

## Immunocytochemical Localization of Hydroxyproline-rich Glycoproteins in Tomato Root Cells Infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*: Study of a Compatible Interaction

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### ABSTRACT

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The accumulation of hydroxyproline-rich glycoproteins (HRGPs) in cell walls of dicotyledonous plants has been correlated with a general defense mechanism against pathogenic attack. An antiserum raised against purified melon HRGPs was found to cross-react specifically with HRGPs from tomato. This antiserum was used to study, by immunogold cytochemistry, the subcellular localization of these glycoproteins in tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. HRGPs were found to be present in low amounts in healthy plant cell walls and were not detected in cultured *F. o. radicis-lycopersici*. Cell walls of infected tomato root tissues, especially those harvested between

96 and 120 hr after inoculation, were markedly enriched in HRGPs. Accumulation of these glycoproteins appeared to be initiated after contact between the pathogen and the plant cell wall and to occur as a result of tissue damage. Although HRGPs were detected in wall appositions formed in the cortical area in response to infection, their restricted occurrence in walls of invaded cells showing signs of pronounced alteration suggests that accumulation of HRGPs in susceptible tomato plants is a late biochemical event. This supports the concept that early enhancement of HRGPs is a prerequisite to their efficiency in the protection of plants.

The production of hydroxyproline-rich glycoproteins (HRGPs) is a well-documented response of many plants to infection by a wide variety of microorganisms (10,13,17,20). The accumulation of these glycoproteins is thought to be part of a general defense mechanism against pathogenic attack (13,15). Support for this concept was drawn by the observations that artificial enhancement or inhibition of HRGP biosynthesis yielded increasing or decreasing resistance of melon plants to *Colletotrichum lagenarium* (Pass.) Ell. & Halsted (13,28). However, the exact function that HRGPs play in the resistance of plants still is unknown. One important facet in ascertaining the significance of HRGPs in plant disease resistance would be the evaluation of their spatial and temporal distribution within infected plant tissues.

In recent years, the interaction of tomato (*Lycopersicon esculentum* Mill.) root cells with the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoem. has received particular attention in relation to physiology (18) and ultrastructure (7,9). Using axenically produced seedlings of the susceptible Bonny Best and slightly tolerant Vendor cultivars, Charest et al (9) reported that *F. o. radicis-lycopersici* colonized the root epidermis within 24 hr, was mainly restricted to the outer cortical zone for 96 hr, and reached the vascular stele by inter- or intracellular growth within 144 hr. Wall appositions, including papillae, were observed frequently in cortical tissues of both cultivars and were interpreted to be a defense reaction to pathogen ingress because the fungus took longer to penetrate the outer cortical than adjacent inner tissues. More recently, these cytological observations have been complemented by cytochemical investigations with gold-complexed agglutinin of *Ricinus communis* L. (3,8). Such studies revealed an accumulation of galactose residues, not only at the periphery of fungal cells in close contact with the plant cell walls, but also in wall appositions and paramural vesicles. In light of the increasing amount of biochemical data reported from other fungal diseases (20), Benhamou et al (3) and Chamberland et al (8) suggested that the accumulation of galactose residues in well-delineated wall areas of infected tomato root cells likely reflected

an increasing level of HRGPs. This assumption was based on the fact that the detected galactose residues might constitute the carbohydrate moiety of these glycoproteins. These findings led us to investigate whether a direct correlation could be made between pathogen attack and HRGP induction in the compatible interaction between tomato and *F. o. radicis-lycopersici*.

An antiserum raised against purified HRGPs from melon (*Cucumis melo* L. 'Cantaloup Charentais') (22) was found to cross-react specifically with purified tomato HRGPs. This antiserum was used to study the subcellular distribution of these glycoproteins in tomato root tissues infected by *F. o. radicis-lycopersici*. Colloidal gold-labeled goat anti-rabbit immunoglobulins, used as second-step reagents, were found to be suitable for accurately revealing antigen-antibody interactions.

### MATERIALS AND METHODS

**Fungus culture.** The isolate of *F. o. radicis-lycopersici* used in this study was obtained from P. O. Thibodeau, Complexe Scientifique, Sainte-Foy, Québec. It was grown on potato-dextrose agar (PDA) medium at 22 C and was reinoculated and reisolated periodically from ripe tomato fruits.

**Inoculation of tomato plants.** Inoculation of tomato plants with *F. o. radicis-lypersici* was performed as described by Charest et al (9). Tomato seedlings (*L. esculentum* 'Bonny Best'), grown in axenic conditions, were inoculated at the three-leaf stage with a suspension of microconidia ( $1.5 \times 10^7$  spores/ml) deposited on the roots with a sterile syringe. This inoculation procedure did not cause wounding of the plants. Control plants were treated with sterile distilled water. All plants were maintained in a growth chamber at 22 C with a relative humidity of 75% and day length of 16 hr. Root samples were collected at 96 and 120 hr after inoculation.

**Tissue processing.** Samples (1 mm<sup>3</sup>) cut from actively growing colonies of *F. o. radicis-lycopersici* and similar samples from infected and healthy tomato root cells were fixed by immersion in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.2, for 2 hr at room temperature. After being washed in the same buffer, samples were dehydrated in a graded ethanol

series and embedded in Epon 812. Some specimens were submitted to a postfixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hr at 4 C before dehydration and embedding.

**Antibody production.** The antigen used for antibody production was the major extensinlike glycoprotein HRGP<sub>2b</sub>, which was extracted from melon cell walls (21). This cell wall glycoprotein was purified by affinity chromatography and characterized as described by Mazau et al (22). Antibodies were raised in New Zealand white rabbits. Preimmune serum was obtained before the first antigen injection. The purified antigen was injected in a three-step procedure, as follows. For the first immunization, 250 µg of purified HRGP<sub>2b</sub> was dissolved in 1 ml of deionized water, emulsified in an equal volume of Freund's complete adjuvant, and injected subcutaneously into several sites along the back. Subsequent booster injections of 180 µg of HRGP<sub>2b</sub> in Freund's adjuvant were given 4 and 6 wk after the first immunization. Seven days after the last boost, aliquots of blood were withdrawn every 5 days over a 1-mo period. The blood was centrifuged at 1,500 g, and the serum, collected and pasteurized at 56 C for 1 hr, was stored at -20 C.

**Dot-immunobinding procedure.** Specificity of the antiserum for tomato HRGPs was tested by dot-immunobinding assays according to a method described by Benhamou et al (4,5). Briefly, strips of nitrocellulose membrane were wetted by capillarity in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and dried. Samples to be tested (purified HRGP<sub>2b</sub> from melon, purified HRGPs from tomato, bovine serum albumin (BSA), and L-hydroxyproline) were dotted onto nitrocellulose in 2 µl droplets containing 1 µg of protein and allowed to dry for 60 min at 40 C. Unoccupied binding sites were blocked by incubating the nitrocellulose strips in 3% (w/v) gelatin in PBS, pH 7.4, for 45 min at room temperature. The strips then were transferred to a solution containing anti-HRGP<sub>2b</sub> diluted 1:200 in PBS and 0.25% ovalbumin for 60 min at 37 C and washed 4 × 15 min in PBS containing 0.05% (v/v) Tween 20. They were, thereafter, incubated in a peroxidase-conjugated goat antiserum to rabbit immunoglobulins diluted 1:500 in PBS-ovalbumin for 60 min at 37 C. After this incubation, strips were washed thoroughly with PBS-Tween and stained for protein detection with 0.03% (w/v) 3-3'-diaminobenzidine (DAB) in PBS containing 0.01% (v/v) H<sub>2</sub>O<sub>2</sub>. Control tests were performed by: 1) treating with preimmune serum, diluted 1:100 in PBS-ovalbumin, instead of antiserum anti-HRGP; 2) omitting the primary antiserum step; and 3) omitting both antibody steps.

**Immunocytochemical labeling.** Ultrathin sections of infected or healthy tomato root samples, collected on formvar-coated nickel grids, first were incubated on a drop of PBS-ovalbumin, pH 7.4, for 5 min at room temperature. They then were transferred to a drop of normal goat serum diluted 1:10 in PBS-ovalbumin, pH 7.4, for 60 min at room temperature and incubated on the primary antibody (rabbit anti-HRGP) diluted 1:100 or 1:200 in PBS-ovalbumin, pH 7.4, for 2 hr at 37 C. Grids were rinsed with Tris HCl, pH 8.2, containing 0.5 M NaCl and 1% BSA and incubated on a drop of colloidal gold (10 nm)-conjugated goat antiserum to rabbit immunoglobulins (GAR-gold antibodies) diluted 1:10 in the rinsing buffer for 30 min at room temperature. After washing with PBS, pH 7.4, and rinsing with double-distilled water, grids were contrasted with uranyl acetate and lead citrate and examined with a JEOL 1200 EX electron microscope (JEOL Co., Japan) at 80 kV.

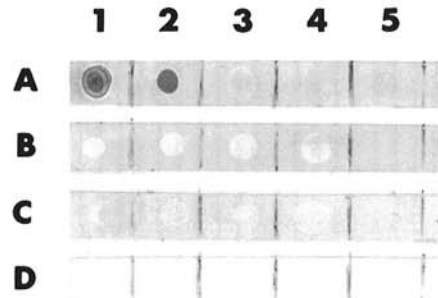
**Immunocytochemical controls.** Specificity of labeling was assessed by the following control tests: 1) incubation with the antiserum antimelon HRGP<sub>2b</sub> to which previously was added purified HRGP<sub>2b</sub> from melon (approximately 1:1 molar ratio); 2) incubation with the antiserum antimelon HRGP<sub>2b</sub> to which previously was added purified HRGPs from tomato (approximately 1:1 molar ratio); 3) incubation with preimmune serum diluted 1:100 in PBS-ovalbumin instead of antiserum anti-HRGP<sub>2b</sub>; 4) incubation with GAR-gold antibodies, the primary antibody step being omitted; and 5) successive incubations with primary antiserum, unlabeled goat antiserum to rabbit immunoglobulins, and finally GAR-gold antibodies.

**Reagents.** Nitrocellulose membranes and gelatin (EIA grade) were purchased from Bio-Rad Laboratories, Mississauga, Ont. Protein A, DAB, BSA, ovalbumin, Tween 20, and gold-conjugated goat antiserum to rabbit immunoglobulins were from Sigma Chemical Co., St. Louis, MO, and Epon 812 from JBS Co., Pointe Claire, Quebec, Canada. Purified HRGPs from tomato were a gift from D. T. A. Lampert, Michigan State University, East Lansing.

## RESULTS

**Characterization of the antiserum raised against melon HRGP<sub>2b</sub> by dot-immunobinding assay.** Specificity of the antiserum raised against melon HRGP<sub>2b</sub> for its corresponding antigen was demonstrated by the dot-immunobinding procedure using peroxidase-conjugated goat antiserum to rabbit immunoglobulins and DAB (Fig. 1) as second-step reagents. As little as 1 µg of melon HRGP was detected by the antiserum at a 1:200 dilution (Fig. 1, lane A, dot 1). Higher dilutions of the antiserum (1:400 and 1:800) yielded also positive reactions although a decreasing dot color intensity was noted (data not shown). In addition to specifically recognizing its corresponding antigen, the antimelon HRGP antiserum was found to cross-react with purified HRGPs from tomato (Fig. 1, lane A, dot 2). No cross-reactivity was observed with BSA (lane A, dot 3), hydroxyproline (lane A, dot 4), and PBS (lane A, dot 5). All control tests including the use of preimmune serum instead of primary antiserum (Fig. 1, lane B), the omission of the primary antiserum step which was replaced by an incubation in PBS (Fig. 1, lane C), and the omission of both antibody steps (Fig. 1, lane D) resulted in a considerable decrease of dot color intensity. This contributed, therefore, to the assessment of the specificity of the antimelon HRGP antiserum towards tomato HRGPs.

**Immunocytochemical labeling.** As mentioned earlier (2,3), success of the indirect immunogold approach depends upon maintenance of protein antigenicity and good ultrastructural preservation. Satisfactory preservation of cellular structures and molecular antigenicity were obtained when glutaraldehyde was used as the only tissue fixative. In contrast, a substantial loss of antiserum-binding sites was noted when tissues were postfixated with osmium tetroxide. In agreement with previous reports (2), these observations indicated that postfixation with osmium tetroxide should be omitted. Another parameter of great importance was found to be the preincubation of ultrathin tissue sections with normal goat serum. Omission of this step always resulted in an accumulation of nonspecific labeling over the entire sections. Finally,

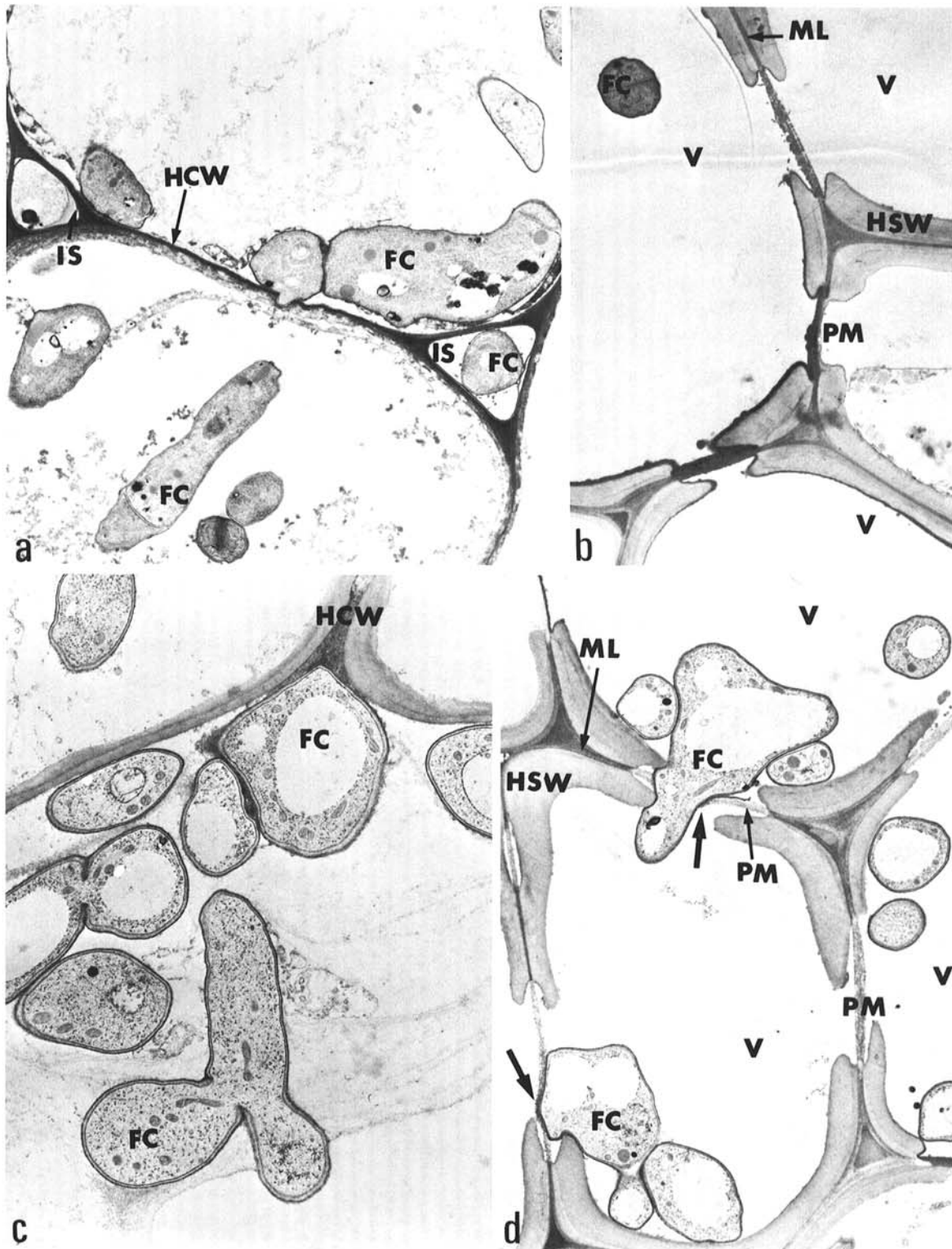


**Fig. 1.** Detection of antigen-antibody interactions by dot-immunobinding assay. Antigens to be tested are as follows: dot 1: hydroxyproline-rich glycoproteins (HRGPs), purified from melon infected by *Colletotrichum lagenarium*, 1 µg/dot; dot 2: HRGP<sub>pl</sub> (= precursor of extensin) purified from tomato, 1 µg/dot; dot 3: bovine serum albumin, 0.5 mg/ml in distilled water; dot 4: L-hydroxyproline, 1 µg/dot; and dot 5: 0.1 M phosphate-buffered saline (PBS), pH 7.4, 2 µl/dot. Primary antiserum is antimelon HRGP antiserum diluted 1:200 in PBS containing 0.25% of ovalbumin (lane A). Second-step reagent is peroxidase-conjugated goat antiserum to rabbit immunoglobulins. Control tests are the use of normal serum instead of antimelon HRGP antiserum (lane B), the omission of the primary antibody step (lane C), and the omission of both antibody steps (lane D).

the use of Tris-HCl buffer, with a high salt concentration (16) and containing BSA as an inert protein, reduced significantly the background staining over both tissue components and embedding resin.

**Ultrastructural aspect of fungal colonization.** From 48 to 96 hr after inoculation, tomato root colonization by *F. o. radices-*

*lycopersici* was intense in the epidermis and started to appear in the hypodermis (Fig. 2A). Fungal hyphae frequently were present within intercellular spaces of the cortex, generally in the vicinity of infected hypodermal cells. Colonization of such cortical cells resulted in severe cell disintegration as judged by the extensive cytoplasm breakdown (Fig. 2A). Penetration of the endodermis

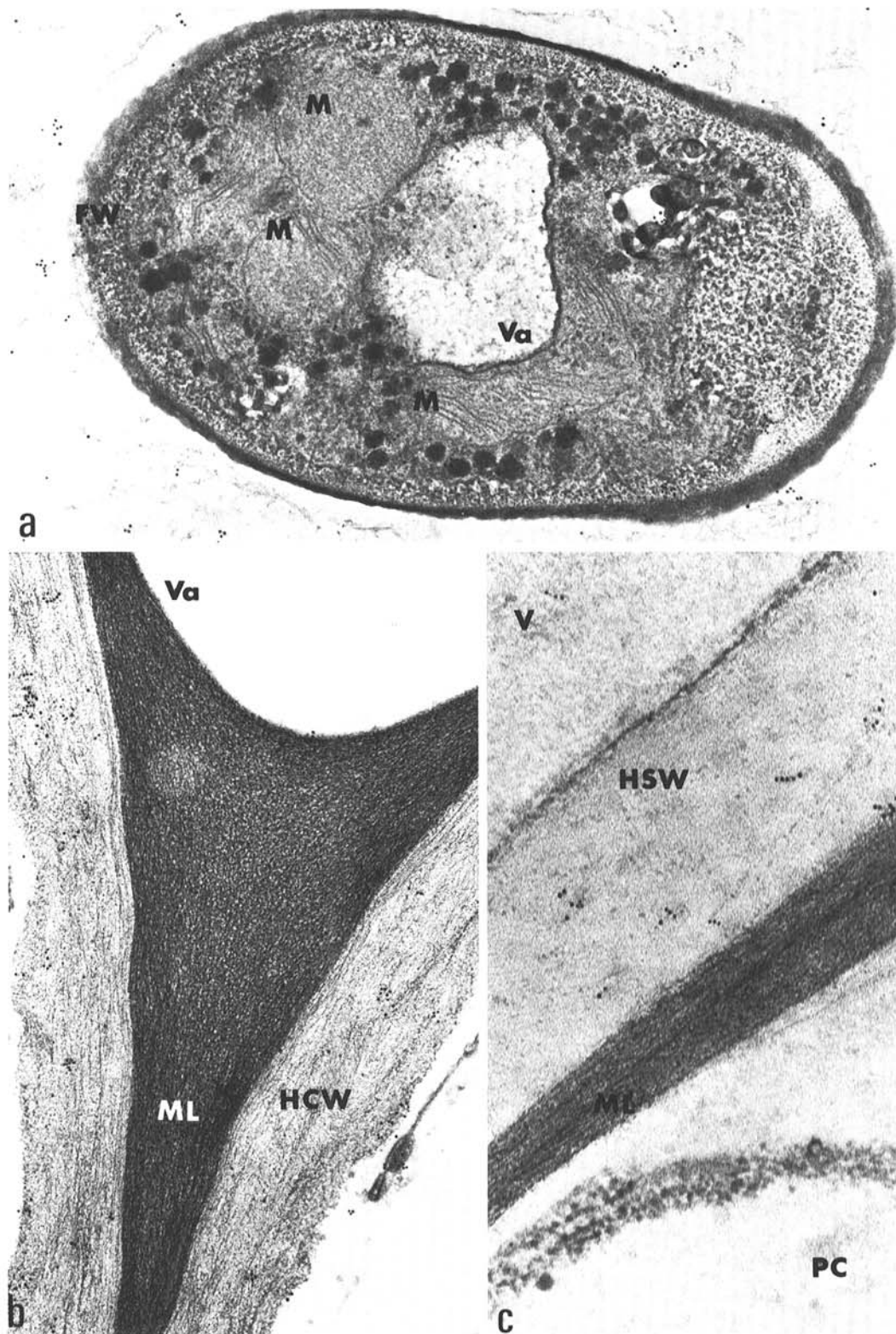


**Fig. 2.** Transmission electron micrographs of tomato root cells, cultivar Bonny Best, infected by *Fusarium oxysporum* f. sp. *radices-lycopersici*. **A**, Cortical area, 96 hr after inoculation. Numerous fungal cells, growing either intra- or intercellularly, are colonizing host cortical cells. Cytoplasmic breakdown is noticeable.  $\times 5,000$ . **B**, Vascular stele, 96 hr after inoculation. Xylem vessels are almost free of fungal colonization.  $\times 5,000$ . **C**, Endodermis, 120 hr after inoculation. Substantial invasion of host endodermis by cells of *F. o. radices-lycopersici* is observed. Host cell cytoplasm shows pronounced disruption.  $\times 6,000$ . **D**, Vascular stele, 120 hr after inoculation. Hyphae occur in xylem vessels. Invasion proceeds mostly through pit membranes (arrows).  $\times 5,000$ . IS = intercellular space; HCW = host cell wall; FC = fungal cell; HSW = host secondary wall; ML = middle lamella; PM = pit membrane; V = vessel.

and the vascular stele was noted occasionally (Fig. 2B), but, usually, paratracheal parenchyma cells and vascular elements were free of fungal colonization.

By 120 hr after inoculation, the fungus had penetrated the endodermis (Fig. 2C), causing pronounced cytoplasm disruption

and wall degradation. Pericycle, paratracheal parenchyma cells, and vascular elements (Fig. 2D) also were invaded by numerous hyphae. Invasion of protoxylem as well as early and late metaxylem proceeded often through the middle lamella and pit membrane (Fig. 2D, arrows). At this time, typical symptoms



**Fig. 3.** Transmission electron micrographs of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in culture and of healthy tomato root cells. Sections were treated with the anti-hydroxyproline-rich glycoprotein antiserum diluted 1:200 and with gold-conjugated goat antiserum to rabbit immunoglobulins. **A**, Labeling is nearly absent over the fungal cell. Cytoplasm, vacuole, mitochondria, and cell walls are almost free of labeling.  $\times 54,000$ . **B**, Cortical area of uninoculated tomato root tissues. Randomly distributed gold particles are associated with cell walls, but labeling is absent over the middle lamella.  $\times 54,000$ . **C**, Vascular area of uninoculated tomato root tissues. A few gold particles occur over secondary thickenings.  $\times 70,000$ . FW = fungal wall; M = mitochondrion; Va = vacuole; HCW = host cell wall; HSW = host secondary wall; ML = middle lamella; PC = parenchyma cell; V = vessel.

including leaf chlorosis and incipient leaf flaccidity started to appear on tube-grown tomato plants. The brief ultrastructural observations reported here are in accordance with those recently described in more detail by Charest et al (9) and Brammall and Higgins (7).

**Immunogold labeling of cells of *F. o. radices-lyopersici* in culture.** Sections from actively growing colonies of *F. o. radices-lyopersici* treated first with the anti-HRGP antiserum and then with GAR-gold antibodies did not exhibit any significant labeling (Fig. 3A). Fungal cell walls, plasmalemma, cytoplasm, and organelles including vacuoles and mitochondria were nearly free of labeling. A few gold particles occasionally were noted over the surrounding agar medium (Fig. 3A). Because these particles were not observed consistently from one experiment to another, they probably were related to a slight nonspecific binding.

**Immunogold labeling of healthy tomato root tissues.** After sections from uninoculated tomato root tissues were incubated with the anti-HRGP antiserum and GAR-gold antibodies, a light and uneven labeling was observed over cell walls (Fig. 3B). Gold particles that often were clustered were randomly distributed; some wall areas were apparently more intensely labeled than others. The expanded middle lamella that occurred at the intersection of adjacent cells usually was unlabeled (Fig. 3B). Very few gold particles, likely corresponding to nonspecific binding, occasionally were seen over the cytoplasm. In the vascular stele, thicker walls characterized by the formation of secondary layers on one or both sides of the compound middle lamella also were labeled by a few clustered gold particles (Fig. 3C).

**Immunogold labeling of infected tomato root tissues.** By 96 hr after inoculation of tomato roots, extensive colonization of the cortical area was noted. Fungal growth was both intracellular (Fig. 4A) and intercellular (Fig. 4B). Treatment of sections with the anti-HRGP antiserum and with GAR-gold antibodies resulted in the labeling of specific host wall areas (Fig. 4A). Gold particles were associated preferentially with host wall areas adjacent to fungal cells (Fig. 4A), whereas other parts of these walls were labeled more unevenly. Such walls often showed a pronounced disintegration as revealed by the noticeable disruption of the inner wall layers (Fig. 4B, arrow). Numerous gold particles usually were associated with fibrils that became detached from the altered host walls in areas neighboring the point of contact with fungal cells (Fig. 4A, arrowheads). Host cytoplasm in these cortical cells was drastically altered. A few gold particles sometimes were found over areas originally filled by the host cytoplasm, but this was not consistent enough to be considered significant. Surprisingly, labeling occurred also over fungal cells. It was, however, preferentially associated with the cell surface (Fig. 4A and B). By 96 hr after inoculation, hyphae that had penetrated the endodermis were rarely observed. However, when this was the case, an increase in host wall labeling at the point of contact with the fungal cell was not noted. The labeling pattern of these host walls was thus similar, in most respects, to that of uninoculated plant root cell walls.

By 120 hr after inoculation, the fungus had ramified through much of the endodermis and had colonized abundantly the vascular stele. Walls of infected inner cortical cells were labeled intensely and specifically after treatment of sections with the anti-HRGP antiserum and with GAR-gold antibodies (Fig. 4C). Gold particles accumulated greatly over the walls of these highly colonized cells but appeared nearly absent over the middle lamella at the intersection of neighboring cells (Fig. 4C). Gold particles, likely associated with disintegrated wall fragments, were observed frequently in the vicinity of altered inner wall layers (Fig. 4C, arrows). Some colonizing hyphae displayed a quite regular labeling, mainly localized at the cell surface (Fig. 4C, FC1 and FC2). Thus, it seemed that at an early stage of contact, while a space was still visible between the walls of both partners (Fig. 4C, arrowhead), the amount of gold particles over the fungal cell surface was quite low. In contrast, when the contact was more intimate (Fig. 4C, FC1), larger amounts of gold particles were concentrated mainly in the vicinity of internal fungal wall layers. Fungal cytoplasm as well as organelles, including nuclei

and mitochondria, were labeled unevenly. In the colonized endodermis, a substantial accumulation of gold particles was noted consistently over host cell walls when these were in close contact with fungal cells (Fig. 4D). Here also, labeling was distributed irregularly over hyphae.

By 120 hr after inoculation, paratracheal parenchyma cells also were invaded by hyphae which grew centripetally towards the vascular elements. Labeling was found associated mainly with wall areas that were contiguous to fungal cells (Fig. 5A). Wall disorganization was pronounced in these tissues. Split walls were observed frequently (Fig. 5B). Gold particles were numerous over both portions of these altered walls, whereas spaces between them were nearly free of labeling. Paratracheal parenchyma cells located in the immediate vicinity of invaded xylem vascular elements were depleted, most often, of cytoplasm. In cells apparently less altered, the plasmalemma was much retracted and the enlarged vacuoles were found frequently to contain bands of amorphous, osmiophilic material (Fig. 5C). Fungal cells, lying in such vacuoles, usually were surrounded by this electron-dense material which was specifically labeled after treatment with the anti-HRGP antiserum and with GAR-gold antibodies (Fig. 5C). In contrast, gold particles nearly were absent over the remaining free parts of the vacuoles. Host cell walls neighboring such invaded vacuoles always were intensely labeled (Fig. 5C).

Secondary thickenings of invaded xylem vessels were labeled heavily (Fig. 5D). In contrast, very few gold particles were found to be associated with the compound middle lamella. Labeling generally was absent over the vessel lumen except in areas close to the innermost secondary wall layers. In these areas, clustered gold particles, likely associated to detached wall fragments, were observed (Fig. 5D, arrowheads). Pit membranes, occurring at the intersection between two invaded vessels, usually were labeled by numerous gold particles, especially when they were in close contact with fungal cells (Fig. 6A). Secondary walls and pit membranes of most invaded xylem vessels were coated by an osmiophilic material which appeared lightly labeled. However, when this material extended in the vessel lumen with a tendency to encircle fungal cells, it was labeled intensely and specifically (Fig. 6B). Hyphae that were in close contact with secondary walls or pit membranes of vessels always were labeled over their cell surface (Fig. 6A).

**Immunogold labeling of specific host cell reactions.** During pathogen ingress through tomato root tissues, various host wall reactions occurred such as a thickening of wall layers by fibrillar components which sometimes were oriented perpendicular to the wall (Fig. 6C). After incubation with the anti-HRGP antiserum and with GAR-gold antibodies, large amounts of gold particles were associated with this fibrillar material.

Another type of wall apposition was characterized by an accumulation of vesicles between the plasmalemma and the host cell wall (6). This reaction, considered to be one of the first prominent host responses to infection, occurred in cortical tissues and in central cylinder parenchyma cells. Sparse gold particles were present over such paramural vesicles (Fig. 6D).

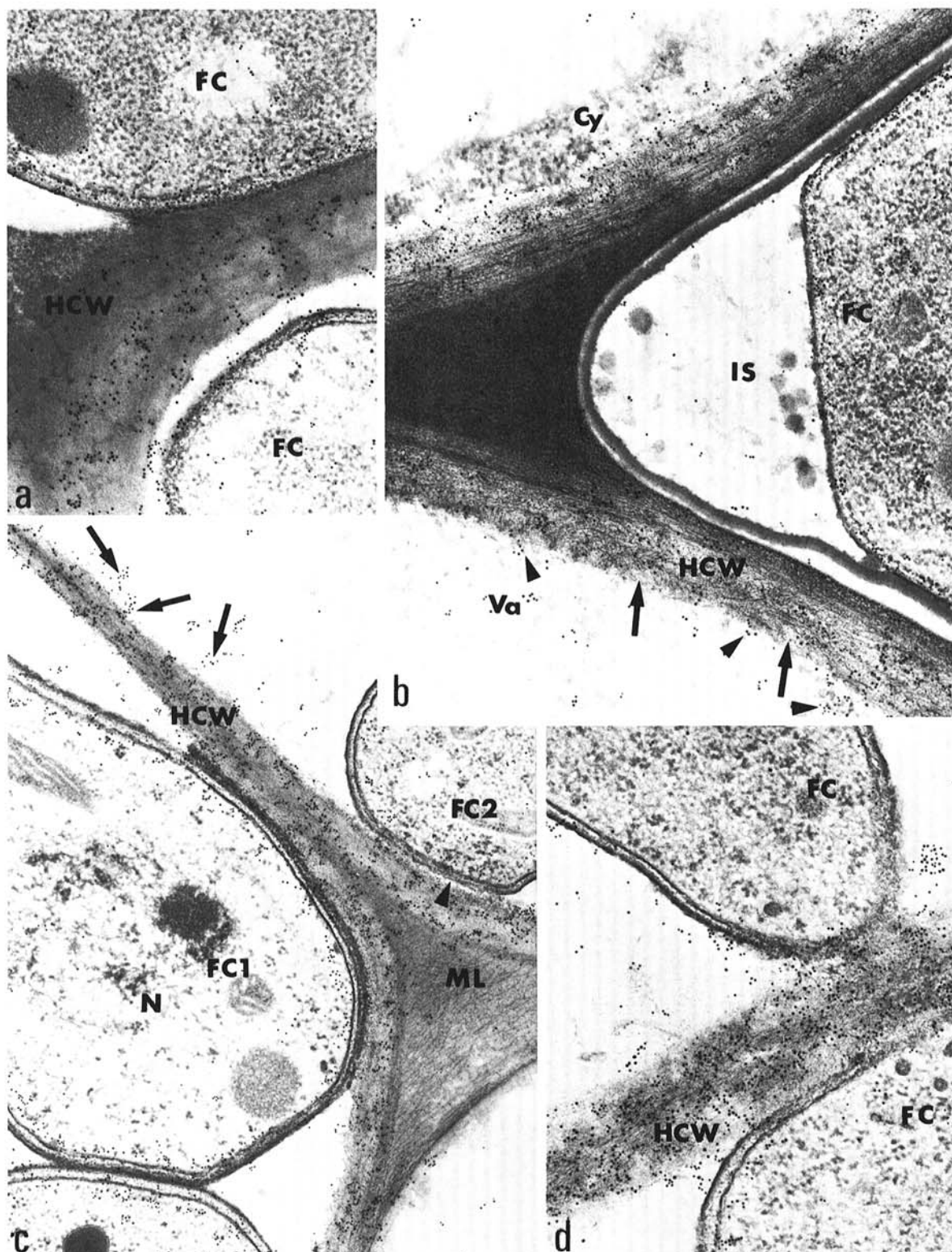
The most common type of wall apposition in cortical tissues and in hypodermis resembled papillae described by others (1,11). These structures were composed of variously shaped areas of different electron density. The plasmalemma surrounding such appositions was hardly discernible because of its severe disintegration (Fig. 6E). A great amount of gold particles was consistently associated with these typical wall appositions (Fig. 6E).

Among the most typical host cell reactions, the gradual occlusion of invaded intercellular spaces commonly was observed (Fig. 7A). Increase in the compactness of the originally deposited, granulo-fibrillar, occluding material led to the progressive formation of dense, amorphous layers (Fig. 7A, arrow). Fungal cells, gradually embedded in this material, thus were greatly limited in their progression. Treatment of sections with anti-HRGP antibodies and GAR-gold antibodies resulted in specific labeling of this material (Fig. 7A). Bubblelike structures, coated with large bands of osmiophilic material, occurred within some xylem vessels (Fig. 7B). A light but specific labeling was observed

over these large "bubbles."

**Immunocytochemical controls.** To test the validity of the labeling patterns obtained with the anti-HRGP antiserum and

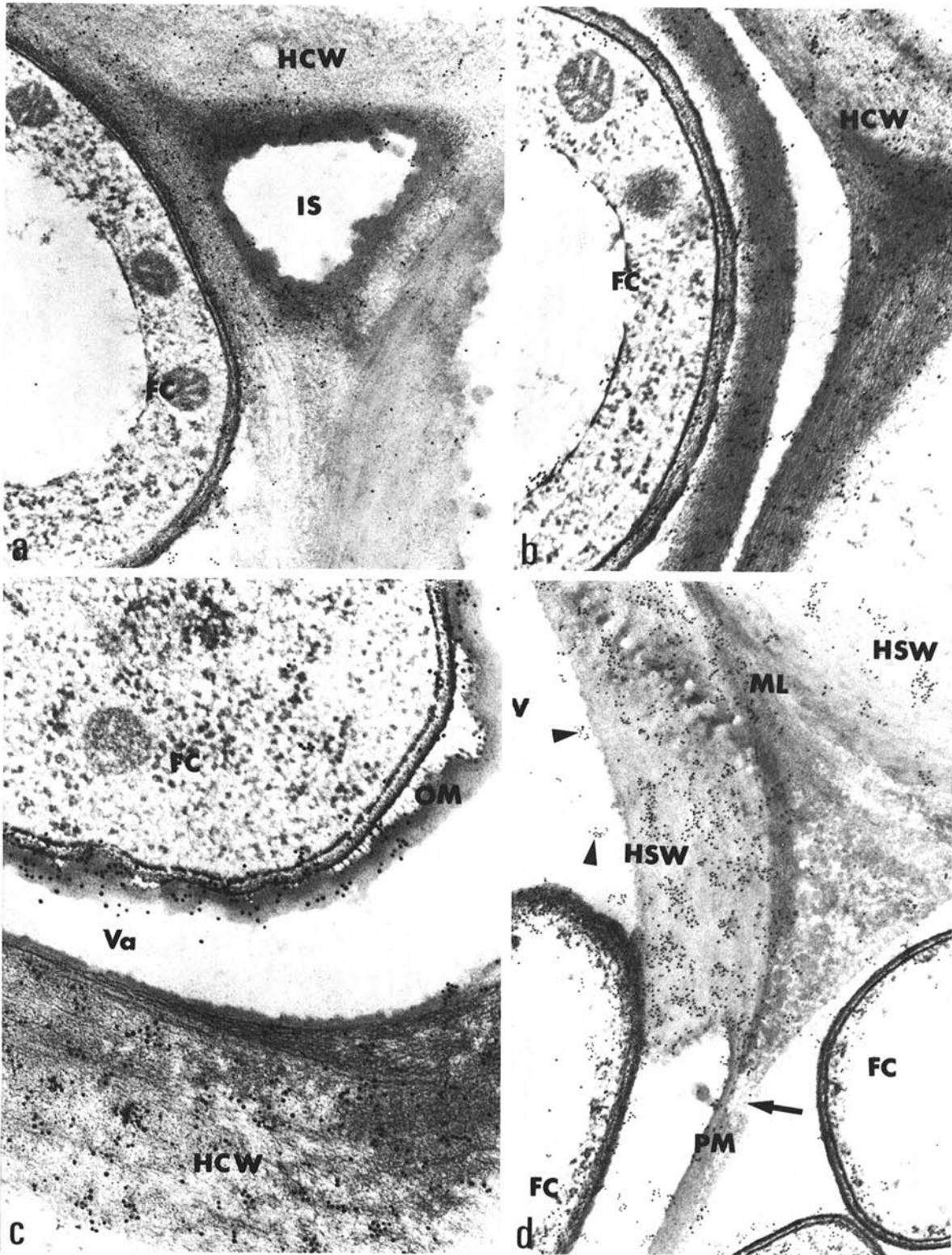
with GAR-gold antibodies, several control tests were performed. Preincubation of the anti-HRGP antibodies with purified HRGPs from melon greatly reduced but did not eliminate total labeling



**Fig. 4.** Transmission electron micrographs of tomato root cells infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Sections were treated with the anti-hydroxyproline-rich glycoprotein antiserum diluted 1:200 and with gold-conjugated goat antiserum to rabbit immunoglobulins. **A**, Cortical area, 96 hr after inoculation. Gold particles mainly are associated with host cell wall areas adjacent to fungal cells.  $\times 70,000$ . **B**, Cortical area, 96 hr after inoculation. A fungal cell is growing in an intercellular space. Host cell walls are labeled intensely. Disruption of these walls is noticeable (arrow). Gold particles are associated with fibrils likely detached from the altered walls (arrowheads). Gold particles occur over the fungus.  $\times 54,000$ . **C**, Cortical area, 120 hr after inoculation. An intense and specific labeling is associated with host cell wall areas neighboring fungal cells. The middle lamella is nearly free of gold particles. Disintegrated wall fragments, in the vicinity of inner host wall layers, are labeled (arrows). Gold particles are present over the cell surface of fungal cells (FC1 and FC2) that are adjacent to the host wall.  $\times 45,000$ . **D**, Endodermis, 120 hr after inoculation. Heavy labeling is present over the host cell wall adjacent to two fungal cells.  $\times 70,000$ . HCW = host cell wall; Va = vacuole; IS = intercellular space; N = nucleus; Cy = cytoplasm; ML = middle lamella.

(Fig. 7C). Similarly, labeling was nearly abolished over sections treated with the anti-HRGP antiserum to which previously was added purified tomato HRGPs (Fig. 8). Incubation with rabbit preimmune serum instead of anti-HRGP antiserum resulted in

a very low labeling of cell walls (Fig. 7D), and treatment with GAR-gold antibodies only (the antiserum step being omitted) yielded negative results (Fig. 7E). All other controls gave negative responses.

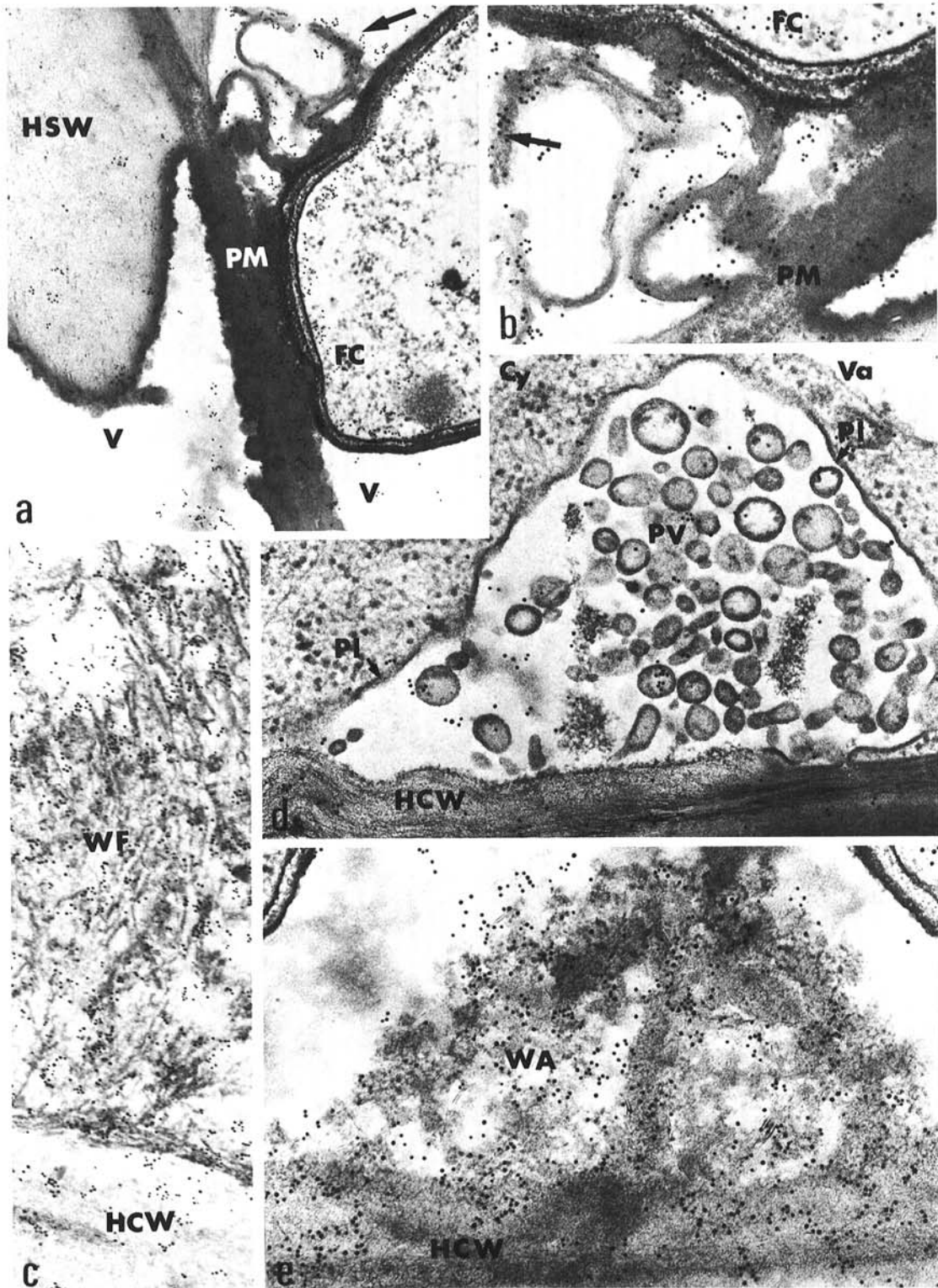


**Fig. 5.** Transmission electron micrographs of infected tomato root tissues. Sections were treated with the anti-hydroxyproline-rich glycoprotein antiserum and with gold-conjugated goat antiserum to rabbit immunoglobulins. **A**, Paratracheal parenchymal cell, 120 hr after inoculation. Labeling is associated with host wall areas that are in close contact with fungal cells.  $\times 45,000$ . **B**, Labeling also is intense over both portions of the split parenchyma cell walls.  $\times 54,000$ . **C**, An enlarged vacuole of a parenchyma cell containing a band of osmiophilic material that appears specifically labeled. This material is apparently encircling a fungal cell. The host cell wall is densely labeled.  $\times 70,000$ . **D**, Vascular stele, 120 hr after inoculation. Secondary thickenings of a xylem vessel are intensely labeled. Labeled fragments of secondary walls are visible in the lumen of the vessel (arrowheads). The pit membrane is altered in some places (arrow).  $\times 45,000$ . FC = fungal cell; HCW = host cell wall; IS = intercellular space; Va = vacuole; OM = osmiophilic material; HSW = host secondary wall; PM = pit membrane; ML = middle lamella; V = vessel.

## DISCUSSION

Since the first demonstration that HRGPs increased in cell walls of melon infected by *C. lagenarium* (12,14), several investigators have suggested that these macromolecules may play a defensive role in various host-pathogen interactions, including

fungal, bacterial, and viral diseases (17,19,23). It was, thus, of particular interest to determine whether such substances accumulated in tomato plants infected by *F. o. radicis-lycopersici* and whether they were involved in defense reactions during compatible interactions. Results of the present immunocytochemical study clearly demonstrate that cell walls of infected



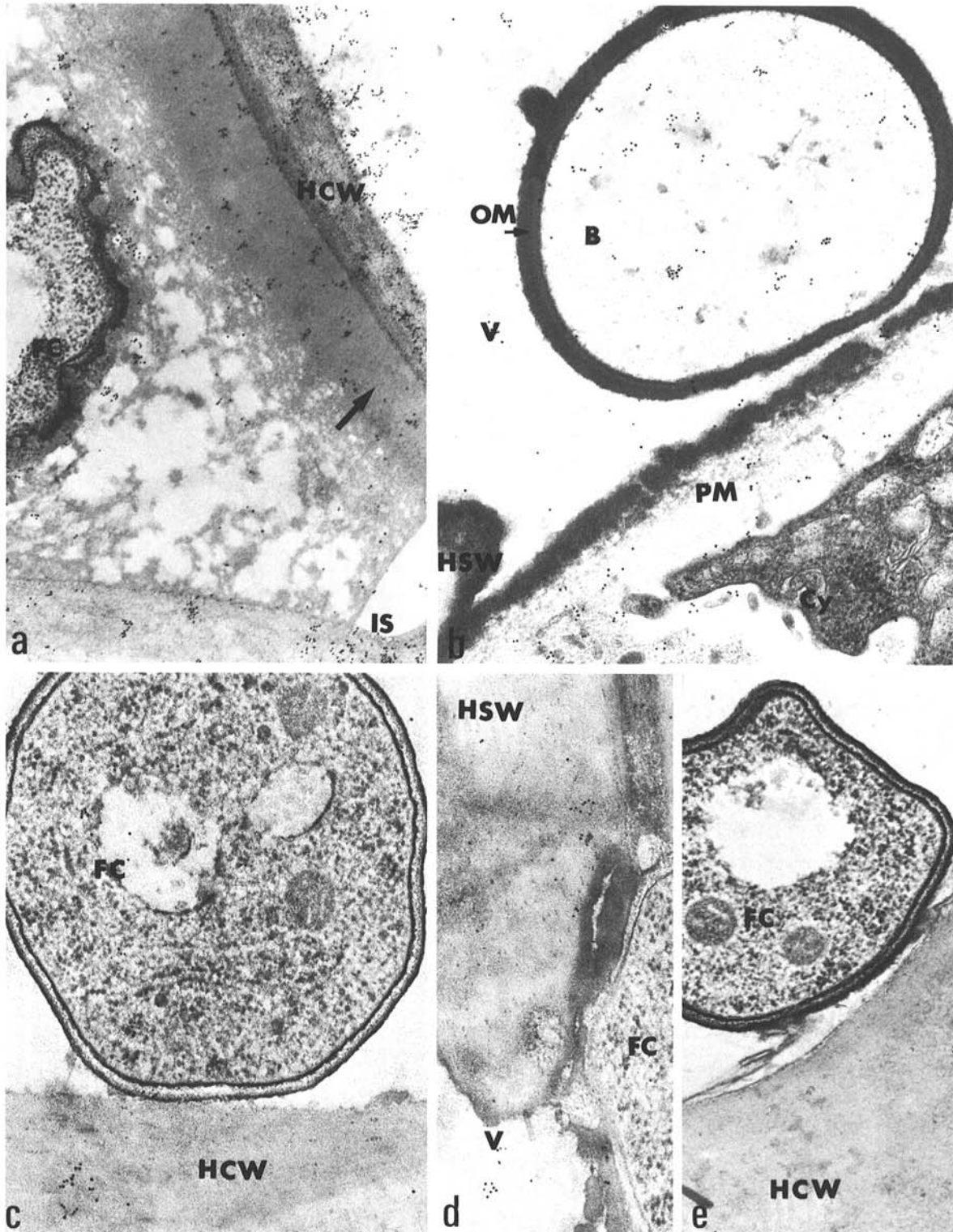
**Fig. 6.** Transmission electron micrographs of infected tomato root tissues. Sections were treated with the anti-hydroxyproline-rich glycoprotein antiserum and with gold-conjugated goat antiserum to rabbit immunoglobulins. **A**, Vascular area, 120 hr after inoculation. Numerous gold particles are associated with a pit membrane adjacent to a fungal cell. Labeling is present over a convoluted band of osmiophilic material (arrow).  $\times 45,000$ . **B**, Enlarged portion of Figure 6A showing the regular association of gold particles with the osmiophilic material that extends in the vessel lumen.  $\times 70,000$ . **C**, Wall-like fibrils, deposited between a fungal cell and the host cell wall, are heavily labeled.  $\times 50,000$ . **D**, Few gold particles are associated with paramural vesicles formed between the plasmalemma and the host cell wall.  $\times 70,000$ . **E**, An intense labeling is present over a wall apposition that shows areas of different electron density.  $\times 70,000$ . HSW = host secondary wall; PM = pit membrane; FC = fungal cell; WF = wall fibrils; HCW = host cell wall; PL = plasmalemma; PV = paramural vesicles; Va = vacuole; WA = wall apposition; Cy = cytoplasm; V = vessel.



tomato root cells are markedly enriched in HRGPs. Occurrence of these macromolecules in structures such as wall appositions, thought to be involved in defense against invasion (1), contributes, therefore, to the proposition that HRGP accumulation is related

to defense reactions in infected tissues (20).

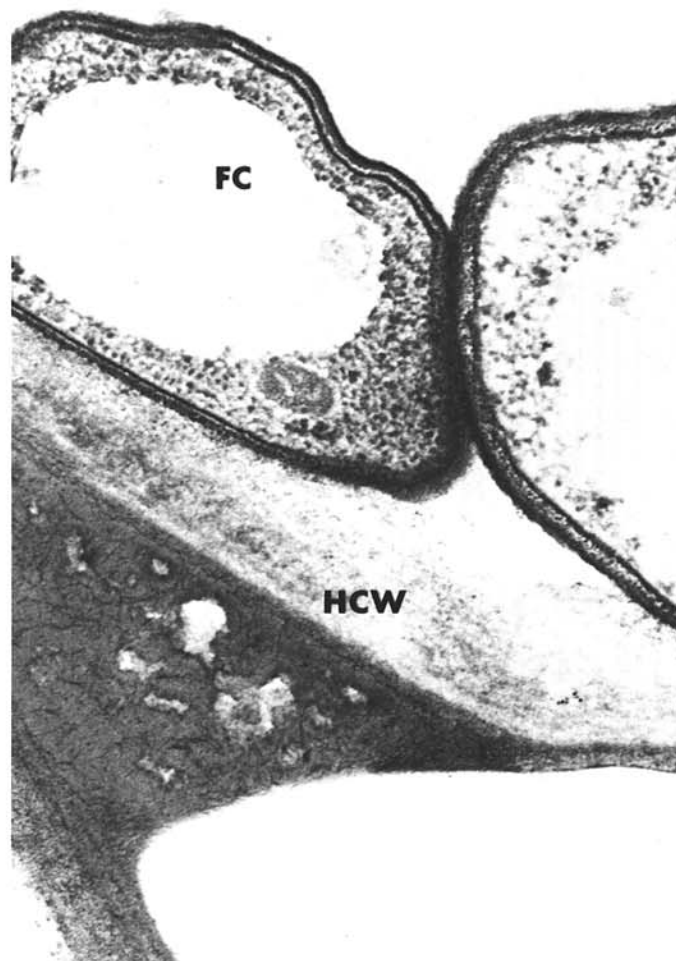
Specific recognition of tomato HRGPs by antibodies raised against melon HRGP<sub>2b</sub> was demonstrated by dot-immunobinding assays. This cross-reactivity was not unexpected because the



**Fig. 7.** Transmission electron micrographs of infected tomato root tissues. Sections were treated with the anti-hydroxyproline-rich glycoprotein (HRGP) antiserum and with gold-conjugated goat antiserum to rabbit immunoglobulins (GAR-gold antibodies) (A and B) or submitted to control tests (C, D, and E). **A**, Endodermis, 120 hr after inoculation. The dense, amorphous material deposited in an intercellular space is significantly labeled (arrow). An intense labeling is present over the host cell wall.  $\times 45,000$ . **B**, Vascular area, 120 hr after inoculation. A bubblelike structure in a xylem vessel contains few gold particles and is surrounded by an osmiophilic layer which is unlabeled.  $\times 45,000$ . **C**, Control test in which the anti-HRGP antiserum has been incubated previously with purified HRGPs. A considerable reduction of labeling is noted over a parenchyma host cell wall.  $\times 54,000$ . **D**, The intensity of labeling over secondary thickenings of infected vessels decreases significantly after incubation of sections with preimmune serum instead of anti-HRGP antiserum.  $\times 45,000$ . **E**, Labeling is absent over sections treated with GAR-gold antibodies alone.  $\times 45,000$ . FC = fungal cell; HCW = host cell wall; IS = intercellular space; HSW = host secondary wall; OM = osmiophilic material; B = bubblelike structure; PM = pit membrane; Cy = cytoplasm; V = vessel.

HRGP<sub>2b</sub> of melon (21) matches the amino acid, sugar composition, and molecular weight (55 kDa after deglycosylation) of the tomato HRGP<sub>p1</sub> (26). Although from different plant species, both glycoproteins are thus similar in nature. The high percentage of sequence similarity between these HRGPs accounts for the occurrence of common epitopes to which the antibodies prepared can bind. Preincubation of the antimelon HRGP<sub>2b</sub> antiserum with purified HRGP<sub>p1</sub> from tomato nearly abolished tissue section labeling, thus confirming that the labeling pattern was indeed due to the specific interaction of the antibodies with tomato HRGPs.

Within healthy tomato root tissues, HRGPs were detected in primary and secondary walls. However, their very low concentration and their random distribution indicated that they were only minor components of plant cell walls. These results contrast with those obtained by Stafstrom and Staehelin (27) who reported the occurrence of large amounts of extensin in cell walls of carrot storage roots. Such differences may reflect variations in HRGPs (= extensin), concentration within plant species, and/or differences in the specificity of the antibodies. Although the extent of labeling was not evaluated quantitatively, a qualitative comparison between labeling patterns of healthy and infected tomato root tissues provided conclusive evidence for a considerable increase of HRGPs in response to infection by *F. o. radialis-lycopersici*. Accumulation of HRGPs in inoculated plants was found to closely parallel pathogen ingress. By 96 hr after inoculation, the level of HRGPs in highly colonized cortical cells had increased quite significantly, whereas it remained similar to that of healthy tissues in uninvaded, inner areas including endodermis,



**Fig. 8.** Transmission electron micrograph of infected tomato root tissues. Control test. Preincubation of the antiserum raised against melon hydroxyproline-rich glycoprotein (HRGP)<sub>2b</sub> with purified HRGPs from tomato results in a near absence of labeling.  $\times 45,000$ . FC = fungal cell; HCW = host cell wall.

paratracheal parenchyma cells, and vascular elements. In root samples collected 120 hr after inoculation, fungal invasion occurred in all tissues, and, in parallel, a considerable cell wall enrichment in HRGPs was easily noticeable. The great accumulation of HRGPs in primary walls of cortical and parenchyma cells and in secondary thickenings of invaded xylem vessels appeared to be initiated after contact between the pathogen and the plant cell wall. In light of such observations, one can suggest that biosynthesis of HRGPs is specifically elicited following recognition between both partners. This interpretation is consistent with that of Roby et al (25) who reported that elicitors, such as glycoconjugates and oligosaccharides isolated from fungal cell walls as well as plant endogenous elicitors, may induce an increased synthesis of HRGPs in several plants. Thus, it is likely that cell surface interaction between plant and pathogen is of crucial importance for the de novo synthesis of HRGPs.

Occurrence of HRGPs in wall appositions formed in response to infection was an indication that these glycoproteins may be involved in plant defense. Their accumulation in such appositions may represent an active barrier against pathogen penetration as previously suggested by Leach et al (19) and Mellon and Helgeson (23). In addition, HRGPs may play the role of matricial substances for the subsequent deposition of lignin and phenolic compounds that are known to occur in wall appositions such as papillae (24,29).

Although HRGPs were not detected in cells of *F. o. radialis-lycopersici* when these were grown in culture, labeling was found to occur in the vicinity of cell wall layers when the fungus was in close contact with the plant cell wall. Whether this reflects the de novo synthesis by the parasite of extensinlike material or indicates a diffusion of these substances through more or less permeabilized fungal cells remains open to question. This intriguing problem deserves to be more fully investigated.

The present study has shown that HRGPs accumulate in tomato root cell walls in response to infection by *F. o. radialis-lycopersici*. Their association with structural barriers such as wall appositions supports their possible role in defense mechanisms. However, their accumulation, in only highly colonized tissues, suggests that HRGP production constitutes a late metabolic response in infected susceptible plants.

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