

## *Fot 1* Insertions in the *Fusarium oxysporum* f. sp. *albedinis* Genome Provide Diagnostic PCR Targets for Detection of the Date Palm Pathogen

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Populations of *Fusarium oxysporum* f. sp. *albedinis*, the causal agent of Bayoud disease of date palm, are derivatives of a single clonal lineage and exhibit very similar *Fot 1* hybridization patterns. In order to develop a sensitive diagnostic tool for *F. oxysporum* f. sp. *albedinis* detection, we isolated several DNA clones containing a copy of the transposable element *Fot 1* from a genomic library of the date palm pathogen. Regions flanking the insertion sites were sequenced, and these sequences were used to design PCR primers that amplify the DNA regions at several *Fot 1* insertion sites. When tested on a large sample of *Fusarium* isolates, including 286 *F. oxysporum* f. sp. *albedinis* isolates, 17 other special forms, nonpathogenic *F. oxysporum* isolates from palm grove soils, and 8 other *Fusarium* species, the primer pair TL3-FOA28 allowed amplification of a 400-bp fragment found only in *F. oxysporum* f. sp. *albedinis*. Sequence analysis showed that one of the *Fot 1* copies was truncated, lacking 182 bp at its 3' terminus. The primer pair BI03-FOA1 amplified a 204-bp fragment which overlapped the *Fot 1* truncated copy and its 3' site of insertion in the *F. oxysporum* f. sp. *albedinis* genome and identified 95% of the isolates. The primer pairs BIO3-FOA1 and TL3-FOA28 used in PCR assays thus provide a useful diagnostic tool for *F. oxysporum* f. sp. *albedinis* isolates.

*Fusarium oxysporum* is a common soilborne fungus which lacks a known sexual stage. Strains of *F. oxysporum* cause vascular wilt disease in many agricultural crops and have been classified into special forms based on their host specificity (1). *F. oxysporum* f. sp. *albedinis* (Killian and Maire) Gordon is the causal agent of Bayoud disease, a devastating disease of date palm (*Phoenix dactylifera* L.) that only occurs in Algerian and Moroccan oases (13). The pathogen is easily spread by exchange of contaminated material; at present, strict phytosanitary rules are applied at borders of date palm-growing countries that are free of Bayoud disease. Detection and identification of *F. oxysporum* f. sp. *albedinis* remain difficult, mainly because time-consuming inoculation tests are required to assess pathogenicity.

We previously showed that *F. oxysporum* f. sp. *albedinis* isolates are genetically closely related and belong to a single clonal lineage (15, 22). Genomic analyses also have shown that the fungus carries repetitive DNA sequences homologous to the DNA transposable element *Fot 1*, first cloned from *F. oxysporum* f. sp. *melonis* and widely distributed in *F. oxysporum* (3). Depending on the strain, 15 to 26 *EcoRI* fragments hybridizing with the *Fot 1* sequence were detected, and several were conserved (15, 22). *Fot 1* hybridization patterns of *F. oxysporum* f. sp. *albedinis* and other, nonpathogenic *F. oxysporum* isolates from palm grove soils were very different, with the nonpathogens exhibiting only zero to eight *EcoRI* hybridizing bands (21).

We hypothesized that at least one *Fot 1* copy is inserted at a genomic location specific for *F. oxysporum* f. sp. *albedinis*. If this hypothesis is correct, this region could be amplified by PCR of *F. oxysporum* f. sp. *albedinis* DNA with primers overlapping the 3' or 5' end of the *Fot 1* copy and its genomic region of insertion.

Testing this hypothesis requires (i) cloning of the *Fot 1* copies present in the genome of the pathogen, together with their flanking genomic sequences, (ii) sequencing of the 3' and 5' insertion regions and designing of primers for PCR amplification, and (iii) testing for amplification of a specific DNA fragment in *F. oxysporum* f. sp. *albedinis* isolates.

In this report, we describe the cloning of several *Fot 1* copies from *F. oxysporum* f. sp. *albedinis*, the characterization of a truncated copy, and the subsequent development of specific oligonucleotides to use as primers in PCR assays for quickly identifying this pathogen.

### MATERIALS AND METHODS

**Fungal isolates.** The isolates examined are listed in Table 1. The *F. oxysporum* f. sp. *albedinis*, *F. oxysporum*, and *F. moniliforme* isolates were collected from different oases in Algeria and Morocco. The *F. oxysporum* f. sp. *elaedis* (6, 14) and *F. oxysporum* f. sp. *vasinfectum* (7) isolates belonged to several vegetative compatibility groups and had different molecular haplotypes. The other *Fusarium* strains examined were obtained either as isolates from the Laboratoire de la Flore Pathogène des Sols (Institut National de la Recherche Agronomique, Dijon, France) or as DNA samples from H. C. Kistler (University of Florida, Gainesville) or S. Bentley (University of Queensland, Brisbane, Queensland, Australia). All cultures were single spore and were maintained in glycerol under liquid nitrogen until use or as mycelia on potato dextrose agar slants.

**Cloning of the *Fot 1* copies.** Genomic DNA from *F. oxysporum* f. sp. *albedinis* GH3 (International Mycological Institute [IMI] accession number 377827) (isolated in Ghardaia, Algeria, in 1993) was isolated and purified by cesium chloride gradient centrifugation as described previously (2). Purified DNA (380 ng) was digested to completion with the restriction enzyme *Clal* (Boehringer Mannheim Biochemicals, Meylan, France) and ligated into *Clal*-restricted pBluescript (500

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TABLE 1. *Fusarium* species tested and results of PCR amplification with the primer pairs designed in this study

<i>Fusarium</i> species	No. of isolates	Source	Geographic origin	PCR result <sup>a</sup> with:	
				BIO3-FOA1	TL3-FOA28
<i>F. oxysporum</i> f. sp. <i>albedinis</i>	208	Date palm leaf	Morocco	198+/10-	+
	3	Date palm root	Morocco	+	+
	5	Palm grove soil	Morocco	+	+
	70	Date palm leaf	Algeria	64+/6-	+
<i>F. oxysporum</i>	28	Palm grove soil	Algeria	-	-
	26	Palm grove soil	Morocco	-	-
<i>F. oxysporum</i> f. sp. <i>basilicum</i>	1	Basil	Australia	-	-
<i>F. oxysporum</i> f. sp. <i>canariensis</i>	17	Ornamental palm	Diverse <sup>b</sup>	-	-
<i>F. oxysporum</i> f. sp. <i>cicerii</i>	1	Chickpea	Morocco	-	-
<i>F. oxysporum</i> f. sp. <i>conglutinans</i>	1	Cabbage	United States	-	-
<i>F. oxysporum</i> f. sp. <i>cubeense</i>	1	Banana		-	-
<i>F. oxysporum</i> f. sp. <i>dianthi</i>	1	Carnation	Australia	-	-
<i>F. oxysporum</i> f. sp. <i>elaedis</i>	6	Oil palm	Diverse <sup>c</sup>	-	-
<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	2	Tomato	Morocco and	-	-
			United States		
<i>F. oxysporum</i> f. sp. <i>matthioli</i>	1	Matthiola	United States	-	-
<i>F. oxysporum</i> f. sp. <i>medicaginis</i>	1	Alfalfa	Australia	-	-
<i>F. oxysporum</i> f. sp. <i>melonis</i>	1	Muskmelon	France	-	-
<i>F. oxysporum</i> f. sp. <i>niveum</i>	1	Watermelon	Australia	-	-
<i>F. oxysporum</i> f. sp. <i>pisi</i>	2	Pea	Australia and	-	-
			United States		
<i>F. oxysporum</i> f. sp. <i>raphani</i>	1	Radish	United States	-	-
<i>F. oxysporum</i> f. sp. <i>vasinfectum</i>	6	Cotton	Diverse <sup>d</sup>	-	-
<i>F. oxysporum</i> f. sp. <i>zingiberi</i>	1	Ginger	Australia	-	-
<i>F. arthrosporoides</i>	1		France	-	-
<i>F. avanaceum</i>	1		France	-	-
<i>F. culmorum</i>	1		France	-	-
<i>F. graminearum</i>	1		France	-	-
<i>F. lateritium</i>	1		France	-	-
<i>F. moniliforme</i>	7	Palm grove soil	Morocco	-	-
<i>F. roseum</i>	1		France	-	-
<i>F. scirpii</i>	1		France	-	-
<i>F. trincitum</i>	1		France	-	-

<sup>a</sup> +, amplification of a DNA fragment; -, no amplification of a DNA fragment.

<sup>b</sup> Canary Islands, France, Italy, Japan, and the United States.

<sup>c</sup> Brazil, Ivory Coast, Ecuador, Ghana, and Zaire.

<sup>d</sup> Brazil, Egypt, India, Sudan, and the United States.

ng) with a ligation kit (Amersham, Les Ulis, France). *Escherichia coli* DH5 $\alpha$  competent cells were prepared and transformed with the ligation mixture (20  $\mu$ l) following the procedure of Inoue et al. (11). Transformed bacterial colonies grown on Luria broth-ampicillin (100  $\mu$ g ml<sup>-1</sup>) plates were transferred to nylon membranes (Nylon N; Amersham) and lysed by standard procedures (17). DNA bound to nylon filters was hybridized with <sup>32</sup>P-labeled *Fot* 1 to detect plasmids containing *Fot* 1.

**Characterization of recombinant plasmids.** Transformed *E. coli* was grown in Luria broth amended with ampicillin (100  $\mu$ g ml<sup>-1</sup>), and plasmid DNA was isolated with a miniprep alkaline lysis protocol (17). Genomic insert sizes were determined by digestion with *Clal* followed by electrophoresis in an 0.8% agarose gel. DNA fragments were blotted onto Nylon N membranes by alkaline vacuum transfer (TE 80 TransVac; Hoefer Scientific Instruments, San Francisco, Calif.) and hybridized with radioactively labeled *Fot* 1.

**Sequencing reactions.** Inserts were sequenced either by automated sequencing (Applied Biosystems, Inc.) with TaqFS polymerase (dye deoxy terminator kit; Perkin-Elmer Cetus, Saint-Quentin en Yvelines, France) or by manual sequencing with <sup>35</sup>S-dATP and T7 polymerase (T7 sequencing kit; Pharmacia LKB

Biotechnology, Orsay, France). <sup>35</sup>S-labeled dATP reaction products were separated by 7% polyacrylamide-urea gel electrophoresis and identified by autoradiography. DNA sequences flanking *Fot* 1 insertion sites in plasmid clones were determined by extension of primers TL3 and BIO3 (Table 2). Primers were synthesized based on the sequence of *Fot* 1 (3): TL3 is located 179 bp from the *Fot* 1 5' end, and BIO3 is located 290 bp from the *Fot* 1 3' end.

***Fusarium* sp. PCR analysis.** Fungal DNA extraction was performed as previously described (7) and with the rapid NaOH procedure described elsewhere (9). The 3' insertion site of the *Fot* 1 copy in plasmid pFalb11 and the 5' insertion site of the *Fot* 1 copy in plasmid pFalb28 were amplified with primer pairs BIO3-FOA1 and TL3-FOA28, respectively (Table 2). Each reaction mixture contained 1.5 mM MgCl<sub>2</sub>; 100  $\mu$ M (each) dATP, dCTP, dGTP, and dTTP; 1  $\mu$ M (each) primers BIO3 and FOA1 or 0.5  $\mu$ M (each) primers TL3 and FOA28; 25 ng of genomic DNA; 2.5  $\mu$ l (or 1.25  $\mu$ l) of 10 $\times$ -concentrated reaction buffer; and 0.5 U (or 0.25 U) of DNA polymerase (Goldstar DNA polymerase; Eurogentec, Seraing, Belgium). Negative controls (no DNA target) were included in every experiment to test for contamination. Amplifications were performed with a DNA thermal cycler (PHC-3 [Techne] or Thermojet [Eurogentec]) programmed

TABLE 2. Sequences of specific primers used for detecting *F. oxysporum* f. sp. *albedinis*

Target clone	Primer	Sequence	<i>Fot</i> 1 matching region	Amplicon size (bp)
pFalb11	FOA1	CAGTTTATTAGAAATGCCGCC	3' flanking	204
	BIO3	GGCGATCTTGATTGTATGTGGTG	3' end	
pFalb28	FOA28	ATCCCCGTAAGCCCTGAAGC	5' flanking	400
	TL3	GGTCGTCCGCAGAGTATACCGGC	5' end	

as follows: 1 cycle for 4 min at 95°C followed by 30 cycles for 30 s at 92°C, 30 s at 60°C, and 30 s at 72°C for the BIO3-FOA1 primer pair and 30 cycles for 30 s at 92°C, 30 s at 62°C, and 45 s at 72°C for the TL3-FOA28 primer pair. One cycle for 15 min at 72°C was conducted after the 30 cycles. After amplification, 8 µl of the reaction mixture was loaded onto a 1.4% agarose gel, separated by electrophoresis, stained with ethidium bromide, and photographed under UV light.

**Fot 1 amplification, probe preparation, and hybridization conditions.** *Fot 1* was amplified either from *F. oxysporum* f. sp. *albedinis* isolates or from the 1.9-kb *Fot 1* sequence provided by Daboussi et al. (3). The primer LTR (5' CCCATG TAACCGACCCCCCTGG 3') homologous to the *Fot 1* inverted terminal repeats was used for PCR with the following conditions: 1 cycle for 4 min at 95°C followed by 30 cycles for 1.5 min at 92°C, 1 min at 60°C, and 1.5 min at 72°C. The amplified fragment was recovered by ethanol precipitation for probe preparation. One hundred nanograms of the purified probe was labeled with 20 µCi of [<sup>32</sup>P]dCTP (Amersham) by random priming according to the manufacturer's specifications (T7 Quick Primer kit; Pharmacia LKB Biotechnology). Membrane-bound DNA was hybridized to the labeled probe at 65°C overnight in 15 ml of buffer (hybridization tablets buffer; Amersham) and washed for 20 min in 50 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) sodium dodecyl sulfate (SDS) at room temperature, 15 min in 50 ml of 1× SSC–0.1% SDS at 65°C, and 15 min in 50 ml of 0.5× SSC–0.1% SDS at 65°C. Hybridization signals were detected by autoradiography at –80°C.

**Nucleotide sequence accession numbers.** The sequences of the flanking regions contained in clones pFalb11 and pFalb28 were deposited at GenBank (accession numbers AF033099 and AF033098).

## RESULTS

**Choice of reference strain and restriction enzyme for cloning of the *Fot 1* copies.** We digested *F. oxysporum* f. sp. *albedinis* DNA with four restriction enzymes (*Cla*I, *Eco*RI, *Xba*I, and *Xho*I) that did not cut in the 1,928-bp *Fot 1* sequence from *F. oxysporum* f. sp. *melonis* (3). We found that the *F. oxysporum* f. sp. *albedinis* *Fot 1* sequence contains an *Eco*RI site not present in the *F. oxysporum* f. sp. *melonis* *Fot 1* sequence. We thus used *Cla*I to construct a genomic library from *F. oxysporum* f. sp. *albedinis* GH3. This strain carries approximately 14 copies of *Fot 1*, and most of the resulting *Cla*I fragments were less than 8 kb long and could be cloned into pBluescript.

**Cloning of the *Fot 1* copies and sequencing of flanking regions.** We identified 42 plasmids that could hybridize with *Fot 1*; 18 of them carried an *F. oxysporum* f. sp. *albedinis* DNA fragment which could be excised from the plasmid by digestion with *Cla*I. The 3' and 5' *Fot 1* insertion sites were obtained for four clones: pFalb11 (insert size, 2.8 kb), pFalb17 (9 kb), pFalb20 (6 kb), and pFalb28 (6.5 kb). With clones pFalb17, pFalb20, and pFalb28, only the 5' *Fot 1*-flanking sequence was obtained, because these plasmids had a *Cla*I site 70 bp from the *Fot 1* 3' end, thus preventing sequencing of the flanking sequence. Oligonucleotides to use as PCR primers were deduced from the 5' *Fot 1*-flanking sequence of all four clones and from the 3' *Fot 1*-flanking sequence of pFalb11 and were tested in combination with TL3 and BIO3, respectively. PCR amplifications of the genomic DNA of *F. oxysporum* f. sp. *albedinis* GH3 were obtained with primer pairs TL3-FOA28 and BIO3-FOA1.

**PCR assays for specific identification of *F. oxysporum* f. sp. *albedinis* strains.** Primer pairs TL3-FOA28 and BIO3-FOA1 were tested on DNA extracted from the *Fusarium* isolates listed in Table 1, including 286 *F. oxysporum* f. sp. *albedinis* isolates, 17 other special forms, nonpathogenic *F. oxysporum* isolates from palm grove soils, and 8 other *Fusarium* species. Primer pair TL3-FOA28 could be used to amplify a 400-bp DNA fragment for all of the *F. oxysporum* f. sp. *albedinis* isolates tested, and primer pair BIO3-FOA1 could be used to amplify a 204-bp DNA fragment for only 95% (270 of 286) of the *F. oxysporum* f. sp. *albedinis* isolates (Fig. 1). No amplification was observed when the DNA template originated from the other *Fusarium* isolates (Fig. 1 and Table 1). When various amounts of DNA from strain GH3 were tested, less than 100 pg of purified *F. oxysporum* f. sp. *albedinis* DNA could be

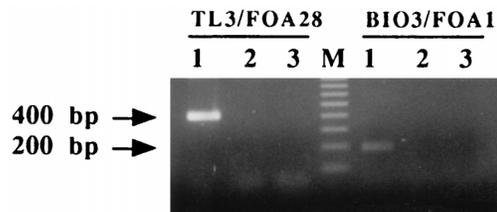


FIG. 1. Ethidium bromide-stained 1.4% agarose gel showing results of PCR experiments with primer pairs TL3-FOA28 and BIO3-FOA1. Lanes: 1 to 3, amplification products from *F. oxysporum* f. sp. *albedinis* isolates, other *F. oxysporum* isolates, and a negative control, respectively; M, molecular size markers (100-bp ladder; Eurogentec). Fragment sizes are indicated on the left. Note the selective amplification of a 400- or 200-bp fragment from *F. oxysporum* f. sp. *albedinis* isolates only.

efficiently detected with primer pairs TL3-FOA28 and BIO3-FOA1.

**Characterization of a 3'-truncated *Fot 1* copy.** The *Fot 1* element carried by clone pFalb11 lacked 182 bp at its 3' end in comparison with the original *Fot 1* sequence (3). With primer TL3, up to 410 bp of the 5' part of the insert was sequenced; 180 bp of this sequence was homologous to the *Fot 1* sequence (positions 1 to 180). Primer BIO3 allowed 235 bp of clone pFalb11 to be read (Fig. 2); the first 75 bp of this sequence were identical to *Fot 1* (positions 1671 to 1746), and the additional 160 bp of this sequence was very different, indicating that pFalb11 apparently carries a 3'-truncated *Fot 1* copy. The 160-bp flanking region of the truncated copy did not show similarity with the sequences listed in the GenBank or EMBL data banks. PCR tests with primer pair BIO3-FOA1 showed that the truncated copy seemed to be present in the genomes of 95% of the *F. oxysporum* f. sp. *albedinis* strains tested (Table 1).

	1621	(BIO3)
Fom24	TGTTATTCAG	TCACAGAAAG <u>GCGATCTTGA</u>
Falb11		
	1651	
Fom24	<u>TTGTATTGTG</u> GTGGATGGGA	AAAGTAGCTC
Falb11		*****
	1681	
Fom24	TGAGTCGGAG	GAAGATCCAG CCCCTGTGCG
Falb11	*****	*****
	1711	
Fom24	CCGATCAACC	CGCGTCAGGC GCGCTACAAA
Falb11	*****	*****
	1741	
Fom24	AATGTATATT	AGACAGGATT TGAGTAGCGA
Falb11	**G***G*AC	*C*GTTAG*A GAGCC**AAC
	1771	
Fom24	AGAGAGCGAT	TGAGAGACCT TTAATAACCA
Falb11	*TCAC*AT**	ATTC*CC*AG **TCGATTAG
	1801	
Fom24	TTTTCTCTGC	ATTTTTCAGCT ATTTATTTGA
Falb11	CAC*GAT*CA	**AACAG**G <u>GCA*T*C*A*</u>
		(FOA1)
	1831	
Fom24	CAAAGTTGTG	TTAAATATT GTAAATAGAT
Falb11	T***C*GT*A	CC*CC**GCA AAC**CTAGA

FIG. 2. Nucleotide sequence of the 3' region of pFalb11 aligned with the 1,928-bp *Fot 1* sequence (Fom24) from *F. oxysporum* f. sp. *melonis* (3). The sequence was obtained with BIO3 as a sequencing primer (underlined). The position of the deduced PCR primer FOA1 is underlined. Asterisks indicate conserved nucleotides, and blank areas indicate missing data.

## DISCUSSION

The transposable element *Fot 1* is widely distributed in the species *F. oxysporum* but displays a discontinuous pattern of conservation in the special forms (4). The aim of this study was to identify copies of the transposable element *Fot 1* inserted at specific loci within the genome of *F. oxysporum* f. sp. *albedinis* isolates and to use them as templates for the PCR characterization of *F. oxysporum* f. sp. *albedinis* DNA.

We characterized two *Fot 1* copies, carried by plasmids pFalb28 and pFalb11. Two primers, FOA28 and FOA1, specific for genomic sequences where the *Fot 1* copies were inserted, enabled the amplification of *F. oxysporum* f. sp. *albedinis* DNAs only when paired with *Fot 1*-specific primers TL3 and BIO3, respectively. These genomic loci are probably common to all *F. oxysporum* isolates, while the presence of *Fot 1* at these loci seems to be *F. oxysporum* f. sp. *albedinis* specific.

Primer pair TL3-FOA28 could be used to amplify a 400-bp fragment from all of the *F. oxysporum* f. sp. *albedinis* isolates. With primer pair BIO3-FOA1, a 204-bp fragment could be amplified from 95% of these isolates. Sixteen *F. oxysporum* f. sp. *albedinis* strains were not amplified with primer pair BIO3-FOA1. Additional hybridization experiments indicated that none of these 16 isolates carried a *Fot 1* insertion (truncated or full length) at the corresponding locus.

In this study, we showed that the *Fot 1* copy in pFalb11 is truncated, lacking 182 bp at its 3' end, and that this truncated copy was carried by 95% of the *F. oxysporum* f. sp. *albedinis* isolates tested. Given the high percentage of Algerian and Moroccan strains that possess the truncated copy and epidemiological data indicating that the Algerian oases were contaminated from Morocco, we think it likely that an ancestral *F. oxysporum* f. sp. *albedinis* strain had a full-length copy that became truncated in one sublineage and was fully lost in another.

We have thus succeeded in obtaining specific oligonucleotides for identifying the date palm pathogen *F. oxysporum* f. sp. *albedinis*. PCR has been successfully used as a sensitive and powerful diagnostic tool for microorganisms in the environment and has been applied to the detection of phytopathogenic and symbiotic fungi in plants or soil (5, 9, 10, 18, 20, 23). This work reports a novel development for a PCR to accurately assign *F. oxysporum* strains to a particular special form. Our study shows the usefulness of transposons and their flanking regions for designing PCR primers specific for the *F. oxysporum* special forms. We have taken advantage of the high level of genetic relatedness among isolates pathogenic for date palm to develop a sensitive PCR assay. Less than  $10^{-10}$  g of purified DNA could be efficiently detected, and further studies were carried out to detect the pathogen directly in host tissues.

This approach differs slightly from approaches that successfully developed PCR primers to identify *F. oxysporum* pathogenic isolates (16, 24). Unless *F. oxysporum* f. sp. *albedinis* shows a remarkable level of genetic homogeneity compared to most of the *F. oxysporum* special forms studied (8, 12, 22), the same strategy could be used for other special forms carrying *Fot 1*. In the same way, in fungal species where several transposable elements have been characterized, such as *Magnaporthe grisea* (19), this transposon-based approach could be used for developing diagnostic PCR tests. Recent characterizations of microsatellite loci in fungi also have proved to be useful for obtaining primers derived from the flanking regions (9) and could provide a novel source of target sequences for PCR tests.

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