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Genetic Differentiation of Strains of *Xanthomonas campestris* pv. *vesicatoria* by Random Amplified Polymorphic DNA (RAPD)

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Random Amplified Polymorphic DNA(RAPD)를 이용한 고추 더뎅이병균 균주의 유전적 분류

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ABSTRACT : Genetic diversity of forty-four strains of *Xanthomonas campestris* pv. *vesicatoria* from diverse geographic origins was investigated using random amplified polymorphic DNA (RAPD) of genomic DNA. One hundred and thirty-seven amplified fragments were produced by polymerase chain reaction with a set of 14 random primers, and the sizes of amplified DNA fragments ranged approximately from 0.3 to 3.2 kb. Cluster analysis of genetic similarity among the strains generated the dendrogram that clearly separated all strains from each other. The 44 strains of *X. campestris* pv. *vesicatoria* were classified into 4 major genomic DNA RAPD groups and 15 subgroups at the genetic similarity of 0.60 and 0.92, respectively. The strains from foreign countries formed discrete subgroups, but the United States strain 87-77 clustered closely with some of Korean strains together. Thirty-nine Korean strains were classified into 11 subgroups, and especially Masan strain Ms93-1 clustered distinctly far from the other Korean strains. RAPD polymorphism suggests strongly the occurrence of genetic differentiation of *X. campestris* pv. *vesicatoria* and the existence of genetically distinctive subgroups among the populations in Korea.

***Key words* : *Xanthomonas campestris* pv. *vesicatoria*, pepper, genetic diversity, random amplified polymorphic DNA (RAPD)**

Xanthomonas campestris, which is the representative species of *Xanthomonas* causing several diseases in plants, comprises at least 125 different pathovars that are distinguished by the diseases they cause (26). Traditional methods for the identification of such a differentiated pathovar have usually depended on a series of tests on their biochemical, serological, and pathological characteristics. The limit of genetically well defined genetic markers has hindered understanding of genetic variability of xanthomonads, but recently several nucleic acid-based techniques were developed to investigate genetic variations. DNA restriction fragment length polymorphism (RFLP) has been most commonly used to differentiate pathovars (13, 14), to identify specific strains (6), and to analyze genetic variation in the populations of xanthomonads including *X. campestris* pv. *vesicatoria* (2, 5, 7, 10). The length po-

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lymorphism can arise from base changes due to insertion, deletion, substitution, or reversions either at or between the restriction sites. The amplification of specific regions of the bacterial genome by the polymerase chain reaction (PCR) may be a sensitive technique in detecting the length polymorphisms without preliminary work, such as isolation of cloned DNA probes, preparation of filters for Southern hybridization, or nucleotide sequencing necessary in RFLP technique (29, 30). This amplification technique or random amplified polymorphic DNA (RAPD) can generate specific DNA fragments useful for genetic mapping, identification of isolates, and application in molecular ecology. For plant pathogenic xanthomonads, RAPD has provided useful markers for identification of species or pathovars and investigation of intraspecific genetic variation (8, 15, 16, 17).

In the present study, we investigated genetic differentiation of genomic DNA in *X. campestris* pv. *vesicatoria* in the strains from the intensively pepper-growing areas in Korea using RAPD, and compared DNA polymorphisms of the Korean strains with those from diverse geographic origins worldwide.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The sources and origins of 44 strains of *X. campestris* pv. *vesicatoria* used in this study are shown in Table 1. Korean strains were isolated in 1991 and 1992 from infected pepper plants growing in various pepper-growing areas of Korea, identified as *X. campestris* pv. *vesicatoria*, and confirmed to be pathogenic on pepper leaves (26). Isolation of the bacteria was carried out on a semiselective Tween medium (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, 0.4 mg tobramycin in 1000 ml deionized water) (19). Other strains were provided by R. E. Stall, Department of Plant Pathology, University of Florida, Gainesville, FL, USA.

All strains of *X. campestris* pv. *vesicatoria* were maintained routinely on yeast-nutrient agar (5 g yeast extract, 8 g nutrient broth, 15 g agar, 1000 ml deionized water) at 28°C, *Escherichia coli* NM522 was maintained on LB (Luria-Bertani) agar at 37°C. All bacteria were also stored for long periods as turbid suspensions in 15% glycerol at − 70°C.

Extraction of genomic DNA and purification. Genomic DNA was extracted from *X. campestris* pv. *vesicatoria* by a modification of alkaline lysis extraction procedure (24). *X. campestris* pv. *vesicatoria* was cultured in 5 ml yeast-nutrient broth for 12 h at

Table 1. Strains of *Xanthomonas campestris* pv. *vesicatoria* used in this study

Code	Strains	Geographic origin
1	87-48	Taiwan
2	87-77	U.S.A.
3	89-8	Australia
4	79-2	Argentina
5	Bv5-4a	Argentina
6	Ds7	Dukso, Korea
7	Ds10	Dukso, Korea
8	Ds13	Dukso, Korea
9	Ds16	Dukso, Korea
10	Ds17	Dukso, Korea
11	Kc3	Kochang, Korea
12	Kc14	Kochang, Korea
13	Kc15	Kochang, Korea
14	Kc23	Kochang, Korea
15	Kc2	Kochang, Korea
16	Kc25	Kochang, Korea
17	Hs1	Hoengsung, Korea
18	Ic1	Inchun, Korea
19	Ky1	Koyang, Korea
20	Yd1	Youngduk, Korea
21	Uj1	Uijeongbu, Korea
22	Ms93-1	Masan, Korea
23	Bs5	Pusan, Korea
24	Cr93-1	Changryung, Korea
25	Cj1	Chungju, Korea
26	Hs93-5	Hwasong, Korea
27	Kj93-1	Kimje, Korea
28	Ds2	Dukso, Korea
29	Ds5	Dukso, Korea
30	Ds8	Dukso, Korea
31	Ds9	Dukso, Korea
32	Ds14	Dukso, Korea
33	Ds18	Dukso, Korea
34	Kc1	Kochang, Korea
35	Kc6	Kochang, Korea
36	Kc7	Kochang, Korea
37	Kc8	Kochang, Korea
38	Kc9	Kochang, Korea
39	Kc17	Kochang, Korea
40	Mw4	Miwon, Korea
41	Sw4	Suwon, Korea
42	As5	Ansung, Korea
43	Ad93-2	Andong, Korea
44	Jc93-5	Jinchon, Korea

28°C, and then harvested by centrifugation for 5 min at 3,500 rpm. Lysis of bacterial cell pellets harvested was performed at room temperature for 20 min in a mixture of 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 2 mg/ml freshly mixed lysozyme (total volume, 3.3 ml). To each DNA sample, sodium dodecyl sulfate (SDS; 334 μl of a 10% solution) was added. After incubation for 10 min at 50°C, 268 μl of RNase A (2.5 mg/ml in 10 mM Tris-HCl, pH 7.6) was added, and the mixture was incubated at 37°C for 1 hr. Then, 340 μl of 0.5 M EDTA was added, and the mixture was incubated for 10 min at 50°C. Pronase (140 μl of a 10 mg/ml solution in 10 mM Tris-HCl, pH 7.6) was added, and the mixture was incubated for 6 hr at 37°C. Each sample was then extracted with one volume of phenol saturated with 0.1 M Tris-HCl (pH 8.0), one volume of a 1 : 1 phenol and chloroform-isoamyl alcohol (24 : 1, v/v) mixture, and one volume of chloroform-isoamyl alcohol. The DNA was precipitated with 1/10 volume of 3 M sodium acetate and two equal volumes of 99.9% ethanol. After drying, the pellet was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C.

DNA amplification. Ten base oligonucleotide primers used were obtained from Korea Biotec Inc. and kits A and B, Operon Technologies Inc. and twelve-base oligonucleotide primers were obtained from kits A and C, Wako Inc. (Table 2). PCR amplifications were carried out by the modified method of Williams *et al.* (30) in 25 μl volumes of reaction mixture in 0.2 ml thinwall tube. Reaction mixture contained; 30 ng of genomic DNA, 18 ng of primer, 0.6 U of *Taq* DNA polymerase (Korea Biotec Inc.), 2 μl 10× reaction buffer, and 10 mM dNTP (dCTP, dGTP, dATP, dTTP). Forty five amplification cycles were performed in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, Conn., U.S.A.) according to the following programs : 15 s of denaturation at 94°C, 30 s of annealing at 45°C, and 45 s of extension at 72°C; the initial denaturation step was 2 min at 94°C, and the last extension step was extended to 5 min. The PCR products were electrophoresed in 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) according to standard procedure (24). The gels were stained with 0.5 μg of ethidium bromide per ml for 40 min and then destained in 1 mM $MgSO_4$ for 1 h and photographed over a UV transilluminator with type 55 Polaroid film.

RAPD data analysis. RAPD patterns of genomic DNAs with each of the random primers used were compared for the strains of *X. campestris* pv. *vesicatoria*. Presence (coded 1) or absence (coded 0) of each fragment was recorded. A dendrogram was constructed based on the binomial data matrix of RAPD analysis, using an unweighted pair group method with an arithmetic average (UPGMA) option in the Numerical Taxonomy System for Personal Computer (NTSYS-pc), version 1.80 (22).

Table 2. Nucleotide sequence of the 14 primers, total number of amplified DNA fragments, and number of polymorphic DNA fragments produced by each primer

Code	Nucleotide sequence (5'→3')	Amplified fragments	Polymorphic fragments	Remark
323	GAC ATC TCG C	8	6	Korea Biotec., Inc.
350	TGA CGC GCT C	11	7	Korea Biotec., Inc.
353	TGG GCT CGC T	11	9	Korea Biotec., Inc.
OPB-01	GTT TCG CTC C	10	6	Operon Tch., Inc.
OPB-04	GGA CTG GAG T	9	7	Operon Tch., Inc.
OPB-07	GGT GAC GCA G	8	6	Operon Tch., Inc.
A-22	GCC TGC CTC ACG	10	5	Wako Co.
A-25	CTC AGC GAT ACG	10	6	Wako Co.
A-28	ATT TGG ATA GGG	10	8	Wako Co.
A-29	GGT TCG GGA ATG	15	12	Wako Co.
A-32	TTG CCG GGA CCA	10	7	Wako Co.
C-43	GGC GGC ACA GGA	12	10	Wako Co.
C-44	CGC AGC CGA GAT	9	5	Wako Co.
C-64	GAG CTC CCG ACA	4	3	Wako Co.
Total		137	97	

RESULTS

RAPD patterns of genomic DNA. The 14 primers which gave clear reproducible bands with genomic DNAs of the strains of *X. campestris* pv. *vesicatoria* were selected for comparative analyses of RAPD patterns. Concentration of DNA template, primer, and dNTP was determined in preliminary trials to get unambiguous amplification patterns. The profiles were reproducible from one experiment to another, with newly extracted DNA from the same culture and from newly cultivated mycelia. All the primers used revealed polymorphisms useful for classifying strains of *X. campestris* pv. *vesicatoria*. One hundred and thirty-seven amplified fragments were produced with 14 primers and the sizes of amplified DNA fragments ranged from approximately 0.3 to 3.2 kb (Table 2 and Fig. 1). Ninety-seven out of the 137 bands were polymorphic. Fig. 1 shows PCR products generated with representative 7 primers; 350, OPB-01, OPB-07, A25, A28, C43, C64, exhibiting varying degrees of polymorphism among strains of *X. campestris* pv. *vesicatoria*.

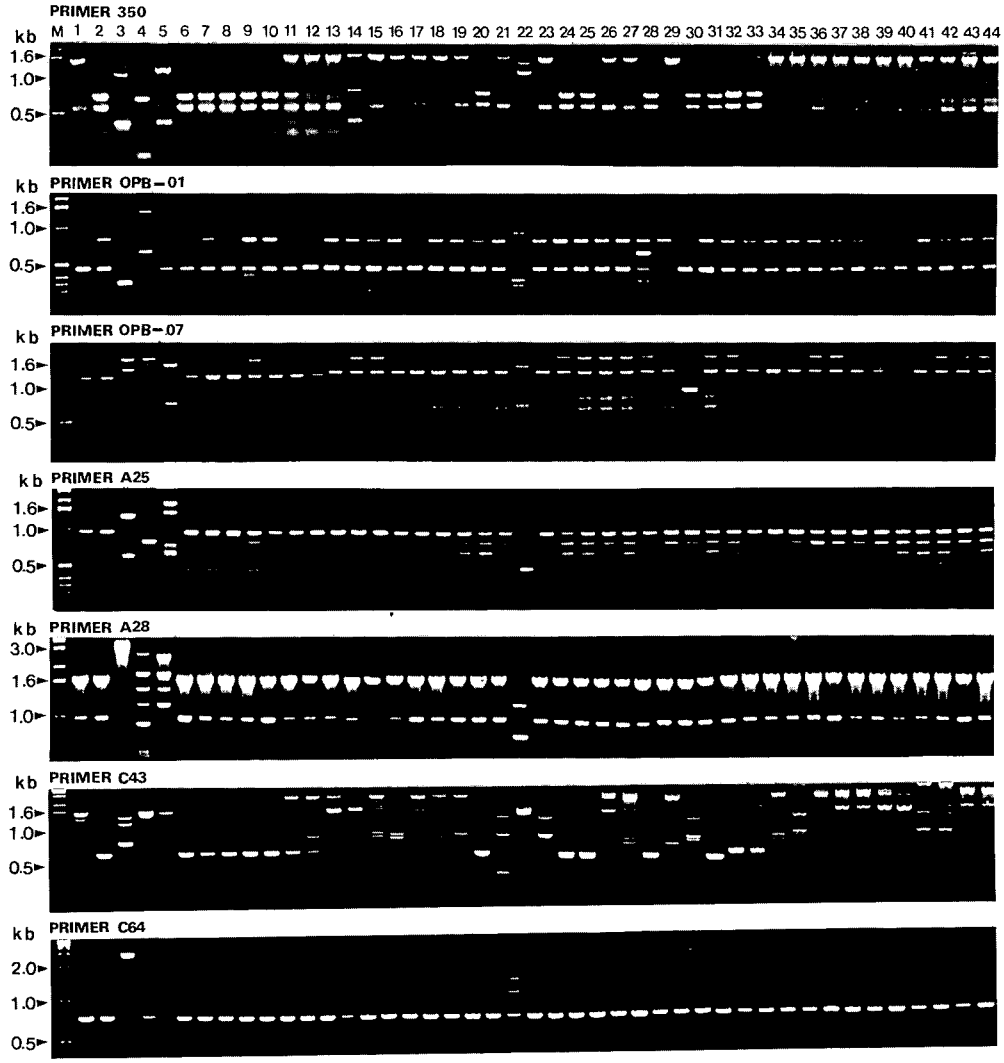

Fig. 1. Random amplified DNA polymorphisms of the 44 strains of *Xanthomonas campestris* pv. *vesicatoria* generated with 7 representative primers. Lanes marked 1 to 44 are strains listed in Table 1. M indicates molecular markers of 1 kb DNA ladder.

Cluster analysis. By combining the results using 14 primers, 137 band positions were scored for presence versus absence (1/0) for all isolates tested. The combined data from 137 RAPD bands were analyzed by a cluster analysis using UPGMA in NTSYS-pc to produce a dendrogram (Fig. 2). At the similarity level of 0.60, four major genomic DNA RAPD groups were differentiated among the 44 strains. RAPD group A included Taiwanese strain 87-48, the United States strain 87-77 and all Korean strains except Ms93-1, and RAPD group B included one strain Ms93-1 collected at Masan in Korea. RAPD group C included Argentine strains 79-2 and Bv5-4a, and RAPD group D included Australian strain 89-8.

The 44 strains of *X. campestris* pv. *vesicatoria* were classified into 15 genomic DNA RAPD subgroups at the similarity level of 0.92 (Fig. 2). The strains from foreign countries formed discrete genomic DNA RAPD subgroups. Thirty-nine Korean strains were classified into 11 subgroups. All Korean strains (RAPD subgroups II~XI) except Ms93-1 (RAPD subgroup XII) fell into a narrow cluster at the similarity level of 0.84, distinctly separated from the foreign strains except the United States strain 87-77 and Taiwanese strain 87-48.

DISCUSSION

We investigated intraspecific genetic variation in the 44 strains of *X. campestris* pv. *vesicatoria* from diverse geographic origins using plasmid and genomic DNA RFLP in the previous studies (2, 10). Korean strains were separated from the other foreign strains and several subgroups were observed within Korean strains with minor differences on the dendrograms generated by cluster analyses of plasmid and genomic DNA.

Cluster analysis of genetic similarity among the strains generated the dendrogram that clearly separated all strains from each other, based on the RAPD profiles of the genomic DNA amplified by polymerase chain reaction with 14 primers in this study. The RAPD profiles of genomic DNA were very similar to those of RFLP in the strains of *X. campestris* pv. *vesicatoria* (2), but the strains clustered in the same genomic DNA RFLP group were reclassified into several subgroups by RAPD analysis. For example, twelve strains

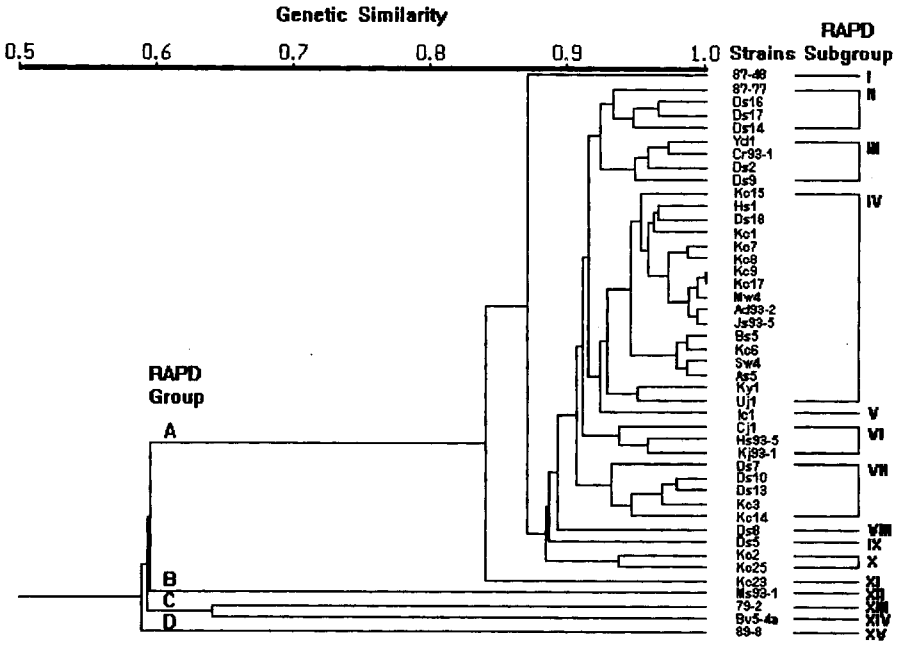

Fig. 2. Dendrogram derived from the RAPD profiles of genomic DNA in the 44 strains of *Xanthomonas campestris* pv. *vesicatoria* with 14 primers using the unweighted pair group method with an arithmetic average program of NTSYS-pc (Version 1.80). A to D indicate 4 major genomic DNA RAPD groups and I to XV indicate 15 genomic DNA RAPD subgroups.

with a same microgeographical origin (Kochang, Korea) were classified into 4 subgroups (RAPD subgroups VI, VII, X, XI) at the similarity level of 0.92 (Fig. 2). This RAPD polymorphism suggests the occurrence of genetic differentiation of *X. campestris* pv. *vesicatoria* and the existence of genetically distinctive subgroups among the populations in Korea.

Kim *et al.* (12) also reported differentiation and distribution of pathovars of *X. campestris* pv. *vesicatoria* in Korea and shift in the race distributions of the pathogen was reported in other countries (1, 3, 9, 21, 23). Such a pathogenic variation was presumed to be due to genetic differentiations in *X. campestris* pv. *vesicatoria*-pepper interactions (25, 27, 28) or in *X. campestris* pv. *vesicatoria*-bactericide interactions (4, 18, 20). The appearance of new virulent races or bactericide-resistant strains of *X. campestris* pv. *vesicatoria* may hamper efforts to manage bacterial spot of pepper and tomato. Therefore, genetic diversity of *X. campestris* pv. *vesicatoria* should be considered in the development program of new bactericides or resistant varieties to control the disease effectively in Korea.

Although the foreign strains formed discrete genomic DNA RAPD subgroups for each country, the United States strain 87-77 had high homology to some of the Korean strains in genomic DNA RAPD profiles. For example, strains Ds14, Ds16 and Ds17 were higher homologous to the United States strain 87-77 than the other ones with a same microgeographical origin (Dukso, Korea). This result reflects that they might have evolved from a common ancestral origin of the strains. In recent years, migration of new races of *X. campestris* pv. *vesicatoria* originated from the United States was detected in Korea (12). Therefore, some of the Korean strains *X. campestris* pv. *vesicatoria* might have been introduced from the United States (2).

Even though RFLP and RAPD are useful molecular markers for pathovar differentiation as well as strain and population analyses of xanthomonads (5~8, 13~17), RAPD is technically easy and rapid, and requires only small amounts of DNA without their prior sequence information (29, 30). Comparison of the RAPD profiles obtained in this study to predetermined RFLP profiles of the strains of *X. campestris* pv. *vesicatoria* showed that RAPD was much more sensitive than RFLP for detection of DNA polymorphisms among the Korean strains of *X. campestris* pv. *vesicatoria* (2). This suggests that RAPD polymorphism could give more convenient phylogenetic grouping for the strains of *X. campestris* pv. *vesicatoria* than that of RFLP.

요 약

우리나라의 주요 고추 재배지와 미국, 대만, 호주, 아르헨티나에서 수집된 44개 고추 더뎅이병균(*Xanthomonas campestris* pv. *vesicatoria*) 균주간의 유전적 다양성을 random amplified polymorphic DNA(RAPD)를 이용하여 분석하였다. 14개의 random primer를 이용하여 genomic DNA를 증폭시킨 결과 0.2~3.2 kb 크기의 총 137개 band들이 증폭되었으며, cluster 분석 결과 44개 균주들은 유사계수 0.60과 0.92에서 각각 4개의 genomic DNA RAPD 집단과 15개의 세부집단으로 분류되었다. 외국 균주들은 각각 다른 집단으로 분류되었지만, 미국 균주 87-77는 우리나라 일부 균주들과 가까운 cluster를 이루었다. 39개 우리나라 균주들은 11개의 세부집단으로 분류되었는데 특히 마산 균주 Ms93-1은 다른 균주들과 원연관계를 나타내었다. RAPD 분석 결과는 restriction fragment length polymorphism(RFLP) 분석 결과와 유사하게 고추 더뎅이병균 균주간의 유전적 분화가 이루어져 우리나라 균주내에 유전적으로 다른 세부집단이 존재함을 시사해 주었다.

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