한국식물병리학회지 11(1) : 53-60(1995)
Korean J. Plant Pathol. 11(1) : 53-60(1995)

Differential Induction of Pathogenesis-Related Proteins in the Compatible and Incompatible Interactions of Tomato Leaves with *Xanthomonas campestris* pv. *vesicatoria*

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Xanthomonas campestris pv. *vesicatoria*와 토마토잎의 친화적, 불친화적 반응에서 병생성관련 단백질의 유도

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ABSTRACT : Inoculation with the compatible strain Ds 1 of *Xanthomonas campestris* pv. *vesicatoria* caused brownish and water-soaked lesions, but incompatible strain Bv5-4a produced hypersensitive symptoms with local necrosis on tomato (cv. Kwangyang) leaves. Bacterial populations of the compatible strain Ds 1 propagated more greatly than the incompatible strain Bv5-4a at the first onset, but no differences were observed 5 days after inoculation. The bacterial infection induced the synthesis and accumulation of soluble proteins in tomato leaves, especially in the incompatible interaction. Native-polyacrylamide gel electrophoresis distinguished the soluble proteins in the tomato leaves infected by the compatible or incompatible strains. A protein of low molecular weight occurred only in the incompatible interaction. Some pathogenesis-related (PR) proteins, especially the 15, 18, 23, 26 and 54 kDa proteins, were detected only in the infected tomato leaves. In the two-dimensional electrophoresis, some proteins with different molecular weights (Mr. 21~29 kDa) and the pI 8~9 appeared more distinctly only in the incompatible interaction. These data suggest that the *de novo* synthesis of some PR proteins in tomato may be significant in defense against *X. c.* pv. *vesicatoria*.

***Key words* : pathogenesis-related (PR) proteins, *Xanthomonas campestris* pv. *vesicatoria*, tomato leaves, defense.**

Xanthomonas campestris pv. *vesicatoria* (Doidge) Dye. is the causal agent of bacterial spot disease on tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annuum* L.). The disease which affects leaves, stems and fruits occurs worldwide when tomato plants are grown under overhead irrigation or during warm, rainy weather. In Korea, the most serious losses are due to the leaf infection that causes defoliation, thereby reducing fruit yield. *X. c.* pv. *vesicatoria* has been reported to survive in association with plant debris, rotation, crops, seed, soil, volunteer plants and weeds, as inoculum sources for recent epidemics (12, 22). In the compatible interactions, infection with *X. c. pv. vesicatoria* gives rise to lesions on leaves, which at first appear water-soaked and later chlorotic. Race-specific resistance to *X. c.* pv. *vesicatoria* is expressed by the hypersensitive reaction (HR) leading to the collapse and desiccation of the infection site (1, 20). The bacterial growth is restricted following the collapse of tissue during the HR (9, 28). The development of the HR requires physical contact between the plant and the pathogen and *de novo* synthesis of proteins in the host plants. The synthesis of some non-constitutive components essential to the initial interaction between the plant and the pathogen and the biochemical events leading to plant cell death explains why protein synthesis is required for the HR induction (4, 27). Although the exact biochemical mechanisms leading to the HR are not known, several physiolo-

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gical changes including callose formation, synthesis of phenylpropanoids, vascular blockage, electrolyte leakage, lignification and changes in respiration rates have been demonstrated to be associated with the cellular collapse and death of the plant cells in the HR. Moreover, particular gene products involved in the plant defence response such as enzymes of the phenylpropanoid pathway (6), enzymes possessing hydrolytic activities (23), cell-wall proteins such as hydroxyproline-rich glycoproteins (18, 26, 29), the pathogenesis-related (PR) proteins (14, 17, 30) have been studied both *in vivo* in plants interacting with pathogens and *in vitro* in cell culture systems treated with biotic and abiotic elicitors of the host defence system.

In the present studies, we examined the multiplication of *X. c.* pv. *vesicatoria* in tomato leaves in the compatible and incompatible interactions. Whether or not the bacterial growth in both tomato-pathogen interactions will affect the *de novo* synthesis and accumulation of individual soluble proteins in tomato leaves were further analyzed by the use of one- and two-dimensional electrophoresis.

MATERIALS AND METHODS

Plant materials. Tomato (*Lycopersicon esculentum* Mill cv. Kwangyang) plants which are being intensively cultivated in Korea were used in this study. Tomato seeds were sown in a plastic tray (55×35×15 cm) containing steam-sterilized soil mix (peat moss : perlite : 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℃ under 16 hr per day illumination.

Bacterial strains and inoculation. Two strains Ds 1 and Bv5-4a of *Xanthomonas campestris* pv. *vesicatoria*, which are compatible and incompatible to the tomato cultivar Kwangyang, respectively, were used in this study. Strain Ds 1 was isolated in Korea from pepper plant in 1991 and the other strain Bv5-4a was kindly supplied by R. E. Stall, Department of Plant Pathology, University of Florida, Gainesvile, U.S.A. The bacteria were grown in yeast-nutrient medium (5 g yeast extract and 8 g nutrient broth per liter H_2O). To prepare bacterial inoculum, the two strains were cultured for 24 hr 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 10^8 cfu/ml, an absorbance of 0.06 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 the leaves appeared water-soaked. The inoculated tomato plants were placed in a moist chamber at 25℃ for 24 hr and returned to the growth chamber with temperature ranging from 23℃ to 27℃ and 16 hr-photo period.

Evaluation of bacterial population in tomato leaves. The bacterial population in the inoculated tomato leaves was recorded for 7 successive days after inoculation with *X. c.* pv. *vesicatoria*. To estimate bacterial populations, tomato leaf segments (4 cm^2 each) were cut and triturated in 10 ml sterile tap water. The resulting suspensions were serially diluted with sterile water. The diluted bacterial suspension of 0.1 ml was spread on Tween media (10 g peptone, 10 g potassium bromide, 0.25 g calcium chloride, 0.3 g boric acid, 10 ml Tween 80, 50 mg cycloheximide, 65 mg cephalexin, 12 mg 5-fluorouracil and 0.4 mg tobramycin per liter H_2O) (19). The inoculated plates were kept in a incubator at 28℃ for 3~4 days. The number of colonies appearing was counted and transformed into the $\log_{10}$ value. The experiments were repeated three times and each experiment had three replications.

Extraction of proteins in tomato leaves. Leaf extracts of tomato plants were obtained at various time intervals after inoculation. The harvested, inoculated leaves (1 g) were homogenized in liquid nitrogen and then extracted with 3 ml of 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℃ 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 (2).

One-dimensional electrophoresis. Before the polyacrylamide gel electrophoresis, proteins in the leaf extracts were precipitated with 4 volumes of acetone overnight at −20℃, and centrifuged at 1,500 g for 15 min at 4℃. After removal of the supernatants,

the remaining pellets were washed three times with 80% acetone, dried in a Speed-Vac and then resuspended in distilled water. Electrophoresis of proteins was carried out under nondenatured condition in 15% polyacrylamide separation gels and 5% stacking gels with 1.5 M Tris-HCl buffer (pH 8.8) (5). SDS-polyacrylamide gel electrophoresis of proteins was carried out in 10~20% SDS polyacrylamide gradient gels using the discontinuous buffer system developed by Laemmli (15). Molecular weight standards (Serva) ranging from 6.5 to 92.5 kDa were used to determine the molecular weights of proteins of interest. For one-dimensional polyacrylamide gel electrophoresis in a vertical slab gel, protein solution (2 μg per lane) was loaded into the slot. The separation of soluble proteins was made at 100 V for 1 hr, and then 200 V for 6 hr at 8℃. Exceptionally for SDS-PAGE, the sample solution was heated for 3 min in a boiling water bath and applied to the separating gel. The separated soluble proteins in polyacrylamide gel were stained with silver nitrate (10).

Two-dimensional electrophoresis. For two-dimensional electrophoresis, proteins were separated by isoelectric focusing in tube gel (100×5 mm). The gel contained 6% acrylamide cross-linked with bisacrylamide (at a ratio of 30 : 1), 3% ampholine (pH 3.5~10.0, Sigma) and 6 M urea. Amounts of protein solutions, each of which was equivalent to 100 μg, were loaded on top and overlaid with the solution containing 3 M urea, 10% sucrose, and 2% ampholine (pH 3.5~10.0). The anode electrode solution was 0.1% H_3PO_4, and the cathode electrode solution was 0.1 N NaOH. Isoelectric focusing was performed at 200 V for 20 min, with increasing from 400 to 1,100 V for 7 hr. Following the first dimensional electrophoresis, each tube gel was equilibrated in the buffer (62.5 mM Tris-HCl, 10% sucrose, 5% mercaptoethanol, 2~3% SDS, 6 M urea, pH 6.8) for 30 min and then placed on the top of a SDS-polyacrylamide slab gel (1 mm thick). Electrophoresis was carried out at 100 V for 1 hr and then at 200 V for 8 hr. Separated soluble proteins were stained by using a silver staining method (10).

RESULTS

Bacterial populations in compatible and incompatible interactions. In tomato leaves infiltrated with the compatible strain Ds 1 of *X. c.* pv. *vesicatoria*, brownish, irregularly shaped and water-soaked lesions were produced at 2 days after inoculation, but no hypersensitive symptom occurred. In contrast, hypersensitive symptoms were observed in the leaves inoculated with the incompatible strain Bv5-4a (no data presented). Bacterial populations in the tomato (cv. Kwangyang) leaves inoculated with the compatible and incompatible strains at the eight-leaf stage are presented in Fig. 1. At the first onset after inoculation, the bacterial cells in the leaves inoculated with the strain Ds 1 were 10~100 fold more than in those inoculated with the strain Bv5-4a. In particular, increase in the number of bacterial cells was much pronounced until 3 days after inoculation with the compatible strain Ds 1. However, 4 days after inoculation, differences in bacterial population between the compatible and incompatible interactions started to diminish, and no differences were observed at 5 days after inoculation.

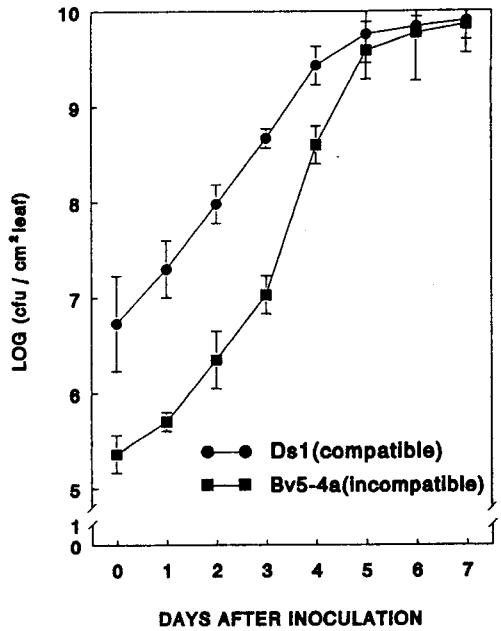

Fig. 1. Time courses for the increase in bacterial populations of the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria* in leaves of tomato cultivar Kwangyang inoculated by foliar spray at the eight-leaf stage. Values are the means of three replications. Vertical bars represent standard deviations.

Assays of soluble proteins after one-dimensional electrophoresis. Changes of soluble protein conce-

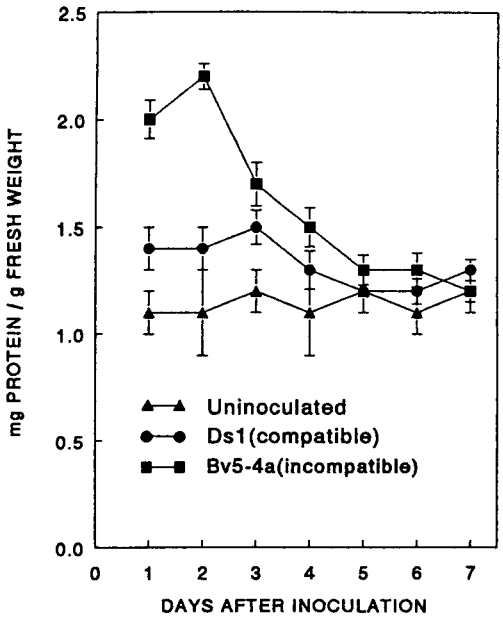

Fig. 2. Changes in soluble protein concentrations in leaves of tomato cultivar Kwangyang uninoculated or inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria*. Vertical bars represent standard deviations.

ntrations in the extracts of tomato (cv. Kwangyang) leaves inoculated with the compatible strain Ds 1 and incompatible strain Bv5-4a and of uninoculated healthy leaves are shown in Fig. 2. Protein concentrations in the uninoculated leaves remained at low levels, whereas protein concentrations increased in the leaves inoculated with each of the two strains. In the compatible interactions, protein concentrations slowly increased, reached maximum at 3 days after inoculation, and then decreased. In contrast, protein concentrations in the incompatible interactions drastically increased at the onset after infection, but thereafter declined to the same levels as the healthy control.

Native-PAGE profiles of soluble proteins in the extracts of leaves inoculated with the compatible strain Ds 1 and the incompatible strain Bv5-4a and of uninoculated leaves are illustrated in Fig. 3. Five different proteins 1, 2, 3, 4 and 7 occurred in the uninoculated leaves, but were not detected in the infected leaves. However, two proteins 6 and 11 were found in the extracts of leaves at the onset of infection by the two strains. In particular, protein 6 was observed at the first day after inoculation

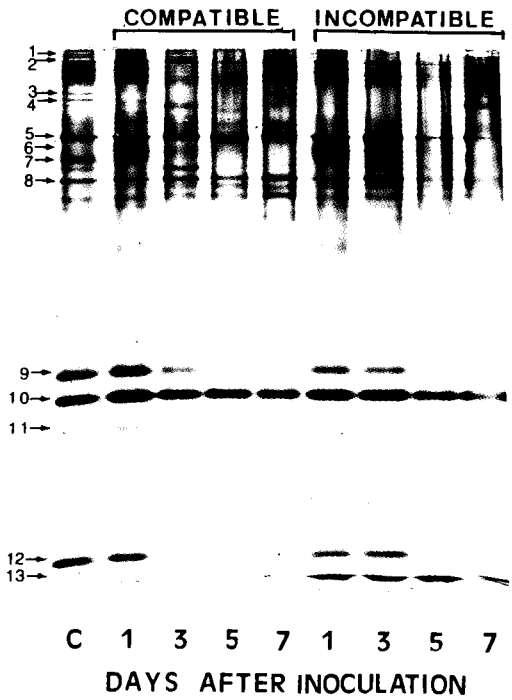

Fig. 3. Native-PAGE of soluble proteins in the extracts from noninoculated, control leaves (C) of tomato cultivar Kwangyang and from leaves infiltrated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria*. Crude protein solutions were electrophoresed in a 15% polyacrylamide gel under undenaturing conditions. Proteins in the gel were stained with silver nitrate.

with either of the strains and then began to disappear at 3 days after inoculation. Protein 11 slightly occurred in the compatible and incompatible responses at 1 or 3 days after inoculation. Since then, the two proteins 6 and 11 disappeared in the infected leaves. Proteins 8 and 10 were found in all leaf extracts. Proteins 9 and 12 gradually disappeared in the infected leaves during the pathogenesis. Protein 13 was found in the extracts of leaves infected by the incompatible strain Bv5-4a.

SDS-PAGE profiles of soluble proteins in the extracts of tomato leaves inoculated with the two strains and of uninoculated leaves are shown in Fig. 4. Soluble proteins in all leaf extracts ranged from 6.5 to 92.5 kDa. The two proteins with molecular weights of 23 and 26 kDa were observed in a high level in the leaves infected by both strains. Particularly, the 23 kDa protein was spotted only 1 day after inoculation. However, the 54 kDa protein ac-

cumulated within 24 hr after inoculation. Protein bands of 15 and 18 kDa were revealed at 3 days after inoculation in the compatible or incompatible responses. No significant differences in the other protein patterns were detected between the uninoculated and compatible or incompatible interactions.

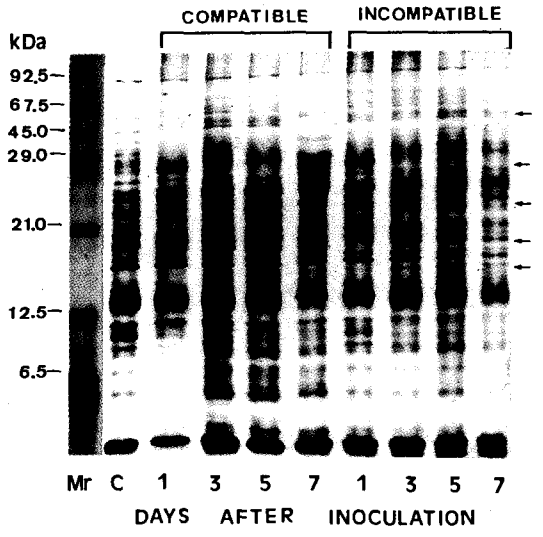

Fig. 4. SDS-PAGE profiles of soluble proteins from noninoculated, control leaves (C) of tomato cultivar Kwangyang and from leaves inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria*. Crude protein solutions were electrophoresed in a 15% SDS-polyacrylamide gel. Proteins in the gel were stained with silver nitrate. Lane Mr. indicates molecular weight markers.

Assays of soluble proteins after two-dimensional electrophoresis. Soluble proteins in the extracts of tomato (cv. Kwangyang) leaves inoculated with each of the compatible strain Ds 1 and incompatible strain Bv5-4a and of uninoculated were separated using two-dimensional electrophoresis (Fig. 5). Electrophoretic patterns of soluble proteins from tomato leaves inoculated with the two strains were markedly different from those of uninoculated leaf extracts. Proteins 2 and 13, which lacked in the inoculated leaf extracts, were present in the healthy tomato leaves. Proteins 6, 8, and 10 were not detected in the extracts of Ds 1-inoculated and uninoculated leaves, but occurred only in the Bv5-4a-inoculated leaves. However, proteins 7, 9, 11 and 14 which showed relatively low levels in the compatible interaction were revealed in the extracts of leaves inoculated with the incompatible strain. Protein 5 present in the compatible response was absent in the incompatible interaction.

DISCUSSION

Differential propagation of bacterial cells in the compatible and incompatible interactions. The propagation of bacterial cells in tomato leaves inoculated with *X. c.* pv. *vesicatoria* was compared in the com-

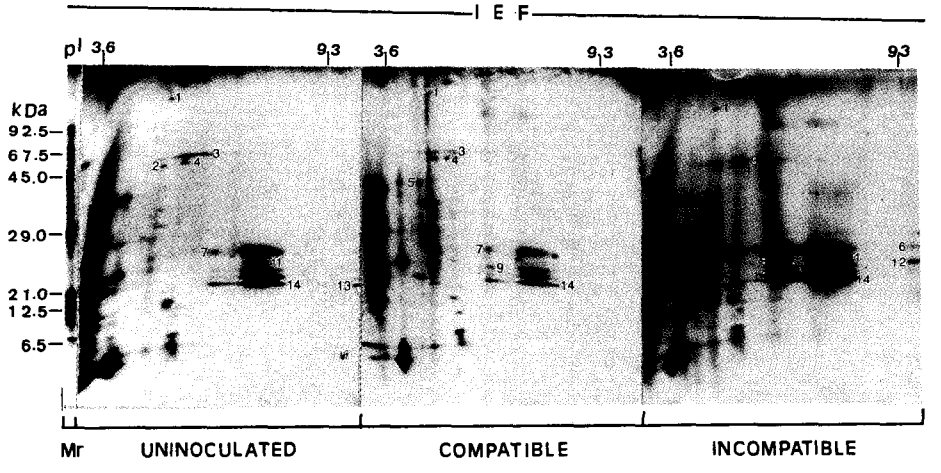

Fig. 5. Two-dimensional analysis of silver nitrate-stained proteins from tomato (cv. Kwangyang) leaves noninoculated or inoculated with the strains Ds 1 (compatible) and Bv5-4a (incompatible) of *Xanthomonas campestris* pv. *vesicatoria*. Amounts of protein equivalent to 100 μg were separated by isoelectric focusing (IEF) in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension.

patible and incompatible interactions. In the compatible interaction, bacterial cells were greatly propagated as compared with the incompatible interaction. However, the populations of both Ds 1 and Bv5-4a were not significantly different 5 days after inoculation. Hypersensitive response (HR) occurred in the tomato leaf tissues inoculated with Bv5-4a. A bacterial strain was classified as avirulent, if it elicited one of the resistance responses within 24 hr when the titer of the inoculum exceeded $10^6 \sim 10^7$ cfu/ml (7). In case of inoculation with *X. c.* pv. *vesicatoria*, resistant plants only show HR when avirulent bacteria are inoculated at a density of about 10^7 cfu/ml or more (33, 36). A bacterial strain was classified as virulent, if it elicited disease symptoms (water-soaked lesion with or without chlorosis) 48 hr after infiltration with a bacterial suspension at a titer of 10^5 cfu/ml (7). A prolonged infection was observed in leaf tissues colonized by the compatible strain Ds 1. Tomato leaf cells within the large water-soaked lesions underwent changes similar to chlorosis occurring during senescence, but few signs of rapid disorganization were observed in the infected leaves. In the incompatible interactions, however, it was postulated that sites of hypersensitive responses were rapidly collapsed and limited nutrients of *X. c.* pv. *vesicatoria* to inhibit its multiplication. Pathogenesis-related proteins (PR proteins) have often been reported to be closely associated with induced resistance, suggesting that they may play a role in defense mechanisms of the plants (31). PR proteins were involved in plant defense reaction like HR (7, 11, 21). Some hydrolytic enzymes in PR-proteins may function only when host cells are lysed during pathogenesis, e.g., when fungal enzymes digest the host cell walls, thereby causing the protoplast to burst, or when the pathogen triggers a hypersensitive response of the surrounding host tissues (34).

Assay of pathogenesis-related proteins in the compatible and incompatible interactions. We found the differential accumulation of soluble proteins in tomato leaves by *X. c.* pv. *vesicatoria* infection. In the incompatible interactions, the more soluble proteins were induced, compared with the compatible ones (Fig. 2). At the initial time after inoculation, soluble proteins accumulated more strongly in the incompatible than in the compatible interactions, suggesting that some proteins may be induced and accumulated so as to be unfavorable for the bacterial multiplication of the incompatible strain. Some proteins were newly detected on the native-PAGE gels. In particular, proteins 6 and 11 were found only in the infected leaves, but not in the healthy ones. In particular, protein 13 was merely present in the incompatible interactions, indicating that the protein is a possible candidate of the defense-related inducible proteins. As shown in Fig. 4, one-dimensional profiles indicated that in the infected tomato leaves, five proteins induced could be related to pathogenesis-related (PR) proteins. The most prominent protein with 26 kDa could be induced in infected tomato plants by *X. c.* pv. *vesicatoria*. When analyzed by two-dimensional electrophoresis, protein 7, 9, 10, and 11 were found in the extracts of leaves inoculated with the incompatible strain. In particular, the four proteins of different molecular weights (Mr. 21~29 kDa) with the pI 8~9 were not present in uninoculated leaves or the compatible interactions. These facts suggest that the induction of *de novo* synthesis of some PR proteins, probably due to activation of defense-related gene by *X. c.* pv. *vesicatoria* infection, may contribute to the expression of defense reaction to the pathogen attack. Therefore, using a method of renaturation of chitinases, we tried to identify which proteins have hydrolytic enzyme activities as PR-proteins. Chitinase isoforms with 26 and 23 kDa were greatly induced after infection by *X. c.* pv. *vesicatoria* (13). Recently, Wubben *et al.* (35) reported that acidic 26 kDa chitinase was usually located in extracellular space of *Cladosporium fulvum*-infected tomato leaves. The 23 kDa protein also has been known to be one of inducible PR proteins in the pathogen-tomato interactions (3). On the other hand, some proteins disappeared gradually in the infected tomato leaves, indicating their possible degradation during *X. c.* pv. *vesicatoria* attack (16). It has been suggested that several induced PR proteins located in extracellular space of infected tobacco leaves might be subjected to degradation by extracellular proteases (25). Recently, Rodrigo *et al.* (24) purified aspartyl proteinase from tomato plants that specifically degrades tomato PR proteins. These protenases may play a significant role in preventing the overaccumulation of PR proteins (8, 25, 32).

요 약

Xanthomonas campestris pv. *vesicatoria*의 친화적 균주 Ds 1을 접종한 토마토 잎에서는 처음에 수침상의 병반이 형성되었고, 갈변하거나 괴저 병반이 나타났으나 과민성 반응은 나타나지 않았다. 그러나 불친화적 균주 Bv5-4a를 접종한 토마토 잎에서는 국부적인 괴저병반인 과민성 병반이 관찰되었다. 초기에는 친화적균주 Ds 1의 개체군의 증가가 불친화적균주 Bv5-4a 보다 뚜렷하였으나 접종후 5일 후에는 두 균주 사이의 차이를 관찰할 수 없었다. 특히 불친화적 상호작용에서, *X. c.* pv. *vesicatoria*의 감염으로 토마토 잎에서 수용성 단백질의 합성과 축적이 유도되었다. Native PAGE 젤에서 친화적 균주와 불친화적 균주에 감염된 토마토 잎에서 수용성 단백질의 차이가 발견되었다. 단지 불친화적 상호작용에서만 낮은 분자량을 지닌 단백질이 유도되었다. 몇개의 병생성관련단백질 (PR-protein), 특히 15, 18, 23, 26과 54 kDa 단백질은 감염된 토마토 잎 추출액에서만 발견되었다. 2차 전기영동법을 이용하여 단백질 패턴을 비교 분석한 결과, pI 8~9을 지니며 분자량(21~29 kDa)이 상이한 몇개의 단백질이 불친화적 상호작용에서 뚜렷하게 나타났다. 이들 데이타는 토마토 잎에서 새로운 병생성 관련단백질(PR-protein)의 합성이 *Xanthomonas campestris* pv. *vesicatoria*에 대한 방어기작에 중요한 역할을 하리라고 시사해 주고 있다.

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