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Bacterial Multiplications and Electrophoretic Patterns of Soluble Proteins in Compatible and Incompatible Interactions of Pepper Leaves with *Xanthomonas campestris* pv. *vesicatoria*

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Xanthomonas campestris pv. *vesicatoria*에 감염된 고추잎의 친화적, 불친화적 반응에서 세균증식과 수용성 단백질의 전기영동 패턴

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ABSTRACT : Typically susceptible lesions were developed on pepper (cv. Hanbyul) leaves inoculated with the compatible strain Ds 1 of *Xanthomonas campestris* pv. *vesicatoria*. The lesions appeared first water-soaked and then turned yellow with a chlorotic area. In contrast, the leaves inoculated with the incompatible strain 81-23 initially turned yellow and then developed local necrosis. Multiplication of *X. c.* pv. *vesicatoria* in pepper leaves also were distinctly different between the two strains. The strain Ds 1 multiplied more greatly than did the strain 81-23 in the infected leaves. *X. c.* pv. *vesicatoria* infection of pepper leaves induced the synthesis of soluble proteins, especially more greatly in the compatible than in the incompatible interactions. Some pathogenesis-related (PR) proteins were detected in the intercellular washing fluid (IWF) and extracts of the infected pepper leaves. In particular, the 32 kDa protein on SDS-PAGE gels appeared intensely in the incompatible interaction. In contrast, some proteins with molecular masses of 65, 71, and 75 kDa disappeared in the infected pepper leaves. Isoelectric focusing could identify the pIs of soluble proteins in infected pepper leaves. The accumulation of the IWF from infected leaves was more conspicuous in the incompatible than the compatible interaction. These results suggest that some extremely acidic and basic proteins were induced and accumulated in the intercellular spaces of infected pepper leaves.

***Key words* : *Xanthomonas campestris* pv. *vesicatoria*, *Capsicum annuum*, bacterial multiplication, pathogenesis-related protein.**

Xanthomonas campestris pv. *vesicatoria* (Doidge) Dye, causes bacterial spot disease in pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill). Development of the disease on these host plants has been known to be favored by plant wounding, high air temperature, rain, and wind (26). Severe plant infection may reduce fruit yield and quality of pepper.

Compatible interaction of plants with pathogenic bacteria is characterized by the phenomena that bacterial growth is very active and susceptible lesions develop (9). Compatible strains inactivate and avoid plant defense responses. Incompatible interaction is characterized by inhibition of bacterial growth and spread, lack of symptom, and hypersensitive reaction. Genetic analysis of host-pathogen interactions can be used to help dissect the process by which plants perceive the presence of a pathogen.

Infections by incompatible microbial pathogens induce accumulation of soluble proteins in plants

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(4, 21, 22). Enzymatic role and way of interacting with pathogen of such proteins remain to be unelucidated. However, such host-synthesized proteins have been known to be usually correlated with development of defense response. When plants are infected with bacteria, fungi or viruses (7, 13, 16, 18, 20), soluble host-encoded proteins are accumulated with symptom development. Since the first discovery of such proteins in tobacco plants hypersensitively reacting with TMV, these have currently been found in several plant species after infection with various pathogens (7, 11, 16, 19, 25). Namely, 'pathogenesis-related (PR)' proteins are accumulated in plant tissues responded to various pathogens. PR proteins are characterized by selective extractions at low pH, relatively low molecular weight, accumulation in the intercellular space, a high resistance to proteolytic activity, easy resolving by electrophoresis in polyacrylamide gel, and extreme isoelectric points.

In the present study, we investigated the changes in amounts and patterns of soluble proteins accumulating in the intercellular washing fluids (IWF) and extracts of pepper (cv. Hanbyul) leaves after inoculation with *X. c.* pv. *vesicatoria* strains Ds 1 (compatible) and 81-23 (incompatible) and of uninoculated leaves. The multiplications of *X. c.* pv. *vesicatoria* in pepper leaves also were compared between compatible and incompatible interactions.

MATERIALS AND METHODS

Plant, bacteria, and inoculations. Seeds of pepper cultivar 'Hanbyul', which differed in susceptibility to *X. c.* pv. *vesicatoria* strains tested (14), 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), sand, and loam soil (1:1:1, v/v/v). Six seedlings at the six-leaf stage were transplanted in each of plastic pots (5×15×10 cm) containing soil mix, sand, and loam soil. Pepper plants were raised up to the second-branch stage in a growth room with temperatures ranging from 23 to 27℃ and 16 h-photo period.

The two strains of *X. c.* pv. *vesicatoria* Ds 1, compatible or 81-23, incompatible to the pepper cultivar Hanbyul were used in this study (14). Strain Ds 1 was isolated in 1991 from pepper plants in Korea. The other strain 81-23 was provided by R. E. Stall, Department of Plant Pathology, University of Florida, Gainsvile. Bacteria were repeatedly purified from single colonies. Broth cultures of bacteria were grown in the yeast-nutrient medium (5 g yeast extract and 8 g nutrient broth per liter H_2O). The bacterial strains were usually stored at −70℃ in the yeast-nutrient broth containing 15% glycerol.

To prepare bacterial inoculum for inoculation in pepper leaves, bacterial strains were grown in yeast-nutrient broth for 24 h. Bacterial inoculum were pelleted by centrifugation at 3,000 g for 15 min. The harvested bacterial cells were then suspended in sterile tap water. Cell suspensions were adjusted to 10^8 colony-forming units per ml (an absorbance of 0.06 at 660 nm) with sterile tap water prior to inoculation. Pepper plants of second-branch stage were inoculated by vacuum-infiltrating the bacterial suspension (10^8 cfu/ml) into the abaxial side of the fully expanded leaves with an atomizer connected to a compressor. The inoculated pepper plants were incubated in a moist chamber with temperature ranging from 23 to 27℃, and 16 h-photo period.

Evaluation of bacterial population in pepper leaves. Test pepper plants were observed for symptom development daily after inoculation. Bacterial population in the inoculated pepper leaves was determined at different time intervals after inoculation by dilution-plating procedure. Two leaf segments (4 cm^2 each) were cut from the inoculated areas and triturated in 10 ml sterile tap water. The resulting suspensions were serially diluted with sterile water. The 0.1 ml bacterial suspension in a dilution series was spread onto Tween media (10 g pepton, 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) (17). The inoculated plates were incubated at 28℃ for 3~4 days. The numbers of colonies appearing were transformed into log10 values. The experiment was repeated twice and each experiment had three replications.

Preparation of intercellular washing fluids (IWF) and leaf extracts. Intercellular washing fluids (IWF) in pepper leaves were obtained at different time intervals after inoculation, according to the method of Klement (8). Entire leaves were vacuum-infiltrated with distilled water at 4℃ for 20 min and then blotted dry with paper towel or filter paper. The infiltrated leaves were rolled and placed in a specially designed centrifuge tube. The IWF was colle-

cted by centrifugation of leaves at 3,000 g for 10 min and stored at −20℃.

Leaf extract was prepared by homogenizing 1 g leaves in liquid nitrogen and extracting the fine leaf powder with 3 ml 0.5 M sodium acetate buffer (pH 5.2) containing 15 mM 2-mercaptoethanol using a prechilled mortar and pestle. The homogenates were centrifuged for 60 min at 20,000 g and the supernatants were stored at −20℃. Aliquots of the clear supernatants from the IWF and leaf extracts were used to determine protein content with the method of Bradford (1), using bovine serum albumin as a standard.

SDS polyacrylamide gel electrophoresis. Before polyacrylamide gel electrophoresis, proteins in intercellular washing fluid and leaf extracts were precipitated with 100% acetone at −20℃ for overnight. IWF and leaf extract were centrifuged at 4℃, 1,500 g for 15 min. The pellet was washed with 80% acetone and resuspended in distilled water. Electrophoresis was performed in 10~20% SDS polyacrylamide gradient gels with an overlayed stacking gels of 5%, according to the method of Laemmli (12). The molecular weights of the various proteins were estimated by coelectrophoresis of marker proteins (Serva) ranging from 6.5 to 92.5 kDa.

The sample solution which consisted of 30 μl protein solution (30 μg protein) and 6 μl sample buffer, was heated for 3 min in a boiling water bath and applied to the gel. Sample buffer contained 0.9 g glycine, 0.1 ml 1% (w/v) bromophenol blue, 1 ml 10% (w/v) SDS and 0.1 ml mercaptoethanol. The electrode buffer (pH 8.3) contained 14.4 g glycine, 1 g SDS and 3 g Tris per liter. SDS-PAGE was carried out at 100 V for 1 h and then at 200 V for 6 h at 8℃. Proteins in the SDS-polyacrylamide gel were stained by Coomassie brilliant blue R 250.

Isoelectric focusing (IEF). Isoelectric focusing of proteins in the IWF and leaf extracts was carried out on 10% polyacrylamide gel containing ampholine (pH 3.5~10, Sigma) according to manufacturer's protocol (LKB). The pI markers, ranging from pI 3.6 to 9.3 (Sigma), were coelectrophoresed to estimate the pIs of the various proteins. Samples (40 μg) were loaded in the center of the IEF gel and run 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 gel was fixed in 20% (v/v) trichloroacetic acid (TCA) for 1 h and then shortly rinsed with distilled water. The gel was stained in Serva blue W and destained in 0.02% (v/v) TCA until background disappeared.

RESULTS

Multiplication of bacteria in the compatible and incompatible responses. Typical bacterial spot lesions were observed daily on pepper leaves inoculated with each of *X. c.* pv. *vesicatoria* strains Ds 1 and 81-23 (Fig. 1). Pepper leaves inoculated with the compatible strain Ds 1 developed susceptible lesions, which appeared water-soaked and turned yellow at 3 days after inoculation. After 6 days, large chlorotic and necrotic areas were developed, turning light-brown. The lesions were slightly sunken on the upper leaf surface, but slightly raised on the lower surface. After 8 days, affected pepper leaves were curled up. Severely affected leaves were dried and dropped. With the incompatible strain 81-23, the affected leaf tissue changed from green to yellow

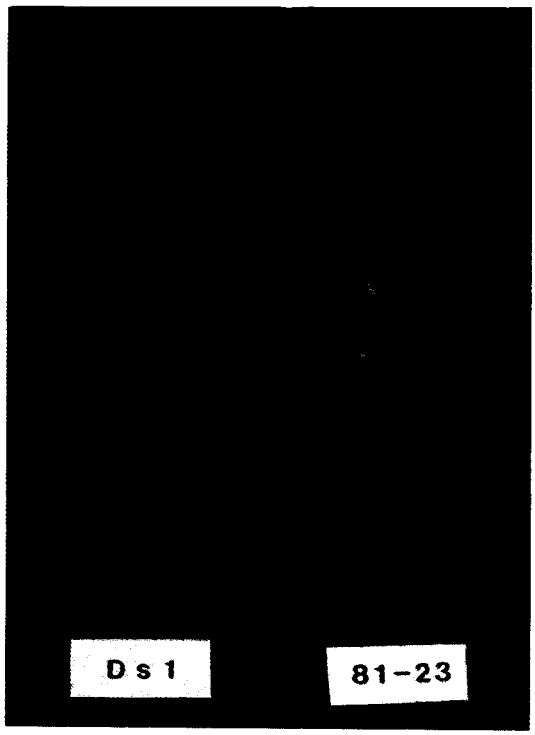

Fig. 1. Differential reactions of pepper (cv. Hanbyul) leaves to the strains Ds 1 (compatible) and 81-23 (incompatible) of *Xanthomonas campestris* pv. *vesicatoria* at the second-branch stage.

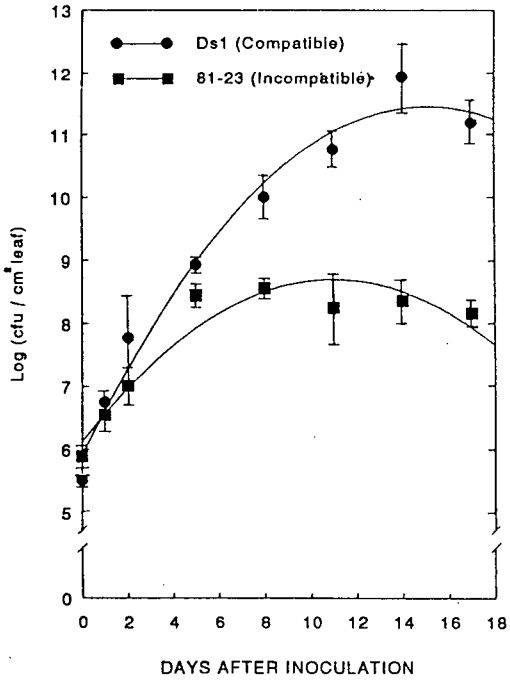

Fig. 2. Time course of bacterial multiplication in pepper (cv. Hanbyul) leaves inoculated with *Xanthomonas campestris* pv. *vesicatoria* strains Ds 1 (compatible) and 81-23 (incompatible) at the second-branch stage. Vertical bars represent standard deviations.

at 3 days after inoculation. After 4 days, localized necrosis appeared at the site of inoculation and faded from green through light-tan to a chalky white color.

Multiplication of *X. c.* pv. *vesicatoria* in pepper (cv. Hanbyul) leaves inoculated with each of the compatible strain Ds 1 and the incompatible strain 81-23 at second-branch stage is presented in Fig. 2. The number of bacteria recovered from lesions on the leaves inoculated with Ds 1 was in general higher than those from lesions on the leaves inoculated with 81-23. However, there were no significant differences between the two compatible and incompatible combinations in bacterial multiplication during the first period of 24 h after inoculation. In the leaves inoculated with compatible strain Ds 1, bacterial population increased to $10^{11}-10^{12}$ cfu/cm^2 leaf by 14 days after inoculation and then declined. In the leaves inoculated with incompatible strain 81-23, bacterial population also increased to 10^8-10^9 cfu/cm^2 area by 8 days and then declined. The compatible strain Ds 1 multiplied 10^3 to 10^4-fold more than of the incompatible strain 81-23 in pepper leaves within 14 days after inoculation.

Comparison of soluble proteins between the compatible and incompatible interactions. Levels of soluble protein concentrations in intercellular washing fluids (IWF) and extracts of pepper (cv. Hanbyul) leaves inoculated with *X. c.* pv. *vesicatoria* strains Ds 1 (compatible) and 81-23 (incompatible) and of uninoculated pepper leaves are presented in Fig. 3. Low levels of protein concentrations remained in IWF and extracts of uninoculated pepper leaves. In contrast, an increase in protein concentrations was observed in IWF and extracts of the leaves inoculated with each of the two strains. Compared to the incompatible interaction, a rapid increase in protein concentrations occurred in the compatible interaction, reaching maximum at 13 days after inoculation. In particular, the differences between the compatible and incompatible interactions in protein accumulations were more pronounced in IWF than in the leaf extracts.

SDS-PAGE of soluble proteins in IWF of leaves inoculated with the compatible strain Ds 1 and the incompatible strain 81-23 and of uninoculated pepper leaves are illustrated in Fig. 4. Proteins of different molecular weights in the range of 6.5~92.5 kDa were detected in IWF of leaves inoculated with the two strains and of uninoculated pepper leaves. The protein with molecular weight of 32 kDa appeared intensely in the IWF of infected leaves. The protein begun to accumulate rapidly within 2 days after inoculation. In the incompatible interaction, the increase in the protein intensity was higher than in the compatible interaction. However, three protein bands (M.W. 65, 71, and 75 kDa) disappeared gradually in the inoculated pepper leaves. On the other hand, five protein bands (M.W. 25, 28, 39, 42, and 53 kDa) became intense in the IWF of inoculated pepper leaves. In the incompatible interaction, the accumulation of these proteins were more pronounced than in the compatible interaction.

SDS-PAGE of soluble proteins in the extracts of pepper leaves inoculated with the strains Ds 1 and 81-23 and of uninoculated pepper leaves is illustrated in Fig. 5. Proteins of different molecular weights were detected in the range of 6.5~92.5 kDa. The protein of 32 kDa occurred in a high level in the extracts of inoculated pepper leaves, as in the IWF. In the inoculated leaves, the accumulation of this

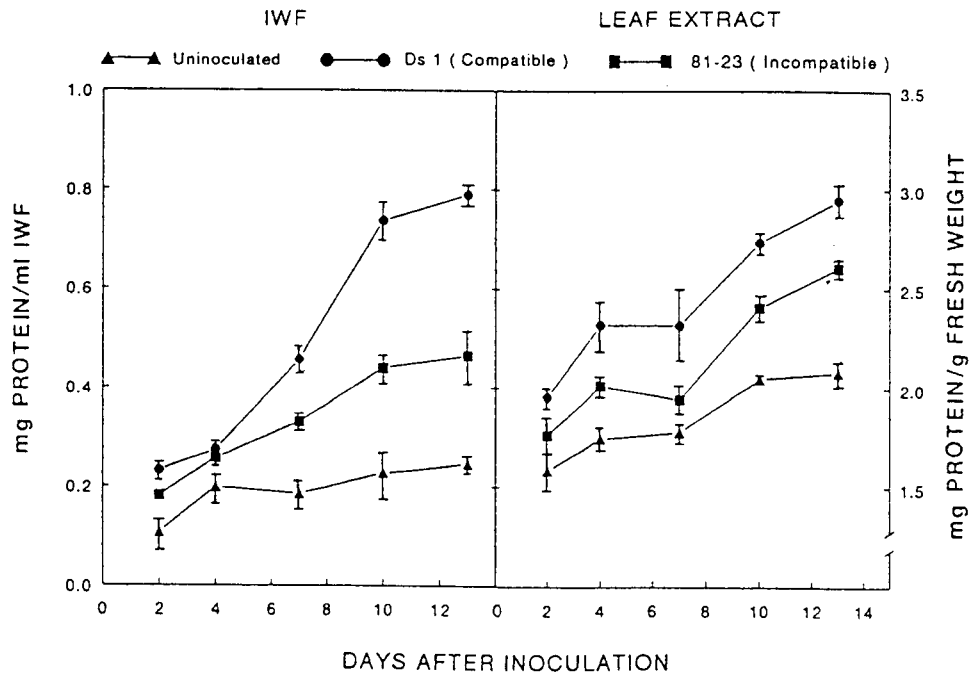

Fig. 3. Time course of soluble proteins in intercellular washing fluids (IWF) and extracts of pepper (cv. Hanbyul) leaves after inoculation with *Xanthomonas campestris* pv. *vesicatoria* strains Ds 1 (compatible) and 81-23 (incompatible) and of uninoculated pepper leaves at the second-branch stage. Vertical bars represent standard deviations.

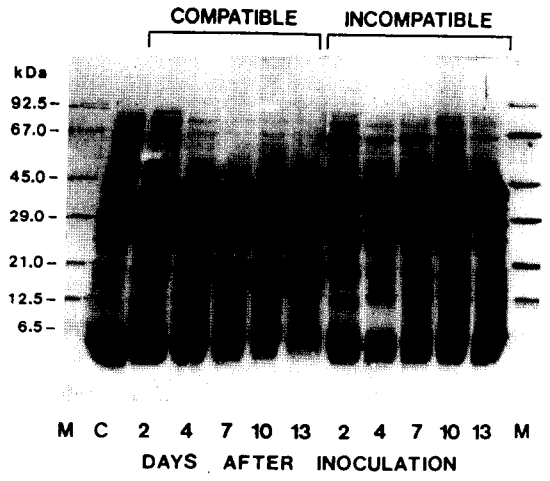

Fig. 4. SDS-PAGE of soluble proteins in the intercellular washing fluids (IWF) of pepper (cv. Hanbyul) leaves obtained 2, 4, 7, 10, and 13 days after inoculation with *Xanthomonas campestris* pv. *vesicatoria* strains Ds 1 (compatible) and 81-23 (incompatible) and of uninoculated pepper leaves (C). Each lane contains 25 μg protein. Lanes 'M' contain Mr. markers. The SDS polyacrylamide gel was stained with Coomassie brilliant blue. Arrows indicate the 32 kDa proteins in the gels.

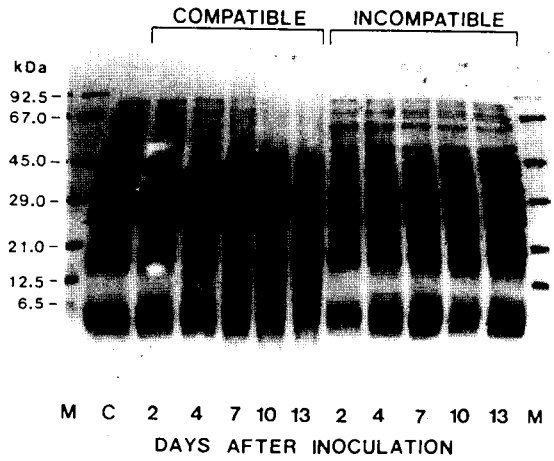

Fig. 5. SDS-PAGE of soluble proteins in the extracts of pepper (cv. Hanbyul) leaves obtained 2, 4, 7, 10, and 13 days after inoculation with *Xanthomonas campestris* pv. *vesicatoria* strains Ds 1 (compatible) and 81-23 (incompatible) and of uninoculated pepper leaves (C). Each lane contains 25 μg protein. Lanes 'M' contain Mr. markers. The SDS polyacrylamide gel was stained with Coomassie brilliant blue. Arrows indicate the 32 kDa proteins in the gels.

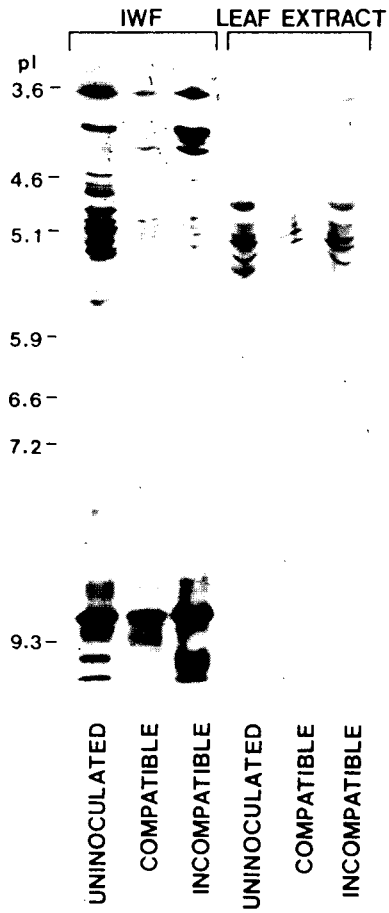

Fig. 6. Isoelectric focusing (IEF) of soluble protein in intercellular washing fluids (IWF) and extracts of pepper (cv. Hanbyul) leaves obtained 7day after inoculation with *Xanthomonas campestris* pv. *vesicatoria* strains Ds 1 (compatible) and 81-23 (incompatible) and of uninoculated pepper leaves (C). Each lane contains 20 μg protein. The IEF gel was stained with Serva blue W.

protein was in general higher in the incompatible interaction than in the compatible interaction. Three proteins of 65 kDa, 71 kDa, and 75 kDa disappeared gradually in pepper leaves 7 days after inoculation with the compatible strain Ds 1. An intense protein band of 20 kDa also appeared in the leaves 13 days after inoculation with the two strains. No significant differences in the other protein patterns were found between the uninoculated leaves, compatible or incompatible interactions.

Patterns of soluble proteins in the IWF and extracts of pepper leaves inoculated with the compatible strain Ds 1 and the incompatible strain 81~23 and of uninoculated pepper leaves after isoelectric focusing (IEF) are illustrated in Fig. 6. A number of proteins with pI in the range of 3.7~9.7 were separated in the IEF gel (Ampholine, pH 3.5~10. Sigma). In the IWF, significant differences in the levels of five proteins with pIs 3.7, 4.0, 4.2, 9.0, and 9.5 were found between the compatible and incompatible interactions. These proteins accumulated more greatly in the incompatible than in the uninoculated and compatible IWF. In the leaf extracts, there were no significant differences in protein patterns between these compatible and incompatible interactions. The two proteins of pIs 4.0 and 4.2 only occurred slightly more in the incompatible than in the uninoculated or compatible leaves.

DISCUSSION

Multiplication of bacteria in compatible and incompatible responses. Typically susceptible lesions were developed on pepper leaves inoculated with the compatible strain Ds 1 of *X. c.* pv. *vesicatoria.* The lesions appeared first water-soaked and then turned yellow with a chlorotic area. Severely affected leaves were dried and dropped. In contrast, the leaves inoculated with the incompatible strain 81-23 initially turned yellow and developed hypersensitive, local necrosis on the site of inoculation. Thereafter, the lesions did not enlarge any more, as previously observed by Stall and Cook (24) and Hibberd *et al.* (5). Multiplication of *X. c.* pv. *vesicatoria* in pepper leaves also were distinctly different between the two strains. In particular, the strain Ds 1 multiplied more greatly than the strain 81-23 in the infected leaves. The population of Ds 1 continuously increased by 14 days after inoculation, whereas in leaves inoculated with 81-23, the increase of bacterial population reached maximum by 8 days after inoculation and thereafter declined. Since the pepper leaves inoculated with 81-23 developed local necrosis at the onset of pathogenesis so as to provide conditions unfavorable for bacterial multiplication, the incompatible bacteria did not multiply actively without the continuous enlargement of the lesions (2, 14, 23, 24).

Hypersensitive reaction (HR) in plants to the pathogenic bacteria seems to be a general event involved in infection processes (10). A basic difference in reactions of hypersensitive and susceptible tissues

is that necrosis occurs much more rapidly in hypersensitive tissue. Interestingly, the multiplication of the incompatible strain 81-23 was greatly inhibited in the hypersensitive, necrotic leaf tissue. Since the Xanthomonad is a facultative saprophyte, it does not seem reasonable that the growth stop or decrease of the strain 81-23 in hypersensitive tissue may result from the altered food relationships after death of plant cell, as suggested by Klement and Goodman (10). The inhibition of bacterial multiplication in hypersensitive leaves may occur after release of bacterial inhibitors such as phytoalexins from hypersensitive plant cells. More recently, the possible involvement of pathogenesis-related (PR) proteins has been demonstrated in the formation of necrotic lesions characteristic of the HR in plant responses to plant pathogenic bacteria (3, 4, 6, 22). Some hydrolytic enzymes may become functional 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 to the surrounding host tissue.

Comparison of soluble proteins between the compatible and incompatible interactions. A *X. c.* pv. *vesicatoria* infection of pepper leaves induced the synthesis of soluble proteins, especially more greatly in the compatible than in the incompatible interactions. Such an accumulation of large amounts of proteins in the compatible interactions suggests that protein metabolism in the compatible leaves may be stimulated to favorably multiply *X. c.* pv. *vesicatoria.* In particular, the presence of high levels of soluble proteins in the intercellular fluid of pepper leaves infected with *X. c.* pv. *vesicatoria* could be explained by the availability of some proteins to colonize and multiply the bacteria in the intercellular space.

Our data of protein patterns on SDS-PAGE indicate that in pepper leaves some pathogenesis-related (PR) proteins could be induced upon inoculation with compatible or incompatible strains of *X. c.* pv. *vesicatoria.* Several new proteins were detected in IWF or extracts of pepper leaves infected by *X. c.* pv. *vesicatoria.* Some of these proteins, of which the 32 kDa protein was an example, were induced in pepper leaves by *X. c.* pv. *vesicatoria.* They could be candidates for PR proteins which have a function specific to host-pathogen interaction. In particular, the 32 kDa protein on SDS polyacrylamide gels appeared intensely in the incompatible interaction. The protein was ascertained as a chitinase on the renaturated gel or by an affinitiy chromatography on a regenerated chitin column (15). In contrast, some proteins with molecular masses of 65, 71, and 75 kDa disappeared from pepper leaves by *X. c.* pv. *vesicatoria* infection especially in the compatible interaction, indicating their possible degradation during pathogen attack. In rice plants infected by *X. oryzae.* pv. *oryzae,* Kim and Yoo (7) demonstrated recently that ten proteins increased in response to infection, one protein decreased during infection, and ten new proteins were induced by infection.

Isoelectric focusing of soluble proteins in the IWF and extracts of pepper leaves showed significantly differences in the levels of some proteins between the compatible and incompatible interactions. In particular, the accumulation of proteins with pIs 3.7, 4.0, 4.2, 9.0 and 9.5 in the IWF of infected leaves was more pronounced in the incompatible than in the compatible interaction. These data suggest that the synthesis of some extremely acidic and basic proteins were remarkably induced in the IWF from the incompatible interaction.

From the SDS-PAGE and IEF, we could observe that acidic and basic proteins with low molecular masses, so called PR-proteins, were induced in pepper leaves following infection by *X. c.* pv. *vesicatoria.* The PR proteins which accumulate in the pathogen-infected plant tissue have been considered to be encoded and synthesized by the host, but not by the pathogen (27). Some of these PR-proteins may function in the disease expression of *X. c.* pv. *vesicatoria* in pepper plants, whereas other PR-proteins may also play a significant role in the induction of a defense against the disease. Further detailed study will be done to elucidate whether or not new proteins occurring in the *X. c.* pv. *vesicatoria*-infected pepper leaves are PR-proteins such as β-1, 3-glucanases and chitinases, and what are their biological functions in the compatible and incompatible interactions of pepper leaves infected by *X. c.* pv. *vesicatoria.*

요 약

고추나 토마토 등에 더뎅이 병을 일으키는 *Xantho-*

monas campestris pv. *vesicatoria*의 친화적 균주 Ds 1을 접종한 고추 잎에서는 전형적인 감수성 병반이 형성되었다. 이것은 처음에 수침상 병반을 나타내다가 시간이 경과함에 따라 황화 병반을 형성하였다. 그러나, 불친화적 균주인 81-23을 접종한 잎에서는 빠르게 조직의 국부적인 괴저가 일어나는 과민성 반응이 나타났다. 또한 고추 잎에서 *X. c.* pv. *vesicatoria*의 증식 역시 두 균주간에 큰 차이를 보여서, 불친화적 균주에 감염된 잎에서보다 친화적 균주에 감염된 잎에서 세균이 훨씬 많이 증식하였다. 고추 잎에서 *X. c.* pv. *vesicatoria*의 감염은 수용성 단백질의 합성을 유도하며 불친화적 상호작용에 비해 친화적 상호작용에서 훨씬 많이 합성되었다. 몇몇 병발생관련단백질(PR-protein)이 감염된 고추 잎의 세포간극 세척액(IWF)과 잎 추출액에서 발견되었다. 특히 SDS-PAGE 젤 상에서 보여지는 32 kDa의 분자량을 갖는 단백질은 불친화적 상호작용에서 강하게 나타났다. Isoelectric focusing을 행하여 감염된 고추잎에 있는 수용성 단백질의 등전점을 알 수 있었다. 또한 감염된 식물 잎의 IWF에 pI 3.7, pI 4.0, pI 4.2, pI 9.0, pI 9.5의 단백질이 친화적 상호작용에서 보다 불친화적 상호반응에서 뚜렷하게 축적되었다. 이러한 실험결과는 강산성 및 강염기성 단백질이 감염된 고추잎의 세포간극에 유도, 축적됨을 시사해 주고 있다.

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