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Temporal and subcellular localization of PR-1 proteins in tomato stem tissues infected by virulent and avirulent isolates of *Phytophthora capsici*

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Summary. Immunoblot analysis and immunogold labeling of PR-1 protein (pathogenesis-related protein 1) in tomato (*Lycopersicon esculentum* Mill.) were performed to examine the temporal and spatial expression patterns of PR-1 protein induced by *Phytophthora capsici* infection. Soluble proteins with molecular masses of 10, 17, 25, 27 and 75 kDa were induced and accumulated in *P. capsici*-infected stem tissues during the compatible and incompatible interactions. Western blot analysis revealed that expression of PR-1 protein (17 kDa), at 12 to 24 h after inoculation, occurred earlier in the incompatible than in the compatible interaction. Immunogold labeling of PR-1 proteins occurred over cell walls and cytoplasm of the host and the oomycete pathogen and at the interface between host and oomycete cell walls at 24 h after inoculation in the compatible interaction. In the incompatible interaction, numerous PR-1 proteins accumulated predominantly over oomycete cell walls and at the interface between host and oomycete cell walls. The quantity of PR-1 proteins deposited in both host and oomycete cells was much less in the compatible than the incompatible interaction. Healthy tomato stem tissue was nearly free of immunogold labeling of PR-1 proteins.

Keywords: Pathogenesis-related protein 1; *Lycopersicon esculentum*; *Phytophthora capsici*; Immunogold labeling; Subcellular localization.

Abbreviations: SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF polyvinylidene difluoride.

Introduction

Induction of pathogenesis-related (PR) proteins 1 in various plant tissues is one of the major biochemical or molecular events when plants are subjected to infection with various pathogens such as viroids, viruses, bacteria, and fungi (Camacho-Henriquez and Sänger 1982, 1984; Cornelissen et al. 1986; Gordon-Weeks et al. 1991; Tahiri-Alaoui et al. 1993; Tornero et al. 1994; Vallélian-Binschedler et al. 1998; Cameron et al. 1999; Lee et al. 2000a). The increase in *PR-1* gene expression also occurred in plant tissues under environmental stresses, including UV irradiation and wounding (Brederode et al. 1991). These findings suggest that PR-1 protein may function in the protection of plants against biotic and abiotic stresses. The presence of PR-1 proteins has been widely reported in many monocots and dicots, and their structures and putative functions have recently been reviewed (Van Loon and Van Strien 1999). Increased tolerance against pathogens of transgenic tobacco plants overexpressing *PR-1* genes and direct antifungal activity of purified PR-1 proteins were tested in vivo and in vitro, respectively (Cutt et al. 1989, Linthorst et al. 1989, Alexander et al. 1993, Niderman et al. 1995). Overexpression of *PR-1* in tobacco provided enhanced resistance to the blue mold pathogen *Peronospora tabacina* and the black shank pathogen *P. parasitica* var. *nicotianae* but not to tobacco mosaic virus (Linthorst et al. 1989, Alexander et al. 1993). Whether or not PR-1 acts as an antimicrobial protein in planta remains to be elucidated. However, in situ immunocytochemical studies of PR-1-type proteins revealed cellular and subcellular localization in plant tissues. In tobacco plants, PR-1 proteins were demonstrated to be deposited in intercellular spaces of leaf tissues treated with salicylic acid able to induce defense reaction against tobacco mosaic virus (Hosokawa and Ohashi 1988). In contrast, PR-1 proteins were found to be present in intercellular spaces, cell walls and secondary wall

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thickenings of xylem, and inter- and intracellular hyphal walls in tobacco root tissues (Tahiri-Alaoui et al. 1993). In germinating maize seeds, another PR-1-type protein, called PRms, was localized at the plasmodesmata or papillae of *Fusarium moniliforme*-infected maize radicles and the fungal cell wall (Cordero et al. 1992; Murillo et al. 1997, 1999).

Many PR-1-type proteins have been studied in tomato plants, a host for a variety of plant pathogens (Nassuth and Sänger 1986, Christ and Mösinger 1989). P14 protein detected in tomato plants was classified as part of the PR-1 family (Cornelissen et al. 1986, Nassuth and Sänger 1986). The amino acid sequence of P14 was similar to that of tobacco PR-1 protein and the proteins serologically cross-reacted with each other (Nassuth and Sänger 1986). Antiserum raised against P14 was used for detection of PR-1-type proteins in tomato leaves infected with *Phytophthora infestans* and *Cladosporium fulvum* (Joosten et al. 1989, Niderman et al. 1995). The cDNA clones encoding the two P14 isomers, P4 and P6, were isolated and characterized (Van Kan et al. 1992). Subcellular localization of PR-1 proteins was also demonstrated in tomato plants. Vera et al. (1989) found that the P1 (P14) protein, which was induced by citrus exocortis viroid infection, targeted both vacuolar and apoplastic compartments of tomato leaf tissues. Deposition of P14 proteins, however, appeared to occur not only predominantly on primary and secondary cell walls but also at a detectable level in intercellular spaces and within wall appositions of tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Benhamou et al. 1991). Subcellular localization of the PR protein P14 in tomato plants infected with *C. fulvum* was revealed in the intercellular spaces of leaf tissues by immunoblot assays (De Wit and van der Meer 1986, De Wit et al. 1986, Joosten and De Wit 1989, Joosten et al. 1990).

Different resistance responses of tomato cultivars were evaluated with isolates of *Phytophthora capsici* from different geographic origins (Hwang and Hwang 1993). Susceptible and resistant responses of tomato stem tissues to *P. capsici* infection also were cytologically confirmed by electron microscopic analysis (Hwang et al. 1994). An accumulation of host cell wall appositions to *P. capsici* infection was a major characteristic of induced structural defense in incompatible interactions of tomato stem tissues with *P. capsici*. More recently, immunogold labeling of PR-1 proteins in pepper stem tissues infected by *P. capsici* has revealed the subcellular localization of the PR-1 protein in the *P. capsici*-pepper interactions (Lee et al. 2000a). In the present study, we have focused our attention on the temporal and spatial expression of PR-1 proteins during *P. capsici* infection in tomato stem tissues in compatible and incompatible interactions. The synthesis and in situ localization of PR-1 proteins in tomato stem tissues in response to infection by virulent and avirulent isolates of *P. capsici* were examined by Western blot analysis and immunogold labeling technique, respectively.

Material and methods

Plant, pathogen, and inoculation

Tomato plants (*Lycopersicon esculentum* Mill. cv. Kwangyang) were grown as previously described (Hwang et al. 1994). Tomato plant stems at the two-leaf stage were used for inoculation of *P. capsici* isolates. Virulent (S197) and avirulent (CBS178.26) isolates of *P. capsici* were cultured as previously described (Hwang et al. 1994). The wounded stems of tomato seedling plants were inoculated by covering them with a small piece of cotton soaked in a zoospore suspension (10^5 zoospores per ml) and bound with plastic tape to maintain moisture. The inoculated tomato plants were placed in the growth room at 28 °C for 4 days, and disease severity was monitored during the incubation period.

Preparation of protein extracts

Tomato stem tissues (2 g of fresh weight) were ground in liquid nitrogen and three volumes of extraction buffer (0.5 M sodium acetate, 15 mM 2-mercaptoethanol, 0.5 M acetic acid, pH 5.2). Homogenates were centrifuged at 20,000 **g** for 1 h at 4 °C. The supernatants were stored at –70 °C until used. The protein in supernatants was precipitated with four volumes of ice-cold acetone at –20 °C overnight, and centrifuged at 1,500 **g** for 20 min at 4 °C. The pellets were washed twice with 80% (v/v) ice-cold acetone, vacuum dried, and suspended in the same extraction buffer. The protein concentration was determined by the method described by Bradford (1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Electrophoresis was performed in a 15% sodium dodecyl sulfate(SDS)-polyacrylamide gel with an overlay stacking gel of 5%, according to the method described by Laemmli (1970). The molecular masses were estimated by coelectrophoresed marker proteins (Serva, Heidelberg, Federal Republic of Germany) ranging from 6.5 to 92.5 kDa. The 1.5 µg of protein in 10 µl of sample buffer (0.9 g of glycerol, 0.1 ml of 1% [w/v] bromophenol blue, 1 ml of 10% [w/v] SDS, and 0.1 ml of mercaptoethanol) was heated for 3 min in boiling water and applied to the gel. Proteins in the SDS-polyacrylamide gel were stained with silver nitrate (Heukeshoven and Dernick 1985).

Immunoblotting of PR-1 proteins

Crude extracts (10 µg of total protein per lane) were denatured, separated on a 15% SDS-polyacrylamide electrophoresis (PAGE) gel, and electrophoretically transferred onto a polyvinylidene difluoride

(PVDF) membrane (Hybond-P; Amersham, Little Chalfont, U.K.) with the Towbin electrotransfer buffer (Towbin et al. 1979) at a current of 200 mA. Molecular-mass markers ranging from 6.5 to 92.5 kDa (Serva) were coelectrophoresed. After protein transfer, the membrane was washed three times with 0.1 M Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST), pH 7.5, for 15 min each and blocked in a 0.5% (w/v) blocking reagent (Boehringer Mannheim, Mannheim, Federal Republic Germany) in TBST overnight at room temperature. The membrane was then incubated with the antiserum raised against the tomato PR-1 (diluted 1 : 1000 in the blocking solution) as a primary antibody, a gift from Dr. Joosten of Wageningen Agricultural University, the Netherlands (Joosten et al. 1990), and then was rinsed three times for 15 min each. Further incubation of the membrane was done for 1 h in an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (diluted 1 : 1000 in the blocking solution) (Sigma, St. Louis, Mo., U.S.A.). Antigen-antibody complexes on the blotted membrane were visualized on X-ray film by chemilluminescent detection with disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4yl) phenyl phosphate (CSPD) (Boehringer Mannheim) diluted in an alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

Tissue preparation and immunogold labeling of PR proteins

At 24 h after inoculation with virulent isolate S197 and avirulent CBS178.26 of *P. capsici*, tomato stem tissues were prepared for the ultrastructural observation of immunogold labeling by electron microscopy. Tissue samples were fixed for 2 h in 1% (w/v) paraformaldehyde and 0.025% (v/v) glutaraldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.2. After washing three times in PBS for 1.5 h each, tissue sections were gradually dehydrated with ethanol and embedded in LR-White resin (The London Resin Co., Theale, Berkshire, U.K.) at –20 °C.

Immunogold labeling was performed according to the previously described method (Lee et al. 2000a). Ultrathin sections were sliced with a diamond knife (Diatome), transferred to nickel grids double-coated with pioloform and carbon, and incubated in a phosphate lysine buffer (20 mM lysine in PBS, pH 7.2). Sections were treated with PBS containing 1% gelatin (w/v) to prevent non-specific binding of the antisera and then incubated for 3 h with the 1 : 400 diluted PR-1 antiserum. After six washes for 10 min each with PBS containing 0.05% (v/v) Tween 20, the sections were incubated with goat anti-rabbit immunoglobulin G conjugated to 10 nm diameter colloidal gold (BioCell, Cardiff, U.K.) diluted 1 : 20 with phosphate buffer containing Triton X-100 and Tween 20. The sections were then washed sequentially with distilled water. They were post-stained with freshly prepared 8% (v/v) uranyl acetate and lead citrate and then examined with a JEOL transmission electron microscope at 80 kV. Negative control test was assessed by incubation with immunoglobulin G-gold antibodies without the antiserum against PR-1 protein.

Results

Temporal accumulation of soluble proteins and PR-1 protein in tomato tissues in compatible and incompatible interactions

Protein profiles of tomato stem tissues at various times after inoculation with virulent S197 and avirulent CBS178.26 isolates of *P. capsici* are presented in Fig. 1 A. A number of soluble proteins were induced and accumulated in tomato stem tissues by *P. capsici* infection. Proteins with molecular masses of 10, 17, 25, 27, and 75 kDa were found to accumulate in the infected stem tissues in both the compatible and incompatible

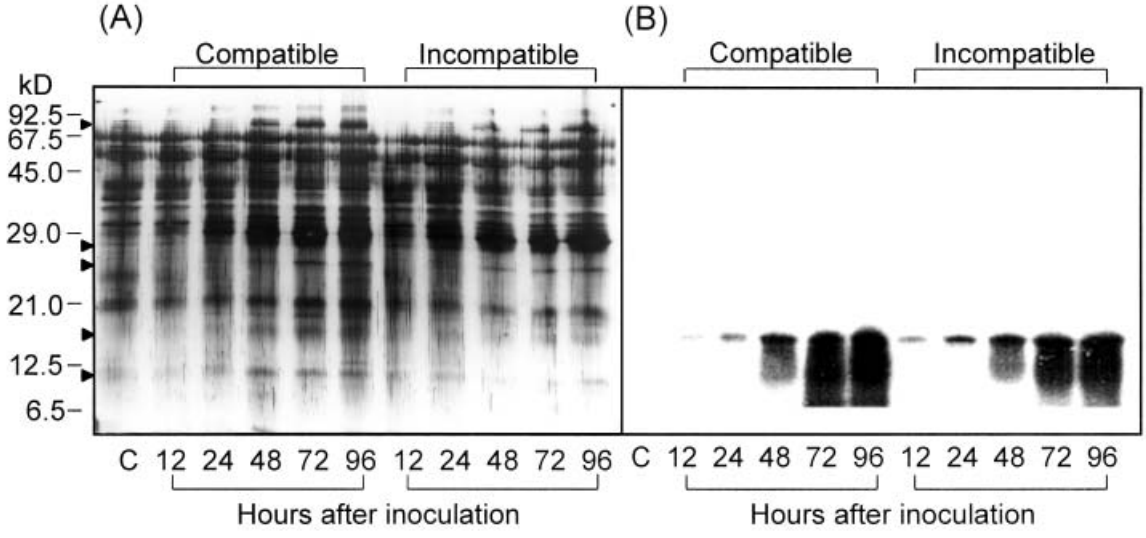

Fig. 1. SDS-PAGE profiles of soluble proteins (**A**) and immunodetection of PR-1 protein (**B**) from stem tissues of tomato plants (cultivar Kwangyang) in compatible and incompatible interactions after inoculation with virulent (S197) and avirulent (CBS178.26) isolates of *P. capsici*, respectively. Crude protein extracts were prepared at the indicated different time intervals after inoculation and electrophoresed in a 15% SDS-polyacrylamide gel. The crude protein extracts (10 µg of protein per lane) were also subjected to SDS-PAGE and immunoblotted with an antiserum raised against tomato PR-1 protein. Arrowheads indicate the de novo inducible proteins, including PR-1 protein (17 kDa)

interactions between 48 and 96 h after inoculation with *P. capsici*. The accumulation levels of these proteins induced by *P. capsici* infection were similar in both interactions. Proteins in the gel identical to that in Fig. 1 A were transferred onto the PVDF membrane. The blot was incubated with the antiserum raised against tomato PR-1. A Western blot following the chemiluminescent detection is presented in Fig. 1 B. Proteins cross-reacting with antisera raised against tomato PR-1 proteins were not found in noninfected, healthy tomato stem tissue. However, single protein bands at a molecular mass of 17 kDa were detected on the immunoblotted membrane, indicating that PR-1 proteins accumulated in the tomato stem tissues infected by *P. capsici*. Slightly higher expression of PR-1 proteins occurred in the incompatible interaction between 12 and 24 h after inoculation, compared to the compatible interaction. During the period of 72–96 h after infection by *P. capsici*, the accumulation of PR-1 proteins in infected tomato stem tissues was relatively lower in the incompatible interaction than in the compatible interaction.

Localization of PR-1 proteins in healthy tomato stem tissues

Examination of ultrathin sections of the tomato stem tissue revealed that the ultrastructure of cell walls and organelles including the nucleus, chloroplasts, mitochondria, and host cytoplasm was well preserved (Fig. 2 A). A few immunogold-labeled PR-1 proteins were detected in the chloroplast, but other cell organelles were nearly free of labeling (Fig. 2 B).

Localization of PR-1 proteins in infected tomato stem tissues during compatible and incompatible interactions

Transverse ultrathin sections were made from *P. capsici*-inoculated tomato stem tissues to examine

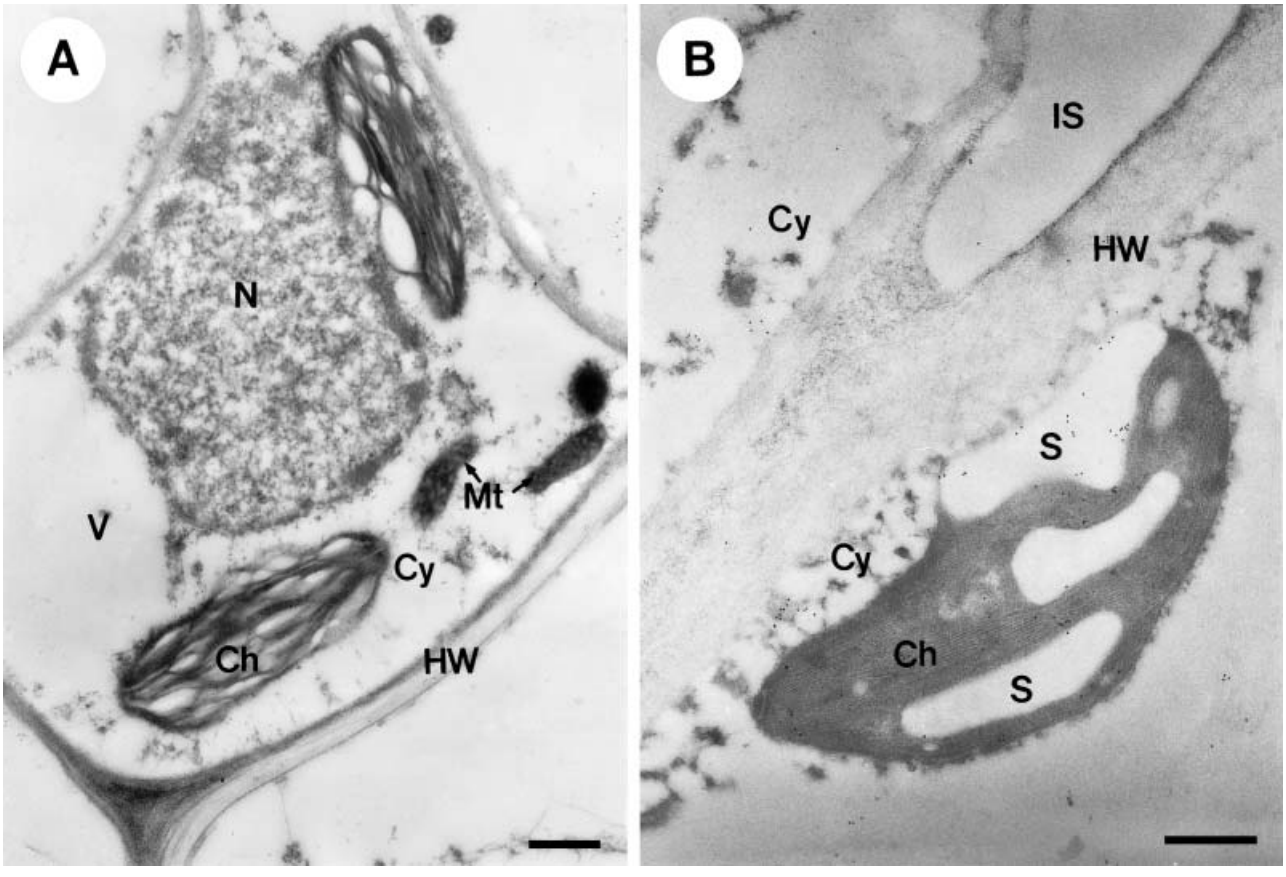

Fig. 2 A, B. Transmission electron micrographs of uninfected, healthy tomato stem tissues labeled with tomato anti-PR-1 antiserum and with goat anti-rabbit gold antibodies. Bars: 500 nm. **A** Gold labeling is nearly absent over the cell organelles such as nucleus, chloroplast, and mitochondria. **B** Some scattered, labeled PR-1 proteins occur on the starch granules of chloroplast. *Ch* Chloroplast; *Cy* cytoplasm; *HW* host cell wall; *IS* intercellular space; *Mt* mitochondria; *N* nucleus; *S* starch granule; *V* vacuole

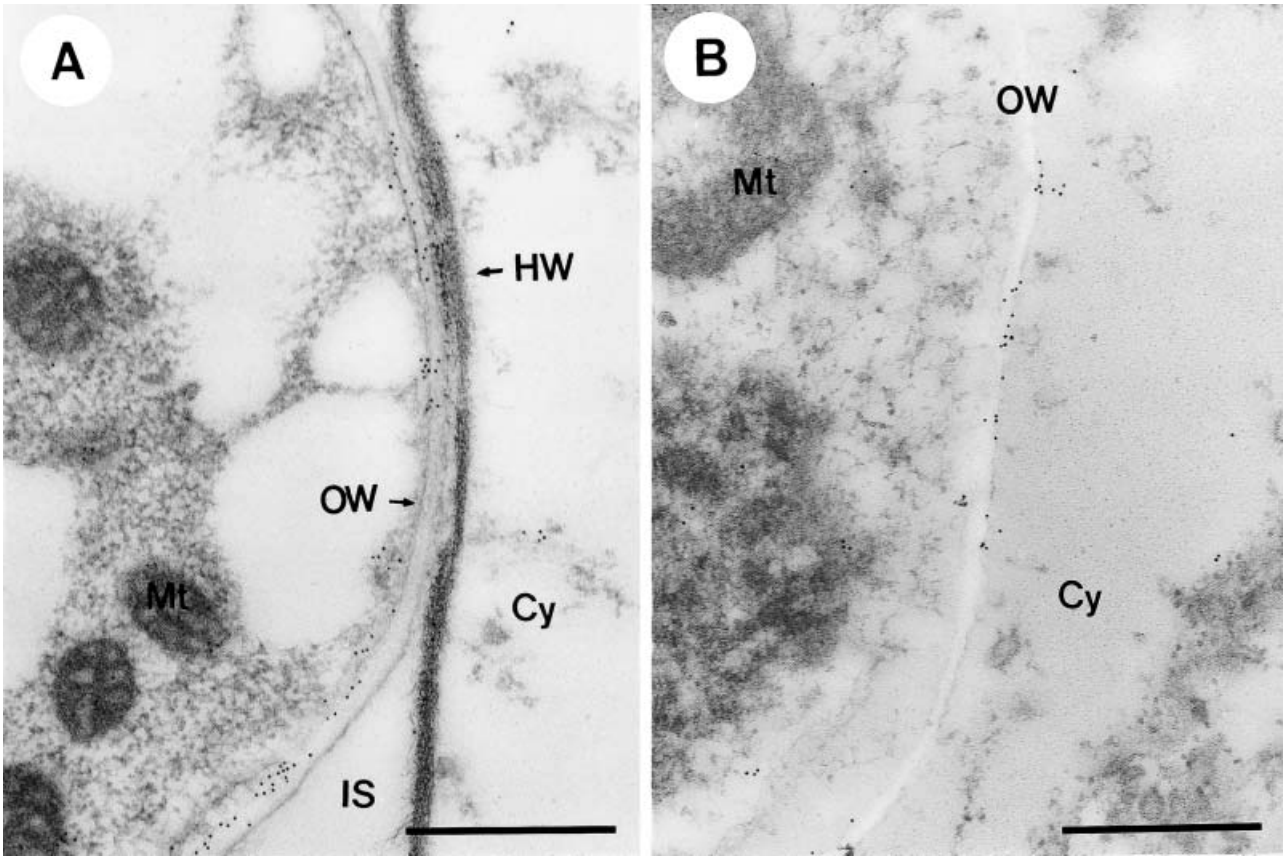

Fig. 3 A, B. Immunocytochemical localization of PR-1 proteins in tomato stem tissues in the compatible interaction 24 h after inoculation with the virulent isolate S197 of *P. capsici*. Bars: 500 nm. **A** Some scattered, labeled PR-1 proteins occur along the oomycete cell wall. **B** Some labeled PR-1 proteins are deposited over oomycete cell wall and in the host cytoplasm. *Cy* Cytoplasm; *HW* host cell wall; *IS* intercellular space; *Mt* mitochondria; *OW* oomycete wall

induction and in situ localization of PR-1 proteins following infection by virulent (S197) and avirulent (CBS178.26) isolates. Transmission electron micrographs at 24 h after infection showed the subcellular localization of PR-1 proteins in tomato stem tissues in both the compatible and incompatible interactions (Figs. 3 and 4). Nonspecifically gold-labeled proteins were not observed in the resin surrounding the stem tissue. As a negative control, immunogold-labeled PR-1 proteins did not appear in any of the sections when the PR-1 antiserum was omitted (data not shown).

Inoculation with a virulent isolate resulted in a rapid colonization of *P. capsici* in tomato stem tissues, although no typical symptoms of Phytophthora blight occurred on the tomato stem surfaces 24 h after infection (data not shown). The ramified oomycete hyphae usually grew in the intercellular spaces and formed numerous haustoria (Hwang et al. 1994). A specific deposition of immunogold-labeled PR-1 proteins was found to occur at the interface between host and oomycete cell walls (data not shown). However, oomycete cell structures such as mitochondria, vacuole, and cytoplasm were nearly free of immunogold labeling. Some scattered labeled proteins were detected along the oomycete cell wall in intimate contact with the host wall (Fig. 3 A). The PR-1 protein usually seemed to be localized in the host cytoplasm as well as in the oomycete cell wall (Fig. 3 B). The quantity of labeled PR-1 proteins deposited in both host and oomycete cells was much less in the compatible interaction than in the incompatible interaction.

In the incompatible interaction, typical symptoms of Phytophthora blight did not occur in tomato stem tissues at 24 h after inoculation with the avirulent isolate CBS178.26 of *P. capsici* (data not shown). Some of the oomycete hyphae were visible in the cortical cells of the infected tomato stem tissue, but the quantity of hyphae observed was less than in the compatible interaction (Hwang et al. 1994). In the incompatible interaction, numerous immunogold-

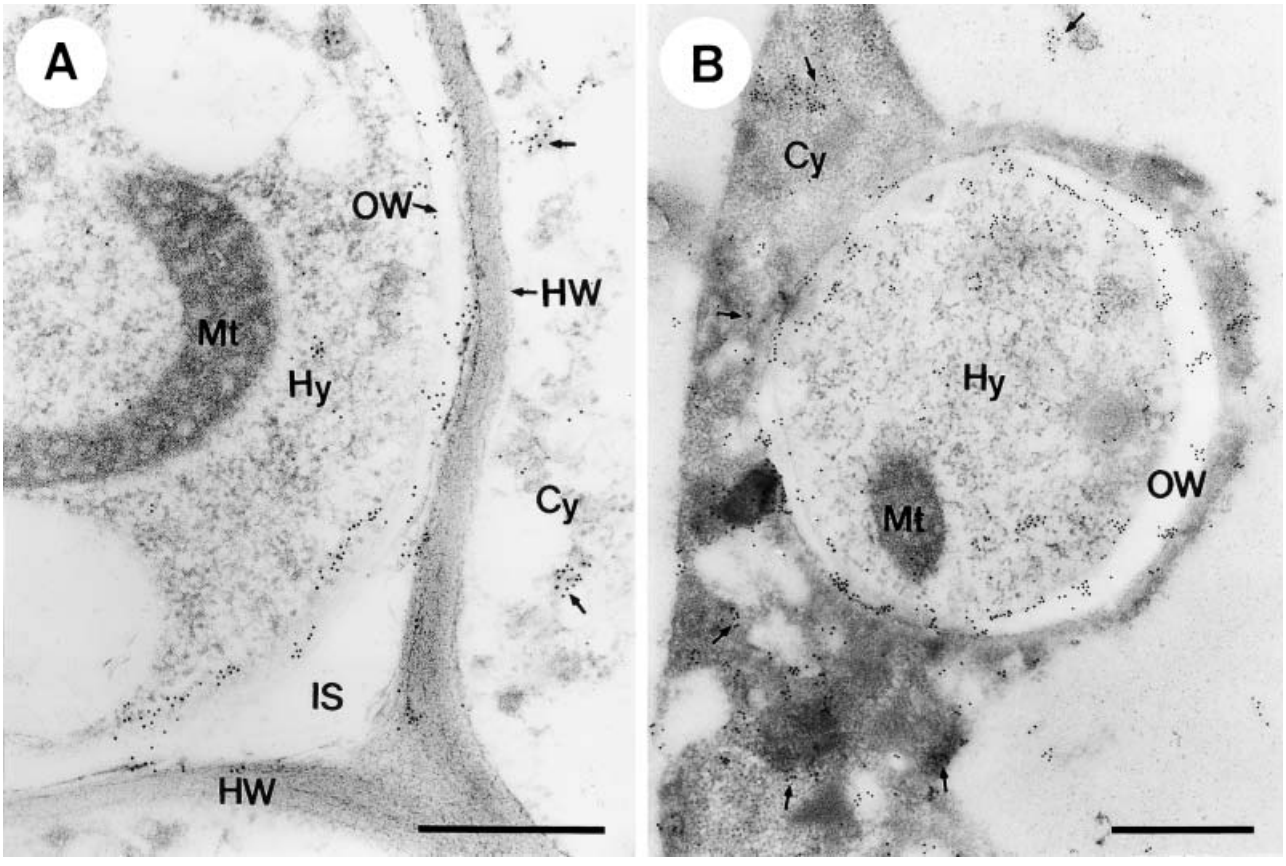

Fig. 4 A, B. Immunocytochemical localizaton of PR-1 proteins in tomato stem tissues in the incompatible interaction 24 h after inoculation with the avirulent isolate CBS178.26 of *P. capsici*. Bars: 500 nm. **A** Numerous gold-labeled PR-1 proteins are deposited over the oomycete cell walls. Some labeled PR-1 proteins (arrows) are localized at the electron-dense materials in the host cytoplasm. **B** Dense accumulation of PR-1 proteins in oomycete cell walls and hyphal cytoplasm. Some labeled PR-1 proteins (arrows) also are seen in the electron-dense host cytoplasm surrounding the oomycete cell. *Cy* Cytoplasm; *HW* host cell wall; *Hy* hypha; *IS* intercellular space; *Mt* mitochondria; *OW* oomycete wall

labeled PR-1 proteins were found to accumulate predominantly over oomycete cell walls and at the interface between host and oomycete cell walls (Fig. 4 A). Some labeled PR-1 proteins were localized at electron-opaque materials in the host cytoplasm. Dense deposits of PR-1 proteins also occurred in the oomycete cytoplasm as well as over the oomycete cell walls (Fig. 4 B). Some gold-labeled PR-1 proteins were visible over the electron-dense host cytoplasm that was in intimate contact with the avirulent oomycete hyphal cells.

Discussion

In the incompatible interaction with the avirulent isolate of *P. capsici*, disease development in the tomato stem tissues was retarded compared to that of the compatible interaction (Hwang and Hwang 1993). The different disease responses in both interactions may be in part due to the different histological and cytological features in the infected stem tissues (Hwang et al. 1994). Colonization of tomato stem tissues by hyphae of the avirulent isolate of *P. capsici* was arrested at the cortical layers, whereas hyphae of the virulent isolate spread throughout the stem tissue through endodermal layers to the pith. In the present study, some soluble proteins, which did not occur in healthy stem tissue, were found to strongly accumulate in the infected stem tissues during compatible and incompatible responses (Fig. 1 A). However, their accumulation was not significantly different between compatible and incompatible interactions. Pathogenesis-related proteins of tomato were detected and studied on their biochemical and immunological characteristics (Christ and Mösinger 1989, Fischer et al. 1989). Eleven soluble proteins with molecular masses in the range of 13–82 kDa were de novo synthesized by *Phytophthora infestans* or *Fulvia fulva* infection. In

response to infection by *P. capsici* in the present study, several PR-proteins with similar low molecular masses were also detectable in tomato stem tissue. Among the host-inducible proteins, drastic induction and accumulation of PR-1 proteins in the tomato stems by either virulent or avirulent isolates of *P. capsici* was revealed by Western blot analysis (Fig. 1 B). The PR-1 proteins seemed to originate from tomato, but not from *P. capsici*, because Joosten et al. (1990) purified and serologically characterized the PR-1 proteins from tomato. The molecular mass of tomato PR-1 protein was estimated to be approximately 17 kDa, which was similar to that of various PR-1 proteins from tobacco leaves and maize seeds (Parent and Asselin 1984, Vera et al. 1989, Murillo et al. 1999). Differences in induction time and in accumulated quantity of tomato PR-1 protein were not found between both interactions, which suggests that PR-1 protein induction in tomato stem tissues may be more significant in the resistance responses against *P. capsici* infection than in disease development. These results are well supported by our previous findings of temporal and spatial expression of PR-1 proteins in pepper stem tissues (Kim and Hwang 2000, Lee et al. 2000a). The *PR-1* gene was activated faster and in greater quantities in the incompatible interaction with *P. capsici*, and a strong accumulation of PR-1 mRNA occurred at vascular bundles in the pepper stem tissue infected by the avirulent isolate of *P. capsici* (Lee et al. 2000a).

In situ localization of the PR-1 protein in the healthy and *P. capsici*-infected tomato stem tissue was examined by the immunogold labeling technique with the antiserum raised against the tomato PR-1 protein. Some gold particles indicating localization of PR-1 proteins were scattered over the starch granules within the chloroplasts in healthy tomato tissue (Fig. 2 B). The role of PR-1 proteins deposited on starch granules in healthy tomato stem tissue seems ambiguous. More recently, the PR-5 group of pathogenesis-related protein AP24 was demonstrated to be localized at the starch granules of tomato leaf tissue (Jeun and Buchenauer 2001). However, although there is the possibility that the immunogold labeling of the chloroplasts was nonspecific, the association of PR-1 protein with chloroplast targeting needs to be studied in greater detail.

The PR-1 protein was mainly deposited over the oomycete hyphal walls of *P. capsici*, host cell wall, and cytoplasm in tomato stem tissues infected with virulent and avirulent isolates (Figs. 3 and 4). In situ localization of the tomato PR-1 protein revealed that the PR-1 protein was induced by the infection of *P. capsici* and localized at the host cytoplasm. The PR-1 protein may play a defensive role against inter- and intracellular hyphae through deposition over the hyphal wall and cytoplasm. Interestingly, the intercellular space of tomato tissues was free of immunogold labeling of PR-1 proteins. Higher amounts of the PR-1 protein were detected in the hyphal walls and oomycete cytoplasm of the avirulent isolate and in the host cytoplasm surrounding the hyphae (Fig. 4). Dense accumulation of PR protein, i.e., PR-1, chitinase, and β-1,3-glucanase found in the resistance responses, was often observed in either tomato leaf tissues infected by *Cladosporium fulvum* (Wubben et al. 1992) or pepper stem tissues infected by *P. capsici* (Lee et al. 2000a, b). Growth of avirulent hyphae in tomato stem tissues was limited to the cortical area (Hwang et al. 1994). The dense deposition of gold particles over the avirulent hyphae in the present study indicates that higher amounts of tomato PR-1 proteins accumulated in the avirulent hyphae locally arrested in the cortical layer in the incompatible interaction.

The tomato PR-1 protein seemed to directly deposit itself at the oomycete hyphal walls in tomato stem tissues. More recently, pepper PR-1 proteins were found to be predominantly localized and accumulated at the electron-dense materials in intercellular spaces and over the interface between oomycete hypha and host cell wall against *P. capsici* infection in pepper stem tissues (Lee et al. 2000a). In the present study, we observed in situ localization of PR-1 proteins in the oomycete hyphal wall and host cytoplasm in tomato stem tissues infected by *P. capsici*. However, deposition of PR-1 proteins in intercellular spaces was not detected in tomato stem tissues. The distinct difference of PR-1 localization between the pepper and the tomato plants suggests that the PR-1 protein may function differently in defense reactions against Phytophthora blight in pepper and tomato stem tissues.

Previously purified tomato and tobacco PR-1 proteins showed antifungal activity against *P. infestans*, that is, germination of zoospores was stopped or zoospore lysis was observed (Niderman et al. 1995). Recently, a possible direct role of PR-1-type proteins was suggested in plant defense (Tahiri-Alaoui et al. 1993, Murillo et al. 1999). In tomato stem tissues infected by *P. capsici*, the PR-1 protein may exert antifungal activity by direct deposition over the oomycete walls, which could be done by acting synergistically

with other PR proteins, including chitinases (Benhamou et al. 1990, Wubben et al. 1992, Lee et al. 2000b), β-1,3-glucanases (Wubben et al. 1992), and osmotin-like AP24 proteins (Jeun and Buchenauer 2001) for the partial degradation of oomycete cell walls. The nature of the Phytophthora cell wall as a binding site of tomato PR-1 protein requires further analysis.

Taken together, PR-1 proteins among various pathogenesis-related proteins were drastically induced in tomato stem tissues by *P. capsici* infection in compatible and incompatible interactions. In particular, a higher expression of tomato PR-1 protein revealed by Western blotting at 24 h after inoculation in the incompatible interaction was consistent with a higher density of immunogold labeling of PR-1 proteins at this time point. In contrast, the high level of PR-1 protein at later times (72–96 h) after inoculation might occur in the compatible tissue, because *P. capsici* had spread much more extensively through the susceptible host tissue at this stage than it had in the resistant host. A strong accumulation of PR-1 protein in the incompatible interaction of tomato with *P. capsici*, especially in oomycete cell walls and host cytoplasm, indicates a possible defensive role of PR-1 proteins in tomato plants as an antifungal activity in combination with other PR-proteins such as β-1,3-glucanase against *P. capsici*. However, the exact functions of tomato PR-1 protein in defense to *P. capsici* infection remain to be further elucidated.

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References

Alexander D, Goodman RM, Gut-Rella M, Glascock C, Weymann K, Friedrich L, Maddox D, Ahl-Goy P, Luntz T, Ward E, Ryals J (1993) Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related proteins 1a. Proc Natl Acad Sci USA 90: 7327–7331

Benhamou N, Grenier J, Asselin A (1991) Immunogold localization of pathogenesis-related protein P14 in tomato root cells infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Physiol Mol Plant Pathol 38: 237–253

– Joosten MHAJ, De Wit PJGM (1990) Subcellular localization of chitinase and of its potential substrate in tomato root tissues infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Plant Physiol 92: 1108–1120

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. Anal Biochem 72: 248–250

Brederode FT, Linthorst HJM, Bol JF (1991) Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol Biol 17: 1117–1125

Camacho-Henriquez A, Sänger HL (1982) Analysis of acid extractable tomato leaf proteins after infection with a viroid, two viruses and a fungus and partial purification of the "pathogenesis-related" protein P14. Arch Virol 74: 181–195

– – (1984) Purification and partial characterization of the major "pathogenesis-related" tomato leaf protein P14 from potato spindle tuber viroid (PSTV)-infected leaves. Arch Virol 81: 263–284

Cameron RK, Pavia NL, Lamb CJ, Dixon RA (1999) Accumulation of salicylic acid and PR-1 transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*. Physiol Mol Plant Pathol 55: 121–130

Christ U, Mösinger E (1989) Pathogenesis-related proteins of tomato I: induction by *Phytophthora infestans* and other biotic and abiotic inducers and correlation with resistance. Physiol Mol Plant Pathol 35: 53–65

Cordero MJ, Raventós D, San Segundo B (1992) Induction of PR proteins in germinating maize seeds infected with the fungus *Fusarium moniliforme*. Physiol Mol Plant Pathol 41: 189–200

Cornelissen BJC, van Huijsduijnen RAMH, van Loon LC, Bol JF (1986) Molecular characterization of messenger RNAs for "pathogenesis-related" proteins 1a, 1b, and 1c, induced by TMV infection of tobacco. EMBO J 5: 37–40

Cutt JR, Harpster MH, Dixon DC, Carr JP, Dunsmuir P, Klessig DF (1989) Disease response to tobacco mosaic virus in transgenic tobacco plants that constitutively express the pathogenesis-related PR-1b gene. Virology 173: 89–97

De Wit PJGM, Van der Meer FE (1986) Accumulation of the pathogenesis-related tomato leaf protein P14 as an early indicator of incompatibility in the interaction between *Cladosporium fulvum* (syn. *Fulvia fulva*) and tomato. Physiol Mol Plant Pathol 28: 203–214

– Buurlage MB, Hammond KE (1986) The occurrence of host-, pathogen, and interaction-specific proteins in the apoplast of *Cladosporium fulvum* (syn. *Fulvia fulva*) infected tomato leaves. Physiol Mol Plant Pathol 29: 159–172

Fischer W, Christ U, Baumgartner M, Erismann KH, Mösinger E (1989) Pathogenesis-related proteins of tomato II: biochemical and immunological characterization. Physiol Mol Plant Pathol 35: 67–83

Gordon-Weeks R, White RF, Pierpoint WS (1991) Evidence of endogenous inducers of the accumulation of pathogenesis-related (PR-1) proteins in leaves of virus-infected tobacco plants and of an interspecific *Nicotiana* hybrid. Physiol Mol Plant Pathol 38: 375–392

Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6: 103–112

Hosokawa D, Ohashi Y (1988) Immunochemical localization of pathogenesis-related proteins secreted into the intercellular spaces of salicylate-treated tobacco leaves. Plant Cell Physiol 29: 1035–1040

Hwang JS, Hwang BK (1993) Quantitative evaluation of resistance of Korean tomato cultivars to isolates of *Phytophthora capsici* from different geographic areas. Plant Dis 77: 1256–1259

– – Kim WK (1994) A light and electron microscopical study of compatible and incompatible interactions between *Phytophthora*

capsici and tomato (*Lycopersici esculentum*). Kor J Plant Pathol 10: 83–91

Jeun YC, Buchenauer H (2001) Infection structures and localization of the pathogenesis-related protein AP24 in leaves of tomato plants exhibiting systemic acquired resistance against *Phytophthora infestans* after pre-treatment with 3-aminobutyric acid or tobacco necrosis virus. J Phytopathol 149: 141–153

Joosten MHAJ, De Wit PJGM (1989) Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as β-1,3-glucanases and chitinases. Plant Physiol 89: 945–951

– Bergmans CJB, Meulenhoff EJS, Cornelissen BJC, De Wit PJGM (1990) Purification and serological characterization of three basic 15-kilodalton pathogenesis-related proteins from tomato. Plant Physiol 94: 585–591

Kim YJ, Hwang BK (2000) Pepper gene encoding a basic pathogenesis-related 1 protein is pathogen and ethylene inducible. Physiol Plant 108: 51–60

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lee YK, Hippe-Sanwald S, Lee SC, Hohenberg H, Hwang BK (2000a) In situ localization of PR-1 mRNA and PR-1 protein in compatible and incompatible interactions of pepper stems with *Phytophthora capsici*. Protoplasma 211: 76–93

– – Jung HW, Hong JK, Hause B, Hwang BK (2000b) In situ localization of chitinase mRNA and protein in compatible and incompatible interactions of pepper stems with *Phytophthora capsici*. Physiol Mol Plant Pathol 57: 111–121

Linthorst HJM, Meuwissen RLJ, Kauffmann S, Bol JF (1989) Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection. Plant Cell 1: 285–291

Murillo I, Cavallarin L, San Segundo B (1997) The maize pathogenesis-related PRms protein localizes to plasmodesmata in maize radicles. Plant Cell 9: 145–156

– – – (1999) Cytology of infection of maize seedlings by *Fusarium moniliforme* and immunolocalization of the pathogenesis-related PRms protein. Phytopathology 89: 737–747

Nassuth A, Sänger HL (1986) Immunological relationship between "pathogenesis-related" leaf proteins from tomato, tobacco and cowpea. Virus Res 4: 229–242

Niderman T, Genetet I, Bruyère T, Gees R, Stintzi A, Legrand M, Fritig B, Mösinger E (1995) Pathogenesis-related PR-1 proteins are antifungal. Plant Physiol 108: 17–27

Parent JP, Asselin A (1984) Detection of pathogenesis-related proteins (PR or *b*) and of other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. Can J Bot 62: 564–569

Tahiri-Alaoui A, Dumas-Gaudot E, Gianinazzi S (1993) Immunocytochemical localization of pathogenesis-related PR-1 proteins in tobacco root tissues infected in vitro by the black root rot fungus *Chalara elegans*. Physiol Mol Plant Pathol 42: 69–82

Tornero P, Conejero V, Vera P (1994) A gene encoding a novel isoform of the PR-1 protein family from tomato is induced upon viroid infection. Mol Gen Genet 243: 47–53

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354

Vallélian-Bindschedler L, Métraux JP, Schweizer P (1998) Salicylic acid accumulation in barley is pathogen specific but not required for defense-gene activation. Mol Plant Microbe Interact 11: 702–705

Van Kan JAL, Joosten MHAJ, Wagemaker CAM, Van den Berg-Velthuis GCM, De Wit PJGM (1992) Differential accumulation of mRNAs encoding extracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol Biol 20: 513–527

Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol 55: 85–97

Vera P, Hernandez-Yago J, Conejero V (1989) "Pathogenesis-related" P1 (p14) protein: vacuolar and apoplastic localization in leaf tissue from tomato plants infected with citrus exocortis viroid: in vitro synthesis and processing. J Gen Virol 70: 1933–1942

Wubben JP, Joosten MHA, Van Kan JAL, De Wit PJGM (1992) Subcellular localization of plant chitinases and β-1,3-glucanases in *Cladosporium fulvum* (syn. *Fulvia fulva*)-infected tomato leaves. Physiol Mol Plant Pathol 41: 23–32