Detection of *Fusarium oxysporum* f. sp. *dianthi* in Carnation Tissue by PCR Amplification of Transposon Insertions

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ABSTRACT

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Strains of the carnation wilt pathogen, *Fusarium oxysporum* f. sp. *dianthi*, can be distinguished by DNA fingerprint patterns, using the fungal transposable elements *Fot1* and *impala* as probes for Southern hybridization. The DNA fingerprints correspond to three groups of *F. oxysporum* f. sp. *dianthi* strains: the first group includes isolates of races 1 and 8; the second group includes isolates of races 2, 5 and 6; and the third group includes isolates of race 4. Genomic DNAs flanking race-associated insertion sites of *Fot1* (from races 1, 2, and 8) or *impala* (from race 4) were amplified by the inverse polymerase chain reaction (PCR) technique. These regions were cloned and sequenced, and three sets of primers overlapping the 3′ or 5′ end of the transposon and its genomic insertion were designed. Using fungal genomic DNA as template in PCR experiments, primer pairs generated amplification products of 295, 564 and 1,315 bp, corresponding to races 1 and 8; races 2, 5, and 6; and race 4, respectively. When multiplex PCR was performed with genomic DNA belonging to races 1 and 8, 2, or 4, single amplimers were generated, allowing clear race determination of the isolate tested. PCR was successfully performed on DNA extracted from susceptible carnation cv. Indios infected with isolates representative of races 1, 2, 4, and 8.

Additional keywords: *Dianthus caryophyllus*, Fusarium wilt, physiological race.

Fusarium oxysporum Schlechtend.:Fr. f. sp. *dianthi* (Prill. & Delacr.) W.C. Snyder & H.N. Hans., causal agent of vascular wilt on carnation (*Dianthus caryophyllus* L.), is the most important pathogen of carnation because it can cause severe losses worldwide (4,18,33). Eight physiological races of *F. oxysporum* f. sp. *dianthi* have been reported in Italy (15,16,20). Races 1 and 8 are associated with the Mediterranean carnation ecotypes and are found in Italy, France, and Spain (5,20). Race 2 is found in all carnation-growing areas (2,5). Race 3 of *F. oxysporum* f. sp. *dianthi* was recently classified as *F. redolens* f. sp. *dianthi* race 3 (5). Race 4 is found on American carnation cultivars in the United States (2,5), Italy (17,20), Israel (6), and Spain and Colombia (5,7). Races 5, 6, and 7 were reported by Garibaldi (17) on diseased carnations from Great Britain, France, and the Netherlands, but only single representatives of these pathotypes are available. Finally, three new races (9, 10, and 11) were described recently on diseased carnations from Australia (race 9 [21]) and the Netherlands (races 10 and 11 [5]).

Physical and chemical soil disinfestation and application of systemic fungicides are not always suitable for control of *F. oxysporum* f. sp. *dianthi*, due to environmental impact, high cost, and limited efficacy. A wide array of resistant cultivars is commercially available, and their use, as well as cultivation of pathogen-free propagative material, offers the most effective approach to Fusarium wilt control (17,18).

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To prevent the introduction of *F. oxysporum* f. sp. *dianthi* into regions free of carnation wilt, a sensitive detection technique is needed to produce certified pathogen-free cuttings. Distinctions between saprophytic *Fusarium* spp. and *F. oxysporum* f. sp. *dianthi* and among different races of *F. oxysporum* f. sp. *dianthi* isolated from diseased plant tissue rely on several techniques, including pathogenicity tests (15–17), vegetative compatibility tests (2,3,5,22), restriction fragment length polymorphisms (RFLP [5,25,26]), karyotype analysis (28), esterase profiles (5), sequence analysis of the ribosomal ITS1 and ITS2 regions (35), and random amplification of polymorphic DNA (RAPD [21,27,30]). Most of these techniques require several weeks to obtain results and are not adapted to large samples. Moreover, all of these methods require isolation of the pathogen from infested soil or plant tissue.

The aim of our study was to develop a polymerase chain reaction (PCR)-based diagnostic tool for identifying *F. oxysporum* f. sp. *dianthi* races 1, 2, 4, and 8, which are the most widespread and are commonly found in Italy. To obtain race-correlated sets of primers, we started from the preliminary evidence that the distribution of repetitive sequences homologous to transposable elements *Fot1* (10) and *impala* (23) in the genome of *F. oxysporum* f. sp. *dianthi* is associated with race. The same grouping was obtained by other molecular techniques: the first group includes isolates of races 1 and 8; the second group includes isolates of race 2 and the single representatives currently available for races 5 and 6; and the third group includes isolates of race 4 (29). As previously demonstrated for *F. oxysporum* f. sp. *albedinis* (14), we speculated that if *Fot1* or *impala* are inserted in a unique genomic region of *F. oxysporum* f. sp. *dianthi*, then this region can be amplified with primers overlapping the 3′ or 5′ end of the transposon and its

TABLE 1. Code, ATCC accession number, race, and geographic origin of *Fusarium oxysporum* f. sp. *dianthi* isolates tested for hybridization to *Fot1* and *impala* probes[a] and PCR results[b]

Isolate	ATCC[c]	Race	Origin	*Fot1*	*Impala*	Reference[d]
1	204207	1	Italy	+	+	2, 5, 17, 28, 30
311	204218	1	Italy	NT	NT	
572	…	1	Italy	+	NT	30
617	204219	1	Italy	+	NT	28
625	…	1	Italy	+	+	30
674	204216	1	Italy	+	+	
676	204208	1	Italy	+	+	30
718	204209	1	Italy	NT	NT	30
732	…	1	Italy	NT	NT	30
746	204210	1	Italy	+	+	19, 28, 30
774	204211	1	Italy	NT	+	30
805	204212	1	Italy	+	+	19, 28, 30
834	…	1	Italy	+	NT	
964	204213	1	Italy	+	+	30
1031	204214	1	Italy	+	+	30
1180	204215	1	Italy	+	NT	30
SM	…	2	Italy	+	NT	
9	…	2	Colombia	+	NT	
17	…	2	Colombia	+	NT	
43	…	2	Colombia	+	NT	
75	…	2	Italy	+	+	2, 5, 17, 19, 28, 30
76	…	2	Colombia	+	NT	
218	204221	2	Italy	+	+	28, 30
451	204222	2	Italy	+	+	19, 30
593	204223	2	Italy	+	+	19, 28, 30
598	204224	2	Italy	+	NT	19, 28, 30
1024	204225	2	Italy	+	NT	30
1027	204226	2	Italy	+	NT	30
1035	204232	2	Italy	+	+	
1041	204227	2	Italy	+	NT	30
1121	204228	2	Italy	+	+	28, 30
1123	…	2	Italy	NT	NT	
1171	204229	2	Israel	+	+	30
1172	…	2	Israel	+	NT	30
1178	204230	2	Israel	+	NT	30
1198	…	2	Japan	NT	NT	30
1223	204233	2	Netherlands	NT	+	
1227	204231	2	Israel	+	NT	30
28	204235	4	Italy	+	+	28, 30
209	204236	4	Italy	+	+	28, 30
245	204237	4	Italy	+	+	28, 30
310	204234	4	Italy	+	+	2, 5, 17, 19, 28, 30
327	204238	4	Italy	NT	+	28, 30
435	204239	4	Italy	+	+	30
445	…	4	Italy	NT	+	
452	…	4	Italy	NT	NT	
481	…	4	Italy	NT	NT	
493	204240	4	Italy	+	+	30
510	204241	4	Italy	+	+	30
738	204242	4	Italy	+	NT	30
752	204243	4	Italy	+	NT	30
757	204244	4	Italy	NT	NT	30
758	204245	4	Italy	+	+	30
761	…	4	Italy	NT	NT	
775	204246	4	Italy	NT	NT	30
814	…	4	Italy	+	+	30
828	204247	4	Italy	+	+	2, 5, 19, 28, 30
165	…	5	France	+	+	2, 5, 17, 28, 30
256	…	6	Netherlands	+	+	2, 5, 17, 28, 30
276	…	8	Italy	+	+	5, 17, 30
316	204248	8	Italy	NT	NT	
325	…	8	Italy	NT	NT	30
617	…	8	Italy	+	NT	30
640	204249	8	Italy	+	+	19, 28, 30
684	204250	8	Italy	+	+	30
788	204251	8	Italy	NT	+	30
812	…	8	Italy	NT	+	
821	204252	8	Italy	+	+	30
834	204253	8	Italy	+	+	5, 19, 28, 30
882	204254	8	Italy	+	+	30
895	204255	8	Italy	+	+	30
902	204256	8	Italy	+	+	30

[a] + indicates hybridization; NT indicates not tested.
[b] Results of the PCR experiment using the race-associated primers developed in this work were all positive.
[c] American Type Culture Collection, Manassas, VA.
[d] Previously published race determinations.

genomic insertion. In this paper, we report on the application of an inverse PCR (IPCR) technique (32,34) for cloning genomic DNA flanking race-associated insertion sites of sequences homologous to *Fot1* or *impala* from different races of *F. oxysporum* f. sp. *dianthi* and on the design of race-correlated primers for detecting the pathogen in infected plant tissue.

MATERIALS AND METHODS

Fungal strains and culture media. A collection of 72 *F. oxysporum* f. sp. *dianthi* isolates from diseased carnations in Italy, Israel, Colombia, the Netherlands, and Japan is maintained at the Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali—Plant Pathology, University of Torino, Italy, on potato dextrose agar (PDA; Merck, Darmstadt, Germany) under mineral oil (Sigma Chemical Co., St. Louis) at 12°C. Table 1 presents the isolates tested, their origins and identities, based on previous studies (2,5,17,19,28,30), and the accession numbers of isolates deposited at ATCC (American Type Culture Collection, Manassas, VA). Nonpathogenic *F. oxysporum* Fo47 (1), single representatives of *F. oxysporum* f. spp. *basilici*, *canariensis*, *cepae*, *cyclaminis*, *gladioli*, *lilii*, *lycopersici*, *melonis*, *pisi*, *radicis-lycopersici*, and *tulipae*, and single representatives of *F. proliferatum*, *F. redolens*, *Phytophthora nicotianae* var. *parasitica*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *S. minor* were included as references.

Genomic DNA isolation. *F. oxysporum* genomic DNA used in Southern blot analysis, IPCR reactions, and PCR reactions was purified from lyophilized mycelium by a miniprep method described previously (31). Briefly, 50 mg of ground mycelium was suspended in 1 ml of 50 mM EDTA, 0.2% sodium dodecyl sulfate (SDS; pH 8.5), and 100 µg of proteinase K and incubated for 1 h at 37°C. After incubation and inactivation of the enzyme at 70°C for 15 min, 100 µl of 5.0 M potassium acetate was added, and the mixture was kept in an ice bath for 30 min. After centrifuging at 13,000 × *g* for 15 min, the supernatant was extracted with 1 volume of phenol/chloroform/isoamyl-alcohol (25:24:1, vol/vol). Nucleic acids were precipitated with 1 volume of isopropanol, rinsed with ethanol, and resuspended in Tris-EDTA (TE; pH 8.0 [24]) buffer.

A shorter method of obtaining DNA from colonies grown on PDA suitable for use in PCR reactions was developed: 1 cm^2 of mycelium was removed from the growing edge of a colony without taking any agar medium and placed in a 1.5-ml tube with a solution of 300 µl of 10 mM Tris-HCl and 0.1 mM EDTA (pH 8), briefly disrupted with a pestle, and boiled for 10 min. After a 5-min spin at maximum speed to pellet cell debris, 1 µl of the supernatant was used as template in PCR reactions.

Extraction of DNA from diseased carnations was performed by grinding 100 mg of plant vascular tissue in a mortar with liquid nitrogen. Extraction buffer (1 ml of 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 1% polyvinylpyrrolidone, and 2% hexadecyltrimethyl-ammonium bromide, pH 8) containing 100 µg of proteinase K was added, and the lysate was incubated for 15 min at 37°C. After centrifuging at maximum speed for 5 min, the supernatant was extracted with phenol, treated with 1 volume of isopropanol, rinsed with ethanol, and in TE (pH 8.0) buffer.

IPCR reaction. Restriction digests were performed, according to the supplier's specifications (New England Biolabs, Beverly, MA), with 5 µg of genomic DNA treated with 20 units of either *Xho*I (which had no restriction site in the *Fot1* element) if amplifying *Fot1* DNA-flanking regions or *Bgl*II (which had no site in the *impala* element) if amplifying *impala* DNA-flanking regions. For self-circularization, each digested product was precipitated, resuspended in 30 µl of ligation buffer (66 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM dithioerythritol, and 1 mM ATP) with 1 unit of T_4 DNA ligase (Boehringer GmbH, Mannheim, Germany), and incubated for 16 h at 8°C. Ligated DNA was precipitated with ethanol and collected by centrifugation.

A total of 0.1 µg of circularized DNA was used as template in IPCR reactions, without prior linearization of circularized molecules.

IPCR was performed in a thermal cycler (Perkin-Elmer Cetus, Emeryville, CA), using the Expand Long Template PCR system (Boehringer). Reactions contained 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)SO_4$, 1.75 mM $MgCl_2$, 0.35 μM each dNTP, 5 μM each primer, Ft2 (5′-CCTTCCTAATGGCGCGTGATCCCCG-3′) and Ft3 (5′-GGCGATCTTGATTGTATTGTGGTG-3′) to isolate *Fot1*-flanking regions or IMP1 (5′-GCGGATCGGTTATGACGG-3′) and IMP2 (5′-AATCCTATAGAGAATCTGTGG-3′) to isolate *impala*-flanking regions, and 2.5 units of *Taq* polymerase in a total volume of 50 μl. Reactions were overlaid with mineral oil and subjected to 35 cycles of denaturation at 94°C for 10 s, primer annealing at 68 to 60°C (2 cycles at 68°C, 2 cycles at 65°C, 1 cycle at 63°C, and 30 cycles at 60°C) for 1.5 min, and extension at 68°C for 2 min, with an autoextension of 10 s during the last 30 cycles. Reactions were completed by extended incubation at 68°C for 7 min prior to analytical gel electrophoresis. A second cycle of nested IPCR was performed, as described above, using oligonucleotides Ft4 (5′-CTCTGCATTTTTAGCTATTTATTTGAC-3′) and Ft5 (5′-CGTCCGCAGAGTATACCGGCATTGTAG-3′) for isolation of *Fot1*-flanking sequences and 1 to 5 μl of the primary reaction as template DNA.

Cloning IPCR products. DNA products obtained by IPCR were fractionated in 0.8% agarose gel (SeaKem, Rockland, MD) and purified with a Quicksorb kit (Genomed, Research Triangle Park, NC). DNA was ligated to the pGEM-T vector (Promega, Madison, WI) following the manufacturer's instructions. Ligated DNA was used to transform *Escherichia coli* XL1 blue (Stratagene, La Jolla, CA), and recombinant clones were screened with a *lac* complementation assay. Positive clones were identified by digestion with *Xho*I or *Bgl*II that resulted in a restriction site in the PCR product but not in the vector's polylinker.

DNA sequencing. DNA sequencing was performed by the Service de Synthèse et d'Analyse, Department of Recherche en Sciences de la Vie et de la Santé (Université Laval, Quebec). Inserts were sequenced automatically with an ABI 373 DNA sequencer (Stretch with XL upgrade, Perkin-Elmer Corp., Norwalk, CT). Fluorescent signals were collected by the ABI's data collection software and analyzed by the ABI's sequence analysis software. The sequences of the *Fot1*- and *impala*-flanking regions identified in the BAR 2, BAR 4, and BIR 8 clones were deposited at GenBank (accession nos. AF113523, AF113525, and AF113524, respectively).

Southern hybridization. Genomic DNA (10 μg) was digested with 50 units of *Xho*I or *Bgl*II at 37°C for 16 h and resolved in a 0.8% agarose gel containing Tris-acetate-EDTA buffer (24). Digested DNA was blotted on Nylon N membranes (Amersham, Little Chalfont, England) by alkaline vacuum transfer (Bio-Rad Laboratories, Hercules, CA) as described by the manufacturer.

IPCR clones were digested with appropriate enzymes to isolate DNA regions missing the *Fot1* or *impala* sequence, labeled using a randomly primed digoxigenin (DIG)-labeling system (Boehringer), and used on Southern blots of *F. oxysporum* f. sp. *dianthi* DNA to verify the identity of specific RFLPs. Filters were hybridized at 65°C in buffer containing 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0), 2% (wt/vol) blocking reagent (Boehringer), 0.1% *N*-lauroylsarcosine, and 0.02% SDS for 16 h, followed by washing twice with 2× SSC and 0.1% SDS and twice with 0.1× SSC and 0.1% SDS at 65°C for 15 min each. Chemiluminescent detection by the nonradioactive DIG DNA detection kit (Boehringer) was performed according to the manufacturer's instructions.

To detect *Fot1* and *impala* polymorphisms in *F. oxysporum* f. sp. *dianthi*, primers FOT1 (5′-AGTCAAGCACCCATGTAACCGACCCCCCCTGG-3′) and IMPALA (5′-CAATAAGTTTGAATACA-3′), complementary to the inverted terminal repeats (ITRs) of transposable elements *Fot1* and *impala* of *F. oxysporum* f. sp *melonis*, respectively (10,23), were used to amplify the corresponding transposon sequences in *F. oxysporum* f. sp. *dianthi* isolates 1035 (race 2) and 684 (race 8; Table 1). Amplification products were cloned (as described previously), labeled, and used as probes in Southern blot analysis performed with *Xho*I- or *Bgl*II-digested genomic DNA of *F. oxysporum* f. sp. *dianthi* races 1, 2, 4, and 8.

PCR reaction. PCR reactions were performed under the conditions described previously for fungal genomic DNA purified from lyophilized mycelium or diseased carnation vascular tissues. Template DNA (1 to 5 μl) was amplified in a solution containing 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.01% (wt/vol) gelatin with the addition of 200 μM each nucleotide, 0.5 μM each primer (Ft3, IMP2, R2.1, R4.2, and R8.1; Table 2), and 0.5 μl of crude recombinant *Taq* polymerase prepared according to Desai and Pfaffle (11). Negative and positive controls were included in all experiments. PCR reactions were performed with a thermal cycler (Perkin-Elmer Cetus) programmed as follows: 1 cycle at 94°C for 5 min, followed by 35 cycles, each consisting of a denaturation step at 94°C for 30 s; an extension step at 72°C for 1 min; and annealing temperatures decreasing during the first 10 cycles from 65 to 55°C for 30 s according to the touchdown program (12). After amplification, the reaction mixture was loaded on a 2% agarose gel, separated by electrophoresis, and photographed under UV light with the Gel Doc 1000 Molecular Analyst (Bio-Rad). Amplification experiments were repeated at least three times.

Plant inoculation. *F. oxysporum* f. sp. *dianthi* isolates 1 (race 1), 75 (race 2), 310 (race 4), and 276 (race 8) were grown in 250-ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (24 g/liter; Difco Laboratories, Detroit) and yeast extract (5 g/liter; Difco) with shaking (150 rpm) at 26°C under constant light. After 7 days, fungal cultures were aseptically filtered through four layers of cheesecloth, and conidia were brought to a final cell density of 10^5 CFU/ml. Inoculum was applied to plant roots by dipping carnation cuttings of susceptible cv. Indios in conidial suspensions at transplantation. A mock-inoculation control was added by dipping carnation cuttings in sterile distilled H_2O at transplantation. Plastic pots (15 liter volume) were filled with a steam-disinfected potting mixture (pH 5.5) containing two parts soil (pH 6.9; P, K, Ca, Mg, Zn, Mn, and Fe at 352, 1,700, 1,500, 415, 29, 8.7, and 130 mg ml^{-1}, respectively) and one part peat moss (vol/vol). Ten plants per isolate were transplanted in each pot. Pots were watered daily, and N (total nitrogen 20%: 7% NH_4^+ and 13% NO_3^-)-K_2O (10%)-P_2O_5 (10%) liquid fertilizer was distributed in irrigation water at a concentration of 0.08%. Pots were kept in a glasshouse (25 to 30°C; 50 to 90% relative humidity; daily light at 50 to 60 Klx/m^2) located at Albenga, a typical carnation-growing area in the Liguria Region (northwestern Italy). At the end of the experiment (≈6 weeks after transplantation), each plant was uprooted and cut lengthwise to evaluate the presence of mild Fusarium wilt symptoms. Diseased plants were stored at 7°C until processing for PCR detection of *F. oxysporum* f. sp. *dianthi*.

TABLE 2. Race-associated primers used for detection of *Fusarium oxysporum* f. sp. *dianthi*

Primer code	Race specificity	Clone[a]	Oligonucleotide sequence	Primer[b]	Product size (bp)
R2.1	2	BAR 2	5′-CTTGTTTCTCGATTTCTGTCTCACG-3′	Ft3	564
R8.1	1 or 8	BAR 8	5′-CGATGAAGTCGGTTTGCGATT-3′	Ft3	295
R4.2	4	BAR 4	5′-GGTGATTGGAGGAGGAATACC-3′	IMP2	1,315

[a] Clone from which the primer was derived.
[b] Primer paired with race-associated primer.

RESULTS

***Fot1* and *impala* polymorphisms in *F. oxysporum* f. sp. *dianthi*.** The distribution of transposable elements *Fot1* and *impala* was determined by Southern hybridization on *F. oxysporum* f. sp. *dianthi* DNA restricted with *Xho*I, having no site in the *Fot1* sequence, and *Bgl*II, having no site in the *impala* sequence. On all isolates tested (Table 1), transposable elements *Fot1* and *impala* showed a race-associated polymorphism with respect to the hybridization profile, i.e., all the isolates belonging to the same race presented identical profiles. *Fot1* hybridization signals varied between one and six, with sizes between 2 and 11 kb (Fig. 1A). For race 4 isolates, a single ≈6-kb band was generated when isolates were hybridized with *Xho*I-digested genomic DNA to the *Fot1* probe. This band always amplified less intensively compared with the corresponding insertion signals present in races 1, 2, 5, 6, and 8 (Fig. 1A) but not compared with the corresponding signals generated by the *impala* probe (Fig. 1B). The *impala* probe generated from three to seven hybridization signals on the genome of *F. oxysporum* f. sp. *dianthi* (Fig. 1B). Only one isolate among those tested, 1-674, showed a difference in its *impala* hybridization profile, lacking a 1.8-kb insertion of *impala* (Fig. 1B).

***Fot1*- and *impala*-flanking regions obtained by IPCR.** The strategy adopted for isolating DNA fragments flanking *Fot1*- or *impala*-homologous sequences in *F. oxysporum* f. sp. *dianthi* by performing PCR with inverse primers is outlined in Figure 2. Amplification with primers Ft2 and Ft3 in the first IPCR cycle allowed

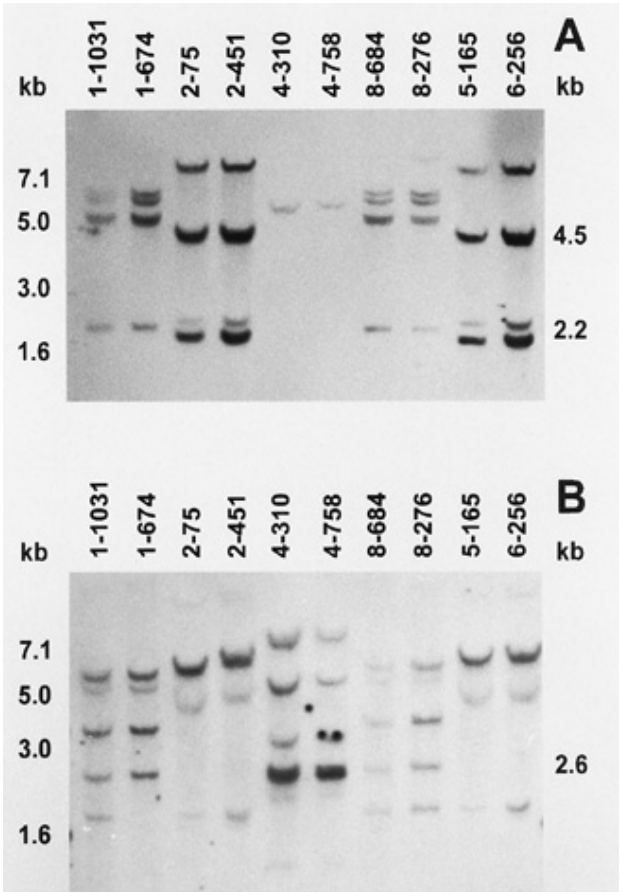

Fig. 1. Distribution of sequences homologous to transposable elements **A,** *Fot1* and **B,** *impala* in the genome of *Fusarium oxysporum* f. sp. *dianthi*. Total genomic DNAs obtained from 10 representative isolates of races 1, 2, 4, 8, 5, and 6 were digested with restriction enzymes **A,** *Xho*I or **B,** *Bgl*II, separated by electrophoresis in 0.8% agarose gels, blotted, and hybridized with *Fot1* or *impala* probes, respectively. The size (kilobases) of selected bands in the marker (1-kb DNA ladder from Life Technologies) are indicated to the left. The size (kilobases) of cloned insertions are indicated to the right.

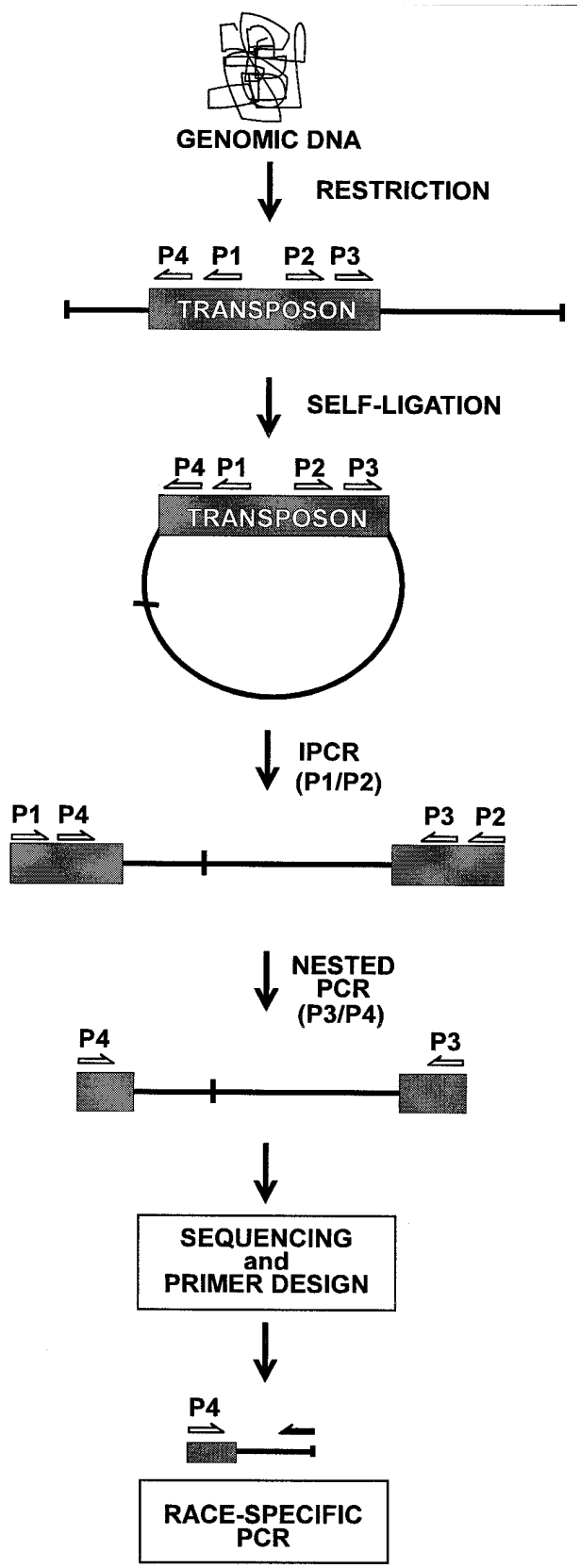

Fig. 2. Strategy for isolating DNA fragments flanking *Fot1*- or *impala*-homologous sequences in *Fusarium oxysporum* f. sp. *dianthi* by inverse polymerase chain reaction (IPCR). P1 and P2 correspond to primers Ft2 and Ft3 or IMP1 and IMP2 for *Fot1* and *impala* sequences, respectively; P3 and P4 correspond to primers Ft4 and Ft5 in the *Fot1* sequence; nested PCR was not performed to isolate *impala*-flanking regions from race 4 isolates.

several bands, ranging in size from 4.6 to 1.6 kb, to be obtained when template DNA was derived from *F. oxysporum* f. sp. *dianthi* races 1, 2, and 8. Nested PCR with primers Ft4 and Ft5 generated a 2.9-kb band for race 2 (corresponding to the 4.5-kb *Fot1* insertion in Figure 1A) and 3.4- and 0.65-kb bands for races 1 and 8 (corresponding to the 5.0- and 2.2-kb *Fot1* insertions in Figure 1A), respectively. The 2.9- and 0.65-kb amplification products were cloned and coded BAR 2 (from race 2) and BIR 8 (from races 1 and 8), respectively.

When *impala*-based primers IMP1 and IMP2 were used in IPCR experiments with *Bgl*II-digested genomic DNA of race 4 as template, one ≈1.7-kb band was obtained, without the need for subsequent nested PCR. This amplimer (corresponding to the 2.6-kb *impala* insertion in Figure 1B) was cloned and coded BAR 4.

Cloned IPCR products deprived of *Fot1* or *impala* sequences were used to probe Southern blots containing genomic DNA of *F. oxysporum* f. sp. *dianthi* races 1, 2, 4, and 8 to determine whether the selected transposon insertion had occurred in a single-copy sequence or a repeated region. All probes tested gave rise to multiband patterns, including the hybridization signal of the expected size (data not shown), accounting for insertion into repeated sequences.

The whole BIR 8 clone and two subclones (coded BAR 2AH and BAR 4SH) obtained from the BAR 2 and BAR 4 clones, respectively, were sequenced. Based on genomic DNA sequences, three primers (coded R2.1, R8.1, and R4.2) were designed (Table 2). The first two, designed on the *Fot1*-flanking region, were used paired with the Ft3 primer, whereas primer R4.2, designed on the *impala*-flanking region, was used with the IMP2 primer.

Race-associated amplification from genomic DNA and infected plant tissue. Genomic DNAs purified from lyophilized mycelia of all *F. oxysporum* isolates tested (Table 1) were used to validate primer specificity in PCR experiments. All isolates tested from forma specialis *dianthi* gave rise to 295-, 564-, or 1,315-bp amplification products, corresponding to race 1 (or 8), 2, and 4, respectively (Figs. 3 and 4). The single representatives of races 5 and 6 produced a 564-bp amplimer that was indistinguishable from the amplimer produced by race 2 isolates (data not shown). The same results were observed when fungal DNA was obtained by the short extraction method with colonies grown in PDA (data not shown).

When multiplex PCR was performed by mixing the three race-associated primer pairs in the same reaction tube with genomic DNA of an isolate belonging to race 1, 2, 4, or 8, a single amplimer was generated, and the differences in the molecular weights of the amplimers allowed clear race identification of each isolate (Fig. 4).

No amplification could be obtained when genomic DNAs from representatives of other formae speciales of *F. oxysporum*, nonpathogenic *F. oxysporum* FO47, or other soilborne pathogens were tested as template, confirming primer specificity for forma specialis *dianthi* (data not shown).

Finally, race-correlated amplification was obtained from the vascular tissue of diseased carnation plants of cv. Indios when inoculated with representative isolates of races 1, 2, 4, and 8. Positive amplification was obtained from plants inoculated with all four races, even in the presence of mild disease symptoms (moderate discoloration of xylem in the absence of external wilt).

DISCUSSION

The aim of our research was to develop race-associated primers for early detection of *F. oxysporum* f. sp. *dianthi* in diseased carnation plant tissue. We postulated that race-correlated PCR amplification could be obtained from the carnation wilt pathogen with primers overlapping the 3′ or 5′ end of transposable element *Fot1* or *impala* and the genomic DNA flanking the element. The hypothesis was based on the results obtained by Fernandez et al. (14) with the date palm pathogen, *F. oxysporum* f. sp. *albedinis*, and on the preliminary observation that repetitive DNA sequences homologous to *Fot1* and *impala* are distributed in a race-correlated pattern in *F. oxysporum* f. sp. *dianthi* (29).

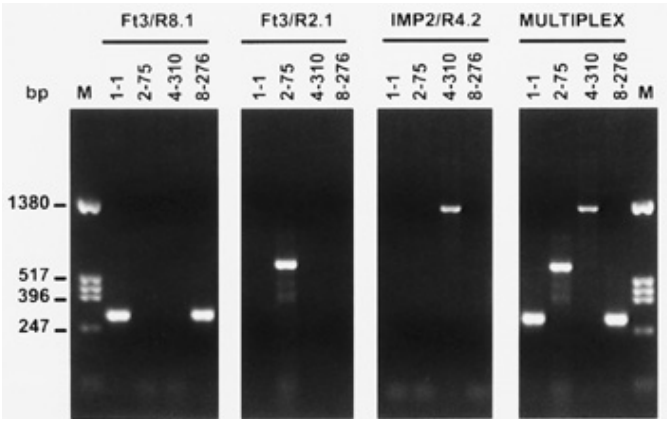

Fig. 4. Agarose gel electrophoresis of polymerase chain reaction (PCR) products from genomic DNAs of four representative isolates of *Fusarium oxysporum* f. sp. *dianthi* races 1, 2, 4, and 8 with Ft3/R8.1, Ft3/R2.1, or IMP2/R4.2 primer pairs. The far right panel shows the result of a multiplex PCR, in which six primers were mixed in the same reaction. M, molecular size marker (*Hin*fI-digested VCS vector, Stratagene); sizes (base pairs) are indicated to the left.

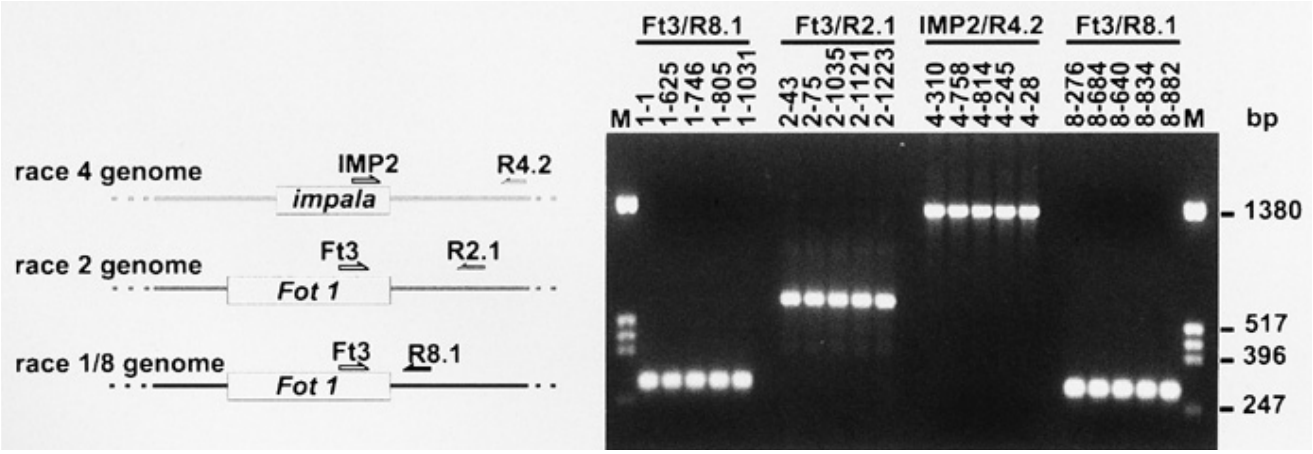

Fig. 3. Agarose gel electrophoresis of polymerase chain reaction products from genomic DNAs of 20 representative isolates of *Fusarium oxysporum* f. sp. *dianthi* races 1, 2, 4, and 8 with Ft3/R8.1, Ft3/R2.1, IMP2/R4.2, and Ft3/R8.1 primer pairs, respectively. A scheme of the race-correlated insertion site of the *Fot1*- or *impala*-homologous sequence with the position of each primer is outlined to the left. M, molecular size marker (*Hin*fI-digested VCS vector, Stratagene); sizes (base pairs) are indicated to the right.

To facilitate cloning of the *Fot1*- and *impala*-flanking regions without constructing a genomic library from each physiological race of *F. oxysporum* f. sp. *dianthi*, we adopted a modified version of the IPCR technique that involves direct amplification of self-ligated restriction fragments without previous linearization of the circular molecules. The possibility of unwanted restriction digestion or nonspecific breakage in flanking genomic DNA through enzymatic digestion or phenol extraction is avoided by IPCR, resulting in larger amplification products. In our experience, the size limit for successful amplification by IPCR technique is 4.6 kb, corresponding to a 5.8-kb insertion of transposable element *Fot1*. Digestion with a wide range of restriction enzymes lacking sites in the transposon sequence may be useful in generating small insertions, which are adequate for amplification and cloning.

When the current study was conceived, we were aware of the fact that a diagnostic system based on the specific insertion of a transposable element would be unreliable if the inserted copy was active and likely to transpose to a different genomic position. Indeed, the possibility cannot be excluded, although analysis of a representative collection of isolates obtained from different geographic areas and at different times should reduce the risk of choosing a mobile copy of the target transposon. Among the isolates analyzed in our study, only one resulted in the loss of an 1.8-kb *impala* insertion signal. This copy, which most probably represents an active transposon of *F. oxysporum* f. sp. *dianthi*, was not considered in our experiments. To increase the reliability of the primers, a deleted copy of the target transposon could be cloned, as was shown for *F. oxysporum* f. sp. *albedinis* (14), avoiding the risk of unexpected transposition of the amplified insertion. Alternatively, two primer pairs targeted at two different insertion sites could be used for each race in the same PCR reaction. The presence of multiple copies of transposable elements *Fot1* and *impala* in the genome of *F. oxysporum* f. sp. *dianthi* makes this approach practical for this pathogenic fungus.

In the case of all race 4 isolates analyzed, a single band was generated when *Xho*I-digested genomic DNA was hybridized to the *Fot1* probe. The band was consistently less intense compared with hybridization signals detected in other races. Moreover, no amplification could be obtained by the ITR-based FOT1 primer, nor in a series of internal primers in PCR experiments or the Ft2 and Ft3 primers in IPCR experiments even at low annealing temperatures. These results led us to conclude that the unique *Fot1* copy present in race 4 could be largely divergent in its sequence or truncated, as was the case for *F. oxysporum* f. sp. *albedinis* (14). Because the scope of the current study was to generate race-associated primers for PCR detection of *F. oxysporum* f. sp. *dianthi*, we decided to clone an *impala* insertion for race 4 and demonstrate that the same technique could be successfully applied regardless of the transposable element provided its sequence is complete and does not diverge at the ends.

Care should be taken not to clone insertions that are present in other formae speciales or in saprophytic *F. oxysporum*. Primer sequences based on these insertions would lead to nonspecific amplification if the organisms are present as contaminants in template DNA. In the case of Fusarium wilt, this possibility is remote because template DNA is extracted from vascular tissue at 20 to 30 cm above the soil, where only the disease incitant should be present (13).

Southern hybridization with the cloned genomic DNA flanking *Fot1* and *impala* demonstrates that these two elements had inserted into repetitive regions in *F. oxysporum* f. sp. *dianthi*. This finding could reflect the preference of these elements to insert into clusters of repeated sequences, or it could be a passive consequence of evolution because of limited deleterious effects. Sequencing flanking regions of newly transposed elements is needed to answer this question.

PCR amplification from plant tissue was accomplished by a simple extraction protocol, allowing purification of template DNA in ≈2 h. The procedure is now routinely applied in our laboratory to detect *F. oxysporum* f. sp. *dianthi* in different carnation cultivar-isolate combinations. By generating amplification products of different sizes, the three race-associated primer pairs can be successfully used in multiplex PCR, allowing both detection of the pathogen in diseased plant tissue and determination of the race in a single reaction. The use of specific primers for one-step detection of the most common determinants of Fusarium wilt on carnation could be adopted for sensitive certification of propagative material and early determination of disease etiology under field and glasshouse conditions.

Isolates of *F. oxysporum* f. sp. *dianthi* races 1 and 8 cannot be distinguished by the proposed PCR technique. Indeed, pathotypes 1 and 8 were previously assigned to the same vegetative compatibility (2) and RFLP groups (25,26) and showed similar electrophoretic karyotypes (28), esterase profiles (5), sequences of ribosomal ITS1 and ITS2 regions (35), and RAPD profiles (30). They also share identical hybridization profiles for *Fot1* and *impala* probes. This confirms the hypothesis that the two pathotypes are closely related, although they differ in virulence (2,16,17) and can be considered near-isogenic (5). The fact that pathotypes 1 and 8 were reported only in the Italian and French Riviera, mainly on cultivars of the Mediterranean ecotype, supports the hypothesis that race 8 may have arisen from race 1 by adaptation to resistant cultivars and only differs by one or a few avirulence genes involved in specific recognition. Similarly, the single representatives of races 5 and 6 currently available were indistinguishable from those belonging to race 2 (2,5,25,26,28,30,35) and gave rise to the same amplified product when using the race 2-specific primer pair, as was expected from the *Fot1* and *impala* hybridization patterns.

The strategy presented here may be applied to develop PCR-based diagnostics for any *F. oxysporum* bearing *Fot1* or *impala* copies within its genome, provided that the sequences are stably inserted at specific sites. Because the distribution of such elements in *F. oxysporum* is widespread (8,9), it should be relatively easy to generate primer sets for direct PCR amplification of most formae speciales and physiological races present in the taxon.

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