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Induction of Phloem Unloading in *Arabidopsis thaliana* Roots by the Parasitic Nematode *Heterodera schachtii*

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Phloem unloading of both the fluorescent probe carboxyfluorescein (CF) and ^{14}C-labeled solutes was induced in *Arabidopsis thaliana* L. roots by the parasitic nematode *Heterodera schachtii* Schmidt. Confocal laser scanning microscopy demonstrated that anomalous unloading of CF from the sieve element companion cell complexes occurred specifically into the syncytium, the nematode-induced feeding structure located within the stele of the root. From this syncytial complex of modified root cells, both fluorescent and radioactive labels were withdrawn by feeding nematodes. Movement of CF was unidirectional from the phloem to the syncytium. A range of low-molecular-weight fluorescent probes (including CF) microinjected into the syncytium stayed in this structure, demonstrating that it is symplastically isolated from the surrounding root tissue. The mechanism of unloading in this host-pathogen relationship therefore appears to be apoplastic. Our results provide unequivocal evidence that sedentary cyst-forming nematodes have direct access to phloem-derived solutes.

Recently, it has been shown that *Arabidopsis thaliana* can act as a suitable host for the study of plant-pathogen interactions (Koch and Slusarenko, 1990; Simpson and Johnson, 1990; Whalen et al., 1991). In addition, Sijmons et al. (1991) have demonstrated that *A. thaliana* can be used as a model host for plant-nematode interactions. These authors devised monoxenic conditions that were suitable for studying the infection and development of several economically important cyst-forming and root-knot nematodes. Furthermore, they showed that the in vitro responses induced in *A. thaliana* were identical to those found in several crop species under field conditions. The small size of the *A. thaliana* root (approximately 100 μm in diameter), together with its simple anatomy (Dolan et al., 1993) and translucency, makes it an ideal choice for imaging the complex relationships that arise during invasion of the root by parasitic nematodes. This has facilitated the advancement of video microscopy of the infection process (Wyss and Grundler, 1992; Wyss et al., 1992).

The feeding structures induced by cyst nematodes have been the subject of numerous anatomical investigations (reviewed by Endo, 1986), but the functional transfer processes that occur between host and pathogen are still a matter of speculation (Sijmons et al., 1994). The cyst nematode *Heterodera schachtii* belongs to a group of nematodes that induce syncytia, special feeding structures within the vascular cylinder of host roots (see Fig. 1A). The infective juveniles select a procambial or cambial cell as an initial syncytial cell. From this cell a syncytium develops by integration of neighboring cells. During this process profound anatomical changes occur. The developing nematode is entirely dependent on the expanding syncytium and withdraws nutrients from it through a feeding tube, a tubular structure attached to its stylet orifice that protrudes deeply into the syncytial cytoplasm (Wyss, 1992). Recently, Böckenhoff and Grundler (1994) injected fluorescent probes of differing molecular weights directly into syncytia induced by *H. schachtii* on *A. thaliana* roots. They showed that fluorescent dextrans of 3, 10, and 20 kD (but not 40 and 70 kD) were ingested freely, and calculated a maximum functional Stoke's radius of about 3.2 to 4.0 nm for molecules to be taken up through the feeding tube. It is interesting that, when injected into the syncytium, low-molecular-weight probes such as LYCH were not detected in root tissue adjacent to the syncytium or in the nearby root vascular elements.

The origin of the nutrients within the syncytium is not clear, although several studies have suggested that the syncytium may act as a xylem-related transfer cell because of the elaborate wall ingrowths at its interface with xylem vessels (Jones, 1981). Bird and Loveys (1975) and McClure (1977) have shown the accumulation of radioactive assimilates in feeding cells and associated root-knot nematodes. The relationship between the phloem and the feeding cells of sedentary nematodes, however, remains unclear. In this respect, the only previous study of phloem transport in nematode-infected roots was by Dorhout et al. (1993), who used CF as a phloem tracer and found that the dye entered the giant cells induced by the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood. They concluded that there must be a symplastic pathway between host cells and giant cells, although the cellular pathway was not studied in detail.

The thin *A. thaliana* root, in addition to its potential for studying host-pathogen interactions, also facilitates the

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Abbreviations: CF, 5(6)-carboxyfluorescein; CFDA, 5(6)-carboxyfluorescein diacetate; CLSM, confocal laser scanning microscopy; LYCH, Lucifer Yellow CH; SE-CC complex, sieve element companion cell complex; SR, sulphorhodamin.

real-time imaging of phloem transport processes using CLSM (Oparka et al., 1994). This noninvasive technique has now been used to image phloem unloading of CF in the root tip (Oparka et al., 1994), the formation of symplast domains in the root epidermis (Duckett et al., 1994), and the establishment of intercellular communication between primary roots and developing lateral primordia (Oparka et al., 1995). Such an approach clearly also has potential for imaging transfer processes during host-pathogen interactions.

Here we use both fluorescence imaging and ^{14}C labeling to study the transfer of solutes between the root phloem of *A. thaliana* and the parasitic nematode *H. schachtii*. The results demonstrate clearly that parasitic nematodes can act as a major sink for phloem-derived solutes. We show that the feeding nematode induces the simultaneous unloading of both CF and ^{14}C from the phloem and that both fluorescent and radioactive labels are subsequently transferred from the syncytium into the nematode's digestive system. Furthermore, we demonstrate that the syncytium is symplastically isolated from surrounding host cells and that unidirectional dye transport occurs only between the phloem and syncytium. Finally, we discuss the potential mechanism(s) by which solutes may be transferred from host to pathogen.

MATERIALS AND METHODS

Infected Plant Material

Seeds of *Arabidopsis thaliana* L. were grown in vitro under conditions supporting root growth and nematode development as described by Sijmons et al. (1991). Special adaptations were employed to optimize growth conditions for microinjection and in vivo observations (Böckenhoff and Grundler, 1994). Approximately 14 d after germination, the seedlings had developed an elaborate root system. Sterile, infective juveniles of the nematode *Heterodera schachtii* Schmidt, harvested from monoxenic stock cultures, were transferred in batches of 400 per plant. Both male and female nematodes were examined. The juveniles readily left the inoculation suspension, entered the agar growth medium, and subsequently penetrated the roots for induction of syncytial feeding sites.

Microinjection

Microinjections into nematode feeding sites were performed as described by Böckenhoff and Grundler (1994) using a modified pressure probe (Oparka and Prior, 1992) for introducing fluorescent probes. The fluorescent probes SR (Aldrich), LYCH (Molecular Probes), and CFDA (Molecular Probes) were injected into syncytia at concentrations of 10 mM in distilled water without additional purification.

Phloem Transport of CF and [^{14}C]Suc

The phloem was loaded with CF by applying 1- to 2-μL droplets of CFDA to gently abraded leaf surfaces (Oparka et al., 1995). The concentration of CFDA was 300 $\mu g\ mL^{-1}$ in distilled water.

Radioactive [^{14}C]Suc (ICN) was freeze-dried and redissolved in 20 μL of CFDA solution to a final concentration of 4 MBq. The solution was applied as above in droplets of 10 μL. This high concentration was necessary to ensure subsequent detection of ^{14}C within individual feeding nematodes.

^{14}C activity in the root systems was determined 18 h after labeling the leaves. Individual roots were scored under a binocular microscope for the presence of feeding nematodes, and the positions of fluorescent and nonfluorescent nematodes were recorded. Under a binocular microscope, root segments were subdivided into equal lengths of (a) the infected root with syncytium, (b) the uninfected region above (closer to leaf) the syncytium, (c) the uninfected region below (closer to root) the syncytium, and (d) individual nematodes. All samples were placed into 250 μL of 80% (v/v) ethanol at 70°C and extracted for 30 min. Four milliliters of scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, GA) was then added and radioactivity was determined on a scintillation counter (model 2000 CA TRICARB, Packard Instruments, Downers Grove, IL).

CLSM

To image CF translocation and microinjection of fluorescent probes, the *A. thaliana* roots were viewed while still in their Petri dishes using a confocal laser scanning microscope (MRC 1000, Bio-Rad). Conditions for imaging CF were identical to those reported previously (Oparka et al., 1994, 1995). For LYCH, an argon laser was employed, which produced blue excitation at 488 nm.

RESULTS

Phloem Unloading of CF in Differentiated Roots Is Restricted to the Syncytium

When the leaves of *A. thaliana* seedlings were labeled with CF, the probe appeared rapidly in the two phloem traces of the root (see also Oparka et al., 1994, 1995). When dye was unloaded into the syncytia at the feeding sites of nematodes, the area became highly fluorescent irrespective of feeding activity. In Figure 1B, three labeled syncytia are apparent, each with attached, feeding nematodes. Consistently, the unloading of CF occurred in mature regions of the root, i.e. within already differentiated tissue in which phloem unloading is not normally observed (Oparka et al., 1994). With continued feeding, the nematodes also became highly fluorescent. Examples of feeding nematodes are shown in Figures 1C and 2, C and D. Note that the dye becomes sequestered from the nematode intestine within a vesicular system.

The unloading of CF in mature root tissue was highly specific and occurred into syncytia only; CF transfer into unmodified, differentiated plant cells was never observed (Fig. 1D). The dye subsequently spread throughout the syncytium, but remained tightly restricted within it. Under the CLSM, the dense cytoplasm of the syncytium became

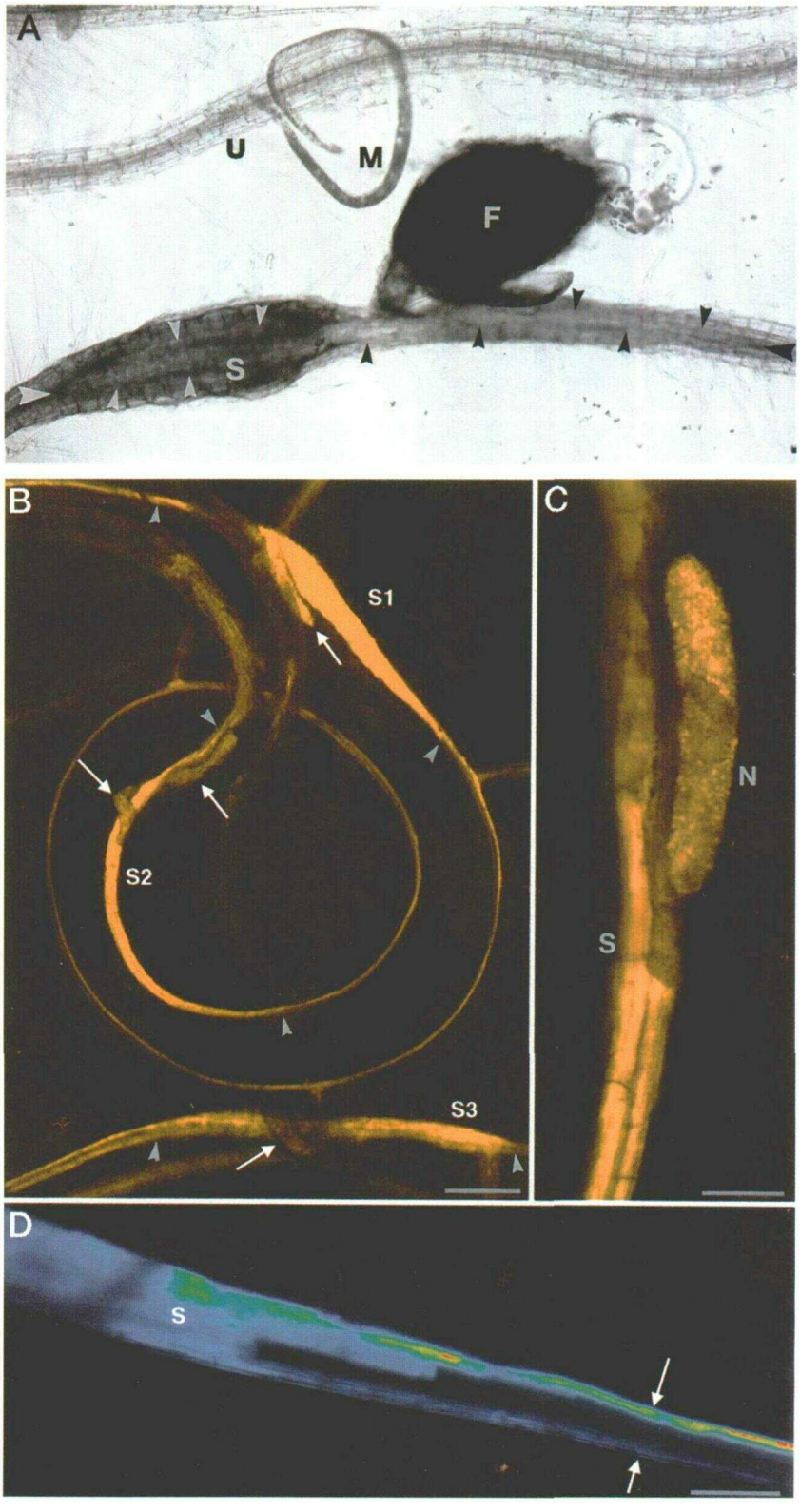

Figure 1. A, Arabidopsis roots uninfected (U, top) and infected (bottom) by the cyst nematode *H. schachtii*. The feeding structure, a syncytium (S), was induced by the associated adult female (F). The boundaries of the syncytium are marked with arrowheads. A mobile male nematode (M) is approaching for mating. Bar = 250 μm. B, Part of an Arabidopsis root system growing in an agar-filled Petri dish. The phloem elements of the roots contain the fluorescent dye, CF, which is unloaded into syncytia (S1, S2, and S3). The dye has accumulated in the syncytia that are strongly hypertrophied and fill almost the whole central cylinder. The boundaries of the syncytia are marked by arrowheads. Nematodes associated with the syncytia are marked with arrows. Note that two nematodes are associated with S2. Unloading into root tips is not shown. Bar = 300 μm. C, Feeding juvenile nematode (N) after uptake of CF from the syncytium (S). The dye is sequestered into small vesicles within the body cavity. Bar = 100 μm. D, A false-color image of a CF-loaded root. The two phloem traces (arrows) conduct CF to the syncytium (S), where it is subsequently unloaded. The red area represents the site of highest fluorescence. Following the light spectrum, the lowest concentration is indicated by blue. Bar = 100 μm.

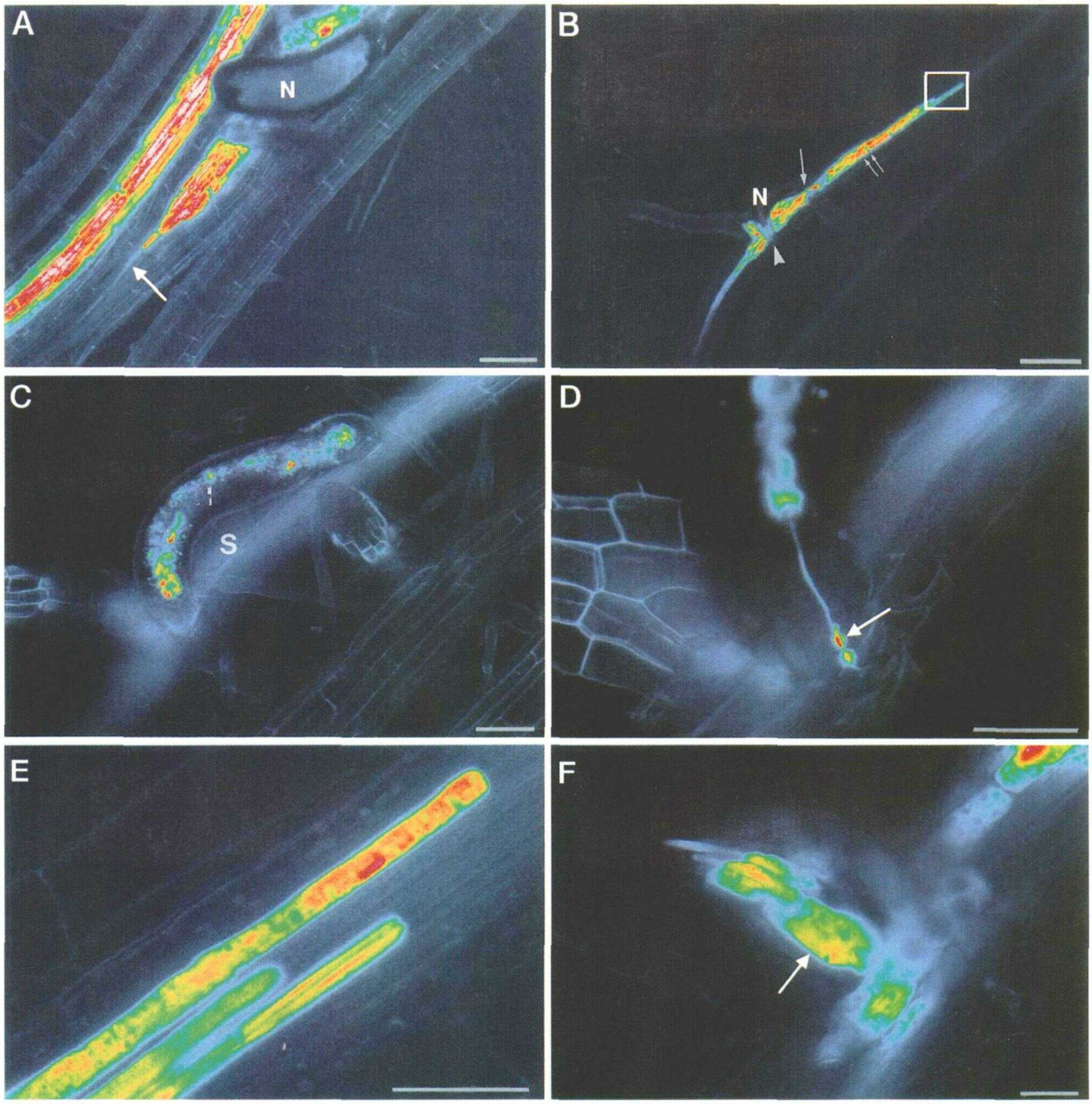

Figure 2. A, Detail of four roots of Arabidopsis arranged in parallel. The two outer roots are not labeled with CF. The phloem of the inner left root translocates CF. The central cylinder of the inner right root contains a fluorescent syncytium (S) with an associated nematode (N). Whereas in the inner left root the phloem transport is continuous, in the infected root the phloem transport is almost completely restricted to the area of unloading into the syncytium. The arrow indicates the distal end of the syncytium. Bar = 100 μm. B, A syncytium in an Arabidopsis root microinjected with LYCH. The dye does not spread to neighboring cells but is taken up actively by the nematode (N). Arrowhead, Nematode anterior end; arrow, nematode posterior end; double arrow, site of injection; box, see E. Bar = 300 μm. C, Juvenile nematode (N) shown in B after uptake of LYCH from the microinjected syncytium (S). The root with the syncytium is out of focus, so the site of injection is not visible in this view. Bar = 100 μm. D, Higher magnification of C; LYCH in the esophageal duct and median bulb (arrow) of the nematode during ingestion. Bar = 20 μm. E, Higher magnification of boxed region in B; the distal end of the syncytium showing fluorescence of microinjected LYCH. The fluorochrome clearly remains confined to the syncytium. Bar = 50 μm. F, A nematode-induced syncytium (arrow) expanding into a lateral root. Bar = 50 μm.

heavily stained (Fig. 1D). The rapid movement of dye throughout the syncytium was probably facilitated by the partially dissolved end walls of the interconnecting cells (see also Böckenhoff and Grundler, 1994). Phloem transport often continued past the syncytium toward the root apex (data not shown), but on a number of occasions it was clearly restricted to the region of the syncytium (e.g. Fig. 2A), indicating either a massive unloading into the syncytium or a blockage of normal phloem transport out of the infected region of the root.

Both CF and ^{14}C Are Unloaded into Syncytia

Following the simultaneous application of [^{14}C]Suc and CF, the root system was allowed to translocate both labels for several hours. Individual roots were then monitored for the appearance of fluorescence, and all nematodes were scored for the presence or absence of CF. Confirmation that only feeding nematodes had accumulated CF was obtained by observing the nematodes at higher magnification under the microscope. Only fluorescent nematodes showed rapid oscillations of the median bulb, an indication of solute withdrawal (Wyss, 1992). After 18 h, the roots containing the feeding and nonfeeding nematodes were dissected under a binocular microscope into three regions: the syncytium (including the surrounding root tissue) and the two adjacent (uninfected) regions of root above and below the feeding site. Finally, individual nematodes were removed and assayed. Determination of ^{14}C within individual syncytia revealed that they consistently contained high levels of ^{14}C (Table I). With one exception, these levels were clearly higher than in the sampled neighboring root areas, indicating an accumulation of ^{14}C exceeding the background activity of the phloem in the infected root area.

CF was not distributed evenly in the root system. Indeed, some roots did not transport CF at all. Although these roots were not fluorescent, they did contain ^{14}C. However, whenever the phloem transported both CF and ^{14}C, both substances were found to be translocated into the syncytia (Table I), suggesting a strong correlation between unloading of CF and ^{14}C. In addition to phloem unloading, the data provide information about translocation processes between syncytia and associated nematodes. For nutrient uptake, nematodes have to feed actively from their syncytia. Considering the volume of nematodes and syncytia, the nematodes contained very high levels of ^{14}C, indicating a progressive accumulation of ^{14}C within the nematode. Nematodes feed in repeating cycles, including a phase without nutrient uptake. Nematodes also did not take up nutrients during molting and, accordingly, those that were not feeding at the time of examination contained only low doses of ^{14}C. The form of this carbon was not determined. Repeatedly, the levels of ^{14}C detected in the region of root below the syncytium (closer to the root apex) were lower than those detected above the syncytium (Table I). This may have been due to a partial blockage of phloem transport out of the syncytium, a feature observed occasionally with CF transport (cf. Fig. 2A).

Relevant statistics for the data shown in Table I cannot be provided because the major parameters, the volumes of the syncytia, the volumes of the associated nematodes, and the amount of nutrients withdrawn by the feeding juveniles, are extremely variable and cannot be determined exactly.

Table I. *^{14}C activity of syncytia (syn), uninfected root segments above and below (closer to the root apex) the syncytium, and individual nematode (nem)*

Activity was determined 18 h after labeling the leaves with CF and [^{14}C]Suc. During this interval both the fluorochrome and ^{14}C were transported via the phloem into the root system. After scoring the individual roots under a binocular microscope for the presence of fluorescent and nonfluorescent feeding nematodes, sample fractions were taken and assayed for ^{14}C.

Fluorescence of syn (CF)	Developmental Stage of nem	^{14}C Activity of the Sample Fractions			
		nem	syn	Above	Below
		% of total cpm			
+	J_3[a]	26.8	32.7	40.2	0.3
+	J_3	30.6	56.6	12.0	0.8
+	J_3	25.1	51.0	16.6	7.3
+	J_3	31.6	44.0	11.2	13.2
+	J_3	17.3	53.8	17.2	11.7
+	J_4[b]	17.9	37.7	33.4	11.0
+	J_3, J_3	35.6	35.1	18.8	10.5
+	J_4, J_3	28.5	47.5	19.3	4.7
+	J_4, J_3, J_3	17.6	36.6	31.8	14.0
+	J_3[c]	2.5	38.4	24.2	34.9
+	J_3[d]	–[e]	47.2	42.7	0.9
+	J_3[d]	–	71.0	24.8	4.2
–	J_3[c]	0.8	42.2	32.9	24.1
–	J_4	38.7	48.5	9.1	3.7
–	J_3, J_3[c]	0.1	62.3	18.7	18.9

[a] J_3, Juvenile of third developmental stage. [b] J_4, Juvenile of fourth developmental stage. [c] No food withdrawal from the syncytium at time of examination. [d] Nematodes destroyed during dissection from root tissue. [e] –, No data.

The Syncytium Is Symplastically Isolated

In a previous report, Böckenhoff and Grundler (1994) showed that LYCH (M_r 457) injected into the syncytium remained confined to this structure for periods of up to 3 weeks without any trace of movement into surrounding plant cells. Nematodes feeding on these syncytia ingested the dye. These results were confirmed in the present study using CLSM to monitor the boundary of the syncytium. In Figure 2B, the syncytium had been injected with LYCH. Note that a feeding nematode is present on the infection site. Within seconds, the dye had spread throughout the syncytium and had begun to accumulate within the feeding nematode. Figure 2C shows a confocal section through the feeding nematode and the accumulation of LYCH inside a vesicular system within the nematode gut. Figure 2D shows the head region of the same nematode; LYCH is seen clearly within the median bulb, the esophageal duct, and the intestine. The end of the syncytium (boxed region in Fig. 2B) is shown in Figure 2E. Note here that the dye is confined exclusively to the cells of the syncytium. Other probes, such as CF (M_r 376) and SR (M_r 552), when injected into the syncytium, behaved identically to LYCH (data not shown). As revealed by injection with LYCH, in several

instances the syncytium of the main root partially entered lateral roots (Fig. 2F).

Movement of CF into the Syncytium Is Unidirectional and Unaffected by Impalement

In one set of experiments syncytia, each with attached feeding nematodes, were microinjected with SR. As found previously, the dye entered the nematodes but failed to leave the syncytium. The same plants were then labeled with CF, which was subsequently translocated via phloem to the roots. CF freely entered syncytia that had previously been microinjected with SR, and both the fluorescent probes accumulated within the feeding nematodes (data not shown). These results confirmed that (a) impalement was not the cause of the inability of microinjected probes to leave the syncytium, and (b) impalement of the syncytium did not affect the ability of the root to unload CF from the phloem. These experiments further highlighted the unidirectional nature of dye transport.

DISCUSSION

The results of the present study show clearly that cyst nematodes can act as major sinks for phloem-derived solutes, with the syncytium acting as an important reservoir for carbon-based compounds prior to their uptake by the nematode. Previous studies of plant-nematode interactions have focused largely on anatomical alterations induced by nematodes (Jones, 1981; Endo, 1986), and have tended to emphasize a role for xylem transport in the delivery of nutrients to the nematode feeding structures (Jones and Northcote, 1972; Sijmons et al., 1994). Bird and Loveys (1975) and McClure (1977) treated host plants of root-knot nematodes with $^{14}CO_2$ and were able to show that assimilates accumulated in feeding sites as well as in feeding nematodes. Recently, a more direct proof of the role of the phloem in the transport of solutes to nematode-induced feeding structures was published for root-knot nematodes (Dorhout et al., 1993).

What Is the Mechanism of Cyst Nematode-Induced Phloem Unloading?

Under normal circumstances, the root phloem of healthy *A. thaliana* seedlings appears to function as an isolated conducting domain, translocating solutes to the root apex, where they are unloaded symplastically into the zone of elongation (Oparka et al., 1994). In the case of developing lateral root primordia, phloem connector elements appear to provide continuity between the phloem of the primary root and the cells of the developing primordium (Oparka et al., 1995). In these studies, CF was used to monitor symplastic unloading. In the present study, *A. thaliana* roots were parasitized by *H. schachtii*, and localized phloem unloading of CF into the syncytia within the root stele was observed. Given the relatively impermeant properties of the CF anion, it would be tempting to suggest that the dye had entered the syncytium symplastically. In general, the common interface of sieve elements and syncytial cells might allow symplastic transport. Such a conclusion was drawn by Dorhout et al. (1993), who observed the entry of CF into giant cells on tomato roots parasitized by the nematode *Meloidogyne incognita*. However, they did not demonstrate a plasmodesmatal pathway (i.e. functional symplast) between the giant cells and the host phloem.

In the nematode *Criconemella xenoplax* (Raski) Luc and Raski, which is not a cyst nematode, plasmodesmata are utilized specifically to facilitate enhanced solute transport into the food cell (Hussey et al., 1992). In this species single cortical cells become modified into "food cells," which display greatly modified plasmodesmata that reach to neighboring cells and have increased diameters and no desmotubules. In addition, an electron-dense "cap" forms over the plasmodesmata in the adjoining cells.

In cyst nematodes such as *H. schachtii*, transfer of CF from the phloem to the syncytium is unlikely to be via a symplastic pathway for two reasons. First, published reports of the structure of the syncytium and its relationships with surrounding host cells would suggest that plasmodesmata are rare or absent at this interface (Jones, 1981). More recently, detailed ultrastructural investigations on syncytia in *A. thaliana* confirmed these reports. Functional plasmodesmata could not be found in syncytial cell walls during the whole course of nematode development (Golinowski et al., 1996; Sobczak, 1996). Furthermore, and more importantly from the point of view of the current investigation, the complete lack of movement of low-molecular-weight, fluorescent probes out of the syncytium would suggest that the syncytium behaves as an isolated symplastic domain within the root stele. One cannot rule out the possibility that existing plasmodesmata, although sparse, might be modified to allow the unidirectional transport of solutes into the syncytium. This would certainly explain the simultaneous appearance of both CF and ^{14}C within the syncytium whenever the phloem contained CF. It would, however, represent an unprecedented mechanism. To our knowledge, no evidence has been advanced to date to suggest that plasmodesmata might operate as "one-way valves."

Consequently, an alternative (apoplastic) step in the transfer of both CF and Suc from the phloem to the syncytium needs consideration. Stimulation of apoplastic unloading from the phloem might be achieved in a number of ways during nematode infection. However, it is clear that any potential scheme for unloading in this system must accommodate the exit of both CF and Suc from the phloem, and the fact that the uptake of both labels into syncytia occurred in a correlated manner.

Localized phloem unloading might be achieved by an inhibition of the proton pump located on the membrane of the SE-CC complex, which would lead to the inability of the SE-CC complex to retrieve Suc, which would then leak passively to the apoplast. From the apoplast, Suc could be transported across the membranes of the syncytial cells by a putative Suc carrier. Such a mechanism of phloem unloading, based on the inhibition of retrieval, is thought to occur naturally in a number of regions in plants, e.g. in growing stems, where radial solute delivery is required

(Van Bel, 1993, and refs. therein). The leakage of CF from the phloem is less likely to be explained by the inhibition of proton pumping. It is questionable whether an inhibition of the proton pump would lead to localized acidification of the sieve-tube sap sufficient to induce the passive leakage of undissociated dye molecules from the SE-CC complex. The mechanism by which the nematode might inhibit the proton pump on the SE-CC complex is as yet obscure.

An alternative hypothesis is that putative, nonspecific solute pores are induced by the nematode during infection, allowing leakage of both CF and Suc from the SE-CC complex into the syncytium. Even less probable is the action of already described channel and carrier proteins, since they were shown to be substrate-specific (Bush, 1993). They cannot, however, explain the simultaneous transport of both sugar and CF.

Clearly, the above schemes are speculative and require further experimentation. However, they explain why under these very specific conditions, in contrast to the physiological conditions in healthy plants, CF could be translocated via the apoplast. Given the almost complete lack of functional transport studies between plant hosts and parasitic nematodes, we believe that the proposed schemes provide a starting point from which to conduct future physiological investigations.

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