한국식물병리학회지 12(1) : 1-10 (1996)
Korean J. Plant Pathol. 12(1) : 1-10 (1996)

Activities and Isoforms of β-1,3-Glucanases and Chitinases in Tomato Leaves Infected by Compatible and Incompatible Strains of *Xanthomonas campestris* pv. *vesicatoria*

Jeong Dong Kim and Byung Kook Hwang*
Department of Agricultural Biology, College of Natural Resources, Korea University, Seoul 136-701, Korea

Xanthomonas campestris pv. *vesicatoria*의 친화적 및 불친화적 균주로 감염된 토마토 잎에서 β-1,3-Glucanases와 Chitinases의 활성과 동위효소

김정동 · 황병국*
고려대학교 자연자원대학 농생물학과

ABSTRACT : *Xanthomonas campestris* pv. *vesicatoria* infection stimulated the synthesis and accumulation of β-1,3-glucanases and chitinases in the tomato leaf tissues, whereas the activities of the two hydrolytic enzymes remained at very low levels in the uninoculated healthy leaves. The activities of the two enzymes were much higher in the incompatible than compatible interactions, suggesting an important role in a defense against *X. campestris* pv. *vesicatoria*. The direct detection of β-1,3-glucanase isoforms on the native-PAGE gels revealed only single acidic isoform Ga 1 and basic isoform Gb 1, which may be very important in the expression of symptom and plant resistance, respectively. Isoelectric focusing identified at least two β-1,3-glucanases of the pIs 6.4 and 8.6 isoforms, especially in the incompatible interactions. The acidic chitinase isoforms Ca 1 and Ca 2 were detected in the extracts of tomato leaves infected with *X. campestris* pv. *vesicatoria*. As the bacterial infection progressed, the activity of Ca 1 gradually declined, indicating its degradation by the pathogen attack. Five basic chitinase isoforms were also detected in infected tomato tissues. In particular, only isoform Cb 3 was induced and accumulated in the tomato leaves infected with the incompatible strain Bv5-4a. Isoelectric focusing detected at least two acidic and four basic isoforms of chitinase in the extracts of infected tomato leaves. The pI 9.5 isoform which corresponded to the chitinase isoform Cb 1 in the native PAGE gel was only identified in the incompatible interaction. The 23 and 26 kDa chitinase isoforms were identified on SDS-PAGE gels after their renaturation by treatment with deionized Triton X-100.

***Key words* : β-1,3-glucanase, chitinase, *Xanthomonas campestris* pv. *vesicatoria*, tomato leaves, resistance.**

Since the initial discovery that β-1,3-glucanases increased in tomato plants in response to pathogen infection (26, 27), several reports have suggested that β-1, 3-glucanases were likely involved in defence reactions in the plant (10, 15). An additional evidence came from the recent study of Joosten and De Wit (15), who reported that the enhancement of β-1,3-glucanases in tomato plants affected by *Cladosporium fulvum* took place earlier and to a higher extent in resistant cultivars than in susceptible ones. When pathogens initially grow in their host plants, they make contact with β-1,3-glucanase molecules. Upon contact, the β-1,3-glucanase is postulated to release oligosaccharide fragments from the β-1,3-glucanase-containing fungal cell wall. Oligosaccharides released by β-1,3-glucanase from isolated fungal cell walls have been demonstrated

*Corresponding author.

to act as elicitors of phytoalexin production (17). Chitinases hydrolyze internal β-1,4-glycosidic linkages of chitin, a homopolymer of N-acetyl-D-glucosamine. Chitin does not occur in higher plant, but is an important component of the exoskeleton of arthropods and nematodes, and along with β-1,3-glucan, of the cell walls of higher fungi. Purified plant chitinase attacks chitin-containing pathogens (14, 17, 33). Chitinases also possess lysozymal activity that is capable of hydrolyzing the peptidoglycan in bacterial cell walls (4).

Lysozyme activities have been detected in various plant species (2). Nearly all known plant enzymes characteristic of lysozyme function are strongly basic proteins and are classified as vacuolar basic chitinase. Tobacco displays a significant extracellular bacterolytic potential (11), when tobacco calli are grown in a lawn of *Erwinia carotovora*. Interestingly, β-1,3-glucanase and chitinase which are detectable in the extracellular, vacuole and intercellular spaces seem to be mostly restricted to the extracellular spaces and vacuoles (9). Moreover, using different bacteria such as *Micrococcus lysodeikticus*, *E. carotovora* subsp. *carotovora*, and *Pseudomonas solanacearum*, a significant variation in enzyme activity towards the individual bacterial species could be detected for potato lysozymes (7). It is suggested that the presence of glucanase, chitinases or lysozymes might represent a component of the plant resistance mechanisms related to the action of these hydrolytic enzymes. Furthermore, β-1,3-glucanases and chitinases act synergistically in the partial degradation of isolated fungal cell walls (20, 33). Several fungi are not inhibited by either β-1,3-glucanases and chitinases alone; however, combinations of the two enzymes strongly inhibited most fungi tested (20). Thus, the coordinated induction of two enzymes may be a part of the defence response of plant tissue. A paralleled increase in the activities of both β-1,3-glucanase and chitinase is important for an optimal function in defence. In order to exploit this defence capacity, optimizing these factors and redirecting highly active lysozymes to the extracellular compartment may convert various susceptible plant-bacteria interactions.

In the present studies, we examined changes in β-1,3-glucanase and chitinase patterns in tomato leaves infected by either compatible strain Ds 1 or incompatible strain Bv5-4a of *Xanthomonas campestris* pv. *vesicatoria*. In addition, we further determined the molecular weights and isoelectric points of these enzymes.

MATERIALS AND METHODS

Plant materials. Tomato (*Lycopersicon esculentum* Mill cv. Kwangyang) plants which are being intensively cultivated in Korea were used in this study. The seeds of tomato cultivar Kwangyang were sown in a plastic tray (55×35×15 cm) containing steam-sterilized soil mix (peat moss, perlite, and vermiculite, 5 : 3 : 2, v/v/v). Six seedlings at the two-leaf stage were transplanted to a plastic pot (5×5×10 cm) containing the same soil mix. The tomato plants were grown up to the eight-leaf stage in a growth chamber at 25±2°C under 16 h per day illumination.

Bacterial strains and inoculation. The two strains Ds 1 and Bv5-4a of *Xanthomonas campestris* pv. *vesicatoria*, compatible and incompatible to the tomato cultivar Kwangyang, respectively, were used in this study. Strain Ds 1 was isolated in Korea from pepper in 1991 and the other incompatible strain Bv5-4a was supplied by R. E. Stall, Department of Plant Pathology, University of Florida, Gainesville, U.S.A. The bacteria were grown in yeast-nutrient medium (5 g yeast extract and 8 g nutrient broth per liter H_2O). The bacterial strains were also maintained in the yeast-nutrient broth containing 15% glycerol at −70°C. To prepare bacterial inoculum, *X. campestris* pv. *vesicatoria* strains were cultured for 24 h in yeast-nutrient broth and centrifuged at 3,000 g for 15 min. The harvested bacterial cells were then suspended in sterilized tap water and diluted to an absorbance of 0.06 (10^8 cfu/ml) at 660 nm prior to inoculation. Tomato plants at the eight-leaf stage were inoculated by infiltrating the cell suspension (10^8 cfu/ml) into the abaxial side of the completely expanded leaves with an automizer connected to a compressor until leaves appeared water-soaked. The inoculated tomato plants were placed in a moist chamber at 25°C for 24 h and returned to the growth chamber with temperature ranging from 23°C to 27°C and 16 h-photo period.

Extraction of proteins from tomato leaves. Leaf extracts from tomato plants were obtained at various time intervals after inoculation. The inoculated leaves (1 g) were harvested and homogenized in liquid nitrogen and then extracted with 3 ml 0.5 M sodium acetate buffer (pH 5.2) containing 15 mM 2-mercaptoethanol using a precooled mortar and pestle. The homogenates were centrifuged at 20,000 g for 60 min and the supernatants were stored at −20°C until used for electrophoresis. Protein contents in the clear supernatant

were measured using bovine serum albumin as a standard, according to the method of Bradford (6).

Assays of β-1,3-glucanase and chitinase activities. β-1,3-Glucanase activity in the tomato leaf extracts was assayed by measuring the release of reducing sugar from laminarin (Sigma) as a substrate. The 0.2 M sodium phosphate-citric acid buffer (pH 5.6) containing laminarin (1 mg/ml) was used as a substrate buffer. The reaction mixtures consisted of 0.4 ml substrate buffer and 0.1 ml enzyme solution of leaf extracts. The reducing sugars generated in the reaction mixture were measured using the method of Nelson (24). Glucose was used as a standard. Enzyme and substrate alone were included as controls. β-1,3-Glucanase activity was expressed in katal. One katal was defined as the quantity of enzyme catalyzing the formation of 1 mole of glucose equivalents per sec.

Chitinase activity in tomato leaf extracts was measured using a colorimetic assay. Before being used as substrate, chitin powder (Sigma) was washed three times with 0.1 M sodium acetate buffer (pH 5.2) to decrease the blank value of the enzymatic test. The reaction mixture contained 0.5 mg washed chitin and various volumes of enzymatic solution in a final volume of 0.5 ml 0.1 M sodium acetate (pH 5.2). The mixture was incubated in a shaking water bath at 37°C for 1 h. The reaction mixture was centrifuged at 10,000 g for 30 min. Of the resulting supernatant, 0.3 ml was incubated at 37°C with 20 μl of 25% glucuronidase to hydrolyze the liberated, water-soluble chitin oligomers to N-acetylglucosamine (GlcNAc). The resulting GlcNAc was determined by a modification of Reissing *et al.* (29) using the internal standard in the assay mixtures for calculations. Prior to heating for 3 min in a boiling water bath, 0.1 ml of 0.6 M potassium tetraborate was added to the mixture. After cooling, 1 ml of the following reagent diluted 1 : 2 with glacial acetic acid was added. The reagent stock solution contained 10% (w/v) 4-(dimethylamino) benzaldehyde in glacial acetic acid/11.5 M HCl, 87.5 ml : 12.5 ml (v/v). Standards of GlcNAc in buffer and enzyme or substrate blanks also were included. Since the amount of the reaction products was not a linear function of enzyme concentration, a dilution series of the enzyme (each in duplicate) was tested, and the activity was calculated for enzyme concentration approaching zero. A katal (kat) was defined as the enzyme activity catalyzing the formation of 1 mole of GlcNAc (or its equivalent) per sec.

Native polyacrylamide gel electrophoresis and isoelectric focusing. Polyacrylamide gel electrophoresis under high pH, nondenaturing conditions on 15% (w/v) polyacrylamide slab gels to resolve acidic proteins was similar to SDS-PAGE, with the exception that SDS was omitted in all buffers. Anodic polyacrylamide gel electrophoresis under native conditions was performed with 1.5 M Tris-HCl buffer (pH 8.8) according to the method of Davis (8). Cathodic polyacrylamide gel electrophoresis under low pH, nondenaturing conditions was performed with 0.3 M potassium acetate buffer (pH 4.3) using 15% (w/v) polyacrylamide resolving gels and 5% (w/v) polyacrylamide stacking gels with 0.01% (w/v) pyronine Y as tracking dye (28). Electrophoresis was run at 4°C for 1 h at 10 mA and for 3 h at 20 mA (anodal separation), or for 1 h at 5 mA and for 5 h at 10 mA (cathodal separation). Isoelectric focusing (IEF) was carried on 10% polyacrylamide gel containing ampholine (pH 3.5~10, Sigma) according to the manufacturer's protocol (LKB). IEF was performed at 200 V for 20 min, 400 V for 1 h, 600 V for 1 h, 800 V for 1 h, 1,000 V for 3 h and 1,100 V for 1 h. The IEF gels were fixed in 20% (v/v) trichloroacetic acid (TCA) for 1 h, thereafter shortly rinsing with distilled water. The gel was stained in Serva blue W and the destained in 0.02% (v/v) TCA until background disappeared. The pIs of proteins of interest were determined by comparison with the following standards obtained from Sigma: amyloglucosidase, pI 3.6; trypsin inhibiter, pI 4.6; β-lactoglobulin A, pI 5.1; carbonic anhydrase II, pI 5.9; myoglobin, pI 6.8 and 7.2; L-lactic dehydrogenase, pI 8.6; trypsinogen, pI 9.3.

Direct detection of β-1,3-glucanase isoforms after native PAGE and IEF. After electrophoresis or isoelectric focusing, the native PAGE or IEF gels were equilibrated with 0.05 M potassium acetate (pH 5.0) for 20 min. They were then incubated at 40°C for 30 min with 25 ml reaction solution mixture containing 0.05 M potassium acetate (pH 5.0) and 0.25 g laminarin dissolved in 25 ml of distilled water, followed by washing three times with distilled water after incubation. After immersing into the glass tray with 0.15 g 2,3,5-triphenyltetrazolium chloride in 100 ml of 1.0 M NaOH, the gels were incubated in a boiling water bath for 5 to 10 min, until red bands appeared. To remove the pink background, the PAGE or IEF gels were stored in 7.5% acetic acid, immediately after the bands of interest thoroughly appeared (25, 26).

Direct detection of chitinase isoforms after native

PAGE and IEF. After electrophoresis or isoelectric focusing, the PAGE or IEF gels were incubated in 0.1 M sodium acetate buffer at pH 5.2 for 10 min. They were then put on a clean glass plate and covered with a 7.5% (w/v) polyacrylamide overlay gel (0.75 mm thick) containing 0.04% (w/v) glycol chitin in 100 mM sodium acetate buffer (pH 5.2). Glycol chitin was obtained by acetylation of glycol chitosan by modification of the method of Molano *et al.* (22). The liquid between the gels and glass plate was eliminated with carefully sliding a test tube over the surface of the overlay gel. The spaces between the separating gels and overlay gels were sealed with 1% (w/v) agarose on the overlay gel around the area of the separating gel. Gels were incubated at 37°C for 2 h in a plastic container under moist conditions. Following incubation, care must be taken to seal the overlay gel to the glass plate. Overlay gels were then transferred into freshly prepared 0.01% (w/v) fluorescent brightener 28 in 500 mM Tris-HCl buffer (pH 8.9) (26). After 10 min, the brightener solution was removed and the overlay gels were incubated for 2 h at room temperature under dark condition in distilled water. Lytic zones produced by chitinases were visualized by placing the overlay gels on an UV transilluminator.

Detection of renatured chitinase isoforms after SDS-PAGE. To detect renatured chitinase isoforms, electrophoresis was performed on the SDS-PAGE gels containing 0.01% (w/v) glycol chitin. After electrophoresis, the gel was incubated for 16 h at 37°C with reciprocal shaking in 100 mM sodium acetate buffer (pH 5.2) containing 1% (v/v) Triton X-100 purified through a mixed-bed resion deionizing column (Bio-Rad mixed bed resin AG 501-X8, 20~50 mesh, fully regenerated) to efficiently remove SDS from the enzymes. The renatured gel was incubated in freshly prepared 0.01% (w/v) fluorescent brightener 28 in 500 mM Tris-HCl (pH 8.9) at room temperature for 10 min. After removal of brightener solution, the renatured gel was also incubated in distilled water in the dark for 5 h. On an UV transilluminator, renatured gel was ascertained to hold chitinase isoforms as dark zones.

RESULTS

Induction of β-1,3-glucanase and chitinase activity. The induction of β-1,3-glucanase and chitinase activities showed a strong and coordinated increase with time during the disease development (Fig. 1). The activities of β-1,3-glucanase and chitinase remained at the low levels in uninoculated leaves, but were stimulated strongly after inoculation of *Xanthomonas campestris* pv. *vesicatoria*. At the onset of pathogenesis, β-1,3-glucanase and chitinase activities increased more greatly in the incompatible than compatible interactions. In the incompatible interaction, the activity of β-1,3-glucanases remained at a high level until 4 days after inoculation, but thereafter declined. However, chitinase activity remained at high levels in both compatible and incompatible interactions during the pathogenesis.

Analysis of β-1,3-glucanase isoforms in the compatible and incompatible interactions. One acidic β-1,3-glucanase isoform (designated Ga 1) in the extracts of tomato (cv. Kwangyang) leaves was revealed as a single band at various times after inoculation with each of the compatible strain Ds 1 and incompatible strain Bv5-4a on a 15% native polyacrylamide gel at high pH (pH 8.8) (Fig. 2A). Activity of isoform Ga 1 occurred at the high levels after inoculation with the compatible and incompatible strains. In the incompatible interactions, the Ga 1 activity was more conspicuous at 1 day after inoculation than that in the compatible interaction. In contrast, no acidic β-1,3-glucanase isoforms were found in the healthy control leaves. Basic β-1,3-glucanase isoforms were present in the leaf extracts from uninoculated and inoculated tomato plants on a nondenaturing, low pH (pH 4.3) gel

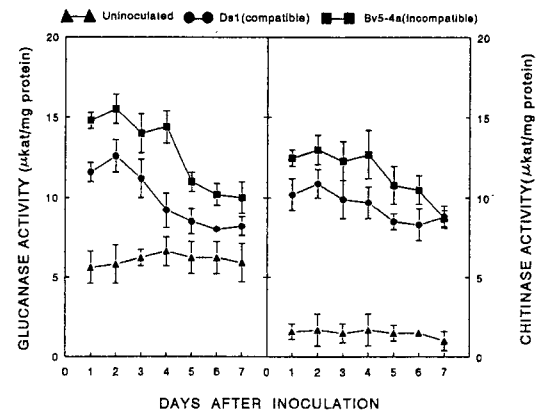

Fig. 1. Time-course induction of β-1,3-glucanase and chitinase activities in leaves of tomato cultivar Kwangyang inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria* and in uninoculated leaves. Vertical bars represent standard deviations.

(Fig. 2B). A distinct band of basic β-1,3-glucanase isoform (designated Gb 1) was found in all leaves infected by the compatible and incompatible strains. Activity of isoform Gb 1 was very intense in the incompatible interactions 1~3 days after inoculation. Any basic β-1,3-glucanase isoform was not found per unit protein amount in the uninoculated leaf tissue.

For the determination of the pI values of β-1,3-glucanase, isoelectric focusing of crude enzymes in the extracts of leaf tissue inoculated with Ds 1 and Bv5-4a and of uninoculated control was carried out on 10% IEF gels with the pH range of 3.5~10.0 (Fig. 3). At least one acidic and some basic β-1,3-glucanase isoforms were detected in the leaf extracts from the compatible and incompatible interactions. Acidic β-1,3-glucanase with pI 6.4 was distinctly induced by *X. campestris* pv. *vesicatoria*, especially in the incompatible interactions. Activity of the pI 8.6 isoform was similar in both compatible and incompatible interactions. Some isoforms appeared as the smeary bands in the range of pI 7.2~8.6, distinctly at first day after inoculation with the incompatible strain. Although there may be more isoforms of β-1,3-glucanase in the tomato leaf extracts, they could not be clearly separated on 10% IEF gels. In the uninoculated control tomato leaves, no β-1,3-glucanase isoforms were detected in the IEF gel.

Analysis of chitinase isoforms in the compatible and incompatible interactions. In the anodic polyacrylamide gels, two acidic chitinase isoforms Ca 1 and Ca 2 were detected in the tomato leaves infected

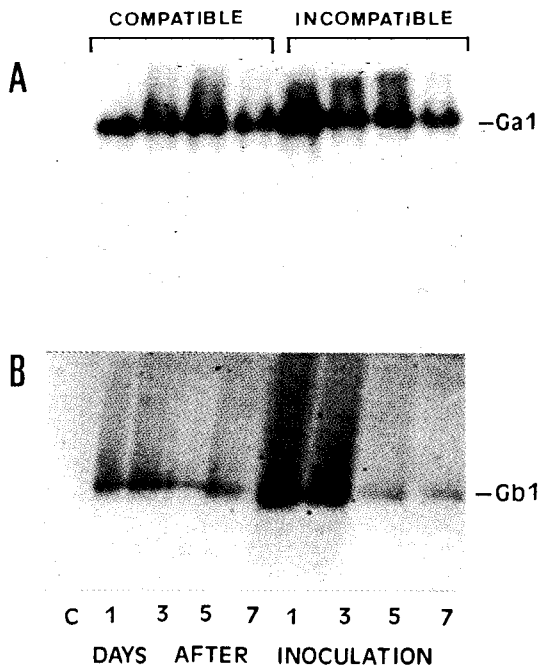

Fig. 2. Detection for acidic (A) and basic (B) β-1,3-glucanase isoforms in the extracts from uninoculated leaves (lane C) of tomato cultivar Kwangyang and in the extracts from leaves inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria*. Crude enzyme extracts (5 μg protein for anodic (A) PAGE, and 20 μg protein for cathodic (B) PAGE) were loaded on 15% PAGE gels and stained for β-1,3-glucanase isoforms.

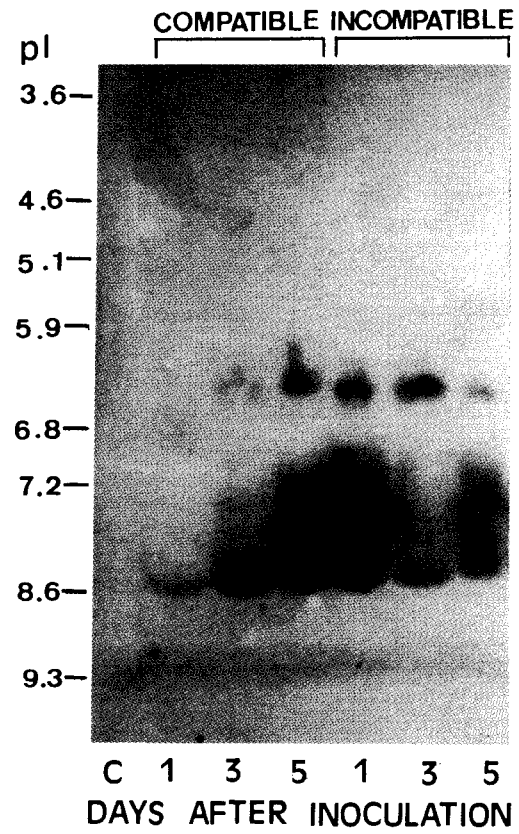

Fig. 3. Isoelectric focusing of β-1,3-glucanase isoforms in the extracts from noninoculated leaves (C) of tomato cultivar Kwangyang and in extracts from the leaves inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria*. Proteins (10 μg per lane) were separated to equilibrium (ampholine: pH 3.5~10.0). The separated gel was incubated with laminarin to detect β-1,3-glucanase activities for 1 h at 40°C.

by *X. campestris* pv. *vesicatoria* (Fig. 4). The activity of isoform Ca 1 was remarkably higher at the onset of pathogenesis, especially in the incompatible interactions. As the bacterial infection became progressed, the activity of Ca 1 gradually declined. The activity of isoform Ca 2 remained consistently at low levels during the pathogenesis in the compatible or incompatible interactions. However, the two acidic chitinase isoforms were not detected in the uninoculated leaves. In the cathodic polyacrylamide gels, five basic chitinase isoforms were found in the extracts of tomato leaves inoculated with either the compatible or the incompatible strain. Basic isoforms Cb 1 and Cb 2 were present in all leaf extracts, irrespective of *X. campestris* pv. *vesicatoria* infection. The other isoforms Cb 3, Cb 4, and Cb 5 were induced in the leaves inoculated with *X. campestris* pv. *vesicatoria*. In the compatible interaction, the two isoforms Cb 1 and Cb 2 gradually disappeared at 3 days after inoculation. Isoform Cb 3 was only induced after inoculation with the incompatible strain Bv5-4a.

To determine pI values of chitinase isoforms in the extracts of leaves inoculated with the two strains, isoelectric focusing was performed on 10% IEF gels of the pH range from 3.5 to 10.0. At least two acidic and four basic isoforms occurred in the extracts of uninoculated and inoculated leaves (Fig. 5). The activity of acidic isoforms with pIs 3.8 and 4.0 steadily re-

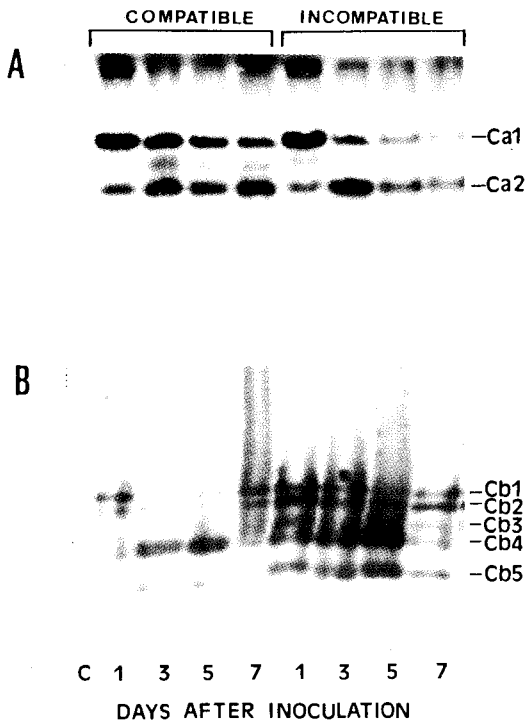

Fig. 4. Direct detection on the overlay gel containing 0.01% glycol chitin of acidic chitinase isoforms (A) and basic chitinase isoforms (B) in the extracts from uninoculated leaves (lane C) of tomato cultivar Kwangyang and in the extracts from leaves inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria* after electrophoretic separation in 15% polyacrylamide gel. Crude protein of 5 μg (A) and 10 μg (B) was loaded into each lane.

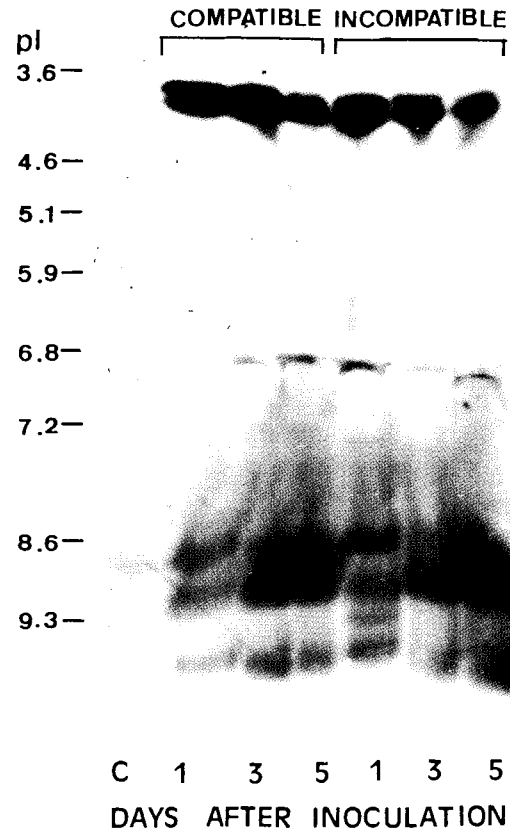

Fig. 5. Isoelectric focusing of chitinase isoforms in the extracts from uninoculated leaves (lane C) of tomato cultivar Kwangyang and in the extracts from leaves inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria*. Proteins (5 μg per lane) were separated to equilibrium (ampholine: pH 3.5~10.0). The separating gel was incubated in contact with the overlay gel containing 0.01% glycol chitin to detect chitinases for 2 h at 40°C.

mained at high levels, as the disease progressed. In uninoculated leaf tissue, three isoforms pIs 4.0, 8.8, and 9.7 were faintly discovered. Basic isoforms with pIs 8.8, 9.3 and 9.7 also were detected in the extracts of inoculated leaves. Their activity was much pronounced in the inoculated leaf tissues. In particular, the pI 9.5 isoform was only identified in the incompatible interaction. Chitinase activity of these isoforms was generally much higher in infected leaves relative to the uninoculated control.

Analysis of chitinase isoforms after SDS-PAGE. Two bands of chitinase isoforms (Mr. 23 and 26 kDa) were observed on SDS-polyacrylamide gels using the detection method of renatured chitinases (Fig. 6). The 26 kDa chitinase lacking in the uninoculated leaves, which corresponded to the 26 kDa protein bands, was intensely stained in the compatible and incompatible leaf extracts. The 23 kDa chitinase isoform also was confirmed by the intensely stained protein of 23 kDa, which were strongly induced by *X. campestris* pv. *vesicatoria* infection.

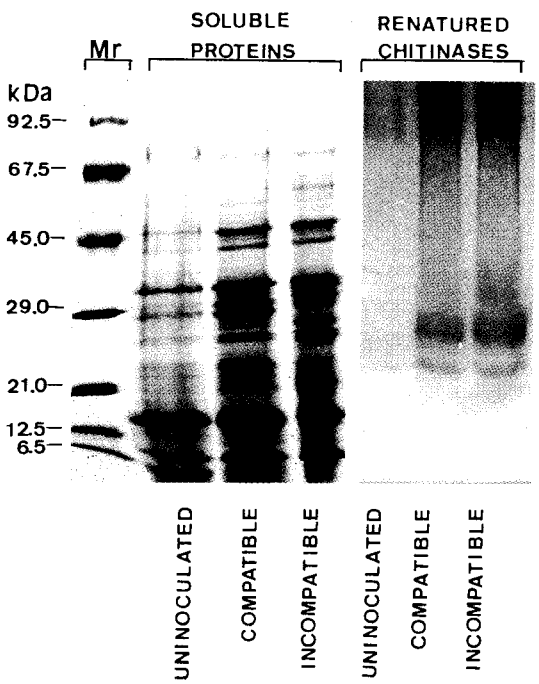

Fig. 6. SDS-PAGE profiles for determination of renatured chitinases in the extracts from the leaves of tomato cultivar Kwangyang inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria* and of uninoculated tomato leaves on the 15% polyacrylamide gel containing 0.01% glycol chitin for chitinases. Soluble proteins (20 μg per lane) were stained with Coomassie brilliant blue.

DISCUSSION

We examined characteristics of β-1,3-glucanase and chitinase in tomato leaves inoculated with the compatible and incompatible strains of *X. campestris* pv. *vesicatoria*. Tomato plants responded to *X. campestris* pv. *vesicatoria* infection, as well as to a wide range of stresses, by the production of β-1,3-glucanase and chitinase classically termed "pathogenesis-related" (PR) proteins. β-1,3-Glucanase and chitinase accumulated to a high level as hydrolytic enzymes in the tomato leaves infected by *X. campestris* pv. *vesicatoria*. Enhanced enzyme activities in the infected tomato plants appear to be host-mediated rather than induced by the *X. campestris* pv. *vesicatoria*, because the bacteria alone did not produce these hydrolytic enzymes (no data presented). Accumulation of the two hydrolytic enzymes was more pronounced in the incompatible tomato-*X. campestris* pv. *vesicatoria* interactions, as observed in other incompatible plant-pathogen interactions (1, 3, 16, 32). Mauch and Staehelin (21) suggested that the two hydrolytic enzymes synthesized after infection are deposited into vacuoles and function as a last line of defense when plant cells are lysed. The advantage of the extracellular localization of the hydrolytic enzymes lies in the possibility of suddenly flooding the invading *X. campestris* pv. *vesicatoria* with potentially lethal concentration of β-1,3-glucanase and chitinase to strongly inhibit the multiplication of the bacterial pathogen, as confirmed by some workers (20, 30).

By the use of the two native-PAGE systems, β-1,3-glucanase isoforms Ga 1 and Gb 1 was only detected in the leaf extracts of tomato plants infected by *X. campestris* pv. *vesicatoria*. The activity of the isoforms Ga 1 and Gb 1 was remarkably high per unit protein amount in the incompatible interactions at 1 or 3 days after inoculation, which suggests the possible role of the hydrolase in defence reaction to *X. campestris* pv. *vesicatoria* during early infection process. An acidic β-1,3-glucanase with pI 6.4 and one basic β-1,3-glucanase with pI 8.6 were detected on the IEF gel, especially with high activity in the incompatible interactions. These β-1,3-glucanase isoforms, e.g., pI 6.4 isoform, may be important in the expression of resis-

tance to *X. campestris* pv. *vesicatoria*, although β-1,3-glucanase with activity on laminarin may not be necessarily endowed with lytic activity on cell walls or alkali-insoluble β-1,3-glucan (13). Two acidic chitinase isoforms Ca 1 and Ca 2 were detected in the inoculated tomato plants, but not in the healthy controls. These data suggest that acidic chitinase isoforms may play a significant role in pathogenesis of *X. campestris* pv. *vesicatoria* infections in tomato leaves. However, basic isoforms Cb 1, Cb 2 and Cb 3 strongly accumulated in the incompatible interactions (Fig. 4B). These basic chitinase isoforms may be involved in active defence of tomato plants against *X. campestris* pv. *vesicatoria*, because of a positive correlation between the appearance of hypersensitive reaction (or necrosis) and the accumulation of these isoforms. In cucumber, tobacco and maize, acidic chitinase has an extracellular localization (5, 23, 31), whereas at least four of the basic chitinases are extracellularly localized in potato (18). It has earlier been reported that specific enzyme activity of basic chitinase was much higher than that of the acidic isoforms (19). The basic isoforms of chitinases may be extracellularly released during the hypersensitive response to bacterial cells in the incompatible interactions. The direct detection of putative chitinases on the overlay gel after isoelectric focusing (pH 3.5~10.0) revealed the two acidic and at least four basic isoforms (Fig. 5). Acidic chitinase isoforms with pIs 3.8 and 4.0 were induced and accumulated by *X. campestris* pv. *vesicatoria* infection, indicating their possible involvement in symptom development. However, no significant differences in the basic chitinase isoforms were found between the compatible and incompatible interactions, except for the pI 9.5 chitinase isoform only identified in the incompatible interaction. The finding suggests that the accumulation of pI 9.5 chitinase, probably isoform Cb 1 in the native PAGE gel, may be most likely the only part of a cascade of defense reactions eventually leading to resistance of tomato plants against *X. campestris* pv. *vesicatoria*. Renaturation of chitinases in SDS-PAGE gels by treatment with deionized Triton X-100 (Fig. 6) could identify 23 and 26 kDa chitinase isoforms, which strongly accumulated in the tomato leaves infected by either compatible or incompatible strain.

In conclusion, increase in β-1,3-glucanase and chitinase activity has positively co-related fashion for the expression of resistance to *X. campestris* pv. *vesicatoria*. However, all the isoforms of the two hydrolases did not seem to be associated with disease resistance. Some isoforms such as the pI 6.4 β-1,3-glucanase may be important in resistance expression, whereas others may be involved in the symptom development. Particularly, we suppose that the combination of β-1,3-glucanase and chitinase may have a lysozyme function to decompose *X. campestris* pv. *vesicatoria*. Nearly all known characterized plant enzymes with lysozyme function have been known to be basic proteins classified as basic chitinases (11, 12). In further studies, β-1,3-glucanases and/or chitinases purified from tomato plants will be tested for their lysozyme ability to degradate cell walls of *X. campestris* pv. *vesicatoria*.

요 약

Xanthomonas campestris pv. *vesicatoria*의 감염으로 토마토 잎조직에 β-1,3-glucanases와 chitinases가 합성, 축적되었다. 그러나 접종되지 않은 건전한 잎에서는 위의 두 가지 가수분해 효소는 매우 낮은 수준으로 유지되었고, 이 두 가지 효소는 친화적 상호작용에서보다는 불친화적 상호작용에서 더욱 높은 수준으로 존재하였다. 이것은 β-1,3-glucanases와 chitinases가 *X. c.* pv. *vesicatoria*의 생육에 대한 방어기작으로서 중요한 역할을 한다는 것을 시사해 주고 있다. Native PAGE 젤 상에서 β-1,3-glucanase를 분리한 결과, 병징 발현이나 저항성 발현에 중요한 역할을 하는 것으로 생각되는 산성 isoform Ga 1과 염기성 isoform Gb 1의 isoform bands만 확인되었다. Isoelectric focusing을 이용하였을 때, 적어도 pI 6.4와 pI 8.6을 지닌 두 개의 β-1,3-glucanases의 isoform을 확인할 수 있었고, 특히 불친화적 상호작용에서 더욱 뚜렷하게 유도되었다. 이것은 병 진전과정에서 *X. c.* pv. *vesicatoria*에 대해 저항성 발현에 관여한다는 것을 나타내고 있다. 산성 chitinase isoform인 Ca 1의 활성은 병원균의 감염이 진전되는 동안 감소하였다. 또한 다섯 개의 염기성 chitinase isoform이 감염된 토마토 잎 조직에서 발견되었는데, 특히 토마토의 방어기작에 관여하여 병원균의 증식을 저해하는데 중요한 isoform Cb 3은 불친화적 균주 Bv5-4a에 감염된 잎에서만 유도, 축적되었다. Isoelectric focusing(IEF)을 이용한 후 적어도 2개의 산성과 4개의 염기성 chitinase isoform이 감염된 토마토 잎 추출액에서 확인되었다. Native PAGE 젤에서 isoform Cb 1에 해당되는 pI 9.5를 지닌 chitinase isoform은 오직 불친화적 상호작용에서만 확인되었다.

이온이 제거된 Triton X-100을 처리하여 renaturation 시킨 후에 SDS-PAGE 겔 상태에서 23 kDa과 26 kDa을 지닌 2개의 chitinase isoform을 확인하였다.

REFERENCES

1. Ahl Goy, P., Metraux J. P. and Meins, F. Jr. 1992. Resistance to disease in the hybrid *Nicotiana glutinosa* × *Nicotiana debneyi* is associated with high constitutive levels of β-1,3-glucanase, chitinase, peroxidase and polyphenoloxidase. *Physiol. Mol. Plant Pathol.* 41 : 11-21.
2. Audy, P., Benhamou, N., Tredel, J. and Asselin, A. 1988. Immunocytochemical localization of a wheat germ lysozyme in wheat embryo and coleoptile cells and cytochemical study of its interaction with the cell wall. *Plant Physiol.* 88 : 1317-1322.
3. Boller, T. 1985. Induction of hydrolases as a defense reaction against pathogens. In : *Cellular and Molecular Biology of Plant Stress*, ed by J. L. Key and T. Konge, pp. 247-262. Alan R. Liss, New York.
4. Boller, T., Gehri, A., Mauch, F. and Vogeli, U. 1983. Chitinase in bean leaves : Induction by ethylene, purification, properties and possible function. *Planta* 157 : 22-31.
5. Boller, T. and Metraux, J. P. 1988. Extracellular localization of chitinase in cucumber. *Physiol. Mol. Plant Pathol.* 33 : 11-16.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72 : 248-254.
7. Cutt, J. R. and Klessing, D. 1992. Pathogenesis-related proteins. In : *Plant Gene Research: Genes Involved in Plant Defense*, ed by T. Boller and F. Meens, pp. 209-243. Springer-Verlag, Berlin.
8. Davis. B. J. 1964. Disc electrophoresis II. Method and application to human serum proteins. *Annals New York Acad. Sci.* 121 : 404-427.
9. During, K. 1993. Can lysozymes mediate antibacterial resistance in plants? *Plant Mol. Biol.* 23 : 209-214.
10. Ferraris, L., Abbattista, G. and Metta, A. 1987. Activation of glycosidases as a consequence of infection stress in *Fusarium* wilt of tomato. *J. Phytopathol.* 118 : 317-325.
11. Glazer, A. N., Barel, A. O., Howard, J. B. and Brown, D. M. 1989. Isolation and characterization of fig lysozyme. *J. Biol. Chem.* 244 : 3583-3589.
12. Howard, J. B. and Glazer, A. N. 1967. Studies on the physicochemical and enzymatic properties of papaya lysozyme. *J. Biol. Chem.* 242 : 5715-5723.
13. Jean G., Claude, P. and Alain, A. 1993. Barley pathogenesis-related proteins with fungal cell wall lytic activity inhibit the growth of yeasts. *Plant Physiol.* 103 : 1277-1283.
14. Joosten, M. H. A. J. and De Wit, P. J. G. M. 1988. Isolation, purification and preliminary characterization of a protein specific for compatible *Cladosporium fulvum* (syn. *Fulvia fulva*)-tomato interactions. *Physiol. Mol. Plant Pathol.* 33 : 241-253.
15. Joosten, M. H. A. J. and De Wit, P. J. G. M. 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3-β-glucanases and chitinases. *Plant Physiol.* 89 : 945-951.
16. Kearney, C. M. and Wu, J. M. 1984. β-1,3-Glucanase and spread of tobacco mosaic virus in *Nicotiana* and *Phaseolus. Can. J. Bot.* 62 : 1984-1988.
17. Keen, N. T. and Yoshikawa, M. 1983. β-1,3-Glucanase from soybean releases elicitor-active carbohydrates from fungus cell wall. *Plant Physiol.* 71 : 460-465.
18. Kombrink, E., Schoder, M. and Hahlbrock, K. 1988. Several 'pathogenesis-related' proteins in potato are 1, 3-β-glucanases and chitinases. *Proc. Natl. Acad. Sci. USA* 85 : 782-786.
19. Legand, M., Kauffman, S., Geoffray, P. and Fritig, B. 1987. Biological functions of pathogenesis-related proteins are chitinases. *Proc. Natl. Acad. Sci. USA* 84 : 6750-6754.
20. Mauch, F., Mauch-Mani, B. and Boller, T. 1988. Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combination of chitinase and β-1,3-glucanase. *Plant Physiol.* 88 : 936-942.
21. Mauch, F. and Staehelin, L. A. 1989. Functional implication of the subcellular localization of ethylene-induced chitinase and 1,3-β-glucanases in bean leaves. *Plant Cell* 1 : 447-457.
22. Molano, J., Duran, A. and Cabib, E. 1977. A rapid and sensitive assay for chitinase using trituated chitin. *Anal. Biochem.* 83 : 648-656.
23. Nasser, W., De Tapla, M. and Burkard, G. 1990. Maize pathogenesis related proteins: characterization and cellar distribution of β-1,3-glucanases and chitinases induced by brome mosaic virus infection or mercuric chloride treatment. *Physiol. Mol. Plant Pathol.* 36 : 1-14.
24. Nelson, N. 1944. A photometric adaption of the somogyi method for the determination of glucanase. *J. Biol. Chem.* 153 : 375-380.
25. Pan, S. Q., Ye, E. X. and Kúc, J. 1989. Direct detection of β-1,3-glucanase isozymes on polyacrlyamide electrophoresis and isoelectrofocusing. *Anal. Biochem.* 182 : 136-140.

26. Pan, S. Q., Ye, E. X. and Kúc, J. 1991. A technique for detection of chitinase, β-1,3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectrofocusing. *Phytopathology* 81 : 970-974.
27. Pegg, G. F. 1976. The occurrence of 1,3-glucanase in healthy *Verticillium albo-atrum*-infected susceptible and resistant tomato plants. *J. Exp. Bot.* 27 : 1093-1101.
28. Reisfeld, R. A., Lewis, U. J. and Williams, D. E. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* 195 : 281-283.
29. Reissing, J. L., Strominger, J. L. and Leloir, L. F. 1955. A modified colorimetric method for the estimations of N-acetylamino sugars. *J. Biol. Chem.* 217 : 959-967.
30. Schlumbaum, A. J., Mauch, F., Voegli, U. and Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. *Nature* 324 : 365-367.
31. Van Den Bulcke, M., Bauw, G., Castresana, C., Van Montagu, M. and Vandekerchove, J. 1989. Characterization of vascular and extracellular β-1,3-glucanases and tobacco: evidence for a strictly compartmentalized plant defense system. *Proc. Natl. Acad. Sci. USA*. 86 : 2673-2677.
32. Woodward, J. R., Keane, P. J. and Stone, B. A. 1980. β-Glucan and β-glucan hydrolase in plant pathogenesis with special reference to wilt-inducing toxins from *Phytophthora* species. In: *Fungal Polysaccharide*, ACS Symposium Series 126, ed by P. Sandford, pp. 113-141. American Chemical Society, Washington.
33. Young, D. H. and Pegg, G. H. 1982. The action of tomato and *Verticillium albo-atrum* glycosidases on the hyphal wall of *V. albo-atrum*. *Physiol. Plant Pathol.* 21 : 411-423.