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In situ localization of PR-1 mRNA and PR-1 protein in compatible and incompatible interactions of pepper stems with *Phytophthora capsici*

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Summary. In situ hybridization and immunogold labeling were performed to examine the temporal and spatial expression pattern of pathogenesis-related protein 1 (*CABPR1*) mRNA and PR-1 protein in pepper (*Capsicum annuum* L.) stem tissues infected by virulent and avirulent isolates of *Phytophthora capsici*. *CABPR1* mRNA accumulation was confirmed in the infected pepper stem tissue by Northern blot analysis and in situ hybridization. Northern blot analysis showed that the temporal expression of *CABPR1* mRNA varied greatly between compatible and incompatible interactions. An earlier expression of the *CABPR1* gene, 6 h after inoculation, was observed in the incompatible interaction. In situ hybridization results revealed that *CABPR1* mRNA was expressed in the phloem areas of vascular bundles in infected pepper stem tissues, but especially strongly in the incompatible interaction. PR-1 protein was predominantly found in the intercellular spaces of pepper stem cells in the compatible and incompatible interactions 24 h after inoculation. Strikingly, the immunogold labeling was associated with fibrillar and electron-dense material localized in the intercellular space. Dense labeling of PR-1 protein was also seen at the interface of the pathogen and the host cell wall, whereas few gold particles were detected over the host cytoplasm. However, PR-1 protein was not detected over the fungal cell wall in either interaction.

Keywords: Pathogenesis-related protein 1; *CABPR1* gene; In situ hybridization; Immunogold labeling; *Capsicum annuum*; *Phytophthora capsici*.

Introduction

Many plants respond to external stimuli, such as environmental stress and pathogen attack. Pathogen attack induces various biochemical responses in plants. Accumulation of phytoalexin, increase in certain hydrolytic enzyme activities, and de novo synthesis of proteins are among the defense mechanisms induced by pathogen infection (Lamb et al. 1989, Keen 1992). A major part of the induced defense response consists of the accumulation of pathogenesis-related (PR) proteins. Accumulation of PR proteins has been observed in several dicotyledonous and monocotyledonous plants (De Wit and Van Der Meer 1986, Cordero et al. 1992). The PR proteins accumulated in plants upon infection by pathogenic organisms (Linthorst 1991, Stintzi et al. 1993, Tornero et al. 1994, Kim and Hwang 1996, Lee and Hwang 1996), abiotic elicitors, UV light, and some chemicals (Green and Fluhr 1995).

PR proteins are characterized by selective extraction at low pH, relatively low molecular weight, accumulation in the intercellular space, high resistance to proteolytic activity, easy resolution by electrophoresis in polyacrylamide gel, and extreme isoelectric points. Recently, Van Loon et al. (1994) proposed classifying PR proteins into 11 families (PR-1 to PR-11) on the basis of their sequence relationships. Accordingly, the term "pathogenesis-related proteins" corresponds to "plant proteins that are induced in pathological and related situations". Thus, the PR-1 family contains tobacco PR-1a that has antifungal activity (Antoniw et al. 1980). However, the old PR-10 is a parsley "PR-1" protein, which has ribonuclease-like activity (Somssich et al. 1986).

Proteins belonging to the PR-1 family were first identified in tobacco mosaic virus-infected tobacco plants (Van Loon 1976). These serologically related proteins have a molecular mass of 14–17 kDa and accumulate in many plant species subjected to

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pathogen infection and external stimuli. Many isoforms of PR-1 have been isolated, and many genes encoding them have been cloned and sequenced (Linthorst 1991, Stintzi et al. 1993). One of the most prominent PR proteins, designated P14, appeared in the intercellular spaces of tomato leaves after infection by viroids, viruses, or fungi (Camacho-Henriquez and Sänger 1982, De Wit and Van Der Meer 1986, Fischer et al. 1989, Vera et al. 1989). This 14 kDa protein was purified from potato spindle tuber viroid-infected tomato leaves and its amino acid sequence was determined (Lucas et al. 1985). P14 appeared to be serologically related to PR-1a, -1b, and -1c from tobacco and to a PR protein from cowpea (Nassuth and Sänger 1986). Joosten et al. (1990) showed that apoplastic fluids from *Cladosporium fulvum*-infected tomato leaves contain three basic PR-1 proteins. No resistance was found to viruses in transgenic tobacco plants expressing PR-1a and PR-1b (Cutt et al. 1989, Linthorst et al. 1989). Recently, increased resistance and significantly reduced disease symptoms were demonstrated in transgenic tobacco expressing protein PR-1a against *Peronospora tabacina* and *P. parasitica* var. *nicotianae* (Alexander et al. 1993). The suggested antifungal activity of a PR-1 protein was confirmed by Niderman et al. (1995), who demonstrated antifungal activity of PR-1 against *Phytophthora infestans* in vitro. It cannot be excluded that proteins of the PR-1 family function directly and/or indirectly in plant defenses against fungal pathogens, although the mechanism of action is still not known.

There is considerable data available on PR proteins in dicotyledonous plants. However, there is more limited information about PR proteins in the pepper plant, especially the PR-1 protein. In previous studies, we showed that some PR proteins could be expressed in pepper plants upon pathogen attack and abiotic-elicitor treatments (Kim and Hwang 1996, Lee and Hwang 1996, Hwang et al. 1997). Lee and Hwang (1996) also demonstrated that the β-1,3-glucanase and chitinase were localized in the intercellular space of pepper leaves in the incompatible interaction between pepper plants and *Xanthomonas campestris* pv. *vesicatoria*. More recently, Hwang et al. (1997) showed the accumulation of PR-1 protein in pepper stem tissues after treatment with DL-β-amino-n-butyric acid and challenge-inoculation with *Phytophthora capsici*. To examine further whether or not the PR-1 gene is induced by pathogen infection, Kim and Hwang (1999) also cloned and characterized the PR-1 (*CABPR1*) cDNA gene from pepper leaves infected by *X. campestris* pv. *vesicatoria*.

In the present study, employing Northern blot analysis, in situ hybridization, and immunogold labeling methods, we focus on temporal and spatial accumulation of PR-1 protein and PR-1 mRNA in compatible and incompatible interactions of pepper stems with *P. capsici*. Northern blot analysis and in situ hybridization can compare the temporal accumulation of PR-1 mRNA in compatible and incompatible interactions, revealing their relationship to resistance reaction. Moreover, the spatial expression of PR-1 protein and PR-1 mRNA can provide a clue to their putative function that may be related to pathogen entry into pepper tissues.

Material and methods

Plant, fungus, and inoculation

Pepper seeds (*Capsicum annuum* L. cv. Hanbyul) were sown in plastic pots (5 by 15 by 10 cm) containing a steam-sterilized soil mix (peat moss, perlite, and vermiculite, 5 : 3 : 2, v/v/v), sand, and loam soil (1 : 1 : 1, v/v/v). Pepper plants were raised in a growth room at 25 ± 3 °C, with 5000 lux illumination for 16 h a day (Lee et al. 1999). The virulent isolate S197 and the avirulent isolate CBS178.26 of *Phytophthora capsici* were used in this study. The fungus was grown on oatmeal agar for 7 days at 28 °C. To sporulate the fungus, the culture plates were irradiated with fluorescent light at 28 °C for 3 days. To release zoospores from mature sporangia, the mycelium-covered plates were incubated with sterile water for 40 min at 4 °C and then 30 min at room temperature. A suspension culture of 1×10^6 zoospores per ml of sterile water was used as inoculum.

The stems of pepper plants at the 2-leaf stage were inoculated by wrapping them with a small piece of cotton soaked in the zoospore suspension and then covering them with plastic tape to retain moisture. The inoculated pepper plants were placed in the growth room at 28 °C until sampled.

RNA isolation

Total RNA was isolated from various pepper tissues, as described by Chomczynski and Sacchi (1987). The acid guanidinium-phenol-chloroform method was used to isolate RNA from pepper tissues. Uninoculated and inoculated stem tissues were harvested from the pepper plants and then ground with a mortar and pestle in liquid nitrogen. The sample was homogenized with 1 ml of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol on ice. 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of phenol (water saturated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49 : 1) were added sequentially to the homogenate. The final suspension was cooled on ice for 15 min. The samples were centrifuged at 10,000 **g** for 20 min at 4 °C. The aqueous phase was mixed with 1 ml of isopropanol and then placed at −20 °C for at least 1 h to precipitate RNA. Sedimentation was again performed at 10,000 **g** for 20 min. The resulting RNA pellet was dissolved in 0.3 ml of the extraction buffer and precipitated with 1 volume of isopropanol at −20 °C for 1 h. After centrifugation for 10 min at 4 °C, the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried for 15 min and dissolved in diethyl pyro-

carbonate-treated distilled water. RNA concentrations and purity were determined spectrophotometrically.

RNA gel blot analysis

RNA electrophoresis and Northern blot analysis were carried out using the methods of Sambrook et al. (1989). Samples containing 20 µg total RNA were denatured in 50% formamide, 1× running buffer (pH 7.0) containing 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA, 6% glycerol at 70 °C for 10 min. RNA samples were separated on 1.2% agarose gel. After electrophoresis, the RNA was transferred onto a nylon membrane (Hybond N⁺; Amersham, Little Chalfont, U.K.), and cross-linked by UV irradiation. To verify equal loading of RNA, the gels were stained with ethidium bromide before transfer to nylon membranes. The cross-linked membrane was prehybridized in a solution containing 0.25 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulfate (SDS) (w/v), 1 mM EDTA, and 5% dextran sulfate for 4 h at 65 °C. By using putative basic PR-1 full-length cDNA gene (*CABPR1*, accession no. AF053343; Kim and Hwang 1999) as a probe, hybridization was performed at 65 °C for 18 h in the same buffer. An *Eco*RI/*Xho*I fragment of the *CABPR1* cDNA was labeled with [α-^{32}P]dCTP by random priming. After hybridization, the membranes were washed twice in 2× SSC-0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 min each at room temperature and then several times in 0.1× SSC-0.1% SDS for 5 min each at 65 °C. Equal loading was checked by reprobing with a *Capsicum annuum* 25S rRNA cDNA probe. The membranes were exposed to X-ray films.

Sample preparation for in situ hybridization

Pepper stem tissues cut into small pieces were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.0, 0.1% Triton X-100 and evacuated for 5–10 min to remove air from the intercellular space. The fixation lasted 2 h at room temperature. The samples were washed three times with PBS for 10 min each. Pepper stem tissues were dehydrated in a graded ethanol series and cleared with xylol. Infiltration was performed by adding small droplets of liquid paraplast. The specimens were embedded in paraplast and hardened at room temperature. The embedded materials were stored at 4 °C until used for in situ hybridization.

In situ hybridization

In situ hybridization of mRNA in the pepper stem tissues was performed according to Hause et al. (1996). Thin sections (10 µm in thickness) were prepared and flattened on microscope slide glasses coated with poly-L-lysine (Sigma, St. Louis, Mo., U.S.A.). They were then baked overnight on a slide warmer set at 42 °C to adhere the sections to the slide. After removing the wax with xylol, the sections were rehydrated by serially diluted ethanol. The sections were rinsed in 0.01 M Tris-HCl, pH 8.0, incubated in 1% bovine serum albumin for 10 min at 37 °C, and then treated with 1 µg of proteinase K (RNase free) per ml for 30 min at 37 °C. The sections were acetylated by incubation with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min at room temperature and washed briefly in 2× SSC. The digoxigenin (DIG) labeling of the insert codes for CABPR1 was performed according to the manufacturer's instruction (Boehringer-Mannheim, Mannheim, Federal Republic of Germany).

Tissue sections fixed on the slides were prehybridized for 30 min at 42 °C in hybridization buffer containing 50% formamide, 2× SSC, 150 µg of tRNA per ml, 0.5% blocking reagent (Boehringer-Mannheim), and 40 units of RNase inhibitor (Boehringer Mannheim) per ml. Hybridization was performed with the DIG-labeled probe in the same hybridization buffer at 42 °C for 16 h in a moist chamber. After hybridization, the sections were washed twice in 50% formamide and 2× SSC for 10 min at 42 °C, 2× SSC for 10 min at 42 °C, and then in distilled water for 5 min.

Immunodetection was performed according to the manufacturer's instructions (Boehringer-Mannheim). After washing in buffer 1 containing 0.1 M Tris-HCl, pH 7.5, for 5 min, the sections were incubated in buffer 2 containing 1% blocking reagent (Boehringer-Mannheim) for 30 min at room temperature. The sections were then incubated in an anti-DIG-alkaline phosphatase solution (1 : 3000 diluted in buffer 2) for 1 h at 32 °C in a moist chamber. The slides were washed twice in buffer 1 for 15 min each and then in buffer 3 (0.1 M Tris-HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5) for 5 min at room temperature. The color reaction was developed overnight in buffer 3 with diluted nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at room temperature. The color reaction was stopped in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Hybridization signals were examined with an Olympus BH2 (Olympus, Tokyo, Japan) microscope equipped with a C-35AD-4 camera (Olympus). Control tests were done without DIG-labeled CABPR1 probes or on the uninoculated stem tissues.

Preparation of pepper stem tissues for immunoelectron microscopical analysis under low-temperature conditions

Pieces of infected and uninfected pepper stem tissues (5 mm in length) were frozen with a high-pressure freezing HPM 010 device (BAL-TEC GmbH, Balzers, Liechtenstein) (Hippe-Sanwald 1995). The stem tissues were cut into small pieces and infiltrated with 8% methanol under low vacuum for 5–10 min in order to infiltrate intercellular space. The high-pressure freezing method was performed under a liquid-nitrogen temperature of –196 °C and a hydrostatic pressure of 2.5×10^8 Pa (2500 bar) (Hippe-Sanwald 1995). The frozen samples were stored in liquid nitrogen until required. After cryofixation, the samples were transferred to the FSU 010 apparatus (BAL-TEC GmbH) for freeze substitution and embedding. The freeze substitution started at –85 °C in 0.5% uranyl acetate and anhydrous methanol. The temperature was raised 1 °C/h after 30 h from –85 °C to –35 °C over 3 days of freeze substitution. The specimens were washed three times in anhydrous methanol for 1 h each. Infiltration in Lowicryl HM20 embedding resin was performed in three stages (1 : 1, 2 : 1 mixtures of methanol to resin and pure resin) over a period of three days. Final polymerization was carried out under UV light (360 nm) for 48 h at –35 °C and for 48 h at room temperature. Thin sections from various blocks were cut with a Diatome diamond knife on an Ultracut E ultramicrotome (Reichert-Jung; Leica, Heidelberg, Federal Republic of Germany). The sections were mounted on pioloform and carbon-coated nickel grids (100 mesh).

Immunogold labeling

The first and second immunogold labeling was done according to Hippe et al. (1989). Polyclonal anti-PR-1 antiserum raised against tomato PR-1 protein, which was used for immunogold labeling, was obtained from Dr. M. H. A. J. Joosten, Wageningen Agricultural University, Wageningen, the Netherlands. Goat anti-rabbit immunoglobulin G (IgG) conjugated to 10 nm colloidal gold particles (BioCell, Cardiff, U.K.) was used as a second antibody. The grids were stained with uranyl acetate for 10 min, followed by poststaining with Reynolds lead citrate for 10 min. The immunostained sections were analyzed with a Philips CM10 (Philips, Eindhoven, the Netherlands) electron microscope operating at 80 kV.

To demonstrate the specificity of the labeling, control tests were performed. The level of nonspecific adsorption of goat anti-rabbit IgG-gold complex was evaluated by omitting the anti-PR-1 antiserum from the standard protocol. The specificity of anti-PR-1 antiserum also was examined on uninfected healthy plants.

Results

Northern blot analysis of CABPR1 mRNA in compatible and incompatible interactions

To determine whether or not PR-1 (*CABPR1*) gene expression is regulated during the course of infection, the *CABPR1* mRNA was analyzed by Northern hybridization using a *CABPR1* cDNA insert as a probe in a *P. capsici*-pepper system (Fig. 1 A). The *CABPR1* cDNA gene was not constitutively expressed in pepper stem tissues. In the compatible interaction, *CABPR1* mRNA was not detected in pepper stem tissue 6 h after inoculation, but the transcript accumulation was significantly triggered 12–24 h after inoculation. 36 h after inoculation, the transcripts had increased dramatically, showing a high level of accumulation. In the incompatible interaction, however, distinct accumulation of the *CABPR1* mRNA started as early as 6 h after inoculation and the transcript level was gradually enhanced over 36 h. 36 h after inoculation, *CABPR1* mRNA accumulation level was similar in both interactions (Fig. 1 B).

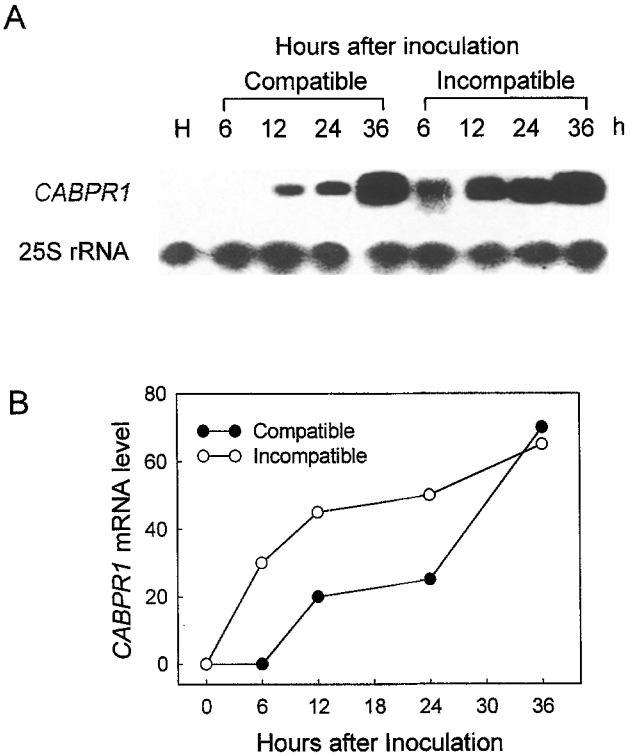


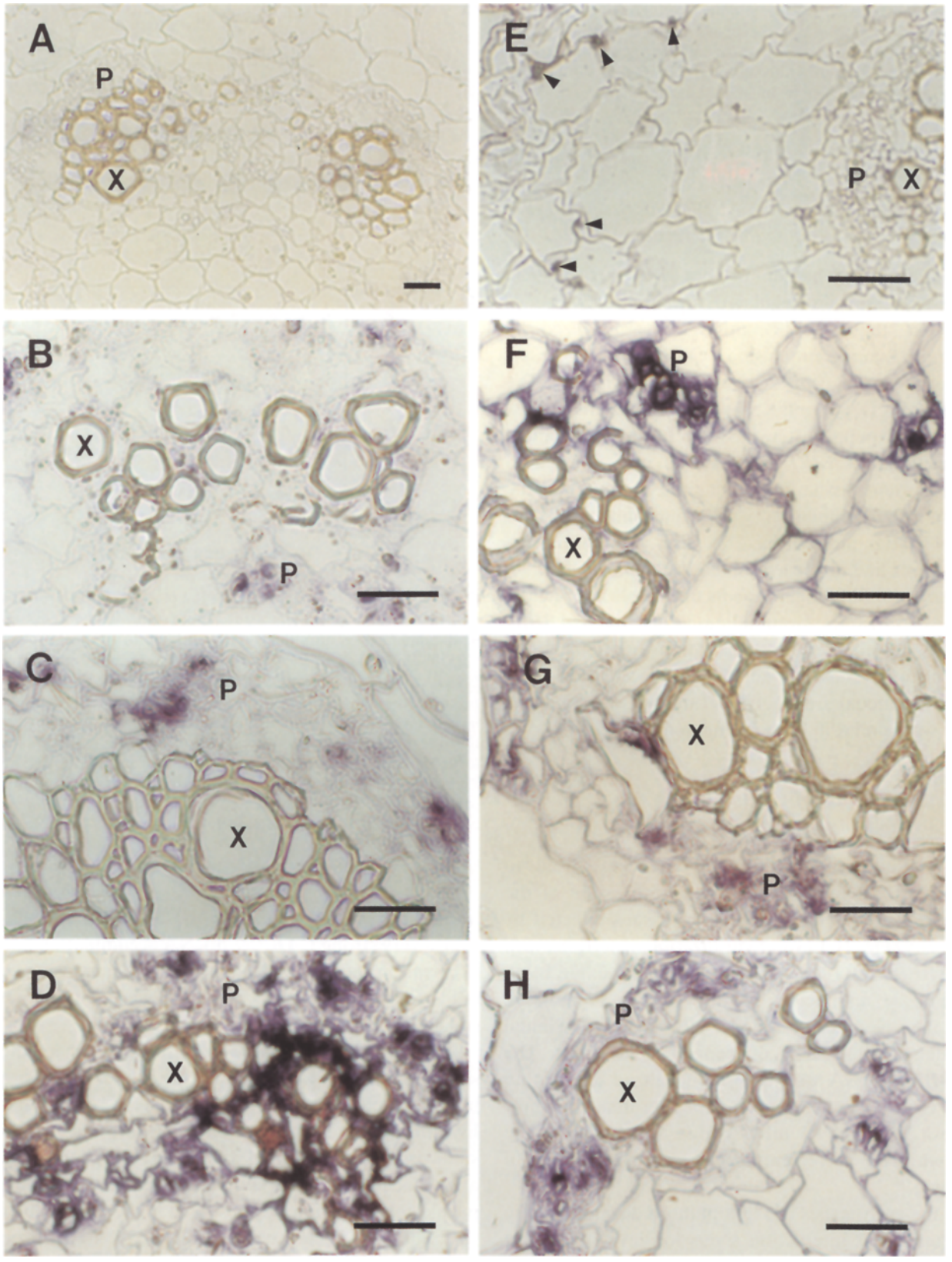
Fig. 1 A, B. Northern blot analysis of PR-1 (*CABPR1*) mRNA accumulation in the pepper stem tissue during compatible and incompatible interactions after infection by virulent S197 and avirulent CBS178.26 isolates of *P. capsici*. **A** Northern blot analysis of *CABPR1* mRNA at various times after inoculation. **B** Densitometric comparison of *CABPR1* mRNA levels between compatible and incompatible interactions. Total RNA was isolated from the stem tissue at the indicated times after infection. Total RNA (20 µg) was loaded in each lane on 1.2% formaldehyde agarose gel. The 0.85 kb *Eco*RI/*Xho*I fragment of pepper PR-1 cDNA insert was used as a probe. The loading of equivalent amounts of RNA was confirmed by hybridization with a 25S rRNA cDNA probe

In situ localization of CABPR1 mRNA in compatible and incompatible interactions

The in situ hybridization technique was used to examine the temporal and spatial expression of the *CABPR1* mRNA in pepper stem tissues infected by *P. capsici* (Fig. 2). In situ hybridization of sections with a *CABPR1* DIG-labeled cDNA probe resulted in no labeling of uninfected, healthy pepper stem tissue (Fig. 2 A). No hybridization signal was observed in the vascular bundle area of pepper stem tissue. The *CABPR1* mRNA was intensely localized only within the vascular bundle of infected pepper tissue. A heavy deposit of *CABPR1* mRNA always occurred in the phloem area, but xylem vessels were almost free of labeling.

In the early stages of Phytophthora development in the compatible interaction, a very low level of labeling was found, confined to phloem cells only (Fig. 2 B). Xylem cells remained unlabeled. 24 h after inoculation, the *CABPR1* mRNA accumulated distinctly around the phloem area (Fig. 2 C). 36 h after inoculation, a very strong *CABPR1* mRNA accumulation had occurred in the phloem cells (Fig. 2 D). However, the epidermal, cortical, and xylem cells were almost free of labeling.

A negative control test was performed to check the specificity of the *CABPR1* cDNA probe for in situ hybridization (Fig. 2 E). All the steps of in situ hybridization were performed using infected pepper stem tissue without the *CABPR1* cDNA probe. 36 h after inoculation in the compatible interaction, a pale purple color reaction was observed in the intercellular space of the infected pepper stem tissues. However, the vascular bundles, especially the phloem cells, were free of labeling (Fig. 2 E). The nonspecific color reaction in the intercellular spaces indicated that the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fungal hyphae had densely colonized in these intercellular spaces 36 h after inoculation, as observed previously by Lee et al. (1999).

In the incompatible interaction, *CABPR1* mRNA usually accumulated in the phloem cells of the vascular bundle. Intense labeling occurred over some phloem cell areas only 6 h after inoculation with the avirulent isolate CBS178.26 (Fig. 2F) and transcript accumulation gradually increased during the Phytophthora development (Fig. 2G, H). Between 24 h to 36 h after inoculation, the *CABPR1* transcripts had not accumulated markedly in phloem cells, but spread over the neighboring host cells. This labeling pattern of infected stem tissues was similar to that observed for corresponding tissues in the compatible interaction. In contrast, marked differences were found with the temporal expression patterns of *CABPR1* mRNA. Transcript induction in the incompatible interaction was earlier and stronger than that in the compatible interaction.

Immunogold labeling control test

Treatment of ultrathin sections of uninfected healthy pepper stem tissues with anti-PR-1 antiserum and goat anti-rabbit IgG antibodies resulted in no labeling over cell materials (Figs. 3 and 4). Some fibrillar material in the intercellular spaces was almost free of labeling (Fig. 3). Only a few scattered gold particles were detected over the host cytoplasm (Fig. 4).

In order to check the nonspecificity of goat anti-rabbit gold antibody for immunogold labeling, a negative control test was performed without anti-PR-1 antiserum. As a result, nonspecific deposition of gold particles was undetected over the entire section of stem tissue 36 h after inoculation in the incompatible interaction (data not shown).

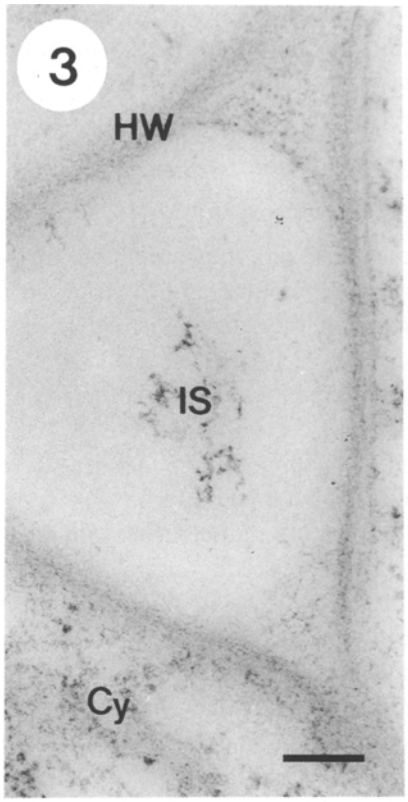
Immunogold localization of PR-1 protein in the compatible interaction

In the *P. capsici*-pepper system, the compatible interaction resulted in rapid development of Phytophthora blight in pepper stems. Although no distinct lesions occurred on the surface of pepper stems 24 h after infection, the fungal hyphae had already colonized all the pepper stem tissues (Lee et al. 1999).

Ultrathin sections of pepper stem tissues were treated with the anti-PR-1 antiserum and goat anti-rabbit IgG antibodies. 24 h after inoculation with a virulent isolate of *P. capsici*, a few scattered gold particles were observed over the cytoplasm of invaded host cells in the compatible interaction (Fig. 5). This labeling was usually visible around the endoplasmic reticulum in the infected cells. Normally, mitochondria, vacuole, and nuclei remained unlabeled in the infected pepper stem tissues (data not shown).

A deposit of gold particles was found in the intercellular spaces and at interfaces between host and fungal cells (Figs. 8 and 9). Intense labeling occurred over intercellular spaces of infected stem cells (Fig. 8). At high magnification, it was noticeable that the labeling was associated with the fibrillar material filling intercellular spaces. However, the numbers of labeled gold particles varied, depending on the presence or absence of filling material in these intercellular spaces. The host cell walls were free of labeling. Some scattered gold particles were visible over the interfaces

Fig. 2. In situ localization of PR-1 (*CABPR1*) mRNA in the stem tissue of pepper plants during compatible and incompatible interactions after infection by virulent S197 and avirulent CBS178.26 isolates of *P. capsici*, respectively. Stems were harvested from infected pepper plants at different times after inoculation and localization of *CABPR1* mRNA in the stem tissue was visualized by in situ hybridization. Bars: 5 μm. **A** Uninfected, healthy pepper stem tissues. All tissues are free of labeling. No labeling is visible in phloem cells. **B–D** In situ localization of *CABPR1* mRNA in the stem tissue of pepper plants during a compatible interaction. **B** Pepper stem tissues 6 h after inoculation. A very low level of label is visible within the vascular bundle, especially in the phloem-related cells. **C** Pepper stem tissues 24 h after inoculation. At this time, PR-1 mRNA accumulates slowly in the phloem cells and more distinct labeling is visible than 6 h after inoculation. **D** Pepper stem tissue 36 h after inoculation. The stem tissue is heavily infected by *P. capsici*. Very strong labeling is seen within the vascular bundle, especially in the phloem cells. **E** Negative control of in situ hybridization. In situ hybridization was performed without *CABPR1* probe on pepper stem tissue of a compatible interaction 36 h after inoculation. The purple-colored structures in the intercellular spaces in the epidermis and cortex represent the invading fungal hyphae (arrowheads). However, all the stem tissue, including phloem cells, are free of labeling. **F–H** In situ localization of *CABPR1* mRNA in the stem tissue of pepper plants during an incompatible interaction. **F** Pepper stem tissues 6 h after inoculation. The label is confined to the vascular bundle. Strong accumulation of *CABPR1* mRNA is visible within the phloem cells. **G** Pepper stem tissues 24 h after inoculation. Intense labeling is seen over the vascular bundle area, including phloem cells. **H** Pepper stem tissues 36 h after inoculation. There is no dramatic increase of *CABPR1* mRNA in phloem cells, but most of the phloem cells are significantly labeled. *P* Phloem, *X* xylem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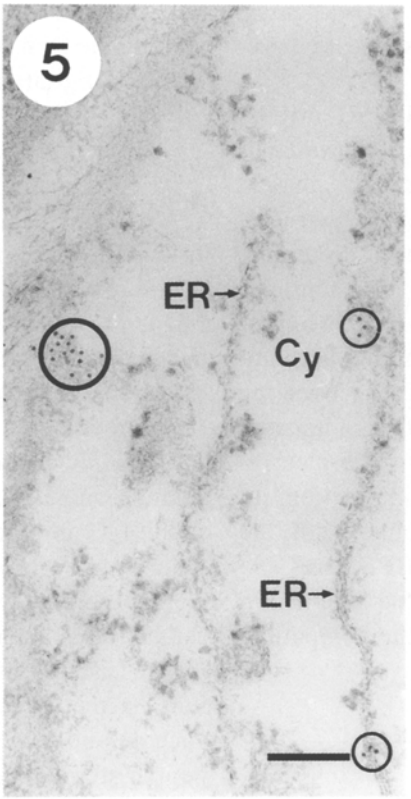
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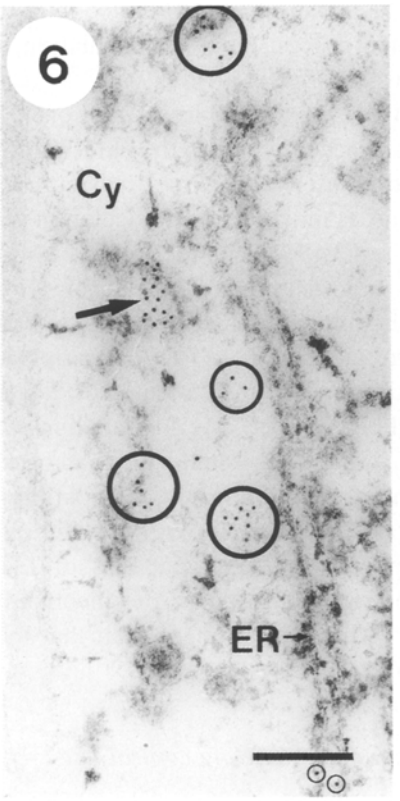
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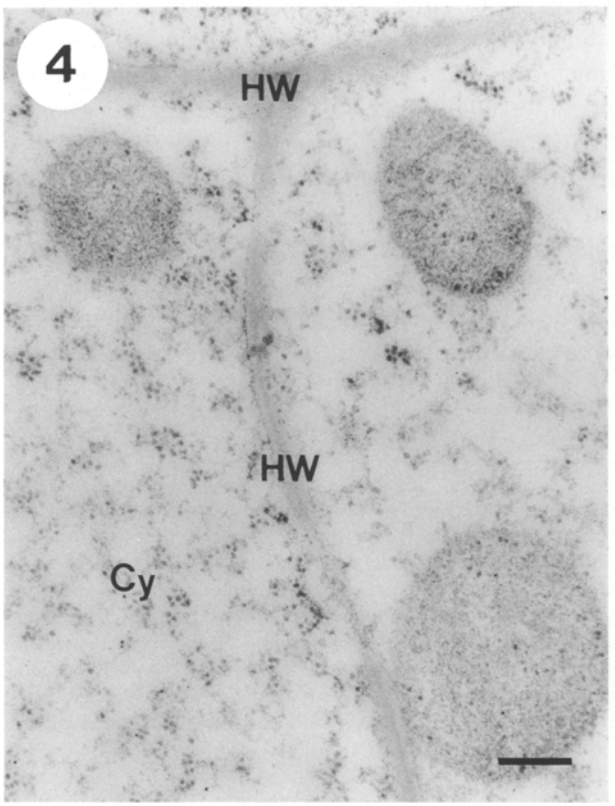
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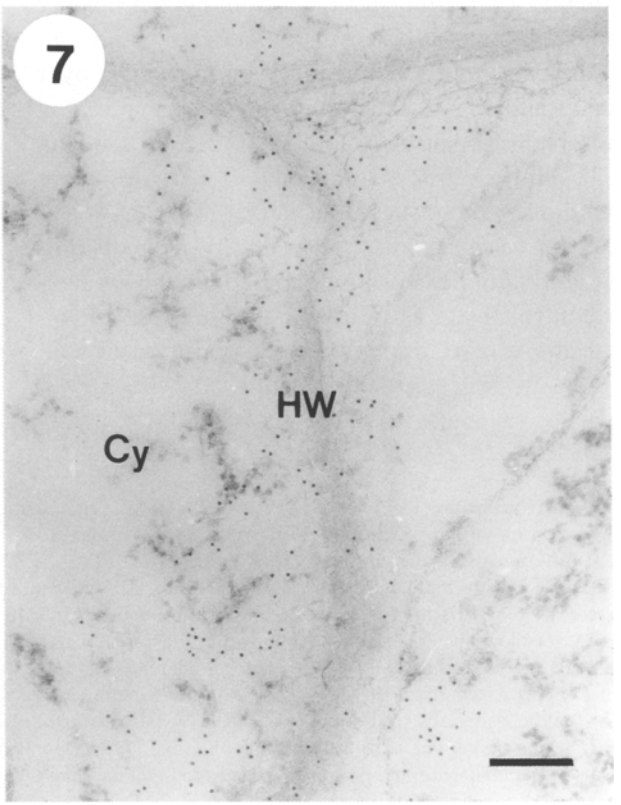
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between host and fungal cell walls (Fig. 9). Gold labeling occurred only at fungal cells that were in intimate contact with host cell walls. Wall appositions were found immediately adjacent to fungal cells. But no label was observed on the wall appositions.

Immunogold localization of PR-1 protein in the incompatible interaction

Typical Phytophthora blight symptoms did not appear on pepper stems in the incompatible interaction 24 h after inoculation with *P. capsici* (Lee et al. 1999). Following treatment with the PR-1 protein antiserum and goat anti-rabbit gold particles, a deposit of gold particles was observed on the host cytoplasm, usually around the endoplasmic reticulum (Fig. 6). This labeling pattern in the incompatible interaction was similar to that observed in the compatible interaction shown in Fig. 5. Preferential label accumulation occurred over host cell wall areas (Fig. 7). Considerable deposits of gold particles were seen over the host cell wall area, however, the labeling was not confined to the host cell wall only but also accumulated in the cytoplasm around the host cell wall.

In the incompatible interaction, the accumulation of gold particles also occurred over the intercellular spaces and at the interface between pepper plant and fungal cells (Figs. 10 and 11). Labeling in the intercellular space was associated with fibrillar or electron-opaque materials (Fig. 10). Observations at higher magnifications revealed that a heavy deposit of gold particles occurred preferentially beside fibrillar materials in the intercellular spaces. The host cell wall and cytoplasm were hardly labeled. There was heavy accumulation of gold particles over the interface area between fungal and host cells (Fig. 11). In fact, the labeling was not confined to the host and fungal cell walls. Interestingly, gold labeling was hardly detected over host cell walls that were not in close contact with fungal cells.

Discussion

In the present study, induction and in situ localization of PR-1 (*CABPR1*) mRNA and PR-1 protein in the *P. capsici*-infected pepper stem tissues were examined by Northern blot analysis, in situ hybridization, and immunogold labeling. In the pepper-*P. capsici* system, Northern blot analysis and in situ hybridization demonstrated earlier PR-1 mRNA expression in the incompatible, compared to the compatible interaction (Figs. 1 and 2). Transcript accumulation in infected pepper stem tissues was rapid in both interactions. *CABPR1* mRNA accumulation started as early as 6 h after inoculation in the incompatible interaction. These findings reveal that PR-1 mRNA accumulation in the incompatible interaction is an early event, associated with limited spread of the fungus. A similar conclusion was reached by De Wit and Van Der Meer (1986), who found that PR-1 protein accumulated earlier and to a greater extent in incompatible interactions between tomato and *Cladosporium fulvum* than in compatible ones.

In the time-course experiment, *CABPR1* mRNA accumulation started as early as 6 h after inoculation, and accumulation levels increased continuously until 36 h after inoculation in the incompatible interaction. In contrast, the transcript level increased dramatically

Figs. 3 and **4.** Immunocytochemical localization of PR-1 protein in uninfected, healthy pepper stem tissues. Sections were treated with anti-PR-1 antiserum, followed by incubation with 10 nm diameter gold-conjugated goat anti-rabbit IgG as a second antibody. Bars: 500 nm

Fig. 3. Cytoplasm (*Cy*), host cell wall (*HW*), and intercellular spaces (*IS*) of pepper plant are free of labeling

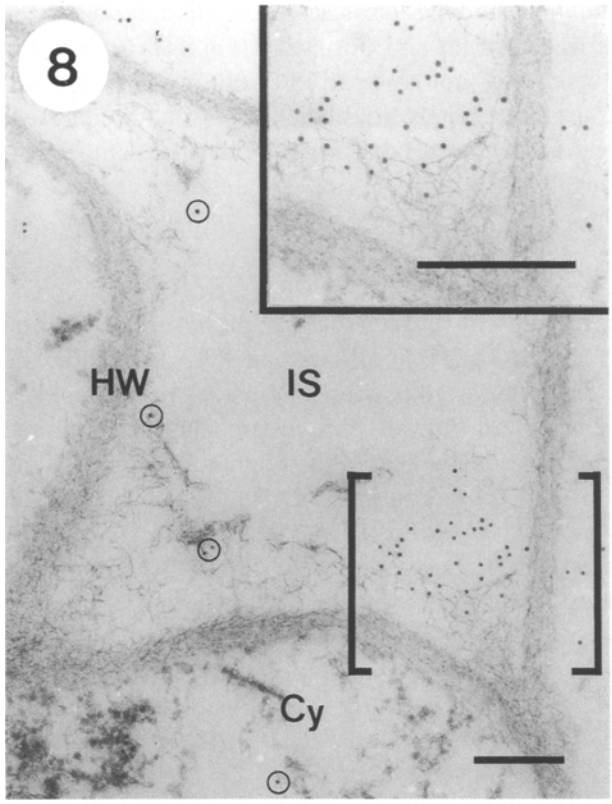
Fig. 4. Host cell wall (*HW*) and cytoplasm (*Cy*) of uninfected, healthy pepper stem tissues are nearly free of labeling

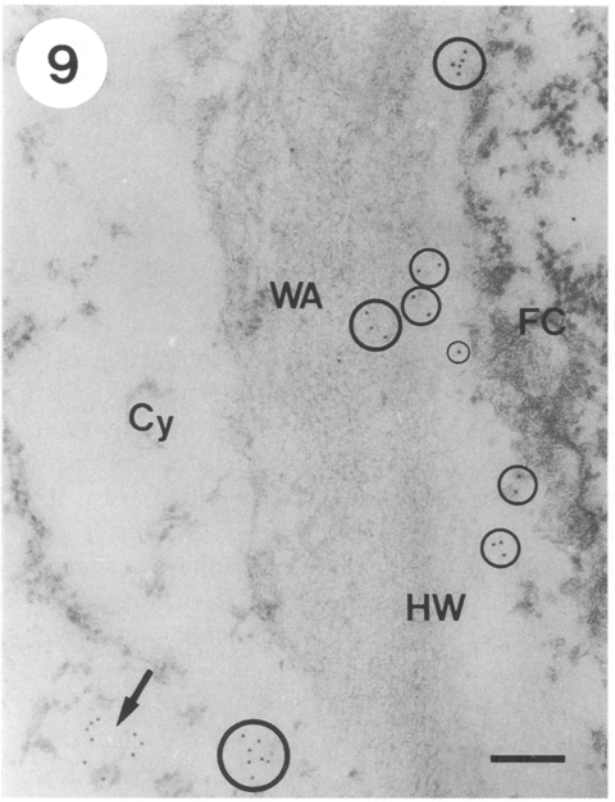
Fig. 5. Immunogold labeling with the polyclonal antibody against PR-1 protein of pepper stem tissue in the compatible interaction 24 h after inoculation with the virulent isolate S197 of *P. capsici*. Some scattered gold particles occur on endoplasmic reticulum (*ER*) and host cytoplasm (*Cy*). Bar: 500 nm

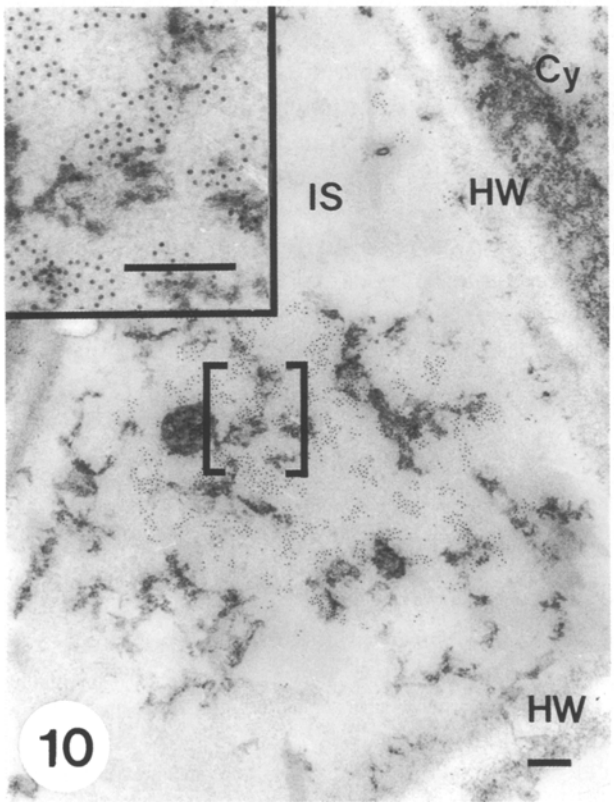
Figs. 6 and **7.** Immunogold labeling with the polyclonal antibody against PR-1 protein of pepper stem tissue in the incompatible interaction 24 h after inoculation with the avirulent isolate CBS178.26 of *P. capsici*. Bars: 500 nm

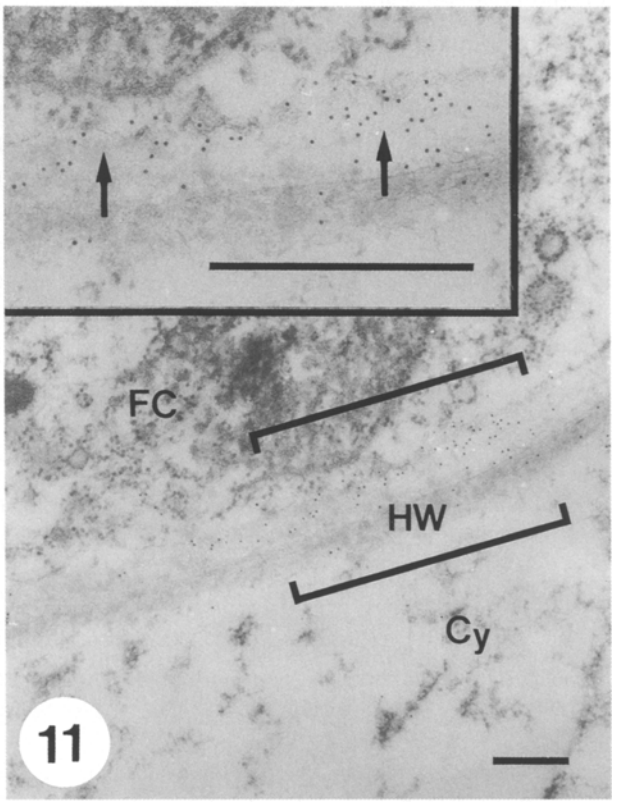
Fig. 6. Gold labeling accumulates preferentially over the host cytoplasm (*Cy*), especially around the endoplasmic reticulum (*ER*) (encircled). Distinct labeling (arrow) is seen near the endoplasmic reticulum

Fig. 7. A heavy deposit of gold particles occurs over host cell walls (*HW*)


8
HW
IS
Cy


9
WA
FC
Cy
HW


Cy
IS
HW
HW
10


FC
HW
Cy
11

36 h after inoculation in the compatible interaction. These results suggest that the PR-1 gene is strongly induced as a defense response against fungal pathogens, as well as in diseased cells (Linthorst 1991, Ryals et al. 1996, Sticker et al. 1997). These suggestions were well supported by recent findings of Kim and Hwang (1999) that the incompatible interaction resulted in a continuous increase of *CABPR1* mRNA in pepper stems 3 days after inoculation with an avirulent isolate of *P. capsici*. 2 days after inoculation, the typical symptoms of Phytophthora blight occurred on the pepper stem in the compatible interaction. In the incompatible interaction, growth of *P. capsici* was inhibited immediately after penetration of the pepper stem tissues, while in the compatible interaction, the fungus continued to grow vigorously (Lee et al. 1999). Accordingly, the amount of accumulated *CABPR1* mRNA could not be correlated with fungal biomass in the pepper stems. Damage to stem tissue may therefore trigger synthesis of the PR-1 protein. Taken together, the resistance response and the susceptible symptoms of Phytophthora blight seemed to trigger PR gene expression in pepper stem tissues differently.

Results of the present in situ hybridization study convincingly revealed that, upon infection PR-1 gene transcripts accumulated mainly in the vascular bundle of pepper stem tissue, especially phloem cells (Fig. 2). Some PR-protein transcripts from other plants were found to be localized in the vascular bundle (Eyal et al. 1993, Breda et al. 1996, Büchter et al. 1997). Recently, Hause et al. (1996) have demonstrated that mRNA synthesis of a jasmonate-inducible protein, JIP-23, occurred in phloem cells during developmentally induced stress. Pathogen attack could be a strong stress to the plant. In this case, a dramatic change in metabolic pathway may occur in infected plant tissue, leading to PR-1 gene expression in the vascular bundle areas. Accordingly, localization of PR-1 mRNA in the phloem cells suggests specific functions of the gene products in these cells. These functions may be associated with pathogen colonization or defense against pathogen attack, because the phloem could be a profitable area for fungal nutrition uptake. However, there is as yet no experimental evidence for this assumption. High levels of PR-1 mRNA in vascular tissue of infected pepper stems could be directed against pathogens and/or be the consequence of higher salicylic acid levels in phloem, as observed in infected tobacco and cucumber leaves (Malamy et al. 1990, Métraux et al. 1990).

Our immunocytochemical observations showed that marked accumulation of PR-1 protein in response to infection by *P. capsici* occurred in the infected pepper stem tissues. This finding is consistent with the well-documented increase in PR-1 protein upon pathogen attack (Camacho-Henriquez and Sänger 1984, De Wit and Van Der Meer 1986, Christ and Mösinger 1989). In particular, marked accumulation of PR-1 protein in intercellular spaces and at interfaces between fungal and plant cell walls supports the assumption that several PR proteins are secreted into extracellular compartments of plant cells (Parent and Asselin 1984, Vera et al. 1988, Lee and Hwang 1996). Intercellular space may be a strategic area for defense against pathogen attack, especially the pathogens growing in this area. Other PR proteins with β-1,3-glucanase

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Figs. 8 and **9.** Immunogold labeling with the polyclonal antibody against PR-1 protein of pepper stem tissue in the compatible interaction 24 h after inoculation with the virulent isolate S197 of *P. capsici*. Bars: 500 nm

Fig. 8. A number of gold particles occur in the intercellular space (*IS*) of the infected pepper stem cells. Host cell wall (*HW*) and cytoplasm (*Cy*) remain unlabeled. The PR-1 protein is related to the fibrillar material in the intercellular space. **Inset** Higher magnification of the labeling

Fig. 9. Wall appositions (*WA*) in the compatible interaction are nearly free of labeling. However, some gold particles are seen over the interface area between host (*HW*) and fungal (*FC*) cell walls (encircled). A few labeled PR-1 proteins are localized in the host cytoplasm (*Cy*) (arrows)

Figs. 10 and **11.** Immunogold labeling with the polyclonal antibody against PR-1 protein of pepper stem tissue in the incompatible interaction 24 h after inoculation with the avirulent isolate CBS178.26 of *P. capsici*. Bars: 500 nm

Fig. 10. Dense accumulation of gold particles in the intercellular space (*IS*) of infected stem tissue. This PR-1 protein accumulation is related to the fibrillar and globular material filling the intercellular space. Host cell wall (*HW*) and cytoplasm (*Cy*) are almost free of labeling. **Inset** Higher magnification of the bracketed area

Fig. 11. Numerous scattered gold particles present at the interface between host (*HW*) and fungal (*FC*) cell walls. Host and fungal cytoplasm are almost unlabeled. **Inset** Higher magnification of the bracketed area

and chitinase activity have also been demonstrated in intercellular spaces, suggesting a putative role in pathogen resistance by direct attack of fungal or bacterial cell walls in this area (Schlumbaum et al. 1986, Lee and Hwang 1996). PR P14 proteins from *Cladosporium fulvum*-infected tomato and P1 (p14) protein from viroid-infected tomato plants have been localized in the intercellular spaces of infected plant tissues (De Wit and Van Der Meer 1986, Vera et al. 1989, Benhamou et al. 1991). In particular, these PR proteins occurred in the intercellular spaces associated with fibrillar electron-dense material, as observed in *P. capsici*-infected pepper stem tissues. The small number of gold particles around endoplasmic reticulum in the cytoplasm (Figs. 5 and 6) might reflect transport of PR-1 proteins through the secretory pathway.

Recently, PR-1 gene expression was found to occur in incompatible interactions related to hypersensitive reaction after infection (Keller et al. 1996). In this respect, in situ accumulation of the PR-1 protein in intercellular spaces and at the interface between fungus and plant cells suggests that PR-1 protein may act as a resistance response against *P. capsici* infection in pepper stems. In particular, PR-1 proteins induced by *P. capsici* infection may exert a direct and/or indirect fungicidal effect that could account for the restriction of Phytophthora development. Alternatively, PR-1 protein may slow the colonization of the pathogens in host tissues or aid in its recognition, thereby allowing the plant to activate additional defense responses that limit the spread of the pathogen.

Taken together, pepper PR-1 mRNA accumulated in phloem cells upon *P. capcisi* infection, whereas the immunolocalized PR-1 proteins were found at the interface of the pathogen and the host cell wall, as well as in the intercellular spaces of pepper stem cells. The difference in localization sites between PR-1 mRNA and PR protein cannot be interpreted precisely, but it seems likely that the PR-1 transcripts may be directionally transported from one infected host tissue to another via the phloem, thus leading to the synthesis of specific PR-1 protein. In the present study, we have also used a polyclonal anti-PR-1 antiserum raised against tomato PR-1 protein. In this context, we could not eliminate the possibility that part of the protein may have skipped or lost its antigenic site in and/or on tissue samples, although labeling in the infected tissues indicates sufficient preservation of the antigenic sites. Thus, the present results may not reflect the full picture for PR-1 proteins produced in response to *P. capsici* infection, but they provide valuable information about its specific distribution in infected pepper stem tissues.

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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 protein 1a. Proc Natl Acad Sci USA 90: 7327–7331

Antoniw JF, Ritter CE, Pierpoint WS, Van Loon LC (1980) Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV. J Gen Virol 47: 79–87

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

Breda C, Sallaud C, El-Turk J, Buffard D, De Kozak I, Esnault R, Kondorosi A (1996) Defense reaction in *Medicago sativa*: a gene encoding a class 10 PR protein is expressed in vascular bundle. Mol Plant Microbe Interact 9: 713–719

Büchter R, Strömberg A, Schmelzer E, Kombrink E (1997) Primary structure and expression of acidic (class III) chitinase in potato. Plant Mol Biol 35: 749–761

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: 181–196

– – (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

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159

Christ V, 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, Raventos D, Segundo BS (1992) Induction of PR proteins in germinating maize seeds infected with the fungus *Fusarium moniliforme*. Physiol Mol Plant Pathol 41: 189–200

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

Eyal Y, Meller Y, Lev-Yadun S, Fluhr R (1993) A basic type PR-1 promoter directs ethylene responsiveness, vascular and abscission zone-specific expression. Plant J 4: 225–234

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

Green R, Fluhr R (1995) UV-B-induced PR-1 accumulation is mediated by active oxygen species. Plant Cell 7: 203–212

Hause B, Demus U, Teichmann C, Parthier B, Wasternack C (1996) Developmental and tissue-specific expression of JIP-23, a jasmonate inducible protein of barley. Plant Cell Physiol 37: 641–649

Hippe S, Düring K, Kreuzaler F (1989) In situ localization of a foreign protein in transgenic plants by immunoelectron microscopy following high pressure freezing, freeze substitution and low temperature embedding. Eur J Cell Biol 50: 230–234

Hippe-Sanwald S (1995) Low temperature technique as a tool in plant pathology. Scan Microsc 9: 881–899

Hwang BK, Sunwoo JY, Kim YJ, Kim BS (1997) Accumulation of β-1,3-glucanase and chitinase isoform, and salicylic acid in the DL-β-amino-n-butyric acid-induced resistance response of pepper stems to *Phytophthora capsici*. Physiol Mol Plant Pathol 51: 305–322

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

Keen NT (1992) The molecular biology of disease resistance. Plant Mol Biol 19: 109–122

Keller H, Bonnet P, Galiana E, Pruvot L, Friedrich L, Ryals J, Ricci P (1996) Salicylic acid mediated elicitin-induced systemic acquired resistance, but not necrosis in tobacco. Mol Plant Microbe Interact 9: 696–703

Kim YJ, Hwang BK (1996) Purification, N-terminal amino acid sequencing and antifungal activity of chitinase from pepper stems treated with mercuric chloride. Physiol Mol Plant Pathol 48: 417–432

– – (1999) Pepper gene encoding a basic PR-1 protein is pathogen and ethylene inducible. Physiol Plant (in press)

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms for activation of plant defense against microbial attack. Cell 56: 215–224

Lee YK, Hwang BK (1996) Differential accumulation of β-1,3-glucanase and chitinase isoforms in the intercellular space and leaf tissues of pepper by *Xanthomonas campestris* pv. *vesicatoria* infection J Phytopathol 144: 79–87

– Hippe-Sanwald S, Hwang BK (1999) Histological and ultrastructural comparisons of compatible, incompatible and DL-β-amino-n-butyric acid-induced resistance responses of pepper stems to *Phytophthora capsici*. Physiol Mol Plant Pathol (in press)

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci 10: 123–150

– 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

Lucas J, Camacho-Henriquez A, Lottspeich F, Henschen A, Sänger HL (1985) Amino acid sequence of the "pathogenesis-related" leaf protein P14 from viroid-infected tomato reveals a new type of structurally unfamiliar proteins. EMBO J 11: 2745–2749

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogeneous signal in the resistance response of tobacco to viral infection. Science 250: 1002–1004

Métraux JP, Signer H, Ryals, J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science 250: 1004–1006

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, Bruyere T, Gees R, Stintzi A, Legrand M, Fritig B, Mösinger E (1995) Pathogenesis-related PR-1 proteins are antifungal: isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. Plant Physiol 108: 17–27

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

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Sterner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8: 1809–1819

Sambrook J, Fritsch EF, Maniatis T (1989) Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In: Ford N, Nolan C, Ferguson M (eds) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY pp 7.35–7.87

Schlumbaum A, Mauch F, Vögeli U, Boller T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365–367

Somssich IE, Schmelzer E, Bollmann J, Hahlbrock K (1986) Rapid activation by fungal elicitor of gene encoding "pathogenesis-related" proteins in cultured parsley cells. Proc Natl Acad Sci USA 83: 2427–2430

Sticker L, Mauch-Mani B, Métraux JP (1997) Systemic acquired resistance. Annu Rev Phytopathol 35: 235–270

Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant pathogenesis-related proteins and their role in defense against pathogens. Biochemistry 75: 687–706

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

Van Loon LC (1976) Specific soluble leaf proteins in virus-infected tobacco plants are not normal constituents. J Gen Virol 30: 375–379

– Pierpoint WS, Boller T, Conejero V (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Rep 12: 245–264

Vera P, Hernandez-Yago J, Conejero V (1988) Immunocytochemical localization of the major pathogenesis-related (PR) protein of tomato plants. Plant Sci 55: 223–230

– – – (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