Xylem – as well as phloem – sustains severe damage due to feeding by the Russian wheat aphid

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Abstract

Investigation of comparative effects of feeding damage by the Russian wheat aphid (RWA, biotype SA1, *Diuraphis noxia* Mordvilko) on leaf blades of susceptible and resistant wheat cultivars (*Triticum aestivum* L. var Betta and Betta-*Dn1* respectively) were carried out to establish the level of ultrastructural damage caused by this aphid and the possible limitation of damage induced which could be ascribed to the resistance gene *Dn1* over the susceptible cultivar. Ultrastructurally, Betta-*Dn1* sustained less damage to the vascular tissue as well as to the mesophyll during the experimental period. Both inter- and intracellular probes resulted in considerable saliva deposition as the aphids probed for suitable feeding sites. Salivary tracks were observed between and within mesophyll, bundle sheath cells as well as the vascular tissue, including the xylem. Disruption of organelles and cytoplasm resulted from cell probing and sheath deposition. Cell and organelle damage was more evident in the non-resistant Betta cultivar. The aphids probed for and fed from thin-walled sieve tubes preferentially. Few thick-walled sieve tubes showed evidence of either aphid probing or feeding-related damage. Saliva was deposited when the aphids probed inter- and intracellularly for feeding sites. The aphids appeared preferentially to probe for and feed from thin-walled sieve tubes, as few thick-walled sieve tubes showed evidence of damage. Vessels, apparently probed for water, contained watery saliva that encased the secondary walls and sealed pit membranes between probed vessels and xylem parenchyma. The xylem probed by the RWA was rendered non-functional, probably contributing to symptoms of leaf roll, chlorosis and necrosis, which were observed within two weeks of infestation in the susceptible Betta cultivar. This damage was limited in the resistant Betta-*Dn1* cultivar during the same time frame.

Introduction

The Russian wheat aphid (RWA-Diuraphis noxia Mordvilko) is a serious insect pest on wheat and barley. The aphid became an important problem in South Africa in 1978 (Walters et al., 1980) and causes major economic losses not only in South Africa, but also in North and South America, and in Australia. RWA has been described as one of the most destructive pests of small grains (Kovalev et al., 1991), causing chlorosis and necrosis upon infestation. The visible damage symptoms of RWA feeding are distinct white, yellow, purple, or at times reddish-purple longitudinal streaks, with severe leaf roll in fully expanded leaves and the prevention of unrolling of developing leaves (Walters et al., 1980; Hewitt et al., 1984; Riedell, 1989). Much efforts have been made to breed for resistance to the aphid, and 10 genes conferring resistance to RWA have been identified from wheat and other related cereals (Liu et al., 2002, 2005). Resistant accessions and near-isogenic lines carrying resistance genes have then been used in various studies in which the aim was to unravel the mechanisms for RWA induced plant damage and the resistance mechanisms. The resistance gene *Dn1* used in this study was first identified in South Africa in the common wheat accession PI 137739 from Iran (Du Toit, 1987, 1988, 1989a,b). It was introduced into wheat cultivars Betta, Tugela and Molopo creating near-isogenic lines (Du Toit, 1989a).

Considerable effort has been directed towards clarifying the effect of *Dn1* which has been reported to be mostly antibiotic, causing a reduction in the feeding aphids', population growth rate, fecundity and aphid biomass on Betta-*Dn1* as compared toBetta (Du Toit, 1987, 1989b; Budak et al., 1999; Heng-Moss et al., 2003). Reduced aphid biomass was also demonstrated on

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Tugela-*Dn1* as compared to the Tugela near-isogenic line (Wang et al., 2004). Some antixenosis were reported in Betta-*Dn1* by Du Toit (1987), but not by Budak et al. (1999). Tolerance effects are absent in wheat lines containing *Dn1* genes (Du Toit, 1989b; Budak et al., 1999). Lines carrying the *Dn1* gene exhibit reduced chlorosis and leaf streak, but the symptom rating is lower than on the corresponding susceptible lines (see Botha et al., 2006 and literature cited). Several studies on the biochemical effects of RWA infestation have been carried out. Pathogenesis-related proteins such as β-1,3-glucanase (Van der Westhuizen et al., 1998a), peroxidase and chitinase (Van der Westhuizen et al., 1998b) have been found to be induced by RWA infestation in the resistant *Dn1* lines. This response is either lacking, is induced later, or to a lower degree in corresponding susceptible lines. These results indicate that RWA induces a hypersensitivity response in resistant lines, an idea that was further supported by the early accumulation of hydrogen peroxide and increased levels of NADPH oxidase activity in RWA-infested resistant Tugela-*Dn1* (Moloi and Van der Westhuizen, 2006). Interestingly, RWA often co-occurs with the non-symptomatic bird cherry-oat aphid (Rhopalosiphum padi L.). RWA-resistant *Dn1* lines do not show any antibiosis effects against this aphid (Messina and Bloxham, 2004), indicating that the resistance mechanism is not activated by the bird cherry-oat aphid or that it acts specifically against RWA.

In a recent study, Saheed et al. (2007) demonstrated differences in cell damage caused by the RWA and bird cherry-oat aphid (BCA) at the ultrastructural level by aphids feeding in vascular bundles of the susceptible barley cultivar cv Clipper. Our results indicated that damage to the conducting elements – including the xylem – could partly explain the severe symptoms caused by RWA infestation and the absence of such symptoms upon infestation by bird-cherry oat aphid. RWA and BCA both tap the xylem for water and in doing so, eject large quantities of watery saliva. However, RWA saliva appears to line the metaxylem vessels with an amorphous non-crystalline saliva matrix, effectively sealing pit membranes between the xylem vessels and xylem parenchyma in the process. BCA ejects less saliva, which is more crystalline in appearance.

We hypothesize that the ejection of saliva into the xylem is a causal factor in the appearance of white and yellow streaks as well as leaf roll, as the known ejection of saliva by RWA could effectively block xylem to xylem parenchyma transfer of water, as well as of nutrients normally exchanged in the leaf during the transpirationally-driven ion exchange and recycling process. In addition, the known hypersensitivity induced by the *Dn1* gene, slower appearance of leaf roll and other effects associated with RWA feeding, are delayed in *Dn1* lines due to lower aphid population growth rates than in the non-resistant Betta.

Materials and methods

Plant material, aphid colony maintenance and treatments
Seeds of the susceptible and resistant wheat cultivars (*T. aestivum* L. cv Betta and Betta-*Dn1* respectively) as well as colonies of the Russian Wheat Aphid (RWA, South African biotype 1 (SA1); D. *noxia* (Mordvilko) Hemiptera: Aphididae, were obtained from the Agricultural Research Council-Small Grain Institute, Bethlehem, South Africa. The wheat seedlings were pre-germinated in Petri dishes and later transferred into potting soil (60:40; peat: vermiculite mixture) in plastic pots 17 cm in diameter. They were fed twice a week with Long-Aston nutrient solution (Hewitt, 1966) in a controlled environment (Conviron S10H, Controlled Environments Limited, Winnipeg, Manitoba, Canada). The Controlled environmental cabinet is set at 24 °C, RH 66% daytime and 22 °C, RH 60% night time, under a 14-h photoperiod for the control plants and 18 °C, RH 66% daytime and 15.5 °C, RH 66% night time, 14-h photoperiod for the aphid-infested plants. Irradiation in the two cabinets were from a combination of fluorescent lamps (F48T12.CW/VHO1500, Sylvania, Danvers, MA) and frosted incandescent 60 W bulbs (Philips, Eindhoven, The Netherlands), with PAR level of 250 μmol m^{-2} s^{-1} 30 cm below the light source.

RWA colonies were maintained on young susceptible wheat (Betta) plants and kept in insect cages in a different controlled environment. For the purpose of this study, 10 replicate plants (one per pot) each of susceptible (Betta) and resistant (Betta-*Dn1*) cultivars of wheat were established for each treatment (control Betta and Betta-*Dn1*, aphid-infested Betta and Betta-*Dn1*). A fine camel's hair brush was used to place five adult aphids on the second or third visible, fully-expanded leaf above the coleoptiles of the replicate plants in the aphid-infested treatment. Single-leaf aphid cages were placed over the infested leaves after allowing the aphids to settle down. Control leaves carried empty aphid cages and were kept and maintained in the control climate chamber as stated above. The aphids were allowed to feed and reproduce for two weeks, after which visible damage was noted before the infested leaves and those of the control were selected for the study of feeding-related damage.

Electron microscopy

Strips of leaf material were cut from each of the control, RWA infested susceptible (Betta) and resistant (Betta-*Dn1*) wheat plants, then carefully trimmed and diced into smaller pieces in cold fixative (6% glutaraldehyde in 0.05 M sodium cacodylate buffer; pH 7) using a sharp clean single-edge razor blade. Leaf segments were transferred to small vials with fresh fixative, subjected to slight vacuum (17 kPa) for 1 h and kept overnight in the refrigerator at 4 °C. The leaf segments were then gently washed in three changes of cold 0.05 M sodium cacodylate buffer, transferred into the 2% osmium tetroxide in 0.05 M sodium cacodylate buffer and kept in the refrigerator overnight, rinsed in the cold buffer and dehydrated in cold graded ethanol series, followed by two changes in propylene oxide. Embedment was in Spurr's epoxy resin. Ulthrathin sections (silver to gold) were cut using a diamond knife and were collected on 300 mesh copper grids. The sections were stained in uranyl acetate and lead citrate and viewed and imaged at 80 KV in a JEOL JEM 1210 transmission electron microscope (JEOL, Tokyo, Japan).

Results

The probing and feeding activities of RWA resulted in chlorosis and necrosis of the leaves as well as leaf rolling which were first observed in the susceptible cultivar Betta within two weeks, while the resistant cultivar Betta-Dn1 only exhibited chlorotic and necrotic spots within the full experimental period.

Control tissue

Fig. 1A and B illustrate aspects of the anatomy of a typical intermediate leaf blade bundle in Betta. One thick-walled (solid dot) and several thin-walled sieve tubes (ST) are visible in this vascular bundle. Fig. 1B shows a detail of a thin-walled sieve tube-companion cell complex (ST and CC respectively) and an associated phloem parenchyma cell (VP). There were no apparent anatomical differences between Betta and Betta-*Dn1*control tissue (not shown).

Penetration of mesophyll cells

Fig. 1C–F illustrates aspects of mesophyll cell damage in Betta leaf blades. The mesophyll tissue showed signs of cell walls having been destroyed and cell contents being disrupted as a result of aphid proving (Fig. 1C–F). The probing resulted also in mesophyll cells being split apart, including cleaving of the plasmodesmatal fields, with the saliva sheath occupying the middle lamella region (Fig. 1E). Such intercellular probing seemed not to affect chloroplasts in neighbouring cells, which appeared intact (Fig. 1E). Mesophyll cell damage was not as severe in Betta-*Dn1* leaf blade mesophyll (data not shown).

Bundle sheath and mestome sheath cells

As with mesophyll cells, the bundle sheath and mestome sheath cells exhibited varying degrees of damage sustained during probing (data not shown). Damage usually resulted in severe plasmolysis and cells usually contained saliva.

Vascular parenchyma

Inter- and intracellular penetration of vascular parenchyma in Betta resulted in severe cell disruption (Figs. 1H, and 2F). In Betta-*Dn1*, intercellular probes of intermediate and small bundles are often obliterated vascular parenchyma (Fig. 1I). In Betta-*Dn1* it appeared as if cell disruption was an isolated event in some instances, with individual, rather than whole groups of cells destroyed during feeding (Fig. 1J).

The phloem

Phloem feeding is the prime activity of RWA. RWA feeds preferentially from the adaxial surface of the leaf, often probing directly through all cells within the feeding pathway, whilst in other instances; a more circuitous route was followed to the phloem. Evidence of probing of both thin- and thick-walled sieve tubes was common.

Thin-walled sieve tubes

In Betta and Betta-*Dn1*, RWA probed the thin-walled sieve tubes preferentially. In Betta, this resulted in severe damage to the phloem, as shown in Fig. 1K where four sieve tubes, a companion cell as well as phloem parenchyma have been punctured. The sieve tubes are plasmolyzed and their plasma membranes have ruptured and torn away from the cell walls in places. Two thin-walled sieve tubes are occluded with sheath material whilst the remaining parenchyma cells are variously plasmolyzed. In punctured, plasmolyzed sieve tubes, callose was sometimes found in the lateral sieve area pores (Fig. 2B). In contrast, Betta-*Dn1* thin-walled sieve tubes usually showed little or no evidence of plasmolysis (Fig. 1J and 2A, D). However, also in Betta-*Dn1*, thin-walled sieve tubes that had become obliterated during probing were occasionally seen.

Thick-walled sieve tubes

In Betta, the thick-walled sieve tubes were sometimes plasmolyzed as a result of aphid probing. One example is illustrated in Fig. 2E where the thick-walled sieve tube (solid circle) was plasmolyzed during this probe. Plasmolysis of thick-walled sieve tubes was not often seen in Betta-*Dn1* leaf blade bundles, even when vascular parenchyma cells adjacent to the thick-walled sieve tubes have been obliterated by stylet probes (Fig. 1J and 2C).

Xylem feeding

There was no apparent difference when RWA probed the xylem in either Betta or Betta-*Dn1*. Xylem element walls in both cultivars probed by RWA were lined with an amorphous, electron-dense layer, which completely occluded pit membrane connections between the vessels and their surrounding xylem parenchyma (Fig. 2G–J). All four images used to illustrate effects to xylem tissue are from Betta, as no apparent differences were observed between Betta or Betta-*Dnl*. It was found that the pit fields between metaxylem vessels as well as those between the xylem and associated xylem parenchyma were occluded and plugged with saliva (Fig. 2G, H).

4. Discussion

RWA subsists primarily on the fluid contents of the plant cells. Its principal feeding sites are the sieve elements, which are reached either via stomata or by puncturing through epidermal and

mesophyll tissue. The route of penetration of the mesophyll of wheat was proposed to be entirely intercellular (Fouché, 1983; Fouché et al., 1984). However, we have found evidence of both intercellular (Fig. 1D–F) and intracellular (Fig. 1C, F) probing of mesophyll tissue. In the susceptible Betta cultivar, inter-as well as intracellular cellular probing resulted in severe structural damage. Mesophyll cells adjacent to salivary material deposits were often damaged, and plasmolysis and disruption of the cytoplasm and its organelles (Fig. 1C–F) was common. Cells containing salivary deposits (SS, Fig. 1C, F) likewise displayed damage, which was less obvious in the resistant Betta-*Dn1* cultivar.

When the aphid stylet reaches the vascular bundles, penetration begins in sequence with the bundle sheath, the vascular parenchyma, xylem elements and sieve element – companion cell (SE–CC) complex (Evert et al., 1973; Fouché et al., 1984; Matsiliza and Botha, 2002). Sealing as a result of stylet clearing events (blowing out and clearing the stylet duct) leads characteristically to sealing of xylem as well as severe phloem damage by RWA, leading to apoplasmic and symplasmic isolation of these conducting elements and may induce leaf roll and streaking. Plasmolysis, damage to the plasma membrane, organelles cytoplasmic content, cell walls and organelles in intracellularly-probed, as well as to adjacent cells grazed by stylets and containing saliva was more evident in the susceptible cultivar (Fig. 1H, K) whereas the resistant line showed less damage to the cytoplasm or organelles (Fig. 1I, J). In grasses, thin-walled sieve tubes and their associated companion cells are the prime target for feeding by aphids (Botha and Matsiliza, 2004). Damage to the thick-walled sieve tubes was minimal (Figs. 1J and 2C). This is not surprising, as thick-walled sieve tubes are known to be symplasmically isolated from the thin-walled sieve tube-companion cell complexes in many grasses (Botha, 2005). This investigation showed that thin-walled sieve tubes suffered the brunt of aphid probing and feeding and that more damage was induced in the susceptible Betta cultivar, (Fig. 1K) than in the resistant Betta-*Dn1* cultivar (Fig. 1J and 2C–D). Symplasmic transport of assimilates could be disrupted due to occlusion of plasmodesmata and callose deposition (Fig. 2F). Formation of ectodesmata is usually associated with severe plasmolysis and is an indicator of damage to the plasmodesmata in question. In these instances, it is likely that cell-to-cell transport of assimilates through the mesophyll cells could be reduced or completely curtailed. Ectodesmata occurred in Betta, but were not detected in Betta-*Dn1*. Only limited plasmodesmatal disruption was observed in the resistant cultivar. Lack of occlusion of plasmodesmata in the Betta-*Dn1* cultivar may perhaps be attributed to β-1,3-glucanase (an enzyme that degrades callose), which has been shown to accumulate to a greater extent in the resistant cultivars (Van der Westhuizen et al., 1998a).

Aphids are known to drink periodically from the xylem (Tjallingii, 1994). We have previously reported that RWA and Bird cherry oat aphid (BCA) tap the xylem in barley plants, presumably to obtain access to water and that they eject salivary material at some stage during this process (Saheed et al., 2007). In barley, RWA deposits a watery, smooth saliva that can completely coat the inner face of the walls of xylem vessels. In contrast, when BCA taps the xylem, the aphids eject a more granular salivary matrix, which does not appear to occlude the pit membrane or pit apertures between metaxylem vessels and adjacent xylem parenchyma. Of great interest was the evidence of the deposition of saliva by RWA, within the metaxylem vessels of all vein classes within Betta and Betta-*Dn1* plants on which RWA had been feeding. Prior to tapping the xylem, RWA regurgitates (excretes) a considerable quantity of saliva into the vessels through its maxillary canal, up to the point where it punctures the plasma membrane. At this stage, the saliva composition may change to a watery one (Martín et al., 1997) as the egestion of saliva into the xylem takes place. It is unlikely that a gelling saliva would spread as evenly or effectively to seal up the pit membranes as is the case when RWA taps for water. The evidence for complete blocking of the pit membranes by saliva (see Fig. 2H) is, we believe, very strong support for the hypothesis that streaking and wilting in Betta and Betta-Dn1 is caused through the prevention of water and nutrient flow to parenchymatous elements from the xylem.

Conclusions

Our study has shown that the RWA-SA1 causes substantially more damage to the phloem of the susceptible wheat cultivar than to its isogenic resistant counterpart, Betta-*Dn1*. The function of the parenchyma cells of the vascular bundles and mesophyll becomes impaired. Tapping the xylem for water results in a salivary ejection that decreases offloading of water to the vascular parenchyma and phloem, thereby increasing water, nutrient and photosynthetic stress and which, in turn, probably results in leaf streaking, curling and necrosis.

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Fig. 1. A–B; Betta control. A: Shows phloem from an intermediate vein. Several thin-walled sieve tubes (ST) associated companion cells (CC), vascular parenchyma (VP) and a solitary thick-walled sieve tube (solid dot). Bar=10 μm. B: Detail of a thin-walled sieve tube-companion cell complex and associated vascular parenchyma. V=vacuole, M=mitochondrion. Bar=5 μm. C: Betta; shows junction between probed mesophyll cells (MES). Cell walls were destroyed and cell contents disrupted and intermixed with salivary material (SS). Scale bar=2 μm. D: Betta; part of a stylet sheath between two mesophyll cells. Note that the cell walls have been punctured and saliva is present within the mesophyll cell to the left (SS). Bar=5 μm. E: Betta; a probe between two mesophyll cells in which the aphid has split the cells (presumably along, or in the middle lamella region) separating plasmodesmata in the process (arrowheads). Mesophyll cells show signs of disruption, yet the chloroplast in the cell to the right (Ch) appears normal. Bar=3 μm. F: Betta; a stylet sheath is in the intercellular space between mesophyll cells (MES). Both walls in contact with the sheath show damage at the contact zone (arrows) and saliva (SS) has leaked into the cells. Bar=5 μm. G: Betta-*Dn1*; detail of sheath deposits (SS) which obliterated several thin-walled sieve tube sieves in the process in this vascular bundle. Bar=5 μm. H: Betta; shows completely disrupted vascular parenchyma in an intermediate vascular bundle. Bar=3 μm. I: Betta-*Dn1*; detail of a probe within a large vascular bundle, near to a large metaxylem vessel (XV). Saliva (SS) was deposited as the aphid appeared to puncture a number of cells sequentially while probing for phloem. Despite the presence of saliva, the punctured xylem parenchyma cell to the right (XVP) does not appear to be damaged or plasmolyzed. Bar=5 μm. J: Betta-*Dn1*; detail of undamaged thick-(solid dot) and thin-walled sieve tubes after being probed In an intermediate vascular bundle. Salivary complex (SS) is vesiculate. Surrounding cells do not appear to have been damaged. Bar=5 μm. K: Betta; detail showing obliteration of phloem tissue due to extended feeding. Two thin-walled sieve tubes (SS) were completely obscured by saliva and plasmolysis was evident in other sieve tubes. Companion cells and phloem parenchyma appear to be severely damaged in this large intermediate vascular bundle. Bar=10 μm.
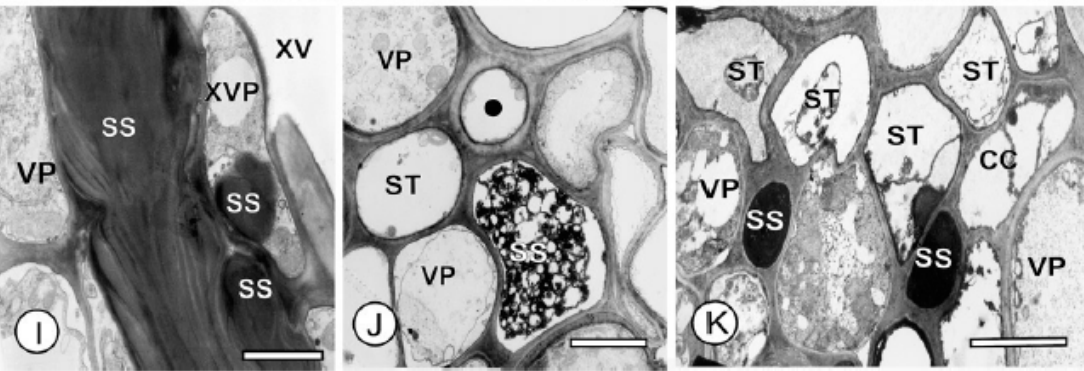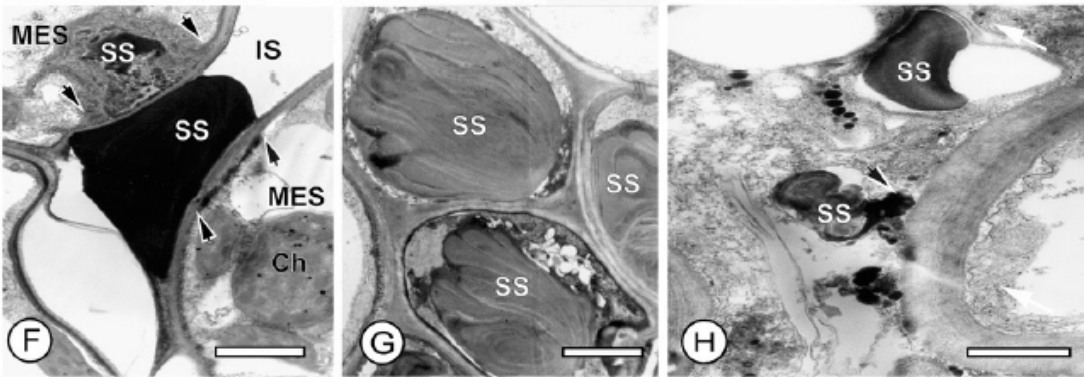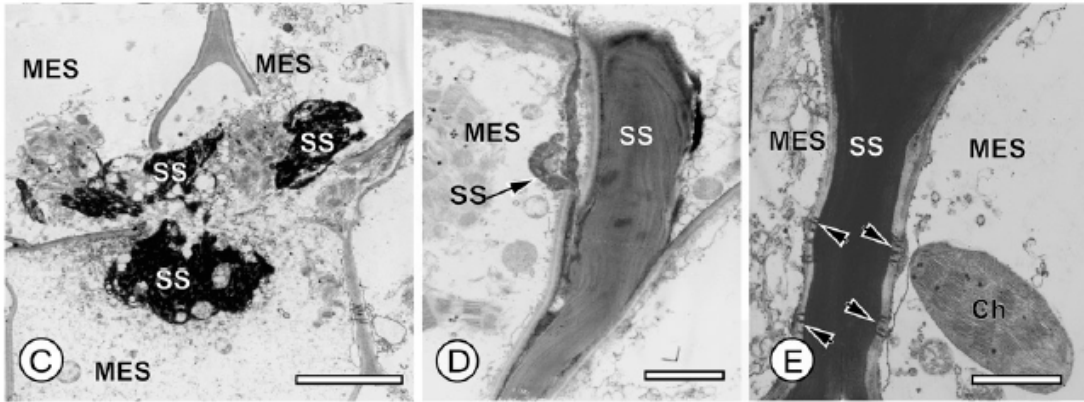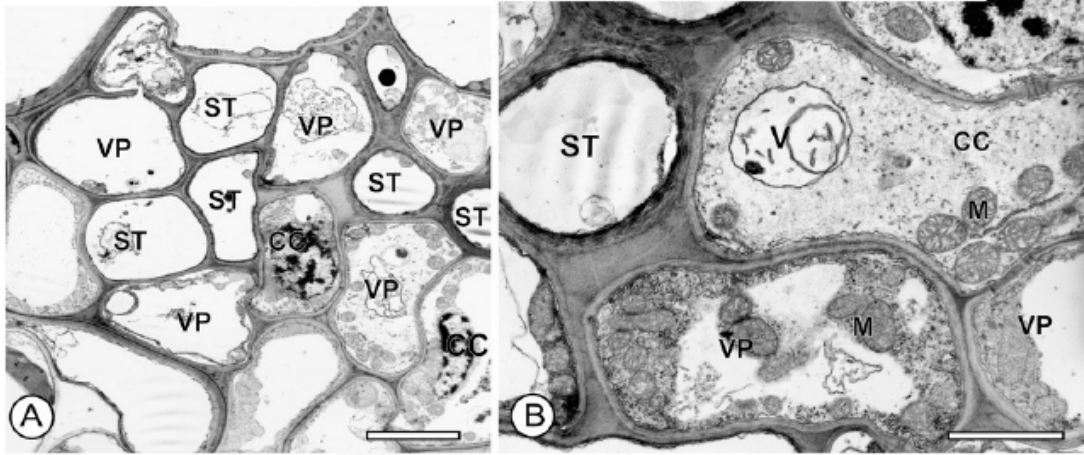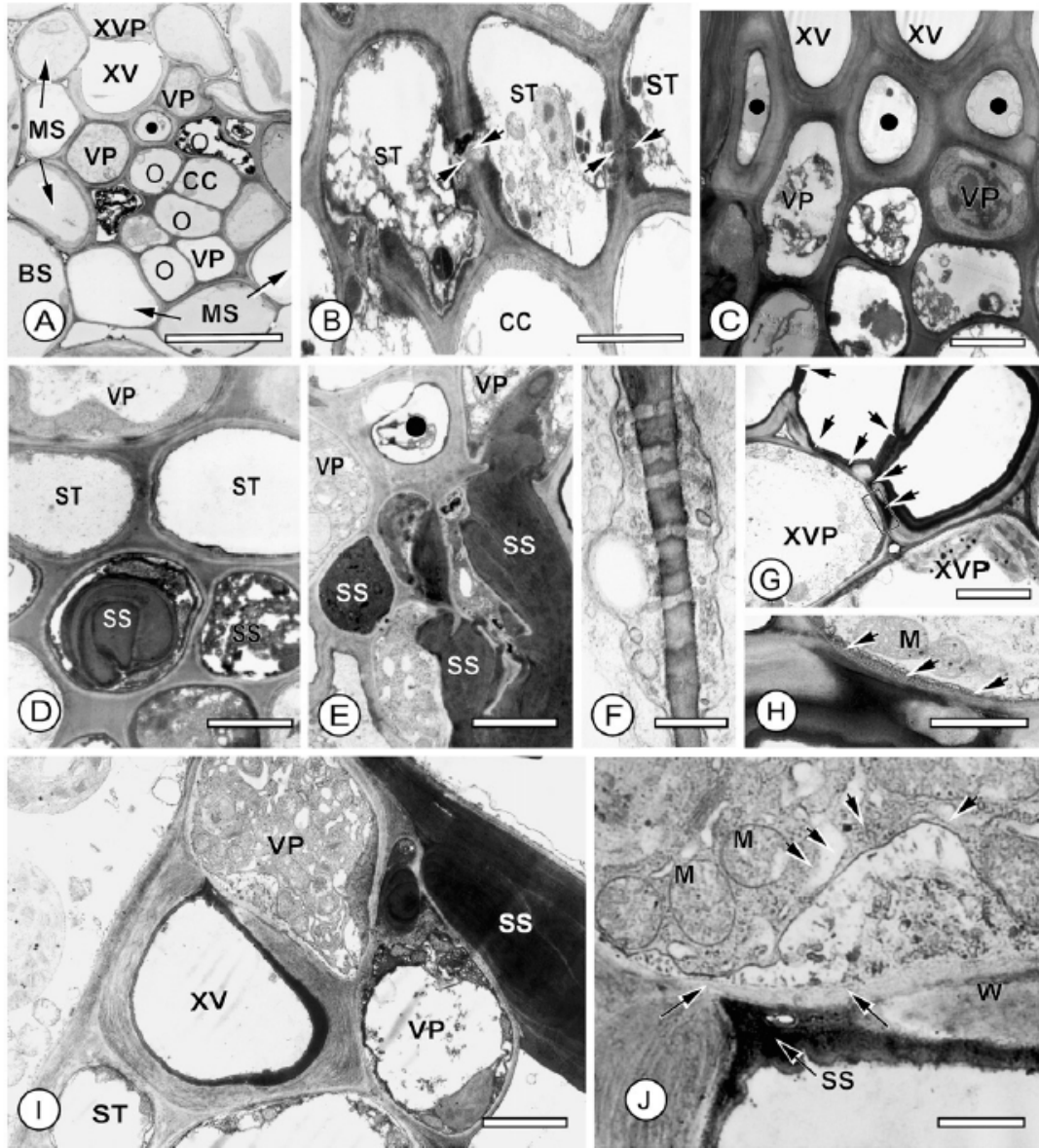
Fig. 2. A: Betta-*Dn1*; shows part of a small intermediate vascular bundle. A metaxylem vessel (XV), several xylem parenchyma cells (XVP), one thick-walled sieve tube (solid dot), several thin-walled sieve tubes (open circles) and associated companion cells (CC) and vascular parenchyma (VP). A mestome sheath (MS) is visible inside the bundle sheath (BS). Bar=15 μm. B: Betta; three adjacent thin-walled sieve tubes (ST) from an intermediate bundle show cytoplasmic damage and callose deposition (arrowheads) in lateral sieve area pores. Bar=5 μm. C: Betta-*Dn1*; detail of undamaged thick-walled sieve tubes (solid dots) adjacent to thin-walled sieve tubes, in which the content has been disrupted and plasmolyzed. Bar=5 μm. D: Betta-*Dn1*; thin-walled sieve tubes in a large bundle are undamaged, in contrast to adjacent elements, which contain saliva. Bar=3 μm. E: Betta; shows widespread probing of the phloem tissue in an intermediate vein. Both thin- and thick-walled sieve tubes were probed. Saliva (SS) has obliterated several cells. Bar=5 μm. F: Betta; plasmodesmatal field between two mesophyll cells. Plasmolysis has resulted in extrusion of the plasmodesmata (forming ectodesmata); some vesiculation of the cytoplasm has occurred. Bar=250 nm. G: Betta; shows part of a group of metaxylem vessels from an intermediate bundle. Note that saliva completely encases the inner walls of the xylem elements and occludes pit membranes between the vessels (arrowheads) as well as the pit membrane between the lowermost vessel and its associated vascular parenchyma cell (paired arrowheads). Bar=5 μm. H: Betta; detail of pit membrane in rectangle in Fig. 2G. Note that the saliva has not crossed the fenestrated wall structure associated with the xylem parenchyma, and that saliva completely blocks the pit membrane on the metaxylem vessel side of the wall. Bar=500 nm. I: Betta; detail of a transverse vein in a leaf blade which was probed, leaving a stylet sheath (SS). The solitary xylem vessel is ensheathed by saliva, as is the pit membrane between this cell and its associated vascular parenchyma cell (VP). The right-most vascular parenchyma cell was punctured — saliva occupies part of the cell (arrowhead) and the cytoplasm has been disrupted. Bar=5 μm. J: Betta; detail of the pit membrane from Fig. 2I. Saliva (SS) has occluded the pit membrane, and ejecta has crossed the membrane, exuding into the parenchyma cell. The plasma membrane has separated from the cell wall (arrowheads point to plasma membrane). The space between the cell wall and the plasma membrane is occupied by saliva and granular, electron-dense material. W=cell wall. Bar=2 μm.