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Subcellular Localization of Chitinase and of Its Potential Substrate in Tomato Root Tissues Infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*[1]

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ABSTRACT

Antiserum raised against a tomato (*Lycopersicon esculentum* Mill.) chitinase (molecular mass of 26 kilodaltons) was used as a probe to study the subcellular localization of this enzyme in tomato root tissues infected with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. A time-course experiment revealed that chitinase accumulated earlier in the incompatible interaction than in the compatible one. However, in both systems, chitinase deposition was largely correlated with pathogen distribution. The enzyme was found to accumulate in areas where host walls were in close contact with fungal cells. In contrast, the enzyme could not be detected in vacuoles and intracellular spaces. The substantial amount of chitinase found at the fungus cell surface supports the view of an antifungal activity. However, the preferential association of the enzyme with altered fungal wall areas indicates that chitinase activity is either preceded by the hydrolytic action of other enzymes such as β-1,3-glucanases or coincides with these enzymes. The possibility that fungal glucans released through the action of β-1,3-glucanases may act as elicitors of chitinase production is discussed.

In recent years, several studies have contributed to the unraveling of the mechanisms that plants have developed to defend themselves against the onslaught of pathogens (4, 20). Results from numerous reports have convincingly demonstrated that the plant response to microbial attack involves rapid mobilization of defense mechanisms in a highly coordinated manner (30–32). These include accumulation of antimicrobial phytoalexins (11), deposition of lignin, callose and phenolic compounds (3), enhancement of wall-bound hydroxyproline-rich glycoproteins (25), as well as synthesis of proteinase inhibitors (7) and hydrolytic enzymes such as chitinases and β-1,3-glucanases (19, 21, 22). The increase in the activity of lytic enzymes has been the focus of considerable interest not only because such hydrolases could degrade fungal wall components causing growth inhibition, but also because they could favor the release of fungal wall fragments that, in turn, might be active elicitors of secondary stress metabolites (16, 18).

Since the initial hypothesis that chitinases and β-1,3-glucanases might be implicated in defense reactions against fungal attack (1), the role of lytic enzymes in restricting pathogen invasion has been emphasized in several reports (9, 20, 26). It has been convincingly established that isolated fungal cell walls are vulnerable to treatment with purified chitinase and β-1,3-glucanase preparations (23) and that, in turn, breakdown products are potential elicitors of specific defense reactions (2, 3, 18). The possibility that plant hydrolases might be active determinants in resistance mechanisms was also supported by the demonstration that increases in chitinase and β-1,3-glucanase activities following infection of tomato plants by *Cladosporium fulvum*, the causal agent of tomato leaf mold occurred earlier and to a higher extent in resistant than in susceptible cultivars (17). These studies together with the observation that lysis of fungal hyphae *in vivo* was concomitant with an increase in plant glycosidase activity (26) stress the antifungal potential of hydrolases induced upon infection. If one considers that chitin and β-1,3-glucans are the main structural components of fungal walls (12), it seems reasonable to assume that these polysaccharides constitute potential substrates for plant chitinases and β-1,3-glucanases. However, demonstration of an antifungal activity *in vivo* is needed to establish a role for hydrolases in disease resistance.

In an attempt to address this question, Mauch and Staehelin (24) studied the subcellular localization of chitinase and β-1,3-glucanase in ethylene-stressed bean leaves. It was found that both enzymes accumulated predominantly in intravacuolar aggregates, and that small amounts of β-1,-glucanase occurred in middle lamellae of plant cell walls. These results led the authors to propose a model outlining the possible implication of these enzymes in defense and recognition events during host-fungus interactions. Although pertinent, this model needs to be supported by further investigations of the spatial distribution of hydrolases in fungus-infected plant tissues. In this context, we have recently examined the spatial localization of β-1,3-glucanases in fungal wilt-diseased plants by immunogold cytochemistry (6). Our observations at various intervals after inoculation revealed that β-1,3-glucanase

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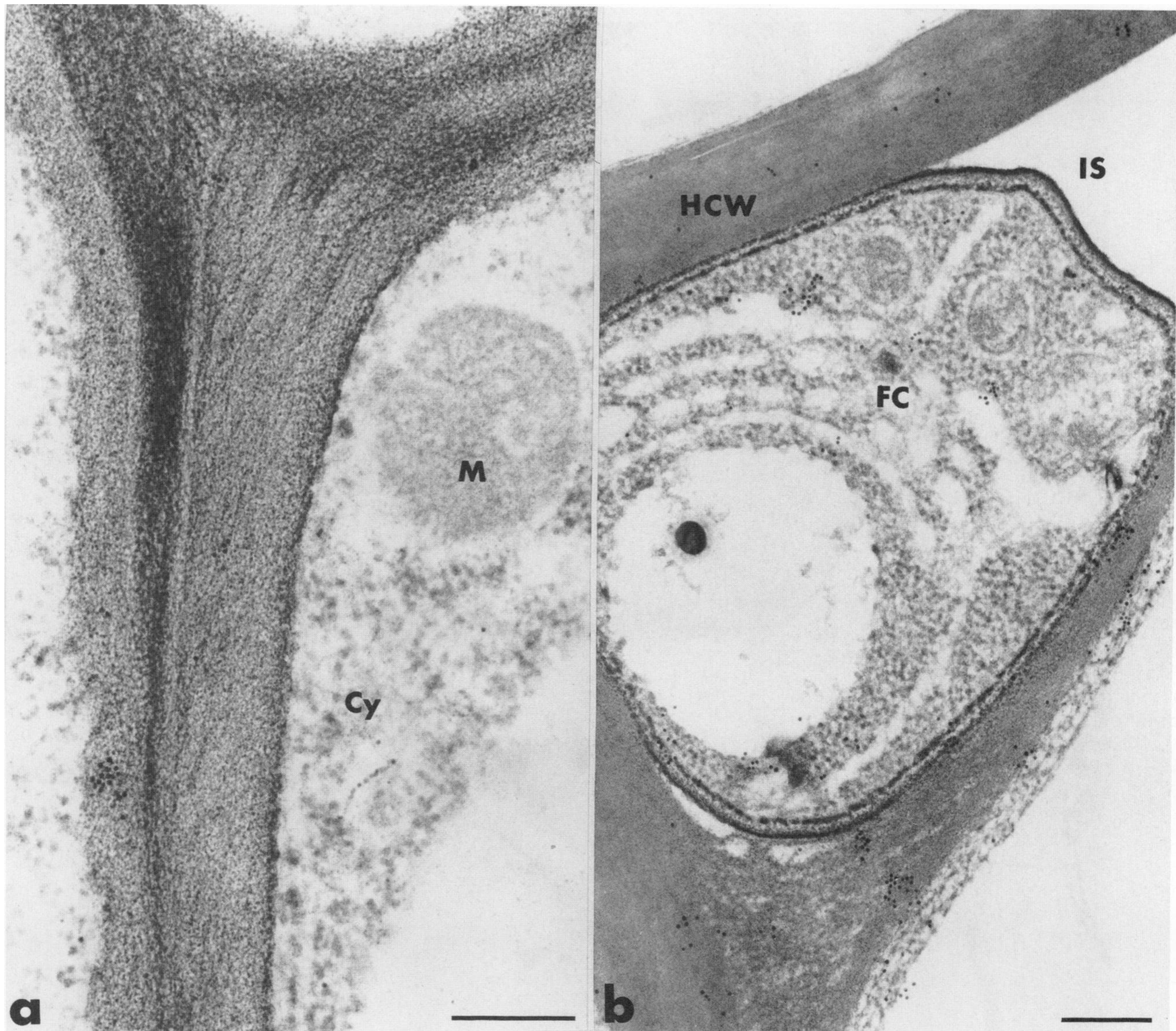


Figure 1. Transmission electron micrographs of healthy (a) and FORL-infected tomato root tissues (b). Labeling with antichitinase antibodies and with GAR-gold antibodies. a, Healthy tissue. Labeling is nearly absent over the cell wall and the cytoplasm. ×45,000; bar = 0.25 μm. b, FORL-infected susceptible tissue: 48 h after inoculation. Very few gold particles occur over the host cell wall. The fungal cell is not specifically labeled. ×45,000; bar = 0.5 μm. Cy, cytoplasm; M, mitochondrion; HCW, host cell wall; FC, fungal cell; IS, intercellular space.

accumulation in resistant plants was an early event associated with the limited spread of the pathogen, whereas in susceptible plants it appeared to occur as a result of successful tissue colonization. These findings together with the observation that β-1,3-glucanase accumulated at the surface of invading fungal cells highlighted the participation of this enzyme in resistance against fungi causing vascular wilt diseases.

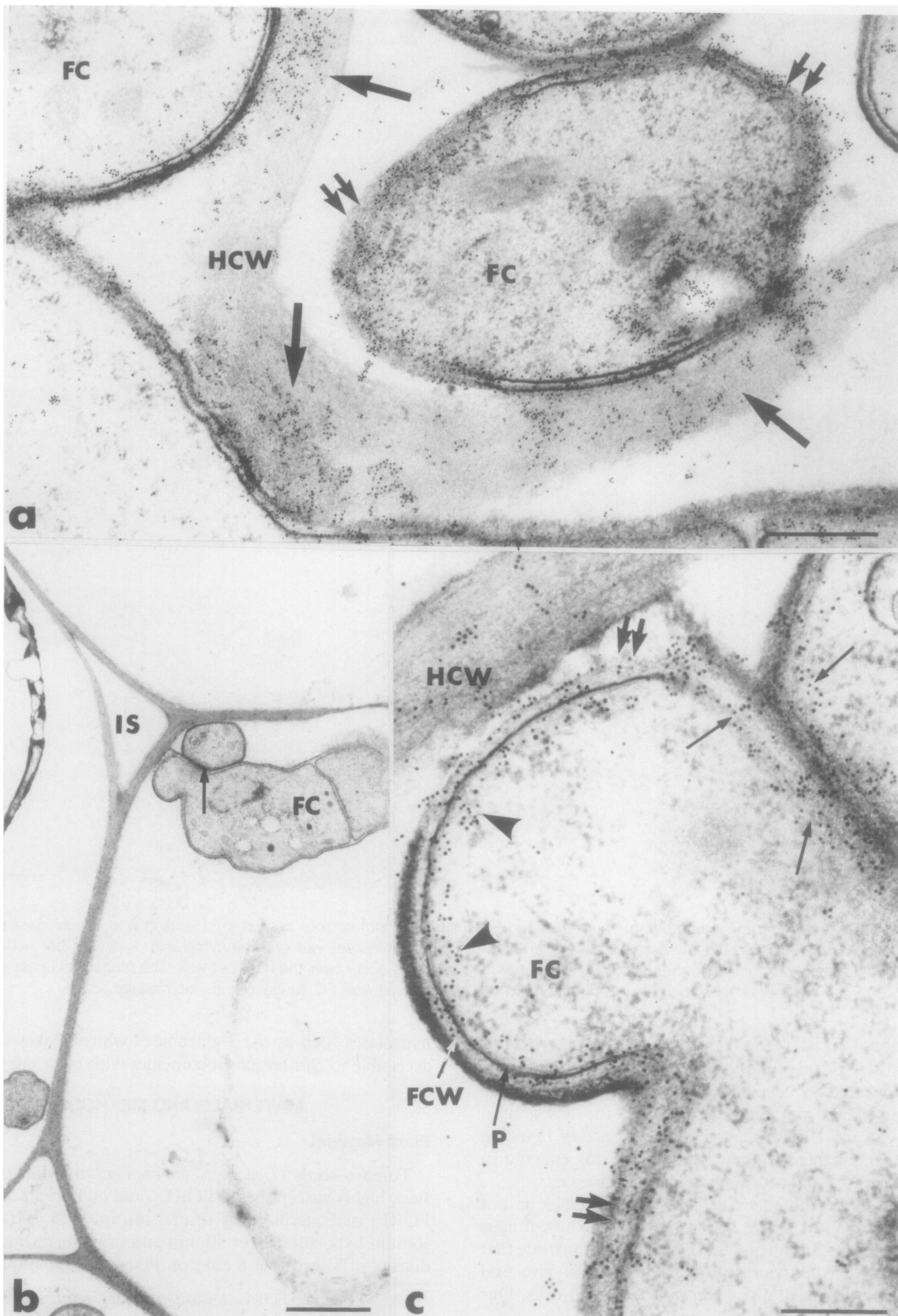
These results prompted us to determine whether or not chitinases also played a role in the outcome of host-fungus interactions *in vivo*. In the present paper, we demonstrate that plant chitinases exhibit an antifungal activity *in vivo* and accumulate in host wall areas adjacent to fungal cells. Our observation that altered fungal wall areas contain larger amounts of chitinase than undamaged ones indicates that the chitinolytic activity is either preceded by the action of other hydrolases such as β-1,3-glucanases which makes the chitin accessible to chitinase or it coincides with these enzymes.

MATERIALS AND METHODS

Plant Material

Tomato seeds (*Lycopersicon esculentum* Mill. cv Bonny Best, highly susceptible to FORL[2], and cv Larma, resistant to FORL) were sterilized by immersion in 0.5% (v/v) aqueous sodium hypochlorite for 30 min and sown in vermiculite at a density of 5 seeds per 6 cm pot. Plants were maintained in a

[2] Abbreviations: FORL, *Fusarium oxysporum* f. sp. *radicis-lycopersici*; PDA, potato dextrose agar; GAR-gold antibodies, gold-conjugated goat antiserum to rabbit immunoglobulins; WGA, wheat germ agglutinin.


FC
HCW
FC
a
IS
FC
b
HCW
FC
FCW
P
c

glasshouse with a soil temperature of about 22°C and a RH of 75%.

The FORL isolate used in this study was kindly supplied by Mr. P. O. Thibodeau, Complexe Scientifique, Sainte-Foy, Quebec. It was grown on PDA medium at 22 ± 2°C. Tomato seedlings at the three-leaf stage were inoculated with a suspension of microconidia (2 × 10^6 spores/mL) deposited on the roots with a sterile syringe. Control plants were treated with sterile distilled water. Root samples from inoculated and control plants were collected every 24 h after inoculation and processed immediately for electron microscope investigations.

Tissue Processing

Samples (1 mm^3) were fixed in 3% (v/v) glutaraldehyde buffered with 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4°C, dehydrated in a graded ethanol series, and embedded in Epon 812. Silver-gold ultrathin sections were collected on formvar-coated nickel grids and processed for immunogold labeling. For each experiment, 10 to 20 sections from five tomato root samples were examined under the electron microscope.

Antichitinase Antiserum

The purity of the 26 kD chitinase used as antigen for antibody production has been convincingly demonstrated in a recent study (17). Specificity of the antiserum raised against this purified enzyme was also assessed by Western blotting after gel electrophoresis of soluble proteins containing chitinases (17). The antiserum was found to specifically react with the 26 kD chitinase from tomato and to also recognize two other protein bands of 30 and 32 kD, respectively (17). These proteins were also detected by antibodies raised against a chitinase (PR-P) from tobacco (21) and antibodies raised against a chitinase isolated from bean leaves (8). In a recent study, Fisher *et al.* (14) purified the 30 kD protein and showed that it was indeed a chitinase. The antiserum raised against the 26 kD tomato chitinase did not react with other proteins including β-1,3-glucanases as recently demonstrated by Joosten and DeWit (17). By virtue of its narrow specificity for tomato chitinases (17), this antiserum was used in a post-embedding immunogold procedure for determining the subcellular localization of these enzymes in infected tomato root tissues.

Immunocytochemical Labeling

Ultrathin sections of infected and noninoculated tomato root samples were floated on a drop of phosphate buffered saline (PBS) (pH 7.2), containing 0.25% (w/v) ovalbumin for 5 min and then transferred to a drop of normal goat serum (diluted 1:10 in PBS-ovalbumin) for 30 to 60 min at room temperature. Sections were incubated for 2 h at 37°C on a drop of the antichitinase antiserum (diluted 1:500 in PBS-ovalbumin) and washed with Tris HCl-BSA-NaCl (0.05 M Tris HCl [pH 8.2], 1% [w/v] BSA, 0.5 M sodium chloride) for at least 15 min. They were then incubated on a drop of colloidal gold (10 nm)-conjugated goat antiserum to rabbit immunoglobulins (GAR-gold antibodies) (diluted 1:10 in Tris HCl-BSA-NaCl) for 1 h at room temperature, washed with PBS (pH 7.4), rinsed with distilled water, and finally contrasted with uranyl acetate and lead citrate. Grids were examined in a JEOL 1200 EX electron microscope at 80 kV.

Immunocytochemical Controls

Specificity of labeling was assessed by the following control tests: (a) incubation with the antiserum against tomato chitinase to which was previously added an excess of its corresponding antigen; (b) incubation with rabbit preimmune serum instead of antichitinase antiserum; and (c) incubation with GAR-gold antibodies, the primary antiserum step being omitted.

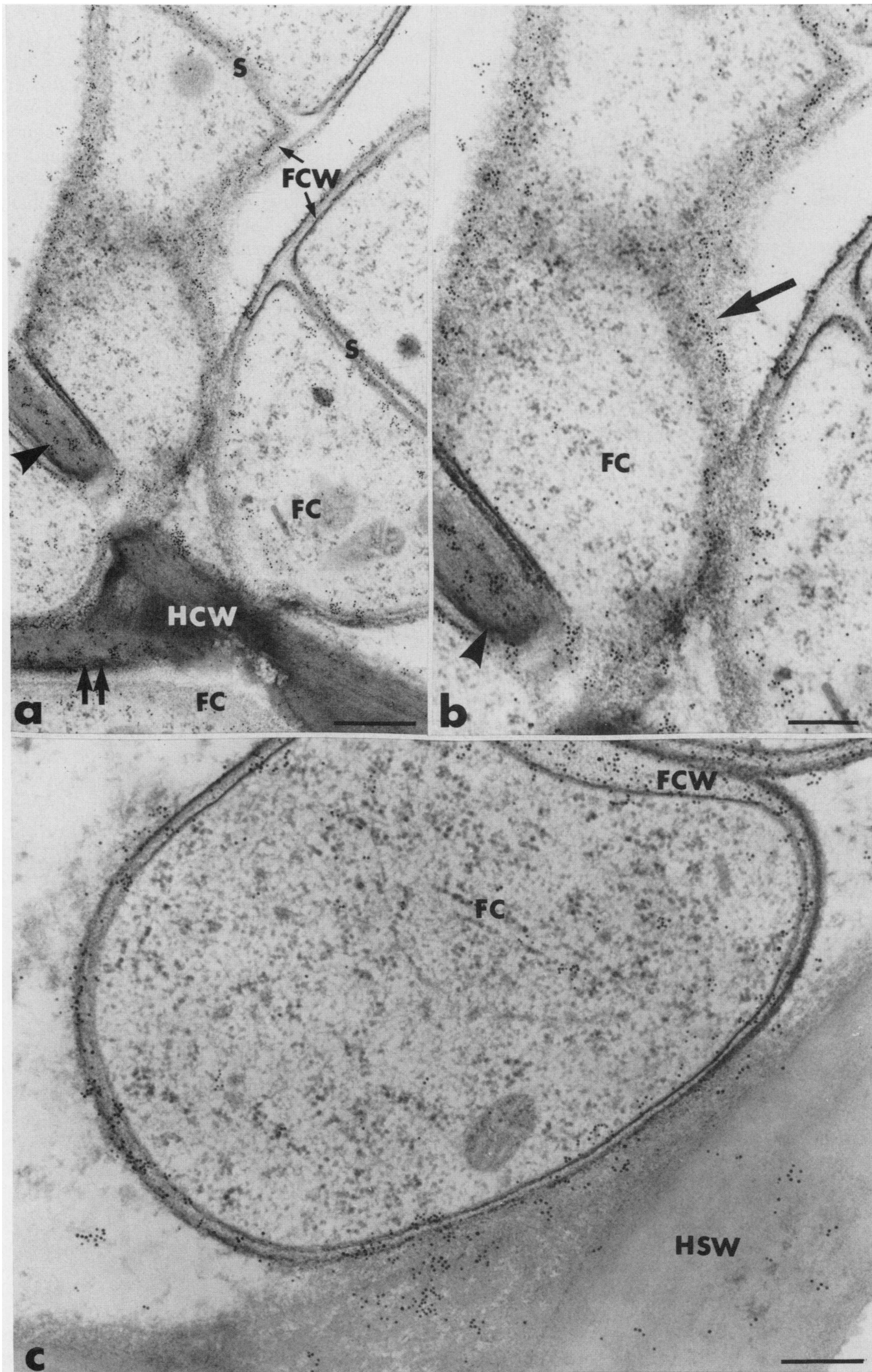
Cytochemical Labeling of *N*-Acetylglucosamine Residues (Chitin)

The colloidal gold suspension was prepared as described by Grandmaison *et al.* (15). Because of its low mol wt, the WGA, a lectin with *N*-acetyl glucosamine-binding specificity, could not be complexed directly to gold and was used in a two-step procedure for studying the distribution of chitin, a linear polysaccharide composed of β-1,4-linked *N*-acetylglucosamine units, within cell walls of invading fungi.

Grids were first incubated on a drop of WGA (25 μg/mL in PBS) for 60 min at room temperature, rinsed with PBS, and transferred to a drop of ovomucoid-gold complex (diluted 1:30 in PBS containing 0.02% (w/v) polyethylene glycol (PEG) 20,000). The ovomucoid was chosen as a second step reagent due to its strong affinity for the WGA (5). Grids were finally contrasted with uranyl acetate and lead citrate. Experiments were repeated five times on 10 sections from different tomato root samples.

Control tests included: (a) incubation with the WGA to which was previously added *N-N′-N″*-triacetyl-chitotriose (1 mg/mL in PBS); (b) incubation with WGA, followed by unlabeled ovomucoid and finally by ovomucoid-gold complex; and (c) direct incubation with the ovomucoid-gold complex, the lectin step being omitted.

Figure 2. Transmission electron micrographs of FORL-infected susceptible tomato root tissues. Labeling with antichitinase antibodies and with GAR-gold antibodies. Cortical area, 96 h after inoculation. a, Gold particles accumulate predominantly over host wall areas adjacent to fungal cells (arrows). The fungal cell surface is intensely labeled, especially in areas exhibiting an apparent alteration (double arrows). ×40,000; bar = 0.5 μm. b, A fungal cell growing intracellularly. The host cell cytoplasm is strongly altered. ×14,000; bar = 1 μm. c, Enlarged portion of (b) showing the intense labeling of the fungal cell surface. The fungus wall is disrupted or altered at places (double arrows). Gold particles accumulate over the degraded wall areas and are also present over the underlying plasma membrane (arrowheads). Inner layers of contiguous fungal cell walls are significantly labeled (small arrows). ×72,000; bar = 0.25 μm. FCW, fungal cell wall; P, plasma membrane; other abbreviations as in Figure 1.


S
FCW
S
FC
HCW
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a
FC
b
FCW
FC
HSW
c

RESULTS

Immunogold Localization of Chitinase in Healthy Tomato Root Cells

Following incubation with the antiserum raised against the 26 kD tomato chitinase and with GAR-gold antibodies, a slight and uneven labeling was observed at both the extra- and intracellular levels (Fig. 1a). Very few gold particles were found to be associated with cell walls, intracellular spaces, or vacuoles.

Immunogold Localization of Chitinase in Tomato Root Cells Infected by FORL

Compatible Interaction: Tomato cv Bonny Best/FORL

As soon as 48 h after inoculation, fungal microconidia had germinated on the root surface, and hyphae had reached the outer cortical layers via the infection of the epidermis and the hypodermis. Fungal growth was most abundant within intercellular spaces below the epidermis, and penetration of hypodermal cells occurred directly through the radial middle lamellae. Incubation with the antichitinase antiserum and with GAR-gold antibodies resulted in a labeling pattern similar to the one observed in healthy tomato root tissues (Fig. 1b). Fungal cells were devoid of any significant labeling. Similar results were obtained with samples collected 72 h after inoculation.

By 96 h after inoculation, the fungus had ramified through much of the cortex, causing pronounced cells alterations including disintegration of the cytoplasm and wall disruption. Fungal growth occurred inter- and intracellularly and in some cases intramurally, causing severe alteration of the fibrillar wall material and middle lamella matrices. From 96 to 120 h after inoculation, tissue colonization was intense, and hyphae were visible in vascular elements. Invasion of xylem vessels occurred mainly through penetration of pit membranes.

When sections from samples collected 96 and 120 h after inoculation were treated with the antichitinase antiserum and with GAR-gold antibodies, a heavy labeling of specific fungal wall areas was observed (Fig. 2, a and c). Gold particles were found to accumulate predominantly over wall portions exhibiting apparent alteration, as judged by the diffuse appearance of such areas (Fig. 2, a and c; double arrows). Disruption of the plasmalemma was frequently observed, and, in most cases, it was not clearly delineated from the surrounding wall portion. Fungal wall areas that appeared unaltered were slightly labeled (Fig. 2c). However, a close examination revealed that a significant amount of gold particles was associated with both the plasmalemma and the cytoplasmic region underlying these undamaged wall areas (Fig. 2c, arrowheads). Labeling was also found to be associated with the inner layers of contiguous fungal cell walls (Fig. 2, b and c, small arrows).

In the invaded cortical cells, gold particles seemed to accumulate over host wall areas in the immediate vicinity of fungal cells (Fig. 2a, arrows). However, a decrease of labeling was observed over wall portions that were not in close contact with fungal cells. Intercellular spaces appeared nearly free of labeling, whereas few gold particles occurred over the space corresponding to the preexisting cytoplasm (Fig. 2b).

In colonized paratracheal parenchyma cells adjacent to xylem vessels, a labeling pattern similar to the one observed in cortical cells was detected (Fig. 3, a and b). These cells as well as primary phloem cells were invaded by hyphae which were found to grow directly through tangential walls toward the stele. Channels of penetration were usually narrower than the average hyphal diameter (Fig. 3a). Following treatment with the antiserum specific for tomato chitinase and with GAR-gold antibodies, walls and septa of the invading fungal cells were intensely labeled (Fig. 3, a and b). However, as already mentioned, gold particles were preferentially associated with severely altered fungal wall areas (Fig. 3b, arrow). Over septa, labeling was evenly distributed, although the inner layers were predominantly covered by gold particles. An accumulation of gold particles occurred over host wall areas either adjacent to the channel of fungal penetration (Fig. 3, a and b, arrowhead) or neighboring fungal cells (Fig. 3a, double arrows).

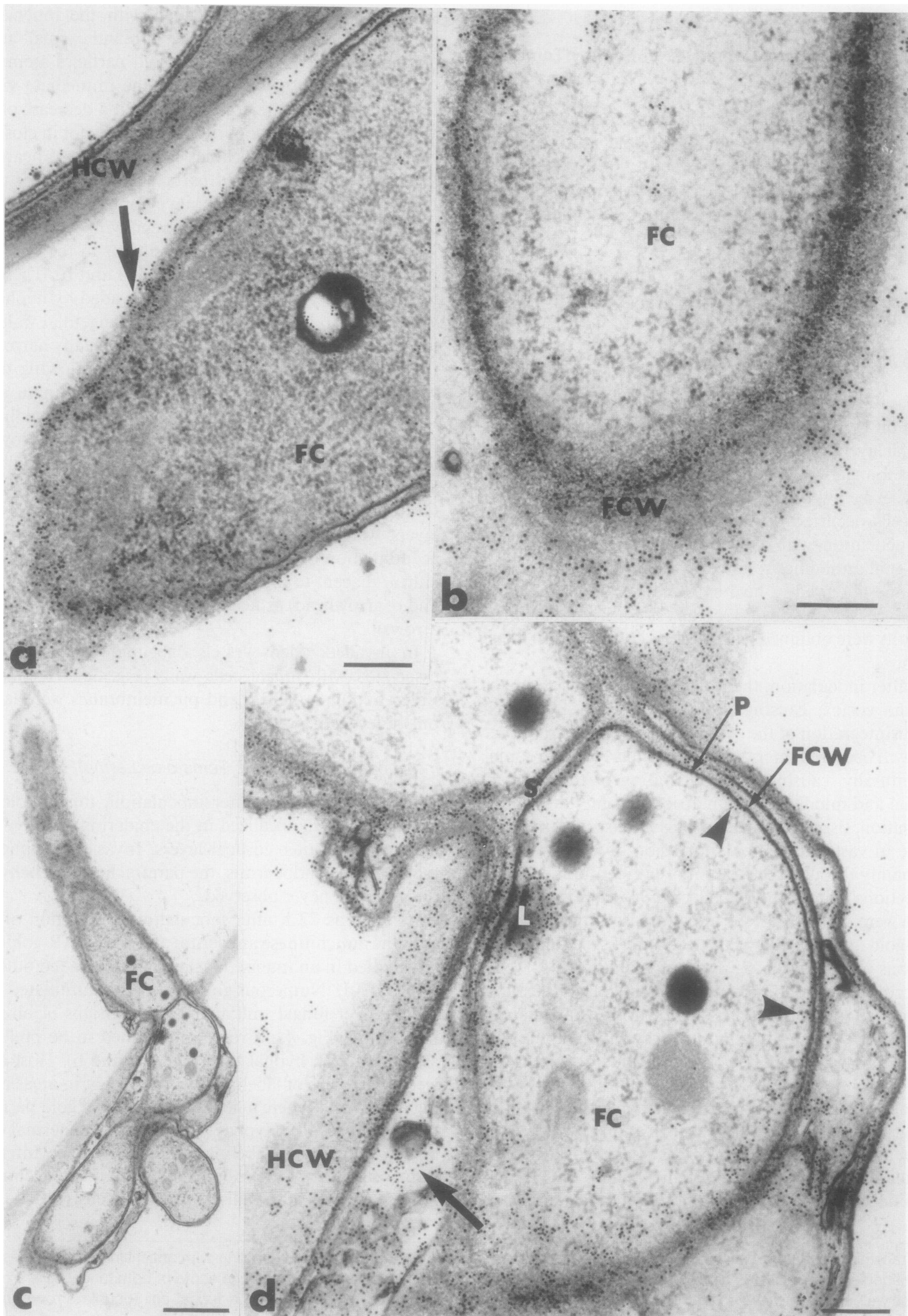
In invaded xylem vessels, labeling was also specifically associated with fungal cells walls (Fig. 3c). Host secondary walls, middle lamellae, and pit membranes were labeled by only a few gold particles.

Incompatible Interaction: Tomato cv Larma/FORL

From 48 to 120 h after inoculation, fungal colonization remained strictly localized in the epidermis, the hypodermis, and the first outer cortical layers. Invasion of inner tissues including the endodermis, the paratracheal parenchyma, and the stele was never observed.

As soon as 72 h after inoculation, incubation of sections with the antichitinase antiserum and with GAR-gold antibodies resulted in an intense labeling of the invaded outer tissues (Fig. 4, a–d). Numerous gold particles accumulated predominantly over fungal wall areas showing signs of obvious disintegration (Fig. 4a, arrow) and seemed to be preferentially associated with hyphal tips (Fig. 4, a and b). Host cell walls were nearly free of labeling (Fig. 4a) except in areas neighboring fungal cells where a heavy deposition of gold particles was observed (Fig. 4d). Hyphae penetrating hypodermal cell walls displayed an intense labeling of their cell surface. Interestingly, numerous gold particles were often found to accumulate in spaces between host walls and fungal cells (Fig. 4d, arrow).

Figure 3. Transmission electron micrographs of FORL-infected susceptible tomato root tissues labeling with antichitinase antibodies and with GAR-gold antibodies. Vascular parenchyma cells (a, b) and vessel (c), 96 h after inoculation. a, Walls and septa of both fungal cells are intensely labeled. Host wall areas adjacent to the channel of fungal penetration (arrowhead) or neighboring a fungal cell (double arrow) are labeled. ×30,000; bar = 0.5 μm. b, Enlarged portion of (a) showing the accumulation of gold particles over apparently altered fungal wall areas (arrow). The host cell wall in the immediate vicinity of the penetration channel is markedly labeled (arrowhead). ×45,000; bar = 0.25 μm. c, The secondary wall of a xylem vessel is labeled by few gold particles. Labeling occurs at the fungus cell surface. ×54,000; bar = 0.25 μm. HSW, host secondary wall; S, septum; other abbreviations as in Figures 1 and 2.


HCW
FC
a
FC
FCW
b
FC
c
P
FCW
S
L
FC
HCW
d

Few gold particles were always associated with the plasmalemma underlying undamaged fungal wall areas (Fig. 4d, arrowheads). Lomasomes, known to result from an invagination of the plasma membrane, were occasionally seen and always found to be intensely labeled (Fig. 4d).

In uninvaded inner tissues, the intensity of labeling was markedly reduced. The few gold particles that were observed, seemed mainly associated with primary (Fig. 5a) and secondary walls (Fig. 5b). Middle lamellae as well as intercellular spaces and cell cytoplasm were nearly free of labeling.

A similar labeling pattern was observed over sections from samples collected at 96 and 120 h after inoculation with FORL.

Cytochemical Localization of Chitin in the Cell Wall of FORL

WGA, a lectin with *N*-acetylglucosamine-binding specificity, was used to detect chitin, a polymer of β-1,4-linked *N*-acetylglucosamine units, in FORL cell walls. Incubation of infected tomato root tissues (collected 96 and 120 h after inoculation) with WGA/ovomucoid-gold complex resulted in a specific labeling of fungal cell walls and septa (Fig. 6a). However, the intensity of labeling was found to vary from one hypha to another according apparently to their specific location in the host tissue. In the susceptible tomato cultivar, gold particles were evenly distributed over the wall of fungal cells that were not in close contact with the host wall (Fig. 6b). Even though disruption of the outermost wall layers of these fungal cells was frequently observed (Fig. 6, c and d, arrows), labeling was not altered as judged by the significant amount of gold particles still occurring over the remaining part of the wall. By contrast, labeling was irregular over fungal cells that were closely appressed to host cell walls (Fig. 7, a and b, arrowheads). Alterations in the labeling distribution were also found to occur over cell walls of hyphae penetrating host walls (Fig. 7c, arrowheads). These changes occurring mainly over hyphae appressed to host cell walls were consistently observed with all sections of tomato root samples examined.

In the resistant tomato cultivar, the labeling pattern of fungal cell walls was similar to that observed in susceptible plants. Fungal cells restricted to the outer host tissue displayed pronounced wall alterations and irregularities in labeling distribution (not illustrated).

Control Tests

Several control tests were performed in order to assess the specificity of both the labeling patterns obtained with the antichitinase antiserum and with the WGA. Preadsorption of the antichitinase antiserum with an excess of purified chitinase prior to section treatment resulted in a near absence of labeling (Fig. 8a). Similarly, incubation of sections with preimmune serum yielded negative results. Specificity of the labeling observed with WGA/ovomucoid-gold complex was assessed through the negative results obtained with all control tests including, among others, the adsorption of WGA with its inhibitory sugar, *N-N′-N″*-acetyl-chitotriose (Fig. 8b).

DISCUSSION

In a recent work, Joosten and De Wit (17) reported that chitinases and β-1,3-glucanases were induced in tomato plants upon infection by *Cladosporium fulvum*. The rapid and intense accumulation of these hydrolases at the site of penetration in incompatible interactions was interpreted by the authors as a biochemical event likely to be involved in resistance against fungal attack. The present immunocytochemical study was undertaken to gain a better insight into the role of chitinases in the outcome of tomato-fungus interactions *in situ*. Our results indicate that, at least in tomato root tissues infected by FORL, chitinases accumulate around damaged hyphae. Their presence in host wall areas adjacent to invading fungal cells suggests that induction is likely mediated by fungal elicitors. The extracellular deposition of chitinases may have a direct significance in the plant defense response by allowing a rapid contact with the pathogen.

Our time-course experiment revealed that chitinases accumulated earlier in the incompatible interaction than in the compatible one. However, in both systems, accumulation of chitinases coincided with host cell invasion by hyphae. In the incompatible interaction, chitinase increase was observed as soon as 72 h after inoculation but remained mostly restricted to invaded outer tissues. In the compatible interaction, the accumulation of chitinase had started by 96 h after inoculation, thus later than in the incompatible one, but was apparent in all tissues soon after. If the induction of chitinases closely parallels fungal distribution, it is possible that, from 4 to more d after inoculation, a higher amount of enzyme is present in susceptible than in resistant tomato plants. Although the extent of gold labeling was not quantitatively evaluated in the present study, the low level of fungal colonization in resistant tomato cultivars as well as the near absence of chitinase in uninvaded inner tissues provide support to the assumption that chitinase is more abundant in susceptible plants. This is in line with previous biochemical studies conducted on tomato—*Verticillium albo-atrum* (27) and tomato—*Fusarium oxysporum* f. sp. *lycopersici* (13) interactions in which the increase in chitinase activity was always higher in susceptible than in resistant plants. However, the present findings contrast strikingly with our recent observations on β-1,3-glucanase

Figure 4. Transmission electron micrographs of FORL-infected resistant tomato root tissues. Labeling with antichitinase antibodies and with GAR-gold antibodies. Epidermis (a, b) and hypodermis (c, d), 72 h after inoculation. a, Numerous gold particles are deposited over the fungus cell surface with a predominant accumulation over areas showing signs of degradation (arrow). The gold particles are present over the host cell wall. ×45,000; bar = 0.25 μm. b, An intense labeling is associated with the area likely corresponding to the hyphal tip. ×54,000; bar = 0.25 μm. c, A hypha is penetrating a hypodermal cell wall. ×11,000; bar = 1 μm. d, Enlarged portion of (c) showing the accumulation of gold particles over the fungus cell wall. Labeling occurs also in the plasmalemma region (arrowheads) and over a lomasome. The host cell wall area neighboring the penetration channel is labeled. Accumulation of gold particles is noticeable in the space between host cell wall and fungal cell (arrow). ×48,000; bar = 0.25 μm. L, lomasome; other abbreviations as in Figures 1 and 2.

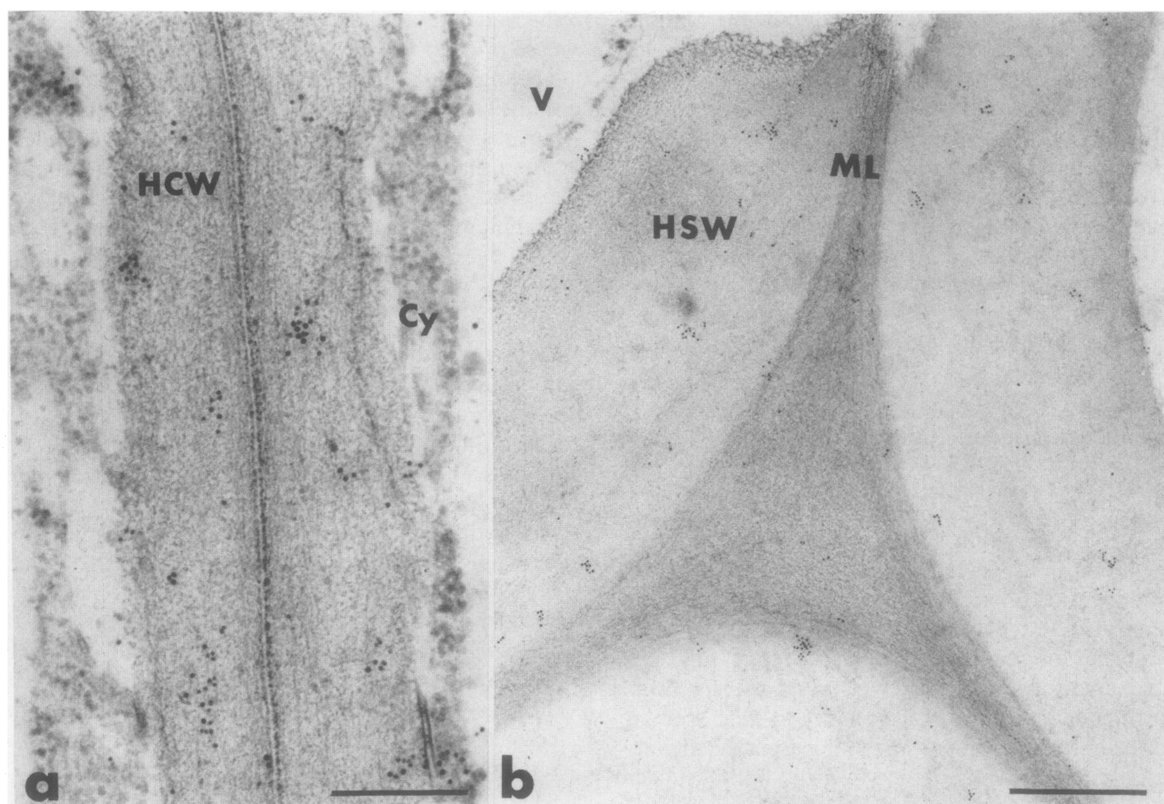


Figure 5. Transmission electron micrographs of FORL-infected resistant tomato root tissues. Labeling with antichitinase antibodies and with GAR-gold antibodies. Endodermis (a) and vascular area (b), 96 h after inoculation. a, Few scattered gold particles are distributed over the wall of an uninvaded endodermal cell. ×72,000; bar = 0.25 μm. b, Few clustered gold particles are associated with secondary thickenings of an uninvaded vessel. ×36,000; bar = 0.5 μm. V, vesicle; ML, middle lamella; other abbreviations as in Figures 1 and 3.

localization in FORL-infected tomato root tissues (6), since this enzyme was found in large amounts in uncolonized tissues of resistant plants. These variations in enzyme distribution suggest that chitinases and β-1,3-glucanases play different roles in the plant defense to fungal attack. In the incompatible tomato-FORL interaction, induction of β-1,3-glucanase is likely to be an early event associated with the protection of plants against fungal invasion, whereas chitinase accumulation may reflect a biochemical response to fungal elicitors released by the β-1,3-glucanase. In this regard, it is interesting to note that a number of plant genes coding for defense-induced molecules are activated by fungal β-1,3-glucans released through the action of plant β-1,3-glucanases (29). A recent molecular analysis of chitinase accumulation in melon plants has shown that the messenger RNAs (mRNAs) which hybridize to a genomic clone of this enzyme increased upon treatment with glucans isolated from the walls of *Colletotrichum lagenarium* (28).

In the present study, chitinase was found to be mainly associated with host wall areas neighboring fungal cells. That the enzyme was not detected in vacuoles and in intercellular spaces is surprising, since these locations are the preferential sites for chitinase accumulation in leaves and cotyledons (8, 24). Whether these variations in the pattern of enzyme distribution correlate with a different mobilization of chitinase according to the elicitor used for induction and the organ studied, or simply reflect a loss of soluble proteins during tissue processing needs to be investigated further. However, the possibility that the enzyme may first accumulate in vacuoles and be rapidly conveyed toward the cell surface for participating to fungal growth inhibition should also be considered.

The substantial amount of chitinase found at the fungal cell surface favors the hypothesis of an antifungal activity. Since chitin is one of the main components of fungal cell walls (12), it is reasonable to assume that this carbohydrate constitutes an ideal substrate for plant chitinases. In this context, the cytochemical observation that fungal chitin is drastically altered during the course of infection provides additional support for the hydrolytic activity of chitinases *in vivo*. However, the main accumulation of chitinase over fungal wall areas exhibiting obvious signs of alteration suggests that this enzyme activity is likely preceded by the hydrolytic action of other enzymes or works in concert with these enzymes. If one considers that chitin is embedded in a matrix of amorphous material (10), it is to be expected that some fungal wall components may hinder access of chitinases to their corresponding substrates. In *Schizophyllum commune*, it has been convincingly demonstrated that preliminary treatment with β-1,3-glucanase renders chitin more susceptible to chitinase (32). This has been interpreted as indicating that chitin is burried in β-glucans, making the chitin inaccessible

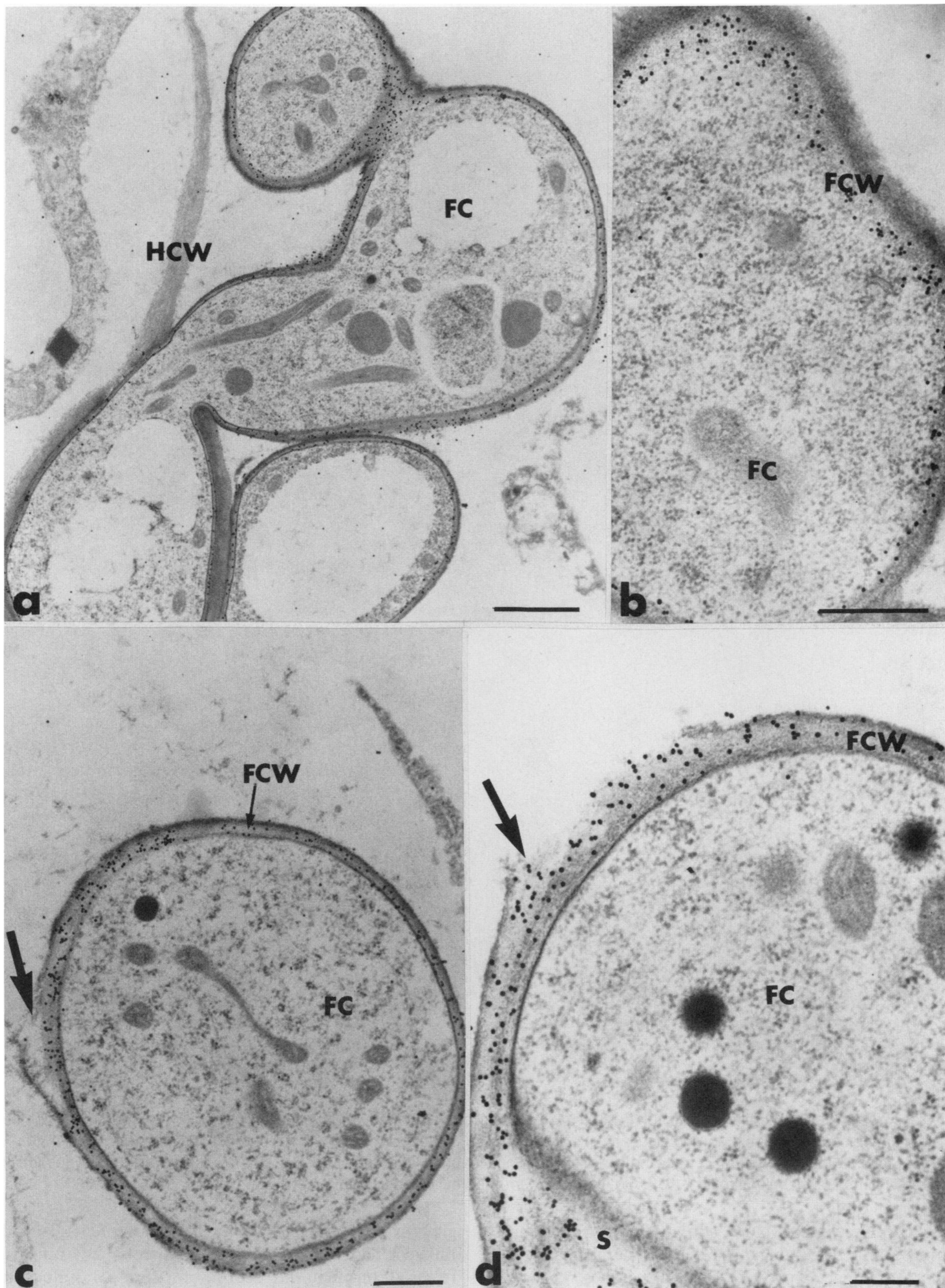


Figure 6. Transmission electron micrographs of FORL-infected susceptible tomato root tissues. Labeling with the WGA/ovomucoid-gold complex specific for *N*-acetylglucosamine residues (=chitin) 96 h after inoculation. a, The wall and the septum of a fungal cell are regularly labeled. ×26,000; bar = 0.5 μm. b, Gold particles are mainly distributed over the innermost wall layers of a fungal cell growing intracellularly. ×34,000; bar = 0.5 μm. c, d, Disruption of the outermost wall layers (arrows) occur in some fungal cells growing intracellularly. Labeling is, however, regularly distributed. c, ×22,000; bar = 0.5 μm; d, ×40,000; bar = 0.25 μm. Abbreviations as in Figures 1 to 3.

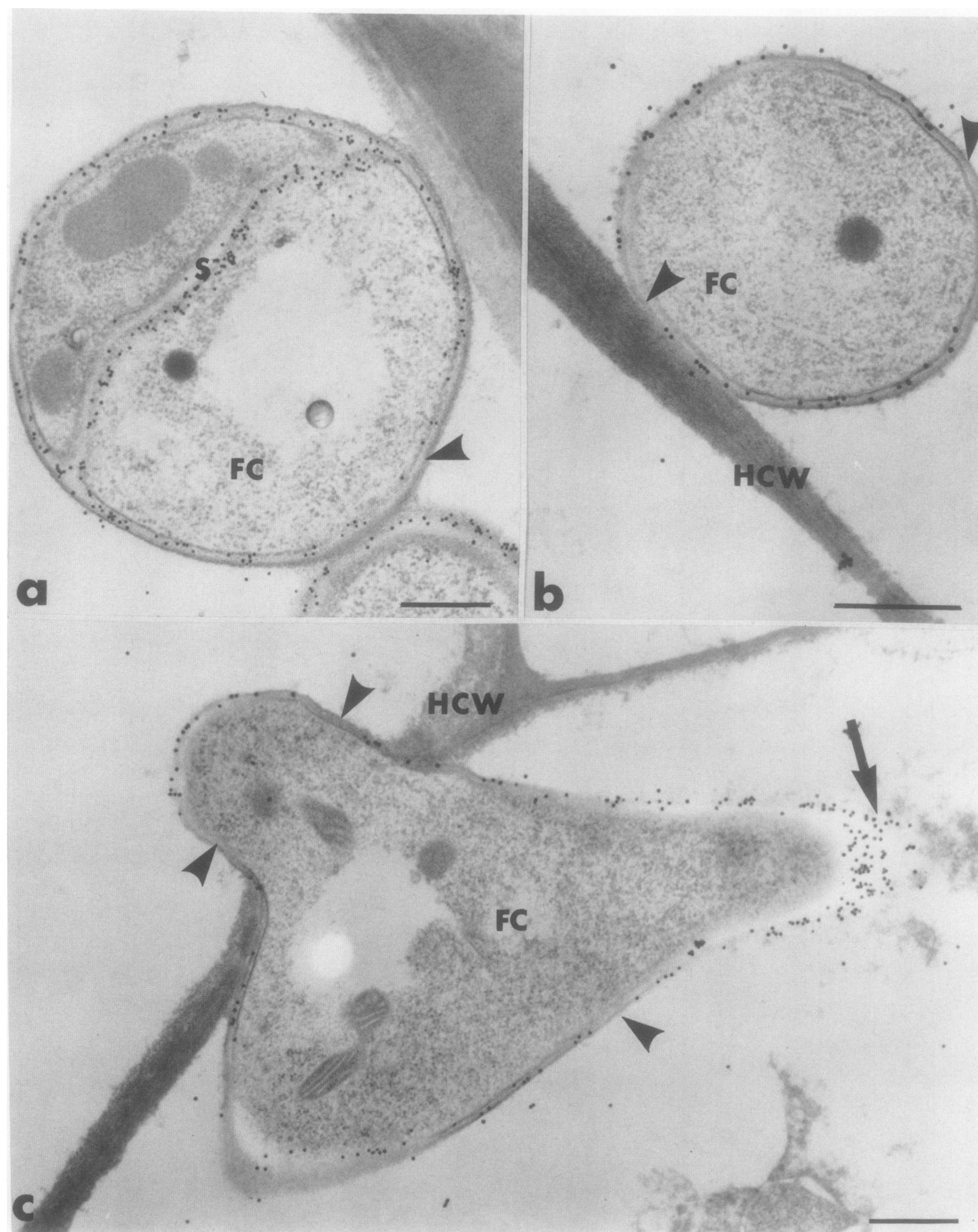


Figure 7. Transmission electron micrographs of FORL-infected susceptible tomato root tissues. Labeling with the WGA/ovomucoid-gold complex, 120 h after inoculation. a, b, Labeling discontinuities (arrowheads) are observed over fungal cells adjacent to host cell walls. a, ×28,000; bar = 0.5 μm. b, ×38,000; bar = 0.5 μm. c, Labeling alterations are noticeable over a fungal cell crossing a host cell wall (arrowheads). However, some areas remain intensely labeled (arrow). ×32,000; bar = 0.5 μm. Abbreviations as in Figures 1 and 3.

to chitinase. More recently, Mauch *et al.* (23) confirmed that plant chitinase and β-1,3-glucanase acted synergistically in the inhibition of fungal growth *in vitro*. These observations indicate that a sequential increase in the activities of β-1,3-glucanase and chitinase is required to reach an optimal function in defense. Treatment of various plant tissues with elicitors of microbial origin has been found to induce plant defense reactions (2, 3, 20, 29). These elicitors have been identified as glucans characterized by the presence of β-1,3- and β-1,6-linked glucosyl residues (2). Whether or not fungal glucans released through the action of plant β-1,3-glucanases are informational molecules for chitinase induction in infected tomato plants in a way similar to the one described in melon plants (28) deserves to be investigated further.

In summary, evidence has been presented in this study that induction of chitinases is a biochemical event of the response

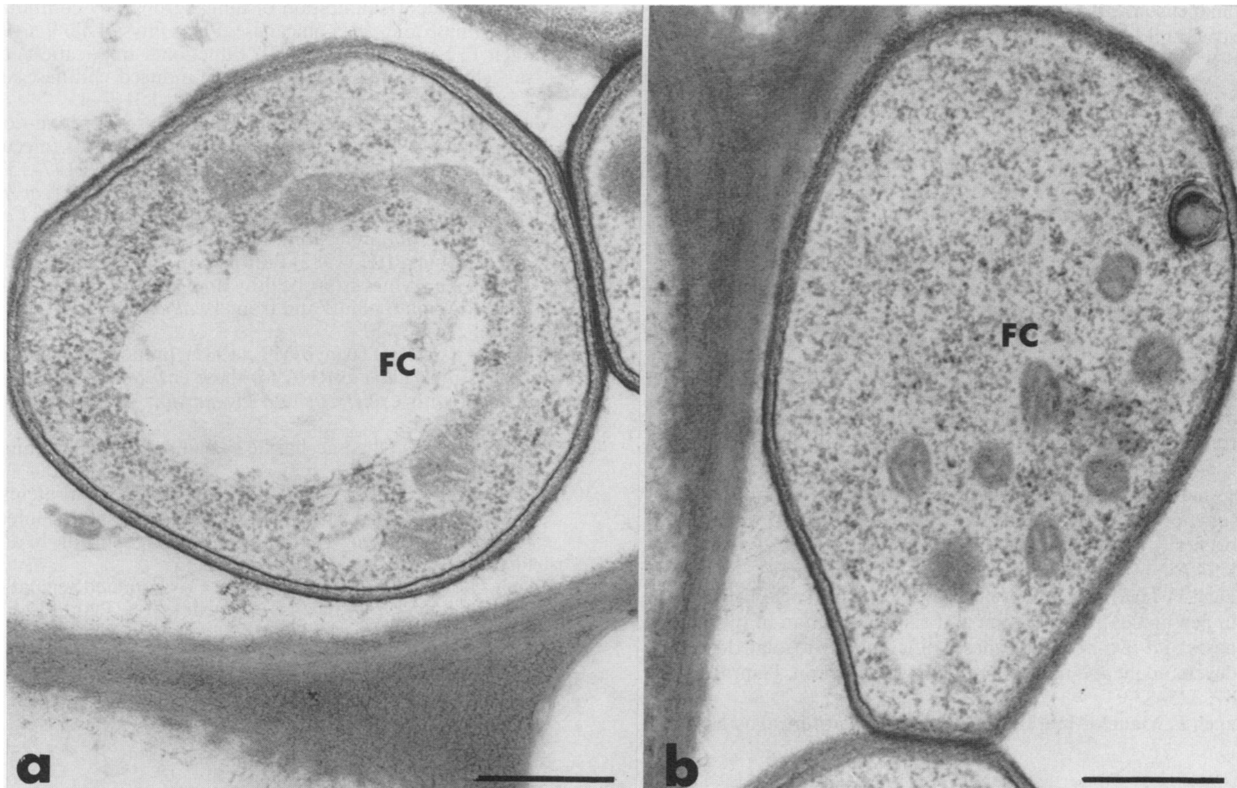


Figure 8. Transmission electron micrographs of FORL-infected susceptible tomato root tissues. Control tests, 96 h after inoculation. a, Preadsorption of the antichitinase antiserum with an excess of purified chitinase from tomato results in an absence of labeling over both fungal cells and host wall. ×40,000; bar = 0.5 μm. b, Labeling is absent over the fungus cell wall after treatment with the WGA which was previously adsorbed with *N-N′-N″*-triacetylchitotriose. ×40,000; bar = 0.5 μm. FC, fungal cell.

of tomato plants to FORL attack. To our knowledge, this study represents the first direct evidence that chitinases accumulate around fungal hyphae *in vivo*. However, it is likely that a coordinated action of chitinase and β-1,3-glucanase is a prerequisite to an effective antifungal defense function. It will now be interesting to determine how chitinases and β-1,3-glucanases are regulated at the level of gene transcription in tomato plants.

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