European Journal of Plant Pathology **110:** 7–11, 2004.

Development of PCR primers for a new *Fusarium oxysporum* pathogenic on Paris daisy (*Argyranthemum frutescens* L.)

Matias Pasquali[1], Alberto Acquadro[1], Virgilio Balmas[2], Quirico Migheli[2], Maria Lodovica Gullino[1] and Angelo Garibaldi[1]
[1]*AGRINNOVA – Centre of Competence for the Innovation in the agro-environmental field, University of Torino, Via Leonardo da Vinci 44, I-10095 Grugliasco (Torino), Italy (Phone: +39 011 6708696; Fax: +39 011 6708541; E-mail: matias.pasquali@unito.it);* [2]*Department of Plant Protection and Center of Excellence for Biotechnology Development and Biodiversity Research, University of Sassari, Via E. De Nicola 9, I-07100 Sassari, Italy*

Accepted 14 July 2003

Key words: transposons, *Fot1*, inverse PCR, molecular diagnosis

Abstract

The inverse PCR technique was applied to clone genomic DNA flanking insertion sites of sequences homologous to the transposable element *Fot1* in the genome of a new pathogenic isolate of *Fusarium oxysporum* obtained from wilted *Argyranthemum frutescens* (Paris daisy). Based on the genomic flanking regions, a primer was designed which when paired to a second primer matching the *Fot1* sequence allowed detection of this pathogen by PCR. The primer pair Mg5/Mg6 could specifically identify nine tested isolates of *F. oxysporum* from *A. frutescens*, when fungal genomic DNA was used as template. Moreover, the primer pair Mg5/Mg6 allowed successful detection of the pathogen in stem and root tissue from asymptomatic plants that were artificially inoculated with a representative isolate of *F. oxysporum* from *A. frutescens.*

Argyranthemum frutescens (L.) (Webb and Berth), commonly known as Paris daisy, is a flower crop grown in many mediterranean countries. In Italy, *A. frutescens*-cultivated areas have increased substantially during the last decade, mostly in the Riviera Ligure (Northwest Italy). In 2000, the production of potted plants in this region reached 12 million units, mainly exported to central and northern Europe (Garibaldi et al., 1998, 2002; Minuto et al., 2000). During the summer of 1997, a new vascular wilt disease caused by *Fusarium oxysporum* was first reported in the Riviera Ligure on the cv. Camilla Ponticelli (Garibaldi et al., 1998). Infected plants show the typical symptoms of vascular disease, such as yellowing and progressive wilting, dark-blue or black basal necrosis and xylem discoloration. The symptoms can be undetectable at low to medium temperatures. This new disease affected more than 30% of plants in several greenhouses (Minuto et al., 2000), thus causing serious concern in the region. Moreover, the appearance of symptoms is limited to the warm season, creating ideal conditions for rapidly expanding epidemics through asymptomatic cuttings (Garibaldi et al., 1998; Minuto et al., 2000). Such situation can cause serious damages to growers and the loss of important markets for this crop. There are no other reports of this new disease besides those described in the Riviera Ligure (Garibaldi et al., 1998; Minuto et al., 2000).

Fusarium wilt management on *A. frutescens* is based on the integration of various control measures such as using disease-free cuttings, soil disinfestation, soil drenching with benomyl, prochloraz, or strobilurines, biological control with antagonistic *Fusarium* spp. (Garibaldi and Gullino, 1990; Garibaldi et al., 1990; Gullino et al., 2002). There is a high probability of the pathogen being disseminated in infected cuttings and of spreading disease in other mediterranean countries where daisy is grown. Consequently, there is a need to design new approaches to allow rapid and reliable detection of *F. oxysporum* pathogenic on Paris daisy mother plants.

The aim of this study was to develop specific primers for the PCR-based identification of this pathogen on plant tissue.

We analyzed a collection of nine *F. oxysporum* isolates, obtained from diseased *A. frutescens* in Italy during 1997–1999 (the reference isolate QUA 1 has been deposited at CBS-Centraalbureau voor Schimmelcultures, Royal Netherlands Academy of Arts and Sciences, Utrecht, the Netherlands, with the accession number CBS 112085). Non-pathogenic *F. oxysporum* used as antagonists against pathogenic *F. oxysporum* (Garibaldi and Gullino, 1990; Minuto et al., 1995, 1997, 2000), and representatives of *F. oxysporum* formae speciales *basilici*, *chrysanthemi*, *cyclaminis*, *dianthi*, *gladioli*, *lilii*, *lycopersici*, *melonis*, *pisi*, *radicis-lycopersici* and *tracheiphilum* were included as control DNAs (Table 1). Genomic DNA was obtained as previously described by Chiocchetti et al. (1999).

In order to obtain polymorphism useful for specific primer design, the distribution of the transposable element *Fot1* was determined by Southern hybridization. The *Fot1* probe was obtained amplifying the corresponding transposon sequence in the

Table 1. Code, American type Culture Collection (ATCC) or Centraalbureau voor Schimmelcultures (CBS) accession number, *forma specialis* or host plant, farm and geographic origin of *F. oxysporum* isolates tested in this work

Code	ATCC or *CBS*	*F. specialis* or host plant	Farm	Geographic origin
QUA 1	*112085*	*Argyranthemum frutescens*	Quarone	Albenga, Savona, Italy
QUA 2	—	*Argyranthemum frutescens*	Quarone	Albenga, Savona, Italy
QUA 3	—	*Argyranthemum frutescens*	Quarone	Albenga, Savona, Italy
VIG 4	—	*Argyranthemum frutescens*	Vigo	Albenga, Savona, Italy
VIG 5	—	*Argyranthemum frutescens*	Vigo	Albenga, Savona, Italy
CRI 6	—	*Argyranthemum frutescens*	Cappello	Albenga, Savona, Italy
PER 7	—	*Argyranthemum frutescens*	Perotto	Albenga, Savona, Italy
REP 8	—	*Argyranthemum frutescens*	Repellini	Albenga, Savona, Italy
CER 9	—	*Argyranthemum frutescens*	CERSAA	Albenga, Savona, Italy
FOB 025*	—	*Basilici*	Besor	Israel
FOC52422	52422	*Chrysanthemi*	Unknown	USA
FOC66279	66279	*Chrysanthemi*	Unknown	California, USA
FOCy	—	*Cyclaminis*	Michero	Albenga, Savona, Italy
FODR1 (race 1)	204207	*Dianthi*	Unknown	Albenga, Savona, Italy
FODR2 (race 2)	204225	*Dianthi*	Unknown	France
FODR4 (race 4)	204234	*Dianthi*	Unknown	Italy
FODR5 (race 5)	—	*Dianthi*	Unknown	Albenga, Savona, Italy
FOG	—	*Gladioli*	Unknown	Ormea, Cuneo, Italy
FOLI	—	*Lilii*	Unknown	Bagnasco, Cuneo, Italy
FOL15	—	*Lycopersici*	Unknown	Albenga, Savona, Italy
FOMK419	—	*Melonis*	Unknown	Tortona, Alessandria, Italy
FOPR3 (race 3)	—	*Pisi*	Unknown	Napoli, Italy
FORL28	—	*Radicis-lycopersici*	Leone	Toirano, Savona, Italy
FOT166608	16608	*Tracheiphilum*	Unknown	Unknown
FOT16609	16609	*Tracheiphilum*	Unknown	Unknown
FOT16610	16610	*Tracheiphilum*	Unknown	Unknown
FOT32724	32724	*Tracheiphilum*	Unknown	Nigeria, Africa
FOT62913	62913	*Tracheiphilum*	Unknown	Georgia, USA
245wt	—	Non-pathogenic	CERSAA	Albenga, Savona, Italy
233/2	—	Non-pathogenic	CERSAA	Albenga, Savona, Italy
251/2	—	Non-pathogenic	CERSAA	Albenga, Savona, Italy
257	—	Non-pathogenic	CERSAA	Albenga, Savona, Italy
152 wt	—	Non-pathogenic	CERSAA	Albenga, Savona, Italy
MSA 35	—	Non-pathogenic	CERSAA	Albenga, Savona, Italy
MSA 32	—	Non-pathogenic	CERSAA	Albenga, Savona, Italy

*Kindly provided by Dr. Talma Katan, The ARO-Volcani Center, Bet Dagan, Israel.

F. oxysporum isolate QUA 1 from *A. frutescens* (Table 1) using the primer FOT1 (5′-AGTCAAGC-ACCCATGTAACCGACCCCCCCTGG-3′), homologous to the inverted terminal repeat of the transposable element *Fot1* of *F. oxysporum* f. sp. *melonis* (Daboussi et al., 1992; Migheli et al., 1999). The amplification product was purified from gel with Quicksorb kit (Genomed, Research Triangle Park, NC, USA), labeled with DIG labeling kit (Roche, Basel, Switzerland) and used as a probe in Southern hybridization performed following Chiocchetti et al. (1999) with *Xho*I-digested genomic DNA of the nine isolates of *F. oxysporum* from *A. frutescens* and of representative isolates of the formae speciales *tracheiphilum* and *chrysanthemi*, that are also pathogenic on *Asteraceae*.

All the isolates from *A. frutescens* (Table 1) presented identical profiles, consisting of at least four *Fot1*-hybridizing bands at 4.7, 5.0, 10.0 and 11.5 kb (Figure 1). The *Fot1*-hybridizing pattern in the *F. oxysporum* isolates obtained from Paris daisy differed from the profiles shown by three of the tested isolates of *F. oxysporum* f. sp. *tracheiphilum*. The two tested isolates of the forma specialis *chrysanthemi* and two out of five representatives of forma specialis *tracheiphilum* lacked *Fot1*-homologous sequences (Figure 1).

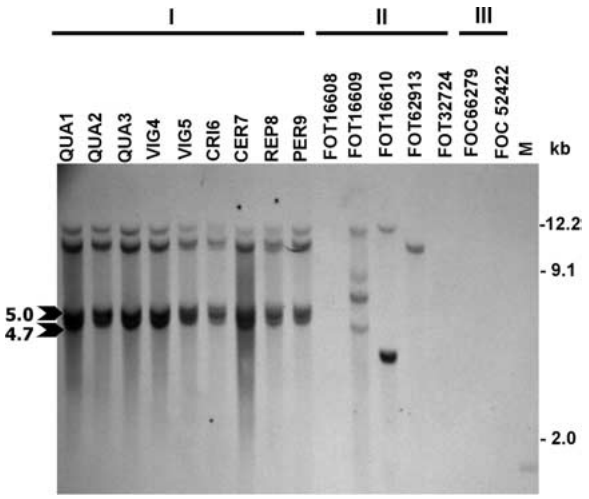

Figure 1. Distribution of sequences homologous to the transposable element *Fot1* in the genome of nine isolates of *F. oxysporum* obtained from *A. frutescens* (I) and of representatives of the formae speciales *tracheiphilum* (II) and *chrysanthemi* (III). Total genomic DNAs were digested with the restriction enzyme *Xho*I, separated by electrophoresis in 0.8% agarose gels, blotted and hybridized to the *Fot1* probe. The size (kb) of selected bands of the marker (1 kb DNA Ladder, Life Technologies, Gaithersburg, MD, USA) is indicated on the right margin. The size (kb) of target insertions is indicated on the left margin.

The distribution of sequences homologous to the transposable element *Fot1* within the genome of the new *F. oxysporum* isolates pathogenic on *A. frutescens* confirms that the Riviera Ligure outbreak has been generated by a clonal population, since the insertion pattern appears identical in all the isolates obtained from this production area. This evidence allowed us to adopt the same strategy already used in the case of *F. oxysporum* f. sp. *albedinis* (Fernandez et al., 1998), and f. sp. *dianthi* (Chiocchetti et al., 1999). We hypothesized that if copies of *Fot1* are inserted in unique positions, the insertion region can be specifically amplified by using primers overlapping the 3′ or the 5′ end of the transposon and its genomic flanks. To obtain the flanking regions we used the IPCR technique (Ochman et al., 1988; Triglia et al., 1988) following the same procedure described by Chiocchetti et al. (1999). Amplification of template DNA from QUA 1 isolate with primers Ft2 (5′-CCTTCCTAATGGCGCGTGATCCCCG-3′) and Ft3 (5′-GGCGATCTTGATTGTATTGTGGTG-3′) in the first IPCR cycle generated two amplicons of 3.5 and 3.8 kb. The nested PCR with primers Ft4 (5′-CTCTGCATTTTTAGCTATTTATTTGAC-3′) and Ft5 (5′-CGTCCGCAGAGTATACCGGCATTGTAG-3′) generated two bands of 3.1 and 3.4 kb (corresponding to the 4.7 and 5.0 kb *Fot1* insertions shown in Figure 1, respectively).

Due to the difficulty encountered in separating the nested PCR products, the two amplicons were eluted from the gel, purified and digested with the restriction enzyme *Xho*I. Four bands were obtained and based on their molecular weight, their respective origin was determined (data not shown). A fragment of 1.5 kb was identified as the genomic sequence flanking the 3′ end of the *Fot1* copy inserted within the 5.0 kb *Xho*I fragment, and cloned as the PAS3A-1 clone (GenBank accession number: AF282999). The sequence was obtained by the Sequencing Service of the Bioindustry Park Canavese s.r.l. (Colleretto Giacosa, TO, Italy) that automatically sequenced by using a CEQ 2000 Analysis System (Beckman Coulter, Inc., Fullerton, CA, USA). This clone, separated from the flanking *Fot1* sequences, was used to probe Southern blots containing genomic DNA of *F. oxysporum* from *A. frutescens* as well as all the control DNAs, in order to confirm its identity. A complex pattern of hybridization was

obtained (data not shown), leading to the hypothesis that the insertion occurred into repeated sequences.

Primer Mg5 (5′-GGGGTCGGTTACATGGGTG-3′), based on the *Fot1* sequence, and primer Mg6 (5′-CAACAACAAGGCGAAGAGGG-3′), matching the PAS3A-1 sequence, were designed by using the program Primer3 (S. Rozen and H.J. Skaletsky, 1998. Primer3 Code available at the website: http://www.genome.wi.mit.edu/genome_software/other/primer3.html).

One-hundred nanograms of genomic DNAs of all the *F. oxysporum* isolates listed in Table 1 was amplified in 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.01% (w/v) gelatin with the addition of 350 mM of each nucleotide (Finnzymes Oy, Espoo, Finland), 0.5 mM of each primer Mg5 and Mg6, and 0.5 μl of crude recombinant *Taq* polymerase prepared according to Desai and Pfaffle (1995) in order to validate primer specificity. PCR conditions were: 1 cycle at 94 °C for 5 min, followed by 32 cycles each consisting of a denaturation step at 94 °C for 10 s, annealing at 68 °C for 30 s and extension at 72 °C for 1 min, and a final extension cycle at 72 °C for 5 min. Amplification experiments were repeated at least two times. Five microliters of the amplification product were loaded in a 2% SeaKem LE agarose (FMC BioProducts) gel and separated as described previously. The expected amplification product of 166 bp, using primer pair Mg5–Mg6, was obtained only from genomic DNAs from all the new isolates highly pathogenic on *A. frutescens* (Figure 2). No amplification was obtained when genomic DNA from all the other isolates listed in Table 1 was tested as template, thus confirming the high level of specificity of the developed primers for the new isolates pathogenic on *A. frutescens*.

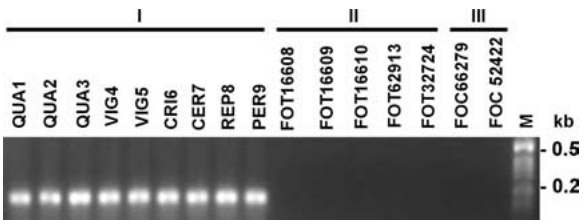

Figure 2. Agarose gel electrophoresis of PCR products from genomic DNAs of *F. oxysporum* by using the primer pair Mg5/Mg6. From left to right: isolates of *F. oxysporum* obtained from *A. frutescens* (I); representatives of the formae speciales *tracheiphilum* (II) and *chrysanthemi* (III); M, molecular size marker (1 kb DNA Ladder, Life Technologies, Gaithersburg, MD, USA). Molecular sizes (kb) are indicated on the right margin.

To verify the efficiency of the PCR reaction also on DNA extracted from infected plants we obtained 10^7 CFU ml^{-1} conidia cell density of *F. oxysporum* isolates VIG 4 and FOT32724 as previously described by Chiocchetti et al. (1999). The inoculum was applied to the plant roots by dipping rooted cuttings of the susceptible cv. Camilla Ponticelli in a conidial suspension of each isolate for 30 s at transplanting. A mock control was added by dipping daisy cuttings in sterile distilled H_2O. Ten days after transplanting (while VIG 4 plants were still asymptomatic) three plants for each treatment were uprooted and cut lengthwise. Roots and stems were isolated, washed with tap water, surface sterilized by dipping once in 3% sodium hypochlorite and twice in sterile distilled water, air dried, weighted, frozen in liquid nitrogen for 5 min and stored at −80 °C for DNA extraction at a later date as described by Chiocchetti et al. (1999) on carnation tissue. There were six replicate plants for each treatment (three plants tested in PCR experiments and three control plants to check the appearance of symptoms) and the experiment was repeated once. The first symptoms (leaf yellowing, xylem discoloration) on control plants appeared 14–18 days after artificial inoculation with isolate VIG 4 and 3–4 days later, these plants were completely wilted (data not shown). Therefore, on asymptomatic plants amplification with primers Mg5/Mg6 generated the expected band at 166 bp, allowing detection of isolate VIG 4 in both root and stem tissues collected from all the inoculated samples (Figure 3). No detectable signal was obtained upon amplification from tissues of plants inoculated with *F. oxysporum* f. sp. *tracheiphilum* FOT32724 or from mock-inoculated controls (Figure 3).

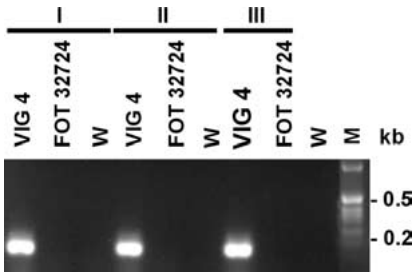

Figure 3. Agarose gel electrophoresis of PCR products obtained by using the primer pair Mg5/Mg6 from: DNA samples extracted from: (I) root or (II) stem of Paris daisy artificially inoculated by dipping in a conidial suspension of isolates VIG 4 or FOT32724 or (W) mock-inoculated by dipping in water; (III) genomic DNA of *F. oxysporum* isolates VIG 4 and FOT32724; (W) negative control. Molecular sizes (kb) are indicated on the right margin.

This protocol allows a rapid and precise identification of propagules of this new pathogen within the cultivated host, thus facilitating the use of pathogen-free propagative material, essential prerequisites for developing control strategies based on prevention.

This work represents a further confirmation that the strategy described here may be applied to develop PCR-based diagnostics for any *F. oxysporum* bearing transposable elements within its genome, provided that these sequences are stably inserted at specific sites (Daboussi, 1997; Daboussi and Langin, 1994). Moreover, the use of universal reverse primers matching the transposon sequence shall facilitate development of multiplex PCR techniques, as previously shown in the case of *F. oxysporum* f. sp. *dianthi* (Chiocchetti et al., 1999).

Acknowledgements

Research supported by Regione Liguria, Ministero dell'Università e della Ricerca Scientifica e Tecnologica and by the National Research Council of Italy (Special Project "Diagnosi precoce di malattie nelle piante di interesse agrario e forestale").

References

Chiocchetti A, Bernardo I, Daboussi MJ, Garibaldi A, Gullino ML, Langin T and Migheli Q (1999) Detection of *Fusarium oxysporum* f. sp. *dianthi* in carnation tissue by PCR amplification of transposon insertions. Phytopathology 89: 1169–1175

Daboussi MJ (1997) Fungal transposable elements and genome evolution. Genetica 100: 253–260

Daboussi MJ and Langin T (1994) Transposable elements in the fungal plant pathogen *Fusarium oxysporum*. Genetica 93: 49–59

Daboussi MJ, Langin T and Brygoo Y (1992) *Fot1*, a new family of fungal transposable elements. Molecular and General Genetics 232: 12–16

Desai UJ and Pfaffle PK (1995) Single step purification of a thermostable DNA polymerase expressed in *Escherichia coli.* BioTechniques 19: 780–784

Fernandez D, Ouinten M, Tantaoui A, Geiger JP and Langin T (1998) *Fot1* insertions in the *Fusarium oxysporum* f. sp. *albedinis* genome provide diagnostic PCR target for detection of the date palm pathogen. Applied and Environmental Microbiology 64: 633–636

Garibaldi A and Gullino M (1990) Disease management of ornamental plants: A never ending challenge. Mededelingen van de Faculteit Landbouwwetenschappen, Rijksuniversiteit Gent 55-2A: 189–201

Garibaldi A, Gullino ML and Aloi C (1990) Biological control of Fusarium wilt of carnation. Proceeding of the Brighton Crop Protection Conference 3A-1: 89–95

Garibaldi A, Minuto A and Gullino ML (1998) First report of Fusarium wilt on Paris daisy (*Argyranthemum* [*Dendranthema*] *frutescens*). (Abstr.) Plant Disease 82: 1403

Garibaldi A, Minuto A, Bertetti D and Bruzzone C (2002) Sensibilità varietale di cultivar di margherita (*Argyranthemum frutescens*) a *Fusarium oxysporum*. Informatore fitopatologico 52(3): 75–77 (in Italian)

Gullino ML, Minuto A, Gilardi G and Garibaldi A (2002) Efficacy of azoxystrobin and other strobilurins against Fusarium wilts of carnation, cyclamen and Paris daisy. Crop Protection 21: 57–61

Migheli Q, Laugé R, Davière JM, Gerlinger C, Kaper F, Langin T and Daboussi MJ (1999) Transposition of the autonomous *Fot1* element in the filamentous fungus *Fusarium oxysporum*. Genetics 151: 1005–1013

Minuto A, Migheli Q and Garibaldi A (1995) Evaluation of antagonistic strains of *Fusarium* in the biological and integrated control of Fusarium wilt of cyclamen. Crop Protection 14: 221–226

Minuto A, Minuto G, Migheli Q, Mocioni M and Gullino ML (1997) Effect of antagonistic *Fusarium* spp. and of different commercial biofungicide formulations on Fusarium wilt of basil (*Ocimum basilicum* L.). Crop Protection 16: 765–769

Minuto G, Bertetti D, Gullino ML and Garibaldi A (2000) Lotta chimica e biologica alla tracheofusariosi della margherita. Atti Giornate Fitopatologiche 2: 323–328 (in Italian)

Ochman H, Gerber AS and Harte DL (1988) Genetic application of an inverse polymerase chain reaction. Genetics 120: 621–625

Triglia T, Peterson MG and Kemp DJ (1988) A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Research 16: 81–86