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Research Papers

# An endopolygalacturonase gene of *Diaporthe helianthi*

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**Summary.** In an attempt to define virulence determinants of *Diaporthe helianthi*, the causative agent of sunflower stem canker, the gene named *Dhpg*, coding for an endopolygalacturonase, was investigated in the highly virulent strain 8/96 isolated from a diseased plant in France. The 1130 bp coding sequence, in addition to 1473 bp from the upstream region, were cloned, sequenced and analyzed. *Dhpg* was most closely related to polygalacturonase genes from phytopathogenic fungi, such as *Ophiostoma ulmi*, *O. novo-ulmi*, and *Gibberella zeae*. A Southern blot RFLP analysis proved that *Dhpg* was represented in single-copy in the genome of the isolate 8/96. Analyses carried out both *in vitro* on liquid cultures and *in vivo* on host infected tissues provided evidence of the constitutive expression of the *Dhpg* transcript under all tested conditions. Moreover, an European collection of *D. helianthi* isolates was screened for the presence of *Dhpg* homologues by PCR, revealing the same single band in all French and Yugoslavian isolates, while one Romanian and all Italian isolates displayed a variable pattern. This genetic variability related to the different geographic origin of *D. helianthi* is consistent with data previously reported for different loci on the same set of isolates. This is the first report of a polygalacturonase gene in *D. helianthi*.

**Key words: c**ell wall degrading enzymes, molecular diversity, *Phomopsis helianthi*, sunflower stem canker.

## Introduction

The degradation of plant cell walls by a battery of polysaccaridases is a major mechanism used by plant pathogens to gain access to host tissues (Herron *et al*., 2000). Of the numerous cell wall degrading enzymes (CWDEs) secreted by pathogenic fungi, pectin-degrading enzymes are of primary importance, because they are typically produced in the initial stages of pathogenesis, in large amounts, and are the only cell wall degrading enzymes (CWDEs) capable of macerating tissues and killing plant cells on their own (Annis and Goodwin, 1997).

Most known fungal endopolygalacturonase (endoPG) sequences belong to phytopathogenic microorganisms, although a report deals with a corresponding gene derived from a biocontrol fungus, *Trichoderma harzianum*, involved in molecular interactions both with plants and fungal pathogens (Morán-Díez *et al*., 2009). All endoPG genes cloned thus far are between 1100 and 1350 bp long, and most of them have one to four introns of 50 to 81 bp. EndoPG-encoding gene families were discovered in *Aspergillus niger*, and in the phytopathogenic fungi *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Bussink *et al*., 1992; Fraissinet-Tachet *et al*., 1995; Wubben *et al*., 1999). The *B. cinerea* endoPG gene family was exploited for evolutionary studies, and the nucleotide diversity of the five endoPG genes was suggested to be involved in differential ecophysiological roles, such as host preference, pathogenicity and environmental fitness (Cettul *et al*., 2008).

In general, comparisons of endoPG DNA sequences among different fungal species have failed

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to reveal relevant homology within the coding regions, except for some genes shared by *Aspergillus* species (Annis and Goodwin, 1997). Even among members of a given endoPG gene family, sequence identity was found to be variable (Wubben et *et al.*, 1999; Parenicova *et al.*, 2000). However, a region of approximately 80 residues with highly conserved amino acids was detected in endoPGs from fungi, bacteria and plants, and shown to contain the active site and to be involved in binding to the substrate (Annis and Goodwin, 1997).

The unveiling of the three-dimensional structure of fungal polygalacturonases (PGs) (Federici *et al.*, 1999) contributed to elucidate aspects of the pathogenetic mechanism at the molecular level (Herron *et al.*, 2000) and the interaction with polygalacturonase inhibiting proteins (PGIPs) (Federici *et al.*, 2001), which are widespread host defence proteins. PGIPs directly interfere with host cell wall degradation performed by pathogen's PG. They may also modulate the PG activity preventing the complete degradation of pectic oligomers, oligogalacturonides (OGs) acting as elicitors of inducible defence systems (Stotz *et al.*, 2000).

The almost universal spread of PGs in phytopathogenic fungi and of PGIPs in plants suggests that the confrontation at the host cell wall level is a general rule in necrotic diseases and that the role of PGs as determinants of pathogen success cannot be neglected. PGs are therefore a primary target of investigation when approaching the study of diseases whose molecular mechanism is still to be understood.

One such disease is sunflower stem canker, caused by the Ascomycete *Diaporthe helianthi* (anamorph *Phomopsis helianthi*) (Muntanola-Cvetkovic *et al.*, 1991). The fungus is among the most important pathogens of sunflower in Europe and can significantly reduce yield and oil content when the environmental conditions are favourable for the disease development, although epidemics have represented an intriguing question (Muntanola-Cvetkovic *et al.*, 1989). *Diapotrthe helianthi* causes serious losses in France and in former Yugoslavia (Says-Lesage *et al.*, 2002; Muntanola-Cvetkovic *et al.*, 1981), while the pathogen is only sporadically recorded in Italy in spite of conducive pedoclimatic conditions (Battilani *et al.*, 2003). In a recent coordinated study, a set of *D. helianthi* isolates collected in different geographic areas was examined with the aim of highlighting different genetic biotypes responsible for epidemiological differences in sunflower stem canker. Genetic variability was evaluated at different genomic regions such as ribosomal intergenic spacers (IGS), internal transcribed spacers (ITS), the mitochondrial ATP6 gene, a polyketide synthase (PKS) homologue and extrachromosomal genetic determinants (as plasmid contents). The complex of investigations performed on the same set of isolates allowed a correlation between *D. helianthi* biotypes, their geographic origin and sunflower stem canker epidemiology (Pecchia *et al.*, 2004; Rekab *et al.*, 2004; Vergara *et al.*, 2004; Vergara *et al.*, 2005).

Despite progress in understanding the characteristics of epidemics, the disease remains poorly understood at the physiological level. The fungus enters host plants through the leaves, progresses towards the petioles along foliar veins, and finally gains entrance to the stems, where the cankers are the expression of an advanced stage of pathogenesis (Muntanola-Cvetkovic *et al.*, 1989; Heller and Gierth, 2001). There are limited data on the mechanisms of pathogenicity and symptom induction by this fungus, although the production of phytotoxic compounds has been demonstrated (Avantaggiato *et al.*, 1999). Recently new hosts have been identified for *D. helianthi* and some fungal isolates originating from these hosts were shown to be pathogenic to sunflower (Vrandecic *et al.*, 2010).

There was no information for *D. helianthi* about endoPG enzymes produced or about their genes. Therefore the aim of the present study was to identify and sequence endoPG coding and promoter regions in a *D. helianthi* isolate derived from diseased plants in France, to analyse the occurrence of the relative gene in the genome and its expression, and to search for endoPG genetic variability in a set of *D. helianthi* isolates from different geographic areas in Europe.

## Materials and methods

### Fungal culture conditions

A French *D. helianthi* strain (F2, collection n. 8/96), isolated from diseased sunflower stem in 1994 from the region Lauragais in France, was employed for most analyses, in addition to an available collection of *D. helianthi* isolates previously tested (Table 1) and a *D. arctii* isolate (142/01). Fungal isolates were routinely grown for appropriate periodsat 24°C under 12 h/12 h light/dark cycles on potato dextrose agar (PDA Difco, USA) plates, and stored under mineral

**Table 1.** *Diaporthe helianthi* isolates.

| Isolate | Origin | Collection/Original strain code[a] |
|---|---|---|
| 2/96 | Former Yugoslavia | CBS 592.81 |
| 3/96 | Italy | CBS 187.87 |
| 4/96 | Romania | DCDSL |
| 7/96 | France | DAPP |
| 8/96 | France | DAPP |
| 9/96 | Italy | DAPP |
| 10/96 | Italy | DAPP |
| 11/96 | Former Yugoslavia | DAPP |
| 68/96 | Italy | DAPP |
| 69/96 | Italy | DAPP |
| 70/96 | Former Yugoslavia | IMI 313865 |
| 101/96 | France | IMI 318861 |
| 102/96 | France | IMI 313866 |
| 769/00 | France | INRA 95006 |
| 770/00 | France | INRA 95007 |
| 771/00 | France | INRA 95015 |
| 772/00 | France | INRA 95010 |
| 773/00 | France | INRA 95016 |
| 774/00 | France | INRA 95044 |
| 775/00 | France | INRA 95042 |
| 776/00 | France | INRA 95051 |
| 777/00 | France | INRA 95084 |
| 778/00 | France | INRA 95100 |

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oil at 4°C. When needed, liquid cultures were set up in a modified basal Fries medium (Vergara *et al*., 2005), at room temperature with 150 rpm shaking.

### PCR analysis

Fungal genomic DNA was isolated from 3–4-day-old mycelium, collected from a cellophane sheet placed on the agar plate surface, by a modified SDS-CTAB method (Vergara *et al*., 2004).

Amplification tests were performed in a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA) with 12.5 μL mastermix and 12.5 μL containing 250 ng each primer, 100 ng genomic DNA and nuclease-free water to a 25 μL final volume. Cycling conditions were: 5 min at 94°C; 1 min at 94°C, 1 min at the annealing temperature (variable depending on primer set) and 1 min at 72°C (35 cycles); 10 min at 72°C. Annealing temperatures were set depending on the use of specific primers (see Table 2). PCR products were resolved by electrophoresis in 1.5% agarose in TBE buffer. A 1 kb DNA ladder (Promega, Madison, WI, USA) was used as molecular weight marker.

Primers pairs used are listed in Table 2; they were all supplied by MWG-BIOTECH (Ebersberg, Germany).

### GenomeWalker library screening

Extension of the sequence from the initially cloned 383 bp DNA fragment was carried out using Universal Genome Walker™ Kit (Clontech Laboratories, Palo Alto, CA, USA). Genomic DNA was extracted using an SDS method (Al-Samarrai and Schmid, 2000). Separate fungal DNA aliquots were thoroughly digested with four different restriction enzymes (*Eco*RV, *Dra*I, *Pvu*II, *Stu*I) leaving blunt ends. Following digestion, each pool of DNA fragments was ligated to adaptors. For each fragment library, a primary PCR amplification using an adaptor primer provided in the kit and an outer, gene-specific primer was carried out. The primary PCR product was then diluted and used as a template for a secondary PCR amplification using a nested adaptor primer (provided with the kit) and a nested gene-specific primer with the high fidelity *Pfu* DNA polymerase (Promega Italia, Milano, Italy). The resulting DNA amplimers were gel purified, cloned using the TA cloning pGEM-T kit (Promega Italia) and sequenced from both ends.

### RFLP

Genomic DNA (5 μg for reaction), isolated as for PCR, was incubated at 37°C for 16 h with 60 Units of restriction enzyme (*Eco*RI, *Bam*HI, *Hind*III, *Sca*I, *Xba*I, *Pvu*II and *Hae*II) in 100 μL final volume of 1× reaction buffer supplied by the manufacturer (Pro-

**Table 2.** PCR primers used in this study.

| Name | Direction | Sequence | Bases | Tm | Use |
|---|---|---|---|---|---|
| EP1 | For | 5′-GYA CIA CYI TKG AYY TSA C -3′ | 19 | 42°C | degenerate PCR |
| EP3 | Rev | 5′-CRA AIS CRT CIG TGT TGT G -3′ | 19 | 50°C | degenerate PCR |
| EPF | For | 5′-AAC GCA CAC GGC ACA GGG-3′ | 18 | 71°C | PCR for coding |
| EPR | Rev | 5′-TCG CCA AGA ATC CAA AAT CC-3′ | 20 | 66°C | PCR for coding |
| PREPF | For | 5′-AGC TAT GGT TGA GGA TAA AG-3′ | 20 | 55°C | PCR for promoter |
| PREPR | Rev | 5′-TGT TCC AAG ACG AAG CCG-3′ | 18 | 65°C | PCR for promoter |
| RTEPF | For | 5′-TCA CCC TCA AGA ACC TCC AG-3′ | 20 | 64°C | RT-PCR |
| RTEPR | Rev | 5′-AGA CCT TGA CGT CCT TCC AG-3′ | 20 | 64°C | RT-PCR |
| ATP6F | For | 5′-TGG ATT ATA TTT AAC AAT AGG TGG A-3′ | 25 | 58°C | ref for Northern |
| ATP6R | Rew | 5′-ATA AAG GTA ATA ATG GCA AAG GAC-3′ | 24 | 59°C | ref for Northern |
| IGS-12a | For | 5′-AGT CTG TGG ATT AGT GGC CG-3′ | 20 | 62°C | standard for PCR |
| NS1R | Rew | 5′-GAG ACA AGC ATA TGA CTA C-3′ | 19 | 56°C | standard for PCR |

mega, USA). Samples were run on 1% agarose gel containing TAE buffer and transferred to a positively charged Nylon membrane (Roche, Mannheim, Germany) by capillary absorption with 10× SSC. All steps (labelling, hybridisation and detection) were performed according to the Roche DIG System Guide. The probe (a PCR product of the 2895 bp *Dhpg* region) was digoxigenin-labelled by random priming and used for hybridisation of restricted samples in 50% formamide at 42°C for 16 h; filters were then washed in 0.5× SSC and 1% SDS at 60°C. Chemiluminescent detection was performed by exposing autoradiographic films (Lumi-films, Roche) impressed by CSPD (DIG DNA Labelling and Detection Kit, Roche).

### Cloning and sequencing

PCR amplification products were excised, eluted from gel, purified by QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned in pGEM-T Easy Vector, according to the manufacturer's instructions (Promega, USA). Plasmid DNA minipreps were tested in restriction analysis for insert excision; selected recombinant clones were then submitted to a sequencing reaction (Genelab service at ENEA, Rome, Italy) using universal T7-SP6 primers and the dye terminator method with an automatic sequencer (ABI-Prism 373, Perkin-Elmer).

Long final (coding and promoter) regions were double-strand re-sequenced by "primer walking" at CRIBI (Padova University, Italy).

### *In silico* analyses

For DNA sequences, sequence homologies were checked by BLAST database search programs (Altschul *et al.*, 1990) and sequence alignments were obtained with ClustalW or ClustalX programs (Larkin *et al.*, 2007). The promoter region was explored by programs HCtata with the Hamming-Clustering procedure (Milanesi *et al.*, 1996) and TFSearch (Heinemeyer *et al.*, 1998) in the database Transfac on a TF-Matrix. For protein analysis the Proscan platform on Prosite database was used as a tool to scan given protein sequences against the protein signatures (Zdobnov and Apweiler, 2001); Prosite is a database of protein domains, families and functional sites (Bairoch *et al.*, 1997). Global identities and similarities were estimated by the Needleman and Wunsch (1970) algorithm using the SRS-tool NeedleP available at EBI (http://srs.ebi.ac.uk/).

### Expression analysis

*In vitro* induced samples were obtained from the fungal isolate grown in liquid cultures: after 5 d in standard medium the mycelium has been filtered

and washed for 24 h in basal mineral medium, then transferred to inductive medium (mineral Fries supplemented with 0.5% w/v apple pectin) for 24, 48, 72 and 96 h. Control cultures were processed similarly, but after washing they were transferred to Fries medium supplemented with 0.5% w/v glucose. All liquid media were buffered at pH 5.0. Final samples were filtered, lyophilised and stored at -80°C.

Another set of induced samples was obtained by infection of host tissues (sunflower stems) with agar plugs colonised by strain 8/96 mycelium. Sunflower stems were treated 24 d after sowing seeds in pot mix, following two procedures: stems were infected by applying colonized agar blocks (0.5 $cm^2$ surface area) directly on plant in correspondence to woodcuts (*in planta*) or on 2 cm long cut off segments laid down on wet paper in plates (stem cuts). Both *in planta* and stem cut samples were collected after 7 and 12 d. Plant experimental control as non-infected samples were collected at 7 days from stem cuts and at 12 days *in planta*; 8/96 mycelium was also rescued from parallel colonized PDA agar plates grown for 7 and 12 d.

Total RNA was isolated from control and induced samples using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987), modified by the addition of 1% w/v soluble polyvinylpyrrolidone, mol. wt. 40 kD (Sigma, St. Louis, MO, USA), in the extraction buffer. RNAs were stored at -80°C.

*RT-PCR.* After DNAse I treatment, 1 μg RNA from mycelium samples and 2 μg RNA from plant tissue samples with 1 μM each specific RT primer (RTEPF and RTEPR, see Table 2), according to manufacturer's instructions for RT-PCR access (Promega, USA), were submitted to a one-step reaction with 60°C as annealing temperature.

*cDNA/DNA hybridization*. cDNAs were synthesized by treating 4 μg total RNAs with 60 U Reverse Transcriptase AMV-RT (Promega) according to manufacturer instructions and stored at -80°C.

An amount of 0.5 μg cDNA from all mycelium samples and of 2 μg cDNA from all plant tissue samples were blotted on a Nylon membrane and then hybridized with DIG-labelled probes: the specific probe was a PCR product of the coding *Dhpg* region while the reference was derived by PCR with primers ATP6F-ATP6R designed on *atp6* gene from *D. helianthi* (see Table 2; Rekab *et al.*, 2004). All hybridization conditions were the same as for RFLP experiments.

## Results

### Cloning and sequencing the *Dhpg* coding region

A short amplimer (383 bp; Figure 1a) was obtained from *D. helianthi* genomic DNA (isolate 8/96) by amplification with degenerate primers (EP1-

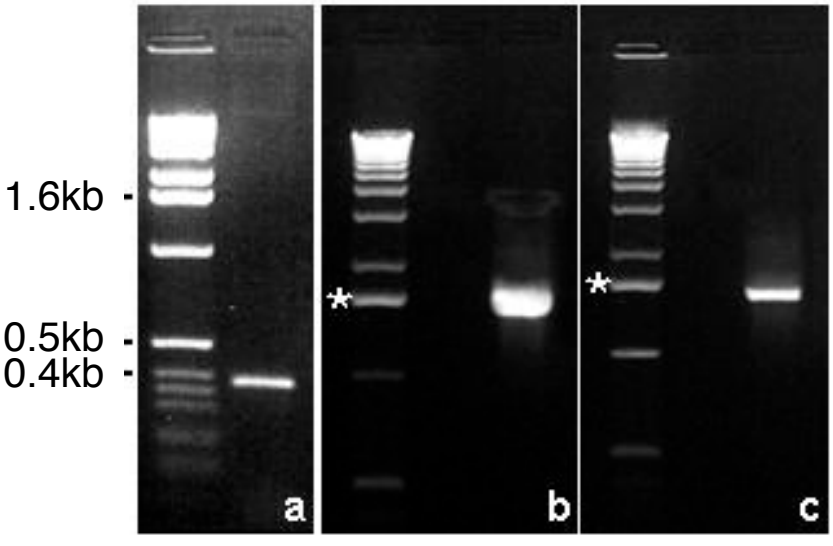


**Figure 1.** PCR on genomic DNA. **a.** Degenerate primers EP1-EP3 (mw 383 bp). **b.** EPF-EPR primers specific for coding region (mw 1624 bp). **c.** PREPF-PREPR primers specific for promoter region (mw 1343 bp). Left lane (each gel) DNA mw marker: 1 kb Ladder (GIBCO-BRL); in **b** and **c:** * 1.6 Kb.

EP3, see Table 2) derived from consensus regions of the fungal endoPG genes. The PCR product, when cloned, sequenced and analyzed with BLAST, displayed a high degree of homology to the fungal endoPG genes available in databases: more than 50 alignments found in tBLASTx search achieved a score between 200 and 100 bits.

A Genome Walker library was prepared with the isolate 8/96 genomic DNA. Using the sequence information from the cloned 383 bp fragment for the development of specific primers, the region was extended by genome walking. Consistent amplifications were obtained from the *Stu*I fragment library, and, based on the sequence of the larger (> 1500 bp) clone ends, specific oligonucleotides were derived for direct amplification and sequencing of genomic DNA. Further extension cycles were performed by genome walking thanks to specific primers designed on the ends of sequenced regions. Finally, two specific primers, EPF and EPR (Table 2), were settled to amplify a region 1624 bp long including the complete coding sequence shortly superimposing to 5′ and 3′ ends (Figure 1b). The amplification product of this region, named coding *Dhpg*, was cloned, sequenced in both directions and submitted to a tBLASTx analysis, with alignments falling regularly inside the regions annotated as coding of fungal endoPG genes. High similarity values resulted from global alignments of the putative translation product with the entries retrieved by blast searches, as shown in Table 3.

### Cloning and sequencing the *Dhpg* promoter region

The Genome Walker library of isolate 8/96 underwent a further screening towards upstream the coding *Dhpg* region. Other specific primers were derived from the 5′ known *Dhpg* sequence and fragments obtained by several rounds of PCR were cloned, sequenced and analysed in BLAST programs. The longest sequence (more than 1600 bp) was selected to be a target for designing specific primers (PREPF- PREPR, see Table 2) for the amplification of a further 1343 bp in the 5′ promoter region (Pr*Dhpg*, Figure 1c). This amplicon was then cloned and double-strand sequenced.

The entire *D. helianthi* endoPG sequence (*Dhpg* 2895 bp), including both promoter and coding regions (1–1474 promoter, 1475–2604 cds and 2605–2895 3′ end), is available from the EMBL data bank with accession number AJ582180.2.

### Bioinformatic analyses

#### *Promoter region*

The 1473 bp Pr*Dhpg* was searched for structural patterns or functional motifs identifying eukaryotic promoters. Automatic searching for prediction of signal sites disclosed several consensus stretches typical for eukaryotic regulative elements. Nine putative TATA-boxes were found using the program HCtata according to the Hamming-Clustering procedure (Milanesi *et al.*, 1996). As much as 278 potential sites for various additional transcriptional factors were revealed by TFSEARCH (Heinemeyer *et al.*, 1998) on a specific distribution matrix (TFMATRIX) in the database TRANSFAC. The most significant of these were: GATA consensus sequences, protein activators, CREB (binding sites for proteins c-AMP regulated), MZF1 (binding elements for zinc-finger proteins), STAT (DNA binding sites), cap (signal for starting transcription) and stuAp (a transcriptional factor in *Aspergillus nidulans*).

**Table 3.** Global identities and similarities of *Dh*EP. Global alignments of putative translated *Dh*EP CAE46647.1 with the following entries of fungal polygalacturonases: AAY82498.1 from *Ophiostoma ulmi*; C05833.1 *Ophiostoma novo-ulmi*; XP_681596.1 from *Aspergillus nidulans*; XP_383370.1 from *Gibberella zeae*. Identity/similarity values were calculated by means of NeedleP.

| AAY82498.1 | AAC05833.1 | XP_681596.1 | XP_383370.1 | CAE46647.1 |
|---|---|---|---|---|
| 99.2-99.5 | | | | |
| 66.9-74.8 | 66.7-76.8 | | | |
| 62.8-74.1 | 63.1-73.8 | 62.2-73.4 | | |
| 56.9-72.3 | 57.2-72.3 | 56.4-72.2 | 60.4-75.9 | |

*Predicted protein*

The comparison of the *D. helianthi Dhpg* sequence (AJ582180.2) with database entries showed that the predicted protein (GenBank: CAE46647.2; 376aa *Dh*EP, in Figure 2a) belongs to the glycosyl hydrolase family 28 (GH28, UniProt entry Q70G74), which includes endoPG, and shares with most PGs from Ascomycetous fungi the conserved domain AAx-AxxxxASxxxIxLxxxxVPxGTTLDL in the N-terminal sequence (bold letters in Figure 2a), (Keon and Waksman, 1990). In the C-terminal sequence of *Dh*PG protein, all amino acid residues catalytically important and strictly conserved in fungal endoPG were found in the same relative position (bold letters in Figure 2a): one His (H234) in the GHGXSIGS structure included in the active site, three Asp (D191 in the box HNTD, D212, D213), a RIK Arg Ile Lys structure (R267, I268, K269) and one Tyr (Y302) essential in *Aspergillus* spp. (Stratilová *et al*., 1996).

A Proscan analysis of the predicted protein, using the Prosite.base as a reference site file, displayed seven different patterns, all typically found in endoPG proteins: the PG active site (position 227–240, SGLCSGGHGLSIGS), N-glycosylation, cAMP- and cGMP-dependent protein kinase phosphorylation, protein kinase C phosphorylation, casein kinase II phosphorylation, N-myristoylation and amidation sites.

Since the endoPG protein derived from *Fusarium moniliforme* (*Fm*PG, accession number AAA74586.1) has been deeply investigated for studies of structure/function relationships (Federici *et al*., 1999), it has been exploited in a comparative analysis in order to disclose *Dh*PG structural features. A Clustal X alignment of the *Dh*PG protein with mature *Fm*PG without the signal peptide, showed a high similarity between sequences (Figure 2b), remarkably in conserved aminoacids for typical secondary structures (the β-helical region and outside the β-helix), in the active site region (italic font aminoacids in Figure 2b) and at interaction sites with PGIP. Relatively to the β-helical region in the *Dh*PG protein, common residues are Phe F193, Phe F254, Tyr Y283 (aromatic residues) inside the β-helix, and multiple Cys polar residues C36-C54, C214-C230, C342-C347 and C366-C376 (the last one shifted by three aminoacids) in disulfide bridges distributed along the β-helix. In the active site surroundings, *Dh*PG contains three Asp in positions D191, D212 and D213 for catalysing the reaction, Lys K269 and Arg R267 needed for substrate binding and involved in PGIP interaction together with His H188 (Federici *et al*., 2001).

**Phylogenetic analysis**

Since many endoPG gene sequences have been deposited from several fungal genera, with gene families known in some genera or species, a phylogenetic comparison with *Dhpg* is possible. In the maximum likelihood full tree built using FastTree (Price *et al*., 2010) with about a thousand PG and rhamnogalacturonase sequences (http://pfam.sanger.ac.uk/family?acc=PF00295) belonging to the GH28 family, *Dhpg* was closely related to genes from *Ophiostoma ulmi* (Q4PJU8_OPHUL), *O. novo-ulmi* (accession O59934_OPHNO), *Gibberella zeae* (Q4IHW4_GIBZE), *Fusarium solani* (C7Z309_NECH7), *Trichoderma harzianum* (B7ZEN3_TRIHA) and other PG genes from *Alternaria* spp. and *Aspergillus* spp.

```
   CAE46647.2  DhPG 376aa
  1 MVYKSGLIAA VLAASVMASP TPITNGDEVM KRASTCTFSG ASGAAQAMKE QASCSTITLK
 61 NLQVPAGTTL DLTKVKANTR IVFEGETTFG YKEWEGPLLS IAGTGLTVEG ASGSVLNDLG
121 EKYWDGKGSN GGKTKPKFFA AHKMMKSSIN NITIKNAPVQ VVSINGCDNL TVNRMHIDNK
181 DGDSKGGHNT DGFDIGSSNN IHINAAQVYN QDDCVAINSG TNIKFDSGLC SGGHGLSIGS
241 VGGRADNTVT GVTFSKSTVQ NSDNGIRIKA KKDTTGNISN IVYEDITLSK IAKYGVLIEQ
301 NYDGGDLHGE PTNKLPISHV TVNGLHGAGA IASGGHNMAI VCGSGSCANW TWKDVKVSGG
361 KKYDSCKNIP GVVGSC
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**Figure 2a.** Aminoacidic sequence of *Dh*PG predicted protein. Aminoacids with bold font: consensus residues in fungal endoPG.

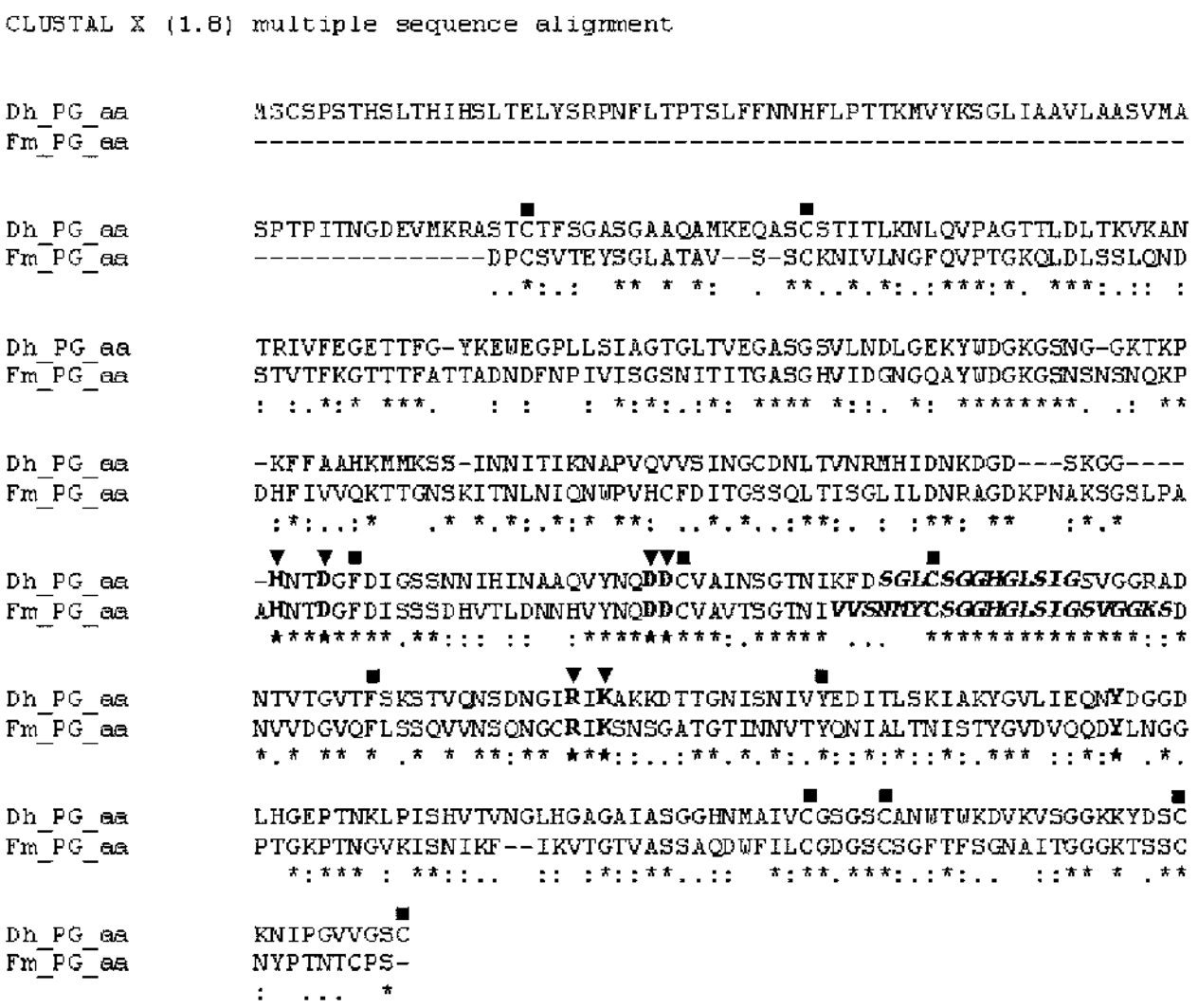


**Figure 2b.** Alignment of *Dh*PG vs *Fm*PG proteins. Active site in bold italics. Aminoacids for specific structure/function: ■ for β-helical region; ▼ for external regions of active site.

### RFLP analysis of the genomic *Dhpg* region

The isolate 8/96 genomic DNA was submitted to RFLP analysis with the complete (2895 bp) *Dhpg* gene as a probe in order to determine its occurrence in the genome. Restriction enzymes were selected based on the restriction map of the 2895 bp *Dhpg* region: *Eco*RI (no sites), *Bam*HI and *Hind*III (one site), *Sca*I and *Xba*I (two sites), *Pvu*II (three sites) and *Hae*II (four sites). Table 4 indicates the location of sites and expected restriction products. Fragments obtained by enzymatic digestions (*Eco*RI uncut >5 kb; *Bam*HI 2.6 and 0.9 kb; *Hind*III 3 and 0.7 kb; *Sca*I 1.6, 1.3 and 0.7 kb; *Xba*I 1.9, 1.6 and 0.2 kb; *Pvu*II 1.3, 0.9, 0.8, 0.4 kb; *Hae*II 1, 0.9, 0.8, 0.6 and 0.2 kb) confirmed predicted patterns, suggesting the presence of a unique sequence recognised by the probe in the total genome (Figure 3).

### Expression analysis

RT-PCR experiments were performed, using RTEPF-RTEPR primers (Table 2) including a 903 bp region in cds, on total RNAs derived from isolate 8/96 mycelium *in vitro* induced by pectin at 24, 48, 72 and 96 h. Control samples were obtained by my-

**Table 4.** Restriction enzymes selected for RFLP. Sites and expected size of products (bp) as inferred by the 2895 bp *Dhpg* sequence.

| Enzyme | Restriction sites | Restriction products (bp) |
|---|---|---|
| *Eco*RI | 0 | >2895 |
| *Bam*HI | 1: 824 | >824, >2071 |
| *Hind*III | 1: 535 | >535, >2360 |
| *Sca*I | 2: 522, 1840 | >522, 1318, >1055 |
| *Xba*I | 2: 1219, 2813 | >1219, 1594, >82 |
| *Pvu*II | 3: 420, 1258, 2597 | >420, 838, 1339, >298 |
| *Hae*II | 4: 609, 1595, 2456, 2711 | >609, 986, 861, 255, >184 |

celium grown in glucose-based medium for the same periods. Results showed a constitutive expression of endoPG under tested conditions, alike in control and induced mycelium at all assayed times, suggesting a non inducible nature of the *Dhpg* gene (Figure 4a).

Furthermore, the Northern hybridization analysis performed with the *Dhpg* coding region as a probe on cDNAs derived from RNAs used in RT-PCR did not show variation of the *Dhpg* transcript abundance among these *in vitro* samples (Figure 4b). Quantitative homogeneity of cDNAs was assayed using a *D. helianthi* probe obtained by PCR from the housekeeping *atp6 synthase* gene (AY263694.1; Rekab *et al*., 2004), as a reference (Figure 4c).

In a different experimental scheme which aimed to investigate *Dhpg* expression in the host, sunflower tissues were also induced by *in planta* and stem cut infection with 8/96 mycelium and submitted to RT-PCR and Northern analysis. The results, as shown in Figure 5a and 5b, confirmed the constitutive expression of *Dhpg* in host tissues.

In order to test for time-dependent transcript abundance variations, an RT-PCR was also performed on the same *in vitro* samples at 24, 48, 72 and 96 h. Again the same pattern was displayed by all transcripts during amplification cycles (data not shown).

### Variability of *Dhpg* in a set of *D. helianthi* isolates from different European regions

EndoPG *D. helianthi* specific primers (EPF-EPR, in the coding region) were tested on genomic DNAs derived from 23 *D. helianthi* isolates (14 French isolates, three Yugoslavian, one Romanian and five Italian) and a reference *D. arctii* (isolate 142/01), in order to compare coding genomic regions among *D. helianthi* of different geographic origins. A conserved profile of a single band (about 1600 bp) was obtained from all French and Yugoslavian isolates, while the Romanian iolate and all those from Italy displayed

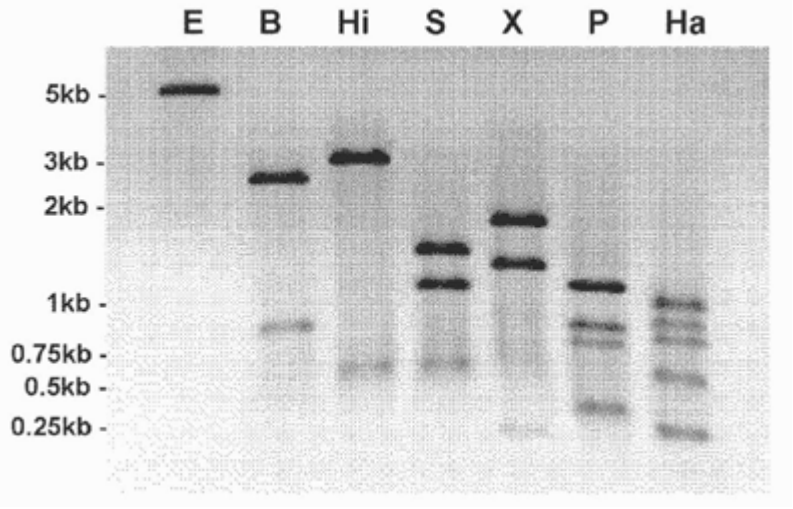


**Figure 3.** RFLP profiles of genomic DNA. Complete *Dh*PG sequence as a probe (PCR product with PREPF-EPR). E, *Eco*RI; B, *Bam*HI; Hi, *Hind*III; S, *Sca*I; X, *Xba*I; P, *Pvu*II; Ha, *Hae*II.

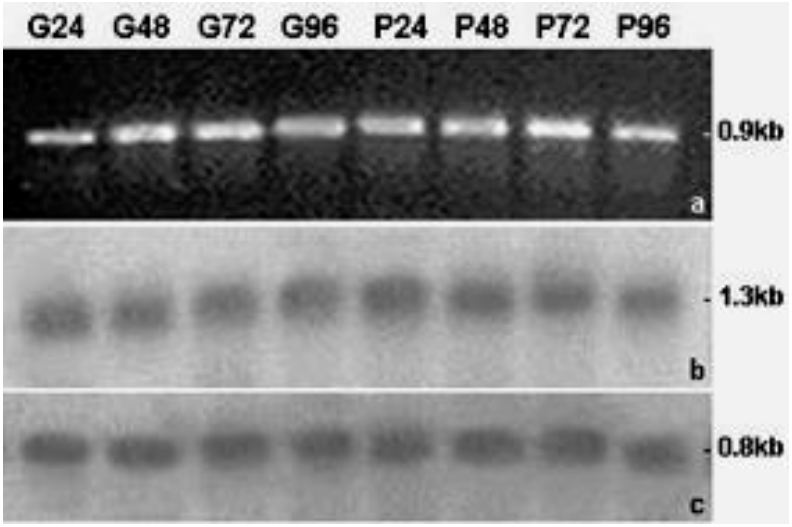


**Figure 4.** *Dhpg* expression on pectin induced mycelium. Samples: glucose (G) cultures at 24 h, 48 h,72 h, 96 h; pectin (P) cultures at 24 h, 48 h,72 h, 96 h. **a.** RT-PCR on RNAs with RTEPF-RTEPR primers, annealing T 63°C. b-c. Northern hybridization on cDNAs with coding *Dhpg* as a specific probe in b (PCR product with EPF-EPR, annealing T 65°C) and *atp6* as a reference probe in c (PCR product with ATP6F-ATP6R, annealing T 58°C).

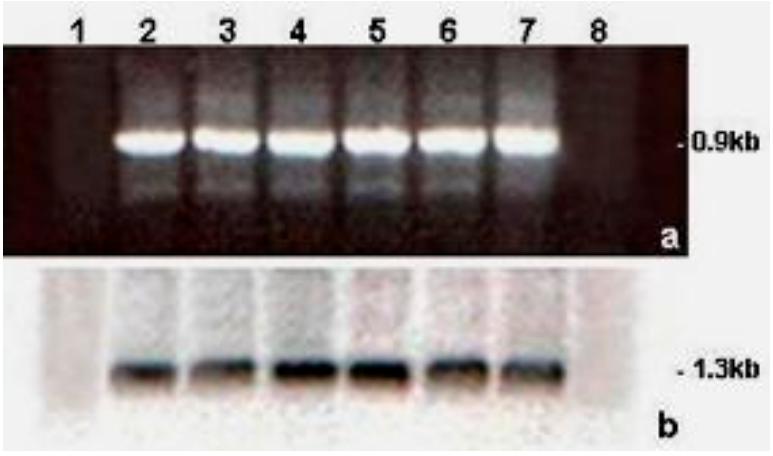


**Figure 5.** *Dhpg* expression on sunflower infected tissues. Lanes 1, 2, 3: stem cuts infection, control stems at 7 d, treated stems after 7 and 12 d respectively; lanes 4, 5: control mycelium grown 7 and 12 d respectively; lanes 6, 7, 8: *in planta* infection, treated stems after 7 and 12 d, control stems at 12 d respectively. **a.** RT-PCR as in Figure 4a. **b**. Northern hybridization as in Figure 4b.

a variable pattern. No amplification was evident in *D. arctii*, showing primer specificity for *D. helianthi* (Figure 6a). These data are consistent with previous studies dealing with the genetic variability inside the same set of *D. helianthi* isolates (Battilani *et al.*, 2003; Pecchia *et al.*, 2004**;** Rekab *et al.*, 2004**;** Vergara *et al.*, 2004**;** Vergara *et al.*, 2005).

A conserved ribosomal region (between distal IGS region and flanking 18S rDNA) was exploited as a reference. The relative region was amplified by

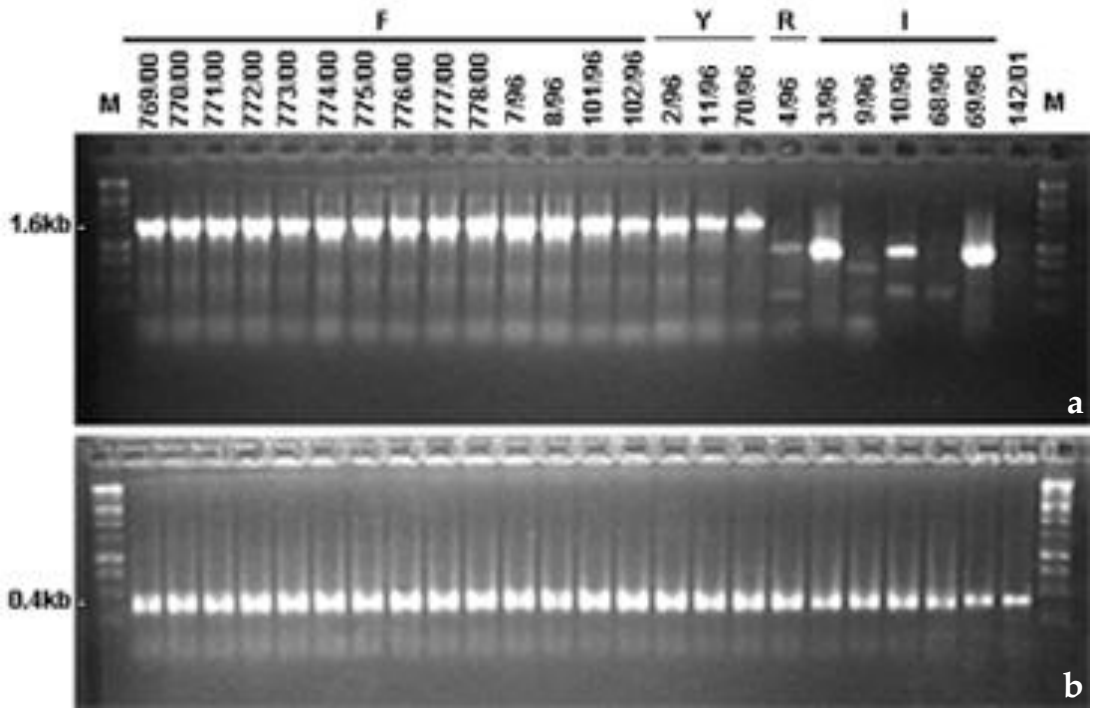


**Figure 6.** PCR analysis on a set of *Diaporthe helianthi* isolates. Genomic DNAs derived from 23 *D. helianthi* isolates (14 French F, three Yugoslavian Y, one Romanian R and five Italian I) and an outgroup *D. arctii* (142/01). **a**. EPF-EPR specific primers; **b.** IGS-12a and NS1R primers as a standard.

specific IGS-12a and NS1R primers (Carbone and Kohn, 1999) (see Table 2) and all DNAs, including the *D. arctii* isolate, produced a 400 bp amplicon as expected (Figure 6b).

## Discussion

*Diaporthe helianthi* is the causal agent of stem canker, an epidemic disease of sunflower in several European countries. We have previously shown that the strains presently classified within this species belong to phylogenetically distinct clades (Pecchia *et al.*, 2004; Rekab *et al.*, 2004; Vergara *et al.*, 2004; Vergara *et al.*, 2005). The strain examined in this work is a member of a tight clade of strains isolated in France and in the Balkans, where the disease has been destructive. Despite the severity of the disease, very little is known about the mechanisms that underly the relationships of *D. helianthi* with its host. Cytological observations of the infection process (Heller and Gierth, 2001) revealed severe cell wall destruction, suggesting the involvement of an enzymatic attack of the host cell walls by the fungus. In many phytopathogenic fungi CWDEs are powerful devices used to attack plants, and among them pectinases are the most relevant and early used weapons (Annis and Goodwin, 1997). Besides their involvement in the first steps of pathogenicity and in colonization ability (Sella *et al.*, 2005), they play a role as virulence determinants allowing the release of OGs from plant cell walls, which are potential elicitors of defence responses in plants (Alghisi and Favaron, 1995; De Lorenzo *et al.*, 1997). Inhibitors of PG activity, the PGIPs, are present in the cell walls of many plants and are able to limit fungal colonization by slowing the hydrolytic activity of endoPG so favouring the accumulation of OGs (D'Ovidio *et al.*, 2004). Pectinases are therefore primary targets for investigation of fungal virulence determinants, particularly in pathogen-host relationships where cell degradation appears to be very effective, as is the case for *D. helianthi* and sunflower (Heller and Gierth, 2001) and in other systems, including *Colletotrichum*-Leguminosae (Hernández-Silva *et al.*, 2007; Ramos *et al.*, 2010).

Despite the hundreds fungal endoPG sequences deposited in public databases, the majority of which belong to phytopathogenic microorganisms, there was no information on *D. helianthi* endoPG before this investigation commenced.

In the present study, a molecular analysis of endoPG genomic regions of the French *D. helianthi* isolate 8/96 was performed both at structural and expression levels, leading to the identification and characterization of a genomic sequence, named *Dhpg* (accession n. AJ582180), including the coding and promoter regions.

In the 1473 bp sequenced upstream the transcription initiation many structural motifs and patterns typical for eukaryotic promoters have been found. The structural properties exhibited by the *Dhpg* coding region are consistent with the expected function: the PG active site region needed for the substrate binding and for PGIP interaction, and secondary specific structures for β-sheets, β-helices and β-strands formation.

Pectic enzymes all share the same parallel β-helix topology: the substrate binding sites are all found in a similar location within a cleft formed externally to the parallel β-helix. Indeed structural information about pectic enzymes can contribute to understanding the multifaceted steps of pathogenesis: the role of multiple, independently regulated pectase isozymes, associated with the heterogeneous nature of substrates, can be involved in the recognition of a unique oligosaccharide in plant cell walls. This knowledge could shed light on specific properties in hosts which render them more sensitive to the attack by specific pathogens (Herron *et al.*, 2000). Structural information concerning pectic enzymes has already been exploited to understand steps of pathogenesis, and to unveil molecular properties of pathogen and host components involved in the first interaction (Misas-Villamil and van der Hoorn, 2008). Common structural features in the N-terminal aminoacid sequences have been reported among PGs from Ascomycetes (Keon and Waksman, 1990); in the C-terminal half of the PG molecule several aminoacidic residues are found strictly conserved in a segment with localized, inter-species homology (Stratilová *et al.*, 1996).

The structural comparisons of *Dh*PG with known endoPG proteins revealed all consensus domains. In the C-terminal region one histidine H residue in a GHGXSIGS stretch, three aspartic acids D (in NTD and DD structures), an arginin-isoleucine-lysine RIK structure and one strictly located tyrosine Y, are all conserved and have been ascribed as catalytic functions. At the N-terminal region the Ascomycetes PG consensus sequence has also been found. In a comparison with the endoPG protein derived from *Fusarium moniliforme* (*Fm*PG), analysed by means of crystallography and X-ray diffraction for studies of structure/function relationships (Federici *et al.*, 1999; Federici *et al.*, 2001), conserved residues at fixed positions in the active site cleft and at its edge, required for substrate binding and involved in the plant PGIP interaction, are present in the *Dh*PG protein.

The sequences that were most similar to *Dhpg* in BLAST analysis derived from other phytopathogens such as *Ophiostoma ulmi*, *O. novo-ulmi*, and *G. zeae*. *Dhpg*, a gene putatively involved in cell wall degradation during infection of sunflower, has been shown here to be single-copy in the *D. helianthi* genome and to be constitutively expressed. These results indicate that *Dhpg* could play a major role in pathogenesis.

The genetic variability of *D. helianthi* isolates from different geographic origins (France, ex-Yugoslavia, Romania and Italy) was investigated in a PCR analysis on *Dhpg* coding regions, putatively most conserved. Data obtained again indicated a common pattern for French and Yugoslavian isolates clustered together, while Romanian and Italian isolates displayed variable amplification profiles. This genetic diversity confirmed a correlation of different *D. helianthi* biotypes with epidemiology of sunflower stem canker in Europe, as previously reported (Pecchia *et al.*, 2004; Rekab *et al.*, 2004; Vergara *et al.*, 2004; Vergara *et al.*, 2005). Thus, *Dhpg* variability might be exploited for diagnostic purposes by PCR with specific endoPG primers.

In conclusion, we report the first *D. helianthi* endoPG genomic sequence. *Dhpg* is a single-copy, constitutive gene and its promoter region has been identified. The potential of such a promoter can be for preparing constructs to study regulation of fungal genes both in homologous and heterologous systems. Furthermore the constitutive expression of *Dhpg* in response to pectin (see *in vitro* data) could be essential for the fungal saprophytic phase and could represent an advantage for its rapid adaptation to pectic substrates (Parenicova *et al.*, 2000).

All properties and connections reported for *Dhpg* are compatible with a gene involved in pathogenesis and in the physical interaction at the host-pathogen interface.

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