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What Does the Movement of the Phloem-mobile Symplasmic Tracer, 5, 6-carboxyfluorescein in Shoots of *Pisum sativum* L. Indicate-the Existence of a Symplasmic Transport System? -A bid to Answer Some Puzzling Questions

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Introduction

Like other members of the Fabaceae, the minor veins of *Pisum* are categorized as a closed system termed type 2 minor vein configuration due to the presence of few or no plasmodesmal connections between the sieve element-transfer cell complex (SE-TCC) and the adjacent cells (Gamalei, 1989; van Bel and Gamalei, 1991). *Pisum* is classified further into the category of type 2 b minor vein configuration due to the presence of transfer cells with the characteristic wall ingrowths in the minor vein phloem (Gamalei, 1989). According to van Bel *et al*. (1992), there is a correlation between minor vein configuration and phloem loading. However, by reason of low plasmodesmatal frequency, the pathway of the flow of assimilates in plants with type 2 minor vein configuration is considered to be apoplasmic (Gamalei, 1989; van Bel and Gamalei, 1991).

Therefore, present reports on the movement of the phloem-mobile 5, 6-carboxyfluorescein, a known symplamically transported compound between pea leaflets raises some doubts on the accession that transport within the phloem in pea is strictly apoplasmic. In this study, we look at different points of arguments and tried to offer our explanation and conclusions on the transport pathways that are likely to exist in *Pisum*.

Is the Movement of 5, 6-CFDA Through the Xylem or the Phloem?

It is possible that some damage could have occurred to the xylem during the abrasion process, to expose underlying xylem to 5, 6-CFDA. If so, it is likely that some 5, 6-CFDA could have been taken up in the transpiration stream directly. If 5, 6-CFDA would be transported passively in the apoplast along the transpiration stream, it would arrive rapidly at the sink. The xylem in vascular bundles of the leaf is surrounded by living parenchyma cells, which are connected to the xylem through pits which are lined by the plasma membrane on the pore side of the pit. The pit membrane is fibrous and permeable on the xylem side and plasma membrane is selectively permeable on the parenchyma side. It has been shown that 5, 6-CFDA can off-load through the pore from the xylem into the parenchyma cells. Once the 5, 6-CFDA has been transported across the pit-plasma membrane interface, the acetate moieties are cleaved by acetyl CoA and 5, 6-CF is released. 5, 6-CF is not capable of crossing membranes and cannot move out from the parenchyma cells back into the transpiration stream. 5, 6-CF can only cross to adjacent living cells if plasmodesmata are present and could conceivably be taken up by the phloem tissue along a symplasmic route (Personal communication, CEJ Botha).

Offloading of 5, 6-CFDA from the xylem into the parenchyma cells could occur anywhere along the translocation stream and is therefore not necessarily confined to distant sink tissues, the transpiration stream is dependent on transpiration rate and is not source-sink dependent. If 5, 6-CFDA

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is cleaved in the xylem, in other words, 5, 6-CF not 5, 6-CFDA is transported in the transpiration stream, logically one will expect to find 5, 6-CF in all terminal veinlets within the leaflet, irrespective of them being source or sink. Botha *et al*. (2005) have shown that where 5, 6-CFDA is allowed to move into cut leaves, it is usually transported along the transpiration stream (xylem vessels) and off loaded via parenchyma cells in all regions of the lamina. Clearly, this is not what was observed in our experiments, as only some class III veins dependent on the sink/source status contain evidence of 5, 6-CF (Fig. II and Fig. II of Ade-Ademilua and Botha, 2006b). Results of the experiments showed that 5, 6-CF is restricted to (if allowed into) certain regions of the lamina when coming from a distant source (Fig. IIB-D of Ade-Ademilua and Botha, 2006a) and even more restricted to the higher order veins in transition and source leaflets, when coming from a local (opposite) source (Fig. IIE and F of Ade-Ademilua and Botha, 2006b). This would seem to rule out the idea that significant quantities of 5, 6-CFDA were transported through the xylem (which is present in all sink and source leaflets) at least in the leaflets presented in these reports. It is apparent that 5,6-CF is within vascular parenchyma cells and possibly sieve elements.

As regards movement of 5, 6-CF between paired leaflets, in order for 5, 6-CFDA to be transported via the xylem across to the opposite leaflets, it would have to move against the transpiration stream in transpiring attached leaflets. It is unlikely that this could occur in such a short period of time (10 min) as reported in this study. Furthermore, it should be noted that 5, 6-CF first appears in the leaflet opposite the fed leaflet, before any basipetal movement occurs.

Evidence for the Existence of a Symplasmic Pathway in P. sativum L.

Wimmers and Turgeon (1991) have shown that wall ingrowths in minor vein transfer cells of *P. sativum* L. facilitate uptake of photoassimilate by increasing plasmalemma surface area as proposed by Gunning *et al*. (1968) in their study of transfer cells of *P. arvense*. According to Gunning *et al*. (1968) two contrasting specializations that are likely to favour efficient absorption of materials into a cell are: (1) enhancement of symplasmic transfer through the development of abundant plasmodesmata and (2) the promotion of capacity uptake from the extracellular environment though increase in the cell's surface:volume ratio. The transfer cells of *P. sativum* are supposedly specialized in the latter direction like those of their counterpart in *P. arvense* (Gunning *et al*. 1968). However, Gunning *et al*. (1968) did not rule out the potential for symplasmic transfer in *P. arvense* via the branched plasmodesmata traversing the wall between transfer cells and their associated sieve elements and also those interconnecting the transfer cells; and since phloem loading is a family-specific multiprogrammed mechanism (van Bel and Gamalei, 1991), transport via symplasmic pathway cannot also be ruled out in *P. sativum*. This is justified even the more, with the results of the experiments reported in our previous papers (Ade-Ademilua and Botha, 20006a, b).

It has been pointed out that plasmodesmatal frequency alone does not make an absolute case for either symplasmic or apoplasmic transport; more importantly because the minimum frequency required for symplasmic movement is yet unknown (Fisher, 1986; Warmbrodt and van der Woude, 1990; Botha and van Bel, 1992). Botha and van Bel (1992) have argued that, plasmodesmata, whether presented as tables or plasmodesmograms, give an indication of the maximum potential pathway of symplasmic transport. They argued further that, frequencies merely represent the number of plasmodesmatal connections determined at a particular time, for a particular interface and at a particular stage of development of the cells making up the interface. According to the researchers, plasmodesmatal frequency per se does not take the transport capacity of the plasmodesmata into account.

The hypothesis has been that plants with the type 2b minor vein configuration translocate assimilates mainly through the apoplasmic pathway have been demonstrated in *P. sativum* L. (Turgeon and Wimmers, 1988; van Bel *et al*., 1992) and other apoplasmic species (van Bel *et al*., 1992),

through the use of the apoplasmic blocker p-chloromercuribenzenesulfonic acid (PCMBS) to block the loading of $^{14}CO_2$. The experiments gave credence to a correlation between minor vein configuration in terms of plasmodesmata frequency and phloem loading pathway. Wimmers and Turgeon (1991) showed that plasmodesmata frequency between the SE-TCC and surrounding cells is low in *P. sativum* and that this cast doubts on the efficacy of a symplasmic pathway in the plant. However, experiments have shown that in any species, the frequency of plasmodesmata is expected to decline from the mesophyll toward the minor-vein phloem to compensate for changes in the surface-area to volume ratio of various tissue types even if the pathway of solute diffusion is entirely symplasmic (Ding *et al*. 1988; Turgeon and Beebe, 1991), therefore Wimmers and Turgeon (1991) agreed that plasmodesmata frequency in the SE-TCC boundary is, in itself, not convincing evidence for an apoplasmic pathway.

Carboxyfluorescein was developed as an advanced form of fluorescein; because though the latter enters the symplast and is transported in the phloem (Wright and Oparka, 1996), it leaks through the plasma membrane at an appreciable rate due to its partial non-dissociation at physiological pHs (Wright and Oparka, 1996). With the presence of an additional carboxyl group on either the 5 or 6 positions on the carboxyfluorescein (CF) molecule, the entry and restriction of 5, 6-CF to the symplast and transportation within the phloem is ensured as it becomes less permeant to biological membranes than fluorescein (Wright and Oparka, 1996). 5, 6-CF has been shown to be transported in this same pattern as ^{14}C assimilates, thus making it an effective phloem-mobile tracer (Grignon *et al*., 1989). When a lamina is loaded with the non-fluorescent ester, 5, 6-CFDA, the compound, will passively cross the cell membranes in the electrically neutral or near-neutral form. Once inside the cells they are subject to cleavage by esterases to form the polar fluorescent compound, 5, 6-CF. 5, 6-CF is then transported within the symplasmic pathway of the phloem (Wright and Oparka, 1996).

According to Wimmers and Turgeon (1991), the relative paucity of plasmodesmata connecting the sieve element-transfer cell (SE-TC) complex to surrounding cells in a plant like P. sativum L., in comparison to species in which the companion cells are not specialized as transfer cells, indicates that the uptake of sucrose by the SE-TC complex is primarily, if not entirely, apoplasmic. However, the results of the experiments we reported earlier showed the transport of 5, 6-CF only within the major and class III veins of importing leaflets. Figure IIH of Ade-Ademilua and Botha (2006a) clearly shows that the unloading of the fluorochrome into the mesophyll was through the class III vein network. The transport and unloading of carboxyfluorescein by the class III vein network has also been demonstrated in Nicotiana benthamiana by Roberts *et al*. (1997). However, no surprise at such movement is expressed, since the plant has a symplasmic minor vein configuration. However, according to Oparka and Santa Cruz (2000), the postphloem symplast does not place major constraints on the exit of solutes or small proteins from the SE-CCC. Oparka and Santa Cruz (2000) pointed out that various experiments have shown that macromolecules like fluorescent solutes, radioactive solutes, GFP and systemic RNA signals all exit the phloem in the same pattern as plant synthesized proteins through the major veins, with the class III vein being the most implicated. Unloading from the major veins has been shown to become reduced and eventually ceases as leaves transit from sink to source (Roberts *et al*., 1997; Imlau *et al*., 1999; Oparka *et al*., 1999; Wright *et al*. 2003). Oparka *et al*. (1999) have demonstrated that the decrease in the transport and unloading of macromolecules via the symplast during sink-to-source transition is as a result of the decrease in plasmodesmal permeability due to the development of single form plasmodesmata into branched forms. Most of the photoassimilates imported by sink leaves is unloaded by moderately large (major) veins and the smallest (minor) veins are relatively or completely unimportant in this regard (Turgeon and Webb, 1976), however, the structure and topology of the major veins have been found to actually facilitate export, rather than redistribution of photosynthate produced by the precociously mature lamina tip during the sink-to-source transition (Larson *et al*., 1972).

The experiments reported earlier demonstrate conclusively that the loading and resultant export of the fluorescent probe through the phloem of major veins in source pea leaflets and the consequent import and unloading through similar major veins of sink and transition leaflets, through the symplasmic pathway despite the so called low frequency of the plasmodesmata within the phloem of *P. sativum* plants. Interestingly, Grignon *et al*. (1992) have demonstrated the movement of 6(5)carboxyfluorescein (6CF) from source to sink regions (leaves and roots) of Glycine max, by showing the transport of the fluorochrome through the phloem of the pulvinus at the base of the petiole. Although Grignon *et al*. (1992) did not show the distribution of the fluorescent probe in the leaflets of *G. max*, the results reported in Ade-Ademilua and Botha (2006a, b) still find support in their work; since *Pisum* is in the same family as Glycine and as stated earlier phloem loading is a family-specific multiprogrammed mechanism (van Bel and Gamalei, 1991) and more so, the fluorescent image of the petiole of leaves used in this experiment showed similar distribution of 5, 6-CF in the phloem.

It is imperative to conclude that though export through the minor veins of *Pisum* may be via the apoplasmic pathway, the evidence presented in these reports point to the existence of a symplasmic pathway in *P. sativum* L. and invariably the functioning of the plasmodesmata that were observed in all cell-cell interface from the mesophyll cells to the SE-TC complex (preliminary studies).

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