Real-time imaging of phloem unloading in the root tip of Arabidopsis

K.J. Oparka1,*, C.M. Duckett2, D.A.M. Prior1 and D.B. Fisher3

1Department of Cellular and Environmental Physiology, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK,
2Department of Cell Biology, John Innes Institute, Colney Lane, Norwich NR4 7UH, UK, and
3Department of Botany, Washington State University, Pullman, Washington, USA

Summary

Confocal laser scanning microscopy (CLSM) has been used to image phloem transport and unloading in the root tip of Arabidopsis. The fluorescent probe 5(6) carboxyfluorescein (CF) was ester loaded into a single cotyledon and the entire seedling placed within an observation chamber under the microscope. Translocation of CF to the root tip was rapid, followed by unloading into discrete concentric files of cells. The position of the prominent unloading ‘zone’ corresponded precisely with that of the two protophloem files of sieve elements, demonstrating a functional role of these cells in symplastic sieve-element unloading. Symplastic transport following unloading was confined to the elongating zone of the root with little basipetal transport to more mature cells. Following photobleaching of the unloading zone, phloem transport was restored immediately into the protophloem sieve elements, followed rapidly by lateral, symplastic sieve-element unloading. The results demonstrate that phloem transport processes can now be imaged in real time, and non-invasively, within an intact plant system.

Introduction

The imaging of phloem transport in vivo remains an extremely difficult subject for plant physiologists to study. This is due mainly to the necessity of using intact (or nearly intact) plants for such work, and to the fragility of the functional transport cells, the sieve elements, the pores of which are acutely sensitive to damage or manipulation (Lucas et al., 1993, and references therein). In addition, the small size of the sieve element–companion cell complexes and their general inaccessibility in plant tissues has hindered investigations of phloem transport, and the related phenomena of phloem loading and unloading, for several years (Oparka, 1990). Studies utilizing 14C- or 3H-labelled isotopes have been used extensively over the last 50 years and have contributed enormously to our understanding of the regulation of phloem transport processes. However, their use for imaging the precise location of transport events requires killing the tissue prior to preparation for autoradiography. This precludes real-time analysis and introduces ambiguity into the sequence, and precise location, of events involved. Strongly emitting isotopes such as 32P (Fisher, 1990), or especially 11C (Minchin and Thorpe, 1992), allow excellent temporal resolution of in vivo transport but lack the spatial resolution for determining the cellular pathway(s) involved in phloem transport phenomena.

The increasing use of fluorescence techniques over the last 10 years has seen the introduction of a range of fluorescent probes, the physicochemical properties of which appear to be ideally suited to movement within the phloem. The use of the relatively permeant dye, fluorescein, as a phloem-transport tracer (Oparka, 1991 and references therein) has become largely superseded by the use of the probe 5(6) carboxyfluorescein, which possesses an optimal combination of dissociation constant ($pK_a$) and oil/water partition coefficient (log $K_{ow}$) to allow ‘trapping’ within the sieve element (Grignon et al., 1989). In this respect, the probe behaves as an ideal phloem-mobile xenobiotic. The studies of Grignon and colleagues (Grignon et al., 1989, 1992) have demonstrated elegantly how this probe remains confined to the phloem and follows a pattern of movement very similar to that of 14C assimilates. Subsequent studies, utilizing the ester form of the probe (CF diacetate) have effectively monitored the postphloem unloading pathways in sink tissues (Wang and Fisher, 1994; Wang et al., 1994). Unfortunately, just as in micro-autoradiography, all of these studies have necessitated cutting the tissue prior to microscopy, and so are subject to the same reservations.

The optical sectioning capacity offered by confocal laser scanning microscopy (CLSM) theoretically makes possible the direct observation of fluorescent probes within the phloem in real time, provided that the conducting cells are sufficiently close to the tissue exterior to allow...
satisfactory imaging. The root of the Arabidopsis seedling, at about only 100 μm in diameter, possesses a remarkably simple anatomy and therefore lends itself to the imaging of phloem transport. There are two phloem poles and these are separated from the epidermis by only three cell layers; the pericycle, the endodermis and a single-celled cortex (Dolan et al., 1993). The whole seedling is extremely small and can be held easily under the microscope as it translocates, allowing the real-time imaging of phloem transport. As pointed out by Schulz (1994), seedlings make particularly simple systems for study since only two major sinks are present, the shoot apex and the root apex (see also Kallarackal et al., 1989). In this paper, we provide the first direct, non-invasive observations of phloem transport and unloading in an intact plant system and demonstrate the major role played by the protophloem in the symplastic unloading of assimilates in the root.

Results

When a single cotyledon of the Arabidopsis seedling was labelled with CF the dye appeared rapidly in the veins of the cotyledon and subsequently as a fine strand running down the hypocotyl (Figure 1). Occasionally, the phloem did not load with CF and the dye moved slowly from cell-to-cell along the outer epidermis only. In such instances the dye was never able to pass across the base of the hypocotyl, encountering a strong symplastic barrier at the junction between the hypocotyl and root (for further details of the nature of these symplast ‘domains’ see Duckett et al. (1994). From a phloem-transport point of view such material was discarded. When the phloem was successfully loaded with dye, CF did not appear in the hypocotyl epidermis. In order to test the potential for xylem movement out of the cotyledon, attempts were made to introduce the xylem-mobile fluorescent probes Lucifer Yellow CH (Oparka and Read, 1994) and sulphorhodamine G (Canny, 1990) into the xylem. Such probes labelled the xylem vessels of the cotyledon but showed a distinct lack of basipetal movement, seldom entering the hypocotyl (data not shown).

Once in the phloem, CF moved rapidly (within 3–5 min) through the hypocotyl into the main root (Figure 1). Two discrete phloem files were usually visible in the main root (Figures 1 and 2), although spiral twisting of the root often revealed only a single phloem pole within a focal plane. Under the CLSM, the two fluorescently labelled phloem files were seen clearly to flank a centrally located xylem pole (Figure 2). In other regions of the root the two xylem poles were clearly visible, and in this case only a single fluorescent phloem trace was apparent (data not shown). These observations confirm that the CF had moved exclusively in the root phloem.

CF moved rapidly along the root and was unloaded

Figure 1. Photomontage of four confocal sections of an Arabidopsis seedling following labelling of a single cotyledon with CF (*). The dye became loaded into the phloem and moved to the opposite cotyledon as well as to the hypocotyl and root. Within the root, the two phloem files are clearly visible (arrow) and undergo considerable twisting. Near the root apex the dye is unloaded into a discrete zone surrounding the protophloem (dart). Scale = 200 μm.
Figure 2. Confocal section of a translocating root.
The fluorescence of CF within the two phloem files is seen superimposed on the bright-field image of the same root. The xylem pole is seen clearly between the phloem files. X, xylem. Magnification × 600.

Figure 3. Time series showing phloem transport into the root tip.
In the uppermost image the two phloem files are seen as the dye enters the root tip. In subsequent images, the dye is seen to spread symplastically into the region of elongation giving the unloading zone a characteristic ‘tear-drop’ appearance. The images were taken at 6 min intervals. Magnification × 50.

Figure 4. The terminus of the two protophloem files is seen within an intact, growing root.
The two files were located at different depths within the root and are shown as two superimposed (red and green false-coloured) confocal ‘sections’ of the root. Note the strong gradient in CF fluorescence between the mature and differentiating sieve elements (darts). Lateral symplastic sieve-element unloading has commenced and the dye has encountered a strong resistance at the endodermis/cortex interface (arrows; see also Figure 7). The thin white lines denote the edge of the root. Magnification × 600.

Figure 5. Arrival and unloading of CF from the protophloem follows a distinctive lateral spread.
Note the lack of basipetal movement following unloading. The images were taken at 6 min intervals. The root tip had extended by 60 μm during the observation period. Magnification × 100.

Figure 6. Fluorescence recovery after photobleaching.
Following arrival of CF and its unloading from the phloem (a), the unloading zone was photobleached for 10 sec with light from a 100 mW Argon laser. Immediately following photobleaching there was a rapid resumption of dye transport along the protophloem (b), followed by a slower lateral spread of dye into adjacent files of cells (c). Magnification × 270.
Phloem unloading

The pattern of phloem unloading was consistent for most of the roots examined; a rapid arrival in the phloem files followed by a slower, lateral spread into adjacent files of cells just distal to the zone of root-hair production (Figures 1, 3, 4 and 5). The location of the unloading zone of the root corresponded closely with that of the protophloem, which in Arabidopsis is the only fully differentiated vascular element found in this rapidly elongating zone of root (Dolan et al., 1993). Metaphloem sieve elements and protoxylem elements differentiate at approximately 1000 μm from the central cells of the root apex, and fully mature xylem elements appear close to the region of root-hair formation (Dolan et al., 1993). Occasionally, files of cells could be seen clearly in contact with the protophloem elements (Figure 4) and appeared to correspond to adjoining pericycle and endodermal cells (the former are closely associated with the differentiating protophloem elements; Dolan et al. (1993)). Indeed, in some species, pericycle cells abutting the protophloem sieve elements may contain P-protein, suggesting a developmental relationship of these cells with the protophloem (Esau and Gill, 1970, and references therein). Occasionally, a strong gradient of fluorescence could be seen at the terminus of each of the protophloem files, and there was a marked decrease in fluorescence at each successive sieve plate. This pattern of decreasing fluorescence probably reflected the movement of CF from mature sieve elements into younger, less differentiated cells where sieve-plate formation was incomplete. In Figure 4 the termini of the two translocating protophloem files were successfully imaged within an intact, growing root. The protophloem files were located at different depths within the root and are shown here as two superimposed confocal sections (false coloured red and green). Note in this figure, also, that dye symplastically unloaded from the protophloem encounters a marked resistance in lateral transport at the endodermis/cortex interface (see also Figure 7). The application of CF to the cotyledon, and its unloading from the phloem in the root tip, had no discernible effects on root-tip growth which continued over the course of the experiments (see also Duckett et al., 1994). For example, in Figure 5 the root tip extended by 60 μm during the course of observation under the CLSM. In some seedlings, in which root-tip growth was slow or arrested prior to dye loading, translocation to the root tip was slowed markedly. However, many seedlings, left for up to 24 h following labelling, showed extensive root growth and continued to unload from the phloem (see also Duckett et al., 1994).

Fluorescence recovery after photobleaching

After the CF had begun to unload from the protophloem files, the unloading zone was photobleached by a short exposure to high laser-light intensity (Figure 6a). Immediately following photobleaching, there was an immediate recovery of fluorescence into the protophloem files (Figure 6b), followed once more by a slower resumption of lateral unloading from the sieve elements into adjacent files of cells (Figure 6c). Thus, longitudinal transport into the unloading zone was considerably faster (as would be expected from phloem transport) than lateral cell-to-cell transport via plasmodesmata. Occasionally, concentric files of cells flanking the protophloem sieve elements were seen to 'fill up' in sequence as the CF re-entered the protophloem after photobleaching. Prolonged exposures to high laser intensities, or repeated photobleachings, were found to be damaging to the cells in the unloading zone and under these circumstances rapid fluorescence recovery did not occur.

Symplastic transport following unloading

Phloem unloading in the root tip followed a remarkably consistent pattern for nearly all of the roots examined. After arrival in the protophloem, CF was unloaded into adjacent files of pericycle and endodermal cells rapidly (Figures 5 and 7). From here the dye slowly entered the differentiating cortex. Once in the cortex dye moved rapidly and longitudinally towards the root apex, often accumulating in cells of the quiescent centre (Figure 7). However, dye movement was relatively restricted between cortex and epidermis. With time, however, all cells in the elongating zone of the root became labelled with CF. Significantly, dye did not move basipetally along files of cortical or epidermal cells, even after several hours of phloem unloading, and the unloading zone retained a characteristic 'tear drop' profile when viewed at low magnifications, i.e. the pattern shown in Figures 3 and 5 was retained as the root elongated. These observations indicate either a marked polarity in dye transport following sieve-element unloading and/or a restriction in basipetal symplastic transport. Thus, the root hairs, and other cells in the zone of cell maturation received no CF from the protophloem due to the basipetal symplastic barrier described above. In addition, no symplastic lateral unloading was observed from the metaphloem. How such observations relate to plasmodesmatal densities and the regulation of intercellular transport in roots remains to be demonstrated.
Discussion

The above observations demonstrate that it is possible, in real time, to observe both phloem transport and phloem unloading in an intact plant system. Provided the phloem is relatively close to the plant surface it can be imaged successfully at the tissue level using CLSM. Living sieve cells of conifer phloem were previously imaged using CLSM by Schultz (1992) who used excised pieces of conifer bark stained with the fluorescent probe DiOC6. However, he did not demonstrate functional phloem transport through the same cells. Micro-injection of membrane-impermeant probes into sieve elements from excised plant tissues has also been used extensively to examine symplastic communication between sieve elements and companion cells (van Bel and Kempers, 1991; van Bel and van Rijen, 1994; van der Schoot and van Bel, 1990). However, the necessity to cut the tissue prior to micro-
injection clearly negates its use for monitoring normal phloem transport.

Our initial observations of phloem transport \textit{in vivo}, open up a number of possibilities for examining, non-destructively, the relative abilities of different sinks to compete for assimilates. The effects of lateral root formation on the distribution pattern of assimilates could be determined, as could the effects of different localized treatments around the root on the pattern of phloem unloading (see e.g. Farrar \textit{et al.}, 1994; Schulz, 1994; Williams \textit{et al.}, 1991). To the extent that fluorescence intensity can be used to assay CF, it should also be possible to quantify its movement. These areas are the subject of further investigation using this system. The ability to label the phloem with a fluorescent probe, which is subsequently unloaded within a discrete region of the root, also offers an experimental tool with which to study intercellular signalling within the root, and can be used as a means to determine how symplast domains are established in the root tip during development (Duckett \textit{et al.}, 1994).

There have been several studies on the unloading of assimilates in roots and these appear to have given rise to a general consensus that the phloem unloading pathway is symplastic (Dick and Ap Rees, 1975; Farrar, 1985, 1992; Geiger and Fondy, 1980 and references therein; Giaquinta \textit{et al.}, 1983; Warmbrodt, 1985). The present study has highlighted an important role for the protophloem in the symplastic unloading of assimilates (for details of the development of this tissue see Esau and Gill, 1973; Melaragno and Walsh, 1976). However, care must be taken when comparing the unloading behaviour of a fluorescent probe with that of sucrose. Although CF is clearly not unloaded into regions of the root where mature metaphloem sieve elements are present, this does not preclude the possibility that sucrose might be unloaded acroplastically via a carrier-mediated mechanism from these cells, a possibility which would not be detected in the present study. Despite these reservations, our observations suggest that symplastic phloem unloading might be confined to only a relatively small proportion of the root. Lateral movement out of the 'transport' metaphloem (see van Bel, 1993) located further back in the root was not observed, an observation consistent with the view that these sieve element–companion cell complexes might, to a large extent, be symplastically isolated. Interestingly, no movement of CF occurred from the unloading zone towards the mature root hairs, suggesting that these cells do not have symplastic access to currently unloaded assimilates (see also Duckett \textit{et al.}, 1994). The cause of the restriction of basipetal symplastic transport clearly deserves further study.

The concept that the protophloem might play a major role in the unloading of assimilates into the root was also suggested recently by Schulz (1994). He labelled the cotyledons of pea seedlings with $^{14}C$ sucrose and found that the most intense unloading of $^{14}C$ occurred into an elongating zone located very close to the root apex. Comparative sections, taken to reveal the anatomy of the root tip, revealed that this zone contained only mature protophloem sieve elements. Thus, although roots are often referred to as 'symplastic unloading sinks', there may clearly be important subzonation within the root, and different sieve elements might well be performing different functions within a given organ. Thus, neither physiological studies of the whole root, nor the use of generalized 'plasmodesmograms' depicting putative phloem unloading pathways (Oparka and van Bel, 1992) may give a true representation of events within localised areas. Real-time imaging of phloem transport events may help resolve these issues further.

\section*{Experimental procedures}

\subsection*{Plant material}

Seeds of \textit{Arabidopsis thaliana} (ecotype 'Columbia') were surface sterilized in 5% sodium hypochlorite and rinsed in water. The seeds were germinated on plates containing Murashige and Skoog (MS) salt mixture, 3% sucrose (pH 5.8) in a thin layer of 1% agarose. After chilling for 24 h at 4°C the plates were incubated in a near-vertical position under continuous light. For the experiments reported here, the seedlings were used at 4 days when a single primary embryonic root was present.

\subsection*{Observation chambers}

Prior to dye loading, individual seedlings (and the underlying agarose) were cut out from the petri dishes and transferred to small (32 mm diameter) plastic petri dishes (Sterilin) which served as observation chambers. Moist filter paper was placed around the seedling to maintain a high humidity within the chamber (Figure 8).

\subsection*{Dye loading}

5(6) Carboxyfluorescein (CF) was ester loaded (see Oparka and Read, 1994) into the phloem by application to the cotyledon of 5(6) carboxyfluorescein diacetate (Molecular Probes, cat. no. C195; stock solution 6 mg ml$^{-1}$ in acetone). CF diacetate was applied to a single cotyledon at a concentration of 60 $\mu$g ml$^{-1}$ in distilled water, as shown in Figure 8. Attempts to introduce the probe through the cuticle were unsuccessful. Gentle abrasion of the cotyledon surface resulted in some phloem 'trapping' of the impermeant, fluorescent form of the dye, although the greatest degree of success was obtained by cutting the tip of the cotyledon away with a sharp scalpel, followed by continuous application of CF diacetate to the cut edge through a microcapillary (Figure 8). Following the successful loading of impermeant CF into the phloem, the lid of the chamber was replaced and the seedling left undisturbed to translocate CF under a light intensity of 370 $\mu$mol m$^{-2}$ sec$^{-1}$. 

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Figure 8: Schematic representation of the unloading zone.}
\end{figure}
Microscopy

During translocation of CF (optimum excitation 490 nm, emission 515 nm), the root was monitored at intervals through the lid of the observation chamber using long working distance objectives (x4, x10, x20; Nikon UK). To minimize water loss from the root, the lid was not removed during microscopy. A Bio-Rad MRC 1000 confocal laser scanning microscope (CLSM) was used to image CF transport along the growing root. Observation periods were kept brief to minimize damage to the root. On some occasions, following import of CF into the root tip, the chamber lid was removed briefly and the root viewed under a coverslip while bathed in MS medium. A 25 mW Krypton/Argon laser (detuned to 15 mW) was used to produce blue excitation at 488 nm (emission filter 560 nm). No significant autofluorescence was detected from the growing roots at these filter combinations. During observation, individual images of the root, with their times of acquisition, were stored on an optical disc. In some instances the root was imaged using a combination of emission light and transmitted light in order to subtract the fluorescence image from the transmission image. This produced a negative image of the fluorescent signal overlayed over a ‘bright field’ image, allowing root anatomy to be visualized whilst maintaining the location of the fluorescent signal.

Fluorescence recovery after photobleaching (FRAP)

On a number of occasions the root tip, following the arrival of CF in the phloem, was exposed to light at 488 nm generated from a 100 mW argon laser for brief periods of time. The laser was concentrated, using a zoom control on the microscope, over clearly defined regions of the root tip. This produced photobleaching of most of the dye in the exposed region of root over a period of 10–20 sec. The laser intensity was subsequently reduced to low, monitoring levels sufficient to image the recovery of fluorescence into the bleached region.

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References


Figure 8. Experimental set-up for labelling a single cotyledon of an Arabidopsis seedling with CF. The seedling is retained within a humid observation chamber and the growing root tip imaged using long-working distance objectives. Inset: details of the labelling procedure. a, nutrient agar block; c, cotyledon; m, microcapillary containing CF diacetate; b, 'blu-tak' support for microcapillary; f, moist filter paper.


