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Scientific Notes

Race differentiation of *Fusarium solani* f. sp. *cucurbitae* by PCR−RFLP

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Abstract: Differentiations between race 1 and race 2 of *Fusarium solani* f. sp. *cucurbitae* by PCR-RFLP analysis of the ITS region including 5.8S rDNA were attempted. Twenty five isolates of *F. solani* f. sp. *cucurbitae* race 1, 7 isolates of race 2, 6 isolates of *F. solani* which were isolated from imported pumpkin and melon fruits, 10 isolates of 6 formae speciales of *F. solani* and 13 isolates of 7 other species of *Fusarium* were used. PCR products of 2 races digested with restriction enzymes, *Afa* I or *Hap* II, had different RFLP patterns between race 1 and race 2 and there was no difference within the same race. Furthermore, race 1 had different RFLP pattern with other *Fusarium* spp. Results of this study suggested that this method was able to replace inoculation test to pumpkin seedling which have been used to differentiate between race 1 and race 2 of *F. solani* f. sp. *cucurbitae*.
Key words: PCR-RFLP, race, *Fusarium solani* f. sp. *cucurbitae*

Introduction

Fusarium solani (Martius) Saccardo f. sp. *cucurbitae* Snyder & Hansen was further classified into two races (TOUSSON and SNYDER, 1960). Race 1 is pathogenic to both pumpkin plant and fruit. The race 1 has been categorized as a pathogen with plant quarantine significance in Japan (KOBAYASHI, 1983). On the other hand, race 2 is pathogenic only to pumpkin fruit. *F. solani* is frequently detected in plant quarantine inspection from pumpkin seed and fruit in Japan. However, it takes a lot of time to identify forma specialis or races of the isolate of *F. solani*. Recently, molecular approach has been reported for rapid identification of some phytopathogenic fungi which are difficult to identify morphologically (TSUGE *et al.*, 1992). In this study, we investigated the possibility of the utilization of PCR-RFLP analysis for the differentiation between *F. solani* f. sp. *cucurbitae* race 1 and race 2.

Material and methods

Fungal isolates

Sixty one isolates of *Fusarium* spp. were used in this study (**Table 1**). Twenty three isolates of *F. solani* f. sp. *cucurbitae* race 1 except ATCC 18278 and ATCC 18280 were isolated in Japan from 1984 to 1989 and identified by morphological characteristics, inoculation tests and mating tests with ATCC 18278 and ATCC 18280 (KINJYO *et al.*, 1989; SHIMONAGANE *et al.*, 1989; OHTO *et al.*, 1989; DAI *et al.*, 1990). Origin of race 2 isolates were U.S.A., New Zealand and Okinawa, Japan. Before PCR-RFLP analysis, pathogenicity of all race 1 and race 2 isolates were confirmed.

DNA extraction

Each isolate was grown in potato dextrose broth (Difco) for 5-6 days at 25℃ in dark. Mycelial mats were collected on paper filter with a Buchner funnel and rinsed with distilled water. After removing excess water with filter paper, the mycelial mats were stored at −80℃ until DNA extraction.

The extraction of total DNA was conducted according to the following modified procedure of GIRARDIN *et al.*, (1994) and LEE *et al.*, (1990). At first, the frozen mycelial mats were grind into powder with a sterilized, pre-cooled motar and pestle at −80℃. Mycelial powder were transferred into microfuge tube containing 0.6 ml lysis buffer (50 mM Tris-HCl, pH8.0, 50 mM EDTA, 3% SDS, 1% mercaptoethanol) and incubated at 65℃ for 1 hour and centrifuged at 12,000×*g* for 10 minutes at room temperature. The supernatant was transferred to a new microfuge tube and then, equal volume of TE saturated phenol-chloroform-isoamylalcohol (25 : 24 : 1) was added. After shaking and centrifugation, the DNA in the aqueous phase was extracted with TE saturated phenol-chloroform-isoamylalcohol(25 : 24 : 1). The DNA in the aqueous phase were precipitated with sodium acetate and isopropanol. After centrifugation, the pellet was washed with 70% ethanol and dried. The DNA pellet was dissolved in TE (10 mM Tris-HCl, pH8.0, 1 mM EDTA) and RNase was added. After incubation at 37℃ for 30 minutes, RNase in this sample was removed with TE saturated phenol-chloroform-isoamylalcohol (25 : 24 : 1). DNA in this sample was precipitated with sodium acetate and isopropanol. After centrifugation, the pellet was washed with 70% ethanol and dried. At last, the extracted DNA was dissolved in 50 μl TE. Its concentration was adjusted 10 μg/ml with TE and stored at −80℃ until PCR.

Polymerase chain reaction (PCR)

By using primer set, ITS-1 and ITS4 designed by WHITE *et al.* (1990), rDNA ITS region including 5.8S rDNA was amplified. Total volume of 50 μl reaction mixture contained 0.2 μM of each primer, 1.25 units of *Taq* DNA polymerase, 0.2 mM dNTP mixture, 1× PCR buffer (10 mM Tris-HCl, pH8.3, 50 mM KCl, and 1.5 mM $MgCl_2$) and 50 ng of DNA template. The mixtures were covered with mineral oil to prevent evaporation. The amplification condition was an initial denaturation at 95℃ for 3 min, followed by 30 cycles of denaturation at 95℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 1 min with the final extension at 72℃ for 10 min (WHITE *et al.*, 1990). For confirming the amplification, 8 μl aliquots of the PCR products were electrophoresed on 1.4% agarose gel in 0.5×TBE buffer at 100 V for 40 minutes. These gels were stained with ethidium bromide and observed.

Restriction fragment length polymorphism (RFLP)

Each PCR product was digested with restriction enzymes, *Afa* I, *Hap* II (Takara shuzo Co. Ltd., Shiga, Japan), according to the manufacturer's instruction. Each digested sample was electrophoresed on 2.0% agarose gels in 0.5×TBE buffer at 50 V for 5 hours. These gels were stained with ethidium bromide, and each RFLP pattern was photographed and compared.

Table 1. *Fusarium* isolates used in this study.

Isolates	Source	Location
F. solani f. sp. *cucurbitae* race 1		
ATCC[1] 18278, ATCC18280	—[8]	U.S.A.
64-1, FII-51-1, FII-57-1, FII-57-2	Pumpkin seedling	Okinawa, Japan
FII-51-2, FII-59-1, FII-60-2, R-1, FII-55-1, FII-59-2		
63.8.3-6, 63.8.4-11, 63.8.4-14, okayama, gen.6.13-4, gen.6.23-1	Pumpkin seedling	Okayama, Japan
F22, E6, Ka A-2, Ka C-3, Ka C-4, Ki A-2, Naka No.3	Pumpkin seedling	Ibaraki, Japan
F. solani f. sp. *cucurbitae* race 2[6]		
S-203	—	U.S.A.
YPPS[2]8-31, YPPS8-25, YPPS8-19, YPPS9-14-1, YPPS9-14-2	Pumpkin fruit	New Zealand
okinawa	—	Okinawa, Japan
F. solani[7]		
YPPS6-4-2, YPPS8-9	Pumpkin fruit	Mexico
YPPS7-30	Pumpkin fruit	Korea
YPPS8-21, YPPS8-22	Pumpkin fruit	New Zealand
YPPS10-46	Melon fruit	U.S.A.
F. solani f. sp. *eumartii*		
905	—	—
F. solani f. sp. *mori*		
SUF[3]235,	Mulberry	Japan
IFO[4]7707, IFO7708	—	—
F. solani f. sp. *phaseoli*		
IFO9974	Kindney bean	Japan
F. solani f. sp. *pisi*		
IFO9425, IFO9975	Pea	Japan
F. solani f. sp. *xanthoxyli*		
IFO7709, IFO7710	—	—
F. solani f. sp *radicicola*		
YPPS11-6	Carrot	Taiwan
F. oxysporum f. sp. *betae*		
ATTC34296	Sugar beet	U.S.A.
F. oxysporum f. sp. *niveum*		
SUF806	—	—
F. oxysporum f. sp. *tuberosi*		
ATTC15650	Potato stem	Scotland
F. oxysporum f. sp. *pisi*		
ATTC16607	Pea	—
F. acuminatum		
MAFF[5]236714	Mascarene grass	Kagawa, Japan
F. culmorum		
MAFF236454	Bentgrass	Ibaraki, Japan
YPPS11-28, YPPS11-27	Pumkin fruit	New zealand
F. equisetii		
MAFF236434	Wheat	Hokkaido, Japan

Isolates	Source	Location
F. graminearum		
MAFF236482	Wheat	Hiroshima, Japan
F. moniliforme		
SUF1000	Rice	—
F. semitectum		
YPPS10-88	Banana fruit	Philippine
YPPS10-54	Pumpkin seed	Thailand

[1] ATCC : American Type Culture Collection, Rockville, MD, U.S.A.
[2] YPPS : Yokohama Plant Protection Station, Yokohama, Japan. These fungi were found at import plant quarantine inspection.
[3] SUF : Culture collection of *Fusarium* in Sinshu University, Japan.
[4] IFO : Institute for Fermentation, Osaka, Japan.
[5] MAFF : Ministry of Agriculture, Forestry and Fisheries, Ibaraki, Japan.
[6] These fungi except 2 isolates, S-203 and okinawa were found at import plant quarantine inspection from 1996 to 1997.
[7] These fungi were found at import plant quarantine inspection from 1994 to 1998. These fungi were non-pathogenic to both pumpkin seedling and fruit.
[8] unknown.

Results and Discussion

PCR

The PCR products were obtained from all isolates used in this study. Their sizes of 60 isolates were approximately 560bp and that of *F. solani* (YPPS8-22) was approximately 520bp (data not shown).

RFLP

Race 1 and race 2 of *F. solani* f. sp. *cucurbitae* had different RFLP patterns of digestion with both restriction enzymes, *Afa* I and *Hap* II. By treatment with *Afa* I, approximately 310 bp and 250 bp fragments were obtained from race 1 (**Fig. 1**), though the PCR products of race 2 were not digested (**Fig. 2**). By treatment with *Hap* II, approximately 350 bp, 110 bp and 100 bp fragments were obtained from race 1, though approximately 350 bp and 210 bp fragments were obtained from race 2 (data not shown).

This result showed that it was able to differentiate the isolates of race 1 and race 2 used in this study by this method. SUGA *et al.* (2000) reported that *F. solani* f. sp. *cucurbitae* race 1 formed different clusters from race 2 by phylogenetic analysis based on the sequence of rDNA-ITS region. In our study, the results also showed that each race which have been stored in our laboratory were divided into different groups molecularly.

This result suggested that PCR-RFLP analysis was able to replace inoculation test to pumpkin seedling which has been used to differentiate between race 1 and race 2 of *F. solani* f. sp. *cucurbitae*.

The RFLP pattern of each race was compared with that of other *Fusarium* species. The other isolates of *Fusarium* spp. had different RFLP patterns from race 1 and most of them had the same patterns with race 2 (**Fig. 3, 4**). SUGA *et al.* (2000) further described that PCR-RFLP analysis was able to distinguish *F. solani* f. spp. *phaseoli*, *cucurbitae* race

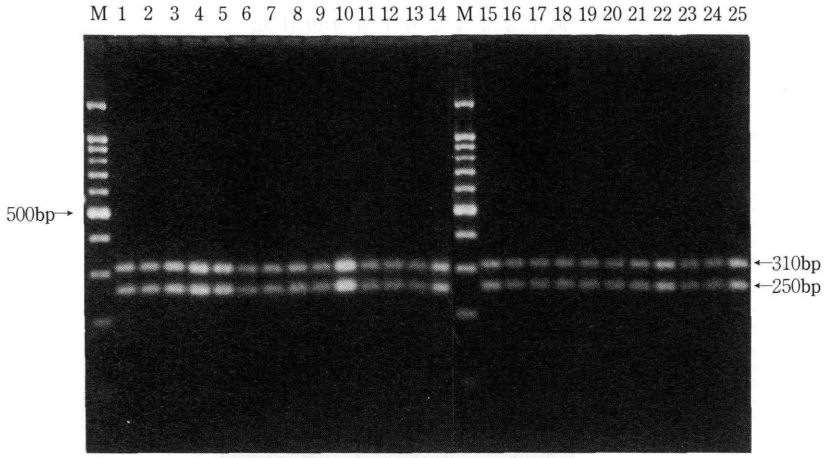

Fig. 1. Comparison of restriction banding patterns of PCR-amplified rDNA-ITS region among 25 isolates of *F. s.* f. sp. *cucurbitae* race 1 digested with *Afa* I.
M: 100bp DNA size marker, Lane 1: ATCC18278, 2: ATCC18280, 3: FII-59-1, 4: FII-51-1, 5: FII-57-1, 6: FII-57-2, 7: FII-60-2, 8: 64-1, 9: gen.6.23-1, 10: 63.8.3-6, 11: 63.8.4-11, 12: okayama, 13: gen.6.13-4, 14: E6, 15: Ki A-2, 16: Ka A-2, 17: Ka C-3, 18: Ka C-4, 19: F22, 20: R-1, 21: 63.8.4-14, 22: FII-59-2, 23: FII-55-1, 24: FII-51-2, 25: Naka No.3.

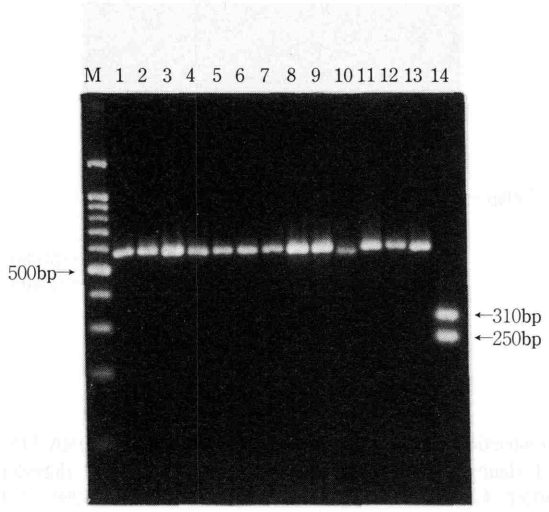

Fig. 2. Comparison of restriction banding patterns of PCR-amplified rDNA-ITS region among *F. s.* f. sp. *cucurbitae* race 1 (lane 14), race 2 (lane 1-7) and *F. solani* (lane 8-13) digested with *Afa* I.
M: 100bp DNA size marker, Lane1: YPPS8-25, 2: YPPS9-14-2, 3: YPPS8-19, 4: YPPS8-31, 5: YPPS9-14-1, 6: S-203, 7: okinawa, 8: YPPS6-4-2, 9: YPPS8-9, 10: YPPS8-22, 11: YPPS7-30, 12: YPPS8-21, 13: YPPS10-46, 14: FII 59-1.

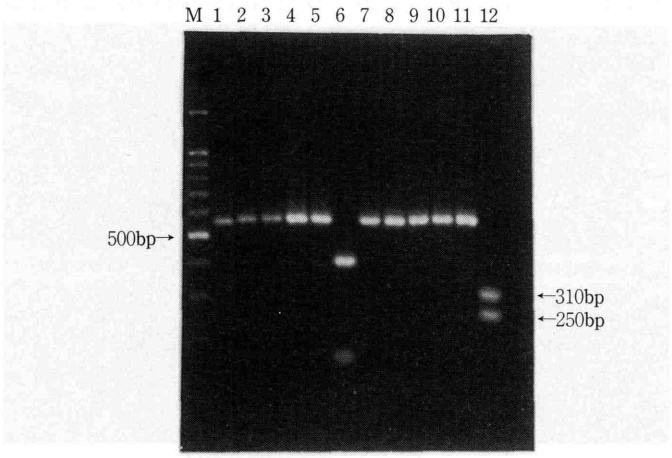

Fig. 3. Comparison of restriction banding patterns of PCR-amplified rDNA-ITS region among *F. s.* f. sp. *cucurbitae* race 1 (lane 12), race 2 (lane 11) and other formae speciales of *F. solani* (lane 1-10) digested with *Afa* I.

M : 100bp DNA size marker, Lane1 : *F. s.* f. sp. *radicicola* YPPS11-6, 2 : *F. s.* f. sp. *eumartii* 905, 3 : *F. s.* f. sp. *mori* SUF235, 4 : *F. s.* f. sp. *mori* IFO7707, 5 : *F. s.* f. sp. *mori* IFO7708, 6 : *F. s.* f. sp. *phaseoli* IFO 9974, 7 : *F. s.* f. sp. *pisi* IFO9425, 8 : *F. s.* f. sp. *pisi* IFO9975, 9 : *F. s.* f. sp. *xanthoxyli* IFO7709, 10 : *F. s.* f. sp. *xanthoxyli* IFO7710, 11 : YPPS8-25, 12 : F II-59-1.

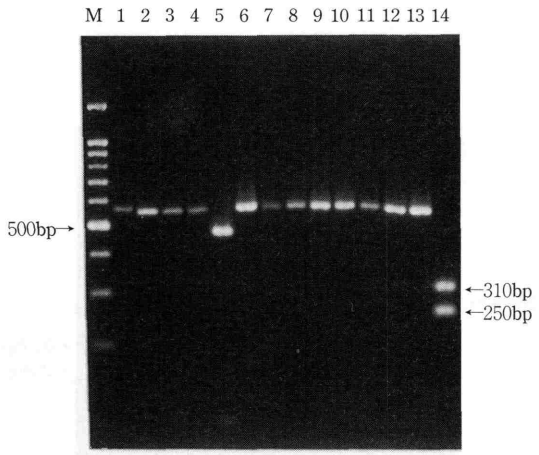

Fig. 4. Comparison of restriction banding patterns of PCR-amplified rDNA-ITS region among *F. s.* f. sp. *cucurbitae* race 1 (lane 14) and other *Fusarium* spp. (lane 1-13) digested with *Afa* I.

M : 100bp DNA size marker, Lane 1 : *F. oxysporum* f. sp. *betae* ATTC34296, 2 : *F. oxysporum* f. sp. *niveum* SUF235, 3 : *F. oxysporum* f. sp. *pisi* ATTC16607, 4 : *F. oxysporum* f. sp. *tuberosi* ATTC15650, 5 : *F. moniliforme* SUF1000, 6 : *F. accuminatum* MAFF236714, 7 : *F. equisetii* MAFF236434, 8 : *F. semitectum* YPPS10-88, 9 : *F. semitectum* YPPS10-54, 10 : *F. culmorum* MAFF236454, 11 : *F. graminearum* MAFF236482, 12 : *F. culmorum* YPPS11-28, 13 : *F. culmorum* YPPS11-27, 14 : F II-59-1.

1, *batatas*, *piperis*, *eumartii*, and *xanthoxyli*.

Race 1 and 2 of *F. solani* f. sp. *cucurbitae* can be differentiated by morphological characteristics, mating specificity and pathogenicity. Our study suggested that PCR-RFLP analysis was also able to use for differentiation as well as them. PCR-RFLP analysis was useful as the method for rapid one.

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和 文 摘 要

PCR-RFLP による *F. solani* f. sp. *cucurbitae* の race 判別

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5.8S rDNA を含む ITS 領域の PCR-RFLP 分析により *F. solani* f. sp. *cucurbitae* の race 判別が可能か検討した。Race1 (25菌株), race2 (7菌株)、輸入カボチャ及びメロン果実由来の *F. solani* (6菌株)、cucurbitae を除く *F. solani* の6分化型(10菌株), *F. solani* を除く *Fusarium* 属菌7種 (13菌株) を供試した。制限酵素 *Afa* I あるいは *Hap* II で処理したとき, 両 race は互いに異なる RFLP パターンを示し, 同一 race 内での差異は認められなかった。さらに、race 1はその他の *Fusarium* 属菌とも異なる RFLP パターンを示した。よって, 本法は, 従来から両 race の判別に利用されているカボチャ苗への接種試験に代用できることが示唆された。