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FUNGAL DISEASES

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Phylogenetic relationships between the lettuce root rot pathogen *Fusarium oxysporum* f. sp. lactucae races 1, 2, and 3 based on the sequence of the intergenic spacer region of its ribosomal DNA

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Abstract The genetic relationship between the vegetative compatibility groups (VCGs) and between physiological races of *Fusarium oxysporum* f. sp. *lactucae* (FOL), the causal pathogen of lettuce root rot, was determined by analyzing the intergenic spacer (IGS) region of its ribosomal DNA. A total of 29 isolates containing a type strain were tested: 24 Japanese isolates, 2 Californian isolates, and 3 Italian isolates. Three races (races 1, 2, and 3) were found in Japan, and race 1 was also distributed in California and Italy. Races 1, 2, and 3 each belonged to a distinct VCG: VCG-1, VCG-2, and VCG-3 (VCG-3-1, VCG-3-3), respectively. Phylogenetic (neighbor-joining) analysis of the IGS sequences revealed that races 1, 2, and 3 coincided with three phylogenetic groups (PG): PG-1, PG-2, and PG-3, respectively. These results indicate that the three races are genetically quite different and have a strong correlation with VCGs and phylogenetic groupings.

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Introduction

The recent outbreak of Fusarium disease in lettuce (*Lactuca sativa* L.) is a serious problem for lettuce-producing farmers in several countries (Fujinaga et al. 2003; Garibaldi et al. 2004; Matheron 2003; Ogiso et al. 2000). It is also one of the most troublesome caused by a soilborne pathogen in Japan, especially in Nagano Prefecture, which is a major area for crisp-head lettuce production (Fujinaga and Wada 1997). A root rot disease of lettuce caused by Fusarium *oxysporum* was first reported in 1960 in Tokyo, Japan (Motohashi et al. 1960), and the causal pathogen was identified as *F. oxysporum* f. sp. *lactucae* (FOL) Matuo *et* Motohashi (Matuo and Motohashi 1967). Recently, Fusarium wilt symptoms that were similar to root rot in Japan have also been reported in California (USA) (Hubbard and Gerik 1993), Taiwan (Huang and Lo 1998), Iran (Millani et al. 1999), and Italy (Garibaldi et al. 2002).

As previously reported (Fujinaga et al. 2003), three races (1, 2, and 3) were found among isolates of FOL. The relationship between race and vegetative compatibility group (VCG) within the same forma specialis has been studied for a few diseases caused by *F. oxysporum*. The races of *F. oxysporum* f. sp. *melonis* could not be differentiated into distinct VCGs (Appel and Gordon 1996; Jacobson and Gordon 1990; Kim et al. 1993; Namiki et al. 1998), although they had different DNA fingerprint patterns (Namiki et al. 1998). Races of *F. oxysporum* f. sp. *lycopersici* were distributed at random among all lineages and appeared to have arisen independently in each VCG (Elias et al. 1993). Thus, *F. oxysporum* f. sp. *melonis* and f. sp. *lycopersici* were thought to be fungi that did not have a relationship between their races and VCGs.

Kawabe et al. (2002, 2005) determined the evolutionary and geographic relationships in Japanese isolates of *F.*

oxysporum f. sp. *lycopersici* using phylogenetic analysis in combination with a classification based on VCG and race. They showed that following multiple cycles of asexual reproduction the isolates subsequently became distinct lineages; that is, isolates of race 1 (with an exception), race 2, and race 3 obtained from Japan belonged to lineages A2, A1, and A3, respectively, suggesting that these three races were introduced to Japan independently.

We determined the relationship between the races and their VCGs in FOL isolates from Japan and other countries. The results showed that races 1 and 2 were associated with two distinct VCGs (VCG-1 and VCG-2, respectively), and race 3 was associated with four VCGs (VCG-3-1, VCG3-2, VCG3-3, VCG3-4) (Nishimura 2003; Ogiso et al. 2002). It has become well accepted that isolates belonging to the same VCG have similar or identical multilocus haploid types and belong to the same clonal lineage. Therefore, the VCG can be a good indicator of genetic similarity (Kistler 1997). Our results also suggested that VCG is a possible indicator of each race, showing its distinct pathogenicity to lettuce.

However, intraspecific and phylogenetic relationships among the three races of FOL remain unknown. To develop a rapid diagnostic tool and method for disease control, we analyzed the sequences of the intergenic spacer (IGS) region of ribosomal DNA and elucidated the relationships between races, VCGs, and IGS sequences using FOL isolates from geographically distinct areas of the world.

Materials and methods

Fusarium isolates and race determination

In total, 29 lettuce strains of FOL were used in this study (Table 1). We collected 24 isolates, including the Japanese type culture SUF-762, from Nagano, Tokyo, Shizuoka, and Fukuoka prefectures in Japan. Two isolates were from the United States (provided by Dr. J.C. Hubbard, University of California), and three were from Italy (provided by Drs. M.L. Gullino and G. Gilardi, University of Torino). The

Table 1. Isolates of *Fusarium oxysporum* f. sp. *lactucae, F. oxysporum* f. sp. *lactucum* and *F. oxysporum* in this study

Species or forma specialis and isolates	Origin	Isolated year	Pathogenicity[a]			Race	VCG[b]
			Patriot	Banchu Red Fire	Costa Rica No. 4		
F. oxysporum f. sp. *lactucae*	Nagano, Japan						
SB1-1	Nagano, Japan	1997	3.0 S	2.9 S	0 R	1	1
FL1101	Nagano, Japan	1998	3.0 S	2.8 S	0 R	1	1
FL1104	Nagano, Japan	1998	3.0 S	3.0 S	0 R	1	1
FL1109	Nagano, Japan	1999	3.0 S	3.0 S	0.1 R	1	1
FL1110	Nagano, Japan	1999	3.0 S	2.8 S	0 R	1	1
FA11	Nagano, Japan	1999	3.0 S	2.8 S	0 R	1	1
FA81	Nagano, Japan	1999	3.0 S	3.0 S	0 R	1	1
12W-B1	Nagano, Japan	2000	2.8 S	3.0 S	0 R	1	1
12W-B2	Nagano, Japan	2000	2.6 S	3.0 S	0 R	1	1
NS-2	Nagano, Japan	2001	2.9 S	3.0 S	0 R	1	1
F9501	Nagano, Japan	1995	3.0 S	0 R	2.7 S	2	2
1-4F	Nagano, Japan	1997	3.0 S	0 R	3.0 S	2	2
KW-H	Nagano, Japan	1997	3.0 S	0 R	2.9 S	2	2
3-1A	Nagano, Japan	1997	3.0 S	0 R	3.0 S	2	2
3A-A	Nagano, Japan	1997	3.0 S	0 R	3.0 S	2	2
GSO3-M	Nagano, Japan	1999	3.0 S	0 R	3.0 S	2	2
OS-20-3	Nagano, Japan	2000	3.0 S	0 R	2.8 S	2	2
K-45	Fukuoka Japan	2000	1.2 S	1.5 S	2.1 S	3	3-1
K-65	Fukuoka Japan	2000	2.0 S	2.6 S	2.8 S	3	3-1
N-85	Fukuoka Japan	2000	1.8 S	1.8 S	3.0 S	3	3-1
N-115	Fukuoka Japan	2000	1.2 S	1.1 S	2.6 S	3	3-1
HS-14	Shizuoka Japan	1992	1.6 S	1.2 S	3.0 S	3	3-3
HS-18	Shizuoka Japan	1992	1.9 S	1.8 S	3.0 S	3	3-3
SUF762[c]	Tokyo Japan	1963	0.5 R	0.6 R	0.8 R	NI[f]	3-1
F. oxysporum f. sp. *lactucae*[d]							
HL-1	California, USA	1990	3.0 S	2.5 S	0 R	1	1
HL-2	California, USA	1990	2.8 S	2.7 S	0 R	1	1
Fusarium oxvsporum[e]							
LE7	Lombardy, Italy	2002	2.8 S	1.4 S	0 R	1	1
LE10A	Lombardy, Italy	2002	1.7 S	1.8 S	0 R	1	1
LE17B	Lombardy, Italy	2002	3.0 S	2.1 S	0 R	1	1

R, resistant; S, susceptible

[a] Race differential system of Fujinaga et al. (2003)

[b] Vegetative compatibility groups determined by Puhalla's methods (1985)

[c] The type culture of *F. oxysporum* f. sp. *lactucae* was obtained from the culture collection of Shinshu University

[d] Carifornia strains were obtained from Dr. J.C. Hubbard, U.S. Department of Agriculture

[e] Italian strains were obtained from Dr. M.L. Gullino, DIVAPRA-Patologia Vegetable

[f] NI, not identified for race determination because of its weak pathogenicity on race determination cultivars

race of each single-spore isolate was identified by a greenhouse pathogenicity test using differential cultivars; Costa Rica No. 4, Banchu Red Fire, and Patriot (Fujinaga et al. 2003). The isolates were grown on 9-cm diameter potato sucrose agar (PSA) plates at 25°C for 2 weeks. Conidial suspensions were then prepared by adding approximately 5 ml of distilled water to each PSA culture plate and scraping the agar surface with a glass slide. The suspension was passed through four layers of cheesecloth and centrifuged at 3000 rpm for 10 min, and then the pellet was resuspended in distilled water. The bud-cell suspension was adjusted to 1×10^5 cells/ml using a hemacytometer and added to autoclaved wheat bran solution (4000 ml) in a round-bottomed flask to give a final concentration of 0.125% (v/v) or 1.25% (v/v) wheat bran; the mixture was incubated at 25°C for 2–3 weeks. The wheat bran culture was then mixed with commercial horticulture soil (Taihei-Engei-BaidoTM; Taihei, Tokyo, Japan) at a rate of 5% (v/v). Lettuce seeds (1 seed/cell) were sown in this soil in each cell (2.5 × 2.5 cm) of a 200-cell plug-tray (Yammer, Osaka, Japan). The trays were kept in a greenhouse maintained at 18°–32°C. Noninoculated plants served as a negative control.

Disease severity was determined 30 days after inoculation using the following scores: 0 (healthy, no symptoms), 1 (leaf yellowing), 2 (leaf necrosis, stunting, and wilt), and 3 (plants died). Disease severity was calculated from the following formula: $[n1 + (n2 \times 2) + (n3 \times 3)]/(n1 + n2 + n3)$, where $n1$ is the number of plants scored as 1; $n2$ is the number of plants scored as 2; and so on. Plants rated with a disease severity of more than 1.0 were regarded as susceptible (S) and those less than 1.0 were considered resistant (R).

Vegetative compatibility group

Nitrate nonutilizing (*nit*) mutants were generated from all isolates of races 1 and 3 on a chlorate-containing minimal medium (MMC) (Puhhalla 1985) and from race 2 isolates on MMC supplemented with biotin (BMMC) 500 µg/l (Ogiso et al. 2002). The phenotype of the mutants was determined as described previously (Correll et al. 1987; Puhhalla 1985). To determine the VCG of each isolate, we examined heterokaryon formation using mutants of standard VCG testers as reported previously (Nishimura 2003; Ogiso et al. 2002).

DNA extraction and PCR amplification

Each isolate was grown in potato dextrose broth (Difco, Detroit, MI, USA) for 2 weeks at 25°C, and the mycelia were collected by filtering through nylon mesh, dried, lyophilized, and ground to a powder. Total genomic DNA was extracted using the procedure reported by Jacobson and Gordon (1990).

The partial IGS region of the ribosomal DNA of *F. oxysporum* was amplified using primers FIGS11 (GTAAGCCGTCCTTCGCCTCG) and FIGS12 (GCAAAATTCAATAGTATGGC) (Kawabe et al. 2005). The polymerase chain reaction (PCR) amplification reactions were performed in 50-µl volumes containing 1.0 µg of DNA template; 1 µM of each primer; 200 µM each of dATP, dGTP, and dTTP; 100 µM dCTP; activated salmon sperm DNA 0.25 mg/ml; 1 mM 2-mercapthoethanol; 2 mM $MgCl_2$; 50 mM KCl; 25 mM TAPS (pH 9.3 at 25°C); and 1.25 units of *Taq* polymerase (TaKaRa, Kyoto, Japan). Amplifications were run in an iCycler (Bio-Rad Japan, Tokyo, Japan) programmed to 94°C for 1 min followed by 35 cycles at 94°C for 1 min, 58°C for 30 s, and 72°C for 1 min, with a final step at 72°C for 10 min. PCR products were electrophoresed on a 1.0% agarose gel in 1x TAE buffer, and the gel was stained with ethidium bromide. Band sizes were estimated by comparison to a 100-bp ladder (Amersham Bioscience, Piscataway, NJ, USA).

DNA sequencing

The PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Chatsworth, CA, USA) and were sequenced directly using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Extension products were analyzed on an ABI 3100 Automated DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Sequences were determined on both strands with primers FIGS11 and FIGS12.

Phylogenetic analysis

For phylogenetic analysis, the sequences were aligned with the program included in Genetyx-WIN v.5.0 (Software Development, Tokyo, Japan). The alignment of all sequences was further optimized manually. The data were analyzed to determine the phylogenetic relationship by the distance and parsimony methods. The distance matrix for the aligned sequences was calculated using Kimura's two-parameter model (1980) and was analyzed with the neighbor-joining (NJ) method (Saitou and Nei 1987) using the program ClustalX v.1.81 (Thompson et al. 1997), excluding positions with gaps and correcting for multiple substitutions. The reliability of the inferred tree was estimated by bootstrap analysis (Felsenstein 1985) using the same program. Parsimony analyses (MP) were done by the Phylogenetic Analysis Using Parsimony (PAUP) program version v.4.0b10 (Sinauer, Sunderland, MA, USA). Confidence limits for branches based on parsimony criteria were estimated by bootstrap analysis of 1000 replicates, and a 50% majority-rule consensus tree was generated. The sequence of *Gibberella fujikuroi* FGSC7610 (obtained from Dr. M. Kawabe, National Institute of Agro-Environmental Sciences) was used as an outgroup for comparison.

Results

Race and VCG determination

Of the 29 isolates tested, 9 Japanese isolates, 2 Californian isolates (HL-1, HL-2), and 3 Italian isolates (LB7, LB10, LB17B) had the same pathogenicity as the standard strain SB1-1 (race 1). Six Japanese isolates had pathogenicity similar to the standard strain F-9501 (race 2), which is pathogenic to cultivars Patriot and Costa Rica No. 4 but not to cultivar Banchu Red Fire. The other six Japanese isolates that were pathogenic to all cultivars tested belonged to race 3. The race of the type strain SUF-762 was not determined because of its weak pathogenicity to cultivar Patriot (Table 1).

The VCGs of 29 isolates were determined using the VCG testers FL1101-n4 (VCG-1, race 1), KM-B-n21 (VCG-2, race 2), N3 (VCG-3-1, race 3), M7 (VCG-3-2, race 3), M104 (VCG-3-3, race 3), and M150 (VCG-3-4, race 3) (Table 1). Fifteen isolates of race 1 were VCG-1, and seven isolates of race 2 were VCG-2. Among six isolates of race 3, four were VCG-3-1, and two were VCG-3-3 (Table 1). The type strain SUF-762 (unknown race) was VCG-3-1.

Phylogenetic analysis

The partial IGS sequence of each strain was analyzed to determine the phylogenetic relationship by the NJ and MP methods. Twenty-nine isolates of FOL belonging to three races, and VCGs were grouped into three phylogenetic groups (PGs) by the NJ analysis as follows: (1) PG-1 consisted of race 1 and VCG-1 that includes Californian and Italian isolates; (2) PG-2 consisted of race 2 and VCG-2; and (3) PG-3 consisted of race 3 and VCG-3-1 or VCG-3-3. The race of the FOL type strain SUF-762 that was isolated in Japan in 1963 was classified as PG-3 (Fig. 1).

In the MP analysis, the 50% majority-rule consensus tree of the optimal trees showed that race 1 and race 2 isolates were monophyletic lineages MG-1 and MG-2, with bootstrap values of 96% and 100%, respectively (Fig. 2). Race 3 isolates were separated into two monophyletic groups, MG-3-1 and MG-3-3 (Fig. 2). Thus, the results by NJ analysis were consistent with those obtained by MP analysis, implying the reliability of these phylogenetic analyses in this experiment.

Race 2 was significantly far from the other races in the NJ tree. Phylogenetic analysis of IGS sequences by NJ and MP methods suggested that race 2 may have been derived from a different ancestor than races 1 and 3.

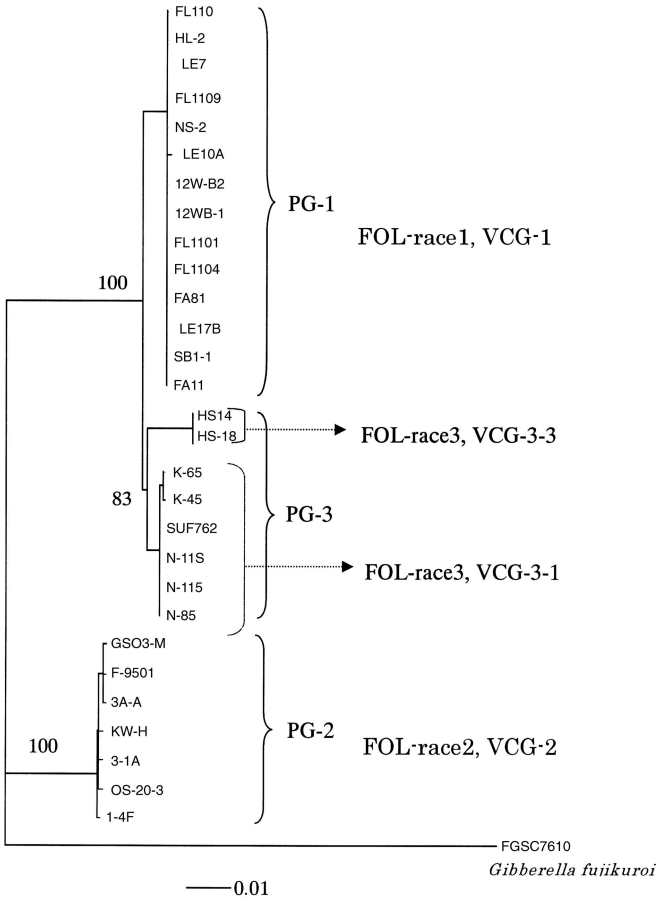

Fig. 1. Neighbor-joining tree illustrating relationships in terms of distance, derived from partial sequences of the rDNA IGS regions in *F. oxysporum* f. sp. *lactucae* and *G. fujikuroi*. Distances were determined by Kimura's two-parameter method. *Gibberella fujikuroi* isolate FGSC7610 was used as the outgroup. *Numbers* alongside branches represent the percentage of congruent clusters in 1000 bootstrap trials when the values were greater than 50%. *Bar* 0.01

Discussion

Fusarium wilt of lettuce resembling root rot disease symptoms in Japan has resulted in a serious problem in several countries. The forma specialis of this pathogen was named f. sp. *lactucum* in California (USA) (Hubbard and Gerik, 1993) but remained unidentified in Italy (Garibaldi et al. 2002). Fujinaga et al. (2003) reported that Japanese and Californian isolates belonged to the same forma specialis based on a study of the races of this pathogen, in which the three races were first reported in Japan. Recently, Matheron (2003) also identified the causal agent of lettuce root rot disease isolated in Arizona (USA) as *F. oxysporum* f. sp. *lactucae*, which belonged to race 1 of FOL that had been isolated in Japan. However, little is known about the phylogenetic relationships between the three races. In this study, phylogenetic analysis of IGS sequences revealed that two isolates, identified as f. sp. *lactucum* and f. sp. *lactucae*, were also confirmed to have an identical genetic background.

Pathogenicity testing on lettuce of 29 isolates, including Californian and Italian isolates, revealed that three races (1, 2, and 3) were observed in Japan and one race (race 1) in the United States and Italy, supporting previous results (Fujinaga et al. 2003; Ogiso et al. 2002). It is noteworthy that races 1, 2, and 3 belonged to VCG-1, VCG-2, and VCG-3 (VCG-3-1, VCG3-2, VCG3-3, VCG3-4), respectively. This result indicates that each race evolved independently with-

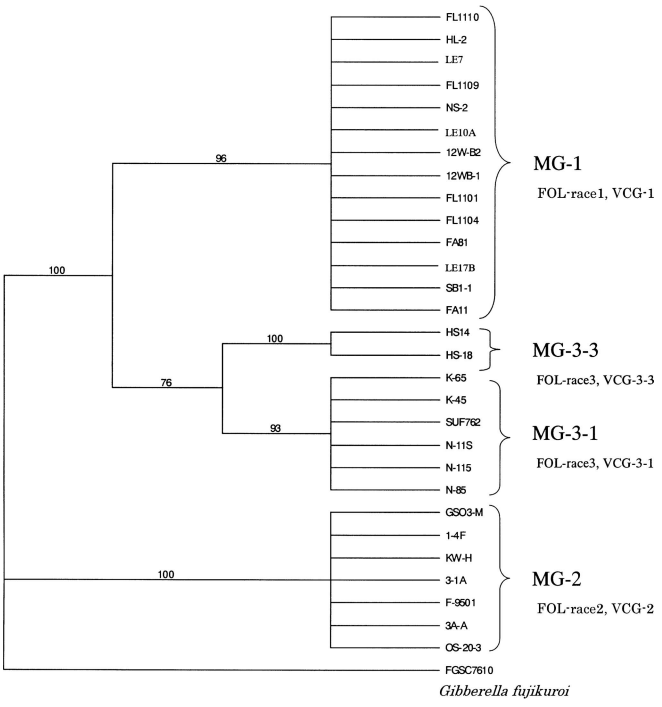

Fig. 2. The 50% majority-rule consensus tree of 45 equally most parsimonious trees (MP tree) generated using the partial sequences of the rDNA IGS region of isolates of *F. oxysporum* f. sp. *lactucae*, with *G. fujikuroi* isolate FSGC7610 as the outgroup. *Numbers* on the branches indicate bootstrap values (%) determined from 10000 replications

out mating with each other. Moreover, these results suggest that Fusarium disease caused by race 1 may be distributed in many geographically separated countries by artificial seed transmission because the same VCG is probably derived clonally from a common ancestor (Kistler, 1997). In contrast, races 2 and 3 have been found in a few restricted regions in Japan, especially race 2, which occurred only in Nagano Prefecture, the major lettuce-producing area of Japan. The reasons for the genetic diversity and geographic distribution of VCG-3 in Japan warrant further investigation.

Analysis of the IGS sequences indicated that the known races (races 1, 2, and 3) of FOL were phylogenetically classified as PG-1, PG-2, and PG-3 by NJ analysis and MG-1, MG-2, and MG-3 by MP analysis, respectively. The isolates in MG-3 were classified into two groups: (1) MG-3-1, which included the type strain SUF-762 and the isolates from Fukuoka Prefecture on Kyushu Island, Japan; and (2) MG-3-3, which contained the isolates from Shizuoka Prefecture on Honshu Island, Japan. These results indicate that the three races are quite different genetically and that the genetic diversity in VCG and rDNA IGS sequences is extremely low within the same race. However, the genetic diversity within VCG-3 should be studied further because VCG-3 was classified into four subgroups by vegetative compatibility testing. In this experiment, we showed that *F. oxysporum* f. sp. *lactucae* is a unique fungus in terms of its relationship between races, VCGs, and IGS groups. Our results suggest that isolates of race 1, race 2, and race 3 evolved independently from different ancestors. Determination of the IGS sequence was a useful indicator of race for this pathogen. Moreover, our results indicate that we should be able to use phylogenetic analysis of the partial sequences of the rDNA IGS region to detect the race of the pathogen and to estimate the geographic distribution of each race.

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