CONFIRMATION OF A SLOW SYMPLASMIC LOADING AND UNLOADING PATHWAY IN BARLEY (*HORDEUM VULGARE* L.) SOURCE AND SINK LEAVES

A thesis submitted in fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

LISA VALENCIA BUWA

February 2003

ABSTRACT

Visualization of the transport pathway in barley (*Hordeum vulgare*) leaves was carried out using a combination of aniline blue and a symplasmically transported fluorochrome, 5,6 carboxyfluorescein (5,6-CF). When applied to a source leaf, basipetal movement of 5,6-CF was observed after 3 h and the fluorochrome front was observed about 3-4cm away from the point of application. The fluorochrome was taken up into the symplasm of the mesophyll and was loaded into the bundle sheath cells and then subsequently the vascular parenchyma and finally into the sieve tubes. In sink leaves, acropetal movement was observed after 3 h and the fluorochrome had moved approximately 3 cm away from the point of application. Unloading of 5,6-CF occurred from all classes of longitudinal veins. Studies on solute retrieval showed that 5,6 CF-diacetate was transferred to xylem parenchyma where it was metabolized. 5,6-CF was then transferred from the xylem parenchyma to the vascular parenchyma cells, and it would appear that thick-walled sieve tubes were the first to show 5,6-CF labeling. Counterstaining with aniline blue demonstrates the presence of plasmodesmata and this suggests a potential symplasmic pathway from the mesophyll to the sieve tubes. Application of 5,6 CF-diacetate revealed a slow symplasmic pathway, which involved transfer of 5,6-CF, which was effected via plasmodesma.

TABLE OF CONTENT

LIST OF FIGURES

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Prof. CEJ Botha, my supervisor, for taking me on as a student and for his skillful assistance during the course of this study. I wish to thank him for his help in the preparation of 5,6-CF retrieval experiments and in the interpretation of the results. His competence, willingness to help and instruct, guidance on completion of the thesis and his advice on many aspects of this work are appreciated.

Sincere thanks are due to Mr RHM Cross, Ms Shirley Pinchuck for their cooperation and technical assistance with electron microscopy. I thank Mr Jay Narsai for helping me with the production of light microscopy slides.

I am deeply grateful to my family for believing in me, and for their unfailing encouragement and sustained interest throughout my studies. I wish to thank the following persons for general support and inspiration: Ntombozuko Matyumza, Baphiwe Ben Qona, Babalwa Matsiliza, Noluzuko Gwayi, Ayanda Ngqandu and lastly, but not least, Nowethu Teyise. My sincere thanks to Nyameko Nondzube for his general support and for help in proof reading the semi-final draft of the thesis.

Lastly, the financial support by the National Research Foundation (NRF, Pretoria, South Africa) who awarded an MSc bursary is acknowledged.

CHAPTER 1: INTRODUCTION

1.1 The phloem loading and unloading pathway

Phloem loading is an important determinant of the growth and development of plants and therefore of crop yield (van Bel 1993). The author further suggested that the process of phloem loading refers to the entire pathway that photosynthates follow from the mesophyll to the sieve element-companion cell complex. The process of phloem loading requires that the major translocated substances are selectively and possibly actively delivered to the sieve tubes in the source region prior to translocation (Geiger 1975). This process is essential not only for the transport of sugars but also for the translocation of all other compounds present in the phloem. The path taken by sucrose from the mesophyll to the sieve elements has been extensively examined. Debate continues on the mode of phloem loading in higher plants, but there is general agreement that the loading process is either symplasmic, apoplasmic, or mixed-mode symplasmic and apoplasmic (Gamalei 1989, 1991; Turgeon and Beebe 1991; Russin *et al.*, 1996 and references therein and Botha *et al.* 2000). Studies by Komor *et al.*, (1996) suggested that there are three different possible ways of sucrose transfer from the medium to the sieve tubes. Firstly, the authors suggested that there is a direct passage of sucrose from the medium through the apoplasm to the sieve tubes where it is taken up. This is direct apoplasmic loading. Secondly, sucrose could be taken up by the mesophyll cells and possibly transferred symplasmically to the veins where it is finally released into the apoplasm from which it is loaded into the sieve tubes. This

process is called indirect apoplasmic loading. Thirdly, sucrose could be taken up from the medium into the mesophyll cells and move through them symplasmically via plasmodesma to the sieve tubes. This process is called symplasmic loading. It has been suggested that the rate controlling factor of symplasmic transport should be diffusion through plasmodesma (Tucker *et al.*, 1989). Conformation of an initial symplasmic route is apparent from a number of ultrastructural studies. Interconnectivity between cells of mature leaves from a wide range of plant species has revealed that all cells within the mesophyll are highly interconnected by plasmodesma (Evert *et al.*, 1978, Fisher and Evert 1982, Robards and Lucas 1990).

The phloem of higher plants forms an essential pathway for the movement of photoassimilates from mature, exporting tissues described as sources to immature developing tissues, which are described in the literature as sinks (Gifford and Evans 1981). Classically, the mature leaf is considered to be a carbon source, whereas the immature leaf is viewed as a net importer of carbon (Biddulph and Cory 1965, Dickson and Larson 1981, 1982, Turgeon 1989). This situation changes as the leaf matures and the sink-source transition progresses basipetally along the leaf (Fellows and Geiger 1974; Larson *et al.*, 1980; Turgeon 1989, 1991). During the transition period, the converted apical portion of the blade begins to export photoassimilates while the basal unconverted portion is still importing them. The process of phloem unloading has been studied extensively but remains a poorly understood process. During phloem unloading, assimilates may leave the SE/CC complex by two potential pathways: through plasmodesmata into vascular parenchyma elements (symplasmic unloading)

Evert 1982; Walsh 1974; Botha *et al.*, 1982b and references cited therein, Dannenhoffer *et al.*, 1990). The first-formed metaphloem consists of thin-walled sieve tubes and associated companion cells and vascular parenchyma cells. The last-formed metaphloem elements differentiate adjacent to the metaxylem vessels. These late-formed sieve tubes are thick-walled and appear to lack companion cells (see Evert *et al.*, 1996 and literature cited). 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 assimilates.

The barley leaf contain a system of longitudinal vascular bundles interconnected by numerous transverse veins (Dannenhoffer *et al.* 1990). Several studies have suggested that the different orders of bundle in the blades of both C_3 and C_4 grasses may have largely different functions (Lush 1976, Altus and Canny 1982, Evert and Mierzwa 1989, Fritz *et al.*, 1989, Evert *et al.*, 1996 and Botha and Cross 1997). Typically, the small and possibly the intermediate bundles have been implicated in the assimilate loading process with the large bundles being primarily involved in longitudinal transport (Evert *et al.*, 1996). Altus and Canny (1982) suggested that transverse veins function in the transfer of assimilates laterally from the intermediate (loading) bundles to large (exporting) bundles. These authors further suggested that these veins contain only one sieve tube and investigations carried out by Botha (pers comm) have shown that the transverse veins contain only one sieve tube and this sieve tube appears to be thin-walled.

Farrar *et al.* (1992) suggested that barley was a potential symplasmic phloem loader. However, subsequent studies undertaken of the small and intermediate bundles of the barley leaf by Evert *et al.* (1996) and then by Botha and Cross (1997) reported that the thick-walled sieve tubes have very few plasmodesmal connections in their common walls with other cell types. In addition, the frequency of plasmodesmal connections between sieve tube-companion cell complexes and other cell types of the leaf, including vascular parenchyma cells and thick-walled sieve tubes, were also rare. The authors therefore concluded that both the sieve tube-companion cell complexes and thick-walled sieve tubes might, based on plasmodesmatal frequencies, be virtually symplasmically isolated from the rest of the leaf. Studies undertaken by Matsiliza and Botha (2002) showed that the aphid *Sitobion yakini* feeds preferentially on thin-walled sieve tubes (ST). These data suggest that SE were a more attractive feeding source than thick-walled sieve tubes and that the thin-walled sieve tubes may be more functional and could play an important role in phloem loading and transport within the small leaf bundles than thick-walled sieve tubes, in source leaf tissue.

1.3 Plasmodesmata and the symplasmic concept

Plasmodesma have been described as the intercellular connections between plant cells that allow cell-to-cell transport of sugars, amino acids, inorganic ions, proteins, virons, nucleic acids and signal molecules (Lucas *et al.*, 1993). An important function of plasmodesma is assumed to be that of symplasmic transport of assimilates from cell to cell to the phloem. Plasmodesma have been intensively studied to determine their

role in the trafficking of assimilates and other molecules for example, between sieve elements and companion cells, and is a clear theme in the recent book by van Bel and van Kesteren (1999). The pore-plasmodesma units (PPUs) that connect the sieve element and companion cells are always branched on the companion cell side of the shared wall only (van Bel 1996). Because plasmodesma provide a potential symplasmic connection pathway between cells, communication and transport within and between these symplasmic domains are intimately linked to the frequency, distribution and function of plasmodesma. The general assumption that is made is that the greater the number of plasmodesma (frequency) at a given interface, the greater is the potential for symplasmic transport across that interface (Botha and van Bel 1992). In contrast, a paucity of plasmodesma in the phloem loading pathway supposedly reflects a higher potential for apoplasmic transfer. Furthermore, there is evidence that blockage of plasmodesma may cause the cessation of symplasmic transport, limit cellular communication and thereby also limit signal transduction between adjacent cells. It is thought that plasmodesmal closure (or gating) is associated with callose deposition in the sieve plates and plasmodesmal aggregates.

Botha (1992) examined the distribution of plasmodesma in C_3 and C_4 grasses. He observed that the plasmodesma where these traverse the suberin lamellae, are constricted at the Kranz mesophyll-bundle sheath (KMS-BS), the bundle sheath-vascular parenchyma (BS-VP), or the bundle sheath-mestome sheath interfaces (BS-MS). A review by Botha and Cross (2001) have shown that control of the phloem loading mechanism in grasses may occur at the junction between the bundle sheath

and the mesophyll. These authors further reported that it is at the mesophyll-bundle sheath interface where many grass species regulate outward water loss and that inward carbohydrate uptake is regulated at the BS-VP. Studies undertaken by Botha and Evert (1986) showed that the suberin lamella regulated the outward water loss which forced a symplasmic pathway through plasmodesma at that interface. They further suggested that in C_3 plants the MS-BS interface assumes greater importance in assimilate transport, especially if suberin lamellae occur at that interface. Plasmodesma are at the heart of cell-to-cell traffic control and regulation.

1.4 Visualization of the transport pathway

One of the major problems in phloem-related studies, remains the visualization of transport processes. Autoradiographic studies have been used extensively in the study of assimilate movement. Sakri and Shannon (1975) made autoradiograms of the distribution of ^{14}C in wheat grains and showed that unloading of assimilates occurs along the entire length of the crease vascular bundle within the seed. Results of a subsequent microautoradiographic study of ^{14}C-photosynthate transport in *Triticum aestivum* leaves indicated that the thick-walled sieve tubes may be involved neither in storage or directly in the transport of photosynthate (Cartwright *et al.*, 1977). The microautoradiographic studies of phloem loading and transport in the leaf of maize was reported by Fritz *et al.*, 1983. These authors showed that in the small and intermediate bundles it was the vascular parenchyma cells that retrieved sucrose from the xylem and transferred it to the thick-walled sieve tubes or released it into the free

space of phloem for uptake by the thin-walled sieve tubes. Based on this study, Fritz *et al.*, (1983) suggested that the thick-walled sieve tubes are not involved in long-distance transport and that the thin-walled sieve tubes appeared to accumulate sucrose and photosynthate from the apoplasm. However, one criticism of microautoradiographic work done in the past, is that not all experiments were conducted on attached leaves, which makes comparison difficult.

Various studies (e.g. Evert *et al.*, 1968, Evert *et al.*, 1973, Botha *et al.*, 1975, Botha and Evert 1978, Matsiliza and Botha 2002) have clearly demonstrated that aphids are useful in the study of phloem functionality. A study of feeding habit of aphids on barley, undertaken by Evert et al. (1973) showed that the aphid preferentially penetrated from the adaxial surface of the leaf. Evert *et al.*, (1973) did not report on which (thin- or thick-walled) sieve tubes were penetrated. However, a recent study by Matsiliza and Botha (2002) showed conclusively that the thin-walled sieve tubes were a more attractive feeding source and that as a result, they may play an important role in phloem loading and transport.

The use of fluorochromes has been limited to a few studies and these have been reviewed by Oparka (1991), and Wright and Oparka (1996). Of particular interest are fluorescent tracers which do not readily cross the plasmalemma but which move from cell to cell by way of permeable junctions such as plasmodesma. These have been used extensively in the study of cell-to-cell communication. The techniques were first described in animal cell studies by Taylor and Wang (1980) for a review of animal

studies; Goodwin and Erwee (1985) for a review of plant studies. Fluorescent dyes are one of the favoured tracers used in phloem studies. *In situ* movement of fluorescein has provided a great deal of information about phloem translocation (Madore *et al.*, 1986; van Kesteren *et al.*, 1988; Madore and Lucas 1989; Goodwin *et al.*, 1990; Turgeon and Beebe 1991; Farrar *et al.*, 1992; Botha *et al.*, 2000; Haupt *et al.*, 2001). Intercellular movement can be monitored by conventional fluorescence microscopy following microinjection of a single cell. From these microinjection studies, convincing evidence for operative symplasmic connections have been presented for a variety of plant tissues. Investigators have microinjected dyes such as Lucifer yellow and carboxyfluorescein into the sieve tubes, mainly to study the loading of assimilates into the phloem and transport related events.

Tyree and Tammes (1975) and Tucker (1982) introduced fluorescent tracers into cells of *Tradescantia* stamen hairs and within a few minutes observed passage into neighboring cells and then further movement through adjacent cells. Van Kesteren et al. (1988) saw dye in a narrow-diameter cell type within the vein of *Commelina* after it was injected into the mestome sheath. Fluorescence microscopy studies on source leaves of SXD-1 mutant *Zea mays* by Botha *et al.*, (2000) revealed the true functional regulation of plasmodesma. These authors have shown that the mutant maize plasmodesma were blocked by callose at the bundle sheath (BS)-vascular parenchyma (VP) interface and this prevented the movement of Lucifer Yellow across the BS-VP interface and, hence, the accumulation of starch was promoted. Studies by Rhoades and Carvalho (1944) demonstrated that in BS cells soluble sugars are

converted into starch and this happens when the rate of sugar accumulation in the BS cells exceeds sugar export into the VP cells.

1.5 Objectives

Although there appears to be general agreement that plants can be broadly grouped into either apoplasmic or symplasmic loaders, debate continues as to the underlying mechanisms involved in the loading process. Studies undertaken by Evert *et al.*, (1996) and subsequently by Botha and Cross (1997) suggest that declining plasmodesmal frequency towards the VP-CCST interface, indicate a potential apoplasmic phloem loading pathway in barley leaves. Botha et al. (2000) further suggested that callose deposition in SXD-1 maize mutant affected symplasmic transport across the bundle sheath-vascular parenchyma interface and this resulted in a major decline in symplasmic transport. A recent study by Haupt et al. (2001) presented data which suggests that unloading in barley sink leaf was symplasmic and it occured via thin- and thick-walled sieve tubes. However, subsequent studies undertaken by Matsiliza and Botha (2002) have shown that aphids selectively feed on only one sieve tube which is thin-walled, and that thin-walled sieve tubes are probably more functional in terms of phloem loading and transport processes.

Many of the data available suggest that phloem loading occurs directly from the apoplasm. However, some authors have recently criticized the experimental approaches that led to this conclusion and suggest that phloem loading might, in part, be symplasmic.

Clearly, the phloem in grasses is complicated by the presence of thin- and thick-walled sieve tubes, whose function in phloem loading and unloading remains unclear. Functionality can only be determined or demonstrated by using symplasmically-transported fluorochromes such as 5,6-CF, to follow the loading process in sink and source leaf tissue. Presence in living cells suggests that it must have entered these cells, via plasmodesmata.

As mentioned, the phloem loading process is one in which assimilates are taken up from the mesophyll and transported to the phloem. Phloem loading is assumed from the literature to be the principal function of the small vein (Botha and Cross 1997, Evert *et al.*, 1996) and transfer from the small vein to the intermediate and large veins must be via the cross veins. Separate injections of Lucifer yellow done by Farrar *et al.* (1992) showed that symplasmic continuity extended from mesophyll to sieve tube, in intermediate veins of barley, though the specific cells in which the dye was moving could not be identified with certainty (see Evert *et al.*, 1996). Research done by Botha and Knoblauch (not published) has shown that 5,6-CF was transported symplasmically via thin-walled sieve tubes. However, based on an ultrastructural study of plasmodesmal frequencies in barley leaf blades, Evert et al. (1996) found that the thick- and thin-walled sieve tube-companion cell complexes were virtually isolated symplasmically, suggesting an apoplasmic pathway. Of immediate interest were the data obtained using confocal microscopy which showed 5,6-CF within sieve tubes of the longitudinal vein and the cross veins in wheat leaves, which suggested an symplasmic loading pathway. Given the fact that barley could be an apoplasmic or a

symplasmic loader, the principal objective of this study was to determine the loading and transport pathways in barley leaves and the key issue relating to this study is the visualization of the role of the sieve element in the cross vein in the overall process. As already mentioned cross vein sieve tube members are apparently thin-walled and as such, connected to the thin-walled sieve tubes in longitudinal veins.

The research questions that arise are therefore:

- Are cross vein sieve tube members connected to both thin- and thick-walled sieve tube members in small and intermediate vascular bundles or only to the thin-walled sieve tube members?

- Was it possible to visualize the symplasmic route? Does 5,6-CF travel in the thin- or thick-walled sieve tube member boundary or is it transported only in thin-walled sieve tube members?

Therefore, the hypothesis of this research is that phloem loading in barley leaves occurs in thin-walled sieve tube members. This is based on evidence of aphid feeding which has shown that thin-walled sieve tubes are preferred over thick-walled sieve tubes as a feeding site and the research by Botha and Knoblauch (not published) have shown transport via thin-walled sieve tubes. If barley is indeed an entirely apoplasmic loader, then no fluorochrome should be evident in sieve tubes (thin- or thick-walled) after feeding the fluorochrome.

Null Hypothesis: If barley is a symplasmic loader, then the fluorochrome should be evident in sieve tubes after feeding.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant material

Seeds of barley (*Hordeum vulgare* L. cv. Clipper) were pre-germinated in petri dishes and thereafter sown in plastic pots containing potting soil. Plants were grown under controlled conditions in a Conviron EF 10H growth chamber at 200 $\mu mol.m^{-2}.sec^{-1}$ light intensity and 350 ppm CO_2 concentration. The plants were watered two to three times per week with a Long-Ashton nutrient solution (Hewitt 1996).

2.2 Light microscopy

Leaf tissue was cut into manageable pieces and fixed in FAA for 24 hours. The leaf tissue was then dehydrated through an alcohol and tertiary butyl alcohol series taking about 12 h in each step. The material was then infiltrated with liquid paraffin and a number of changes of paraplast wax over three days, in an embedding oven at 60°C. Blocks were mounted and trimmed for transverse and paradermal sectioning. Serial sections were cut at 15µm using a Minot rotary microtome (Leitz Wetzlar, Germany). Sections were stained in Safranin and Fast green. The stained sections were mounted onto slides using Canada Balsam and hardened in an oven at 37°C for three weeks. Selected sections were photographed using an Olympus DP-10 camera (Olympus, Japan), mounted in a Zeiss Std Junior 18 Microscope.

2.3 Transmission Electron microscopy

Plant tissues were cut into small pieces and fixed in cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2), for 24 h and then postfixed in 2% osmium tetroxide in 0.05 M cacodylate buffer overnight in a refrigerator. Leaf tissues were dehydrated in a graded ethanol series, followed by two 30 min changes of propylene oxide and finally infiltrated and embedded in Araldite Taab 812 resin. Thin sections were cut into water using a glass knife and collected on microslides and dried down at 60^0C. Sections were stained in toluidine blue for 1 min, rinsed in distilled water and then mounted under coverslips in DPX. Ultrathin sections (silver to gold) were cut using a diamond knife (Drukker, Netherlands) and were collected on 300 mesh copper grids (SPI suppliers, Philadelphia, USA). They were then stained in uranyl acetate and lead citrate. Sections were viewed and photographed using a JEM 1210-EX (JEOL, Tokyo, Japan) transmission electron microscope.

2.4 Fluorescence microscopy

2.4.1 Aniline blue fluorescence microscopy

The abaxial surface of the leaf strip was gently scraped to remove the cuticle and the epidermis in part, thus exposing underlying tissue. Leaf material was mounted in 0.05% (w/v in water) aniline blue solution and examined after 5-10 min for the presence of callose, and photographed using a Zeiss fluorescence microscope using a Plan-Neofluar 25X (NA, 0.8) and 40X (NA, 0.9) water-glycerine-oil immersion optics. A special filter set, specifically for aniline blue comprising a BP: 425-440; FT: 475; and LP: 460DLCP (Chroma Technology Corp, Battleboro, USA) was used to image callose.

2.4.2 Preparation of 5,6 CFDA

1 ml DMSO was added 100 mg 5,6 CFDA to make the stock solution. 1-2 µl aliquots of stock solution was used and diluted to 1.0 ml distilled water, to make working strength 5,6 CFDA. Until used, working stock was stored at -5°C.

2.4.3 Leaf tissue preparation for 5,6 CFDA

Immature (11 day-old) and mature (31 day-old) plants were transported to the laboratory where attached sink and source leaves, and selected portions, were immersed in cold MES buffer. Small areas of the leaves were gently scraped with a sharp single-edged razor blade to remove the cuticle and underlying epidermal tissue from the adaxial surface, effectively opening small "windows" into the mesophyll. 5,6 carboxyfluorescein diacetate (5,6 CFDA) was then introduced intracellularly into the exposed cells and the exposed area of the fed leaf was covered with a coverslip to prevent evaporation of the dye and dehydration of the exposed tissue. After three hours leaf strips (3 mm X 5 cm) were gently cut out, the abaxial epidermis was gently scraped and the section was immediately examined in 0.1M MES buffer under a coverslip. The experiments were either performed at a light intensity of 200 $\mu mol.m^{-2}.sec^{-1}$ or in the absence of light. The experiments were repeated more than twenty times.

2.4.4 5,6 CFDA retrieval

Leaf segments, about 6 cm long, were taken from the apical half of the leaf blade. The basal end of each leaf segment was placed in 5,6 CFDA at a light intensity of 200 $\mu mol.m^{-2}.sec^{-1}$. 5,6 CFDA was allowed to be taken up in the transpiration stream for 25, 120, and 180 min. After each of the retrieval times, the whole leaf was removed and examined under epifluorescence to locate fluorescence. Transverse sections were cut from the region of interest, region with strong 5,6-CF label and the region with the

fluorochrome front, with a tissue chopper (The Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey) at 15-25 µm into silicon oil and examined in silicon oil under coverslip.

2.4.5 Microscopic examination of 5,6 CFDA transport

Specimens were viewed with either a Zeiss 25X (NA, 0.8), or 40X 0.9 NA, water-glycerine-oil immersion objective. High resolution digital images were captured using an Olympus DP-10 camera (Olympus, Japan), fitted to the microscope. Subsequently a specialized filter set was used with the following characteristics: BP: HQ 500/20; FT: HQ 535/30M; LP: Q515 (Chroma Technology Corp, Battleboro, USA). Images were stored for later manipulation as tagged image file format (TIFF) files.

2.4.6 5,6-CF and aniline blue fluorescence microscopy

In order to be able to recognize the phloem (through callose deposition), it was necessary to carry out experiments in which the leaves were first exposed to 5,6 CFDA , and then counterstained in aniline blue. Attached leaves were gently prepared as for leaf tissue preparation for 5,6 CFDA and the dye was introduced via flap feeding. After 3 hours leaf strips were removed and the abaxial surface was gently scraped and immediately mounted in 0.05% aniline blue solution and observed with a Zeiss epifluorescence microscope using 25X and 40X water-immersion optics. High-

resolution digital images were captured using an Olympus DP-10 camera (Olympus, Japan), fitted to the microscope.

CHAPTER 3: RESULTS

3.1 Anatomy of the leaf blade

According to Dannenhoffer et al., (1990); Dannenhoffer and Evert (1994) the leaf blade of barley has typical Pooid grass anatomy, with a system of longitudinal vascular bundles interconnected by numerous transverse veins (Fig. 1A). The vascular bundles are widely separated by loosely arranged mesophyll (Fig. 1B). The longitudinal strands are surrounded by two bundle sheaths, an inner mestome sheath and an outer parenchymatous sheath (Evert et al., 1996). In paradermal and transverse sections of the leaf, three orders of longitudinal vascular bundles are present, namely, large (first order), intermediate (second order), and small (third order) (see Fig. 1A-B and Fig. 2). These can be recognized on the basis of their size, the composition of their xylem and phloem, and the nature of the tissues in contact (Dannenhoffer et al., 1990; Dannenhoffer and Evert 1994). As seen in the transverse section (Fig. 2A, B and C), the large bundles are characterized by the presence of protoxylem lacuna and two large metaxylem vessels, one on either side of the lacuna. Protophloem and metaphloem are also present, but in mature bundles only metaphloem can be seen. The intermediate bundles lack protoxylem and the large metaxylem vessels which are characteristic of the large bundles. Large and intermediate bundles are associated with hypodermal sclerenchyma strands (Dannenhoffer et al., 1990). Small bundles are smaller than intermediate bundles and their vascular tissue consist entirely of

metaxylem and metaphloem. The metaphloem of all three orders of longitudinal bundles typically contain both thin- and thick-walled sieve tubes.

As seen in the paradermal section, the longitudinal bundles are linked by transverse veins (Fig. 1A), which usually contain only one sieve tube which is a thin-walled sieve tube. The transverse veins are small and are all more or less similar in size. They are very simple in construction. They consist of a single file of tracheary elements and a single file of sieve tube members (Blackman 1971). The two files are in direct contact with each other. Sieve plates can be detected on the end walls of the sieve tube members and the vessel members develop spiral thickening (Fig. 1C and D). Two parenchyma cells are also present and may adjoin both sieve tube members and tracheary elements.

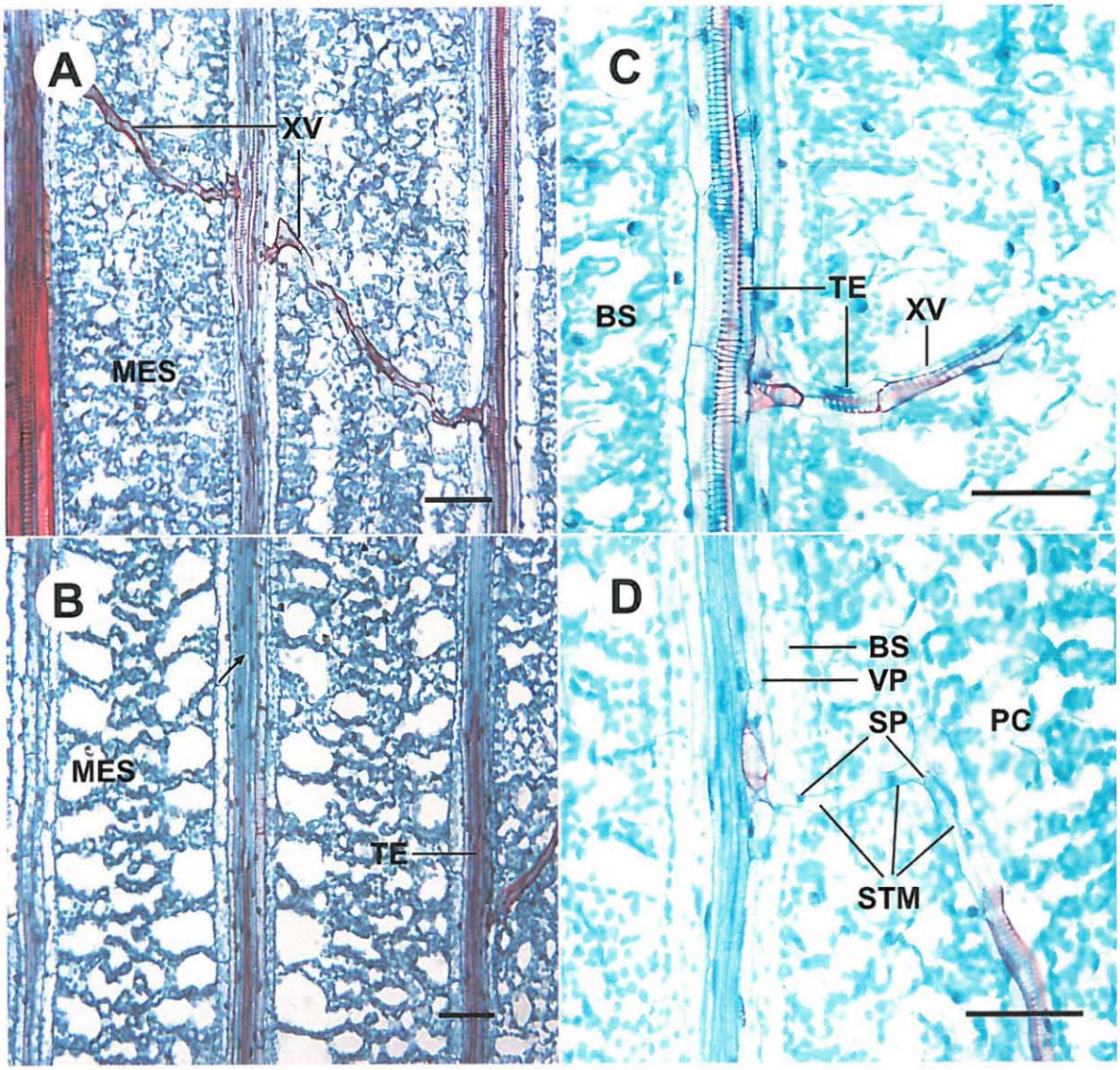

A
XV
MES
C
BS
TE
XV
B
MES
TE
D
BS
VP
SP
PC
STM

Figure 1 Shows paradermal sections of barley leaf. **(A)** Three longitudinal bundles- one large bundle (left) an intermediate bundle (center), and a small bundle (right)- interconnected by two transverse veins. The transverse veins are at the same level. Note the longitudinal bundles are surrounded by the parenchymatous bundle sheath and the mestome sheath. **(B)** Shows three longitudinal vascular bundles separated by loosely arranged mesophyll. Sieve plates (arrows) and tracheary elements can be identified. Note the cross vein joining an intermediate bundle (right). **(C)** Shows part of a cross vein joining to a longitudinal vascular bundle. Note the connecting tracheary element joining longitudinal tracheary element with a pitted wall. **(D)** Shows a cross vein (right) joining a longitudinal vascular bundle (left). Sieve plates and nucleus in the vascular parenchyma, and the tracheary element can be identified. Note the parenchymatous cell abut the sieve element. The bundle sheath and the mestome sheath are visible. BS, bundle sheath; MES, mesophyll; PC, parenchyma cell; SP, sieve plate; STM, sieve tube member; TE, tracheary element. (A-B) Bars = 20 µm, (C-D) Bars = 10 µm.

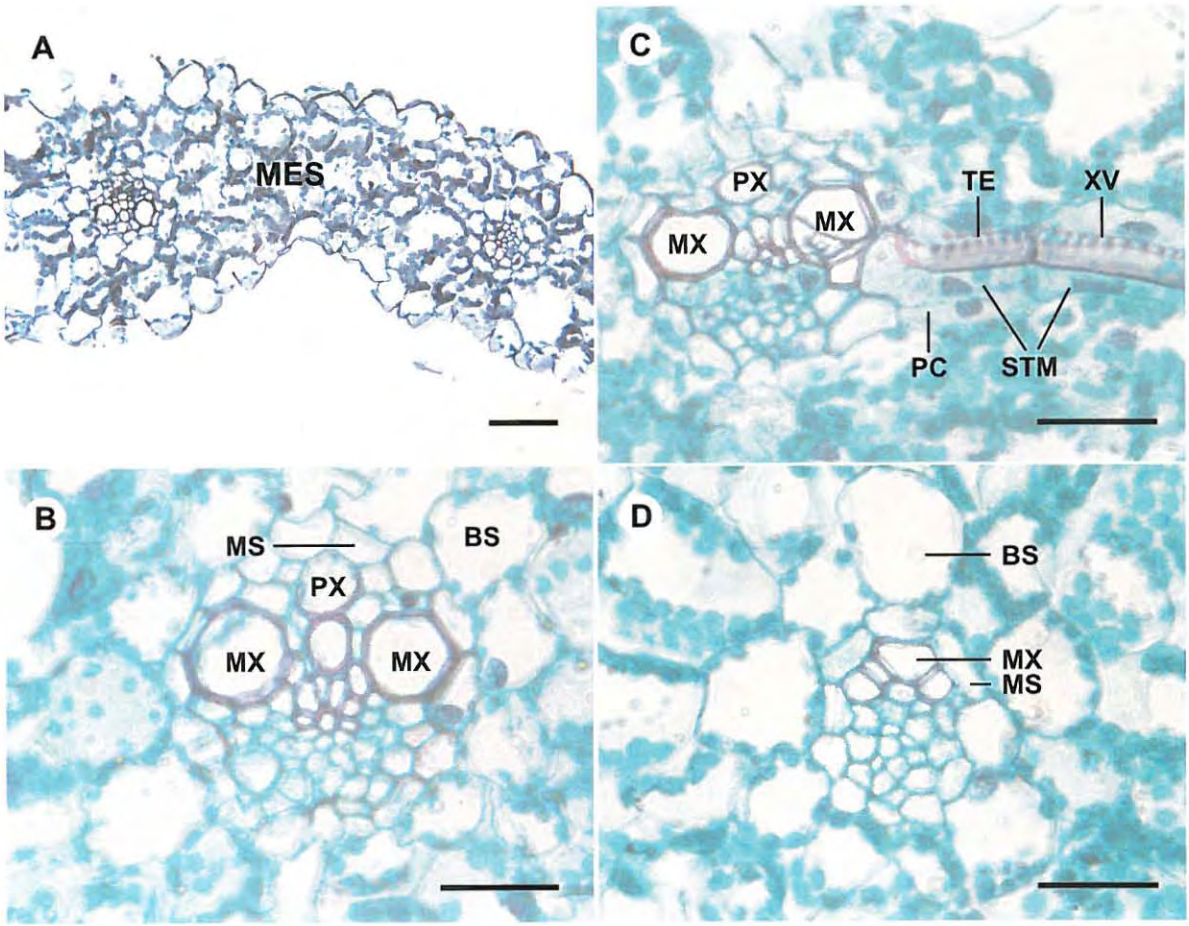

A
MES
C
PX
MX
MX
TE
XV
PC
STM
B
MS
BS
PX
MX
MX
D
BS
MX
MS

Figure 2 Shows transverse sections of barley leaf. **(A)** Shows a transverse section of blade containing large (left) and small intermediate (right) bundles. Note that the bundles are separated by mesophyll. **(B)** Photomicrograph of large bundle. Note that this bundle is characterized by the presence of large metaxylem vessels, one on either side of the protoxylem, metaphloem and protophloem. An outer parenchymatous bundle sheath surrounds a complete mestome sheath. **(C)** Shows a cross vein joining a large vascular bundle. In the connecting region a tracheary element can be identified. Sieve plates of the cross vein sieve element and the connecting cells can also be identified. A parenchymatous cell is present and it abut sieve tube members and vessel members. **(D)** Shows a micrograph of an intermediate bundle which consists entirely of metaxylem and metaphloem. An inner mestome sheath, which completely surrounds the bundle, is bordered by an outer parenchymatous bundle sheath. BS, bundle sheath; MS, mestome sheath; MES, mesophyll; MX, metaxylem; PX, protoxylem; STM, sieve tube member; SP, sieve plate; TE, tracheary element; XV, cross vein. (A) Bar = 20 µm, (B-D) Bars = 10 µm.

3.2 Structure of the leaf blade as shown by aniline blue fluorescence

Figure 3 illustrates fluorescence images taken of barley leaf blade tissue after aniline blue staining for callose localization. When freshly prepared barley leaf blade material treated with aniline blue was examined under blue light, callose was observed, associated with sieve plate pores in the cross veins and in longitudinal bundles, and with the plasmodesmal fields at the SE-CC complex. Figures 3A and 3B show two longitudinal bundles, a large and a small bundle, connected by a cross vein which contains a single file of sieve tube members. Sieve plate pores, lateral sieve areas and pore-plasmodesmal aggregates fluoresce brightly in these micrographs. The parenchymatous bundle sheath and the vascular parenchyma can be identified.

Figure 3C shows the connection between a large exporting vein and a cross vein. Callose-occluded sieve plates are visible in the sieve elements, and multiple callose-occluded pore-plasmodesmal fields can be seen in the phloem of a large bundle and a faint fluorescence in a cross vein indicates the presence of callose-occluded pore-plasmodesmal fields. The parenchymatous bundle sheath can be identified.

Figure 3D shows callose occluded sieve plates in a small longitudinal vein to the right and in its connected cross vein. Many callose-occluded pore-plasmodesmal fields can be seen throughout the phloem tissue, and lateral sieve areas and pore-plasmodesmal aggregates are brightly fluorescing. Figure E is a magnification view of a cross vein after staining with aniline blue. Sieve plate pores, lateral sieve areas and pore-

plasmodesmal aggregates are brightly fluorescing, and the distribution of callose deposits can be seen associated with pore-plasmodesmal fields between the sieve element and the companion cell. The parenchymatous sheath can be identified.

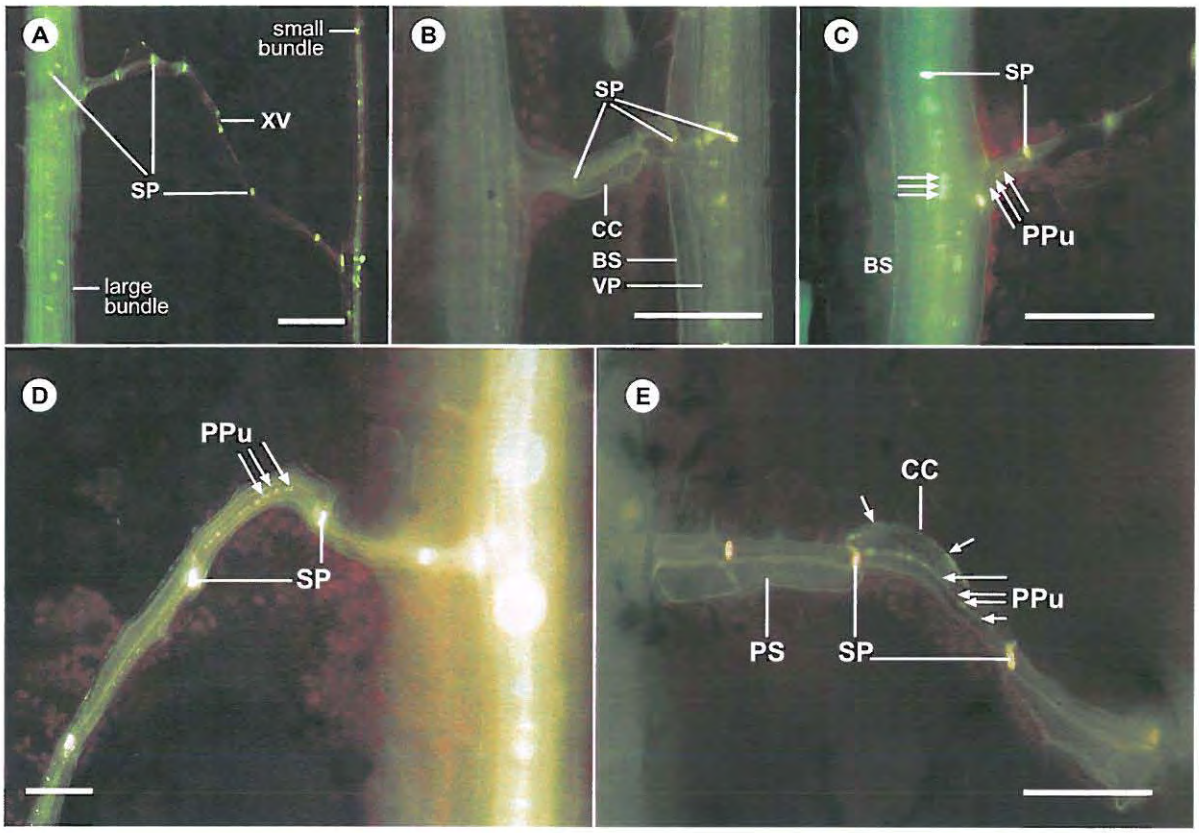

A
small bundle
XV
SP
large bundle
B
SP
CC
BS
VP
C
SP
PPu
BS
D
PPu
SP
E
CC
PPu
PS
SP

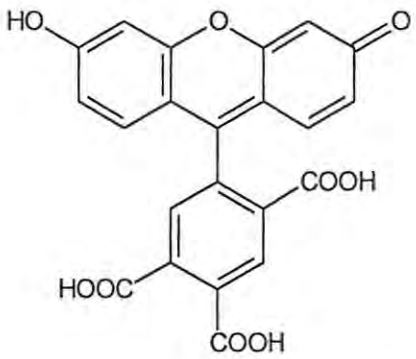
Fig. 3 Shows the localization of callose in a barley leaf blade after aniline blue staining. **(A)** Shows a longitudinal leaf section stained in aniline blue, showing sieve plates in a large transport bundle (left) and a small bundle (right) connected by a cross vein containing only one sieve tube. Note the brightly fluorescing sieve plates which indicate the distribution of callose deposits. The parenchymatous bundle sheath is visible. **(B)** Higher magnification view, showing callose distribution after aniline blue staining. It shows a small and a large vein connected by a short cross vein at center. Note the distribution of callose which associated with the sieve plates. Non-fluorescing sieve plate in a cross vein indicates an absence of callose. **(C)** Shows the junction between a large bundle and a cross vein. Note callose deposits associated with the sieve plates in the phloem. Abundant bright fluorescence indicates the presence of many callose-occluded pore-plasmodesmal units (arrows). **(D)** Longitudinal section of the leaf showing sieve plates in a small bundle and in a cross vein. Arrows indicate many callose-occluded pore-plasmodesmal fields throughout the phloem and arrowheads indicate pore-plasmodesmal aggregates. **(E)** Detail image of a cross vein, showing sieve plates after aniline blue staining for callose distribution. Note callose deposits associated with pore-plasmodesmal fields (arrows). Arrowheads indicate callose-occluded pore-plasmodesmal aggregates. The parenchymatous sheath is visible. BS, bundle sheath; CC, companion cell; PPu, pore-plasmodesmal units; PS, parenchymatous sheath; SP, sieve plate; VP, vascular parenchyma. (A and D) Bars = 20 µm, (B, C and E) Bars = 10 µm.

CHAPTER 4: 5,6 CARBOXYFLUORESCEIN UPTAKE

4.1 Introduction

Fluorescent compounds have been used to study transport pathways within plants. A number of authors used fluorescent dyes such as 5,6-carboxyfluorescein to trace both symplasmic and apoplasmic (reviewed in Canny 1990) transport pathways within plants.

5,6-carboxyfluorescein (5,6-CF) has been described by several authors (Grignon *et al.*, 1989; Oparka 1991; Wright and Oparka 1996) as a mixture of 5- and 6-carboxysubstituted fluoresceins.



5,6 carboxyfluorescein (after Wright and Oparka 1996)

5,6-CF has been used to monitor cell-cell fusion or similar events (Szoka *et al.*, 1979; Ralston *et al.*, 1981), intercellular transport (Erwee and Goodwin 1983, Goodwin 1983) and to study symplasmic continuity in leaves and stems (Erwee and Goodwin 1985; Grignon *et al.*, 1989). Subsequently, the ester form of a probe (CFDA) has been utilized to monitor the post-phloem unloading pathways in sink tissues (Wang and Fisher 1994; Wang *et al.*, 1994; Oparka *et al.*, 1994; Duckett *et al.*, 1994). CFDA has been used extensively in plant research as a marker for symplasmic transport (Grignon *et al.*, 1989; Oparka *et al.*, 1994). Non-fluorescent esters such as CFDA are able to cross membranes passively in the electrically neutral form. Once inside the cells they are subject to cleavage by esterases to form polar fluorescent compounds such as CF.

In early studies, fluorescein was shown to be able to enter the symplasm and be transported in the phloem. Studies undertaken by Socolar and Loewenstein (1979) suggested that the fluorescein may leak through the plasmamembrane at an appreciable rate since it remains partially undissociated at physiological pH's. The presence of an additional carboxyl group in either position 5 or 6 on the carboxyfluorescein molecule means that it is considerably less permeant to biological membranes than fluorescein. Thus, Grignon *et al.* (1989) described 5- (and 6-) carboxyfluorescein as a useful phloem-mobile xenobiotic. The authors suggested that since the dye will readily cross the membrane in the undissociated form if the apoplasmic pH is sufficiently low its description as 'membrane impermeant' in many studies should be treated with suspicion. However, a handbook from the manufacturer

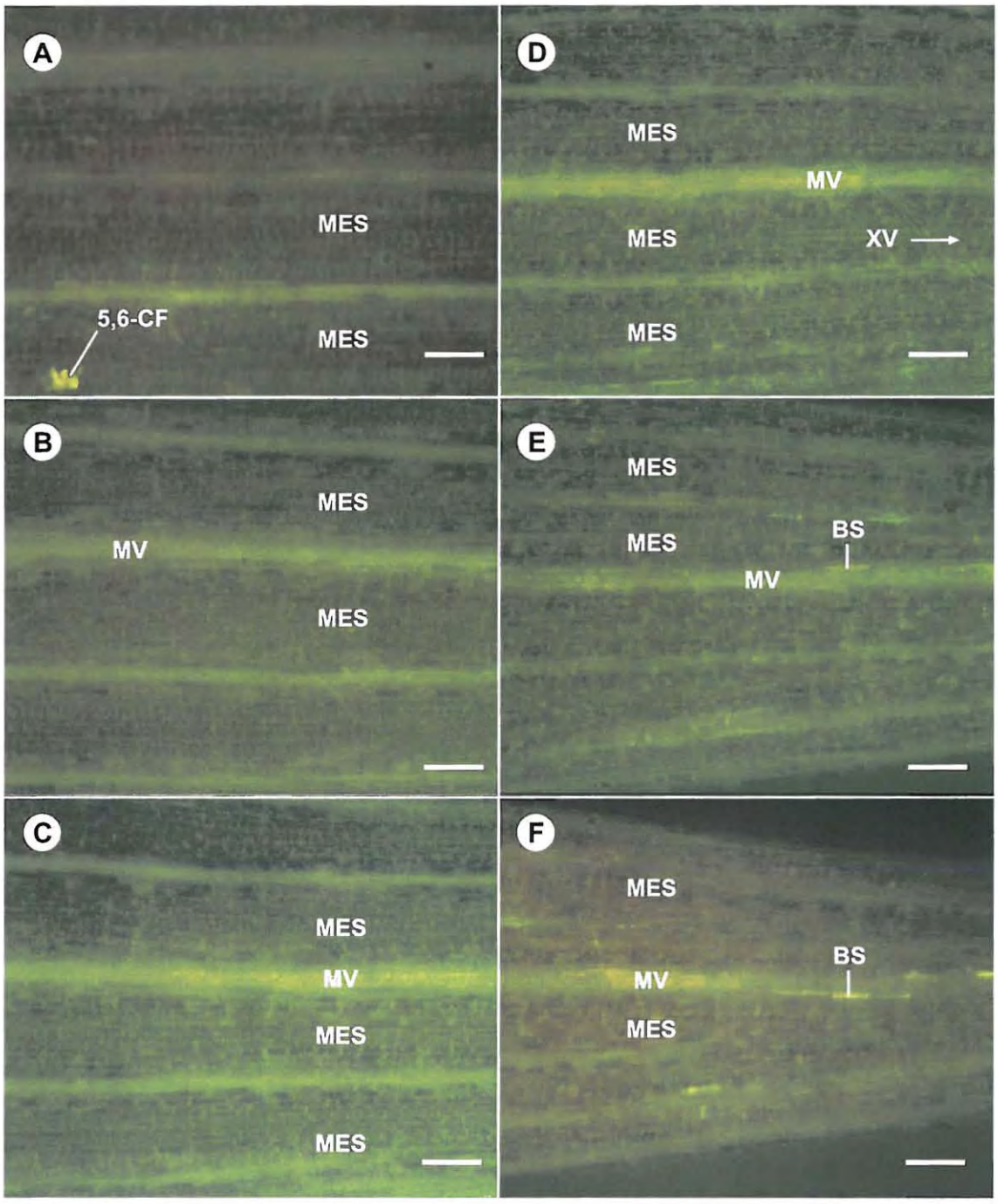
(Molecular Probes, Eugene, OR, USA) state that 5 (6) carboxyfluorescein can cross the plasmamembrane in the diacetate form and that cleavage by cytosolic enzymes produces membrane-impermeant carboxyfluoresceins (cited by Knoblauch and van Bel 1998).

4.2 Results

These experiments focused on the acropetal and basipetal movement of 5,6 carboxyfluorescein (5,6-CF) in the longitudinal bundles, as well as the role of the transverse vein system in the assimilate loading and transfer processes, based upon examination of the movement of 5,6-CF within the phloem tissue.

When 5,6 CFDA was applied to a sink leaf, acropetal movement of the dissociated fluorochrome (5,6-CF) was observed. With time the fluorochrome appeared to be unloaded symplasmically, and moved from the sieve tubes, into the mesophyll tissue via vascular parenchyma and the bundle sheath. By 30 min the fluorochrome had moved 2 cm away from the application point. 5,6-CF unloading was extensive and very rapid. A typical unloading sequence is shown in Figure 4. The sink leaf was 5 cm long and the fluorochrome was applied in the lower 3rd of the leaf, approximately 3 cm away from the apex. 5,6-CF was unloaded in the sink leaf within 30 min after the application and showed a discontinuous pattern of unloading. After 3 h the fluorochrome had moved approximately 3 cm away from the point of application. Following an initial rapid phase, lasting approximately 1 h, the rate of uptake

decreased and then remained constant for at least 2 h. Figure 4 shows the unloading of the fluorochrome at a lower magnification whereas Fig. 5 shows unloading at higher magnification. Figs. 4A & 5A shows the leaf material 3 h after introducing 5,6-CF. They show the approximate point of application. 5,6-CF unloading occurred from major longitudinal veins (Figs. 5B and C) and the distribution of the fluorochrome was discontinuous along the length of a single vein (Figs. 5E). The transverse veins appeared to be involved in unloading of the fluorochrome (Figs. 5D and F). After 3 h the fluorochrome was unloaded and it appeared to move laterally from the veins (Figs. 5B, C and D) into the mesophyll (Figs. 5E and F). All cells of the importing leaf eventually became labeled with unloaded carboxyfluorescein (Figs. 5F).


A
MES
5,6-CF
MES
D
MES
MV
MES
XV
MES
B
MES
MV
MES
E
MES
MES
BS
MV
C
MES
MV
MES
MES
F
MES
MV
BS
MES

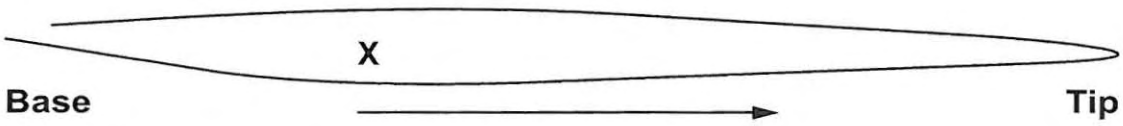

X
Base
Tip

Figure 4 Shows a typical unloading of 5,6-CF in barley sink leaves. Note a discontinuous pattern of dye unloading. The sink leaf was 5 cm long. **(A)** Shows the leaf material 3 h after the application of 5,6 CFDA. The image shows the approximate point of application in the mesophyll. The fluorochrome is distributed within the mesophyll and there is uptake within the mesophyll. **(B)** Shows unloading of the fluorochrome about 1-2 cm away from the point of application. Not much fluorochrome evidence seen between 1 and 2 cm away. A discontinuous pattern of the fluorochrome unloading is evident. **(C) & (D)** Show 5,6-CF unloading about 2-4cm away from the point of application. Extensive spread of the fluorochrome is evident from the major vascular bundles into the minor bundles and into the mesophyll. The cross vein is evident. A discontinuous pattern of dye unloading is also evident. **(E) & (F)** Show the progression of 5,6-CF unloading into a sink leaf of barley. Shows unloading of the fluorochrome in the apical region of a sink leaf. The fluorochrome was unloaded and moved laterally from the veins into the mesophyll. Note that the cells in the apical region are filled with unloaded carboxyfluorescein. BS, bundle sheath; MES, mesophyll; MV, midvein; XV, cross vein. Bars = 20 µm.

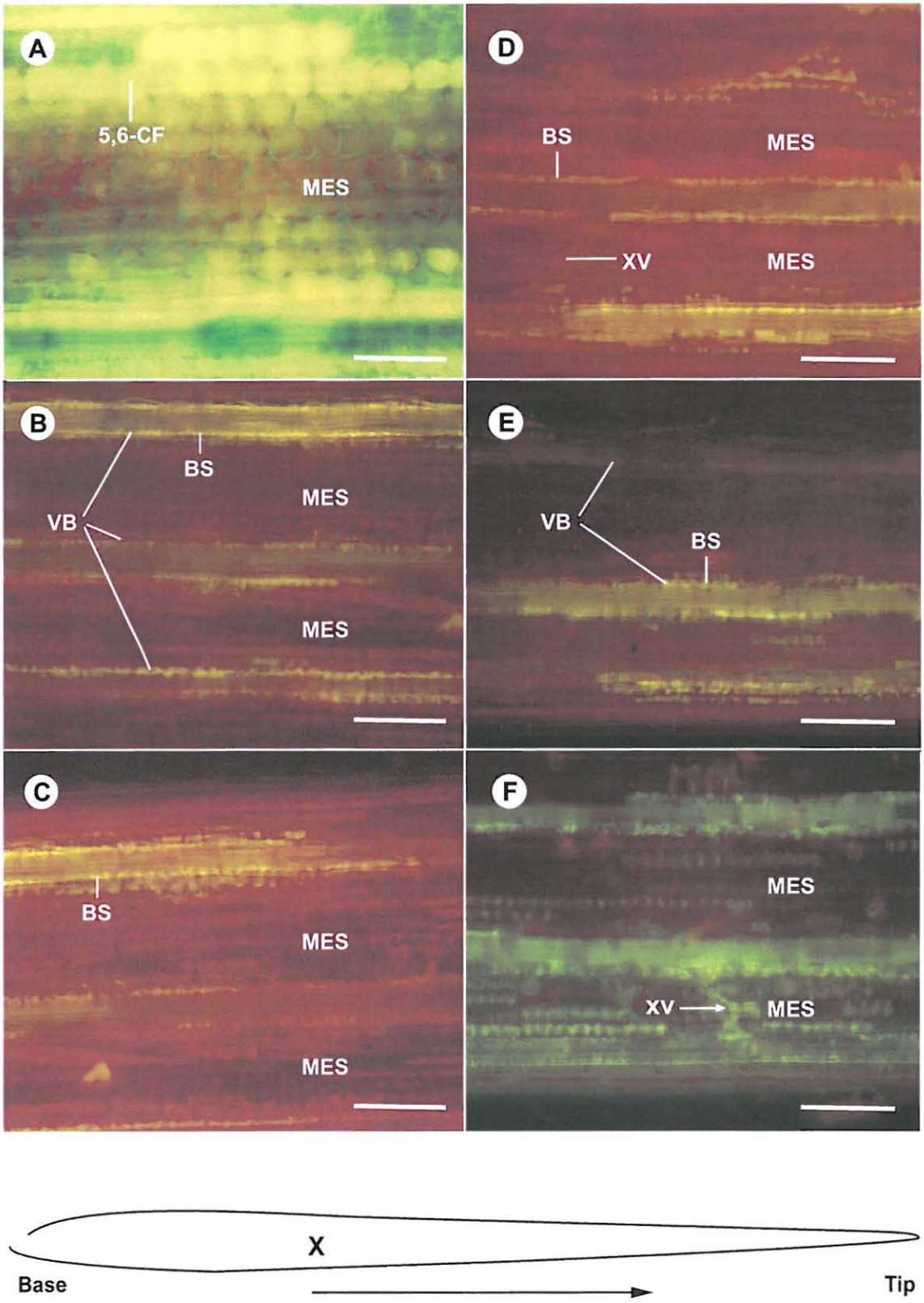

A
5,6-CF
MES
D
BS
MES
XV
MES
B
BS
VB
MES
MES
E
VB
BS
C
BS
MES
MES
F
MES
XV
MES
X
Base
Tip

Fig. 5 Shows a typical unloading sequence at higher magnification. **Fig. 5A** shows the leaf material 3 h after introducing 5,6-CF. It shows the approximate point of application. Note that the fluorochrome is distributed and loaded within the mesophyll cells. **(B)** Shows 5,6-CF unloading about 1-2 cm away from the application point. The extensive fluorochrome spread is evident from the bundle sheath in the major and minor longitudinal bundles. A discontinuous pattern of 5,6-CF unloading is evident. **(C)** 5,6-CF unloading about 2-3 cm away from the point of application. Extensive fluorochrome spread is evident from the major longitudinal bundle into the mesophyll. Note the discontinuous exit of the fluorochrome from the major and intermediate bundles. **(D)** Shows the distribution of 5,6-CF unloading in a barley sink leaf, about 3-4 cm away from the point of application. Note the extensive spread of the fluorochrome in the vicinity of the large and intermediate bundles, the bundle sheath, and into the mesophyll is evident in the micrograph. The cross vein is evident and it appears to be involved in 5,6-CF unloading. **(E)** Shows 5,6-CF unloading about 3-5 cm away from the point of application near the apical region of a sink leaf. The fluorochrome was unloaded and moved laterally from the longitudinal bundles into the mesophyll. BS, bundle sheath; VB, vascular bundle; MES, mesophyll; XV, cross vein Bars = 10 µm.

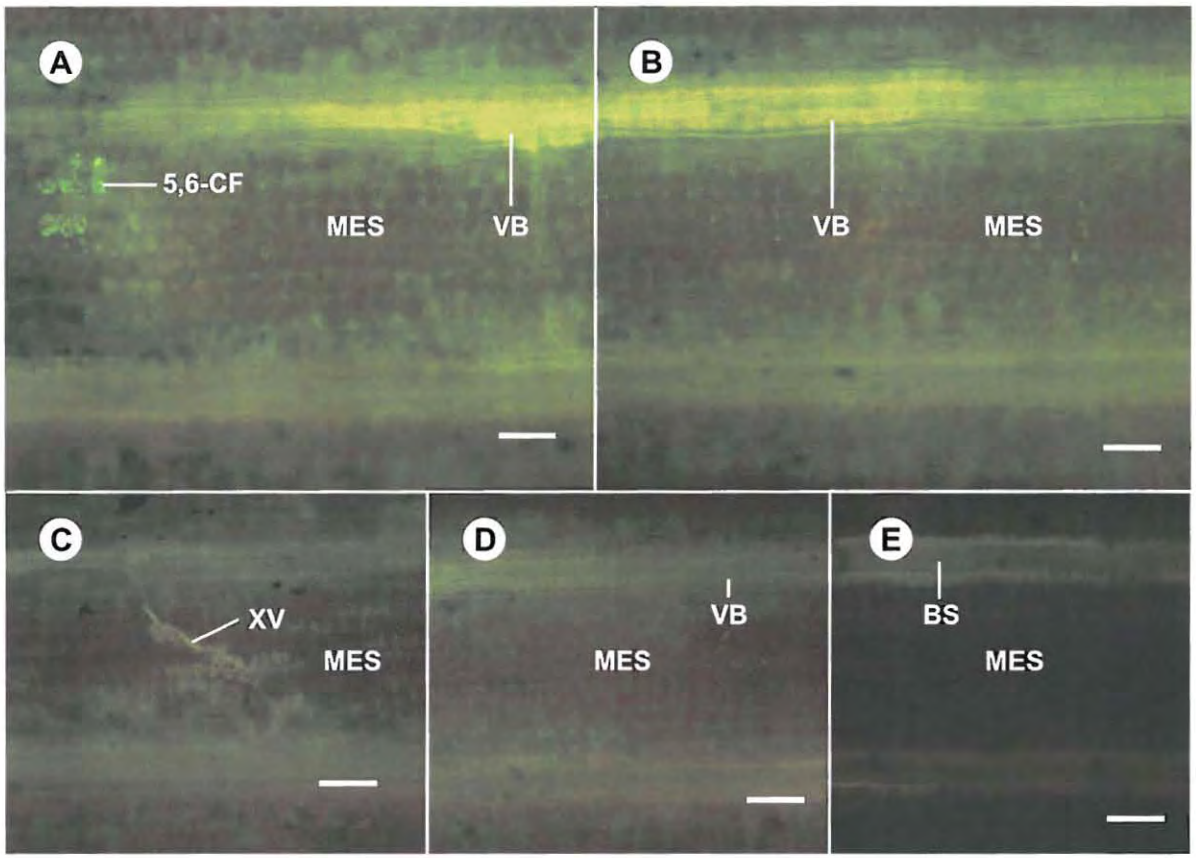
4.2.1 5,6-CF loading

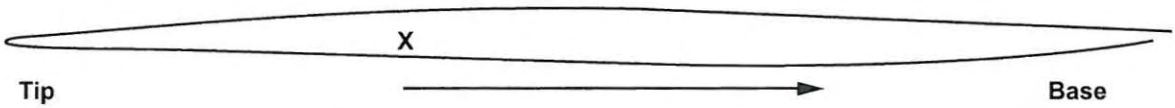
When 5,6 CFDA was applied to barley source leaves basipetal fluorochrome movement was observed (Figs.6 and 7). Based on observations of repeated experiments, loading was seen to take place from the small and intermediate veins and 5,6-CF seemed to move towards the large (exporting) vein (Fig. 6 and 7B). After 3h, 5,6-CF had moved approximately 3-4 cm away from the point of application. In all instances, loading of 5,6-CF took several hours before movement was noticed.

Under light the fluorochrome moved approximately ½ cm away from the application point by 30 min and after 3h, the fluorochrome had moved approximately 4 cm away from the point of application. This contrasts with dark-adapted loading where the symplasmic transport was slow, the fluorochrome moved 2 cm away from the application point after 3h. By 60 min, minor veins 1.5 cm away from the application point showed considerable fluorescence in the symplasm of the leaf. After 3 h the fluorochrome had moved 2 cm away from the point of application, meaning that symplasmic transport was very fast in the first hour and with increase in time it became slow.

A typical loading sequence is shown in Fig. 6. Phloem loading is shown in detail in Fig. 7. Figure 7A shows the leaf material 3 h after the application of 5,6 CFDA. This micrograph shows the approximate point of application in the mesophyll and it shows that the fluorochrome was able to enter the symplasm from damaged cells, where it

was introduced. The dissociated fluorochrome is distributed within the mesophyll and there is uptake within the mesophyll cells. The fluorochrome filled the mesophyll and moved to neighboring cells. Figures 7C-D clearly show longitudinal transport, via the bundle sheath, into the vascular parenchyma and lateral transfer via the cross vein into an adjacent intermediate vein. In Figs. 7E, and Figs. 8A-D the fluorochrome appears to be associated the sieve tube-companion cell complex, in a cross vein, as well as with the vascular parenchyma (Figs. 7E and 8D).


A
5,6-CF
MES
VB
B
VB
MES
C
XV
MES
D
VB
MES
E
BS
MES


X
Tip
Base

Figs. 6 Shows the basipetal movement of 5,6-CF in a barley source leaf. It shows a typical loading sequence. The used source leaves were ranging between 17-19 cm in length. **(A)** Is the leaf material 3h after introducing 5,6-CF. It shows the approximate point of application in the mesophyll. The fluorochrome is distributed within the mesophyll and there is loading from the flap fed mesophyll into the underlying cells. **(B)** Shows 5,6-CF loading between 1-2 cm away from the application point. There is an extensive fluorochrome spread from the mesophyll into the small longitudinal bundles. **(C)** Shows loading of 5,6-CF between 2-3 cm away from the point of application. Extensive spread of the fluorochrome is evident from the mesophyll into the adjacent loading vein and lateral transfer via the cross vein. A discontinuous pattern of 5,6-CF loading is evident. **(D-E)** Shows the progression of 5,6-CF loading about 3-5 cm away from the point of application. Note that there is an extensive longitudinal transport into the longitudinal bundles. A discontinuous pattern of 5,6-CF loading is evident. In (E) there is not much fluorochrome evidence seen. MES, mesophyll; VB, vascular bundle; XV, cross vein. (A-E) Bars = 20 µm.

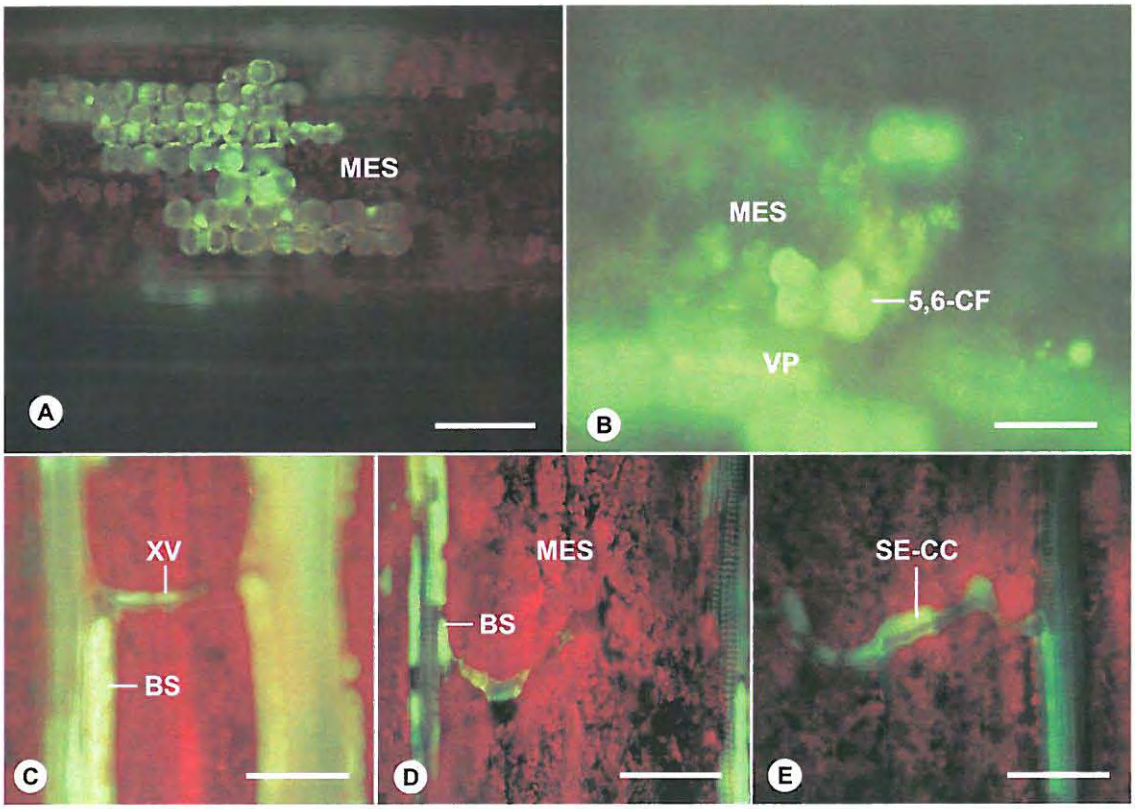

MES
A
MES
5,6-CF
VP
B
XV
BS
C
MES
BS
D
SE-CC
E

Fig. 7 is a higher magnification of the loading sequence. **(A)** Shows the location of fluorochrome 10 min after application, near the point of application of 5,6 CFDA. It demonstrates symplasmic uptake and transport within 10-15 min. Note that 5,6-CF is located in the cytoplasm of the mesophyll cells. **(B)** Shows symplasmic uptake, then loading at higher magnification. 5,6-CF is taken up into the symplasm of the mesophyll cells, within 20 min it was loaded in the vascular parenchyma. **(C)** Shows a small and an intermediate longitudinal vein, connected by a cross vein, containing only one sieve tube at center. Note 5,6-CF is present in the mesophyll and is concentrated in the bundle sheath. The fluorochrome appears to have transferred, laterally, via the cross vein. **(D)** Shows part of a small longitudinal vascular bundle with 5,6-CF located in vascular parenchyma and deeper-seated sieve tubes. Note the bundle sheath connection to the cross vein which contain brightly fluorescing 5,6-CF. **(E)** Shows detail of part of a cross vein, and connection to an intermediate longitudinal vein (right) showing 5,6-CF distribution. The fluorochrome appears to be associated with the vascular parenchyma and SE-CC complex in the cross vein. BS, bundle sheath; MES, mesophyll; SE-CC, sieve element-companion cell; VP, vascular parenchyma; XV, cross vein. (A-E) Bars = 10 µm.

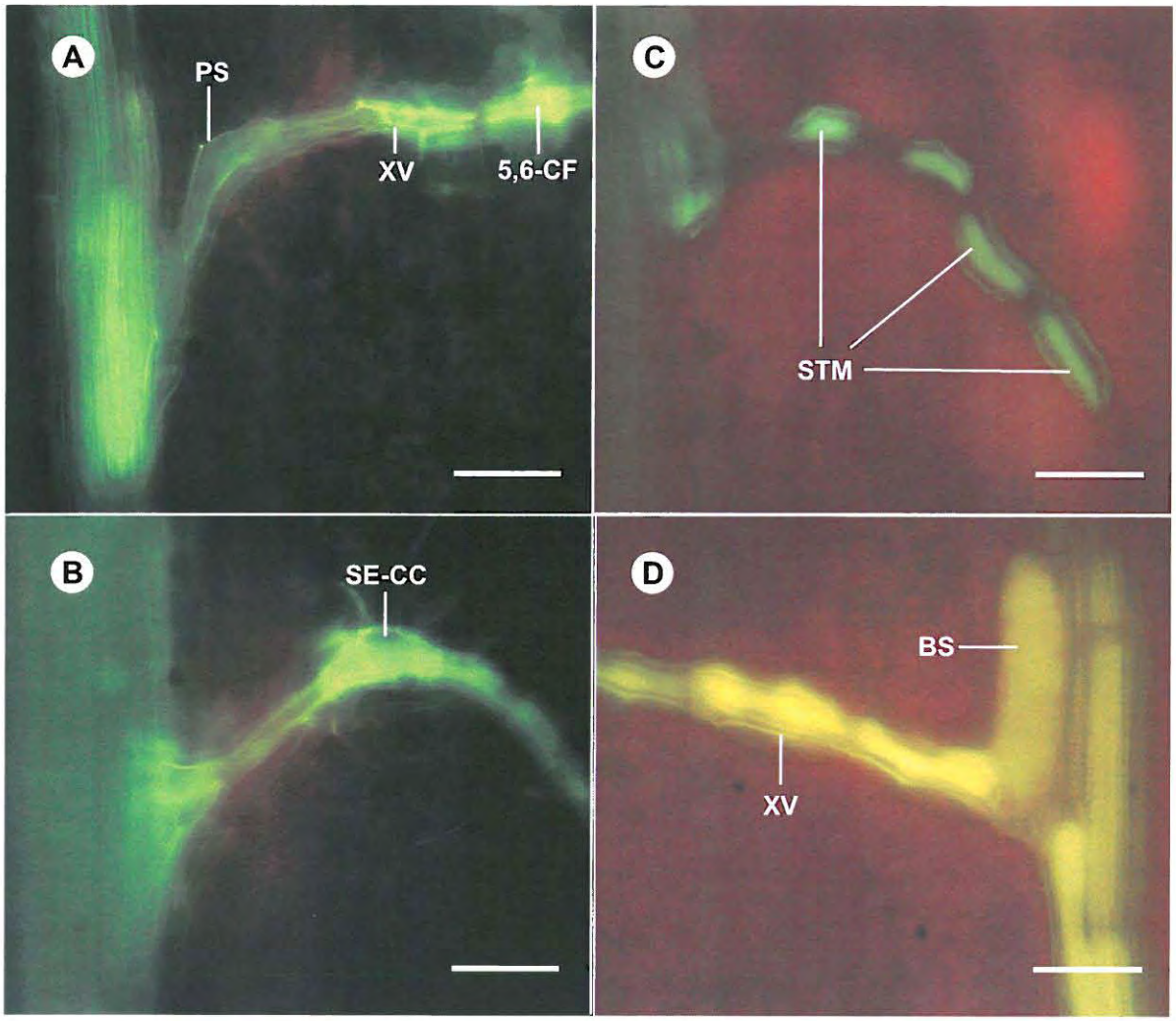

A
PS
XV
5,6-CF
C
STM
B
SE-CC
D
BS
XV

Fig. 8A Shows connection between the cross vein (upper right) and an intermediate vein (left). 5,6-CF appears to be associated with the sieve tube. **(B)** Shows bright fluorescence apparently associated with the sieve tube-companion cell complex as well as with the vascular parenchyma. **(C)** Shows 5,6-CF distribution in part of a small longitudinal bundle (left) and in a cross vein. In this micrograph the fluorochrome is contained within the sieve tube. **(D)** Shows connection between a cross vein and an intermediate vein. Note 5,6-CF is located in the bundle sheath and it appears to be associated with the vascular parenchyma and sieve tube-companion cell complex. BS, bundle sheath; SE-CC, sieve element-companion cell; STM, sieve tube member; PS, parenchymatous sheath; XV, cross vein. (A-D) Bars = 10 µm.

4.2.2 5,6-CF and aniline blue fluorescence

Based upon the results that have been presented here, it was not possible to identify the sieve tubes conclusively in 5,6-CF transport experiments. As a result, it was decided to examine the 5,6-CF co-localized with aniline blue staining, where the latter would offer positive identification of sieve tube members.

The principal results obtained in this study are illustrated in figures 8A-C. Fluorescence images showing 5,6-CF loading and transport in sieve tube companion cell complex in a cross vein of a barley source leaf. When 5,6 CFDA was flap fed into the mesophyll the fluorochrome was loaded to the bundle sheath and into the loading vein (Fig. 9A) and it was then laterally transferred via the cross vein (Figs. 9B and C) into an adjacent vein. Thus movement of the fluorochrome from the mesophyll to the underlying cells after flap feeding most probably occurred symplasmically, since the dyes do not pass the plasmalemma. The specimen was counterstained in 0.05% (w/v) aniline blue to show up the sieve plate pores. 5,6-CF is clearly associated with the sieve tubes. Pore-plasmodesmal units between the sieve tube member and the companion cell (Fig. 9D) are clearly visible.

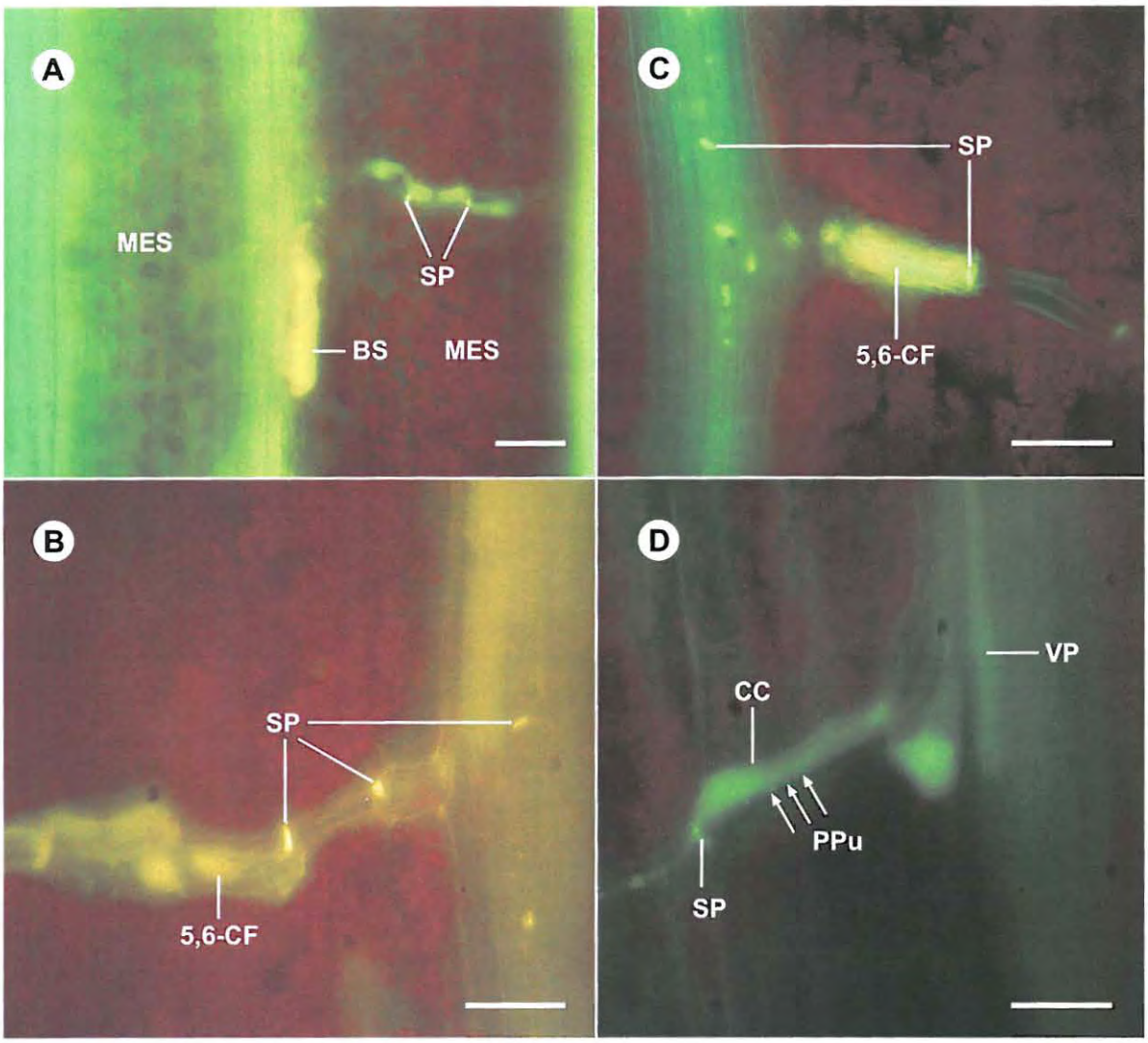

A
MES
SP
BS
MES
C
SP
5,6-CF
B
SP
5,6-CF
D
VP
CC
PPu
SP

Fig. 9 Shows co-localization of 5,6-CF with aniline blue. **(A)** Shows longitudinal transport of 5,6-CF in a small loading vein and lateral transfer via the cross vein. Note the brightly fluorescing sieve plates and also note that the fluorochrome is associated with companion cells. **(B)** Shows the connection between the cross vein and an intermediate vein. The fluorochrome is transported longitudinally and there is evidence of some lateral transfer via the cross vein. Note brightly fluorescing sieve plates, in a cross vein and in the intermediate vein, which are occluded by callose. **(C)** Shows the connection between an intermediate bundle and a cross vein. Evidence of lateral transfer can be seen via the cross vein. Note the distribution of callose associated with sieve plates in the longitudinal vein as well as in the cross vein. 5,6-CF is in the phloem. **(D)** Shows 5,6-CF distribution located in the longitudinal bundle and 5,6-CF appears to be associated with the vascular parenchyma as well as with the companion cell in a cross vein. Note the distribution of callose deposits associated with pore-plasmodesmal units as well as with the sieve plate. BS, bundle sheath; CC, companion cell; MES, mesophyll, PPu, pore-plasmodesmal units; SP, sieve plate; VP, vascular parenchyma. (A) Bar = 20 µm, (B-D) Bars = 10 µm.

4.2.3 Solute retrieval from the xylem

Previous experiments demonstrated that 5,6-CF can enter the symplasm from the damaged cells (where it was applied). Whilst the previous experiments showed conclusively that 5,6-CF was transported to the phloem and that a slow symplasmic phloem transport mechanism was implied by this, there were still a few questions unanswered with respect to the route(s) available for 5,6-CF transport and there still remained the issue about where 5,6 CFDA was dissociated. If all the 5,6 CFDA was dissociated prior to entry into the phloem, then the uptake pathway could logically be argued to be under symplasmic control. However, what was not clear at this stage, was the role of the xylem in 5,6-CF retrieval. Could 5,6 CFDA be taken up by the xylem and if so, where was it cleaved? Was this before or after entry into the phloem tissue? If before, this would add credence to the hypothesis that symplasmic dye movement was occurring. These experiments would also hopefully, indicate if the 5,6-CF was, in fact, taken up in the sieve tubes as well.

Previously, studies undertaken by Fritz et al., (1983) demonstrated that ^{14}C-sucrose was retrieved by xylem, and taken up from the apoplasm, first via thick-walled sieve tubes, and then via thin-walled sieve tubes in *Zea mays*. So, if sugars can be retrieved directly from the apoplasm, then what about 5,6 CFDA?

The results that follow clearly show that 5,6 CFDA was taken up in the transpiration stream. Figure 10A shows an example of the distribution of 5,6-CF after 25 min uptake

via cut leaf ends and translocation in a leaf material. Lateral movement of the fluorochrome between bundles is evident, suggesting that 5,6-CF was capable of movement in longitudinal and transverse veins. Based on a number of replicates, it would appear that 5,6 CFDA was taken up via the xylem, transported and that 5,6 CFDA crossed over to the xylem parenchyma via the pit membrane region, where it was cleaved (no fluorescence was seen in the xylem, only in living parenchyma cells). 5,6-CFDA was transferred to the xylem parenchyma (Fig. 10B) where it was cleaved. The dissociated 5,6-CF was then translocated via the xylem parenchyma. Only a few xylem parenchyma cells associated with the vessels were not labeled and few of them were lightly labeled. Fig. 10B shows that the unlabeled xylem parenchyma and Figs. 10C and 10D show lightly labeled xylem parenchyma. From the xylem parenchyma, the fluorochrome was then transferred to the vascular parenchyma cells bordering the vessels including those associated with the phloem, followed by thick-walled sieve tubes. Fig. 10E shows one of the few bundles from the 25 min feeding experiments in which the thick-walled sieve tubes and the sclerenchyma strands were labeled.

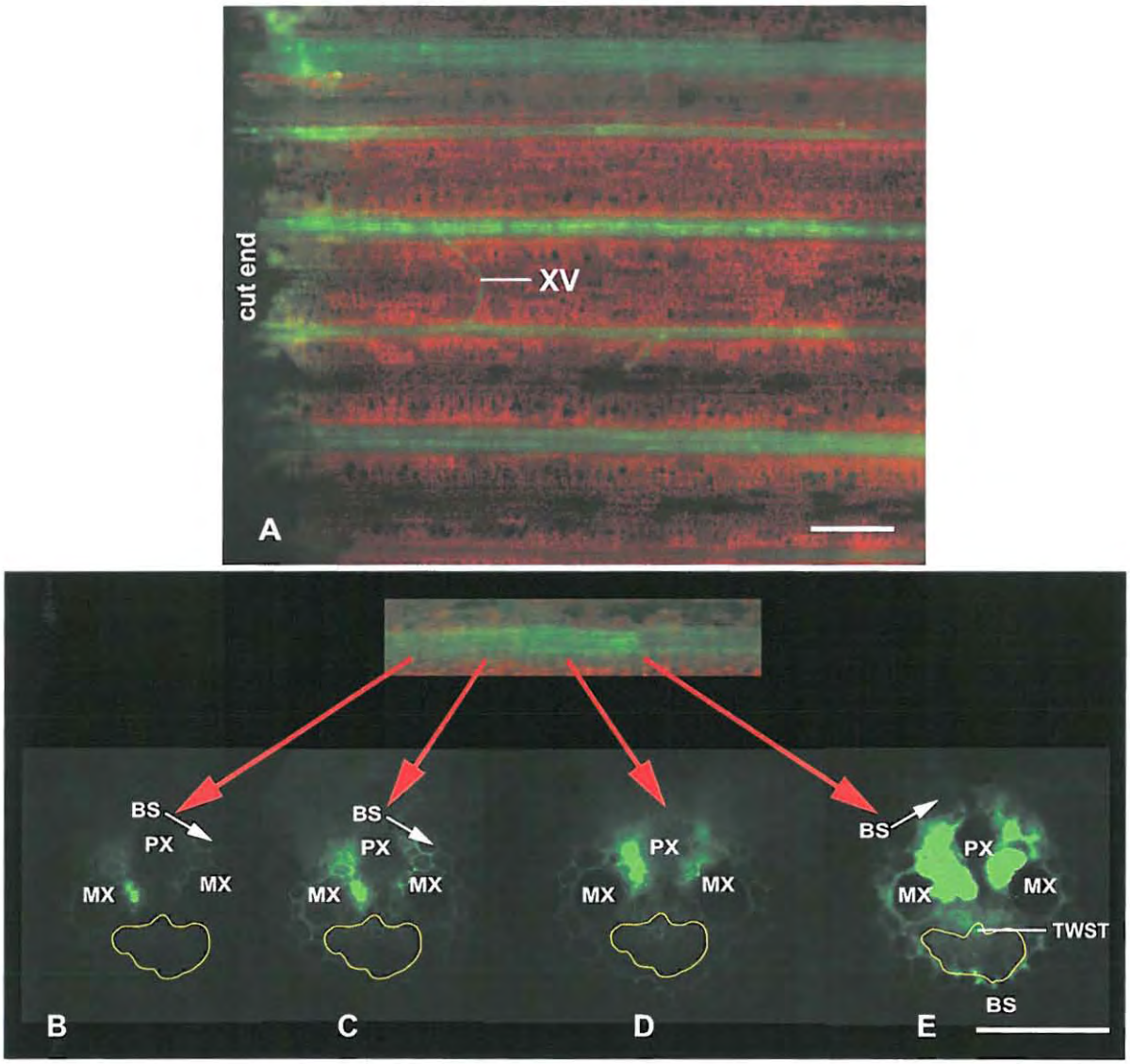


Fig. 10A Shows the cut end (left) of a leaf which had been immersed in a 5,6 CFDA solution. It shows the distribution of 5,6-CF after 25 min translocation. Lateral movement of the fluorochrome between the bundles is evident. **Fig. 10B-E** Series of sections "downstream" of 5,6-CF movement showing the distribution of fluorescence after 25 min uptake. All images from the same vein. Fig. 10B shows the vein with little evidence of fluorescence, with the exception of one xylem parenchyma cell. This suggests that 5,6 CFDA was cleaved in the xylem parenchyma cell. Figs. 10C-D show increased 5,6-CF fluorescence within the xylem parenchyma. Fig. 10E shows evidence of retrieval by phloem tissue. BS, bundle sheath; PX, protoxylem; MX, metaxylem; TWST, thick-walled sieve tubes.(A) Bar = 20 µm, (B-E) Bars = 10 µm.

CHAPTER 5: DISCUSSION

The data presented in the thesis clearly support the hypothesis that the thin-walled sieve tubes are primarily involved in loading and transport of assimilates.

5.1 The vein loading pathway

When freshly prepared barley leaf blade material treated with aniline blue was examined under blue light, callose was observed associated with sieve plate pores and with the plasmodesmal aggregates at the sieve tube-companion cell complexes, confirming that the symplasm is normally contiguous at this point. The presence of callose-occluded plasmodesmal fields at the sieve tube-companion cell complexes in Figs. 3C, D and E suggest that the plasmodesmata at this location are normally gated open. Studies by Hughes and Gunning (1980) and Lucas *et al.*, (1993) suggested that the closure of plasmodesma is due to the callose deposits which are the result of the activation of wounding response due to the loss of turgor pressure.

Studies undertaken previously by Evert *et al.* (1996) and Botha and Cross (2001) on plasmodesmal frequencies have shown that in barley leaf blade plasmodesma are most abundant at the mesophyll-bundle sheath, bundle sheath-vascular parenchyma interfaces, and between the companion cell-thin-walled sieve tube interface other than the interface between vascular parenchyma cells. The frequency data suggest that a symplasmic pathway is possible from mesophyll to vascular parenchyma, but that the low frequency at the vascular parenchyma-companion cell or vascular parenchyma -

thin-walled sieve tube or vascular parenchyma -thick-walled sieve tube interfaces precludes a major symplasmic pathway across the VP to phloem interfaces. The data presented in the thesis on the uptake of the fluorochrome is supportive of the presence of high numbers of plasmodesma between the mesophyll-bundle sheath-vascular parenchyma interfaces, but the fact that sieve tube-companion cell complexes and thick-walled sieve tubes are virtually symplasmically isolated from the rest of the leaf does not correspond with the data obtained. 5,6-CF uptake demonstrated that the fluorochrome was taken up into the symplasm of the mesophyll (see Fig. 7A) and it was loaded into the bundle sheath cells to the vascular parenchyma (see Fig. 7B) and into the sieve tubes. These results are consistent with the view that the assimilates undergo lateral transport from the mesophyll to the conducting bundle, and are then loaded into the phloem (Delrot 1989).

The finding of the fluorochrome within the vascular bundles after flap feeding into the mesophyll agrees with the dye transfer reported by van Kesteren *et al.*, (1988) from the mesophyll to the sieve tube in *Commelina benghalensis* and by Farrar *et al.*, (1992) in barley. A technical difference that may be of account is that Farrar *et al.*, (1992) found dye in sieve tubes after injection into the mestome sheath or parenchymatous bundle sheath, but not in the mesophyll whereas the data presented in this study shows that 5,6-CF was found in sieve tubes after application into the mesophyll. The flap fed 5,6-CFDA, in the mesophyll, is unable to pass the plasmalemma; hence, movement of the fluorochrome must occur in the symplasm via the plasmodesma. 5,6-CF is able to move freely between the mesophyll and

underlying cells, suggesting that these cells are linked together. Movement of photosynthetic metabolites between mesophyll cells occurs mainly in the symplasm (Giaquinta 1983). Mesophyll cells are reported to be well linked by plasmodesma (Gamalei and Pakhomova 1982) and in barley the plasmodesmata are functional as indicated by the fluorochrome movement between mesophyll cells. Schmitz *et al.* (1987) suggested that the presence of adequate numbers of plasmodesma between the sieve tube-companion cell complex and the mesophyll is a prerequisite for symplasmic transport. If the leaf apoplasm is subdivided by suberised walls, separating mesophyll and vascular bundles, water-soluble compounds can traverse this barrier along a symplasmic route only. This is apparently the case in wheat (Kuo *et al.*, 1974) and in maize (Evert *et al.*, 1977), where numerous plasmodesma perforate cell walls between mesophyll and bundle sheath cells. Plasmodesmal connections may however be rare or absent between sieve tube-companion cell complexes and adjacent cells (Evert *et al.*, 1978; Fisher and Evert, 1982), indicating loading from the apoplasm by a membrane-transport process.

The early review presented by Fondy and Geiger (1977) suggested that the mesophyll cells may act as a collecting area for photosynthates. The authors further suggested that the absolute sucrose uptake was 80% higher in mesophyll cells than in veins. Studies undertaken by Maynard and Lucas (1982a) also proposed that most of the sucrose uptake in *B. vulgaris* is performed by the mesophyll and that the sieve tube loading merely presents a general retrieval system.

5.2 The unloading pathway

Studies undertaken by Ding *et al.*, (1988) on tobacco suggested that photoassimilate was unloaded primarily from class III veins and that smaller veins, i.e. class IV and V, do not transport or unload photoassimilate in sink tissue because the sieve elements of these veins are immature until after the tissue stops importing. The authors further suggested that in class III veins, the sieve tube-companion cell complexes are surrounded by phloem parenchyma which abuts the bundle sheath, and the frequency of plasmodesma increases at the interfaces along the unloading route, from the SE-CC complex to phloem parenchyma to bundle sheath to mesophyll cells. As mentioned, the loading process can, based upon plasmodesmal frequency, be assumed to be symplasmic. Eschrich (1970) suggested that phloem unloading is the reverse process of phloem loading. It could therefore be argued that loading could mirror the unloading process in some grasses.

Based on their microscopic evidence Haupt *et al.* (2001) concluded that CF unloading only occurred from longitudinal major veins and that the transverse veins did not support CF unloading. This contrasts sharply with the data presented here which provided overwhelming support for 5,6-CF unloading which occurred from all classes of longitudinal veins. Furthermore, the data presented here show that transverse veins were involved in the unloading of the fluorochrome to the mesophyll. In fast-growing young leaves phloem unloading is rapid, as indicated by rapid 5,6-CF unloading from the veins to the mesophyll (Figs. 4 and 5). As the leaf matures, both structurally and physiologically, there is a decline in the rate of phloem unloading leading to the

relatively rapid movement of solutes and water from sieve elements. When mature, a photosynthesizing barley leaf is a source (Fromm and Eschrich 1989). Observations have shown that a symplasmic phloem unloading pathway operates in barley sink leaf.

5.3 Solute retrieval

A microautoradiographic study undertaken by Heyser *et al.* (1977) showed that, when applied to one end of a maize leaf strip, ^{14}C-labeled sucrose was taken up initially by the xylem vessels and then loaded into the phloem and transported to the opposite end which was immersed in diluted Hoagland's nutrient solution. Subsequent studies by Fritz *et al.* (1983) suggest that sucrose can be retrieved from the xylem and loaded into thick- and thin-walled sieve tubes. The authors further suggested that the vascular parenchyma cells are probably the first cells of the bundles to retrieve photosynthates from the xylem and transfer it to thick-walled sieve tubes or release it into the free space of the phloem for uptake by the thin-walled sieve tubes. Based on this study, the authors suggested that thick-walled sieve tubes are not involved in long-distance transport and that the thin-walled sieve tubes appeared to accumulate assimilates from the apoplasm.

Evert *et al.* (1978) presented data that suggests that photosynthates arriving at the vascular parenchyma cells from the bundle sheath cells or retrieved from the vessels must be unloaded into the free space before they can be loaded into the thin-walled sieve tubes. The subsequent data presented by Fritz *et al.* (1983) suggests that

perhaps the vascular parenchyma cells adjoining the vessels are able to both retrieve (load) sucrose from the xylem free space and to unload it into the free space of the phloem, or the sucrose may have moved directly from the vessels to the sieve tube-companion cell complexes in the free space. The data presented in this study showed that in the small and intermediate bundles, 5,6-CF was initially taken up by the xylem parenchyma and then transferred to the vascular parenchyma cells abutting the vessels. The first sieve tubes to show 5,6-CF labeling were thick-walled sieve tubes adjoining heavily-labeled vascular parenchyma cells. Our results show that after a short-term feeding period of about 25 min few thick-walled sieve tubes were labeled with 5,6-CF. These results are supportive of the findings by Fritz *et al.* (1983) that thick-walled sieve tubes were the first to exhibit ^{14}C-labeling. The authors further suggested that with increasing [^{14}C] sucrose-feeding periods, greater proportions of both thick- and thin-walled sieve tubes became labeled. Therefore, if we had increased feeding periods by 120-180 min greater proportions of both thin- and thick-walled sieve tubes would have been labeled, and these findings would have been supportive of those by Fritz *et al.* (1983). Importantly, Fritz *et al.* (1983) suggested that sucrose can be retrieved from the xylem and loaded into thick- and thin-walled sieve tubes.

5.4 aniline blue and 5,6-CF fluorescence

Treatment of barley leaf blade with aniline blue showed callose associated with sieve plates and pore-plasmodesmal fields at the sieve tube-companion cell complex. These results are consistent with those of van Bel and Kempers (1991) that the fluorescence

of plates might depend on the presence of callose. The large array of callose deposits at the CC-SE interface in Figs. 3D and E, and in Fig. 9D is overwhelming evidence for callose localization at or near plasmodesma. Figures 9A-D provide some evidence that symplasmic transport had taken place in the sieve tube-companion cell complex and these observations are consistent with the high plasmodesmal frequency at the CC-SE interface. The presence of abundant plasmodesmal fields demonstrates at least the potential for a symplasmic transport pathway in that interface. Flap feeding of 5,6 CFDA into the mesophyll demonstrated that cleaved 5,6-CF was symplasmically loaded from the flap fed mesophyll into the sieve tubes.

Counterstaining 5,6-CF with aniline blue confirmed that callose was associated with sieve plates and plasmodesmal fields. It is known that most, if not all, of the callose found in the pores of sieve elements is deposited there in response to injury during tissue preparation. Callose has been described by Currier (1957) as amorphous and colorless, insoluble in water, ethanol, solutions of alkali carbonates, and cuprammonia (Schweitze's reagent). Aniline blue in aqueous solution has long been known to stain callose a clear and glistening blue (Currier 1957). Movement of tracer dyes has been shown to be controlled by changes in cellular concentrations of ions such as Ca^{2+} and aromatic amino acids (Erwee and Goodwin 1983, 1984). Calcium, in the form of the Ca^{2+} cation, is involved in the regulation and synthesis of callose (Kauss 1987). Schulz (1999) suggested that if Ca^{2+} remains at a high level, callose synthesis becomes activated, and the result is permanent closure of plasmodesmal canals. It was noted before (van der Schoot and van Bel 1989, 1990) that plasmodesma often shut off in

response to excision of the tissue. The authors further suggested that plasmodesmal closure as a result of wounding may thus explain the poor mobility of dye between the phloem parenchyma cells which contrasts with the ready dye exchange between sieve tube and companion cell.

The data presented in the thesis indicate that there have to be fully functional symplasmic connections along the entire pathway from the mesophyll to the sieve tubes. What is intriguing is the fact that 5,6-CF appeared so slowly and moved so slowly from the point of application. Slow transport (uptake?) is indicative of few connections resulting, perhaps, in a low diffusion rate from the vascular parenchyma to the sieve tube, because of higher turgor in the sieve tubes. What was evident was a 5,6-CF diffusion pathway via the symplasm. Without doubt, application of 5,6-CFDA revealed that there is a slow symplasmic pathway which occurs via plasmodesma, in the vein loading and unloading processes.

5.5 Conclusion

In summary the following information was demonstrated in this thesis:

- 5,6-CF was taken up from the mesophyll in source and sink leaves.
- Phloem loading and unloading had a symplasmic component. Evert and Russin (1993) and Evert *et al.* (1996) suggested that phloem unloading in sink maize leaves could not be symplasmic. This statement was based on plasmodesmal frequency studies which showed that the ST-CC complex was virtually isolated

symplasmically from the rest of the leaf. This would indicate an apoplasmic loading step. Farrar *et al.* (1992) suggested that barley leaf (intermediate veins) loaded symplasmically. Subsequent studies undertaken by Botha and Cross (1997) based upon the electrophysiology as well as intracellular injection finding suggested that phloem loading in barley does not occur directly from the symplasm. In contrast, the present study shows clear evidence of symplasmic unloading of 5,6-CF in sink leaves. Unloading of the fluorochrome occurred from all classes of veins into the mesophyll and this is in direct contrast to the findings by Haupt *et al.* (2001) that unloading only occurred from large vein classes. It is also interesting to note that transverse veins were involved in the unloading of the fluorochrome.

- The presented data confirm transport of 5,6-CF in sieve tube members.
- The null hypothesis proposed to this study was proved. The data presented confirm symplasmic involvement, it suggests that symplasm is a potential (slow) transport pathway in barley source and sink leaves.
- Finally, the presence of a symplasmic loading pathway does, as suggested by Farrar *et al.* (1992), not suggest that the symplasmic pathway seen here occurs under normal (photosynthetically driven) field conditions. It simply shows that it is there.

REFERENCES

Altus DP, Canny MJ (1982) Loading of assimilates in wheat leaves. I. The specialization of vein types for separate activities. Aust J Plant Physiol **9**: 571- 581

Anderson LS, Dale JE (1983) The sucrose of carbon for developing leaves of barley. J Exp Bot **34**: 405-414

Biddulph O, Cory R (1965) Translocation of ^{14}C metabolites in the phloem of the bean plant. Plant Physiol **40**: 119-129

Blackman E (1971) The morphology and development of cross veins in the leaves of bread wheat (*Triticum aestivum* L.) Ann Bot **35**: 653-665

Botha CEJ, Evert RF, Walmsley RD (1975a) Studies on *Gomphocarpus physocarpus*: further evidence of preferential feeding by the aphid, *Aphis nerii* on the internal phloem. Protoplasma **84**: 345-356

Botha CEJ, Evert RF, Walmsley RD (1975b) Observations of the penetration of the phloem in leaves of *Nerium oleander* (Linn.) by stylets of the aphid, *Aphis nerii* (B. de F). Protoplasma **86**: 309-319

Botha CEJ, Evert RF (1978) Observations of preferential feeding by the aphid, *Rhopalosiphum maidis* on abaxial phloem of *Cucurbita maxima*. Protoplasma **96**: 75-80

Botha CEJ, Evert RF, Cross RHM, Marshall DM (1982b) Comparative anatomy of mature *Themeda triandra* Forsk. Leaf blades. A correlated light and microscopy study. S Afr J Bot **48**: 311-328

Botha CEJ, Evert RF (1986) Free space marker studies on *Saccharum officinarum* and *Bromus unioloides*. S Afr J Bot **52**: 335-342

Botha CEJ (1992) Plasmodesmatal distribution, structure and frequency in relation to assimilation in C_3 and C_4 grasses in southern Africa. Planta **187**: 348-358

Botha CEJ, van Bel AJE (1992) Quantification of symplasmic continuity as visualized by plasmodesmograms: diagnostic value for phloem loading pathways. Planta **187**: 359-366

Botha CEJ, Cross RHM (1997) Plasmodesmatal frequency in relation to short-distance transport and phloem loading in leaves of barley (*Hordeum vulgare*). Phloem is not loaded directly from the symplast. Physiol Plant **99**: 355-362

Botha CEJ, Cross RHM, van Bel AJE, Peter CI (2000) Phloem loading in the sucrose-export-defective (SXD-1) mutant maize is limited by callose deposition at plasmodesmata in bundle sheath-vascular parenchyma interface. Protoplasma **214**: 65-72

Botha CEJ, Cross RHM (2001) Regulation within the supracellular highway-plasmodesma are the key. S Afr J Bot **67**: 1-9

Canny MJ (1990) What becomes of the transpiration stream? Tansley Review No. 22. New Phytol **114**: 341-368

Cartwright SC, Lush WM, Canny MJ (1977) A comparison of translocation of labeled assimilate by normal and lignified sieve elements in wheat leaves. Planta **134**: 207-208

Colbert JT, Evert RF (1982) Leaf vasculature in sugarcane (*Saccharum officinarum* L). Planta **156**: 136-151

Currier HB (1957) Callose substance in plant cells. Am J Bot **44**: 478-488

Dannenhoffer JM, Ebert W, Evert RF (1990) Leaf vasculature in Barley, *Hordeum vulgare* (Poaceae). Am J Bot **77**: 636-652

Dannenhoffer JM, Evert RF (1994) Development of the vascular system in the leaf of barley (*Hordeum vulgare* L.). Int J Plant Sci **155(2)**: 143-157

Delrot S (1989) Loading of photoassimilates. In Baker DA, Milburn JA (eds) Transport of photoassimilates. John Wiley & Sons Inc., New York, pp 167-205

Dickson RE, Larson PR (1981) ^{14}C fixation, metabolic labeling patterns, and translocation profiles during leaf development in *Populus deltoides*. Planta **152**: 461-470

Dickson RE, Larson PR (1982) Translocation pathways in the petioles and stem between source and sink leaves of *Populus deltoides* Bartr. ex Marsh. Planta **156**: 345-358

Ding B, Parthasarathy MV, Niklas K, Turgeon R (1988) A morphometric analysis of the phloem-unloading pathway in developing tobacco leaves. Planta **176**: 307-318

Duckett CM, Oparka KJ, Prior DAM, Dolan L, Roberts K (1994) Dye-coupling in the root epidermis of *Arabidopsis* is progressively reduced during development. Development **120**: 3247-3255

Erwee MG, Goodwin PB (1983) Characterization of the *Egeria densa* Planch. leaf symplast: inhibition of the intercellular movement of fluorescent probes by group II ions. Planta **158**: 320-328

Erwee MG, Goodwin PB (1984) Characterization of the *Egeria densa* leaf symplast: response to plasmolysis and to aromatic amino acids. Protoplasma **122**: 162-158

Erwee MG, Goodwin PB (1985) Symplastic domains in extrastelar tissues of *Egeria densa* Planch. Planta **163**: 9-19

Eschrich W (1970) Biochemistry and fine structures of phloem in relation to transport. Annu Rev Plant Physiol **21**: 193-214

Evert RF, Eschrich W, Medler JT, Alfeiri FJ (1968) Observation of the penetration of linden branches by the stylets of the aphid *Longistigma caryae* (Ham). Am J Bot **55**: 860-874

Evert RF, Eschrich W, Eichhorn SE, Limbach ST (1973) Observations on penetration of barley leaves by the aphid *Rhopalosiphum maidis* (Fitch). Protoplasma **77**: 95-109

Evert RF, Eschrich W, Neuberger DS, Eichhorn SE (1977) Tubular extensions of the plasmalemma in leaf cells of *Zea mays* L. Planta **135**: 203-205

Evert RF, Eschrich W, Heyser W (1978) Leaf structure in relation to solute transport and phloem loading in *Zea mays* L. Planta **138**: 279-294

Evert RF, Mierzwa RJ (1989) The cell wall-plasmalemma interface in sieve tubes of barley. Planta **177**: 24-34

Evert RF, Russin WA (1993) Structurally, phloem unloading in the maize leaf cannot be symplastic. Am J Bot **80**: 1310-1317

Evert RF, Russin WA, Botha CEJ (1996) Distribution and frequency of plasmodesmata in relation to photoassimilate pathways and phloem loading in the barley leaf. Planta **198**: 572-579

Farrar SC, Farrar JF (1985) Carbon fluxes in leaf blades of barley. New Phytol **100**: 271-283

Farrar J, van der Schoot C, Drent P, van Bel A (1992) Symplastic transport of Lucifer yellow in mature blades of barley: potential mesophyll-to-sieve tube transfer. New Phytol **120**: 191-196

Fellows RJ, Geiger DR (1974) Structural and physiological changes in sugar beet leaves during sink to source conversion. Plant Physiol **54**: 877-885

Fisher DG, Evert FR (1982) Studies of the leaf of *Amaranthus retroflexus* (Amaranthaceae): ultrastructure, plasmodesmatal frequency, and solute concentration in relation to phloem loading. Planta **155**: 377-387

Fondy BR, Geiger DR (1977) Sugar selectivity and other characteristic of phloem loading in *Beta vulgaris* L. Plant Physiol **59**: 953-960

Fritz E, Evert RF, Heyser W (1983) Microautoradiographic studies of phloem loading and transport in the leaf of *Zea mays* L. Planta **159**: 193-206

Fritz E, Evert RF, Nasse H (1989) Loading and transport of assimilates in different maize leaf bundles. Digital images analysis of ^{14}C-microautographs. Planta **178**: 1-9

Fromm J, Eschrich W (1989) Correlation of ionic movements with phloem unloading and loading in barley leaves. Plant Physiol Biochem **27(4)**: 577-585

Gamalei YV, Pakhomova MV (1982) Distribution of plasmodesmata and parenchyma transport of assimilates in the leaves of several dicots. Sov Plant Physiol **29**: 649-661 (not seen)

Gamalei YV (1989) Structure and function of leaf minor veins in trees and herbs: a taxonomic review. Trees **3**: 96-110

Gamalei YV (1991) Phloem loading and its development related to plant evolution from trees to herbs. Trees **5**: 50-64

Geiger DR (1975) Phloem loading. In Zimmermann MH, Milburn JA (eds) Transport in plants I. Phloem transport, Encyclopedia of Plant Physiol pp. 395-431. Berlin, Heidelberg, New York, Springer-Verlag

Giaquinta RT (1983) Phloem loading of sucrose. Annu Rev Plant Physiol **34**: 347-387

Gifford RM, Evans LT (1981) Photosynthesis, carbon partitioning and yield. Annu Rev Plant Physiol **32**: 485-509

Goodwin PB (1983) Molecular size limits for movement in the symplast of the *Elodea* leaf. Planta **157**: 124-130

Goodwin PB, Erwee MG (1985) Transport across the tonoplast in relation to cell communication in plants. In: Beilby MJ, Walker NA, Smith JR (eds) Transport across membranes of plants and fungi, Sydney University Press, Sydney, pp. 303-306

Goodwin PB, Sheperd V, Erwee MG (1990) Compartmentation of fluorescent tracers injected into the epidermal cells of *Egeria densa* leaves. Planta **181**: 129-136

Gordon AJ, Ryle GHA, Mitchell DF, Powell CE (1982) The dynamics of carbon supply from leaves of barley plants grown in long and short days. J Exp Bot **33**: 241-250

Grignon N, Touraine B, Durand M (1989) 5(6) Carboxyfluorescein as a tracer of phloem sap translocation. Am J Bot **76(6)**: 871-877

Haupt S, Duncan GH, Holzberg S, Oparka KJ (2001) Evidence for symplastic phloem unloading in sink leaves of barley. Plant Physiol **125**: 209-218

Heldt HW, Lohaus G, Winter H (1992) Phloem transport of amino-acids and sucrose in relation to their cytosolic levels in barley leaves. Photosynth Res **34**: 241 (not seen)

Heyser W, Heyser R, Eschrich W, Fritz E (1977) The influence of externally supplied sucrose on phloem transport in the maize leaf strip. Planta **137**: 145-151

Hughes JE, Gunning BES (1980) Glutaraldehyde- induced deposition of callose. Can J Bot **58**: 250-258

Kauss H (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu Rev Plant Physiol **38**: 47-72

Knoblauch M, van Bel AJE (1998) Sieve tubes in action. Plant Cell **10**: 35-50

Komor E, Orlich G, Weig A, Köckenberger W (1996) Phloem loading- not metaphysical, only complex: towards a unified model of phloem loading. J Exp Bot **47**: 1155-1164

Kuo J, O'Brien TP (1974) Lignified sieve elements in the wheat leaf. Planta **117**: 349-353

Kuo J, O'Brien TP, Canny MJ (1974) Pit-field distribution, plasmodesmatal frequency, and assimilate flux in the mestome sheath cells of wheat leaves. Planta **121**: 97-118

Larson PR, Isebrands JG, Dickson RE (1980) Sink to source transition of *Populus* leaves. Ber Dtsch Bot Ges **93**: 79-90 (not seen)

Lucas WJ, Ding B, van der Schoot C (1993) Plasmodesma and the supracellular nature of plants. New Phytol **125**: 435-476

Lush WM (1976) Leaf structure and translocation of dry matter in a C_3 and a C_4 grass. Planta **130**: 235-244

Madore MA, Lucas WJ (1989) Loading of photoassimilates between leaf cells. In Baker DA, Milburn JA (eds) Transport of photoassimilates, John Wiley and Sons Inc., New York, pp 49-78

Madore MA, Oross JW, Lucas WJ (1986) Symplastic transport in *Ipomea tricolor* source leaves: demonstration of functional symplastic connections from mesophyll to minor veins by a novel dye-tracer method. Plant Physiol **82**: 432-442

Matsiliza B, Botha CEJ (2002) Aphid *Sitobion yakini* investigation suggests thin-walled sieve tubes in barley (*Hordeum vulgare*) to be more functional than thick-walled sieve tubes. Physiol Plant **115**: 137-143

Maynard JW, Lucas WJ (1982a) A reanalysis of the two-component phloem loading system in *Beta vulgaris*. Plant Physiol **69**: 734-739

Maynard JW, Lucas WJ (1982b) Sucrose and glucose uptake into *Beta vulgaris* leaf tissues. A case for general (apoplastic) retrieval systems. Plant Physiol **70**: 1436-1443

Oparka KJ (1990) What is phloem unloading? Plant Physiol **94**: 393-396

Oparka KJ (1991) Uptake and compartmentation of fluorescent probes by plant cells. J Exp Bot **42**: 565-579

Oparka KJ, Duckett CM, Prior DAM, Fisher DB (1994) Real time imaging of phloem unloading in the root tip of *Arabidopsis*. Plant J **6**: 759-766

Overall RT, Gunning BES (1982) Intercellular communication in Azolla roots. II. Electrical coupling. Protoplasma **111**: 151-160

Ralston E, Hjelmeland LM, Klausner RD, Weinstein JN, Blumenthal R (1981) Carboxyfluorescein as probe for liposome-cell interactions. Effect of impurities, and purification of the dye. Biochim Biophys Acta **649**: 133-137 (cited by Grignon et al.,)

Rhoades MM, Carvalho A (1944) The function and structure of the parenchyma sheath plastids of the maize leaf. Bull Torrey Bot Club **71**: 335-346 (cited by Botha et al., 2000)

Riens B, Lohaus G, Winter H, Heldt HW (1994) Production and diurnal utilization of assimilates in leaves of spinach (*Spinacia oleracea* L.) and barley (*Hordeum vulgare* L.). Planta **192**: 497-501

Robards AW, Lucas WJ (1990) Plasmodesmata. Annu Rev Plant Physiol Plant Mol Biol **41**: 369-419

Russin WA, Evert RF, Vanderveer PJ, Sharkey TD, Briggs SP (1996) Modification of a specific class of plasmodesmata and loss of sucrose export ability in the sucrose export defective1 maize mutant. Plant Cell **8**: 645-658

Sakri FAK, Shannon JC (1975) Movement of ^{14}C-labeled assimilates into kernels of wheat. Plant Physiology **55**: 881-889

Schmalstig JG, Geiger DR (1985) Phloem unloading in developing leaves of sugar beet I. Evidence for pathway through the symplast. Plant Physiol **79**: 237-241

Schmalstig JG, Geiger DR (1987) Phloem unloading in developing leaves of sugar beet. II. Termination of phloem unloading. Plant Physiol **83**: 49-52

Schmitz K, Cuypers B, Moll M (1987) Pathway and assimilate transfer between mesophyll cells and minor veins in leaves of *Cucumis melo* L. Planta **171**: 19-29

Schulz A (1999) Physiological control of plasmodesmal gating. In: van Bel AJE, van Kesteren WJP (eds) Plasmodesmata: structure, function, role in cell communication, Springer, Berlin Heidelberg, New York, Tokyo, pp 173-204

Socolar SJ, Loewenstein WR (1979) Cell-to-cell junctional transmission. In: Korn ED, ed. Methods in membrane biology 10. New York: Plenum Press, 129-179 (not seen)

Szoka FC, Jacobson K, Papahadjopoulos D (1979) The use of aqueous space markers to determine the mechanism of interaction between phospholipid vesicles and cells. Biochim Biophys Acta **551**: 295-303 (cited by Grignon N, Touraine B, Durand M (1989) 5(6) Carboxyfluorescein as a tracer of phloem sap translocation. Am J Bot 76(6): 871-877

Taylor DL, Wang YL (1980) Fluorescently labeled molecules as probes of the structure and function of living cells. Nature **284**: 405-410

Trivett CL, Evert RF (1998) Ontogeny of the vascular bundles and contiguous tissues in the barley leaf blade. Int J Plant Sci **159(5)**: 716-723

Tucker EB (1982) Translocation in the staminal hairs of *Setcreasea purpurea*. I. A study of cell ultrastructure and cell-to-cell passage of molecular probes. Protoplasma **113**: 193-201

Tucker JE, Mauzerall D, Tucker EB (1989) Symplastic transport of carboxyfluorescein in staminal hairs of *Setcreasea purpurea* is diffusive and includes loss to the vacuole. Plant Physiol **90**: 1143-1147

Turgeon R (1987) Phloem unloading in tobacco sink leaves: insensitivity to anoxia indicates a symplastic pathway. Planta **171**: 73-81

Turgeon R (1989) The sink-source transition in leaves. Annu Rev Plant Physiol Plant Mol Biol **40**: 119-138

Turgeon R, Beebe DU (1991) The evidence for symplasmic phloem loading. Plant Physiol **96**: 349-354

Tyree MT, Tammes PML (1975) Translocation of uranin in the symplasm of staminal hairs of *Tradescantia*. Can J Bot **53**: 2038-2046

van Bel AJE, Kempers R (1991) Symplastic isolation of the sieve element-companion cell complex in the phloem of *Ricinus communis* and *Salix alba* stems. Planta **183**: 69-76

van Bel AJE (1993) Strategies of phloem loading. Annu Rev Plant Physiol Plant Mol Biol **44**: 253-281

van Bel AJE (1996) Interaction between sieve element and companion cell and the consequences for photoassimilate distribution. Two structural hardware frames with associated physiological software packages in dicotyledons. J Exp Bot **47**: 1129-1140

van Bel AJE, van Kesteren JP (1999) Plasmodesmata: structure, function, and role in cell communication. Berlin, Heidelberg, New York, Springer-Verlag

van der Schoot C, van Bel AJE (1989) Glass microelectrode measurements of sieve tube membrane potentials in internodes and petioles of tomato (*Solanum lycopersicum*). Protoplasma **149**: 144-154

van der Schoot C, van Bel AJE (1990) Mapping membrane potentials and dye coupling in internodal tissues of tomato (*Solanum lycopersicum* L.). Planta **182**: 9-21

van Kesteren WJP, van der Schoot C, van Bel AJE (1988) Symplastic transfer of fluorescent dyes from mesophyll to sieve tube in stripped leaf tissue and partly isolated minor veins of *Commelina benghalensis*. Plant Physiol **88**: 667-670

Walsh MA (1974) Late formed metaphloem sieve elements in *Zea mays*. Planta **121**: 17-25

Wang HL, Offler CE, Patrick JW, Ugalde TD (1994) The cellular pathway of photosynthate transfer in the developing wheat grain. I. Delineation of a potential transfer pathway using fluorescent dyes. Plant Cell Environ **17**: 257-266

Wang N, Fisher DB (1994) The use of fluorescent tracers to characterize the post-phloem transport pathway in maternal tissues of developing wheat grains. Plant Physiol **104**: 17-27

Wright KM, Oparka KJ (1996)The fluorescent probe HPTS as a phloem-mobile, symplastic tracer: an evaluation using confocal laser scanning microscopy. J Exp Bot **47**: 439-445

APPENDIX A

The full Long-Ashton nutrient solution used in this study as taken from Hewitt (1996)

Salt	Wt used (g)	V. stock sol. (ml)	V. stock sol. Dil. In 25 L (ml)	Conc. In final V. of 25 L (ml)
Macronutrient				
KNO_3	101	500	25	2
K_2SO_4	43	500	25	1
$Ca(NO_3)_2$	164	500	25	4
$CaCl_2$	111	500	25	4
$MgSO_47H_2O$	92	500	25	1.5
$NaH_2PO_4.2H_2O$	104	500	25	4
Micronutrient				
$MnSO_4.4H_2O$	11.20	500	2.5	0.02
$CuSO_4.5H_2O$	1.25	500	2.5	0.002
$ZnSO_4.7H_2O$	1.45	500	2.5	0.002
H_3BO_3	15.50	500	2.5	0.05
$Na_2MO_4.2H_2O$	0.605	500	2.5	0.0005
NaCl	29.30	500	2.5	0.1
Fe-Citrate (3H2O)	29.30	500	2.5	0.6

APPENDIX B

Summary of the dehydration (a) and wax embedding (b) series and the staining procedure (c) used for tissue preparation for light microscopy (from Botha 1994).

(a) Dehydration series

Solution	Time, h
FAA	24
50% EtOH	12
70% EtOH	12
35% BuOH	12
55% BuOH	12
75% BuOH	12
100% BuOH	3X12

(b) Wax embedding series

100% n-Butanol (liquid paraffin)	3 X 12

(c) Staining series

Stage 1	
Solution	**Time**
Xylol 1	3 min
Xylol 2	3 min
Xylol 3	3 min
Xylene/Alcohol 50:50	3 min
Absolute alcohol	2 min
90% EtOH	2 min
70% EtOH	2 min
50% EtOH	2 min
Safranin	24 h
Stage 2	
70% Alcohol	2 min
90% Alcohol	2 min
90% Alcohol + Picric acid	Dip and drain
90% Alcohol + Ammonia	Dip and drain
Absolute alcohol I	2 min
Absolute alcohol II	2 min
Fast green	1 min
Clove oil	Dip and drain
Clearer	Dip and drain
Xylol 1	2 min
Xylol 2	2 min
Xylol 3	max. 5-6 min