Development of PCR Primers to Identify *Fusarium oxysporum* f. sp. *fragariae*

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Abstract

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Fusarium oxysporum f. sp. *fragariae* is a fungal pathogen causing Fusarium wilt on strawberry. Polymerase chain reaction (PCR) primers that can discriminate *F. oxysporum* f. sp. *fragariae* from nonpathogenic *F. oxysporum* would greatly assist pathogen identification. In order to develop a molecular diagnostic tool for this pathogen, transposable elements in the pathogen were characterized and used for designing a specific set of PCR primers. Portions of the transposable elements *Fot3*, *Han*, *Hop*, *Hornet1*, and *Skippy* were detected in all 33 strains of *F. oxysporum* f. sp. *fragariae* tested by PCR, whereas *Foxy* was detected in 32 strains and *Impala* sequences were detected in 30 strains. Two types of sequences were detected for *Hop*, two types for *Impala*,

and three types for *Skippy*. The genomic region between *Han* and *Skippy* was amplified by an inter-retrotransposon amplified polymorphism technique, and PCR primers (FofraF and FofraR) to specifically identify *F. oxysporum* f. sp. *fragariae* were designed from this region. The developed PCR primers discriminated *F. oxysporum* f. sp. *fragariae* strains from nonpathogenic *F. oxysporum* strains and five other formae speciales. Conidia of *F. oxysporum* f. sp. *fragariae* could be detected in brown lowland-type soil by PCR using the primers. After preculturing the soil sample on FoG2 medium, 1×10^2 conidia/g of soil could be detected; without preculturing, 1×10^3 conidia/g of soil were detected.

Strawberry (*Fragariae* × *ananassa* Duch.) is an economically important crop grown worldwide. However, most of the strawberry cultivars are highly susceptible to several destructive and economically important soilborne diseases such as Phytophthora rot, Verticillium wilt, and Fusarium wilt. Fusarium wilt was first reported in Australia in 1965 (25) and has since been reported in Japan (14), Korea (16), China (26), Spain (1), and the United States (10). The causal agent, *Fusarium oxysporum* f. sp. *fragariae* Winks & Y.N. Williams, is a haploid mitospore fungus and is difficult to control because the pathogen survives as chlamydospores in soil for many years. Early detection and diagnosis of the pathogen in plants and soils is essential for development of an effective disease control strategy.

F. oxysporum is distributed in soils worldwide and causes vascular wilt or root rot disease in over 100 plant species. However, hosts for individual strains of this pathogen are confined to a single or a few plant species. Therefore, *F. oxysporum* is subdivided into formae speciales based on host specificity. Furthermore, some formae speciales include strains that only cause disease on certain varieties of a plant species and are subdivided into races. In addition, pathogenic strains of *F. oxysporum* are morphologically indistinguishable from nonpathogenic strains. Selective media are useful for isolating *Fusarium* spp. from plants, soils, and air. However, species identification is quite difficult using only colony morphology, and some *Fusarium* spp. such as *F. oxysporum* include nonpathogenic strains. Despite the importance of rapid pathogen detection for disease control, identification of formae speciales within *F. oxysporum* often requires a troublesome and time-consuming test.

Molecular markers to identify formae speciales or races of *F. oxysporum* would accelerate pathogen identification. Although host-determining factors are ideal markers, none have been identified for *F. oxysporum* except for the *secreted in xylem* (*SIX*) gene of *F. oxysporum* f. sp. *lycopersici*. Forma-specialis- or race-specific markers have been developed by using random amplified polymorphic DNA (RAPD) analyses followed by sequencing to design primers for sequence-characterized amplified regions or transposable elements (12).

Many types of transposable elements have been identified in *F. oxysporum*, and approximately 5% of the *F. oxysporum* genome appears to be composed of transposable elements (4). Whole-genome sequencing of *F. oxysporum* f. sp. *lycopersici* revealed that about 28% of the entire genome was composed of repetitive sequences, and many types of transposable elements were detected. Five distinct superfamilies have been characterized: *Tc1-mariner* with the *Pogo* and the *Impala* families, *hAT* with 13 distinct families, *Mutator*-like with 5 families, miniature inverted repeat transposable elements (MITEs) with 6 distinct families, and a *Helitron*-like family (13).

Molecular markers specific for *F. oxysporum* f. spp. *albedinis* (6) and *chrysanthemi* (18) were developed with a transposable element, *Fot1*. Another transposable element, *Impala*, was used to develop a molecular marker for *F. oxysporum* f. sp. *dianthi* race 4 (3). The inter-retrotransposon amplified polymorphism (IRAP) technique was used to develop molecular markers for *F. oxysporum* f. sp. *lactucae* race 1 (19).

In this study, our objectives were to (i) characterize transposable elements in *F. oxysporum* f. sp. *fragariae* and (ii) develop diagnostic polymerase chain reaction (PCR) primers for pathogen-specific elements to be used in pathogen detection. Developed primers were validated using pathogenic and nonpathogenic *Fusarium* strains obtained from the area in Japan where strawberry plants are cultivated and were used to detect artificially infested conidia of *F. oxysporum* f. sp. *fragariae* in soil.

Materials and Methods

Fungal strains. In total, 44 strains of *F. oxysporum* (Table 1) were used for PCR detection of transposable elements; these strains included 33 strains of *F. oxysporum* f. sp. *fragariae* from

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*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary table is included in the online edition.

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geographically dispersed prefectures in Japan, 1 strain each of five other formae speciales, and 6 strains that were isolated from strawberry root but did not show pathogenicity to strawberry (i.e., non-pathogenic strains). *F. fujikuroi* and *F. raminearum* s. str. were also used to confirm the specificity of diagnostic PCR for *F. oxysporum* f. sp. *fragariae*. In all, 157 *Fusarium* strains isolated from the area where strawberry plants are cultivated in Nara prefecture, Japan in 2008 and 2009 (Supplementary Table S1) with FoG2 medium (17) were used for validation of diagnostic PCR developed in this study. Strains were maintained on potato dextrose agar (PDA) and kept at 6°C for short-term storage and at -80°C in 50% glycerol for long-term storage.

Genomic DNA extraction. Each strain was cultured in potato dextrose broth (PDB) for 3 to 4 days. The resulting mycelial mat (2 to 3 cm in diameter) was air dried and, thereafter, ground to a powder by vortexing with small pieces of stainless steel wire in a microtube (22); then, DNA was extracted using a MagExtractorPlant-

Genome kit according to the manufacturer's instructions (Toyobo). The final DNA pellet was dissolved in 100 µl of water and kept at -30°C until used.

PCR detection of transposable elements. Selected regions of transposable elements were amplified by PCR with the primer sets shown in Table 2. Forward (F) and reverse (R) primers were designed from sequences of *Foxy*, *Impala*, and *Hornet1* in GenBank, and primers used in amplification of the other elements were obtained from previously published sequences (Table 2). Complementary sequences of Gagi and Gaga primers (19) were used as SkippyF and -R, respectively. Amplification reactions were carried out in a 10-µl reaction mixture that consisted of 1× PCR buffer (Applied Biosystems), 200 µM dNTPs, 1 µM each primer, 0.025 units of Taq DNA polymerase (Applied Biosystems), and 1 µl of genomic DNA. PCR was performed in an iCycler thermal cycler (Bio-Rad Laboratories), using the following cycling parameters: 94°C for 2 min and 25 cycles of 94°C for 1 min, 63°C (54°C only

Table 1. Strains used in this study

Species, strain	Origin ^b	Year ^c	Source ^d	forma specialis	Path ^e	Primer pair ^a										
						Fot3FR	HanF, HanR	HopF, HopR	Hornet1F, Hornet1R	SkippyF, SkippyR	FoxyF, FoxyR	ImpalaF, ImpalaR	HanRC, SkippyFC	FofraF, FofraR	PFO3, PFO2	
<i>Fusarium oxysporum</i>																
MAFF305557	Unknown	1978	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
MAFF305558	Unknown	1978	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
MAFF727510	Nara	Unknown	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
MAFF744009	Fukuoka	Unknown	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF1	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF10	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF11	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF12	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	-	-	+	+	+	+
FOF30	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF50	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF68	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF84	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF85	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF87	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF97	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF98	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF100	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF103	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	-	+	+	+	+
FOF104	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF120	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF123	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF126	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
FOF127	Tochigi	2000	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
KMK	Tochigi	2006	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
UKA1	Tochigi	2009	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
UKA2	Tochigi	2009	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
UKA3	Tochigi	2009	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	-	+	+	+	+
NRF0501	Nara	2005	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
NRF0508	Nara	2005	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
NRF0604	Nara	2006	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
NRF0801	Nara	2008	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
NRF0804	Nara	2008	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
NRF0820	Nara	2008	Strawberry	<i>fragariae</i>	+	+	+	+	+	+	+	+	+	+	+	+
MAFF727519	Shizuoka	1984	Strawberry	Nonpathogenic	-	-	+	+	+	+	+	+	+	-	-	+
FOF13	Tochigi	2000	Strawberry	Nonpathogenic	-	-	+	+	-	+	-	-	+	-	-	+
FOF25	Tochigi	2000	Strawberry	Nonpathogenic	-	-	+	+	-	+	-	-	+	-	-	+
FOF55	Tochigi	2000	Strawberry	Nonpathogenic	-	+	+	-	+	+	-	+	-	-	-	+
FOF113	Tochigi	2000	Strawberry	Nonpathogenic	-	-	+	-	-	+	-	+	-	-	-	+
FOF121	Tochigi	2000	Strawberry	Nonpathogenic	-	-	+	+	-	-	-	-	-	-	-	+
GUS77	Unknown	Unknown	Unknown	<i>cucumerinum</i>	NT	-	+	-	+	+	+	+	-	-	-	+
GUS80	Unknown	Unknown	Unknown	<i>laganariae</i>	NT	+	+	+	+	+	+	+	+	-	-	+
GUS76	Unknown	Unknown	Unknown	<i>melonis</i>	NT	+	+	+	+	+	+	+	+	-	-	+
GUS126	Gifu	Unknown	Tomato	<i>radicis lycopersici</i>	NT	+	+	+	+	+	+	+	+	-	-	+
GUS78	Unknown	Unknown	Unknown	<i>spinaciae</i>	NT	+	+	+	+	+	+	+	+	-	-	+
<i>F. fujikuroi</i>																
NRF0812	Nara	2008	Strawberry	...	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-
<i>F. graminearum</i> s. str.																
0407011	Fukushima	2004	Wheat	...	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-

^a Presence (+) or absence (-) of PCR product with the size of amplified length indicated in Table 2. NT = not tested.

^b Geographic origin (prefecture of Japan).

^c Year of isolation.

^d Isolation source.

^e Result of pathogenicity test in this study.

for *Hop*) for 1 min, and 72°C for 1 min. PCR products were detected by electrophoresis in a 1.0% agarose gel in Tris-borate-EDTA buffer. Band size was estimated by comparison to a 100-bp ladder marker (New England Biolabs). The DNA bands were visualized under UV light after staining the gel in ethidium bromide at 0.5 µg/ml.

Sequencing of transposable elements. PCR products of the expected size for each transposable element from strain FOF10 were sequenced with the same primers that were used for PCR (Table 2). Gels containing the expected size of PCR products after electrophoresis with 1% SeaKem GTG Agarose (Takara Bio Inc.) were used for purification by the Nucleospin Gel and PCR Clean Up kit (Takara Bio Inc.). At first, purified PCR products (except for *Fot3*) were directly sequenced by BigDye terminator V3.1 cycle sequencing kits (Applied Biosystems) and then run on an ABI 3100 genetic analyzer (Applied Biosystems). *Foxy*, *Han*, and *Hornet1* showed clear electropherogram traces whereas *Hop*, *Impala*, and *Skippy* showed mixed signals (multiple peaks) at some portions of the trace. In order to obtain clear signals, these PCR products were cloned into a plasmid, pCR4-TOPO (Life Technologies Inc.). Plasmids were extracted with an ABI PRISM Miniprep Kit (Life Technologies Inc.) according to the manufacturer's instructions. For each transposable element, 7 to 12 colonies in which insert DNA was confirmed were selected for further screening by restriction enzyme digestion. Insert DNA was amplified by PCR with primer pairs corresponding to each transposable element (Table 2) and then digested by individual restriction enzymes: *AluI*, *HhaI*, *HinfI*, *MseI*, *RsaI*, and *Sau3AI*. Fragment patterns were compared among colonies of each transposable element with 2.5% agarose gel electrophoresis. Plasmids with different patterns were used for sequencing with M13M4 (5'-GTTTCCAGTCACGACGTTG TAAA-3') and M13RV (5'-CAGGAAACAGCTATGACCATG ATTA-3') primers. The PCR product of *Fot3* was also cloned into the pCR4-TOPO vector (Life Technologies) and sequenced with M13M4, M13RV and HS475 primers (5'-CAGTTTCCAGTCACT CTCCA-3') because the PCR product has the same primer sequence at both ends. Nucleotide sequences were edited and aligned using Sequencher (version 3.1.1; Gene Codes) and Genetyx (version 4.0; Genetyx). Sequences have been deposited in GenBank (Table 2).

IRAP-PCR. Complementary sequence primers (FC or RC) for *Fot3*, *Han*, *Hop*, *Hornet1*, and *Skippy* were used for IRAP-PCR

(Table 2). In total, 23 primer combinations out of 60 possible combinations were examined using *F. oxysporum* f. sp. *fragariae* strain FOF10, five other formae speciales (strains GUS76, -77 -78, -80, and -126), and a nonpathogenic *F. oxysporum* strain FOF13. Five combinations assumed the presence of the same type of transposable element in the same direction: *Fot3*FC only, *Han*FC and RC, *Hop*FC and RC, *Hornet1*FC and RC, and *Skippy*FC and RC. Eight combinations assumed the presence of the same type of transposable element in the inverse direction: *Han*FC only, *Han*RC only, *Hop*FC only, *Hop*RC only, *Hornet1*FC only, *Hornet1*RC only, *Skippy*FC only, and *Skippy*RC only. Ten combinations assumed the presence of different types of transposable elements in the same or the inverse direction: *Fot3*FC and *Hop*FC, *Fot3*FC and *Hop*RC, *Han*FC and *Skippy*FC, *Han*FC and *Skippy*RC, *Han*RC and *Skippy*FC, *Han*RC and *Skippy*RC, *Hornet1*FC and *Hop*FC, *Hornet1*FC and *Hop*RC, *Hornet1*RC and *Hop*FC, and *Hornet1*RC and *Hop*RC. The reaction mixture was the same used for transposable elements PCR. PCR was performed by the following cycling parameters: 94°C for 2 min and 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. PCR products were detected by 1.0% agarose gel electrophoresis.

Designing *F. oxysporum* f. sp. *fragariae*-specific primers and multiplex PCR with *F. oxysporum*-specific primers. The 268-bp DNA detected from *F. oxysporum* f. sp. *fragariae* strain FOF10 by IRAP-PCR with *Han*RC and *Skippy*FC primers was sequenced with the same primers (Fig. 1). *FofraF* and *FofraR* primers (renamed from HS430 and HS432 primers by Suga et al. [21]) were newly designed from the revealed sequence to avoid extra DNA amplifications (Fig. 1; Table 2).

Optimum conditions for multiplex PCR with *FofraF* and *FofraR* primers and *F. oxysporum*-specific primers (PFO2 and PFO3) (5) were established using *F. oxysporum* f. sp. *fragariae* strain FOF10, five other formae speciales (strains GUS76, -77 -78, -80, and -126), nonpathogenic *F. oxysporum* strain FOF13, *F. fujikuroi*, and *F. graminearum* s. str. (Table 1). Annealing temperatures and primer concentrations were optimized, and final amplification reaction conditions were determined in a 10-µl reaction mixture that consisted of 1× GoTaq buffer (Promega Corp.), 200 µM dNTPs, 2 µM each *FofraF* and *FofraR* primers (Table 2), 1 µM each PFO2 and PFO3 primers (5), 2.5 units of Go Taq polymerase (Promega Corp.), and 2 µl of genomic DNA. PCR was performed using the

Table 2. Primers used in this study

	Primer sequence (5'-3') ^a			Length (bp)	
	Forward	Reverse	Target sequence	Expected ^b	Amplified ^c
Fot3FR	CCGGCAAGCAAAAAAGTTTC (Fot3, Hua-Van et al. [8])	Fot3FR was used as reverse primer	Fot3	2,117 (AF076631)	2,133 (JX204293)
Fot3FRC	GAAACTTTTTTTTGCTGCCGG		Between Fot3		
HanF	TGAATGTTGGAGGGTTCACC (S1, Hua-Van et al. [8])	HanR CAGATATTGAGGTGAATCGTG (S2, Hua-Van et al. [8])	Han	200 (AF076629)	200 (JX204294)
HanFC	GAACCTCCAACATTCAACA (Hani, Pasquali et al. [19])	HanRC CACGATTCACCTTAATATCTGACA (Hana, Pasquali et al. [19])	Between Han		
HopF	AAGAGGCATAGGTGGCCTTAT	HopR TTACACAGGCTTTCCTTTCG	Hop	394 (AY267761)	394 (JX204295), 412 (JX204296)
HopFC	ATAAGGCCACCTATGCCTCTT	HopRC TTATTCGCACGACCGTGGTG	Between Hop		
Hornet1F	TGTGGATCCATATCCATTCCA	Hornet1R TGATCGTTGACGATCGAGAA	Hornet1	792 (AF076626)	798 (JX204297)
Hornet1FC	TGGAATGGATATGGATCCACA	Hornet1RC TTCTCGATCGTCAACGATCA	Between Hornet1		
SkippyF	TGTTACGACCTAGCGGTTA	SkippyR TGTGACGGTTGGTTCC	Skippy	429 (L34658)	396 (JX204298), JX204299, JX204300)
SkippyFC	TAACCGCTAGGGTCGTAACA (Gagi, Pasquali et al. [19])	SkippyRC GGGAACCAACCGTCACA (Gaga, Pasquali et al. [19])	Between Skippy		
FoxyF	CTGGGTGGCTGAACGAAG	FoxyR GGAATTTGGAGAGTTCGCCT	Foxy	483 (AJ250814)	460 (JX204301)
ImpalaF	GCCTCAAAGCTYAYAGAAGA	ImpalaR TAGATGCKMTGGCCTTGA	Impala	459 (AF282722), AF363434, AF363409)	459 (JX204302), 464 (JX204303)
SkippyFC	Described above	HanRC Described above	Between Han and Skippy		268 (JX204304)
FofraF	CAGACTGGGGTCTTAAAGTT	FofraR AACCGTAGGGTCGTAACAAA	Between Han and Skippy		239
PFO3	CGGGGGATAAAGGCGG (Edel et al. [5])	PFO2 CCCAGGTATTACACGGT (Edel et al. [5])	18S rDNA		70

^a Primers previously published are shown with the reference in parentheses. Others were designed in this study.

^b Length was as expected based on the GenBank accession number as shown in parentheses.

^c Length amplified from *Fusarium oxysporum* f. sp. *fragariae* strain FOF10. GenBank accession number is shown in parentheses.

following cycling parameters: 94°C for 2 min and 30 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min. PCR products were detected by 2.5% agarose gel electrophoresis. Expected sizes of DNA fragments amplified by the FofraF and FofraR primer pair and PFO2 and PFO3 primer pair were 239 bp (Fig. 1) and 70 bp of DNA (5), respectively. The sequence of the 239-bp DNA fragment amplified from *F. oxysporum* f. sp. *fragariae* strain FOF10 was confirmed by sequencing with FofraF and FofraR primers (Table 2).

PCR detection of *F. oxysporum* f. sp. *fragariae* in soils. Brown lowland-type soils were artificially infested with conidia of *F. oxysporum* f. sp. *fragariae* strain FOF10. Conidia produced on synthetic low-nutrient agar medium were harvested with sterile water and adjusted at 1×10^6 /ml. This suspension was sequentially diluted to obtain 10^5 , 10^4 , or 10^3 conidia/ml and then 20 μ l of each or sterile water was mixed with 0.2 g of brown lowland-type soil (i.e., 0, 10^2 , 10^3 , 10^4 , or 10^5 conidia/g of soil). DNA was extracted from the soil containing conidia according to Kageyama et al. (9), with some modifications. For testing the effect of preculturing, 40 μ l of FoG2 medium without agar (17) was added to the soil containing conidia and incubated for 4 days at 25°C before DNA extraction. RNase A was not used in this study, and the mixture was kept at 60°C for 15 min instead of at 50°C for 60 min. After incubation, 150 μ l of 3 M NaOAc was added and the samples were kept on ice for 15 min. The suspension was centrifuged at $18,000 \times g$ for 10 min, the upper layer (300 μ l) was transferred to a clean tube, and DNA was further purified with a MagExtractor-PlantGenome kit (Toyobo). The upper layer of suspension was mixed with 600 μ l of absorbent and 40 μ l of magnetic bead suspensions. Then, purification steps were continued according to the manufacturer's instructions. Finally, DNA was eluted with 50 μ l of water and 1 μ l was used for PCR. PCR conditions were the same as the multiplex PCR, with the following modifications: only FofraF and FofraR primers were used, 0.4 mg of bovine serum albumin was included in the reaction mixture, and the thermal cycling number was increased from 30 to 40. The experiment from preparation of the soil containing conidia was repeated twice.

Pathogenicity test. Pathogenicity tests were performed in a greenhouse during July and August in Japan. Strains were cultured in 100 ml of PDB at 25 to 28°C for 10 days. Conidial suspensions obtained by culture filtration with two layers of gauze were adjusted to approximately 1×10^5 /ml with water, and 10 ml was poured into a pot (9 cm in diameter) in which nursery-grown strawberry (*Fragariae* $\times$ *ananassa* Duch. 'Asukarubi') plants were growing. The pots were returned to a greenhouse and foliar distortions, a typical symptom of Fusarium wilt, were observed 1 month after inoculation.

Results

Transposable elements in *F. oxysporum* f. sp. *fragariae*. PCR was used to amplify the following transposable elements from *F. oxysporum* f. sp. *fragariae*: *Fot3*, *Foxy*, *Han*, *Hop*, *Hornet1*, *Impala*, and *Skippy* (Table 1). DNA fragments of the expected sizes for *Fot3*, *Han*, *Hop*, *Hornet1*, and *Skippy* were obtained for all 33 strains of *F. oxysporum* f. sp. *fragariae* tested, whereas those for *Foxy* were amplified in all but 1 strain and those for *Impala* were amplified in all but 3 strains of *F. oxysporum* f. sp. *fragariae* (Table 1).

The PCR products for each transposable element (except for *Fot3* from strain FOF10) were directly sequenced. The sequences of *Foxy*, *Han*, and *Hornet1* could be determined, whereas the sequences for *Hop*, *Impala*, and *Skippy* showed mixed signals (multiple peaks) at some portions of the trace. Therefore, PCR products were cloned into a plasmid vector, and the plasmids that showed different restriction enzyme patterns for each transposable element were sequenced. Two types of sequences were detected for *Hop*, two types for *Impala*, and three types for *Skippy* (Table 2). The PCR product of *Fot3* was also sequenced after cloning into a plasmid. A BLAST search showed that all of the PCR products amplified from strain FOF10 using transposable element primer pairs have the highest similarities to the corresponding transposable elements of *F. oxysporum* in GenBank.

The expected sizes of DNAs for each transposable element examined were amplified in some nonpathogenic strains and also in five formae speciales (Table 1). Therefore, *F. oxysporum* f. sp. *fragariae* could not be uniquely identified by these PCR primer pairs.

IRAP-PCR. In all, 23 primer combinations were examined using *F. oxysporum* f. sp. *fragariae* strain FOF10, each strain of five other formae speciales (strain GUS76, -77 -78, -80, and -126), and a nonpathogenic *F. oxysporum* strain FOF13. *Fot3*, *Han*, *Hop*, *Hornet1*, and *Skippy* were detected from all 33 strains of *F. oxysporum* f. sp. *fragariae* tested, and their complementary sequence primers (FC or RC) were used for IRAP-PCR. Nine primer combinations resulted in no amplification of these seven strains. Fourteen combinations amplified DNAs in at least one strain. The HanRC and SkippyFC combination amplified a 268-bp DNA fragment only in *F. oxysporum* f. sp. *fragariae* strain FOF10, although additional DNAs were also amplified from this strain and the other strains (Fig. 2). Specific amplification of the 268-bp DNA fragment was confirmed in all 33 strains of *F. oxysporum* f. sp. *fragariae* (Table 1).

Designing *F. oxysporum* f. sp. *fragariae*-specific primers and multiplex PCR with *F. oxysporum*-specific primers. FofraF and FofraR primers were designed to avoid unspecific DNA amplifica-

Fig. 1. Positions of FofraF and FofraR primers in the *Fusarium oxysporum* f. sp. *fragariae*-specific 268-bp DNA amplified by polymerase chain reaction (PCR) with the HanRC and SkippyFC primers. Lowercase characters indicate the positions of the HanRC and SkippyFC primers. FofraF and FofraR primers amplify a 239-bp DNA fragment by PCR.

tions in *F. oxysporum* f. sp. *fragariae* (Fig. 1), and conditions for multiplex PCR with *F. oxysporum*-specific primers (PFO2 and PFO3) (5) were standardized (Fig. 3). The expected sizes of DNAs, 239 bp for the FofraF and FofraR primer pair and 70 bp for the PFO2 and PFO3 primer pair, were amplified from all 33 strains of *F. oxysporum* f. sp. *fragariae*, whereas only a 70-bp DNA fragment was amplified in the five other formae speciales and the nonpathogenic *F. oxysporum* (Table 1).

Validation of developed multiplex PCR. The accuracy of diagnosis by the multiplex PCR was confirmed by testing 157 *Fusarium* strains from the area where strawberry plants are cultivated in Nara prefecture. The results of the multiplex PCR were compared with the results of a pathogenicity test. The multiplex PCR determined that 79 strains were *F. oxysporum* f. sp. *fragariae* based on amplification of both the 239- and 70-bp DNA fragments. Of the 79 strains, 73 showed typical disease symptoms on strawberry, whereas 6 strains did not show any symptoms on strawberry by the pathogenicity test. The pathogenicity test was repeated twice for these six strains but did not produce symptoms in the subsequent tests. The multiplex PCR determined that the remaining 58 strains were nonpathogenic *F. oxysporum* based on amplification of only the 70-bp DNA fragment, and 20 strains were some species other than *F. oxysporum* based on no amplification. None of these 58 strains produced symptoms on strawberry by the pathogenicity test.

PCR detection of *F. oxysporum* f. sp. *fragariae* in soils. Conidia of *F. oxysporum* f. sp. *fragariae* were mixed in soils at 0, 10², 10³, 10⁴, or 10⁵ conidia/g of soil, and DNA was extracted immediately or after preculturing in FoG2 medium (17). Positive amplifications were observed in samples where the conidia levels were 10³, 10⁴, and 10⁵ conidia/g of soil without preculturing by PCR with the FofraF and FofraR primer pair (Fig. 4). When samples were precultured, positive amplifications were observed in samples where the conidia levels were 10², 10³, 10⁴, and 10⁵ conidia/g (Fig. 4).

Discussion

In this study, several transposable elements (*Fot3*, *Han*, *Hop*, *Hornet1*, and *Skippy*) were detected in *F. oxysporum* f. sp. *fragariae* by PCR. At the least, several sequences of *Hop*, *Impala*, and *Skippy* are present in a single strain of *F. oxysporum* f. sp. *fragariae*. Based on these PCR results, IRAP-PCR was performed and a PCR method was developed to identify *F. oxysporum* f. sp. *fragariae*.

Soilborne diseases of strawberry cause serious economic losses worldwide and rapid detection is important for optimal disease control. PCR primers have been developed for detection of Phytophthora rot pathogens *Phytophthora nicotianae* and *P. cactorum* (11) and Verticillium wilt pathogen *Verticillium dahliae* (2) but not for the Fusarium wilt pathogen. In the case of Phytophthora rot and

Verticillium wilt, PCR primers that can identify the fungus at species level are sufficient for pathogen identification. However, such primers are inadequate for identification of the Fusarium wilt pathogen because saprobic *F. oxysporum* is ubiquitous and only *F. oxysporum* f. sp. *fragariae* causes Fusarium wilt on strawberry. PCR primers that can discriminate *F. oxysporum* f. sp. *fragariae* from nonpathogenic *F. oxysporum* are required for pathogen identification.

Diagnostic PCR rather than pathogenicity testing is more effective for the quick determination of pathogenic strains. PCR primers to discriminate forma specialis or race of *F. oxysporum* have been designed using RAPD analyses and transposable elements (12). Recently, Southwood et al. (20) developed diagnostic primers for two vegetative compatibility groups of *F. oxysporum* f. sp. *cepae* by IRAP-PCR. In this study, HanRC and SkippyFC primers amplified a 268-bp DNA fragment from *F. oxysporum* f. sp. *fragariae* by IRAP-PCR (Fig. 2). A BLAST search indicated that the nucleotide sequence of approximately 200 bp of amplified DNA at the SkippyFC site was similar to the IRAP-Two sequence obtained from *F. oxysporum* f. sp. *cepae* (20). Similar sequences were also found in the genomic sequences of *F. oxysporum* f. sp. *melonis* and *F. oxysporum* II5 (Fusarium comparative sequencing project, Broad Institute of Harvard and MIT; <http://www.broadinstitute.org/>). When the remaining 43-bp sequence without the HanRC primer sequence was used for the BLAST search, the longest match was 29 bp in the *F. oxysporum* PHW808 genome sequence (Fusarium comparative sequencing project, Broad Institute of Harvard and MIT; <http://www.broadinstitute.org/>). Similar sequences of 23 and a 27 bp were also detected in the genome of *F. oxysporum* f. sp. *melonis*. Consequently, *F. oxysporum* f. sp. *melonis* has sequences similar to both to approximately 200 bp and the remaining 43 bp of the target sequence for *F. oxysporum* f. sp. *fragariae* diagnostic PCR. However, the supercontigs were different for the similar sequences. In addition, no PCR product was detected with the FofraF and FofraR primer pair in *F. oxysporum* f. sp. *melonis* (Table 1).

Transposable elements are enriched in the virulence chromosomes in tomato wilt pathogen *F. oxysporum* f. sp. *lycopersici* (13,24) and the pathogenicity cluster of the pea root rot pathogen *Fusarium solani* f. sp. *pisi* (7,23). Compared with nonpathogenic strains, this study revealed that all six formae speciales, including *F. oxysporum* f. sp. *fragariae*, have many types of transposable elements. Because more than 100 formae speciales are known in *F. oxysporum*, primers used for IRAP-PCR in this study may be useful to develop a molecular marker for identification and detection of other forma specialis and also to investigate the virulence region of the *F. oxysporum* genome.

Transposable elements are grouped into two classes: class I elements mobilize via an RNA intermediate and class II elements via a DNA cut-and-paste mechanism. IRAP-PCR targets the region

Fig. 2. Inter-retrotransposon amplified polymorphism polymerase chain reaction (PCR) with the HanRC and SkippyFC primers. PCR products separated by electrophoresis in a 1.0% agarose gel. Lane 1, *Fusarium oxysporum* f. sp. *fragariae* strain FOF10; lane 2, nonpathogenic *F. oxysporum* strain FOF13; *F. oxysporum* f. sp. *melonis*; lane 4, *cucumerinum*; lane 5, *spinaciae*; lane 6, *laganariae*; lane 7, *radicle lycopersici*; lane M, 100-bp ladder. An arrow indicates *F. oxysporum* f. sp. *fragariae*-specific 268-bp DNA fragment.

Fig. 3. Multiplex polymerase chain reaction (PCR) with *Fusarium oxysporum* f. sp. *fragariae*-specific primers and *F. oxysporum*-specific primers. PCR products separated by electrophoresis in a 2.5% agarose gel. Lane 1, *F. oxysporum* f. sp. *fragariae* strain FOF10; lane 2, *F. oxysporum* f. sp. *radicle lycopersici*; lane 3, nonpathogenic *F. oxysporum* strain FOF13; lane 4, *F. fujikuroi*; lane 5, *F. graminearum* s. str.; lane M, 100-bp ladder. White and black arrows indicate an *F. oxysporum* f. sp. *fragariae*-specific DNA fragment and an *F. oxysporum*-specific DNA fragment, respectively.

Fig. 4. A, Polymerase chain reaction (PCR) detection of *Fusarium oxysporum* f. sp. *fragariae* in soils without preculturing. PCR products with the FofraF and FofraR primers separated by electrophoresis in a 1.5% agarose gel. Lane M, 100-bp ladder; lane 1, *F. oxysporum* f. sp. *fragariae* strain FOF10 genomic DNA and lanes 2–6, conidia of the strain FOF10 were mixed in soils at rates of 0, 10^2 , 10^3 , 10^4 , or 10^5 conidia/g of soil, respectively. **B,** PCR detection of *Fusarium oxysporum* f. sp. *fragariae* in soils after preculturing. Same as A, except DNA was extracted after preculturing in FoG2 medium for 4 days at 25°C.

between the long terminal repeat of class I transposable elements. This region is not influenced by transposition events as can be the case with active class II transposable elements and, therefore, is more suitable for use as a molecular marker. The FofraR primer is very similar to the SkippyFC primer (Gagi primer in Pasquali et al. [19]), whereas the FofraF primer was designed from an internal region between HanRC and SkippyFC. Consequently, the PCR specificity with these primers should be stable because no false negatives were detected by the diagnostic PCR in this study. However, six strains determined as *F. oxysporum* f. sp. *fragariae* by diagnostic PCR with FofraF and FofraR primers did not produce symptoms on strawberry by the pathogenicity test. The results of both diagnostic PCR and pathogenicity tests were repeatedly confirmed. There are two possibilities for this incongruence. One possibility is attributed to the aggressiveness of the strains. The aggressiveness in *F. oxysporum* f. sp. *fragariae* varies among strains, and low aggressiveness may not be detected by the pathogenicity test using only one strawberry cultivar, Asukarubi, in this study. Otherwise, degeneration during culturing on media may have contributed to the loss in pathogenicity of these strains. Another possibility is that diagnostic PCR may have generated a false positive. The stability of the diagnostic PCR could also be associated with the genetic variation of *F. oxysporum* f. sp. *fragariae*. Fusarium wilt of strawberry has occurred worldwide recently and RAPD analyses showed a high level of genetic variation in Korea (15,16). However, genetic variation of this pathogen has not been well investigated to date. Therefore, further studies will be needed to analyze the accuracy of the diagnostic PCR developed in this study using field isolates with more varied geographic origins.

Selective media have been used to isolate *F. oxysporum*, and a time-consuming pathogenicity test is required to determine the identity of pathogens after isolation. The diagnostic PCR developed in this study is beneficial for control of Fusarium wilt of strawberry because it can quickly determine the identity of the pathogen and can handle many samples simultaneously. This technique may also be useful for the direct detection of *F. oxysporum* f. sp. *fragariae* from field soil, though PCR detection was demonstrated by using artificially prepared soil. PCR amplification was successfully observed with brown lowland-type soil containing more than 10^3 conidia of *F. oxysporum* f. sp. *fragariae* per gram of soil. Generally, the sensitivity of PCR detection of pathogens from soil is influenced by many factors, especially the purity of extracted DNA (9). Preculturing might increase the sensitivity of PCR detection because a 10-fold increase was observed in this study, although the effect may be limited depending on soil types or pathogen source. Not only the presence of pathogens but also the concentration of pathogens is important for control of soilborne

pathogens. Our future studies will be focused on the quantitative detection of *F. oxysporum* f. sp. *fragariae* from soil using the PCR methods developed in this study.

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