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CONVERSION OF THE SEARCHING HYPHAE OF DODDER INTO XYLIC AND PHLOIC HYPHAE: A CYTOCHEMICAL AND IMMUNOCYTOCHEMICAL INVESTIGATION

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Dodders are obligate parasites, requiring both water and carbohydrate to be supplied by the host plant. To achieve the successful flow of both carbohydrate and water, the searching hyphae of the dodder must penetrate the host and transform into xylic or phloic hyphae, depending on the cell type that the hyphae encounter. The phloic hyphae develop a massive handlike appendage that surrounds the host phloem, whereas the xylic hyphae are directly connected to the host. In this study, changes in wall composition and structure are monitored via structural, cytochemical, and immunocytochemical techniques that allow one to discriminate changes in the hyphae as they make the transition into functional xylem and phloem. Although the terminal structure of the phloic hyphae has been termed a transfer cell, it bears little resemblance to those cell types; few if any wall ingrowths are noted. Rather, an extensive array of smooth endoplasmic reticulum (ER) is aligned at right angles and adjacent to the host phloem cells; the wall of the phloic hyphae becomes reduced in cellulose and xyloglucans and enriched in pectins. This loosening of its own wall (by loss of cellulose-xyloglucan and increased pectin) and the presence of abundant smooth ER facilitates the transfer of saccharides apoplastically into the parasite. In contrast to the phloic hyphae, xylic hyphae achieve a direct connection between the host and parasite, so that a flow of water is maintained between the host and the parasite. Xylic hyphae do not form secondary walls as do normal xylem elements. Rather, areas of the xylic hyphae have areas where the secondary wall is produced evenly along their length as well as in less regular true tracheid-type secondary walls. Similarly, the xylans that are characteristic of secondary walls are found both in the secondary thickenings and along extensive stretches of the mature xylic hyphae. In some xylic hyphae, both continuous and sporadic thickenings are observed. The host xylem and the xylem of the haustoria have xylans limited to secondary thickenings only, however. These data indicate that although the xylic and phloic hyphae mimic xylem and phloem elements, they retain some unique qualities to facilitate their parasitic function.

Keywords: dodder, parasitic plants, wall composition, xylic and phloic hyphae.

Introduction

Dodders (*Cuscuta* spp.) are obligate parasites and, as such, require all of their nutrition from the host plant. The relatively low rates of photosynthesis in these plants are below what would be required for normal maintenance (Malik and Singh 1979; Dawson et al. 1994; Sherman et al. 1999), and the plant essentially has no roots to conduct water on its own (Lyshede 1986). Because of this situation, dodders must obtain both polysaccharides and water through connections to the host xylem and phloem. This is achieved by the conversion of searching hyphae into specific xylic hyphae that establish a xylem bridge between host and parasite and specific phloic hyphae that mimic sieve elements and make a similar phloem-to-phloem connection.

Searching hyphae transform from tip-growing cells into cells that resemble the cell type to which contact has been made. This is one of the few examples in plants of a cell recognition phenomenon. Amazingly, the touch response results in differentiation from opposite directions, depending on the host vascular element contacted (Kujit 1969; Kujit and Toth 1976). Contact with host xylem elements results in a basipetal conversion of the searching hyphae into xylic hyphae. In contrast, contact with a phloem cell results in an acropetal conversion of the searching hyphae into phloic hyphae, also known as absorbing hyphae (Dörr 1990; Dawson et al. 1994).

Although structural studies have laid the groundwork for understanding this parasitism (Dörr 1968*a*, 1968*b*, 1969, 1987, 1990; Dawson et al. 1994), several major questions remain in understanding how these cells function in achieving such a highly functional parasitic union. Although phloic hyphae are relatively large and, as such, have a large surface area, they do not have traditional transfer-type cell walls with many invaginations to facilitate uptake of carbohydrate from the host. How, then, does the parasite achieve such an efficient flow of saccharide without these ingrowths? Moreover, more recent studies (Haupt et al. 2001) indicate that there might be more substantial bridges between host and parasite, allowing uptake of green fluorescent protein (MW ca. 27 kD) that theoretically should not move apoplastically. In the case of the xylic hyphae, there have been relatively few

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structural studies undertaken compared to those of the more elaborate phloic hyphae (the studies by Dawson et al. [1994] are the only substantial ones at the electron microscopy level). Do the cell walls go through the same sort of modifications that normal xylem elements do in production of their regularly thickened cell walls?

In earlier studies, we used immunocytochemical techniques to determine the nature of the cement that allows attachment of the dodder to the host (Vaughn 2002) and the cell wall modifications that occur during the movement of the hyphae through the host cells (Vaughn 2003). In this study, data from light and electron microscopy, cytochemistry, and immunocytochemistry are combined to revisit some of the structural studies in a system that fixes easily for transmission electron microscopy and the use of antibodies to characterize specific cell wall modifications.

Material and Methods

Plant Material

Impatiens plants (*Impatiens balsaminea*) were grown in a Conviron growth chamber at ca. 450 μmol/m^2 PAR in a potting mix consisting of peat, Perlite, and finely ground pine bark (at a ratio of ca. 2 : 1 : 2). Seeds of dodder (*Cuscuta pentagona* L.) were acid scarified, neutralized with sodium bicarbonate, and placed in the pots of the impatiens to germinate. After 5–7 d, the dodder had begun to wrap around the host. Samples for these experiments were collected 10–17 d after host invasion so that mainly successful parasitic unions were studied. Some blocks from previous studies that concentrated on earlier phases (Vaughn 2002, 2003) were also monitored to determine possible induction of xylem and phloem in the host in the presence of the dodder and for earlier developmental stages in xylic and phloic hyphae formation.

Microscopy

For transmission electron microscopy, sections of impatiens stems parasitized by dodder were fixed in 6% (v/v) glutaraldehyde in 0.05 M PIPES buffer (pH 7.4) for 2 h at room temperature, washed in 0.10 M sodium cacodylate buffer (pH 7.2) (2 × 15 min), and postfixed in 2% (w/v) osmium tetroxide in cacodylate buffer for 2 h. After a brief water rinse, the samples were stained en bloc with 2% (w/v) uranyl acetate for 18 h at 4°C. Samples were then brought through an acetone series at 25% increments, transferred to propylene oxide, and embedded slowly over 3 d in a 1 : 1 mixture of Spurr's resin and Epon. Samples were polymerized at 68°C in flat embedding molds and then mounted on stubs for sectioning at right angles to the long axis of the impatiens stem or leaf. Semithin (0.35 μm) sections were cut with a Delaware histological knife and poststained with aqueous 1% (w/v) toluidine blue in 1% (v/v) borax. Thin (ca. 100 nm) sections were cut with a Delaware diamond knife on a Reichert Ultracut ultramicrotome and mounted on slot grids coated with Formvar. Sections were poststained with uranyl acetate (7 min) and Reynold's lead citrate (5 min) before observation with a Zeiss EM 10 CR electron microscope operating at 60 kV. In most cases, serial light sections were made from a block face, with thin sections cut as structures of interest were encountered. This allowed for a better determination of the interaction between host and parasite tissues because serial sections could follow individual hyphae along their lengths.

An alternate protocol for fixation used for the enhanced staining of membranes, especially endoplasmic reticulum (ER), was the osmium-ferrocyanide protocol of Hepler (1981). Fixation follows the protocols exactly as described by Hepler or with a higher level of calcium (20 mM) added to all of the fixation and wash buffers (examples in Vaughn and Harper 1998). Dehydration, embedding, and sectioning were as described as above for standard microscopy protocols.

An alternate poststaining protocol involved grid staining of sections in acidified 1% (w/v) phosphotungstic acid (PTA) or silicotungstic acid (STA) for 30 min at room temperature (Roland and Vian 1992). Sections were mounted on uncoated gold 300-mesh grids and were bleached with 1% (w/v) periodic acid before the poststaining to remove some of the surface osmium so that areas stained with STA or PTA would be more apparent. Grids were also processed for periodic acid–thiocarbohydrazide–silver proteinate (PATAg) staining (Roland and Vian 1992).

For scanning electron microscopy, the protocols of Vaughn (2002) were used. Tissues were cut with a razor blade in fixative at an orientation such that the haustorium was cut in longitudinal section and the stem of the impatiens was cut in cross section. Five to seven pieces were cut from each haustorium. This exposed many profiles of hyphae crossing through tissue and invading the host vascular tissue. After critical-point drying, the samples were examined with a dissecting microscope, and samples displaying good xylem or phloem connections were mounted on aluminum stubs so that this surface of interest could be observed. Samples were coated with gold-palladium and observed with a JEOL scanning electron microscope operating at 15 kV.

Immunocytochemistry

Two different protocols were used to fix and embed samples for immunocytochemistry. One was performed as above for standard microscopy, whereas one involved a less drastic sequence of fixation and embedding protocols, described here. Impatiens stem, leaf, and petiole segments infected with dodder were fixed in 3% (v/v) glutaraldehyde in 0.05 M PIPES buffer (pH 7.4) for 1–2 h at room temperature and washed in two exchanges of 4°C PIPES buffer. Samples were dehydrated in an ethanol series (through 75% at 4°C and 100% at −20°C). Samples were embedded in LR white resin, with the amount of resin increased at 25% increments each day. After two exchanges of 100% resin at −20°C, the samples were warmed to room temperature and rocked on a platform rocker for 24 h. The samples were then transferred to BEEM capsules and polymerized at 50°C in a vacuum oven for ca. 3 h. Sections for light and electron microscopy were obtained as above.

Protocols for light immunogold-silver, transmission electron microscopy immunogold, and associated statistical analysis were as described previously (Vaughn 2002, 2003). Antibody specificities are described in more detail in earlier studies from my lab (Vaughn 2003, 2006).

Results

Structural Studies

After successful penetration of the hypha through the host tissue (fig. 1, inset), the hypha contacts either xylem or phloem elements (fig. 1*A*) and begins a transformation from a tip-growing cell into a structure resembling the vascular tissue with which it has made contact. Sections through successful host-parasite connections reveal numerous xylic and phloic hyphae in leaves, stems, and petioles of the host (fig. 1*B*). Although the process described below is a developmental process, we are hampered in that these are static images of a dynamic process of hyphal development. However, these observations are consistent through many sets of serial and nonserial sections.

Differentiation of the xylic hyphae starts near the tip and proceeds to the base of the hypha, where it meets the xylem of the haustorium (fig. 1*C*). The first indications of xylic hyphae formation are the movement of the hyphae into areas adjacent to host xylem elements (fig. 2*A*). At an early phase of this process, the hyphal cytoplasm and the tip of the hyphal wall often contain numerous osmiophilic particles (fig. 2*A*), which contain expansin. These particles probably aid in the loosening of the host xylem wall and penetration of the hyphae. Unlike previous incursions of hyphae into or through host cells, the movement of the hypha into xylem cells does not involve a coating wall from the host except at very early stages (fig. 2*B*) but rather involves just penetration of the hypha into the host xylem element. The lack of a coating host wall in the host xylem may be because these cells have no sort of wall synthesis machinery, lacking nuclei or any other organelles.

During differentiation, the xylic hyphae lose their tonoplast and eventually all organelles. Although the hyphae are generally unbranched in the early stages of the searching phase, as the xylic hyphae approach xylem, often branches of the hyphal tip will contact two or more places in the host xylem or even adjacent elements (not shown but revealed in serial sections). Xylic hypha walls are thickened, some into irregularly thickened walls (fig. 1*C*, 1*D*) and others into walls with annular rings resembling those in haustorial or host xylem elements (figs. 1*B*, 3). These two different patterns of secondary wall formation may occur in the same hypha and even in fairly close segments of the same hypha (fig. 1*D*). Xylic hyphae are highly variable in morphology. Take, for example, the three xylic hyphae in figure 1*C*. Each of these xylic hyphae, although connected to the haustorial xylem and presumably ontogenetically complete, is distinct. Cell *1* has extensive and irregular secondary wall ingrowths, cell *2* has a rather even and thin secondary wall, and cell *3* has a thicker wall but no secondary wall invaginations.

The nature of the opening between the host xylem and the xylic hyphae seems to be dependent on the angle and orientation of the hyphae with respect to the host xylem. In one extreme, the opening connecting the host to the parasite appears only at the very tip of the xylic hyphae (fig. 3*B*), when hyphae appear to grow straight into the xylem cells and penetrate only for short distances. In others, a broad side of the hyphal tip is opened to the host xylem (fig. 1*B*), and rather extensive penetration of the xylic hyphae into the host xylem is observed. At an early stage of host xylem to xylic hyphae connection, the ends of the hypha walls appear to perforate or become thinned in preparation for making an opening (fig. 2*C*). Occasional bits of membrane (residual plasma membrane of the host?) are found at the interface between the xylic hyphae and host xylem even after an opening has been made (fig. 3*B*). Some disruption of host annular ring morphology/orientation is noted at the points where xylic hyphae invade the host xylem (fig. 1*B*). At the haustorial end of the xylic hyphae, open connections are observed between the ends of xylic hyphae and haustorial xylem (fig. 1*C*). Thus, xylic hyphae provide bridges for solute delivery between the host and dodder xylems.

In contrast to xylic hyphae differentiation, the differentiation of phloic hyphae starts at the base of the hypha near the haustorium and proceeds toward the tip. The tip enlarges greatly, engulfing the phloem of the host in a handlike grip (figs. 4, 5*A*). Although the phloic hypha has been described as having transfer cell–type walls, the walls of phloic hyphae display essentially no wall invaginations or wall ingrowths as are noted in traditional transfer-type cells (e.g., fig. 4). Rather, the odd shape of this cell (fig. 4) has caused wall accommodations that might accompany any sort of intrusive growth. Aside from the large amount of surface contact between dodder and host phloem, the internal anatomy of the phloic hyphae also seems to assist the uptake of material from the host. Along the entire host/parasite surface, an extensive array of smooth ER is aligned at right angles to the wall surface (figs. 4, 5*B*). Classic differentiation of phloem-type plastids and mitochondria (fig. 5*B*) is also noted, and P-protein is noted in the cytoplasm of these cells (fig. 5*C*). Thus, in some ways, the phloic hyphae have the characteristics of sieve elements (smooth ER, plastids, and mitochondria typical of phloem; P-protein) without a neighboring companion cell. Haustorial phloem is connected at maturity to the phloic hyphae, but the method of connection has not been established, partly because the differentiation of the phloic hyphae starts near the haustorium and proceeds toward the host phloem, making early stages of the process difficult to recognize. However, the termini of phloic hyphae observed appear to be strictly connected to haustorial sieve cells/companion cell pairs.

Although there have been reports of the growth or differentiation of xylem and phloem cells in the host in response to the invading dodder, no evidence of such growth was observed in the stem segments of the impatiens. The vascular tissue in the impatiens stem is very regular, and no cells outlying this well-defined band were noted. For the xylic hyphae, the localizations of xylans in the dodder and host xylems (see below) gives a clear view of the very regular host xylem and the lack of outgrowths of these structures even at sites of conversion of host and parasite. No evidence for host xylem expansion is noted, even with this very sensitive probe for xylem differentiation. Serial sections were cut completely through more than 20 dodder stem invasions, and no evidence of phloem or xylem outside the normal band of vascular tissue was observed. When a massive hyphal invasion had occurred, there was some obvious displacement of host vascular tissue and occasional evidence of hypertrophied cells. In leaves, however, there were pockets of phloem cells that seemed to be displaced

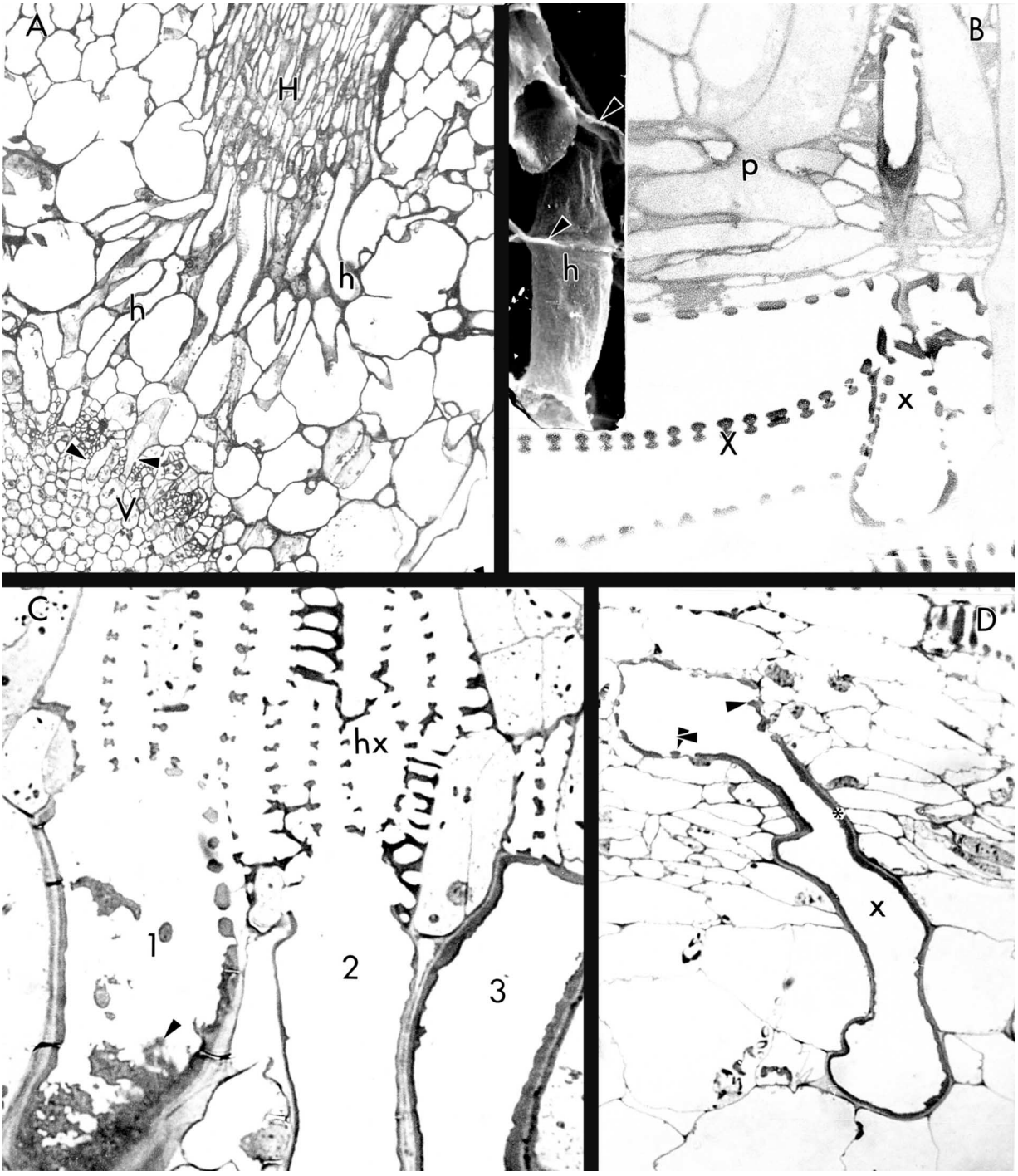


Fig. 1 Light and scanning electron microscopy of the invasion of *Impatiens* tissue with dodder. *A*, Low-magnification light micrograph showing haustorium (*H*) and hyphae (*h*) growing through host tissue toward the host vascular tissue (*V*). Arrowheads mark hyphae in intimate contact with the host vascular tissue; ×80. *B*, Higher-magnification light micrograph showing the sinuous phloic hyphae (*p*) and a xylic hypha (*x*) that has grown into and connected with the host xylem (*X*). The haustorium is growing in at a ca. 90° angle to the host xylem (*X*); ×360. *C*, At the convergence of the haustorial xylem (*hx*) are three xylic hyphae with quite different morphologies. Cell *1* has very irregularly thickened walls (marked with arrowhead), cell *2* has a relatively thin wall, and cell *3* has a uniformly and highly thickened wall; ×660. *D*, Longisection through a xylic hypha showing areas of this single hypha with uniform thickenings (asterisk) and with more typical xylem thickenings (arrowheads); ×330. Inset is a scanning electron micrograph (×500) of two hyphae (*h*) growing through the host tissue and connected to the vascular at a point deeper in the host tissue. The upper hypha has been cut open to show the hollow interior. Arrowheads mark host cell walls that have been traversed by the hyphae.

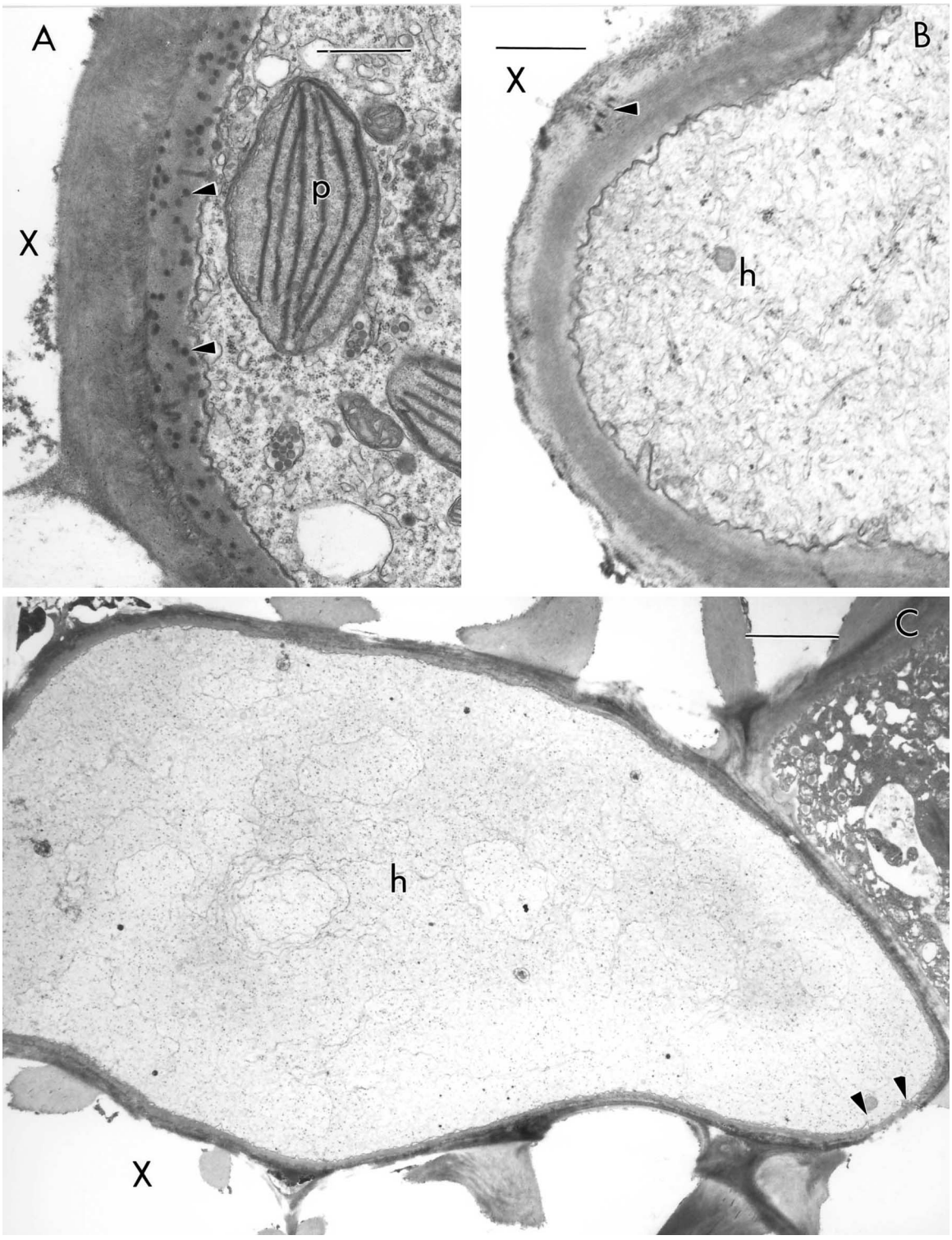
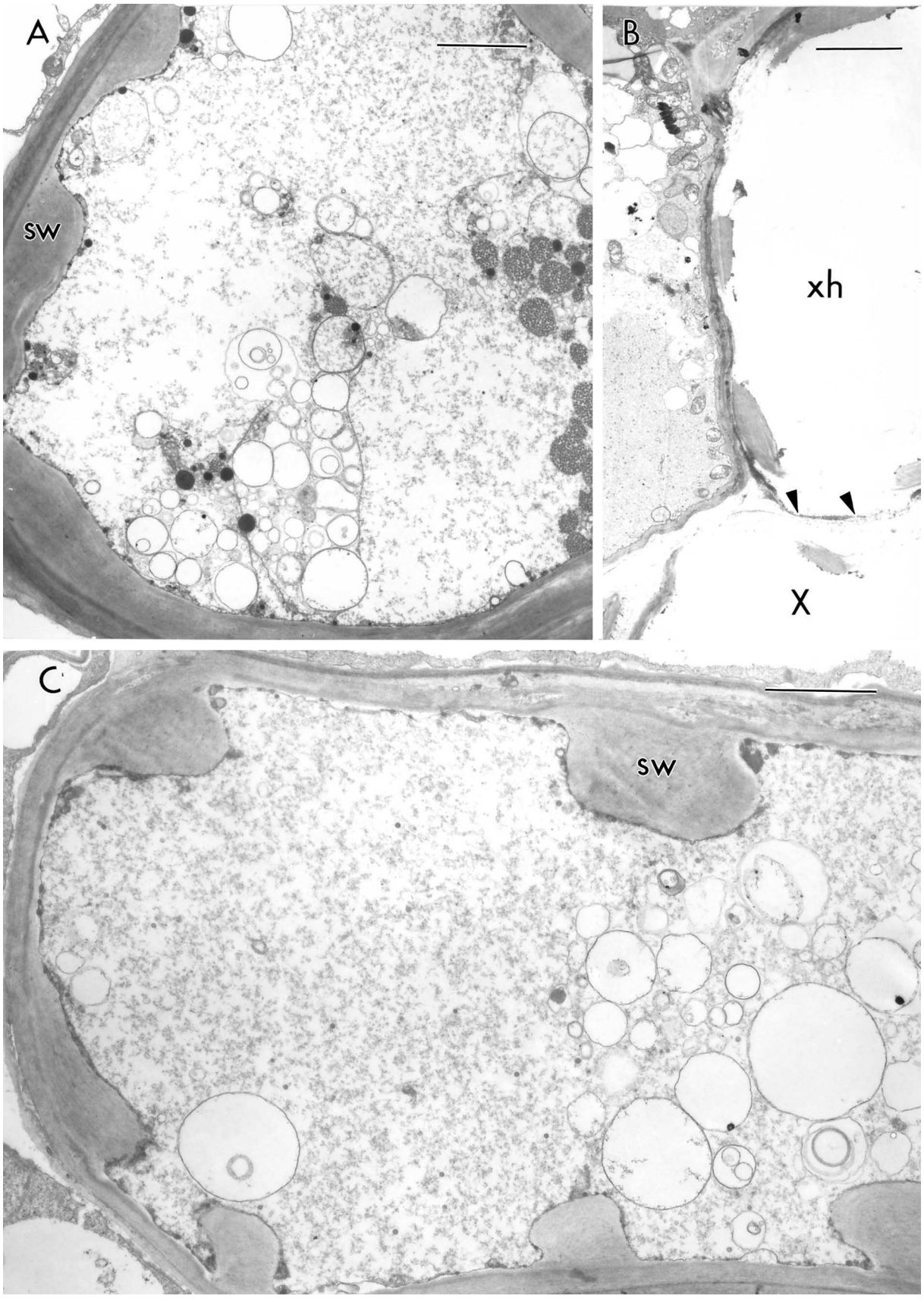


Fig. 2 Electron micrographs illustrating some of the early steps of xylic hyphae formation. *A*, First association of the hypha with a host xylem (*X*) element. Near the tip of the hyphae, wall-associated osmiophilic particles (two marked with arrowheads) are characteristic of walls at this stage. *p* = plastid. *B*, Early stages of hyphal penetration into the host xylem (*X*). The host wall is being pushed by the expanding hypha (*h*). Arrowhead marks plasmodesmata in hyphal wall. *C*, A well-developed hypha (*h*) in the host xylem (*X*). Thinning of the xylic hyphae wall is noted at one end (arrowheads). Bars = 0.3 μm (*A*, *B*) and 4 μm (*C*).


A
sw
B
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X
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sw

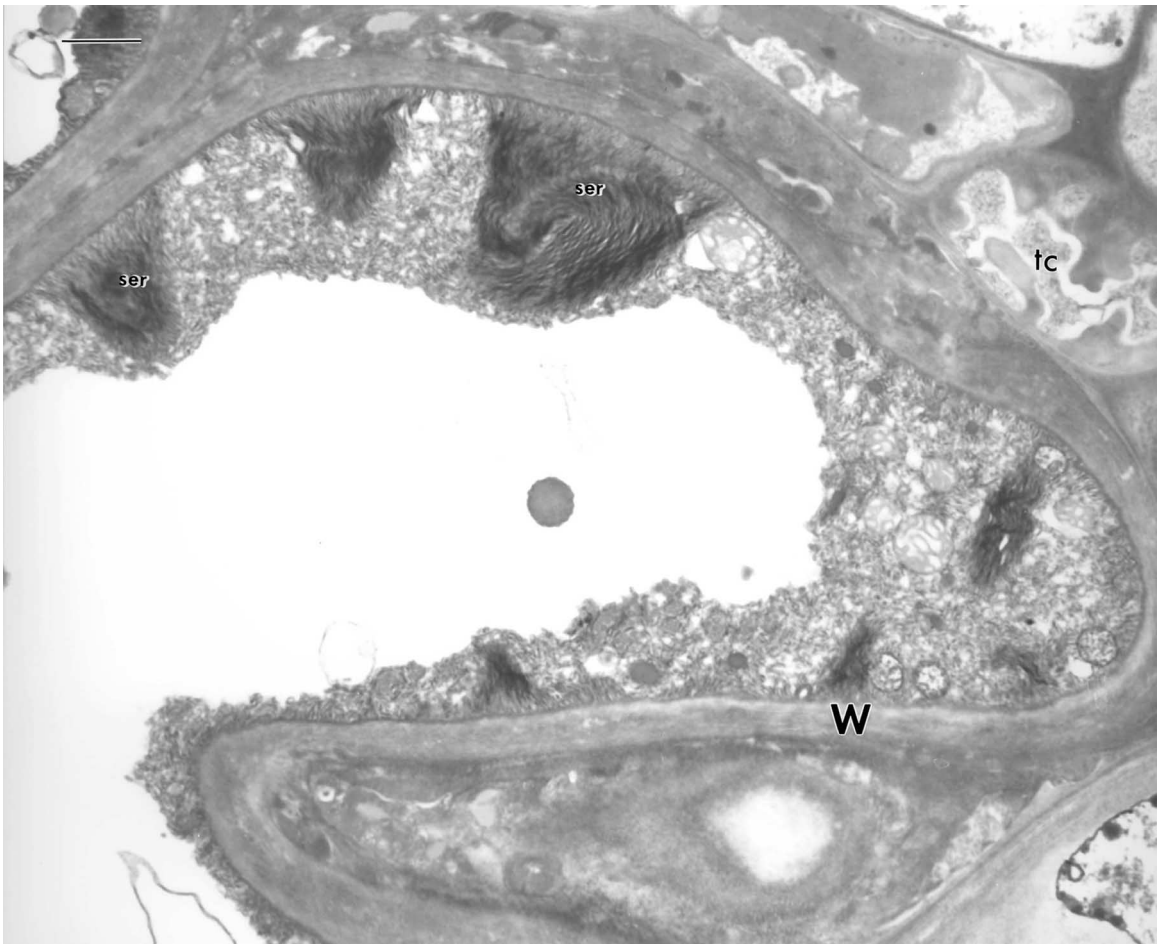

Fig. 4 Transmission electron micrograph of a mature phloic hypha cell associated with transfer cells (*tc*) of the host. Although the phloic hypha wall (*W*) is obviously undulated so as to envelop the host phloem, it displays none of the wall ingrowths of the transfer cells. Abundant smooth endoplasmic reticula (*ser*) are prominent along the cell walls associated with the phloem cells. Bar = 1 μm.

from their normal locations; whether this was due to de novo production of phloem in the host (a possible example is shown in fig. 6*A*) in response to dodder invasion or due to the displacement of host tissue by the relatively massive haustorium through the leaf tissue is not known.

In addition to these cases of possible vascular differentiation in the host, there were also some possible misidentifications of searching hyphae for a vascular element of the host. Occasional xylic hyphae would form next to or in cells that were clearly not xylem, especially in leaf tissue (14 out of more than 230 individual hyphae that were traced by serial sections in leaf invasions). An example is the xylic hypha shown in figure 3*A*, which terminates in photosynthetic parenchyma but already has well-developed secondary walls. Whether this is due to a signal in these cells types perceived by the dodder as a xylem or just differentiation in the absence of a signal is not known. The thicker walls of xylic hyphae are more rigid than those of searching hyphae because of the presence of secondary walls, thus enhancing the dodder's hold into the host tissue. This more rigid structure might be needed in tissue such as leaves, where the tissue itself is not as sturdy as that in stem tissue. Moreover, of the 14 "miscued" xylic hyphae, 11 were from hyphae that extended more than 300 μm. These very extended hyphae that failed to reach xylem or phloem may have just formed a terminal differentiation, and xylic hyphae formation may represent the "default" pathway. No misplaced phloic hyphae were found in the thousands of sections that were monitored,

Fig. 3 Electron micrographs of later stages of xylic hyphae formation. *A*, Xylic hypha in leaf tissue with well-developed secondary walls (*sw*). This hypha ended bluntly in a mesophyll cell rather than being associated with host xylem. *B*, Opening between the xylic hyphae (*xh*) and the host xylem (*X*) in mature xylem. Remnant membranes and wall fragments are still found (arrowhead). *C*, Near terminal stage of xylic hyphae formation ca. 120 μm from the host xylem interface. This area displays relatively regular secondary walls (*sw*). Bars = 3 μm (*A*), 4 μm (*B*), and 2 μm (*C*).

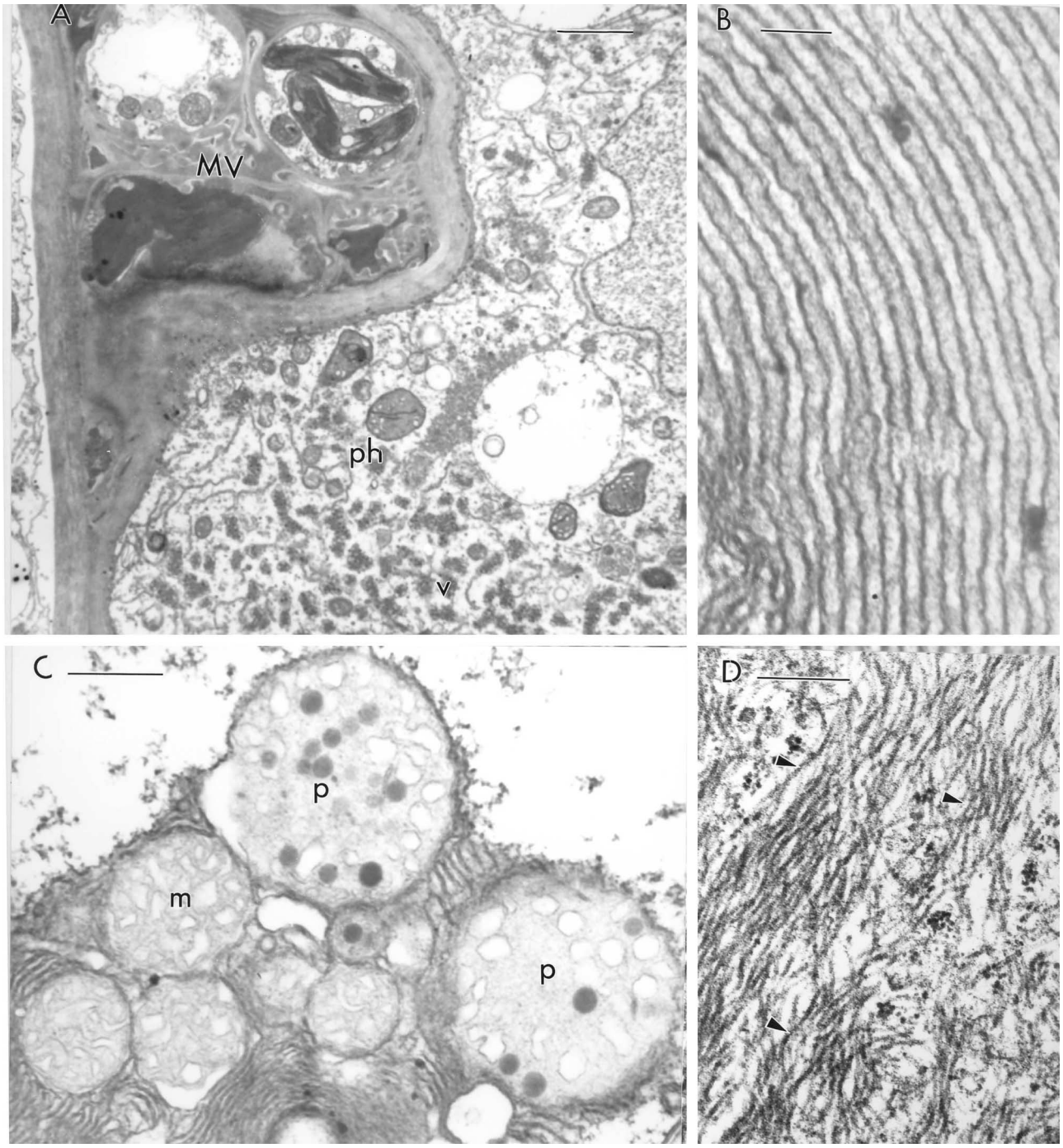

Fig. 5 Transmission electron micrographs of phloic hyphae. *A*, A minor vein (*MV*) in a leaf that appears to be displaced and/or induced compared to the normal deposition of phloem in leaves. The immature phloic hyphae (*ph*) has already enveloped this tissue. *B*, High magnification of the smooth endoplasmic reticulum after osmium-ferrocyanide fixation. Although membranes are well contrasted, there is little indication of filling in the lumen as occurs in some cell types. *C*, Mitochondria *(m)* and plastids (*p*) are typical of sieve cell plastids. *D*, P-protein strands (arrowheads) are prominent in the phloic hyphae. Bars = 2 μm (*A*), 0.2 μm (*B*), and 0.5 μm (*C*, *D*).

further supporting this idea. However, if the searching hyphae encountered xylem or phloem, then the correct type of hypha was formed in all of the hyphae that were examined by light serial sections (230 in leaves/petioles and 617 in shoots).

Cytochemistry

Two cytochemical reactions were used to detect changes in the xylic and phloic hyphae walls and plasma membranes. The PATAg reaction labels vicinal —OH groups in polysaccharides,

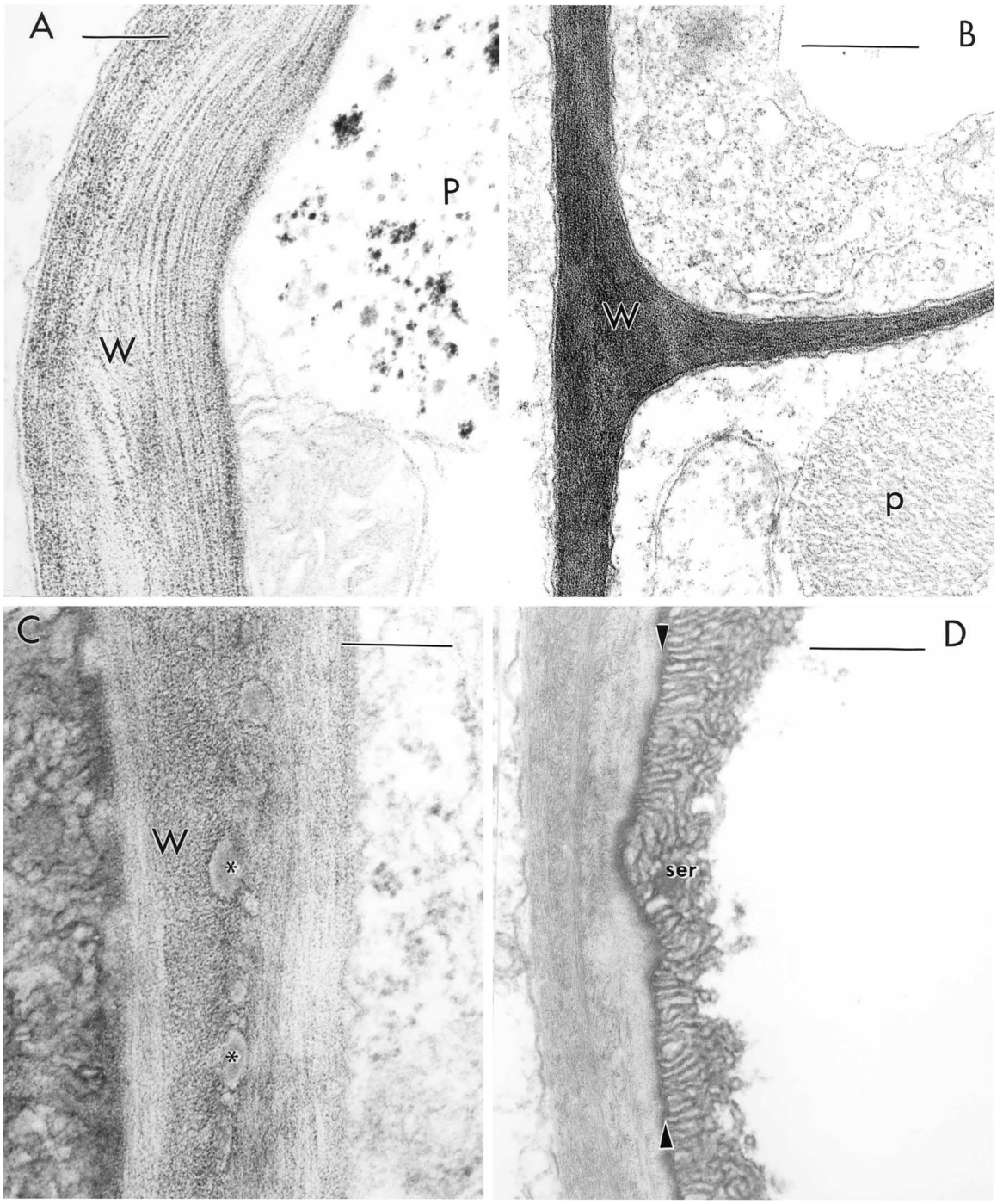

Fig. 6 Wall cytochemistry of phloic hyphae (*A*, *C*, *D*) and host phloem (*B*) cells. *A*, Periodic acid–thiocarbohydrazide–silver proteinate (PATAg) reaction in the wall of the phloic hyphae. Note the lamellate staining and large gaps between reactive strands. Starch in the phloic hyphal plastid is also strongly reactive. *B*, Strong and dense PATAg staining in a host phloem cell wall (*W*) and associated other cells away from the invasion of phloic hyphae. *p* = cluster of P-protein. *C*, An oblique section of a phloic hyphal wall (*W*). Unusual, negatively stained ovular structures are found within the wall (asterisks). *D*, Phosphotungstic acid stain reveals a strongly stained and nonundulated plasma membrane (arrowheads). *ser* = smooth endoplasmic reticulum. Bars = 0.2 μm (*A*, *C*) and 0.5 μm (*B*, *D*).

whereas the STA or PTA stains react with glycoproteins in the plasma membrane and cell wall.

PATAg staining of the phloic hyphae revealed an unusual staining of the fibrillar structures within the wall (fig. 6*A*). Instead of a relative tight and intense labeling, the phloic hyphae and, to a lesser extent, the adjoining host phloem had lamellate bands of reaction, separated by nonstaining areas. In appearance they were similar to walls that had been

extracted with enzymes or chemical treatments before PATAg staining or to the cellulose-poor, pectin-rich walls of cultures grown on the cellulose biosynthesis inhibitor dichlobenil. Walls in the haustorium, host, and nonphloic hyphae gave characteristic staining with PATAg (e.g., fig. 6*B*). Here, the walls were strongly reactive throughout, with no indication of a lamellate wall. In addition to the lamellate walls observed in phloic hyphae, in obliquely sectioned walls there were ovular patches of wall that reacted differently (or less) to the PATAg stain than other areas (fig. 6*C*). These patches tended to occur at the interface between hyphal and host walls.

The unusual arrangement and large amount of smooth ER in the phloic hyphae prompted questions of whether this might in fact be a highly invaginated plasma membrane (which would radically increase the surface area) rather than ER. To test this, PTA and STA were used to stain the plasma membrane of phloic hyphae cells. In these sections, although both plasma membrane and wall were stained more than the levels of staining of the osmicated sample, the smooth ER was not similarly enhanced (fig. 6*D*). This observation also substantiated the contention that there were no wall ingrowths or associated plasma membrane ingrowths in the phloic hyphae (see above). Attempts to stain the ER with the osmium-ferrocyanide stains were also negative in this tissue, as is often found with this technique with single-celled appendages such as the hyphae. Instead, there was a simple enhancement of the membrane reactivity but no filling in of the ER lumen (fig. 5*B*). Because of the species- and tissue-specific nature of the ER stain, no conclusions could be made about the nature of the smooth ER in the phloic hyphae. However, it is clear from the PTA and STA stains that it was not invaginated plasma membrane.

Immunocytochemistry

Sections embedded in LR White resin were cut as serial sections from a number of block faces and probed with a battery of antibodies to identify constituents and arrangements of the wall components in xylic and phloic hyphae. These same techniques have proven to be of critical importance in identifying wall modifications in the dodder hyphal tips as they crossed through host cells. A summary of the localizations in these cell types is given in table 1.

For xylic hyphae, the most striking antibody localizations were the antixylan antibodies LM 10 and LM 11. In the impatiens and dodder tissue, only the xylem elements were labeled with these antibodies, so xylem tissue of any type could be easily detected at the light level from the mass of other cells in the section (fig. 7*A*–7*C*). In the host tissue, only the secondary walls on the tracheary elements were labeled, and these were very formed very evenly, allowing their distinction from the more irregularly formed xylic hyphae (fig. 7*A*). In the xylic hyphae, broader areas of apparent secondary wall as well as less well-defined rings were labeled, thus almost resembling scalariform tracheids (fig. 7*A*–7*C*). These secondary wall extensions occurred as patches and were sometimes adjacent to what appear to be true annular rings (fig. 7*C*). Although the xylic hypha has a very irregular secondary wall as marked by the LM 10 or LM 11 antibodies, the haustorial xylem produced very regular helical secondary wall ingrowths, and only the ingrowths were labeled with these antixylan monoclonals (fig. 7*B*).

Table 1

Distribution of Labeling of Xylic and Phloic Hyphae by a Battery of Wall Antibodies

Antibody	Xylic hyphae	Phloic hyphae
Pectins:		
JIM 5	+	++
JIM 7	+	++
CCRCM2	+	++
LM 5	+	++
LM 6	++	++
PGA polyclonal	+	++
Xyloglucans:		
CCRCM1	++	±
Xyloglucan polyclonal	++	±
Xylans:		
LM 10	++	−
LM 11	++	−
Callose:		
Monoclonal	−	++
Polyclonal	−	++
Extensins:		
LM 1	++	−
JIM 11	+	−
Cellulose:		
Cellulase-gold	++	±

Note. Summary of light immunogold-silver reactions. Data are from at least three replications. + = present; ++ = greater reaction than parenchyma; ± = weak reaction; − = no reaction.

The only other somewhat surprising result was the strong reaction of xylic hyphae with the LM 6 antibody that recognizes a 1→5 arabinan side chain on rhamnogalacturonan-1 (fig. 7*D*). Neither host nor haustorial xylem cells react with this antibody much beyond background levels, whereas the xylic hyphae are labeled strongly in both their primary and secondary walls. Searching hyphae do react with the LM 6 antibody, although even there, the reaction is less than in the xylic hyphae (not shown). Thus, despite resembling a xylem element in some respects, the xylic hypha wall is compositionally and structurally unique from true xylem of host or parasite.

Light-level probing of phloic hyphae with a battery of antibodies did not reveal any such unique polysaccharide epitope that was specific for the phloic hyphae. Rather, these cells showed stronger reactions for pectic epitopes (JIM 5, JIM 7, LM 5, LM 6, and PGA polyclonal) and callose (monoclonal and polyclonal) but weaker reactions for xyloglucans (CCRCM1 and xyloglucan polyclonal) and cellulose than the surrounding host cells (table 1). Because of this, transmission electron microscopy sections were prepared to obtain counts of the colloidal gold labeling of these cell types to more accurately determine the relative amounts of label.

Labeling with the cellulase-gold probe resulted in a relatively weak label along the interface wall separating the host cells from the phloic hypha (fig. 8*A*). When one counts the gold particle densities along the phloic hyphae/host phloem interface, the label in the phloic hypha was 12% of that in host walls and 27% of that in hyphal walls not associated

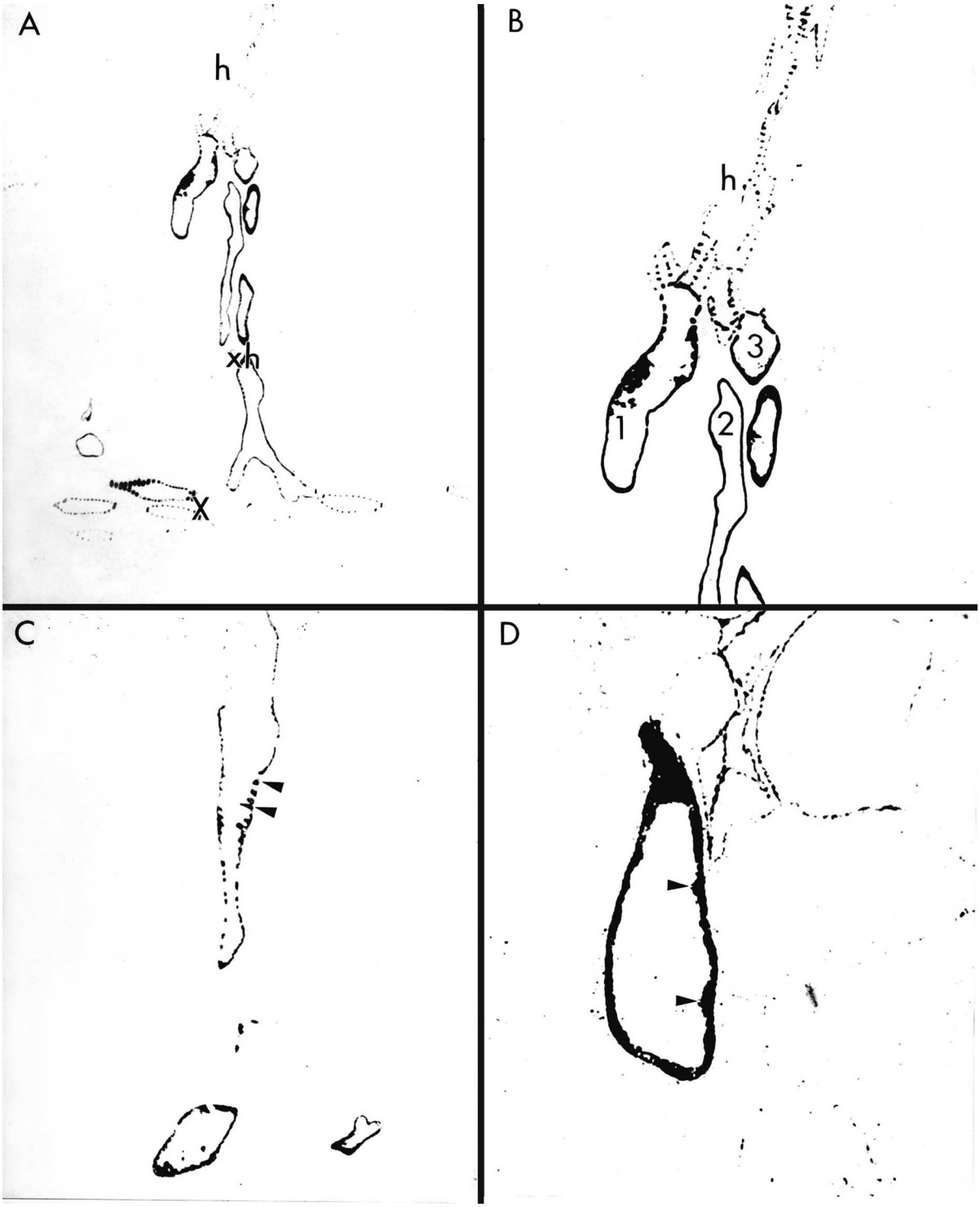

Fig. 7 Immunogold-silver localization of xylans (*A–C*) and a 1→5 arabinan side chain of RG-1 (*D*) in xylic hyphae. *A*, Low-magnification micrograph revealing the bridge between the host and haustorial xylems immunolabeled with the LM 10 antibody. Xylic hyphae (*xh*) connect host xylem (*X*) to haustorial xylem (*h*). Note the lack of reaction in all other tissues and the even reaction in the xylem compared to the uneven reaction in the xylic hyphae; ×80. *B*, Higher magnification of fig. 1 and a nonconsecutive serial section to the cell in fig. 1*C*, showing the same three hyphae. Although xylem cells in the haustoria contain xylans, each cell contains different amounts and has distributed the xylan differently. Cell *1* has extensive depositions, extending far from the wall, cell *2* has a thin but uniform coating, and cell *3* has a thick and uniform coating; ×220. *C*, LM 11 labeling of a xylic hypha, revealing areas of uniform secondary thickenings (arrowheads) as well as more uniform label in the same cell; ×330. *D*, Intense immunolabeling with the LM 6 antibody. Although neighboring cells have a reaction, the xylic hypha is strongly labeled in both its primary and secondary walls. Arrowheads mark sites of secondary wall thickening; ×460.

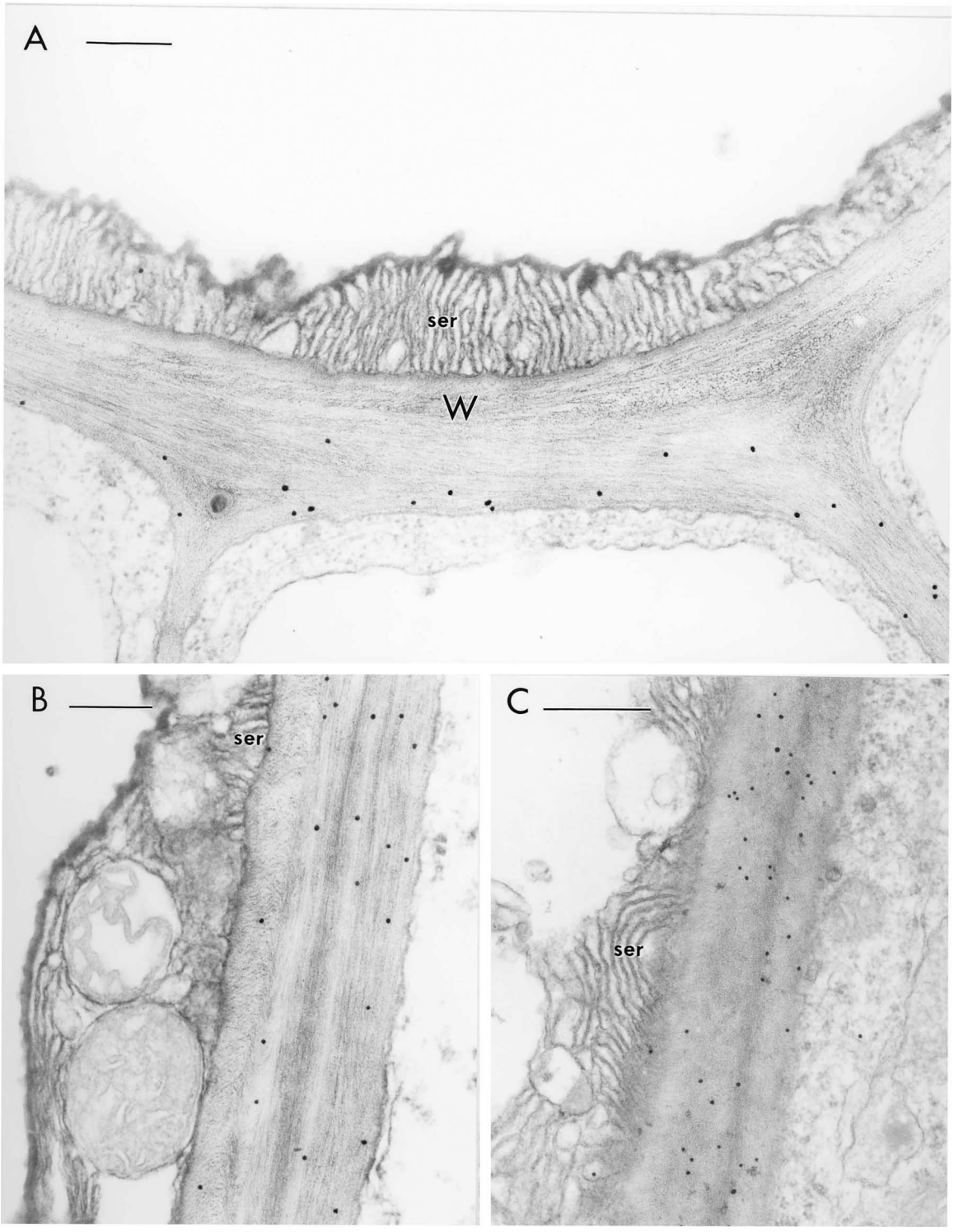

Fig. 8 Cellulase-gold (*A*) and immunogold (*B*, *C*) of phloic hyphae walls. *A*, Cellulase-gold labels the wall (*W*) of the phloic hypha very sparingly compared to walls of the adjacent host cells. This lightly poststained section also reveals the differences in wall organization between the phloic hypha wall and the surrounding walls of the host. *ser* = smooth endoplasmic reticulum. *B*, Immunogold labeling of xyloglucan. The wall of the phloic hypha has less labeling than the adjoining host cell. *C*, JIM 5 label of the phloic hypha and adjoining host wall is actually enhanced compared to noncontacted control cells. Bars = 0.3 μm (*A*, *B*) and 0.5 μm (*C*).

Table 2

Quantification of Immunogold Labeling on Xylic and Phloic Hyphae

Antibody	Xylic hyphae	Phloic hyphae
Pectins:		
PGA polyclonal	3	47
JIM 5	8	52
JIM 7	11	50
Xyloglucans:		
CCRCM1	26	8
Xyloglucan polyclonal	31	9
Xylans:		
LM 10	67	0
LM 11	54	0
Callose:		
Monoclonal	3	17
Polyclonal	5	21
Cellulose:		
Cellulase-gold	39	14

Note. Data are from 18–20 micrographs and are expressed as gold particles per square micrometer. Values are rounded to the nearest whole integer.

with the phloem (table 2). A similar relative change was observed when comparing xyloglucan immunolabeling (fig. 8*B*) in the phloic hyphae and the host (18%) or nonphloic hyphae (22%) walls.

In contrast, label of the phloic hyphae walls with any of the pectin antibodies was much higher in the phloic hyphae walls than in any of the host or other types of hyphae (table 2). The greatest change was in the labeling associated with the PGA polyclonal and the JIM 5 monoclonal (fig. 8C). Labeling of these pectin antibodies is often restricted to the cell corners and middle lamellae, but both of these antibodies labeled throughout the phloic hyphae wall, indicating a strong enrichment (230%–670%) in highly de-esterified homogalacturonans. There also appeared to be some reduction of cellulose and xyloglucan in the adjoining host phloem cells relative to noninvaded phloem and parenchyma cells, with 27% and 41% reductions, respectively, based on immunogold and affinity-gold labeling.

Discussion

Changes in Hyphal Wall Composition Accompany Conversions to Xylic and Phloic Hyphae

Dodder hyphae undergo an amazing conversion from a thin-walled tip-growing cell to cell types resembling xylem and phloem in the course of several days. This conversion is accompanied by a concomitant change in wall composition and differentiation of the cytoplasm to facilitate the use of these hyphal cells in their new roles of water and assimilate conduction. Both of these vascular connections are required for the sustenance of the obligate parasite dodder.

In the xylic hyphae, the major wall change involves the incorporation of xylans into the secondary walls (fig. 7*A*–7*C*). Xylans are found in many secondary or thickened walls in dicots and more extensively through primary walls in noncommelinoid monocots. Xylans in secondary walls act as pectins and xyloglucans do in primary walls of dicots, ensheathing the cellulose microfibrils. In the xylem cells of the host, the xylans are restricted to the annular rings in the xylem elements (fig. 7*A*). In xylic hyphae, the distribution of xylan is much more dispersed (fig. 7*A*–7*C*). When annular rings are produced, these structures have xylans, but the more general thickening that occurs randomly along the xylic hyphae does also. It is well known that microtubules are involved in annular ring morphology in developing xylem elements (Seagull and Falconer 1991). In xylic hyphae, the predominant tip-growing arrangement of microtubules in the searching hypha (K. C. Vaughn, unpublished data) may not be completely converted to this mode during the secondary wall formation, allowing a more general dispersal of the xylans in the xylic hyphae than is noted in true xylem. In the observations of numerous xylic hyphae at varying stages of differentiation, only sparse microtubules are observed in areas away from the tip, although more are generally found if a wall ingrowth is forming (not shown). Because tip-growing searching hyphae within the same section have a well-preserved microtubule array, the difference is less likely to be caused by fixation of these structures than to result from real differences in quantity and organization of the microtubules. Thus, the absence of microtubules (or their presence in poorly organized arrays) may explain the more random or haphazard nature in the distribution of secondary walls in the xylic hyphae. Extreme variations observed in xylic hyphae formation (e.g., fig. 1*C*) might be related to the degree to which microtubule arrays are reorganized during this transition. Alternately, the xylic hyphae might be forming a more scalariform type of vascular element that has secondary thickening at points other than the annular or helical thickenings. Merger of the xylic hyphae with the haustorial xylem reveals a wide-open connection between these two parasite xylem types as well as their very different morphologies (e.g., fig. 1*C*).

In phloic hyphae, there appears to be a shift of wall components from the majority of the wall being composed of cellulose and xyloglucan in a pectic matrix to a composition in which the pectin components predominate, accompanied by a general loosening of wall structure. This shift may serve two purposes in the phloic hyphae. The conversion of the searching hyphae to the phloic hyphae requires the hyphal tip to convert from a narrow tip to an enlarged structure encircling the host phloem elements (fig. 5*C*). Often this involves considerable contortions to the cell to achieve the coverage and the shape change. A pectic matrix and, in general, a looser matrix may allow the cells a greater amount of bending and accommodation than the relatively rigid cellulose-xyloglucan matrix, making this morphological modification of the phloic hyphae possible. A comparable situation occurs in laticifer cells that grow intrusively, and these cells are also enriched in pectins compared with the surrounding nonlaticifers (Serpe et al. 2004). Structurally, the lamellate wall of the phloic hypha resembles the cell walls of cultures grown on the cellulose biosynthesis inhibitor dicholbenil (Sabba et al. 1999), again indicating a greater porosity and plasticity than in the typical cellulose-xyloglucan cell wall. An additional advantage may lie in the movement of metabolites across the walls. Unlike in the connection of xylic hyphae to host xylem, no direct flow of metabolites from the host phloem to

the phloic hyphae is possible. Flow must be primarily or exclusively apoplastic, and the conversion of the walls to a much looser texture with the loss of much of the cellulose-xyloglucan backbones might also facilitate the flow of sucrose across the apoplasts of host phloem and dodder phloic hyphae. Smooth ER in sieve cells of other species has been shown to have an ATPase activity that aids in transmembrane movement. Compared to other sieve elements in both the dodder and the host, the phloic hyphae cells have a much more abundant smooth ER at the tip of the phloic hyphae (fig. 4), indicating a considerable potential enhancement of that activity.

Although the absorbing or phloic hypha has been termed a transfer cell, the cells in the dodder-*Impatiens* parasitic union displayed no wall ingrowths, although true transfer cells of the host displayed wall ingrowths immediately adjacent to these phloic hyphae (fig. 4). This system was chosen for its relative ease of fixation compared to that of many other dodder-host interactions. *Impatiens* tissue is soft and fixes well in the relatively high percentage of glutaraldehyde required to fix the dodder and the host. The use of PIPES buffers and high percentage of glutaraldehyde has similarly improved the fixation of plant cells in almost every case (Salema and Brandao 1973), as has the mix of Spurr's and Epon resins, which combines the best quality of both resins and is considered the best for embedding plant cells (Bozzola and Russell 1991). Thus, the fixations herein describe the best sort of traditional fixations (e.g., not involving cryotechniques, which would be nearly impossible on this bulky and watery tissue). Phloem tissue is difficult to fix even under the best of conditions. From these considerations, it could be argued that previous structures designated as wall ingrowths (e.g., Dawson et al. 1994) might be considered artifactual separations of the plasmalemma from the wall in these cells rather than a transfer-type cell. The high osmotic potential of these cells relative to neighboring ones might facilitate the chances for fixation artifacts. It should be noted that the data I describe are for only the *Impatiens*-dodder parasitic union, and it is possible that in other systems (or other dodder species), transfer-type phloic hyphae may indeed exist, although the data herein indicate that those observed previously are probably artifactual.

Does Dodder Induce New Vascular Synthesis in the Host?

Previous reports (summarized in Dawson et al. 1994) have indicated that the presence of a dodder hypha induces the host to form new vascular elements near the dodder, facilitating its parasitism. In this study, I searched for evidence to support this theory by complete serial sectioning of more than 20 haustoria on stems and about half that number on petioles and leaves, plus partial sets of serial sections (from about one-fourth to one-half) of many other haustoria. In *Impatiens*, the vascular arrangement of the stem is very regular. A ring of vascular tissue lies all around the stem, with xylem and phloem in close proximity. At no time were any extensions or new vascular tissues initiated toward the haustorium at any stage of dodder invasion. In the dodder invasion of the leaves, there was some indication that new vascular elements were being formed and/or that the vascular system was changed in position because the huge haustorium had displaced other cells in this tissue. In these oddly positioned host phloem cells, the suspect host cells were always like those in minor veins (e.g., fig. 5*A*). Also, in leaves, the hyphae sometimes grow between the cells rather than through them, as is typical of almost all of the hyphal penetration in the stem (Vaughn 2003). This cell-cell separation caused by the hyphal growth may cause shifts in the tissue. Alternately, it is well known that cytokines and auxins induce vascular differentiation (Church and Galston 1988). Levels of these might be altered by the hyphal invasion, and these hormones might cause a cascade of effects resulting in more vascular differentiation. Age might also be a factor in whether there is an induction of host vascular tissue. The *Impatiens* stem is older than the leaves and petioles and, as such, might be less amenable for the induction of new vascular tissue formation.

Similar conclusions can be reached for induction of xylem formation in the stem of the *Impatiens* plant. In the material stained with the LM 10 and LM 11 antibodies, only xylem of the host and the dodder are labeled. The host has a very regular distribution of xylem elements. Sections cut perpendicular to the long axis of the impatiens stem clearly show the regular distribution of xylem and the irregular xylic hyphae joining the xylem elements. There appears to be little or no new growth of the host xylem. Rather, there appears to be either a clean opening with no disruption of the host xylem or some misarrangement of the host xylem elements after the hyphae has grown through the host and the wall has then been broken by the dodder. Dodder is well equipped with a battery of wall-degrading hydrolytic enzymes (Nagar et al. 1984; Chatterjee and Sanwal 1999), so such a wall breach is quite plausible. These same enzymes might be involved in wall loosening and modification that occurs in the phloic hyphae as well.

Does the Morphological Evidence Support the Evidence from Flow of Markers?

Haupt et al. (2001) and Christensen et al. (2003) have used fluorescent markers to monitor the flow of material from the host to the dodder. In the case of xylic hyphae, there is a clear opening between the xylem elements of the host and the dodder. Not surprisingly, movement of the xylem-mobile dye Texas red was also noted (Christensen et al. 2003). However, movement of this marker was fairly slow, possibly because of the low transpiration rates of the parasite. There was relatively little movement of phloem-mobile carboxyfluoresceine before 2 d in parasitized geranium stems (Christensen et al. 2003), although movement of this phloem-mobile marker did eventually occur. Unexpectedly, green fluorescent protein, which should not cross intact plant membranes, did cross from phloem cells of a transformed host into the dodder (Haupt et al. 2001).

The movement of the Texas red dye from host to parasite is well substantiated by the open xylic hyphae to host xylem connection that is presented here and by others. However, the data for phloem transfer of a phloem-expressed green fluorescent protein (MW ca. 27 kD) indicate that a similar cell-to-cell opening must occur in the phloic hyphae as well.

Serial sections through phloic hyphae and host phloem cells reveal no such connections (results herein and elsewhere). Could it be that the modifications to the cell wall, essentially resulting in the replacement of the standard cellulose-xyloglucan network with one enriched in pectin, could account for this phenomenon? At earlier stages, there are cell-to-cell connections through plasmodesmata in searching hyphae, although as the hyphae develop, these connections appear to be occluded (e.g., Vaughn 2003), indicating their temporary nature. Similarly, the mature phloic hypha has no interspecies plasmodesmatal connections at its tip that would facilitate this exchange, although they did exist up until the time of the searching hypha's contact with the host phloem. Unfortunately, the level of detection of the fluorescent probes does not enable any discrimination of possible entry sites, such as possibly extant plasmodesmatal connections. As the hypha is growing, ectodesmata are produced on the hyphal surface (Vaughn 2003) and become functional plasmodesmata as they link with host plasmodesmata. However, these remnant ectodesmata might also serve as conduits from leaky or compromised host phloem and the haustoria. The movement of phloem-mobile viruses between dodder and hosts has been well established (Bennett 1944), also indicating that apoplastic flow, possible movement through extant ectodesmata, or a reconnection of plasmodesmata must occur. Considering the high sink strength of the dodder tissue (Wolswinkel 1978), it might be possible that even ephemeral cytoplasmic connections between not fully occluded plasmodesmata might allow for transfer of phloem-mobile compounds, despite limited apoplastic flow.

Dodder Uses Wall Modification in All Facets of Its Parasitic Life

In this and the companions in this series of articles (Vaughn 2002, 2003; K. C. Vaughn, unpublished manuscript), dodders appear to be master manipulators and users of cell wall modifications to accomplish all phases of parasitism. In the attachment phase, the cement that aids in the attachment of the dodder to the stem is enriched in highly de-esterified pectins (Vaughn 2002). As the hyphae traverse the host tissue, the dodder excretes expansin into the host tissue, allowing for the loosening of the existing wall and the synthesis of a new coating wall (Vaughn 2002, 2003) by the host. This coating wall contains pectin constituents not noted in the host wall (Vaughn 2003). In the final conversion of the searching hyphae, the extensive modification to both the hyphal anatomy and wall composition described herein occurs. Xylic hyphae accumulate xylans and form secondary walls in a cell that had been until then a tip-growing cell. Openings into the host must be created with cellulytic enzymes (Nagar et al. 1984). In phloic hyphae, loosening of the cellulose-xyloglucan cell wall facilitates not only the severe shape change required of these cells but also the transfer of saccharides apoplastically. These transformations may make dodder one of the master manipulators of wall components to facilitate its parasitism.

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