores in susceptible Betta leaves. Figure. 1c shows part of a leaf blade of susceptible Betta that has been extensively fed by aphids. The vascular parenchyma and phloem elements within these veins show signs of damage,

which is evidenced by extensive callose deposition, most notably associated with the sieve plates and lateral sieve areas.

Figure. 1d. Illustrates the transport of the phloem-mobile fluorophore, 5, 6-carboxyfluorescein (5, 6-CF) in longitudinal as well as cross veins in control leaf blade material in uninfested Betta. Bright fluorescence in cross veins, as well as in longitudinal leaf blade bundles, suggests that the fluorophore has trafficked in the phloem tissue.

Leaves from Betta plants which were infested with aphid colonies, demonstrated reduced 5, 6-CF trafficking through the phloem tissue (Fig. 1e). The patchy appearance of the 5, 6-CF in the phloem suggests that structural and/or functional damage is caused by aphid feeding. In contrast, resistant Betta-Dn leaves show very little sign of callose synthesis, in uninfested (Fig. 1f) as well as infested (Fig. 1g) leaves. Some callose is associated with the sieve plates in the infested leaf (Fig. 1g), but appeared to be limited to sieve plates only.

Betta-Dn leaves without (Fig. 1h) and with (Fig.1i) aphids show good trafficking of 5, 6-CF. In these figures, the distribution of 5, 6-CF in the vascular parenchyma and sieve tubes in intermediate leaf blade bundles in resistant Betta Dn leaves is evident in both cases. Phloem transport does not seem to have been impeded in leaves on which aphids had been feeding.

Transport of 5, 6-CF can be seen throughout intermediate and cross veins (Fig 1d). In aphid-infested Betta leaves (Fig. 1c), callose is markedly more apparent than in aphid-free plants (Fig. 1a). In Fig. 1c, callose can be seen in both intermediate (IV) and cross veins (CV). Callose deposition is associated with sieve plates and with pore-plasmodesmal units (Fig. 1b). Arrest of 5, 6-CF transport is apparent in intermediate veins (IV), where it is terminated. 5, 6-CF transport was halted in cross veins (CV) as well (Fig. 1e).

Discussion

The general effects of aphid infestation on plants are well-known: plants tend to be smaller due to loss of assimilates (Mallot & Davy 1978). Resistant strains are better able to cope with infestation and show less physical damage (Nkongolo *et al* 1990). Both susceptible and resistant cultivars of wheat reduce the numbers of proteins produced in response to aphid feeding, but resistant plants tend to maintain a higher production of proteins (van der Westhuizen *et al*. 2002; van der Weshuizen & Pretorious 1995). In addition, secondary compounds in wheat play an essential role in resistance to aphids (Leszczynski *et al.* 1989). However, little is known about the effects on the phloem of leaf or stem tissue.

Our results demonstrate that aphid infestation of the susceptible Betta, increased extensive callose production when compared to aphid-free plants. The results agree with an earlier study (Botha and Matsiliza, 2004) in which an obsolete cultivar of highly susceptible wheat (Adamtas) showed extensive wound callose formation as a result of

probing and feeding by *D. noxia.* In Adamtas, the damage to the phloem was extensive and apparently long-term. Aniline blue staining for callose in the present study, suggests that callose synthesis is impeded in the Betta-Dn cultivar, but not in the susceptible Betta cultivar. Clearly, callose deposition is promoted by the presence of aphids, but in the resistant Betta-Dn cultivar this is a reduced reaction.. In this case, callose deposition is a wound response directly attributable to the feeding aphids. These results show that there is an obvious difference in the wounding response elicited by the aphid, on Betta and Betta-Dn cultivars. The work reported here will enable us to examine the potentially more devastating structural damage, which we hypothesise that the Russian wheat and Birdcherry oat aphids will inflict on the same cultivars.

Leszczynski *et al.* (1989) describes three different resistant mechanisms against aphids; antixenosis, antibiosis and tolerance. Antixenosis occurs when resistant plants deter aphids from settling and colonizing them. Antibiosis occurs when the plant restricts the aphid's rate of increase and finally, tolerance occurs when the plant is able to tolerate the nutrient drain imposed by the aphids. Plant response to aphid feeding may be largely species-specific (Gill & Metcalf 1977); the signals that elicit plant responses derive from the aphid itself, particularly the saliva secreted during stylet penetration and subsequent feeding.

As other studies have reported an increase in the production of proteins in strains resistant to aphid infestation, it must be assumed that these proteins play a role in eliciting the response of resistant plants. In our study, the relative absence of callose in the resistant

cultivar (Fig. 1f), when compared to its susceptible counterpart (Fig. 1c), shows that the action of this resistance may be the sites at which deposition or breakdown of callose take place.

A noted response in both barley and wheat is the induction of ?-1, 3-glucanases in both resistant and susceptible cultivars, but in much larger quantities in resistant cultivars (van der Westhuizen *at al.* 2002; Forslund *et al.* 2000). This indicates an important function of ?-1, 3-glucanases in the defence system for aphids (Forslund *et al* 2000). ?-Glucanases are implicated with hydroxamic glucosides in a defence system against sap-sucking insects in wheat (Nikus & Jonsson 1999). As these enzymes are responsible for the breakdown of callose, their presence in infested plants is important (Nikus & Johnson 1999). Our results support this notion, and we suggest that the function of resistance may not be in the formation of callose, but rather in the breakdown of existing callose, thereby allowing the aphids to continue feeding despite the drain that this imposes on resources.

We have demonstrated that the transport of assimilates in the phloem is not at all halted in infested Betta-Dn plants (Fig. 1i), as little or no callose is deposited in response to the aphid's feeding. In contrast, deposition of callose is more obvious in Betta, (Fig. 1c), and the result is manifested by reduction in the transport of assimilates in the phloem of these young leaves. This response in Betta must, in the long-term, lead to a drastic reduction of available phloem sap. The generally observed lack of callose in the resistant cultivar leads us to speculate that the response to feeding may be one of tolerance, rather than of resistance: the plants are effectively tolerating the nutrient drain imposed by the actively

feeding aphids. It is possible that the synthesis of ?-1, 3-glucanases is curtailed in the susceptible Betta, thereby preventing callose breakdown, whilst the resistant Betta-Dn may either express elevated ?-1, 3-glucanase activity, thereby enhancing callose degradation or, alternatively, ?-1, 3-glucan synthase activity is down-regulated, thereby down-regulating callose synthesis.

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Legends to text Figures.

Figs. 1a-i. Shows aspects of the effect of aphid feeding on the leaf blades of *T. aestivum*.

Fig. 1a. The distribution of callose, in the absence of colonies of feeding aphids on Betta. Note that there is little if any evidence visible at this magnification, of any would callose formation. Intermediate (IV) and small veins (SV) are connected via a cross vein (CV). Aniline blue stain**.**

Fig. 1b. Details the extensive damage observed is sieve tubes of a cross vein in the susceptible Betta leaf. Massive callose deposits occur within sieve tubes (unlabelled arrowhead), especially associated with the sieve plates as well as with lateral sieve area pores. Aniline blue stain**.**

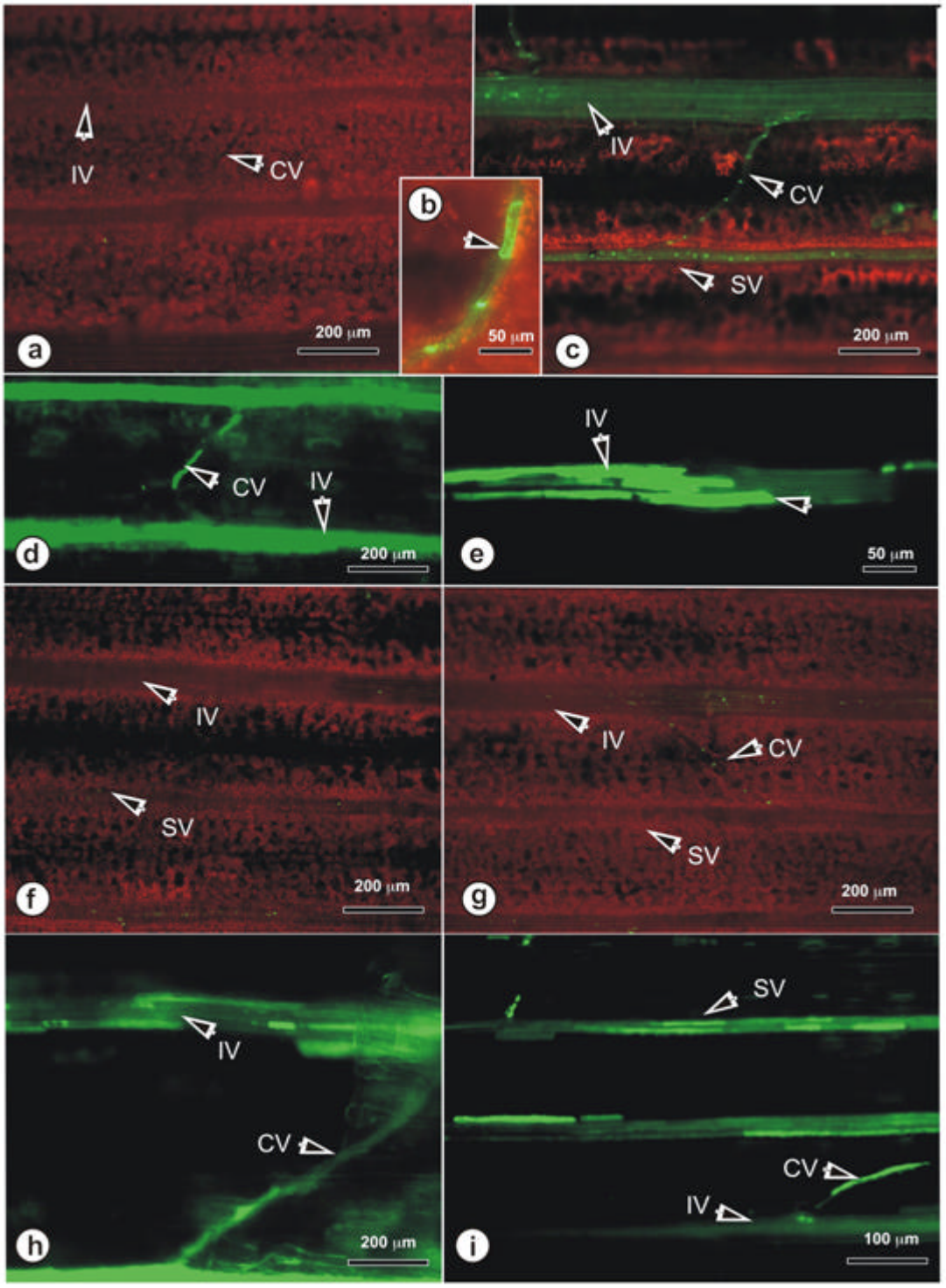
Fig. 1c. Part of a leaf blade of susceptible Betta, which had been extensively fed upon by the aphids. Note that the vascular parenchyma and phloem elements in these veins shows signs of extensive damage, evidenced by callose deposition, which is most notable, associated with the sieve plates (detail in Fig. 1b).

Fig. 1d. The transport of the phloem-mobile fluorophore, 5,6-carboxyfluorescein (5,6-CF) in longitudinal as well as cross veins in control Betta leaf blade material. Bright fluorescence in the cross vein is associated with the file of sieve tubes, and in the longitudinal veins, mostly with the sieve elements and possibly, with associated parenchymatous cells as well.

Fig. 1e. The longitudinal transport of 5,6-CF through the vascular parenchyma and sieve tubes in an intermediary vein. In this infested, susceptible Betta leaf, it is evident that phloem transport is affected markedly, as evidenced by the patchy distribution of the fluorophore. Unlabelled arrowhead points to disrupted transport of 5,6-CF through a file of sieve tube members.

Figs. 1f & g. Longitudinal images of the resistant cultivar, Betta Dn, showing distribution of would callose in the absence (Fig. 1f), and the presence (Fig. 1g) of feeding aphids. Note that callose synthesis appears to be limited in the resistant (Fig. 1g) compared with susceptible cultivar (Fig. 1c). Aniline blue stain**.**

Figs. 1h & i. The distribution of 5,6-CF in the vascular parenchyma and sieve tubes in intermediate leaf blade bundles in resistant Betta Dn leaves, without (Fig. 1h) and with (Fig. 1i) feeding aphid colonies. In both cases, phloem transport between longitudinal and cross veins is evident, with only some of the veins in the aphid-infested leaves showing reduced fluorophore trafficking.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