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Differentiation of *Fusarium oxysporum* f. sp. *fragariae* Isolates by Random Amplified Polymorphic DNA (RAPD) Analysis

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Random Amplified Polymorphic DNA(RAPD)를 이용한 딸기 시들음병균(*Fusarium oxysporum* f. sp. *fragariae*)의 분류

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Abstract : We used random amplified polymorphic DNA (RAPD) markers to assess genetic similarity among 24 isolates of *Fusarium oxysporum* f. sp. *fragariae*. The amount of genetic variation was evaluated by polymerase chain reaction amplification with a set of 16 random 10-mer primers. All amplifications revealed scorable polymorphisms among the isolates, and a total of 231 band positions were scored by presence versus absence (1/0) for the 16 primers tested. Genetic similarities between the isolates were calculated. Cluster analysis was used to generate a phenogram showing relationships between the isolates. The isolates were clustered into two groups, RAPD I and II. The RAPD group I included isolates Y1, K 1, K2, K3, K4, N2, N3, N4-1, N6-1, N6-2, N8, N9, N10 and M1-2-1 assigned to vegetative compatibility group (VCG) A, isolates N1 and Y2 assigned to VCG C and isolate M4-1 assigned to VCG B. The RAPD group II included isolates M1-1, M2-2-1, M2-4-1, M2-4-2, M3-2 and M3-3-2 assigned to VCG B and isolate N11 assigned to VCG D. The genetic similarity between RAPD group I and II was 0.310.

***Key words* : cluster analysis, *Fusarium oxysporum* f. sp. *fragariae*, random amplified polymorphic DNA (RAPD) marker, vegetative compatibility group (VCG).**

Fusarium oxysporum is a common soilborne plant pathogen with a worldwide distribution, and it causes severe economic damage to agricultural crops. Within the species, however, there is high level of host specificity (3). This specificity led Snyder and Hansen (22) to classify strains of the fungus into formae speciales on the basis of the host plants attacked.

Winks (24) reported that wilt of strawberry is caused by a new form of *F. oxysporum*, *F. oxysporum* Schlecht. ex Fr. f. sp. *fragariae*, and the Fusarium wilt of strawberry was reported in Korea (2, 10).

Determination of both host specificity and genetic diversity of *F. oxysporum* f. sp. *fragariae* populations is of great importance in plant breeding for resistance. Assessment of genetic diversity in *F. oxysporum* f. sp. *fragariae* is needed to obtain molecular markers for differentiating them.

The modified polymerase chain reaction (PCR) with a single primer of arbitrary nucleotide sequence has been proved useful in detecting intraspecific polymorphisms among organisms (23) and useful for genome mapping and identification of isolates (9). For plant pathogenic fungi, random amplified polymorphic DNA (RAPD) analysis can provide markers to differentiate races of *F. solani* f. sp. *cucurbitae* (4), *F. oxysporum* f. sp. *pisi* (7), aggressive and nonaggressive isolates of *Phoma lingam* (21) and *F. oxysporum* f. sp. *dianthi* (16), and isolates with different geographic origins of *Colletotrichum graminicola* (8). Additionally, RAPD markers are used for tracking strains of *F. graminearum* in field experiment (19) and for investigating evolutionary processes and genetic linkage in the barley powdery mildew pathogen (17).

The aim of this study was to examine the dif-

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ferentiation of *F. oxysporum* f. sp. *fragariae* isolates by RAPD markers and the relationships among vegetative compatibility groups, isozyme polymorphism groups and RAPD markers.

MATERIALS AND METHODS

Isolates of *F. oxysporum* f. sp. *fragariae*. All isolates of the pathogen were obtained from strawberry plants showing typical disease symptoms (wilt and xylem discoloration). Twenty-four isolates; 14 isolates assigned to VCG A, 7 to VCG B, 2 to VCG C and 1 to VCG D which were grouped by pairing of complementary nitrate-nonutilizing (*nit*) mutants in the previous test, were used for this study (Table 1).

DNA extraction. DNA was extracted from mycelium by modified Yoon's method (25). Approximately 100 mg of fresh mycelium was ground using a precooled mortar and pestle with 1 ml of lysis buffer (50 mM Tris-HCl; pH 7.2, 50 mM EDTA; pH 7.2, 3% sodium dodecyl sulfate and 1% 2-mercaptoethanol). The ground mycelium was transferred into a 2.0 ml precooled microfuge tube and incubated at 65°C for 1 hour, occasionally inverting the tube to ensure complete lysis. The tube was added one volume of chloroform, gently inverted several times, and centrifuged at 14,000 g for 15 min at room temperature. To precipitate DNA, the upper phase was transferred to a new 1.5 ml tube, added 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol, mixed, and centrifuged at 14,000 g for 5 min. The supernatant was decanted and the pellet was rinsed with ice-cold 70% ethanol. The pellet was resuspended in 200 µl TE (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA, pH 8.0), added 100 mg of CsCl, and centrifuged at 14,000 g for 15 min at room temperature. The upper phase was transferred to a new tube and added 2 volumes of TE. The tube was added one volume of isopropanol and centrifuged at 14,000 g for 5 min at room temperature. The pellet was rinsed with ice-cold 80% ethanol, dried, resuspended in 100 µl TE, and stored at −20°C.

PCR conditions. PCR was carried out in 25 µl of

Table 1. Host cultivars, geographic origins, vegetative compatibility groups, isozyme electrophoretic types and random amplified polymorphic DNA (RAPD) groups of isolates of *Fusarium oxysporum* f. sp. *fragariae*

Isolate	Host cultivar	Geographic origin	VCG[a]	I.P.T.[b]	RAPD[c]
Y1	Hokowase	Chungchongnam-do Yongi	A	I	I
K1	Hokowase	Kyonggi-do Kimpo	A	I	I
K2	Hokowase	Kyonggi-do Kimpo	A	I	I
K3	Hokowase	Kyonggi-do Kimpo	A	I	I
K4	Hokowase	Kyonggi-do Kimpo	A	I	I
N2	Hokowase	Chungchongnam-do Nonsan	A	I	I
N3	Hokowase	Chungchongnam-do Nonsan	A	I	I
N4-1	Hokowase	Chungchongnam-do Nonsan	A	I	I
N6-1	Hokowase	Chungchongnam-do Nonsan	A	I	I
N6-2	Hokowase	Chungchongnam-do Nonsan	A	I	I
N8	Hokowase	Chungchongnam-do Nonsan	A	I	I
N9	Hokowase	Chungchongnam-do Nonsan	A	I	I
N10	Hokowase	Chungchongnam-do Nonsan	A	I	I
M1-2-1	Sistakara	Kyongsangnam-do Miryang	A	I	I
M1-1	Sistakara	Kyongsangnam-do Miryang	B	IV	II
M2-2-1	Sistakara	Kyongsangnam-do Miryang	B	IV	II
M2-4-1	Sistakara	Kyongsangnam-do Miryang	B	IV	II
M2-4-2	Sistakara	Kyongsangnam-do Miryang	B	IV	II
M3-2	Sistakara	Kyongsangnam-do Miryang	B	IV	II
M3-3-2	Sistakara	Kyongsangnam-do Miryang	B	III	II
M4-1	Leiko	Kyongsangnam-do Miryang	B	I	I
N11	Hokowase	Chungchongnam-do Nonsan	C	IV	II
N1	Hokowase	Chungchongnam-do Nonsan	D	I	I
Y2	Hokowase	Kyonggi-do Kimpo	D	I	I

[a] Vegetative compatibility group. [b] Isozyme polymorphism type. [c] RAPD group.

a solution containing 1~5 ng of *F. oxysporum* f. sp. *fragariae* genomic DNA; 1 mM $MgCl_2$; 0.3 M of primer; 0.5 U of *Taq* DNA polymerase (Finnzymes Inc., Finland); 100 μM each of dCTP, dGTP, dATP and dTTP (Promega Corporation, U.S.A); 100 mM KCl; 0.1 mM EDTA; 1 mM DTT; 160 μg/ml BSA; 50% glycerol and 0.1% Triton X-100 under 20 μl of mineral oil. Amplification was performed in a thermal cycler (Perkin-Elmer Cetus) programmed for one cycle of 2 min at 95°C, followed by 45 cycles of 30 sec at 94°C, 1 min at 40°C, and 2 min at 72°C and a final incubation at 72°C for 5 min. After PCR, 12.5 μl of the product was electrophoresed in 1.4% agarose gel and visualized by ethidium bromide staining. One Kb DNA ladder (Gibco BRL) was used as the molecular weight markers.

RAPD assays. Ten-base oligonucleotide primers were obtained from kit A, Operon Technologies, Inc. (Alameda, CA 94501, USA). Amplification reactions were conducted with each primer on the DNA of the 24 isolates of *F. oxysporum* f. sp. *fragariae*.

All amplification reactions were conducted at least twice in two separate tests.

Cluster analysis. Each profile for each primer was compared on the basis of the presence versus absence (1/0) of RAPD products of the same distance. Bands of the same distance were scored as identical. Analyses were based on the simple matching index, which measures the proportion of common discrete data (either 0 or 1) between the isolates. A dendrogram was derived from the similarity matrix by the unweighted pair-group method algorithm contained in NTSYS-pc (20).

Table 2. Code and sequence of the 16 primers were used, with the total number of amplified DNA fragments and size of polymorphic DNA fragments obtained with each primer in random amplified polymorphic DNA (RAPD) experiments

Code	Sequence 5′ to 3′	Amplified fragments	Size of fragments (bp)
OPA-01	CAGGCCCTTC	11	1600~400
OPA-02	TGCCGAGCTG	21	2000~350
OPA-03	AGTCAGCCAC	12	1500~500
OPA-04	AATCGGGCTG	9	1700~850
OPA-05	AGGGGTCTTG	17	2100~500
OPA-06	GGTCCCTGAC	20	2900~300
OPA-07	GAAACGGGTG	14	1500~300
OPA-08	GTGACGTAGG	13	2800~350
OPA-09	GGGTAACGCC	9	1900~650
OPA-10	GTGATCGCAG	15	1800~500
OPA-11	CAATCGCCGT	16	2000~400
OPA-12	TCGGCGATAG	11	1600~400
OPA-13	CAGCACCCAC	17	2800~800
OPA-14	TCTGTGCTGG	19	2600~400
OPA-15	TTCCGAACCC	13	3200~700
OPA-16	AGCCAGCGAA	14	1700~400

RESULTS

RAPD patterns were established for the 24 isolates of *F. oxysporum* f. sp. *fragariae* with the 16 primers (Table 2). Concentrations of DNA template, primer, $MgCl_2$ and dNTP were determined in preliminary trials to get unambiguous amplification patterns. The profiles were reproducible.

The size of amplified DNA fragments generated

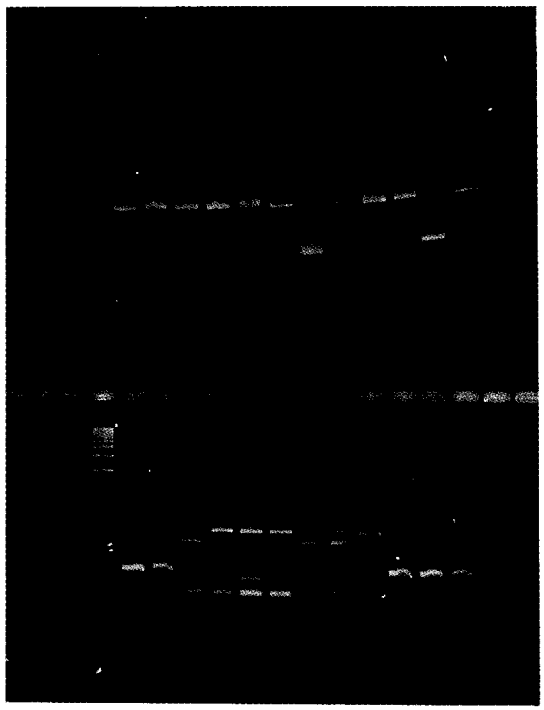

Fig. 1. Electrophoretic patterns showing amplification products generated from the *Fusarium oxysporum* f. sp. *fragariae* isolates with primer OPA-04 on 1.4% agarose gel. Lanes from left to right and upper to lower, show amplification products from isolates Y1, K1, K2, K3, K4, N2, N3, N4-1, N6-, N6-2, N8, N9, N10, M1-2-1, M1-1, M2-2-1, M2-4-1, M2-4-2, M3-2, M3-3-2, N11, N1, Y2 and M4-1. The last lanes on the left show 1 kb DNA ladder (Gibco BRL) for molecular size marker.

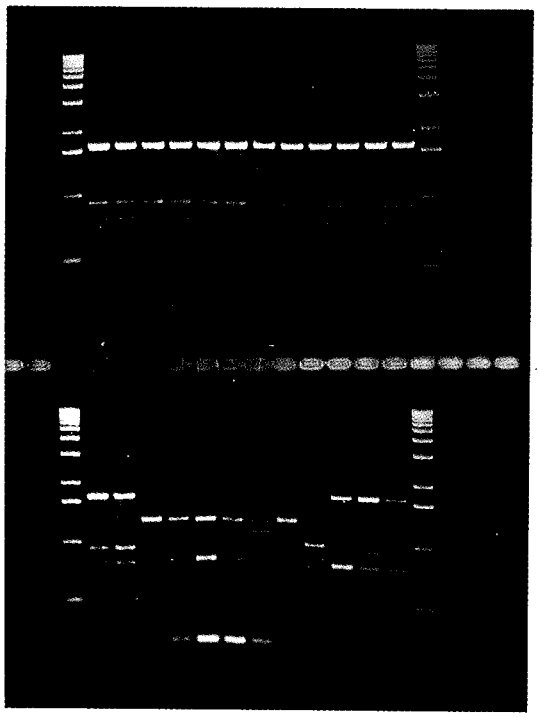

Fig. 2. Electrophoretic patterns showing amplification products generated from the *Fusarium oxysporum* f. sp. *fragariae* isolates with primer OPA-06 on 1.4% agarose gel. Lanes, from left to right and upper to lower, show amplification products from isolates Y1, K1, K2, K3, K4, N2, N3, N4-1, N6-1, N6-2, N8, N9, N10, M1-2-1, M1-1, M2-2-1, M2-4-1, M2-4-2, M3-2, M3-3-2, N11, N1, Y2 and M4-1. The last lanes on the right and left show 1 kb DNA ladder (Gibco BRL) for molecular size marker.

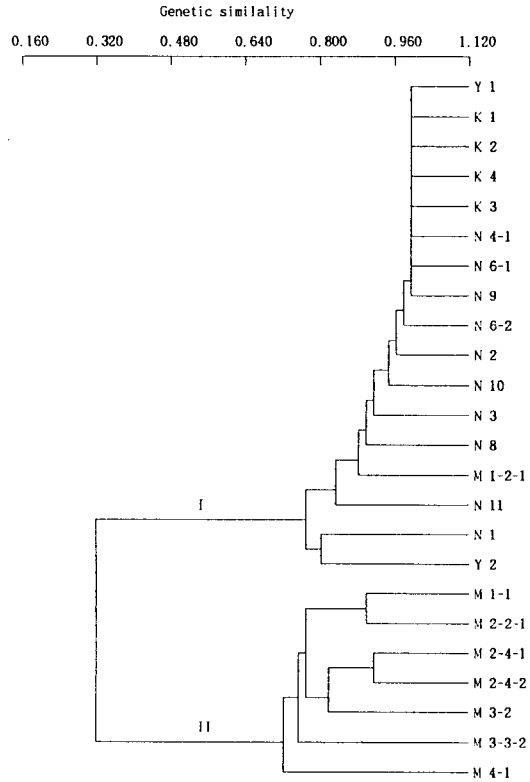

Fig. 3. Phenogram showing relationships among the 24 *Fusarium oxysporum* f. sp. *fragaria* isolates. Genetic similarities were obtained by random amplified polymorphic DNA analysis with 16 primers.

with the 16 primers ranged from 0.3 to 3.2 Kb. All the primers revealed polymorphisms useful for classifying isolates. The total number of amplified fragments and the number of polymorphic fragments produced with each primer were shown in Table 2.

Amplification patterns for isolates Y1, K1, K2, K3, K4, N2, N3, N4-1, N6-1, N6-2, N8, N9, N10, M1-2-1, N1, Y2 and M4-1 were very distinct from those of the isolates M1-1, M2-2-1, M2-4-1, M2-4-2, M3-2, M3-3-2 and N11 (Figs. 1, 2). By combining the results using 16 primers, 231 band positions were scored for presence versus absence (1/0) for all the isolates studied.

The combined data from all isolates were analyzed by a simple matching coefficient to produce a phenogram (Fig. 3). At genetic similarity of 0.334, two distinct groups were differentiated among the 24 *F. oxysporum* f. sp. *fragariae* isolates by RAPD markers.

The first group, RAPD I, included isolates Y 1, K 1, K 2, K 3, K 4, N 2, N 3, N 4-1, N 6-1, N 6-2, N 8, N 9, N 10, and M 1-2-1 assigned to VCG A, isolates N 1 and Y 2 assigned to VCG C, and isolate M 4-1 assigned to VCG B. The isolates included in RAPD I only exhibited slight differences in RAPD products. A second group, RAPD II, included isolates M 1-1, M 2-2-1, M 2-4-1, M 2-4-2, M 3-2, and M 3-3-2 assigned to VCG B and isolate N 11 assigned to VCG D, and the genetic similarities between isolates was 0.724 to 0.926.

The genetic similarity between RAPD group I and II was 0.310.

DISCUSSION

Since the RAPD technique was introduced (23), it has been widely used to detect genetic polymorphisms in various organisms, including fungi (21) and nema-

todes (6).

In a previous test, four major vegetative compatibility groups by means of a nitrate reductase complementation test and four patterns of isozyme polymorphism by electrophoresis were found among 32 isolates of *F. oxysporum* f.sp. *fragariae* (12).

We observed genetic diversity within a collection of 24 *F. oxysporum* f. sp. *fragariae* isolates, based on RAPD markers. Random amplified DNA patterns produced from genomic DNA reliably and unambiguously distinguished isolates of *F. oxysporum* f. sp. *fragariae* assigned to each of VCGs A and B except isolates M4-1 and M3-3-2. It may be attributed that the isolates in VCG B weakly paired with some other isolates in the same VCG. And if more number of mutants are tested, the isolates in VCG D may be included in VCG A by results of RAPD assay.

Most of the studies based on restriction fragment length polymorphism (RFLP) analyses of nuclear or mitochondrial DNA failed to characterize races (5, 11, 13, 14), and RAPD analysis conducted on isolates of *F. oxysporum* f. sp. *pisi* allowed differentiation of only one of four races studied (7). On the other hand, Nicholson *et al.* (18) differentiated the three races of *Bipolaria maydis* and Assigbetse *et al.* (1) found the races of *F. oxysporum* f. sp. *vasinfectum* corresponded to their RAPD markers. In other phytopathogenic fungi, RAPD analyses have been proved useful for detecting genomic polymorphisms directly related to host specialization (21).

Our results provide evidence that RAPD analysis can be used for differentiating isolates of *F. oxysporum* f. sp. *fragariae*, and RAPD markers were correlated with VCGs and isozyme polymorphism groups but not perfectly. Therefore, we suggest that RAPD markers may be used as a quick and reliable alternative for differentiating isolates of *F. oxysporum* f. sp. *fragariae*.

요 약

본 실험은 이병 딸기의 조직에서 분리 동정된 시들음병균(*Fusarium oxysporum* f. sp. *fragariae*) 균주들의 유전적 변이를 random amplified polymorphic DNA (RAPD) marker들을 이용하여 조사하였다. 총 24개의 딸기 시들음병 균주들의 DNA를 주형으로 하여 16개의 random 10-mer primer들을 사용하여 증폭시킨 결과 총 231개의 marker들이 합성되었으며 이들 RAPD marker들을 이용하여 유전적 변이를 조사해 본 결과 크게 RAPD I과 RAPD II의 2개 그룹으로 나눌 수 있었다. RAPD I 그룹에 속하는 균주는 VCG A에 속하는 Y1, K1, K2, K3, K4, N2, N3, N4-1, N6-1, N6-2, N8, N9, N10, M1-2-1 균주, VCG B에 속하는 M4-1 균주 그리고 VCG C에 속하는 N1, Y2 균주들이었고, RAPD II그룹에는 VCG B에 속하는 M1-1, M2-2-1, M2-4-2, M3-2, M3-3-2 균주와 VCG D에 속하는 N11 균주가 속하였다. 이들 2그룹 간에는 31%의 유사성이 있었다.

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