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Dodder hyphae invade the host: a structural and immunocytochemical characterization

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Summary. Dodder (*Cuscuta pentagona*) hyphae are unique amongst the parasitic weeds for their ability to apparently grow through the walls of the host plant. Closer examination reveals, however, that the hyphae do not grow through the host but rather induce the host to form a new cell wall (or extend the existing wall) to coat the growing hypha. This chimeric wall composed of walls from two species is even traversed by plasmodesmata that connect the two cytoplasms. Compositionally, the chimeric wall is quite different from the walls of either the host or in other cells of the dodder plant, on the basis of immunocytochemical labeling. The most striking differences were in the pectins, with much stronger labeling present in the chimeric wall than in either the host or other dodder walls. Interestingly, labeling with monoclonal antibodies specific to arabinan side chains of rhamnogalacturonan I pectin fraction was highly enriched in the chimeric wall, but antibodies to galactan side chains revealed no labeling. Arabinogalactan protein antibodies labeled the plasma membrane and vesicles at the tips of the hyphae and the complementary host wall, although the JIM8-reactive epitope, associated with very lipophilic arabinogalactan proteins, was found only in dodder cells and not the host. Callose was found in the plasmodesmata and along the forming hyphal wall but was found at low levels in the host wall. The low level of host wall labeling with anticallose indicates that a typical woundlike response was not induced by the dodder. When dodder infects leaf lamina, which have more abundant intercellular spaces than petioles or shoots, the hyphae grew both intra- and extracellularly. In the latter condition, a host wall did not ensheath the parasite and there was clear degradation of the host middle lamellae by the growing hyphae, allowing the dodder to pass between cells. These data indicate that the chimeric walls formed from the growth of the host cell wall in concert with the developing hyphae are unique in composition and structure and represent an induction of a wall type in the host that is not noted in surrounding walls.

Keywords: Dodder; *Cuscuta pentagona*; Hypha; Parasitic weed; Polysaccharide; Immunocytochemistry.

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Introduction

Dodders are the most important group of parasitic weeds in the world, inhabiting virtually every continent and causing untold damage to both crop and noncrop species (Kujit 1969, Malik and Singh 1979, Dawson et al. 1994). Because dodders are obligate parasites, requiring both water and all other nutrients from the host, these plants must attack the host rapidly and achieve contact with the host xylem and phloem before their own seed reserves are depleted. Thus, the dodder must perform the delicate balancing act of an aggressive invasion of the host while at the same time not damaging it sufficiently to cause host defense responses nor wounding it so much as to interfere with the host's ability to conduct water or produce photosynthate. Despite the ubiquity of this group of weeds, relatively little is known of the mechanisms by which dodder invades the host. In a previous report from my laboratory (Vaughn 2002), the mechanisms by which dodder attaches to the host are described, whereas in this report the mechanisms of host penetration and host response to this invasion are investigated.

Light and more limited electron microscopic studies (Dörr 1968a, 1968b, 1969; Lee and Lee 1989; and summarized in Kujit and Toth 1976, Dawson et al. 1994) have elucidated the basic steps of the dodder invasion. After attachment to the host, the lower haustorium (Lee and Lee 1989) penetrates the host tissue, making a fissure in the host by either mechanical or enzymatic means. Epidermal cells of the lower haustorium begin to elongate (now called searching hyphae) and penetrate the host tissue on their way to contact with the xylem and phloem of the host. These tip-growing

hyphal cells may extend up to 800 µm in this searching process. Light microscopic studies of the dodder hyphae reveal an apparent growth of the hyphae right through the host tissue (e.g., Lee and Lee 1989). However, electron microscopic examination revealed that the hyphae apparently did not grow through the host but were coated with a layer of "stretched" (Dawson et al. 1994) host wall. This mechanism allows the dodder to penetrate the host, yet essentially still be extracellular, thus minimizing stress to the host. Because dodder relies on the host totally (or nearly so, see Sherman et al. 1999) for both fixed carbon and water throughout its life cycle, it is imperative that the dodder does not stress the host by disrupting the flow of either water or photosynthate.

In this study, the characteristics of the dodder invasion of the host are investigated by microscopic, cytochemical, and immunocytochemical techniques. These data reveal the varying methods by which the dodder is able to penetrate the host and the unique composition of the chimeric wall formed in the interaction of host and parasite as the hyphae move through the host tissue. Some of these data have been recently presented in abstract form (Vaughn et al. 2001).

Material and methods

Plant material

Plants of impatiens (*Impatiens sultanii*) were grown as described previously under constant illumination in a Conviron growth chamber (Vaughn 2002). Dodder (*Cuscuta pentagona*) seeds were scarified with concentrated sulfuric acid, washed extensively in bicarbonate and water (as per Sherman et al. 1999), and then placed on top of the potting medium in which the impatiens plants are growing. Materials for this study were fixed during the initial stages of dodder invasion of the host and after a successful parasitic union had been completed. Haustoria associated with stems, petioles, and leaves were investigated.

Immunogold-silver light microscopy

Sections of tissue containing developing and mature pieces of dodder invading the host tissue were fixed in 3% (v/v) glutaraldehyde in 0.05 M piperazine-N, N′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.4) at room temperature for 2 h. The samples were washed in two exchanges of PIPES buffer, 15 min each, and dehydrated in an ethanol series at 4 °C. After two exchanges of 4 °C absolute ethanol, the samples were transferred to a –20 °C freezer and embedded in London Resin White resin, by increasing the concentration of London Resin White by 25% increments each day. The samples were left in 100% London Resin White resin for 2 days at –20 °C and then allowed to warm to room temperature and placed on a rocking shaker for 24 h. The segments were then transferred to BEEM capsules, the capsules sealed, and placed at 50 °C for 2–3 h to effect polymerization.

Sections (0.35 µm thick) were cut with a diamond histoknife (Delaware Diamond Knives, Bear, Del., U.S.A.) and mounted on clean glass slides coated with chrome-alum to enhance section adhesion. Rings were drawn around the sections with wax pencils to maintain the fluid around the sections in subsequent steps of the protocol. The slides were then moved to an incubation chamber with high relative humidity for the incubation steps. The steps and times are as follows: 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS), 30 min; primary antiserum diluted 1 : 8 to 1 : 80 depending upon the antibody or antiserum, 4 h; three exchanges of PBS-BSA; 1 : 20 dilution of secondary antibody-gold (EY Labs, San Mateo, Calif., U.S.A.) in PBS-BSA; three washes of PBS. The slides were then rinsed with double distilled water from a squirt bottle and then three to six short incubations of water alone to remove residual chloride ions, which would interfere with the silver intensification.

For silver intensification, the sections were incubated in freshly prepared solutions from the Amersham InstenSe (Amersham, Arlington, Ill., U.S.A.) silver enhancement kit. A 50–100 µl aliquot of the solution was pipetted on each slide and the slides returned to the humid chamber for 15–30 min. After the silver had developed, the sections were washed thoroughly with distilled water from a squirt bottle, dried with a stream of compressed air, and then mounted with Permount. Sections were photographed with a Zeiss Axioskop light microscope. Sections serial to those used for immunocytochemistry were stained for 1 min with a 1% (w/v) Toluidine blue in 1% (w/v) sodium borate to assess structural preservation and to determine the positions of hyphae in treatments where hyphae were unlabeled with the particular antibody.

Immunogold transmission electron microscopy

Preparation of samples for immunogold transmission electron microscopy (TEM) follows the fixation and embedding protocols described above for immunogold silver (above) or for transmission electron microscopy (below). Sections from these block faces were cut with a Delaware diamond knife at 99 nm (pale gold reflectance) and mounted on 300-mesh gold grids. The grids were then processed through the localization protocol exactly as described for the slides in the immunogold silver protocols before the intensification steps (above), except that the grids are floated on 4 µl drops of the solutions. After the water wash, the samples are dried and then stained for 2 min in 2% (w/v) uranyl acetate and 30 s in Reynolds lead citrate before observation with a Zeiss EM 10 CR electron microscope. Sections from several block faces were used in the examination and 20 random micrographs were counted to determine the density of gold particles over structures of interest.

Electron microscopy and standard light microscopy

Samples were fixed in 6% (v/v) glutaraldehyde in 0.05 M PIPES buffer (pH 7.2) for 2 h at room temperature and then washed in two exchanges of 0.10 M cacodylate buffer (pH 7.2) for 15 min each. Postfixation was carried out in 2% (w/v) osmium tetroxide in 0.1 M cacodylate buffer for 2 h at room temperature. After a brief water rinse, the samples were incubated in 2% (w/v) uranyl acetate overnight at 4 °C. After a water rinse to remove residual uranyl acetate, the samples were dehydrated in an acetone series and then transferred to 100% propylene oxide. Embedding was carried out slowly by infiltrating in increasing increments of 25% plastic resin (1 : 1 mixture of Spurr resin and Epon), 2–4 h/step. The propylene oxide was allowed to evaporate through tiny holes in the lid of the sample vials from the 75% plastic step overnight, gradually increasing the concentration of resin. After reaching 100% resin, fresh resin was added to the sample vials and the samples were rocked on a rotating platform for 24–48 h to enhance plastic infiltration. BEEM tear-away molds were used to flat embed the specimens and poly-

merization of the plastic took place at 68 °C. Pieces of tissue were cut from the polymerized plastic with jeweler's saws and mounted on acrylic rods for sectioning.

Thin sections (with silver to pale gold reflectance colors) were cut with a Delaware diamond knife and collected on 300-mesh copper grids or Formvar-coated slot grids (serial sections). Sections were stained for 7 min each in 2% (w/v) uranyl acetate and Reynolds lead citrate before observation with a Zeiss EM 10 CR transmission electron microscope.

For light microscopy, sections (0.35 μm thick) of the same block faces used for TEM analysis were cut with a histological diamond knife and mounted on chrome-alum coated slides. Some block faces containing abundant hyphae were cut and mounted individually in serial sections so as to trace the paths of individual hyphae as they grew in the host tissue. Sections were stained with 1% (w/v) Toluidine blue in 1% (w/v) sodium borate for 3–5 min. After mounting in Permount resin, the sections were photographed with a Zeiss Axioskop light microscope.

Antibodies and specificity

Most of the antibodies and antisera used in this study have been described in other papers using these technologies (Sabba et al. 1999, Vaughn 2002) and are presented here briefly. To eliminate nonspecific binding of the secondary antibody (sometimes a problem with walls), some controls were run in the absence of primary antibody. For some of the other antibodies and the cytochemical probe cellulase-gold, the antibodies or probes were incubated in pachyman (callose), highly esterified citrus pectin (JIM7), or carboxymethyl cellulose (cellulase-gold). All of these preincubations essentially eliminated any labeling of the sections. For characterization of pectins, the monoclonal antibodies that recognize chiefly an esterified pectin epitope (JIM7) or chiefly de-esterified pectin epitope (JIM5) and a polyclonal antiserum that recognizes the de-esterified polygalacturonic acid (PGA) backbone (Moore and Staehelin 1988) were utilized. Recent investigations by Willats et al. (1999, 2001) indicate that the assigned specificities of these two monoclonal antibodies may not be as exact as previously presumed. However, in material embedded for TEM by standard protocols, JIM5 seems to recognize only a highly de-esterified pectin epitope and these labeling patterns are very similar to those obtained with the anti-PGA backbone polyclonal serum. Pretreatment of the sections with 0.1 M sodium carbonate prior to the labeling protocol eliminated labeling by the JIM7 monoclonal antibody but greatly enhanced the labeling of both JIM5 and the PGA backbone serum. The LM5 and LM6 monoclonal antibodies recognize the 1→4-galactan and 1→5-arabinan side chains of rhamnogalacturonan-I (RG-I), respectively (Willats et al. 1999, 2001). These monoclonal antibodies were raised to the purified polysaccharide and are highly specific. The arabinogalactan protein (AGP) rat monoclonal antibody JIM8 and the CCRCMM7 and the AGP mouse monoclonal (BioSystems Australia) antibodies are highly specific to the plasma membrane-associated AGPs. Similarly, the polyclonal antibodies to xyloglucan and monoclonal antibody to fucosylated xyloglucan (CCRCMM1) are well characterized and specific for each of these polysaccharides (Moore and Staehelin 1988, Freshour et al. 1996).

Results

Growth of dodder hyphae within host tissue

After the successful attachment of the parasite to the host, a large mass of dodder tissue known as the inner or lower haustorium (Lee and Lee 1989) invades the host tissue by creating a fissure in the host stem tissue (Fig. 1). From the inner haustorium tissue, epidermal cells known as searching hyphae begin to elongate dramatically, apparently growing through the host tissue (Fig. 1). The searching hyphae may extend up to 800 μm before contact with the host phloem or xylem cells (Dawson et al. 1994, this study). In a single 0.35 μm thick section, a single hypha can be seen to traverse several cells, extending about 100–200 μm. In addition to their "searching" action, their extensive growth may aid in anchoring the parasite securely to the host plant.

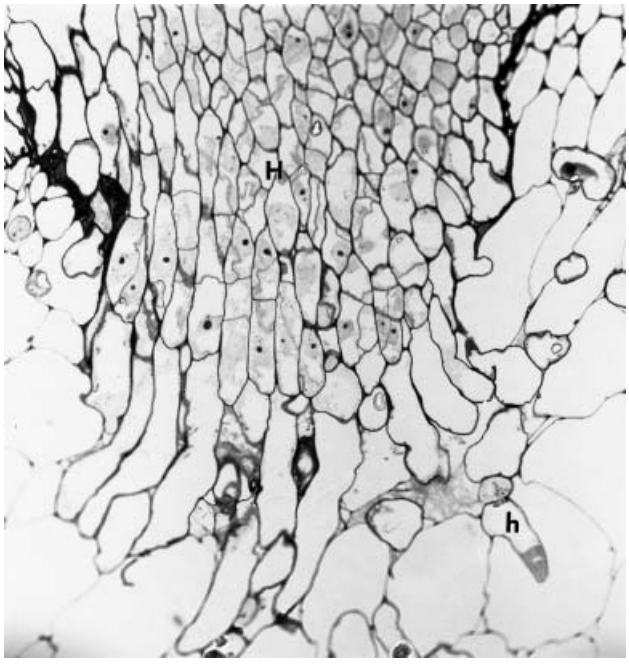

Fig. 1. Light micrograph of dodder invading a stem of impatiens. The lower haustorium (*H*) is a peg of tissue inserted into the impatiens tissue. From this haustorial peg, elongated epidermal cells called hyphae (*h*) appear to grow through the host cells. ×150

Searching hyphae are tip-growing cells, in some ways similar to pollen tubes or fungal hyphae in structure. The growing end of the hypha (Fig. 2 A, B) is enriched in Golgi-derived vesicles with larger organelles (plastids and mitochondria) and has a prominent very lobed (and probably polyploid) nucleus, progressively back from the tip. Large strands of actin are found throughout the ends of the developing hyphae and are in contact with the abundant vesicles present in the cytoplasm. Although observations at the light microscopic level indicate that the searching hyphae appear to grow through the host tissue, electron microscopic observations reveal a

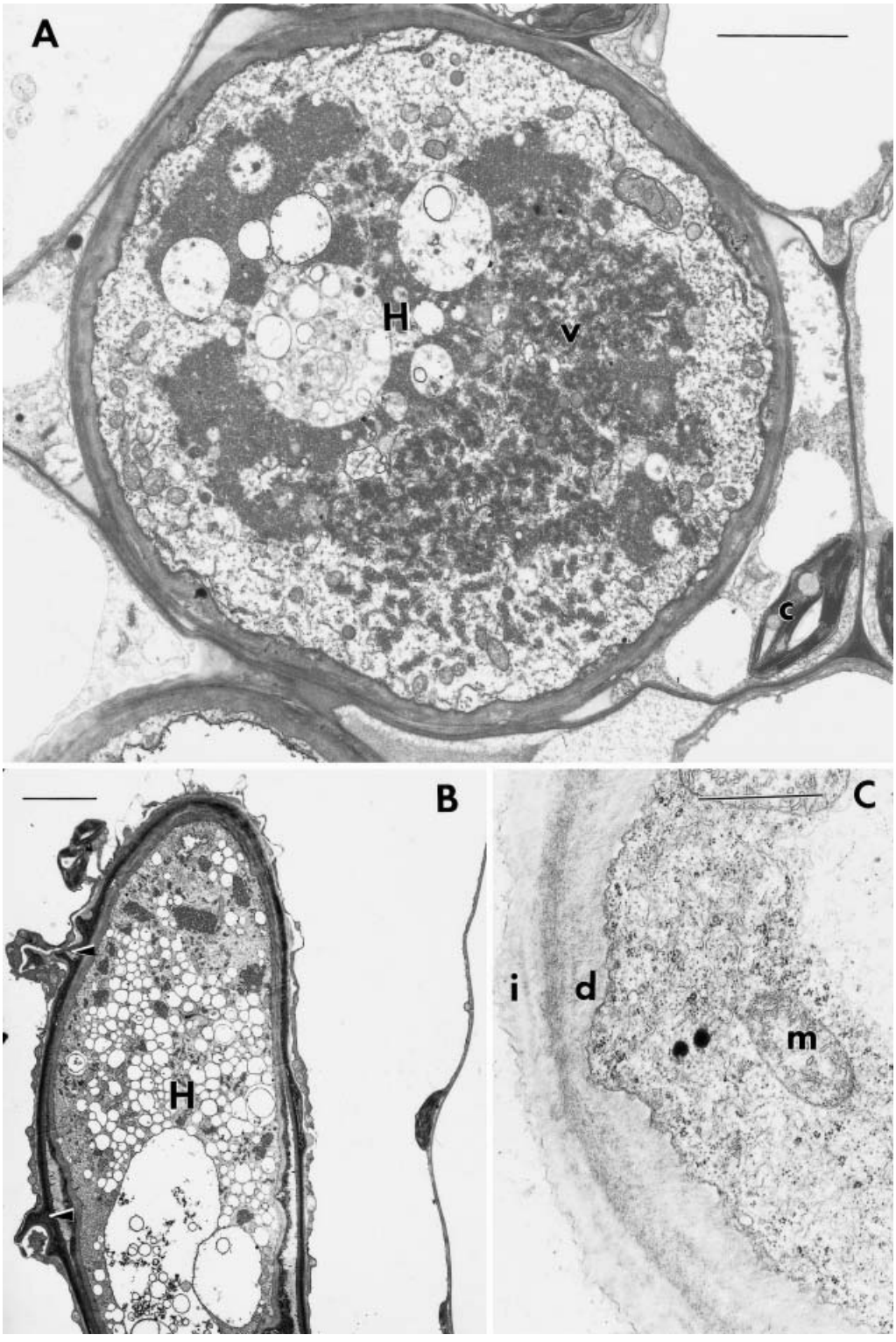

Fig. 2 A–C. Electron micrographs of hyphae invading the host tissue. **A** Cross-sectional view of a hypha (*H*) invading a leaf cell of impatiens at a point just behind the growing tip. Vesicles (*v*) and particles, some of which are membrane bound, have accumulated in the cytoplasm. *c* Chloroplast. **B** Longitudinal section of a hypha (*H*) growing through a leaf cell showing the typical tip growth of these cells. Appositions of the host wall are noted along the growing hypha (arrowheads). **C** Hypha growing through the stem tissue of dodder, showing the bilayered wall composed of a common wall of both impatiens (*i*) and dodder (*d*). *m* Mitochondrion. Bars: A and C, 1 µm; B, 2 µm

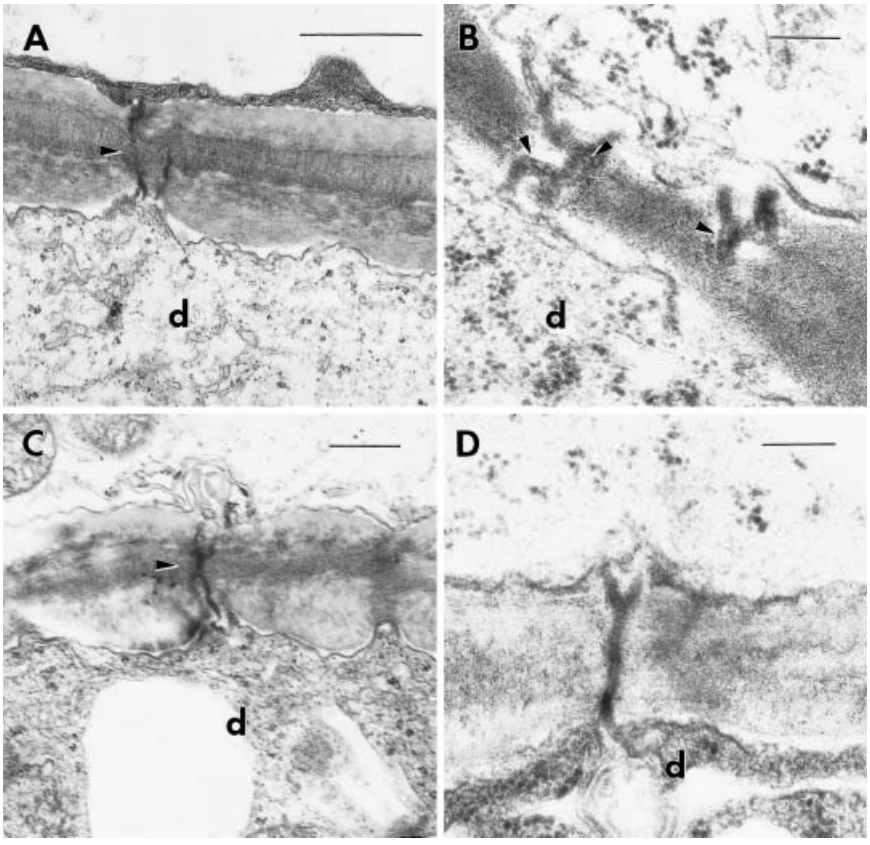

Fig. 3 A–D. Electron micrographs of chimeric dodder-to-impatiens plasmodesmata. **A** Simple plasmodesma (arrowhead) connecting the dodder (*d*) to the host. **B** Branched plasmodesmata with small electron-translucent particles (arrowheads) noted along the desmotubules. **C** Branched plasmodesma (arrowhead) connecting the dodder (*d*) and host cytoplasms. **D** Plasmodesma with fine fibrillar spokes radiating from the desmotubule; *d* dodder cytoplasm. Bars: A, 1.0 mm; B and D, 0.2 µm; C, 0.5 µm

significantly different mechanism. The hyphae are in fact not growing through the cells per se but are actually coated with a layer of host cell wall (Fig. 2 A, B). Although in most cases the wall of the host makes a rather uniform thin coating around the searching hyphae, occasionally there are appositions of the host wall (Fig. 2 B), similar to those formed in host tissue during fungal hyphae invasion. Appositions are less frequently observed in the hyphal wall. A more electron-opaque zone that corresponds to the middle lamellae can clearly be discerned separating the two walls (Fig. 2 C). Although, in general, the coating host wall appears to be thinner than the dodder wall, certain of these walls display a thickness similar to or greater than the hyphal wall (e.g., Fig. 2 C). Thus, it is unlikely that the wall is simply stretched during the invasion process, rather some new cell walls must be produced to cover a hypha growing through a host cell. Also the volume of coating wall surface must be rather extensive to cover a new wall that is nearly equal in volume to the preexisting host wall. In some cases, such as through multiple invasions in a single cell, the volume of the coating walls must greatly exceed that of the preexisting walls.

Unlike in pollen tube cells, plasmodesmata (or ectodesmata, as they are formed on the surface, not via cell division) are found abundantly along the walls of the developing hypha (Fig. 3). Connections between host and dodder cytoplasms occur frequently in the areas near the hyphal tip but are relatively rare in later stages of hyphal differentiation further back from the tip. Both relatively simple unbranched plasmodesmata (Fig. 3 A, C) and more complex branched structures are found between the host and parasite. Examples of collars, particles (Fig. 3 B), and radial spokes (Fig. 3 D) are found in these plasmodesmata, much as has been noted in other plasmodesmata that do not connect species to species. Plasmodesmata appear to degenerate in these older portions of the hyphae, with occlusions and hairpin turns back into the dodder cytoplasm, indicating their loss of function and possible recycling.

Variations in dodder invasion

What is described above is true for virtually all of the hyphae in dodder invasions of stem or petiole tissue. These host tissues have relatively few extracellular air

spaces so that cells occupy the majority of the tissue volume and the hyphae are in intimate contact with cells during virtually the entire invasion sequence. In leaf tissue, however, there are large air spaces between cells, especially in the spongy mesophyll. The dodder invasion in leaf tissue takes advantage of the air spaces, so that both extracellular and intracellular growth of the hypha occur (Fig. 4 A). Serial sections of a given hypha reveal that both extra- and intracellular growth may occur on a single hypha (Fig. 4 A). The extracellular growth of the hyphae is similar to that found in the intracellular growth except that the hyphae are not coated with any host cell wall material. Areas between adjacent cells of the leaf palisade are normally connected tightly by the middle lamellae. In those areas where hyphae are present, however, the area occupied by middle lamellae is now replaced by a meshlike electron-opaque network and is spread through a greater area than the relatively compressed middle lamella (Fig. 4 B, C). The growth of the searching hyphae intracellular in the leaf lamina, however, is identical to that in the petioles or stems, with a coating wall of the host surrounding the hyphae (e.g., Fig. 2 A).

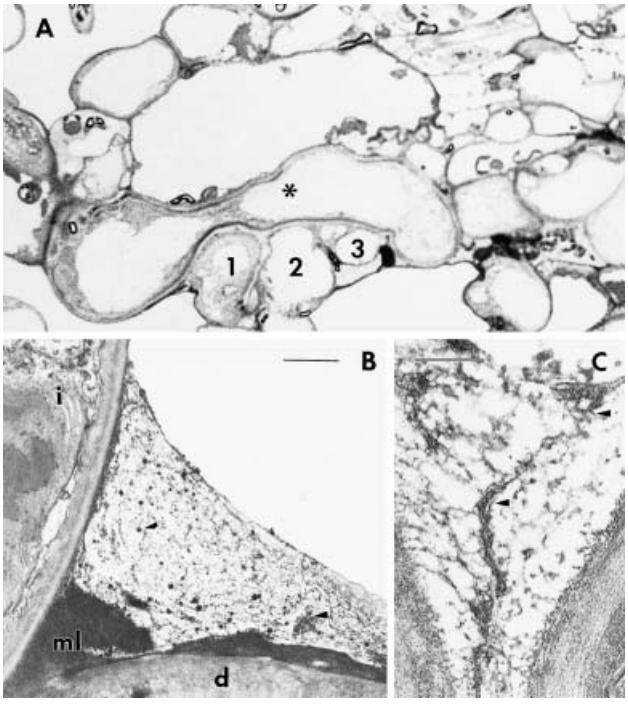

Fig. 4 A–C. Micrographs illustrating the extracellular growth of hyphae in leaf blade tissue. **A** Light micrograph revealing a prominent hypha growing extracellularly (arterisk) and three hyphae growing in an adjacent mesophyll cell. ×400. **B** Details of the intracellular space between an impatiens (*i*) mesophyll cell and an invading dodder (*d*) hypha. The middle lamella (*ml*) is mostly intact at the point of cell-to-cell contact, but a large area of fibrillar and granular material (arrowheads) now occurs in this area as well. **C** Higher-magnification details of the fibrillar material (arrowheads) that accumulates in the space normally occupied by middle lamellae. Bars: 0.5 μm

Characterization of the chimeric wall

To determine the composition of the unusual chimeric hypha and coating host wall, we probed sections of impatiens stem tissue infected with dodder with a battery of antibodies and probes to polysaccharides and wall proteins (Table 1). The technique of immunogold-silver-enhanced light microscopy was used for all of these antibodies to obtain a more "global" picture of the distribution of these epitopes in the tissue, and these observations were confirmed by immunogold

Table 1. Distribution of cell wall components in the dodder invasion of the host as determined by immunocytochemistry

Compound and antibody or antiserum	Labeling intensity[a] on host or dodder structure[b]			Additional site of labeling[c]
	Host wall	Dodder		
		wall	hypha	
Pectins				
JIM7	+	+	++	
JIM5	+	+	+	cement
CCRCMM2	+	+	++	
PGA polyclonal	+	+	+	cement
AGPs				
JIM8	–	+	+	PM
MAC207	+	+	+	PM
CCRCMM7	+	+	+	PM
Xyloglucans				
CCRCMM1	+	+	±	
xyloglucan polyclonal	+	+	±	
Extensins				
LM1	±	+	±	VT
JIM11	±	+	±	VT
JIM12	±	+	±	VT
extensin polyclonal	±	+	±	VT
Others				
LM5	+	+	–	
LM6	+	+	++	
callose monoclonal	+	+	++	PD
callose polyclonal	+	+	++	PD
cellulase-gold	+	+	±	

[a] +, labeling typical of plant cell walls; –, label absent; ±, label present at low levels; ++, label present at higher levels than typical plant cell wall

[b] Host wall, impatiens cell wall at sites not around hyphae; dodder wall, walls of cells except hyphae; dodder hypha, coating wall not included

[c] Cement, pectic substance secreted by dodder to attach to host; PM, plasma membrane; VT, vascular tissue; PD, plasmodesmata

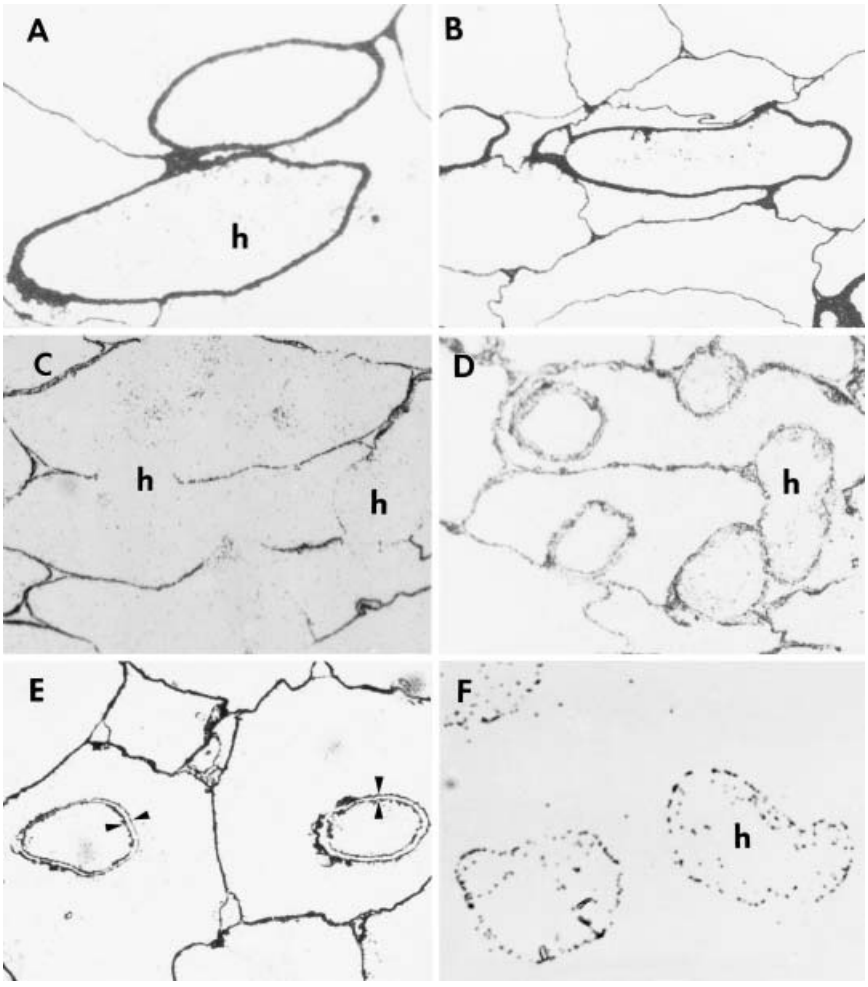

Fig. 5 A–F. Immunogold-silver light micrographs of dodder hyphae invading host stem tissue. **A** Two hyphae strongly labeled with the JIM5 monoclonal antibody, which recognizes primarily de-esterified pectins. **B** Hyphae labeled strongly with the JIM7 monoclonal antibody, which recognizes primarily highly esterified pectin. **C** The LM5 antibody, which recognizes 1→4 galactans, strongly labels the impatiens walls, although the walls of the hyphae (*h*) are unlabelled or very weakly labeled. **D** The LM6 antibody, which recognizes the 1→5 arabinans, labels both the host and hyphal walls strongly. **E** CCRCMM7 antibody labels AGPs in both the host and the dodder. The double-ring structures (arrowheads) are the result of label on both the dodder and host plasma membrane. **F** The JIM8 antibody, which recognizes highly lipophilic AGPs, labels on the dodder side of the growing hyphae. The reaction with this antibody is much more punctate than the distribution of the reaction with the CCRCMM7 or other AGP antibodies. A and F, ×550; all others, ×400

localizations at the TEM level on sections from these same block faces. Other antibodies were used on thin sections prepared for traditional TEM studies to allow high-resolution detection of their reactive sites. Four different block faces from four different fixations were probed with the complete battery of these antibodies and cytochemical probes at the light microscopic level. The results described herein are consistent both between and within a block face. In some cases serial thick sections were probed with several different antibodies so that the wall composition of a given hypha could be determined.

Pectins are a diverse group of polysaccharides and a number of antibodies and antisera are available to detect specific epitopes present in the wall. Almost all of these antibodies and antisera revealed an enhanced labeling of the chimeric wall compared to the host or nonhyphal dodder walls. Label with the monoclonal antibodies JIM5 and JIM7, which recognize primarily esterified or de-esterified pectins, respectively, and the polyclonal serum to the PGA backbone, strongly label the chimeric wall (Fig. 5 A, B). At the TEM level, a strong reaction at the dodder and host middle lamellae could be detected, especially with the JIM5 and PGA backbone probes (not shown). A strong reaction was noted throughout this wall when sodium bicarbonate treatment to de-esterify the pectins was utilized and the sections probed with either the JIM5 monclonal or the PGA backbone antiserum (not shown). This treatment eliminated labeling with the JIM7 monoclonal antibody, however.

RG-I is the major pectic fraction in the primary wall. Two different modifications, a galactan and an arabinan can be added as side groups that modify the properties of the RG-I. Monoclonal antibodies LM5 and LM6 specifically label RG-I modified with either 1→4-galactan or 1→5-arabinan, respectively (Willats et al. 1999, 2001). When sections are probed with LM5,

the host and nonhyphal dodder walls react strongly, but the chimeric wall of host and parasite is unlabeled (Fig. 5C). In contrast, labeling with LM6 monoclonal results in very intense label over the chimeric wall, but staining is less intense in the surrounding host walls (Fig. 5D). These data indicate that unlike other walls in both the host and the dodder, the chimeric wall has RG-I that is substituted only with arabinans, not galactans.

AGPs are associated with the plasma membrane, especially around areas of new cell wall biogenesis (Freshour et al. 1996, Gaspar et al. 2001). The label with these antibodies was especially distinctive in that a double ring formation could be detected when the chimeric wall was cut at right angles to the planes of the section, representing label of both the host and dodder plasma membranes. This pattern was observed for CCRCMM7 and an AGP monoclonal antibody raised by Anderson et al. (1984) (Fig. 5E). An exception to this pattern is the label with the JIM8 antibody, which labels only the dodder tissue, leaving the host cells unlabeled (Fig. 5F).

The host often responds to pathogen invasion by producing callose and extensin. Despite the extensive invasion of the host by the dodder, the host tissue produces relatively little callose and only relatively scattered patches of labeling was detected at the light microscopic level. Callose is found in association with the extensive plasmodesmata that are produced along the searching hyphae (Fig. 6A) and in all other plasmodesmata throughout both the host and parasite, confirming that the punctate labeling at the light microscopic level is indeed due to plasmodesmata. The tips of the growing searching hyphae are callose-enriched and a thin layer of wall containing callose extends further back in the hyphae, perhaps as remnants of this tip area of callose (not shown). Appositions formed on either the host or hyphal walls were labeled with anticallose (Fig. 6B, C). The only host structures exhibiting intense callose labeling were those walls that had been crushed in the penetration of the lower haustorium into the host tissue (Fig. 6D). These remnant cell walls were uniformly labeled. Likewise, extensin, another component of thickened or hardened walls after pathogen invasion, was probed for with a polyclonal serum and three different monoclonal antibodies. None of these label the chimeric walls or any other wall strongly with the exception of the vascular systems of both host and dodder (Table 1).

Although both the fucosylated xyloglucan monoclonal and the xyloglucan polyclonal antibodies label all the wall surfaces, there was a lower density of labeling over the chimeric wall than in the host walls (Table 1). A similar pattern was noted for the distribution of labeling with the affinity gold probe cellulase-gold. Because the chimeric walls are enriched in pectins, these polysaccharides must make up most of the bulk of the chimeric wall rather than the normal cellulose-xyloglucan matrix.

In the growth of hyphae through the air spaces in leaf tissue, the hyphal tip has a distinctly bilayered wall with an outer zone enriched in de-esterified pectins and an inner zone composed primarily of cellulose-xyloglucan (Fig. 7A, B). The threadlike material present in the areas where the hypha has grown between cells reacts only with antibodies to de-esterified pectin, indicating that these represent degraded areas of middle lamellae, as predicted by their location in this extracellular area (Fig. 7C, D).

Discussion

Hyphae induce growth of host wall in intracellular invasion but degrade middle lamellae in extracellular invasion

Dodder hyphae have long intrigued botanists and plant pathologist and even the term “hypha” was first applied to the structure in dodder haustorium, not to the more familiar and similar tip-growing cell in the fungi (Kujit 1969, Kujit and Toth 1976). Earlier light microscopic investigations reported that the hyphae grow through the host cells (reviewed in Kujit 1969, Malik and Singh 1979). However, it is clear that the hyphae grow through the cells encased in a layer of host cell wall that either is stretched (Tripodi and Pizzolongo 1967, Dörr 1968b, Dawson et al. 1994) or is de novo synthesized in concert with the expanding hyphae. Although the thickness of the coating wall is often less than that of the hyphae, there are ample cases of the walls of the host being equal to the thickness of that of the hyphae (e.g., Fig. 2B, C). Moreover, the presence of a wall of different composition between the coating wall and the surrounding host walls (e.g., Fig. 5) indicates that the new wall must be made de novo or involves the selective stretching of certain components of the old host wall.

Because dodders are obligate parasites, they rely on the host plant for both water and photosynthate to

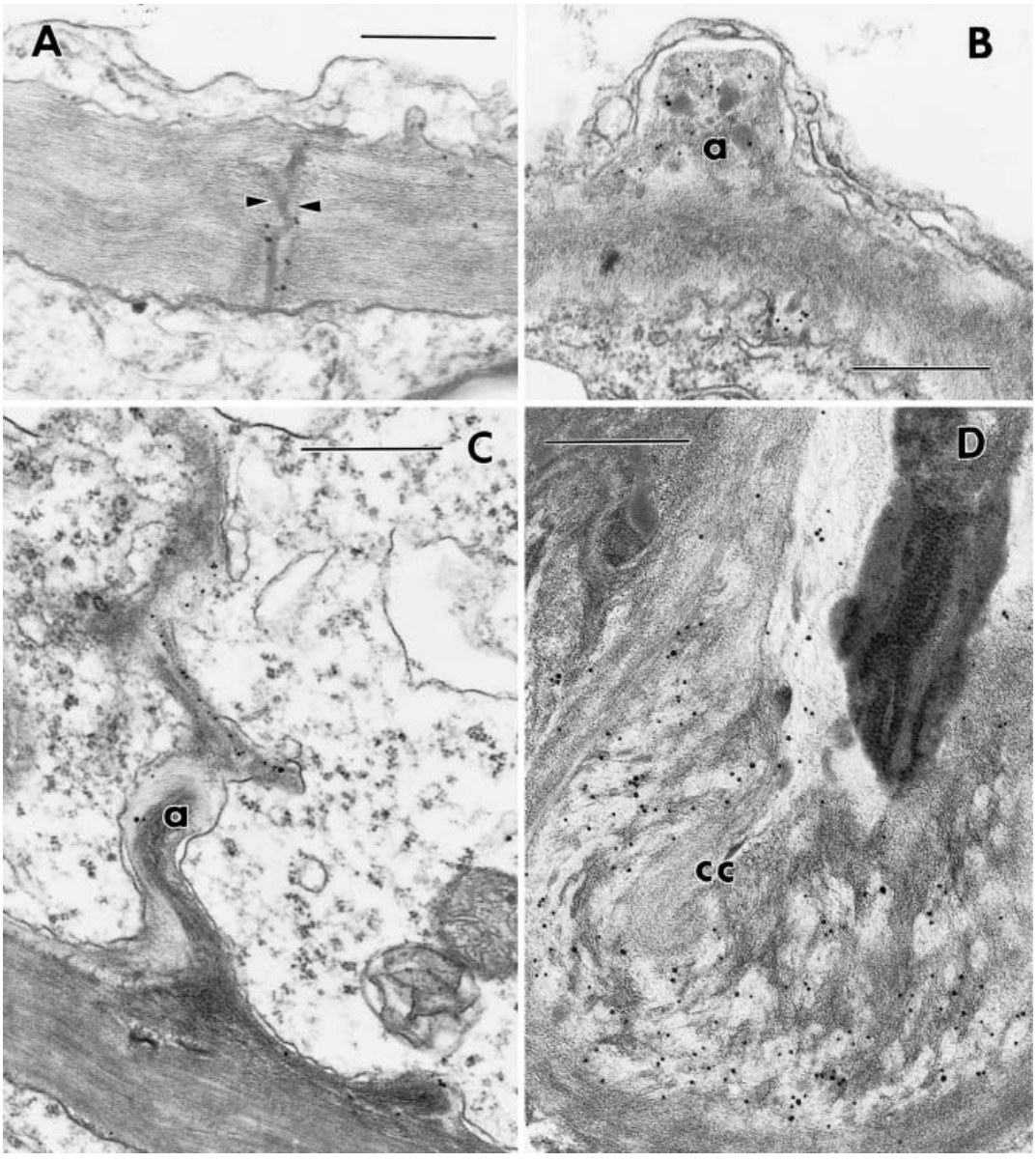

Fig. 6 A–D. Electron micrographs of immunogold labeling of anticallose. **A** Cross-species plasmodesmata connection (arrowheads) is labeled with anticallose along its length. **B** An apposition (*a*) in the host wall. **C** A longer, more filamentous apposition in the host wall. **D** A crushed cell (*cc*) of impatiens is strongly labeled. Bars: 0.5 µm

maintain their growth. Thus, the dodder must invade the plant rapidly but at the same time perturb the host minimally so that the host continues to function normally to sustain the parasite. In contrast, most pathogenic fungi would not require a functional host after a successful invasion. Because the host and dodder are physically separated by a wall during the invasion process, the host appears to be less perturbed than in the invasion by fungi, as there are few or no indications of wound-type responses of either callose or extensin production in the host walls (Fig. 6). The occasional wall appositions (Figs. 2 B and 6 B, C) are the only indication of any perturbation to the host around the growing hyphae. In contrast, fungal invasion almost always results in the extensive production of both callose and extensin by the host.

Remarkably, the dodder hypha appears to "sense" the difference between growth in cells and that between the cells. When the dodder grows between cells, the hypha appears to degrade the cell-to-cell cement in the middle lamellae and is thus more similar to the growth patterns described for the hyphae of the

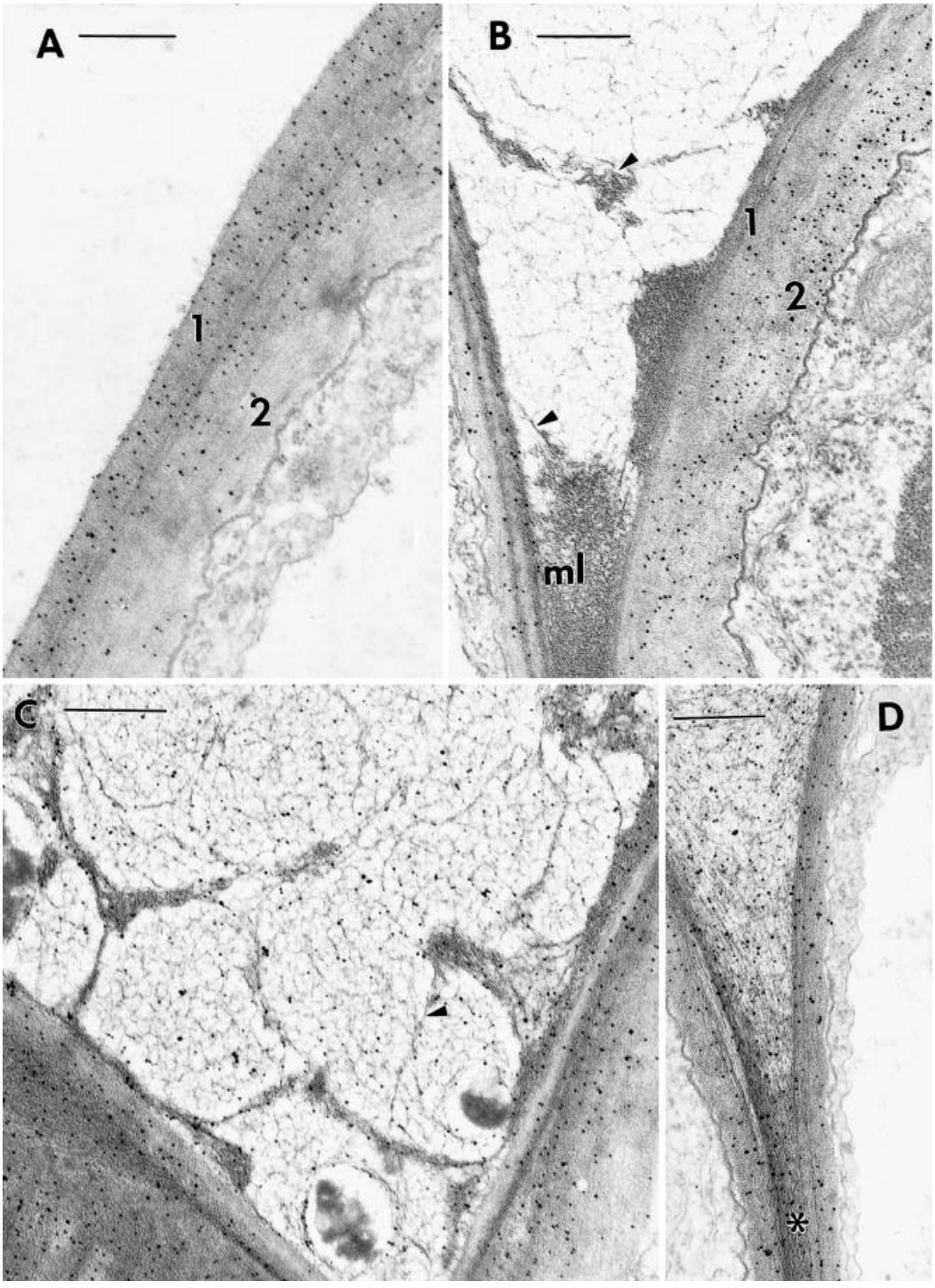

Fig. 7. Electron micrographs showing the dodder hyphae invading the host leaf blade extracellularly and the labeling with antibodies to the PGA backbone (**A**, **C**, and **D**) or xyloglucan (**B**). **A** Hyphal cell wall with distinct bilayered structure reveals a strong labeling in the outer layer (*1*) and a weak labeling in the inner layer (*2*). **B** Hypha with a bilayered wall in which the inner layer (*2*) is strongly labeled with antixyloglucan. The middle lamella (*ml*) is degraded and replaced by a meshlike network (arrowhead). **C** A large expanse of broken down middle lamella labeled with anti-PGA antibodies after chemical de-esterification of the section with sodium carbonate. Although the filaments are labeled with the antibodies, their densities are much less than on intact middle lamellae or in the walls. **D** A contact zone in which cell-to-cell contact is near normal (asterisk) and an area towards the top of the micrograph in which the middle lamellae has obviously been degraded. Bars: 0.5 μm

parasitic weed *Orobanche* spp. (Losner-Goshen et al. 1998). Few or no plasmodesmata are formed (or are degraded quickly) in the extracellular hypha of the dodder, even though they are formed extensively in hyphae that traverse the host cells. The hyphal wall in this growth pattern resembles the wall composition and organization of expanding epidermal cells, essentially a bilayered wall with the outer portions enriched in de-esterified pectin (Vaughn and Turley 1999, Vaughn 2002). Thus, depending upon whether the hypha grows through the wall or through air space, the hypha is able to modify its composition and organization of the wall and the machinery for invasion and penetration. In the case of intracellular invasion the host wall is induced to expand and in extracellular invasion the middle lamella is degraded. Both allow expansion of the hyphae but by radically different routes.

Composition of the chimeric wall

The composition of the chimeric wall formed from the dodder hypha and the coating host cell wall is unique and distinct from both the host and nonhyphal walls of the dodder.

Pectins are altered both quantitatively and qualitatively in the chimeric wall formation (Fig. 5 A–D and Table 1). Both the hyphal wall and the coating wall are enriched in both esterified and de-esterified pectins relative to the surrounding host walls (Fig. 5 A, B). In addition, there is a dramatic difference in the modifying side chains of the RG-I pectins in the chimeric walls. In the preexisting host walls, RG-I is modified both by the 1→4-galactan and the 1→5-arabinan side chains (Fig. 5 C, D). Label with both the LM5 and LM6 antibodies is also noted on all other walls of the dodder (not shown). In the hypha and coating host wall, however, there is label associated with the arabinan, not the galactan (Fig. 5 C, D). Arabinan side chains are associated with laticifer walls, which are flexible and/or invasive in growth (Serpe et al. 2001), and perhaps it is these characteristics of the RG-I so modified that are necessary for the circuitous movement of the hypha through the host tissues. Moreover, the presence of a new host wall with differences in RG-I side group composition compared to the surrounding host walls indicates that these walls are just not merely "stretched" but are also altered in composition. Those data indicate that the coating host wall is a de novo synthesized wall (or at least the pectins in that wall) in response to hyphal penetration.

Although AGPs as a group have been suggested to be involved in signaling processes, indicating where new walls are to be produced, and other developmental regulation, the functions of a given AGP or epitope(s) recognized by an AGP antibody are unknown (Gaspar et al. 2001). The presence of strong reactions of the CCRCMM7 (Freshour et al. 1996) and the AGP monoclonal antibody of Anderson et al. (1984) in both the dodder and the host, compared to the exclusive labeling of the dodder by JIM8, indicates a difference in AGPs between host and parasite. Recently, Gaspar et al. (2001) confirmed that the AGP recognized by JIM8 recognizes a very lipophilic AGP, whereas MAC207 and JIM13 antibodies recognize "general AGP epitopes". The growing dodder hypha secretes large numbers of Golgi-derived vesicles and it may be that a very lipophilic AGP is involved in an interaction between the lipophilic Golgi vesicles and the plasma membrane. Certainly the very punctate labeling of the JIM8 AGP monoclonal antibody may indicate regions of specific membrane fusions.

The low levels of labeling with either extensin or callose antisera in the host indicate that neither of the wall components frequently associated with wounding is induced by dodder invasion. Exceptions to this are the crushed, cytoplasm-less cells and tissues at the site of lower haustorium formation (Fig. 6 D), in which the cytoplasm exists only in the remnant form of degraded organelles or not at all. These data indicate that the host does not recognize the dodder hyphal invasion of its tissues as a pathogenic attack, either through or between cells. This apparent lack of host response is in striking contrast to the induction of pathogenesis-related proteins in the parasitic attack of *Orobanche* spp. (Joel and Portnoy 1998, Westwood et al. 1998), a parasite that does not induce a coating wall formation by the host.

Possible roles of plasmodesmatal species-to-species connection

The dodder hyphae-to-host plasmodesmata connection is one of the rare instances of direct species-to-species cytoplasmic contact in the plant kingdom (Dörr 1968a, 1968b, 1987; Blackman and Overall 2001; Ehlers and Kollman 2001). At the tip of growing hyphae, plasmodesmata (or ectodesmata) are produced in great numbers and the chimeric plasmodes-

mata are labeled by callose antibodies (Fig. 6 A), as are same-species plasmodesmata in cells of both the host and the dodder. Some of the plasmodesmata establish cross-species bridges between the host and the parasite, whereas others appear to be degraded or to meander through the cell walls, sometimes forming hairpin loops. In areas of the hyphae back from the tip, these connections become increasingly more rare, indicating that the connections are ephemeral. The presence of these cross-species plasmodesmata is unlikely to be mere happenstance and certainly begs questions as to messages flowing between the species. For example, the production of a coating host wall of a composition unique compared to the preexisting host walls indicates the induction of a new-wall-synthesizing machinery in a cell that has long stopped producing new wall. Whether mRNA, protein, or small molecules are involved in this reaction is not known, but it does represent an intriguing aspect of this unique parasitism, requiring further investigation.

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