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Molecular Approaches to Evaluate the Role of Some Genes Required for Plant Pathogenicity of *Xanthomonas campestris* pv. *campestris*

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Xanthomonas campestris pv. *campestris*의 병원성 관련 형질 탐색에 관한 연구

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ABSTRACT : *Xanthomonas campestris* pv. *campestris*, causal agent of Black rot of crucifers, were isolated and identified from crucifer host. In order to determine the characters of *X. c.* pv. *campestris* associated with pathogenicity, Tn5 mutagenesis was carried out by conjugating with *E. coli* pJB4J1. Transconjugants were plate-assayed for missing cellulase, protease and amylase activity. A cellulase negative mutant was selected and tested for pathogenicity. Light microscopy and Scanning electron microscopy revealed that substomatal tissues were colonized by mutant, but was far less extensive than those by wild type. Stomatal surface and substomatal tissue appeared to have degraded by only wild type in 24 hrs and progression of pathogenesis was distinct in 48 hrs. In 6 days, wild type proliferated well in the tissue facilitated by cellulase activity. As a result, cellulase was determined as the important factor in pathogenesis.

***Key words* : *Xanthomonas campestris* pv. *campestris*, Tn5 mutagenesis, Cellulase negative mutant**

Xanthomonas campestris pv. *campestris* (Pammel, Dowson) Dye is the causal bacterium of Black rot disease of crucifers. The typical large V-shaped chlorotic lesions on the leaf of adult hosts, originating from the point of bacterial entry, are suggestive of number of extracellular enzyme activities being involved during the pathogenesis. This pathogen is seedborne and invades the vascular tissue of leaves, stalks, stumps and roots through hydathodes and wound. However, according to the Sherf & Macnab (12), stomatal infections are common for young seedlings. Later inoculations take place through hydathodes along leaf margins, through insect feeding injuries, and in very susceptible crops such as cauliflower, directly through stomata. Infection on the xylem vessels of succulent radish root accompanies blackening of xylems, followed by deterioration of inner root tissues until the black cavity develops. This was further supported by the epidemiological observation that unseasonally warm weather during growth period in autumn are conducive to epidemic in Korea. Recently, Daniel's group (2, 3, 6, 14) have been working on the role of extracellular enzyme in pathogenicity of this pathogen. We have attempted to isolate and identify *Xanthomonas campestris* pv. *campestris* from diseased crucifer in Korea and to establish the role of individual extracellular enzymes associated with plant pathogenicity.

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MATERIALS AND METHODS

Isolation and identification of *Xanthomonas campestris* pv. *campestris*. Periodically collected sample taken from diseased crucifer were dillution-plated on YDC media (Yeast extract, 10 g; Dextrose, 20 g; $CaCO_3$, 20 g; Bactoagar, 15 g per liter distilled water)

and incubated at 30°C for 3 days. Only virulent isolates were selected for further standard identification procedure.

Pathogenicity test. The pathogenicity of *Xanthomonas campestris* pv. *campestris* was tested on several crucifers including Chinese cabbage and radish. Plants were grown for 4 to 6 weeks in a growth chamber prior to inoculation. The overnight culture of bacteria upto 10^8 cfu/m*l* in nutrient broth at 30°C was used as an inoculum and sprayed on each plant with spray bottle. The spray-inoculated plants were kept at high humidity covered with clear plastic dome for one day and transfered and kept uncovered in greenhouse. The plants showed progression of symptoms over a period of 4 to 5 days.

Mutagenesis of *Xanthomonas campestris* pv. *campestris*. The *E. coli* pJB4J1 and *Xanthomonas campestris* pv. *campestris* (Rif^r) grown to log-phase were pelleted by centrifugation (3,500 rpm, 20 min) and suspended in 1 m*l* of 0.85% NaCl. The cells of *E. coli* pJB4J1 and *Xanthomonas campestris* pv. *campestris* were mixed in equal proportion and pelleted by centrifugation (3,500 rpm, 20 min). The mixture resuspended in 1 m*l* of sterile water was spread onto a sterile membrane filter (Millipore 0.45 μm pore size) on LB plate (Bactotryptone, 10 g; Yeast extract, 5 g; Sodium chloride, 10 g; Bactoagar, 15 g per liter distilled water) and incubated for overnight at 30°C. And 50 μ*l* portions of cells suspended in 1 m*l* of LB broth spread on LB agar supplemented with Kanamycin (10 mg/m*l*) and Rifampicin (10 mg/m*l*).

Screening of transconjugants for enzyme activity. Several thousand *Xanthomonas campestris* pv. *campestris* transconjugants were screened by indicator media for protease (Bactopeptone, 5 g; Skimmed milk, 10 g; Bactoagar, 15 g per liter distilled water), amylase (K_2HPO_4, 0.7 g; KH_2PO_4, 0.2 g; $(NH_4)_2SO_4$, 1 g; $FeSO_4$ $7H_2O$, 0.01 g; $MnCl_2$, 0.1 g; Tryptone, 0.125%; Yeast extract, 0.125%; Starch, 0.5%; Bactoagar, 15 g per liter distilled water) and cellulase (Tryptone, 5 g; Yeast extract, 5 g; $CaCl_2$ $2H_2O$, 1 g; Carboxymethylcellulose, 5 g; Bactoagar, 15 g per liter distilled water) according to the Kluepfel (8).

Semithin Light Microscopy. Sample were fixed in 0.05 M potassium phosphate (pH 7.0) containing 3% glutaraldehyde for 90 min or overnight at 4°C. After rinsing with the distilled water, the leaf blades were fixed in 1% OsO_4 (Osmium tetraoxide, Sigma, USA) in 0.05 M phosphate buffer (pH 7.0) for at room temperature. The specimens were washed with distilled water, subjected to serial dehydration with ethanol gradient (50, 60, 70, 80, 90, 100%) and embedded in Epon 812. The specimen blocks trimmed to an appropriate shapes were semithin-sectioned to 0.3 μm thick with Ultramicrotome (Reichert Jung, Ultra cut), stained with toluidine blue and observed with a light microscope at 400× and 1000× magnification.

Scanning Electron Microscopy (SEM). The scanning electron microscopy (JSM-6400. Jeol, Japan) was used to investigate the infected tissue by cellulase negative mutant vs. wild type. The healthy and/or infected tissues were sampled with a razor blade in 12 hrs, 24 hrs, 48 hrs, 4 days, 6 days after spraying bacterial suspension (10^8 cfu/m*l*). Leaf blade samples were fixed in 3% glutaraldehyde (pH 7.5) in 0.05 M phosphate buffer (pH 7.0) for 90 min at 4°C. And the specimens were fixed 1% OsO_4 (Osmium tetraoxide, Sigma, USA) in 0.05 M phosphate buffer (pH 7.0) for at room temperature. The specimens were rinsed carefully three times with distilled water, dehydrated through a series of ethanol gradient (50, 60, 70, 80, 90 and 100%) and 100% Isoamylacetate twice. The dried specimen were subjected to the gold coating and observed with scanning electron microscopy in 2000× or 5000×.

RESULT

Identification of pathogenic bacteria. The bacteria isolated from Chinese cabbage was yellow colonies on yeast extract-dextrose-calcium carbonate (YDC) agar and, gram negative and aerobic growth on Miller-Schroth (MS) agar, negative in fluorescent pigment on King's B media. Currently, five species of *Xanthomonas* are known and these could be easily differentiated. *Xanthomonas campestris* could be differentiated from other four species by growth at 36°C, protein digestion, and also by utilization of arabinose, cellobiose and galactose (Table 1). Pathovar of this bacterium was identified as *campestris* based on the growth on semiselective agar media, BSCAA or SX media (Table 1).

Pathogenicity test. The bacteria grown at 30°C was suspended in distilled water at a concentration of about 10^8 cfu/m*l* and used as the inoculum. This pathogen was highly virulent on Kale, Cabbage and Chinese cabbage. Radish was rather moderately susceptible

Table 1. Identification of *Xanthomonas campestris* pv. *campestris*

Tests	*X. c. c.* No.1	*X. c. c.* No.2	*X. c. c.* No.3	Bergey's manual
Yellow colonies on YDC media	+	+	+	+
Growth on MS media	−	−	−	−
Fluorescent pigment on KB	−	−	−	−
Anaerobic growth	−	−	−	−
Gram reaction	−	−	−	−
Growth at 36°C	+	+	+	+
Protein digestion	+	+	+	+
Acid from : Arabinose	+	+	+	+
Cellobiose	+	+	+	+
Galactose	+	+	+	+
Glucose	+	+	+	+
Mucoid growth on NA+5% glucose	+	+	+	+
Growth on : BSCAA media	+	+		
SX media	+	+		

YDC : Yeast extract-dextrose-calcium carbonate Agar, MS : Miller – Schroth medium, KB : King's B medium, NA : Nutrient Agar, BSCAA : Semi-selective media especially useful for isolating *X. c.* pv. *campestris*, SX : Semi-selective and several other starch positive for plant tissues and soil

(Table 2).

Screening of transconjugant. One cellulase negative transconjugant was obtained from seven thousands Tn5 mutants by replica plating on 1% carboxymethylcellulose plate, which were incubated at 30° C for four days, stained with 0.1% Congo Red for 30 min washed with distilled water (Fig. 1).

Light Microscopy and Scanning Electron Microscopy. Light microscopy and Scanning electron microscopy (SEM) revealed bacterial cells of the wild type were present in the stomatal and substomatal cell in 12 hrs and 24 hrs and these bacteria were getting numerous in 48 hrs, but few bacterial cell of cellulase negative mutant were detected (Fig. 2, Fig. 3). Such a difference in bacterial colonization precedes the macroscopic symptom development. Stomatal surface and substomatal tissue appeared to have been degraded by only wild type *Xanthomonas campestris* pv. *campestris* in 24 hrs and progression of pathogenesis was distinct in 48 hrs. In 6 days, wild type pathogen proliferated well in tissue facilitated by cellulase activity (Fig. 4).

Table 2. Pathogenicity of *Xanthomonas campestris* pv. *campestris* isolated from diseased Chinese cabbage to several crucifers

Plant		Pathogenicity		
Common name	Scientific name	*X. c. c.* No.1	*X. c. c.* No.2	*X. c. c.* No.3
Kale	*Brassica oleracea* var. *acephala*	+++	+++	+++
Cabbage	*Brassica oleracea* var. *capitata*	+++	+++	+++
Chinese cabbage	*Brassica campestris* subsp. *napus* var. *pekinensis*	+++	+++	+++
Radish	*Raphanus sativus* var. *hortensis* for *acanthiformis*	++	++	++

+++ : very susceptible, ++ : moderately susceptible

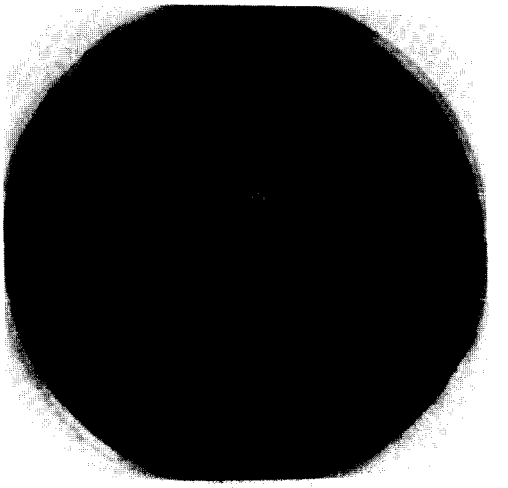

Fig. 1. Selection of cellulase – negative by Tn5 mutagenesis on Shin's media containing carboxymethycellulose (1%). Incubated plates for 4 days at 30°C were stained with Congo Red (0.1%) for 30 min and washed 1 N HCl or 1 N NaOH.

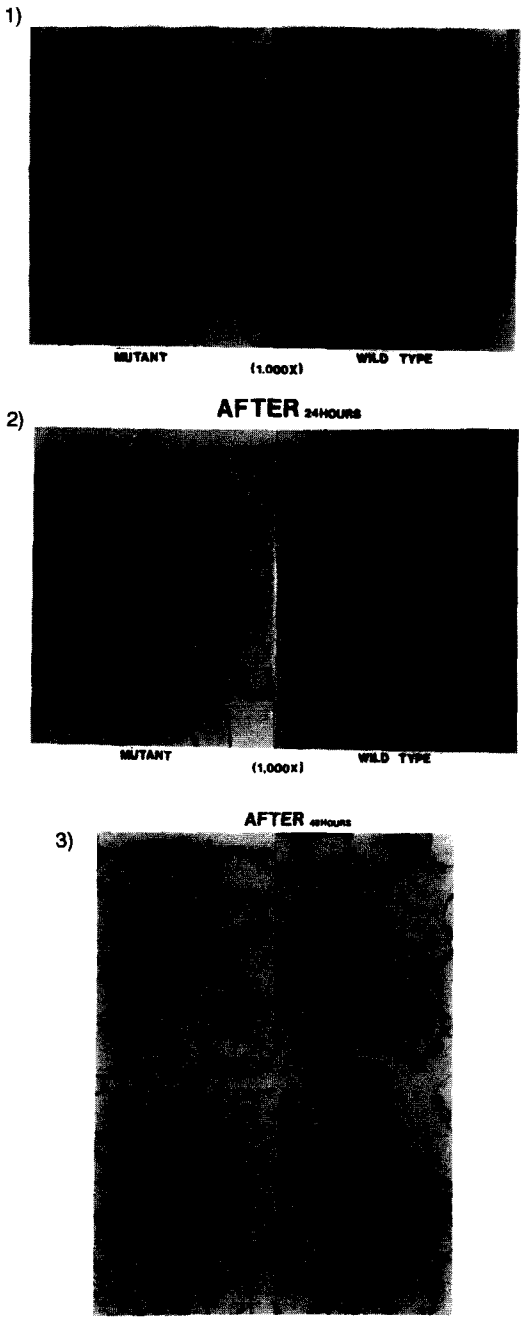

Fig. 2. Light microscopy of Chinese cabbage infected tissue by wild type vs. cellulase negative mutant of *Xanthomonas campestris* pv. *campestris*. 1) After 12 hrs 2) After 24 hrs 3) After 48 hrs, A and B; Stomatal tissues infected by mutant and wild type respectively (1000×), C; Subjacent tissue below the substomal tissue of the wild type (400×), D; Close-up view of subjacent tissue (C) under high magnification (1000×).

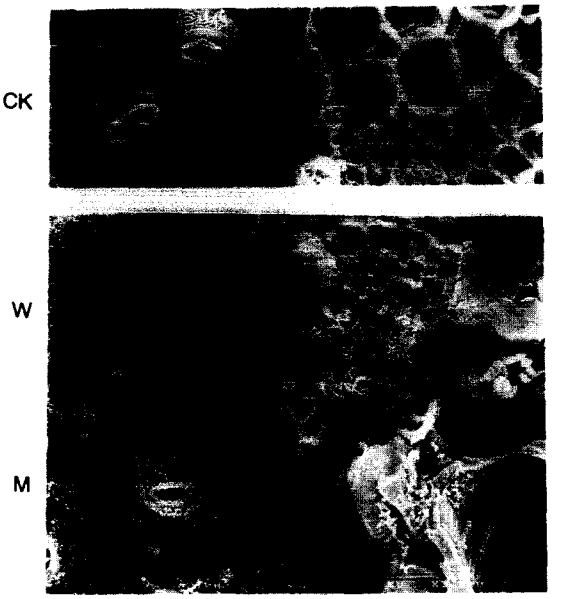

Fig. 3. Scanning Electron Microscopy of healthy and infected tissue by wild type vs. cellulase negative mutant of *Xanthomonas campestris* pv. *campestris* 12 hours after inoculation. Stomata and substomatal tissue infected with wild type (W), mutant (M) and untreated (CK).

This result suggested that the cellulase gene help the plant pathogenicity.

DISCUSSION

Currently, comprehensive way of molecular genetic techniques is available for gram-negative bacterial pathogen. Such approaches clearly have much to offer for the understanding the complex phenomena of pathogenesis. Recent development in molecular genetic has made it possible for researchers with only limited experience in this field to successfully employ a variety of new approaches and techniques to clone genes and characterize their products (9, 11). Bacterial plant pathogens produce a range of extracellular enzymes which may be associated with symptom development and pathogenesis. The role in pathogenesis of enzyme produced by *Xanthomonas* has not been extensively worked out compared to *Erwinia* and *Pseudomonas*. Daniel's group reported that the broad host-range cosmid pLAFR1 could be used as a gene cloning vector in *Xanthomonas campestris* pv. *campestris*. An advantage of broad host-range vector is that genes may be cloned in a convenient host such as *E. coli* and

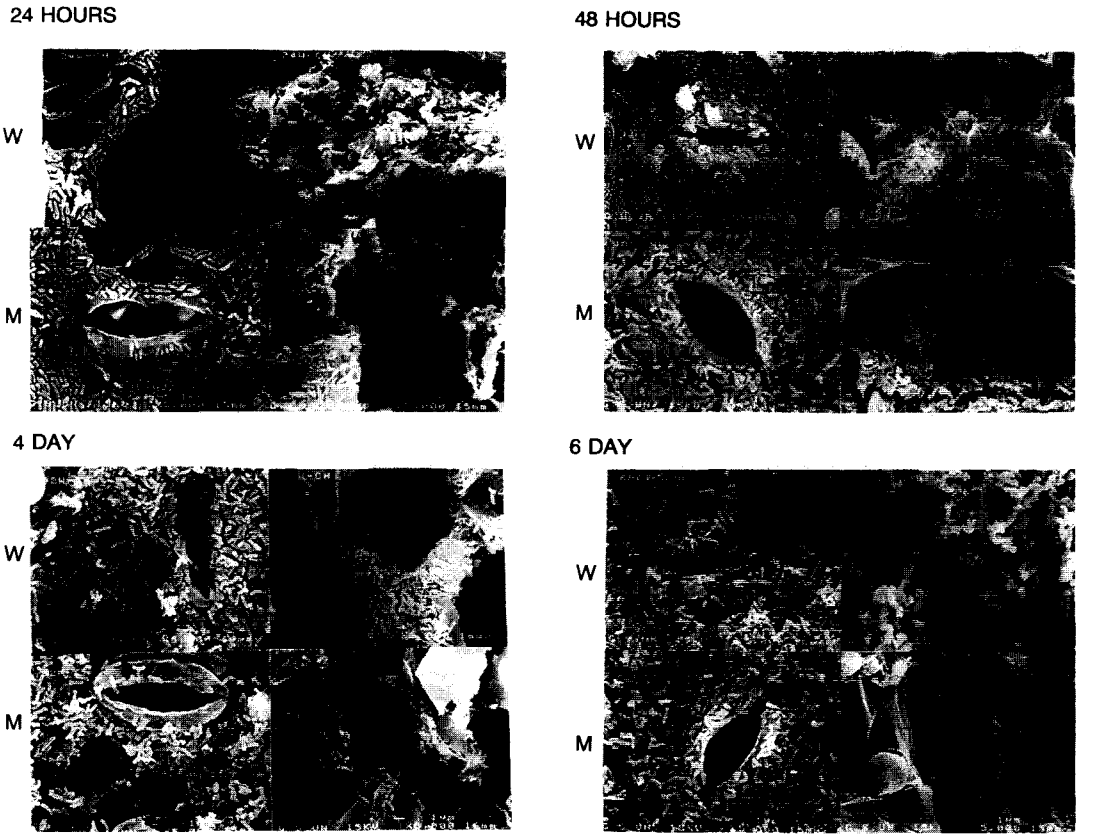

Fig. 4. Scanning Electron Microscopy of infected tissue by wild type (W) vs. cellulase negative mutant of *Xanthomonas campestris* pv. *campestris* (M) observed at 24 hr, 48 hr, 4 day, 6 day after inoculation.

therefore be accessible to molecular genetical techniques developed for that organism, at the same time the cloned genes may be transferred into original host species for functional tests (2-5, 15).

Non-pathogenic mutant 8237 of *Xanthomonas campestris* pv. *campestris* was found to be defective in producing protease, and polygalacturonate lyase (4). Tang *et al.* (14) showed that the protease is not critically important for the pathogenicity of *Xanthomonas campestris* pv. *campestris* on turnip plants and may play a minor role in disease development. Sawczyc *et al.* (10) observed that *Xanthomonas campestris* pv. *translucens* clone restored the pathogenicity of mutant to turnips and production of protease by mutant 8237 (11). Dow *et al.* (6) suggested that isozyme I of polygalacturonate lyase, gene of which was cloned by expression in non-pectolytic *Xanthomonas campestris* pv. *translucens* is not absolutely necessary for black rot pathogenesis indicating a redundancy amongst the three major isozymes resolved by ion-exchange chromatography. Bae (1) reported extracellular α-amylase, protease, and cellulase by *Xanthomonas campestris* pv. *campestris*. Hu *et al.* (7) characterized the gene required for secretion of extracellular enzymes across the outer membranes of non-pathogenic mutant of *Xanthomonas campestris* pv. *campestris*. Yun *et al.* (16) suggested the cellulase gene might play a important role in *Xanthomonas campestris* pv. *oryzae*-rice interaction. Therefore, it is very complicated or even puzzled to interpret the results of previous workers regarding the presumed involvement of enzyme activities of *Xanthomonas campestris* pv. *campestris* in pathogenesis. The genes identified by their involvement in the synthesis of known determinants of pathogenicity probably represent only a fraction of the set required for full pathogenicity.

Through microscopic examination of inoculated tissues, we evaluated the role of cellulase genes in the pathogenicity by comparing the cellulase negative mu-

tant with wild type *Xanthomonas campestris* pv. *campestris* for difference in infection through stomata, migration and proliferation through substomatal tissues and also macroscopic symptom development. Cellulase negative mutant certainly did migrate through substomatal tissue after invading via stomata but less proliferated than the wild type did. One may claim that stomata are not the natural route of infection for *Xanthomonas campestris* pv. *campestris*. However, that is the case with later infection. That is, the seed-borne inocula on the newly emerge seedlingly pass from cotyledon to young leaves either directly or through stigmata. Particularly for very susceptible crop, cauliflower, invasion always occurs directly through stomata (12).

We have provided substantial evidences that this bacteria invade the Chinase cabbage through stomata and the role of cellulase activities in the ongoing process of pathogenesis, even though further works along this line are still necessary to verify this result. Pathogenicity genes whose functions appear to be essential for the sustained growth of the pathogen and expression of disease symptom in a susceptible host are also of considerable interest. Molecular work is in progress to characterize cel gene according to the strategy of Staskawicz *et al.* (13).

요 약

십자화과 작물에 발생하는 검은썩음병(Black rot or Black vein of crucifer)의 병원성 세균인 *Xanthomonas campestris* pv. *campestris*를 분리, 동정하고 병원성을 검정하였다. 이 *X. c.* pv. *campestris*는 3가지 종의 Chinese cabbage에 병원성을 나타내었고, 병원성과 관련된 특성을 결정하기 위하여 Tn5 mutagenesis를 실시 cellulase negative mutant를 선발하여 병원성 검정하였다. 선발된 cellulase negative mutant를 배추에 분무 접종하여 광학 현미경과 전자현미경으로 관찰한 결과 cellulase negative mutant는 wild type와 함께 기공표면과 기공하부조직에서 정착하였지만 그 밀도는 낮았다. 반면 접종 24시간 이후 wild type은 기공표면과 기공하부조직이 lysis되기 시작하여 48시간 이후에는 병원성의 진전으로 보다 많이 lysis되었다. 6일 후, wild type은 cellulase활성에 의해 식물체 조직에서 높은 증식력을 보이며 조직을 lysis 시키고 또한 조직 깊숙이 침입, 정착하는 것을 관찰하였다. 이 결과로 *X. c.* pv. *campestris*의 cellulase는 병원성에 관여하는 중요한 요인으로 생각된다.

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