# A DIVERGENT POLYGALACTURONASE OF *FUSARIUM PHYLLOPHILUM* SHOWS SEQUENCE AND FUNCTIONAL SIMILARITY TO THE ENZYME OF *F. VERTICILLIOIDES*

**L. Mariotti[1], M. Casasoli[1], C. Caprari[2] and G. De Lorenzo[1]**

[1] *Dipartimento di Biologia Vegetale, Università di Roma La Sapienza, Piazzale A. Moro 5, 00185 Roma, Italy*
[2] *Dipartimento di Scienze e Tecnologie per l'Ambiente e il Territorio, Università del Molise, Contrada Fonte Lappone, 86090 Pesche (IS), Italy*

**SUMMARY**

Endopolygalacturonases (endoPGs) are fungal enzymes secreted during the infection in order to degrade plant cell wall pectins. In *Fusarium* spp., endoPGs are among the first enzymes produced during infection and play a crucial role in plant tissue penetration and colonization. The endoPG of *F. phyllophilum* strain FC-10, previously classified as *F. verticillioides*, is the best characterized *Fusarium* cell wall degrading enzyme. In this work we have carried out a phylogenetic analysis of the endoPG (*pg*) gene sequence that confirms the classification of the FC-10 strain as *F. phyllophilum*, and also shows an unexpected divergence of the *pg* gene of *F. phyllophilum* strain NRRL 25305. This gene and the biochemical characteristics of the encoded product appear more closely related to those of *F. verticillioides pg*. This observation and the evidence that endoPGs have experienced positive selection indicate that selective pressure acting on these enzymes may limit the use of their gene sequences as reliable markers for phylogenetic studies.

*Keywords*: *Fusarium phyllophilum*, polygalacturonase, PGIP, taxonomy.

## INTRODUCTION

Pathogenesis by fungi often involves degradation of the mechanical barrier represented by the plant cell wall as a crucial event both to release compounds that are used as carbon sources by the fungus and to facilitate invasion, colonization and in many cases maceration of the host tissue. *Fusarium* spp. produce a great variety of cell wall degrading enzymes (CWDE) (Caprari *et al.*, 1996; de las Heras *et al.*, 2003; Jenczmionka and Schafer, 2005; Ospina-Giraldo *et al.*, 2003). Endopolygalacturonases [poly (1,4-a-D-galacturonide) glycanohydrolase, EC 3.2.1.15; endoPGs] are among the first enzymes produced during infection (Garcia-Maceira *et al.*, 2001). EndoPGs degrade homogalacturonan to tri-, di-, and monogalacturonic acid as final products; however, partial degradation leads to the release of oligogalacturonides (OGs), which are elicitors of plant defence responses (Hahn *et al.*, 1981). The interaction of endoPGs with plant cell wall polygalacturonase-inhibiting proteins (PGIPs) modulates their enzymatic activity and may favour the accumulation of elicitor-active OGs (Cervone *et al.*, 1989; De Lorenzo and Ferrari, 2002). PGIPs are characterized by 9-10 repeats of a leucine-rich sequence (LRR), indicated as responsible for binding PGs (De Lorenzo *et al.*, 1994; De Lorenzo *et al.*, 2001; Di Matteo *et al.*, 2003; Federici *et al.*, 2006).

Several *Fusarium* endoPGs have been characterized biochemically and genetically. They are very variable, with multiple isoforms (Caprari *et al.*, 1993a; Chimwamurombe *et al.*, 2001; Fernandez-Pol *et al.*, 1993; Niture and Pant, 2004), different modes of action (Bonnin *et al.*, 2002) and different pectin substrates (André-Leroux *et al.*, 2005). The endoPG of *F. phyllophilum* strain FC-10, previously classified as *F. verticillioides* (Mariotti *et al.*, 2008) is one of the best studied PGs. This enzyme, herein indicated as FpPG, is encoded by a single copy gene expressed only in the presence of pectin as the sole carbon source in the medium and consists of four different glycoforms (Caprari *et al.*, 1993a; De Lorenzo *et al.*, 1987). The plant inhibitors PvPGIP2 of *Phaseolus vulgaris* and GmPGIP3 of *Glycine max* are the only known inhibitors for this endoPG (D'Ovidio *et al.*, 2006; De Lorenzo *et al.*, 2001; Leckie *et al.*, 1999). These features distinguish FpPG from the enzymes of *F. verticillioides* strains PD and 62264, which show only two glycoforms and are unaffected in the presence of the inhibitor (Raiola *et al.*, 2007; Sella *et al.*, 2004).

The 3-D structure of FpPG, deduced by crystallographic analysis, shows the typical pectic enzyme structure, a right-handed parallel beta helix, formed by 10 coils with tandem repetition of 3-4 beta strands for coil, and the active site located in a deep cleft on one side of the enzyme (Federici *et al.*, 2001; Herron *et al.*, 2000). FpPG amino acid residues involved in binding of PGIP have been identified by site-directed mutagenesis studies (Federici *et al.*, 2001; Raiola *et al.*, 2007). From the

*Corresponding author*: C. Caprari
Fax: +390874404123
E-mail: claudio.caprari@unimol.it

evolutionary point of view, a molecular co-evolutionary race between endoPGs and PGIP proteins may be envisaged: PGIPs continually adapt in response to variation of pathogen-derived PGs that, on the other hand, evolve to escape PGIP recognition. Variation of specific residues (named positively selected sites), which are likely important for recognition, has been shown to occur in both proteins (Stotz *et al.*, 2000; Bishop, 2005).

The mode of interaction of FpPG with PvPGIP2 has been studied in detail. Surface plasmon resonance analysis and site-directed mutagenesis allowed characterization of the amino acid residues in the LRR domain of PvPGIP2 that are responsible for recognition of this PG (Leckie *et al.*, 1999). Furthermore, molecular docking studies have predicted a mode of interaction of this PG with PvPGIP2 that is in agreement with kinetic analysis showing competitive inhibition (Federici *et al.*, 2001, 2006).

Since an earlier paper proposed the use of the endoPG-encoding gene (*pg*) as a marker for taxonomic analysis in *Fusarium* (Posada *et al.*, 2000), we assessed whether phylogenetic analysis of the *pg* genes of the different *Fusarium* strains and species examined to reclassify the FC-10 strain as *F. phyllophilum* would confirm the results obtained with other taxonomic markers (Mariotti *et al.*, 2008). Here we show that the analysis supports the identification of strain FC-10 as *F. phyllophilum*; however, it revealed an unexpected divergence of the *pg* gene of *F. phyllophilum* strain NRRL 25305. This gene and the biochemical characteristics of the encoded product appeared more closely related to those of *F. verticillioides pg*. A maximum likelihood analysis confirmed that *pg* genes have experienced positive selection. We therefore suggest caution in the use of the *pg* gene sequences as molecular markers for phylogenetic analysis.

## MATERIALS AND METHODS

**Fungal strains and growth conditions.** *F. phyllophilum* sp. strain FC-10 was provided by Prof. F. Cervone (University La Sapienza, Rome, Italy); *F. phyllophilum* strains NRRL 25218, NRRL 25219, NRRL 25305 were provided by Dr. K. O'Donnell (USDA - NCAUR, Peoria, USA); *F. fujikuroi* strain PVS Fu-103 was provided by Dr. V. Balmas (University of Sassari, Italy); *F. verticillioides* strain PD was provided by Prof. F. Favaron (University of Padova, Italy); *F. verticillioides* strain 62264 was provided by Dr. L. Daroda (E.N.E.A., Casaccia, Italy). The strains were routinely grown on potato dextrose agar (PDA, Merck, Darmstadt, Germany) under natural day/night conditions at 21°C for 15 days.

**Genomic DNA isolation.** Fungal mycelium was inoculated in 100 ml PDB medium and grown for 6 days at 21°C in a 500-ml flask without shaking. Filtered mycelium (500-800 mg) was homogenized in liquid nitrogen and resuspended in extraction buffer (50 mM Tris-HCl pH 6.8, 20 mM EDTA, 2% sarcosyl, 8 M urea, 100 mM NaCl). A 1:1 phenol-chloroform solution was added to the homogenate, which was then centrifuged at 3000 *g* for 15 min. The aqueous phase was added to 0.11 vol of 3 M sodium acetate pH 5.8 and 2.5 vol of 100% ethanol and centrifuged at 30000 *g* for 30 min. The pellet was washed with 70% ethanol, recovered by centrifugation at 30000 *g* for 30 min and then redissolved in sterile water.

**PCR amplification and sequencing of endoPG (*pg*) genes.** Amplicons corresponding to the entire *pg* gene were obtained using oligonucleotides (FmPGEcofw: 5'-ATCGGAATTCGATCCCTGCTCCGTGACTGAG-TAC-3' and FmXbarv: 5'-ATCGTCTAGACTAGCTG GGGCAAGTGTT-3') matching the genomic sequence of *F. phyllophilum* FC-10 *pg* gene (Caprari *et al.*, 1993a). PCR performed with Taq DNA polymerase according to manufacturer's instructions (Promega, USA), was set up as follows: 1 cycle at 94°C for 4 min, 25 cycles consisting of a denaturation step at 94°C for 2 min, an annealing step at 56°C for 1 min, an elongation step at 72°C for 1 min and a final cycle at 72°C for 7 min. PCR products were analysed by electrophoresis on a 1% agarose gel. Amplified DNA was purified using a GFX™ PCR DNA gel band purification kit (GE Healthcare, USA), and sent to MWG (Germany) for sequencing.

**Sequence analysis.** Sequences of *pg* genes used for analysis were either determined as described above or obtained from NCBI (accession numbers are reported in Fig.1). Sequence alignments and phylogenetic analyses were performed using MEGA 3 software version 3.1 (Kumar *et al.*, 2004). Phylogenetic trees were constructed using the Neighbour-Joining (NJ) and the Kimura–2P distance algorithms. Construction of trees was also performed with Minimum Evolution and UPGMA algorithms and results were concordant with the trees obtained with the NJ method. Statistical support of trees was tested by bootstrap analysis using a 2000 random re-sampling and a 50% threshold (Kumar *et al.*, 2004).

**Southern blotting analysis**. Genomic DNA was extracted from mycelium grown in liquid medium (PDB) and aliquots of 10 mg were digested separately with 60 U of *Eco*RI, *Bgl*II and *Bam*HI restriction enzymes. Digested DNA was separated by electrophoresis on a 0.8% agarose gel and then transferred onto positively charged. Nucleobond nylon membrane (Amersham, USA). The membrane was incubated for 12 h at 65°C in hybridization buffer (DIG-easy Hyb, Roche, USA) with

an endo-PG-specific digoxigenin-labeled probe. The probe was obtained by amplification of a *Fusarium* FC-10 *pg* gene fragment with the primers SeqPGFW 5'-CTTGCCCGCTGCGC-3' and FmXbarv 5'-ATCGTC-TAGACTAGCTGGGGCAAGTGTT-3'. Digoxigenin (50 mM; Roche, USA) was directly included in the PCR mix, unincorporated Digoxigenin was removed using the High Pure PCR Cleanup Micro Kit (Roche, USA). The membrane was treated and developed according to the manufacturer's instructions (DIG Wash and Block buffer set, Roche, USA).

**Induction of endoPGs.** For *in vitro* induction of endoPGs, fungi were grown in 50 ml CZAPEK-DOX medium containing 0.2% glucose for 2 days with shaking (80 rpm) at 21°C, before addition of 1.5% citrus pectin to the culture medium as previously described (De Lorenzo *et al.*, 1987). After 3 days of growth cultures were filtered to remove mycelium and to collect the crude filtrate. This was dialyzed in sodium 20 mM acetate and stored at 4°C.

**Western blotting analysis.** SDS-PAGE of crude filtrates obtained from pectin-induced fungal cultures, was done with 10% polyacrylamide gels. Proteins were transferred to Hybond-C nitrocellulose membrane (Amersham, USA) at 100 V for 1 h. The membranes were blocked for 2 h at room temperature in blocking buffer (3% bovine serum albumin BSA, 0.1% Tween 20, 1% PBS), washed in 10% PBS and 0.2% Tween 20 (Sigma, USA) (Wash buffer), and incubated 12 h at room temperature with primary antibody mAb-antiFmPG (1:7000 dilution) in wash buffer containing 0.5% BSA. After washing, the peroxidase-conjugated anti-rabbit secondary antibody (Amersham, USA) (1:10000 diluition) was added to the membrane in a wash buffer containing 0.5% BSA, then incubated for 2 h at room temperature. Peroxidase activity was detected by enhanced chemoluminescence (ECL, Amersham, USA).

**Agar diffusion assay.** EndoPG enzymatic activity was assayed as described in Ferrari *et al.* (2003). An aliquot of crude filtrate from fungal cultures was loaded in 20-ml wells made in 30 ml plates of solid substrate (0.5% polygalacturonic acid, 0.8% agarose, 100 mM $CH_3COONa$) and incubated o/n at 28°C to allow diffusion. After adding 6 M HCl, degradation haloes revealed endoPG enzymatic activity. PG activity was expressed as agarose diffusion units, with 1 unit defined as the amount of enzyme producing a halo of 0.5 cm radius (external to the inoculation well). The inhibition assay was performed loading the fungal crude filtrate and PvPGIP2 in the same well and incubated o/n at 28°C to allow diffusion. The agarose inhibition unit was defined as the amount of PvPGIP2 causing 50% inhibition of 1 agarose diffusion unit at pH 4.7.

**Analysis of codon substitution patterns.** Maximum-likelihood models of codon substitution, implemented in CODEML [PAML software, (Yang *et al.*, 2000)], were used to analyse a data set of sequences in order to test for heterogeneous selection pressure among amino acid sites. Sequence and tree input files were generated by MEGA3 software version 3.1 (Kumar *et al.*, 2004). Six different models, which allow the non-synonymous/synonymous rate ratio ($d_N/d_S$, i.e. the w ratio) to vary among codons, were used to test for the presence of sites under diversifying selection (with $w > 1$). Model M0 (one ratio) assumes one w for all sites; model M1 (neutral) assumes two classes of sites: a proportion $p_1$ of conserved sites at which $w = 0$ and a proportion $p_2 = 1 - p_1$ of neutral sites at which $w = 1$; model M2 (selection) adds a third class of sites with $w > 1$, estimated from the data, and with a frequency $p_3 = 1 - p_1 - p_2$; model M3 (discrete) uses a discrete and unconstrained distribution with three site classes, with both proportions and w ratios estimated form the data. Model M7 (beta) uses a beta distribution of site classes that can take various shapes (such L, J, U and inverted U shapes) in the interval (0.1), depending on two parameters p and q (Yang *et al.*, 2000). Finally, model M8 (beta&w>1) adds an extra class of sites to the M7 model, with the proportion and the ratios estimated from the data. A likelihood ratio test (LTR) was then performed to compare models that allow for positive selection with those that do not (M0 vs. M3, M1 vs. M2 and M7 vs. M8). For each comparison, twice the log-likelihood difference was compared with a $c^2$ distribution with the degrees of freedom equal to the difference in the number of parameters between the two models (Yang *et al.*, 2000).

## RESULTS

**Structure of the PG gene.** To assess the validity of *pg* gene sequences as taxonomic markers for classification of *Fusarium* species, as previously proposed by Posada *et al.* (2000), a phylogenetic analysis of the *F. phyllophilum* strains FC-10, NRRL 25218, NRRL 25219, NRRL 25305 and IHEM 10241, *F. verticillioides* strains 62264 and PD, and *F. fujikuroi* strain PVS Fu-103, previously used to reclassify the strain FC-10 as *F. phyllophilum* (Mariotti *et al.*, 2008), was performed. Fragments corresponding to the entire *pg* genes were amplified and directly sequenced. Notably, the *pg* sequence of *F. verticillioides* 62264 did not correspond to the previously published one (Daroda *et al.*, 2001) and was more similar to that of strain PD (99% instead of 94%).

In *Fusarium* spp., the *pg* gene is ~1400 bp long and has 5 exons and 4 introns (Caprari *et al.*, 1993a; Posada *et al.*, 2000) (Fig. 1A). An NJ tree was generated using the spliced *pg* sequences and including *pg* sequences of four other *Fusarium* species and *Ophiostoma novo-ulmi*

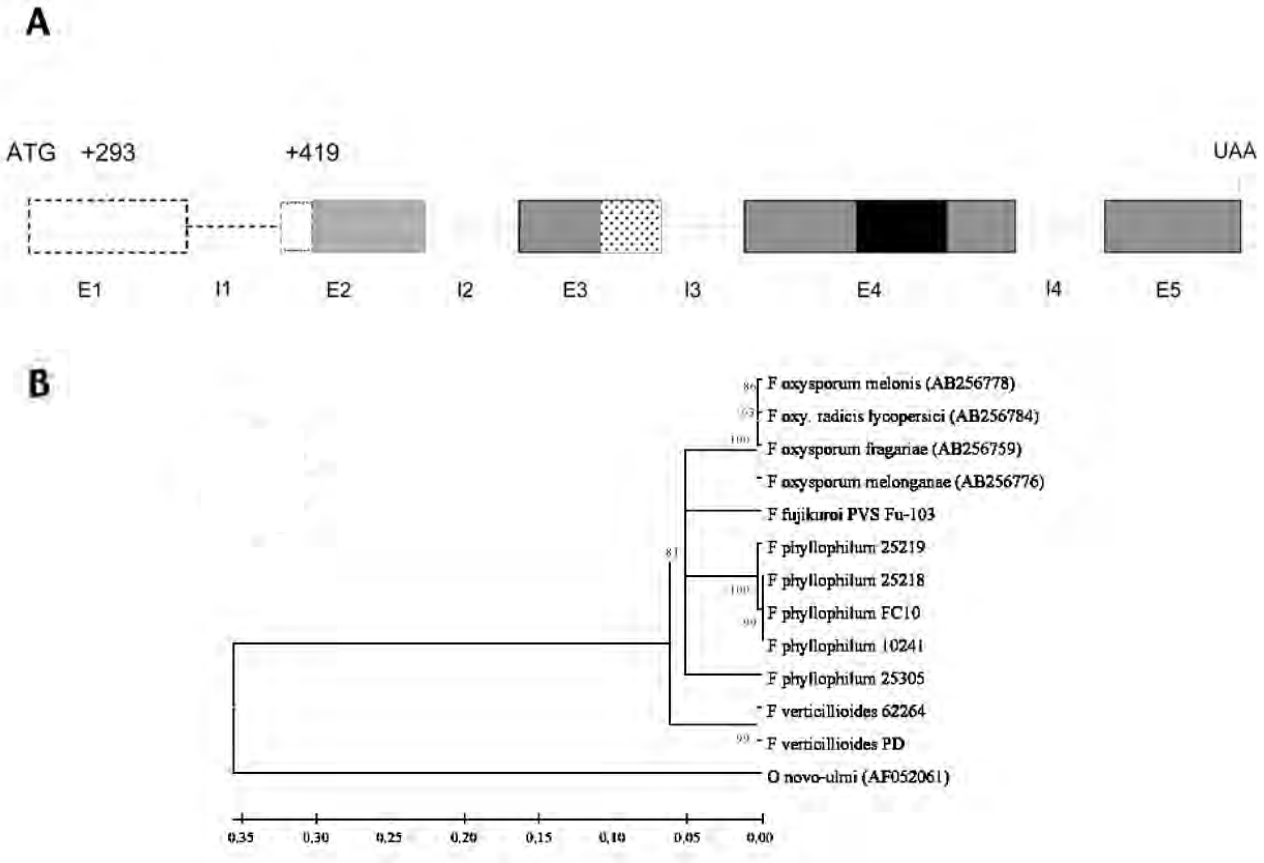


**Fig. 1.** Phylogenetic analysis of *Fusarium pg* genes. A. Schematic representation of the *pg* gene of *Fusarium* strain FC-10 (not to scale). Exons (E) are in grey and introns (I) are the lines. Region +293/+418 encodes for the signal peptide for secretion. The black box and the dashed box indicate sequences corresponding to the active site and the loop structures, respectively, described in Federici *et al.* (2001). Sequences of exon 1, intron 1 and the 5' portion of intron 2, shown with dotted lines, were not included in the analysis. B. Phylogenetic tree of *Fusarium pg* nucleotide sequences, generated using the Neighbour-Joining method employing the Kimura 2-parameter distance. Clade support was evaluated by a bootstrap analysis (2000 re-samplings). Branch lengths are proportional to distance as indicated by the scale bar and bootstrap support is as indicated for each branch. *Ophiostoma novo-ulmi* was included as outgroup. GenBank accession number of each sequences are in parenthesis (oxy.=oxysporum).

as an outgroup. The tree resolved two main clades, one grouping the *F. verticillioides* strains, and the other including *F. oxysporum* and *F. phyllophilum* strains NRRL 25219, IHEM 10241 and FC-10. Notably, *F. phyllophilum* NRRL 25305 and *F. fujikuroi* PVS Fu-103 did not belong to either clade (Fig. 1B).

Intron and exon sequences of the *pg* genes of *Fusarium* strains, including NRRL 25305, were separately analyzed, with particular attention to introns 3 and 4 and exon 4, previously used by Posada *et al.* (2000). Only sequences corresponding to the mature protein were considered, therefore excluding the region encoding the signal peptide for secretion, which is 24-amino acids long in FC-10; thus, exon 1, intron 1 and part of exon 2 were not included (Fig. 1A). Table 1 shows details of variable and parsimony informative sites detected aligning all sequences in the different endoPG exons and introns.

All trees constructed using the different *pg* gene regions showed that strain FC-10 grouped with the *F. phyllophilum* clade (Fig. 2). Unexpectedly, NRRL 25305, which belongs to the *F. phyllophilum* group according to morphological characteristics, growth conditions, and molecular marker analysis (Mariotti *et al.*, 2008), grouped in the *F. phyllophilum* clade only in the tree constructed with exon 2. Trees constructed with intron 2 and the contiguous exon 3, as well with exon 5, grouped this strain with the *F. verticillioides* strains, while in the trees constructed with intron 3 and exon 4, NRRL 25305 was alone in a separate clade, showing diversification of these gene regions.

The genomic organization of the *pg* gene in *F. phyllophilum* FC-10, NRRL 25305, IHEM 10241 and NRRL 25218 and *F. verticillioides* 62264 was compared by Southern blot analysis of genomic DNA separately digested with the *Eco*RI and *Bgl*II restriction enzymes, under high stringency. A digoxigenin-labelled fragment of 580 bp, amplified from the cloned FC-10 *pg* gene, was used as a probe. All strains shared a single *Eco*RI band of ~4.3 kb. A single *Bgl*II band of ~4.1 kb was observed

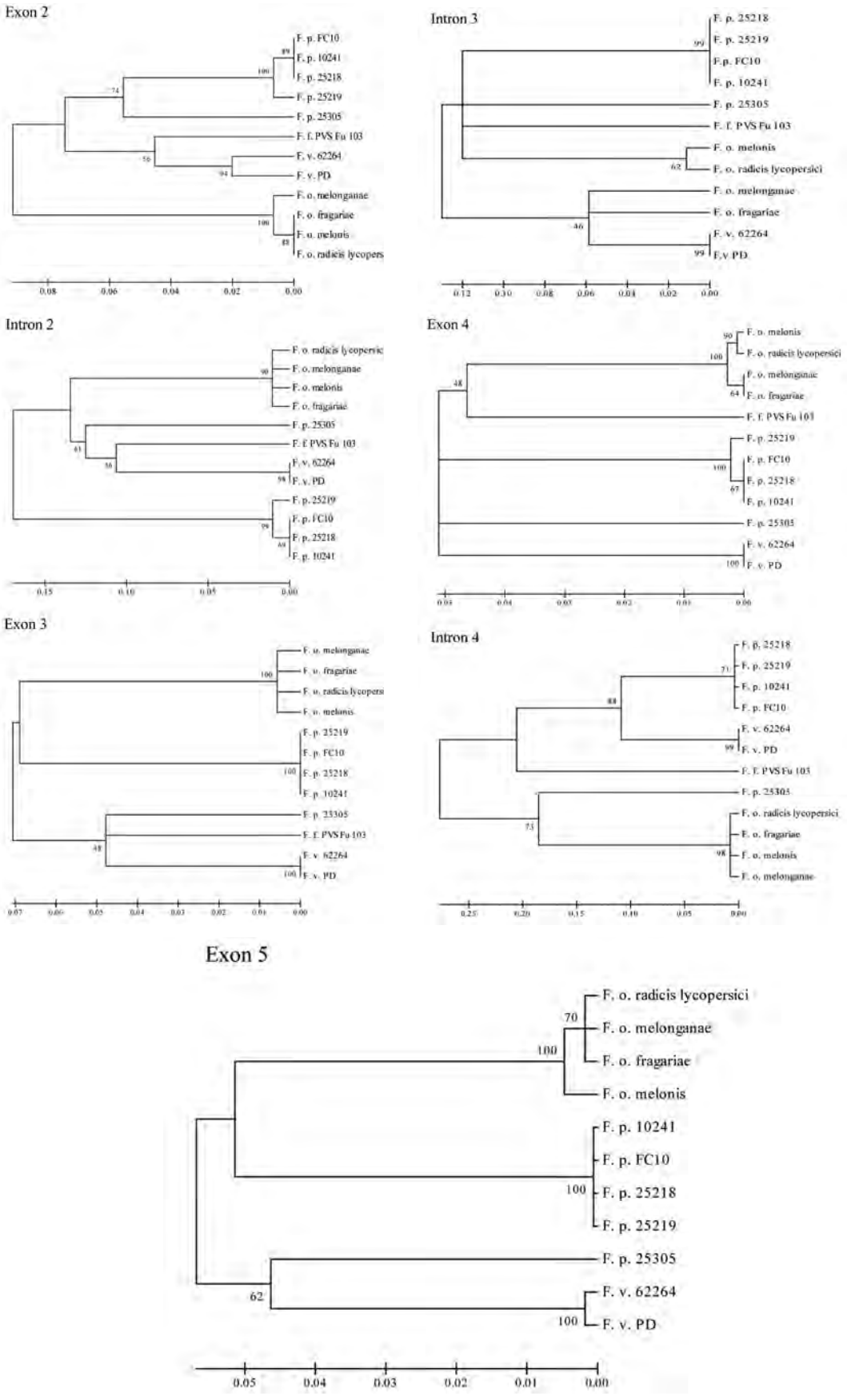


**Fig. 2.** Phylogenetic trees of introns and exons of *Fusarium* spp. *pg* genes. Intron and exons were analysed individually. Trees were generated using the NJ method and the Kimura 2-parameter distance. Clade support was evaluated by a bootstrap analysis (2000 re-samplings). Branch lengths are proportional to distance as indicated by the scale bar and bootstrap support is indicated for each branch. (F.p.= *F. phyllophilum*, F.v.= *F. verticillioides*, F.o.= *F. oxysporum*, F.f.= *F. fujikuroi*).

**Table 1.** Parameters estimated from the analysis of nucleotide and deduced amino acid sequences of exons and introns of *pg* genes (see Materials an Methods).

| | Exon 2 | Intron 2 | Exon 3 | Intron 3 | Exon 4 | Intron 4 | Exon 5 |
|---|---|---|---|---|---|---|---|
| Length (bp) | 153 | 53 | 149 | 55 | 455 | 54 | 290 |
| Variable sites | 43 | 28 | 39 | 24 | 96 | 30 | 60 |
| Parsimony informative sites | 35 | 19 | 29 | 17 | 64 | 25 | 45 |
| Non-synonymous substitutions /total amino acids | 14/51 | NA[a] | 11/49 | NA[a] | 20/151 | NA[a] | 10/96 |

[a]NA: not applicable

in *F. phyllophilum* FC10, IHEM 10241 and NRRL 25219, while *F. phyllophilum* NRRL 25305 and *F. verticillioides* 62264 showed single *Bgl*II bands of 5.5 kb and 5.8 kb, respectively (Fig. 3).

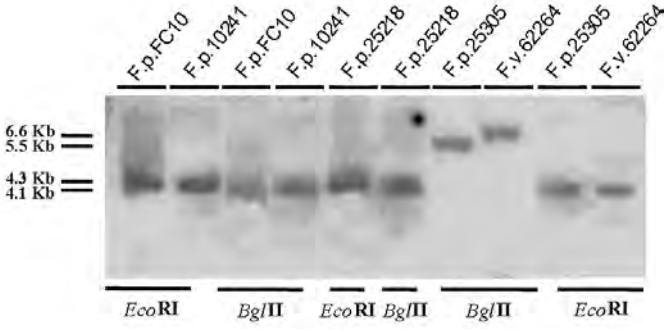


**Fig. 3.** Southern blot of *Fusarium* spp. and strains. Genomic DNA (10 mg) was digested with the restriction enzymes *Eco*RI and *Bgl*II, electrophoretically separated and analysed by Southern blotting using a digoxigenin-labelled probe. Hybridization was conducted under high stringency (65°C). F.p.= *F. phyllophilum*, F.v.= *F. verticillioides*. DNA fragment size (bp) is indicated on the left.

**Table 2.** Inhibitory activity of *Phaseolus vulgaris* PGIP2 (*Pv*PGIP2) against 10 µl of crude extract of *Fusarium* strains.

| Strain PG | PvPGIP2 [a] (ng) |
|---|---|
| *F. phyllophilum* FC-10 | 9 |
| *F. phyllophilum* 10241 | 9 |
| *F. phyllophilum* 25219 | 9 |
| *F. phyllophilum* 25218 | 9 |
| *F. phyllophilum* 25305 | ∞ |
| *F. verticillioides* 62264 | ∞ |
| *F. verticillioides* PD | ∞ |

[a]Amount of PvPGIP2 that determines 50% inhibition of 1 agarose diffusion unit (see Materials and Methods) of the indicated PGs at pH 4.7. The symbol ∞ indicates no inhibition for amounts up to 4 µg. Amount of PG in the different crude extracts was comparable (see Results).

**Analysis of deduced endoPG amino acid sequences.** Amino acid multi-alignment showed that PGs of *F. phyllophilum* were identical whereas NRRL 25305 PG was only 91% similar. The ratio between non-synonymous substitutions and total amino acids showed that exons 2 and 3 of the *pg* genes are the most variable ones (Table 1 and Fig. 4), while exon 5 is the most conserved. Exon 4, which comprises the substrate binding site and the active site of the enzyme, showed intermediate variability; in this region however, amino acid replacements did not involve residues known to have functional importance. Out of 30 amino acids that distinguish *F. phyllophilum* NRRL 25305 and FC-10 endoPGs, 13 are singletons, while 17 are identical to the corresponding residues in *F. verticillioides* endoPGs. Ten out of these 17 are in the portion encoded by exon 3 (Fig. 4), in agreement with the phylogenetic analysis that groups this exon (as well as intron 2 and exon 5) in the *F. verticillioides* clade.

**Characterization of endoPGs.** The crude culture filtrates of *F. phyllophilum* FC-10, IHEM 10241, NRRL 25305 and *F. verticillioides* PD, 62264 were used for SDS-PAGE and Western blotting analyses using an antibody raised against FC-10 endoPG. *F. phyllophilum* FC-10 and IHEM 10241 showed four glycoforms (Fig. 5), in agreement with previous observations (Caprari *et al.*, 1993a). Instead, *F. phyllophilum* NRRL 25305 crude filtrates gave a band pattern similar to that of *F. verticillioides* PD and 62264 with two putative glycoforms of ~40 kDa. Additional immunolabelled bands were observed in culture filtrates of *F. verticillioides* PD (70 kDa) and 62264 (48 kDa), as well as of NRRL 25305 (50 and 65 kDa).

The same crude filtrates were tested for inhibition of PG activity by *P. vulgaris* PvPGIP2. Aliquots corresponding to 1 agarose diffusion unit and different amounts of PvPGIP2 were employed in the assay. EndoPGs of *F. phyllophilum* FC-10, IHEM 10241, NRRL 25218, NRRL 25219 were completely inhibited (Table 2). On the contrary, endoPGs from *F. verticillioides* PD and 62264

were not inhibited, in agreement with previous data (Sella *et al.*, 2004), and neither was the enzyme from *F. phyllophilum* NRRL 25305. These results indicate that biochemical features of *F. phyllophilum* NRRL 25305 are more similar to those of *F. verticillioides* than *F. phyllophilum* endoPGs.

**Maximum likelihood analysis of codon evolution for endoPG sequences.** The use of a gene as a phylogenetic marker could be inappropriate if this gene has experienced positive selection, as suggested in the case of *pg* by Stotz *et al.* (2000). Using a maximum likelihood analysis of codon evolution (Yang *et al.*, 2000), we wanted evaluate the hypothesis of positive selection acting on endoPG on a data set comprising 17 *pg* sequences [the 14 *pg* sequences indicated in Fig. 1B and *pg* sequences of *F. graminearum* (XM391184), *Gibberella circinata* (AF207825), *Aspergillus niger* (X58893). Table 3 shows the results obtained when different models of codon evolution were applied. LRT tests showed evidence of a non-homogenous distribution of the ω ratio (*dN/dS*) values across the protein sites (model M3 fits the data better than M0) with most of the sites under very strong purifying selection (ω ratio near zero). Two different tests were performed to detect positive selection: M2 versus M1 and M8 versus M7 (see method section). Only in one case (M8 versus M7) was the test significant, giving evidence of positive selection. Nevertheless, even in the case of model M8, the analysis detected only seven sites with ω ratio estimates greater than 1, four of which showed a posterior probability greater than 0.80 (Asn97, Ser43, Gln33 and Gln137) to be under positive selection. None of them were amino acid sites previously indicated as positively selected sites (Stotz *et al.*, 2000).

## DISCUSSION

EndoPGs are important virulence factors in fungi (Isshiki *et al.*, 2001; Kars *et al.*, 2005; Oeser *et al.*, 2002; Shieh *et al.*, 1997; ten Have *et al.*, 1998) and bacteria (Huang and Allen, 2000), and control of their activity by PGIPs limits fungal colonization in dicots (Ferrari *et al.*, 2003; Manfredini *et al.*, 2005; Powell *et al.*, 2000) and monocots (Janni *et al.*, 2008). Due to the co-evolutionary race between pathogen and host, these enzymes are likely subject to strong selective pressure. Evidence of positive selection acting on PGs, as well as on their plant inhibitors (PGIPs), have been reported (Stotz *et al.*, 2000), suggesting a typical evolutionary arms race between these two interacting proteins.

PG is encoded by a single gene in many *Fusarium* species [*F. verticillioides* (Caprari *et al.*, 1993b; Daroda *et al.*, 2001; Sella *et al.*, 2004), *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *radicis-lycopersici* (Patiño *et al.*, 1997), *F. episphaeria* and *F. solani* (Posada *et al.*, 2000)]. For this reason, the *pg* gene has been proposed as a molecular marker for taxonomic studies of *Fusarium* spp. (Posada *et al.*, 2000). However, the use of a gene as a phylogenetic marker could be inappropriate if this gene has experienced positive selection. In this work we have found that phylogenetic trees generated using sequences of the *pg* genes, as well as of individual *pg* exons and introns, confirm the classification of the FC-10 as *F. phyllophilum*, previously obtained using *TEF* and *chb-C* se-

**Table 3.** Parameter estimates and log-likelihood values under models of variable ω ratios among sites.

| Model | Parameter estimation | L[a] | LRT[b] | Positively selected sites (Pr > 0.95) |
|---|---|---|---|---|
| M0 (one ratio) | $\omega = 0.072$ | -4928.909 | - | None |
| M1 (neutral) | $p_1 = 0.89$ $p_2 = 0.11$<br>$\omega_1 = 0.029$<br>$\omega_2 = 1.00$ | -4875.209 | - | Not allowed |
| M2 (selection) | $p_1 = 0.89$ $p_2 = 0.02$ $p_3 = 0.09$<br>$\omega_1 = 0.029$<br>$\omega_2 = 1.00$ $\omega_3 = 1.00$ | -4875.209 | M2-M1: ns | None |
| M3 (discrete) | $p_1 = 0.38$ $p_2 = 0.53$ $p_3 = 0.09$<br>$\omega_1 = 0.002$<br>$\omega_2 = 0.06$<br>$\omega_3 = 0.75$ | -4815.421 | M3-M0: 227.0 ($p<0.001$) | None |
| M7 (beta) | $p = 0.33$ $q = 3.13$ | -4822.494 | - | Not allowed |
| M8 (beta&ω>1) | $p_1 = 0.94$ ($p_2 = 0.06$)<br>$p = 0.41$ $q = 7.62$<br>$\omega = 1.00$ | -4816.948 | M8-M7: 11,1 ($p<0.01$) | None[c] |

[a] L : likelihood of the model.
[b] LRT: the model comparison, the $\chi 2$ and the p value are shown, with 2 d.f. (M2-M1, M7-M8) or 4 d.f. (M0-M3).
[c] Positively selected sites with Pr (Bayesian posterior probability) > 0,8: 33Q (0.830); 43S (0.860); 97N (0.848); 137Q (0.933).

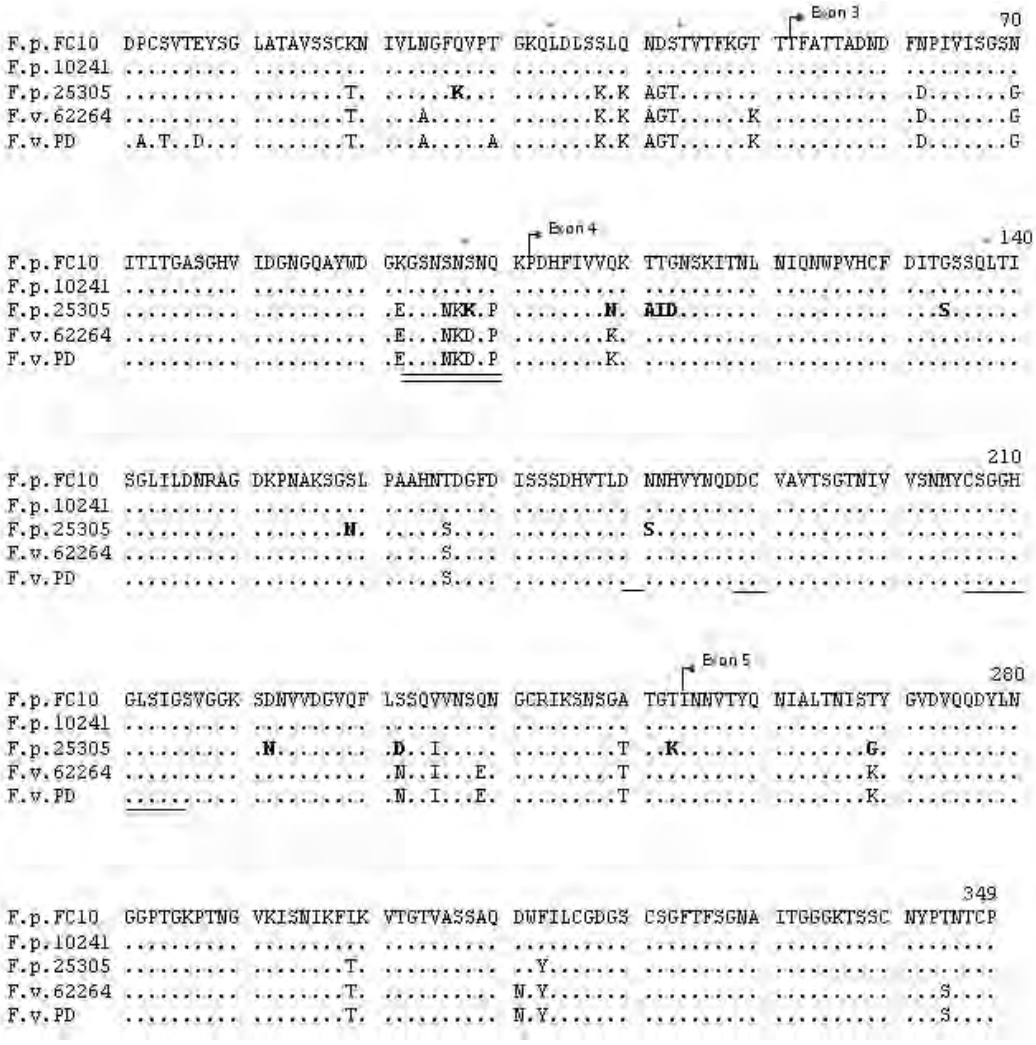


**Fig. 4.** Alignment of endoPG deduced amino acid sequences. *F.p.= F. phyllophilum*, F.v.= *F. verticillioides*. EndoPG amino acid sequences of *F. phyllophilum* NRRL 25218 and NRRL 25219 sequence were not included because they were 100% identical to those of FC-10. Dots indicate residues identical to those in FC-10 endoPG. Residues in bold are NRRL 25305 singletons. The start of each exon is indicated by an arrow above the sequence. An asterisk (*) above the sequence indicates putative positively selected sites. Residues forming the active site and loop 1 (Federici *et al*., 2001) are single and double underlined, respectively.

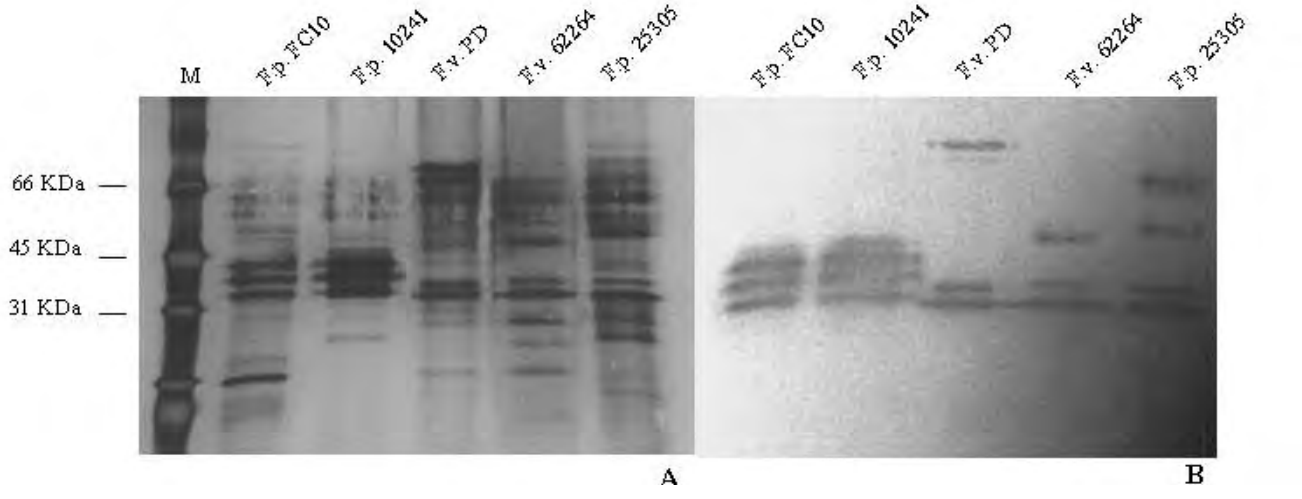


**Fig. 5.** SDS-PAGE (A) and Western Blot (B) analysis of culture filtrates of *Fusarium spp*. and strains grown in the presence of pectin to induce endoPG expression. A. 10 ml of the crude culture filtrate of *F. phyllophilum* (F.p) strains FC-10, IHEM 10241 and NRRL 25305, and *F. verticillioides* (F.v.) strains PD and 62264, separated by 10% acrylamide gel and stained. B. Immunodetection using a polyclonal rabbit antibody against endoPG of FC-10. Lane M, molecular weight marker.

quences as molecular markers (Mariotti *et al.*, 2008). The identification of strain FC-10 as *F. phyllophilum* has the important implication in that no PGIP significantly active *in vitro* against a natural *F. verticillioides* endoPG has been so far identified (De Lorenzo *et al.*, 2001) and prompts the search for efficient inhibitors of PG of this important pathogen to devise a control strategy. Both the isolation and characterization of novel natural inhibitors or the development of variants by directed evolution (Zhao, 2007) represent promising approaches to this aim.

Our analysis also showed that the *F. verticillioides* 62264 *pg* sequence was more similar to that of the PD strain than previously reported (Daroda *et al.*, 2001). This is in agreement with the extremely low level of *pg* variation observed within *F. verticillioides*, where only 3 amino acid substitutions were detected in comparison of 11 strains. A very high level of similarity at the amino acid level (94-100%) was also observed in 16 strains belonging to different species within the *G. fujikuroi* complex (Raiola *et al.*, 2007).

Surprisingly, in our analysis of *pg* sequences strain NRRL 25305 was excluded from the *F. phyllophilum* group. This strain was isolated from the leaves of *Gasteria excavata*, a *F. phyllophilum* host (Nirenberg and O'Donnell, 1998), and it belongs to the *F. phyllophilum* group according to morphological characteristics and growth conditions, as well as to phylogenetic analysis carried out using *TEF* and *cbh-C* (Mariotti *et al.*, 2008). Puzzlingly, PGs of this strain showed only 91.3% similarity with PGs of *F. phyllophilum*, which were all identical, and it was more similar to the *F. verticillioides* enzymes in terms of amino acid sequence, number of glycoforms (two) and lack of inhibition by PGIP (Caprari *et al.*, 1993a; Sella *et al.*, 2004). Phylogenetic analysis of intron 2, the contiguous exon 3 and exon 5 of NRRL 25305 *pg* confirmed the closer relationship with *F. verticillioides pg*, whereas it showed a higher divergence of intron 3 and exon 4. Only the analysis of exon 2 grouped NRRL 25305 in the *F. phyllophilum* clade. Variation therefore appears heterogeneously distributed along the NRRL 25305 *pg* sequence, suggesting a selection pressure acting differentially on the different regions of this endoPG.

A sexual stage is unknown for *F. phyllophilum* and so far there have been no reports of hybrids between *F. phyllophilum* and other species of the *G. fujikuroi* complex, either in nature and or in laboratory (K. O'Donnell, personal communication). Convergent evolution is therefore a possible explanation for the similarity of the NRRL 25305 PG with that of *F. verticillioides.* An alternative mechanism, such as horizontal gene transfer (HGT) of a region encompassing the *pg* gene, may also plausibly explain the divergence of NRRL 25305 *pg*. The genomic environment of the NRRL 25305 *pg* gene, which is different from that of the other *F. phyllophilum* strains, may support this hypothesis. HGT is more important in the evolution of fungi than of other eukayotes (Friesen *et al.*, 2006; Rosewich and Kistler, 2000). For example, HGT has been proposed to explain the patchy taxonomic distribution across the genus *Fusarium* of the transposable element *Fot1* (Daboussi *et al.*, 2002) or the presence of a virulence-related genomic region of at least 8 kb comprising the genes *SIX1*, *SIX2* and *SHH1* in all isolates belonging to *F. oxysporum* f.sp. *lycopersici*, but not in other *formae speciales* and putative non-pathogenic isolates (van der Does *et al.*, 2008).

Diversification of PGs is likely driven by several factors: the need to adapt to the different structure of the cell wall pectin in different plant species, different changes occurring in the apoplast of different plant hosts during infection (for example pH or ion concentration changes), and the need to avoid recognition by different PGIPs. It is likely that not only are different sets of endoPG amino acid sites able to evolve at different rates, but also that certain parts of the gene are under selection in some species but not in others, depending on the variation of selective pressures. Exon 3, for instance, encodes a particular protein region characterized by a loop structure, loop 1, which is located at the edge of the active site and is likely to influence interactions with plant inhibitors (Federici *et al.*, 2001). This exon corresponds to one of the most variable portions of the NRRL 25305 PG and contains a putative positively selected site, Asn97, which in FC-10 endoPG is predicted by molecular docking analysis to interact with residues located on the concave surface of *Pv*PGIP2 (Federici *et al.*, 2006). Notably, the replacement of residue 97 (a lysine) with an alanine or a glutamine in the endoPG of *F. verticillioides* strain PD, leads to inhibition by bean PGIP (Raiola *et al.*, 2007). A different putatively selected residue, Ser43, is enclosed in the putative N-glycosylation consensus sequence, while for Gln33 and Gln137 there is no evidence yet for an interaction with plant inhibitors.

In conclusion, our results indicate an unexpected divergence of the *F. phyllophilum pg* gene, casting doubts on the usefulness of *pg* sequences as molecular phylogenetic markers. This finding supports the conclusion that taxonomic classification in fungi requires an accurate choice of gene sequences and multigene analyses.

## ACKNOWLEDGEMENTS

We thank Kerry O'Donnell for providing *F. phyllophilum* strains and for useful information on *F. phyllophilum* host range and genetics, and Prof. Luca Federici for help in elucidating the endoPG 3D structure and PG-PGIP interaction model.

Work supported by MIUR Prin 2007-2007K7KY8Y.

## REFERENCES

André-Leroux G., Tessier D., Bonnin E., 2005. Action pattern of *Fusarium moniliforme* endopolygalacturonase towards pectin fragments: Comprehension and prediction. *Biochimica et Biophysica Acta* **1749**: 53-64.

Bonnin E., Le Goff A., Korner R., Vigouroux J., Roepstorff P., Thibault J.F., 2002. Hydrolysis of pectins with different degrees and patterns of methylation by the endopolygalacturonase of *Fusarium moniliforme*. *Biochimica et Biophysica Acta* **1596**: 83-94.

Bishop J.G., 2005. Directed mutagenesis confirms the functional importance of positively selected sites in polygalacturonase inhibitor protein. *Molecular Biology and Evolution* **22**: 1531-1534

Caprari C., Bergmann C., Migheli Q., Salvi G., Albersheim P., Darvill A., Cervone F., De Lorenzo G., 1993a. *Fusarium moniliforme* secretes four endopolygalacturonases derived from a single gene product. *Physiological and Molecular Plant Pathology* **43**: 453-462.

Caprari C., Richter A., Bergmann C., Lo Cicero S., Salvi G., Cervone F., De Lorenzo G., 1993b. Cloning and characterization of the gene encoding the endopolygalacturonase of *Fusarium moniliforme*. *Mycological Research* **97**: 497-505.

Caprari C., Mattei B., Basile M.L., Salvi G., Crescenzi V., De Lorenzo G., Cervone F., 1996. Mutagenesis of endopolygalacturonase from *Fusarium moniliforme*: Histidine residue 234 is critical for enzymatic and macerating activities and not for binding to polygalacturonase-inhibiting protein (PGIP). *Molecular Plant-Microbe Interactions* **9**: 617-624.

Cervone F., Hahn M.G., De Lorenzo G., Darvill A., Albersheim P., 1989. Host-pathogen interactions. XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. *Plant Physiology* **90**: 542-548.

Chimwamurombe P.M., Wingfield B.D., Botha A.M., Wingfield M.J., 2001. Cloning and sequence analysis of the endopolygalacturonase gene from the pitch canker fungus, *Fusarium circinatum*. *Current Microbiology* **42**: 350-352.

D'Ovidio R., Roberti S., Di Giovanni M., Capodicasa C., Melaragni M., Sella L., Tosi P., Favaron F., 2006. The characterization of the soybean polygalacturonase-inhibiting proteins (*Pgip*) gene family reveals that a single member is responsible for the activity detected in soybean tissues. *Planta* **224**: 633-645**.**

Daboussi M.J., Davière J.M., Graziani S., Langin T., 2002. Evolution of the *Fot1* transposons in the genus *Fusarium*: discontinuous distribution and epigenetic inactivation. *Molecular Biology and Evolution* **19**: 510-520.

Daroda L., Hahn K., Pashkoulov D., Benvenuto E., 2001. Molecular characterization and *in planta* detection of *Fusarium moniliforme* endopolygalacturonase isoforms. *Physiological and Molecular Plant Pathology* **59**: 317-325.

De la Heras A., Patiño B., Posada M.L., Martinez M.J., Vazquez C., Jaen M.T.G., 2003. Characterization and in vitro expression patterns of an exopolygalacturonase encoding gene from *Fusarium oxysporum* f.sp. *radicis lycopersici*. *Journal of Applied Microbiology* **94**: 856-864.

De Lorenzo G., Cervone F., Bellincampi D., Caprari C., Clark A.J., Desiderio A., Devoto A., Forrest R., Leckie F., Nuss L., Salvi G., 1994. Polygalacturonase, PGIP and oligogalacturonides in cell-cell communication. *Biochemical Society Transactions* **22**: 396-399.

De Lorenzo G., D'Ovidio R., Cervone F., 2001. The role of polygacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annual Review of Phytopathology* **39**: 313-335.

De Lorenzo G., Ferrari S., 2002. Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. *Current Opinion in Plant Biology* **5**: 295-299.

De Lorenzo G., Salvi G., Degra R., D'Ovidio R., Cervone F., 1987. Induction of extracellular polygalacturonase and its mRNA in the pathogenic fungus *Fusarium moniliforme*. *Journal of General Microbiology* **133**: 3365-3373.

Di Matteo A., Federici L., Mattei B., Salvi G., Johnson K.A., Savino C., De Lorenzo G., Tsernoglou D., Cervone F., 2003. The crystal structure of PGIP (polygalacturonase-inhibiting protein), a leucine-rich repeat protein involved in plant defense. *Proceedings of the National Academy of Sciences USA* **100:** 10124-10128.

Federici L., Caprari C., Mattei B., Savino C., Di Matteo A., De Lorenzo G., Cervone F., Tsernoglou D., 2001. Structural requirements of endopolygalacturonase for the interaction with PGIP (polygalacturonase-inhibiting protein). *Proceedings of the National Academy of Sciences USA* **98**: 13425-13430.

Federici L., Di Matteo A., Fernandez-Recio J., Tsernoglou D., Cervone F., 2006. Polygalacturonase inhibiting proteins: players in plant innate immunity? *Trends in Plant Sciences* **11**: 65-70.

Fernandez-Pol J.A., Klos D.J., Hamilton P.D., 1993. Cytotoxic activity of fusaric acid on human adenocarcinoma cells in tissue culture. *Anticancer Research* **13**: 57-64.

Ferrari S., Vairo D., Ausubel F.M., Cervone F., De Lorenzo G., 2003. Tandemly duplicated Arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. *The Plant Cell* **15**: 93-106.

Friesen T.L., Stukenbrock E.H., Liu Z., Meinhardt S., Ling H., Faris J.D., Rasmussen J.B., Solomon P.S., McDonald B.A., Oliver R.P., 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics* **38**: 953-956.

Garcia-Maceira F.I., Di Pietro A., Huertas-Gonzalez M.D., Ruiz-Roldan M.C., Roncero M.I., 2001. Molecular characterization of an endopolygalacturonase from *Fusarium oxysporum* expressed during early stages of infection. *Applied Environmental Microbiology* **67**: 2191-2196.

Hahn M.G., Darvill A.G., Albersheim P., 1981. Host-pathogen interactions. XIX. The endogenous elicitor, a fragment of plant cell wall polysaccharide that elicits phytoalexin accumulation in soy beans. *Plant Physiology* **68**: 1161-1169.

Herron S.R., Benen J.A., Scavetta R.D., Visser J., Jurnak F. , 2000. Structure and function of pectic enzymes: virulence factors of plant pathogens. *Proceedings of the National Academy of Sciences USA* **97:** 8762-8769.

Huang Q., Allen C., 2000. Polygalacturonases are required for rapid colonization and full virulence of *Ralstonia solanacearum* on tomato plants. *Physiological and Molecular Plant Pathology* **57**: 77-83.

Isshiki A., Akimitsu K., Yamamoto M., Yamamoto H., 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *Molecular Plant-Microbe Interactions* **14**: 749-757.

Janni M., Sella L., Favaron F., Blechl A.E., De Lorenzo G., D'Ovidio R., 2008. The expression of a bean polygalacturonase-inhibiting proteins in transgenic wheat confers increased resistance to the fungal pathogen *Bipolaris sorokiniana*. *Molecular Plant-Microbe Interactions* **21**: 171-177.

Jenczmionka N.J., Schafer W., 2005. The Gpmk1 MAP kinase of *Fusarium graminearum* regulates the induction of specific secreted enzymes. *Current Genetics* **47**: 29-36.

Kars I., Krooshof G.H., Wagemakers L., Joosten R., Benen J.A., van Kan J.A., 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *The Plant Journal* **43**: 213-225.

Kumar S., Tamura K., Nei M., 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150-163.

Leckie F., Mattei B., Capodicasa C., Hemmings A., Nuss L., Aracri B., De Lorenzo G., Cervone F., 1999. The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed β-strand/β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. *The EMBO Journal* **18**: 2352-2363.

Manfredini C., Sicilia F., Ferrari S., Pontiggia D., Salvi G., Caprari C., Lorito M., De Lorenzo G., 2005. Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. *Physiological and Molecular Plant Pathology* **67**: 108-115.

Mariotti L., Casasoli M., Migheli Q., Balmas V., Caprari C., De Lorenzo G., 2008. Reclassification of *Fusarium verticillioides* (syn. *F. moniliforme*) strain FC-10 as *F. phyllophilum*. *Mycological Research* **112**: 1010-1011.

Nirenberg H.I., O'Donnell K., 1998. New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. *Mycologia* **90:** 434-458.

Niture S.K., Pant A., 2004. Purification and biochemical characterization of polygalacturonase II produced in semi-solid medium by a strain of *Fusarium moniliforme*. *Microbiological Research* **159**: 305-314.

Oeser B., Heidrich P.M., Muller U., Tudzynski P., Tenberge K.B., 2002. Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea*/rye interaction. *Fungal Genetics and Biology* **36**: 176-186.

Ospina-Giraldo M.D., Mullins E., Kang S., 2003. Loss of function of the *Fusarium oxysporum* SNF1 gene reduces virulence on cabbage and Arabidopsis. *Current Genetics* **44**: 49-57.

Patiño B., Posada M.L., González-Jaén M.T., Vázquez C., 1997. The course of pectin degradation by polygalacturonases from *Fusarium oxysporum* f.sp. *radicis lycopersici*. *Microbios* **91**: 47-54.

Posada M.L., Patiño B., De la Heras A., Mirete S., Vazquez C., Gonzalez-Jaen M.T., 2000. Comparative analysis of an endopolygalacturonase coding gene in isolates of seven *Fusarium* species. *Mycological Research* **104**: 1342-1347.

Powell A.L., van Kan J., ten Have A., Visser J., Greve L.C., Bennett A.B., Labavitch J.M., 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. *Molecular Plant-Microbe Interactions* **13**: 942-950.

Raiola A., Sella L., Castiglioni C., Balmas V., Favaron F., 2007. A single amino acid substitution in highly similar endo-PGs from *Fusarium verticillioides* and related *Fusarium* species affects PGIP inhibition. *Fungal Genetics and Biology* **45**: 776-789.

Rosewich U.L., Kistler H.C., 2000. Role of horizontal gene transfer in the evolution of fungi. *Annual Review of Phytopathology* **38**: 325-363.

Sella L., Castiglioni C., Roberti S., D'Ovidio R., Favaron F., 2004. An endo-polygalacturonase (PG) of *Fusarium moniliforme* escaping inhibition by plant polygalacturonase-inhibiting proteins (PGIPs) provides new insights into the PG-PGIP interaction. *FEMS Microbiology Letters* **240**: 117-124.

Shieh M.T., Brown R.L., Whitehead M.P., Cary J.W., Cotty P.J., Cleveland T.E., Dean R.A., 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. *Applied Environmental Microbiology* **63**: 3548-3552.

Stotz H.U., Bishop J.G., Bergmann C.W., Koch M., Albersheim P., Darvill A.G., Labavitch J.M., 2000. Identification of target amino acids that effect interactions of fungal polygalacturonases and their plant inhibitors. *Physiological and Molecular Plant Pathology* **56**: 117-130.

ten Have A., Mulder W., Visser J., van Kan J.A., 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. *Molecular Plant Microbe Interaction* **11**: 1009-16.

van der Does H.C., Lievens B., Claes L., Houterman P.M., Cornelissen B.J., Rep M., 2008. The presence of a virulence locus discriminates Fusarium oxysporum isolates causing tomato wilt from other isolates. *Environmental Microbiology* **10 (6)**: 1475-1485

Yang Z.H., Swanson W.J., Vacquier V.D., 2000. Maximum-likelihood analysis of molecular adaptation in abalone sperm lysin reveals variable selective pressures among lineages and sites. *Molecular Biology and Evolution* **17**: 1446-1455.

Zhao H., 2007. Directed evolution of novel protein functions. *Biotechnology and Bioengineering* **98**: 313-317.

Received July 19, 2008
Accepted September 12, 2008