

DIFFERENTIAL TRANSCRIPTS IN *PYRENOPHORA GRAMINEA* AND *PYRENOPHORA TERES* PUTATIVELY RELATED TO PATHOGENICITY

M. Vergara, C. Cristani and G. Vannacci

*Dipartimento di Coltivazione e Difesa Specie Legnose "G. Scaramuzzi", Sezione di Patologia Vegetale,
Via del Borghetto 80, I-56124, Pisa, Italy*

SUMMARY

Non radioactive Differential Display Reverse Transcription-PCR (DDRT-PCR) was used to screen molecular variants at expression level in *Pyrenophora graminea* and *Pyrenophora teres*, grown *in vitro* with glucose or plant cell walls as carbon sources. Several differential transcripts from both species were identified, either constitutive or induced by barley leaf cell walls. Three cloned species-specific transcripts were sequenced: one was constitutively expressed in *P. graminea* and absent in *P. teres*, two were specifically induced by host leaf cell walls either in *P. graminea* or in *P. teres*. For all three, sequence analysis showed significant homology with known pathogenicity-related genes available in databases, coding for toxic metabolites, cell wall degrading enzymes and regulative factors as kinases. These data are discussed in relation to the biology of the two pathogens under study.

Key words: DDRT-PCR, pathogenicity, *Pyrenophora graminea*, *Pyrenophora teres*.

INTRODUCTION

Pyrenophora graminea Ito and Kurib. [anamorph *Drechslera graminea* (Rabenh. ex Schlecht.) Shoem.] and *Pyrenophora teres* Drechs. [anamorph *Drechslera teres* (Sacc.) Shoem.] are serious seed-borne pathogens of barley, causing different diseases.

P. teres is the causal organism of net blotch of barley and occurs in two forms which differ in the type of symptoms incited on the host. The net form *P. teres* Drechs. f. *teres*, produces spots and streaks which expand to narrow, dark brown longitudinal and transverse necrotic streaks, forming a characteristic net-like pattern. The spot form, *P. teres* Drechs. f. *maculata* Smedeg., produces dark brown elliptical or fusiform lesions surrounded by a chlorotic zone of varying width.

P. graminea is the causal agent of barley leaf stripe. In contrast to *P. teres*, *P. graminea* cannot successfully infect

barley leaves by direct penetration and stripe symptoms always arise from systemic infection derived from infected seed (Husted, 1994). On leaves of infected plants one or more long chlorotic stripes develop parallel to the leaf ribs. Subsequently, the yellow stripes become necrotic and the leaf blade may split and present a frayed appearance. Although *P. teres* and *P. graminea* produce different symptoms on barley and traditionally constitute two different species, it has been demonstrated that they are able to intercross and produce fertile progeny, indicating that they are closely related. The close relationship is further emphasized by the presence of striking similarities in many of their morphological and biological traits, so that the range of morphological variability of their asexual reproductive structures overlaps. This makes their identification difficult (Smedegaard-Petersen, 1983).

Research on fungal processes involved in pathogenesis has focused mainly on production of substances toxic to plant cells which have been characterized both in *P. teres* (Smedegaard-Petersen, 1977; Friss *et al.*, 1991) and in *P. graminea* (Haegi and Porta-Puglia, 1995), while little is known at the molecular level on the mechanisms by which these pathogens attack the host (Porta-Puglia *et al.*, 2000).

Differential display reverse transcription-PCR (DDRT-PCR) (Liang and Pardee, 1992; Liang *et al.*, 1993) is a powerful technique to compare expression patterns in different biological conditions, providing a random collection of all differential transcripts in samples of interest. For this reason, it represents a suitable method to approach the study of host pathogen interaction and has been successfully used in several biological systems, such as medically important fungi and plant pathogens (Sturtevant, 2000). This technique has been exploited to isolate genes specifically induced during pathogenesis in several interactions, such as *Botrytis cinerea*-tomato (Benito *et al.*, 1996), *Phytophthora capsici*-pepper (Muñoz and Baily, 1998) and *Phytophthora infestans*-potato (Collinge and Boller, 2001).

Due to the close phylogenetic relationship between the two *Pyrenophora* species and the differences in their biology, the study of the molecular bases of the host-pathogen interaction should be useful for better understanding the mechanisms involved in their infection process. We started focusing our attention on cell wall degrading enzymes, as the ability to degrade plant cell

walls may be an essential requirement for virulence. In fact, fungi degrade plant cell wall polymers to obtain nutrients and to aid in penetrating cells and spreading through plant tissue (Annis and Goodwin, 1997). Thus, our aim was to identify variable transcripts from both species when they interact with host leaf cell walls. Media containing purified plant cell walls have been previously used to study, *in vitro*, the induction of various cell wall degrading enzymes (Favaron and Marciano, 1992; Tonukari *et al.*, 2000).

Here we report on the application of DDRT-PCR to search for differential transcripts, either specifically induced by plant cell wall fragments or constitutively present. Differential inducible transcripts could allow the detection of changes arising from the interaction with host, *e.g.* cell wall degrading enzymes (CWDE) or receptors for plant signals (Alghisi and Favaron, 1995; Annis and Goodwin, 1997; De Lorenzo *et al.*, 1997), while differential constitutive transcripts may reflect basic molecular differences between the two species.

MATERIALS AND METHODS

Fungal strains and culture conditions. Fungal strains used were *P. graminea* (Pg2) and *P. teres* Drechs. f. *teres* (Pt4), both isolated from infected *Hordeum vulgare* in Italy. Pg2 and Pt4 mycelia were routinely grown in liquid cultures, at 24°C with shaking at 180 rpm, in basal Fries medium (Pringle and Braun, 1957) supplemented with 0.4% w/v glucose.

Inductive media. Barley cell walls were extracted from leaves according to Nevins *et al.* (1967). Induction by cell wall fragments was carried out on 5-days old standard cultures, which were washed for 24 h in glucose-depleted Fries medium and then incubated for 40 hours in basal Fries medium supplemented with cell wall preparation (0.4% w/v). Control cultures were processed similarly but were then incubated for the last 40 hours in 0.4% w/v glucose.

DNA and RNA extraction. Mycelium samples were collected from liquid cultures, frozen with liquid N₂ and lyophilised for five days. Genomic DNAs were extracted by an SDS-CTAB method (Kim *et al.*, 1990). Total RNAs were isolated from control or induced mycelium by guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987), modified by the addition of 1% w/v soluble polyvinylpyrrolidone, mol. wt. 40 kD (Sigma, Missouri, U.S.A.), in the extraction buffer. RNAs were stored at -80°C.

Non radioactive differential display (DDRT-PCR). RNA was treated by Dnase I and then reversely transcribed using the one-base 3' anchored oligo-dT primer 5'-AAG CTT TTT TTT TTT C -3', according to Liang *et al.* (1994). For amplification of cDNA, five random

primers were used combined with the anchored oligo-dT: 149 (5'-CAC GTG GAC G -3'; annealing temperature 34°C), 150 (5'-CGG CCC CTG T -3'; annealing temperature 36°C), 152 (5'-GGT AGC AGT C -3'; annealing temperature 32°C), 220 (5'-CAG CAC CCA C -3'; annealing temperature 34°C) and 222 (5'-AGG TGA CCG T -3'; annealing temperature 32°C).

In the PCR reaction mixture, 50 ng cDNA was amplified with 200 µM dNTPs, 2 µM anchored oligo-dT, 0.25 µM random primer, 1.5 units Taq DNA Polymerase (Promega, Madison, WI, USA) and 2.5 µl 10x Taq DNA Polymerase buffer with 25 mM MgCl₂, to a final volume of 25 µl. Cycling parameters were: 40 amplification cycles at 94°C for 30 sec, at specific annealing temperatures (32°C, 34°C or 36°C) for 1 min, at 72°C for 1 min, and 1 elongation cycle at 72°C for 5 min, in a Perkin Elmer (Norwalk, USA) 9600 thermocycler. Samples were run on a 3.5% non-denaturing polyacrylamide sequencing 1.5-mm-thick gel, in Tris-borate buffer. The gel was stained in 4 µg ml⁻¹ ethidium bromide for 15-20 min and finally exposed to UV light (302 nm) to visualize the band pattern (Boschi and Vergara, 1998). Bands of interest were excised from the gel and stored at -20°C. In order to reduce the number of false positives, each experimental process was repeated twice and only reproducible bands were rescued.

Cloning of differential transcripts. The selected bands with amplified cDNAs were eluted from polyacrylamide gel in nuclease-free water for 16-24 hours at room temperature and reamplified under the same conditions previously described. PCR products were re-excised from agarose gel, purified with QIAquick gel extraction kit (Qiagen, Milan, Italy) and cloned in pGEM-T Easy vector (Promega, Madison, WI, USA), following manufacturer's instructions.

RNA and DNA slot blot. Total RNAs (2 µg each sample) or genomic DNAs (100 ng each sample) were heat denatured, transferred to positively charged Nylon membranes (Roche Diagnostics, Basel, Switzerland) by a slot blot apparatus Minifold II (Schleicher & Schuell, Dassel, Germany) (Sambrook *et al.*, 1989) and then hybridised to DIG-labelled probes (cDNA clones). Hybridization was performed in 50% formamide overnight at 42°C and membranes were washed in 0.5x SSC at 65°C. Chemiluminescent detection steps with CSPD (disodium 3-(4-methoxy-spiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3,3.1.1.^{3,7}] decan}-4yl)phenyl phosphate) were carried out according to manufacturer's instructions (DIG system; Roche Diagnostics, Basel, Switzerland).

Sequence analysis. Clones carrying differential transcripts were submitted to a sequencing reaction (Genelab Service at ENEA, Rome, Italy) using T7 and SP6 universal primers and the dye terminator method with an automatic sequencer (ABI-Prism 373, Perkin-Elmer, Norwalk, USA). Sequence homologies of transcripts were

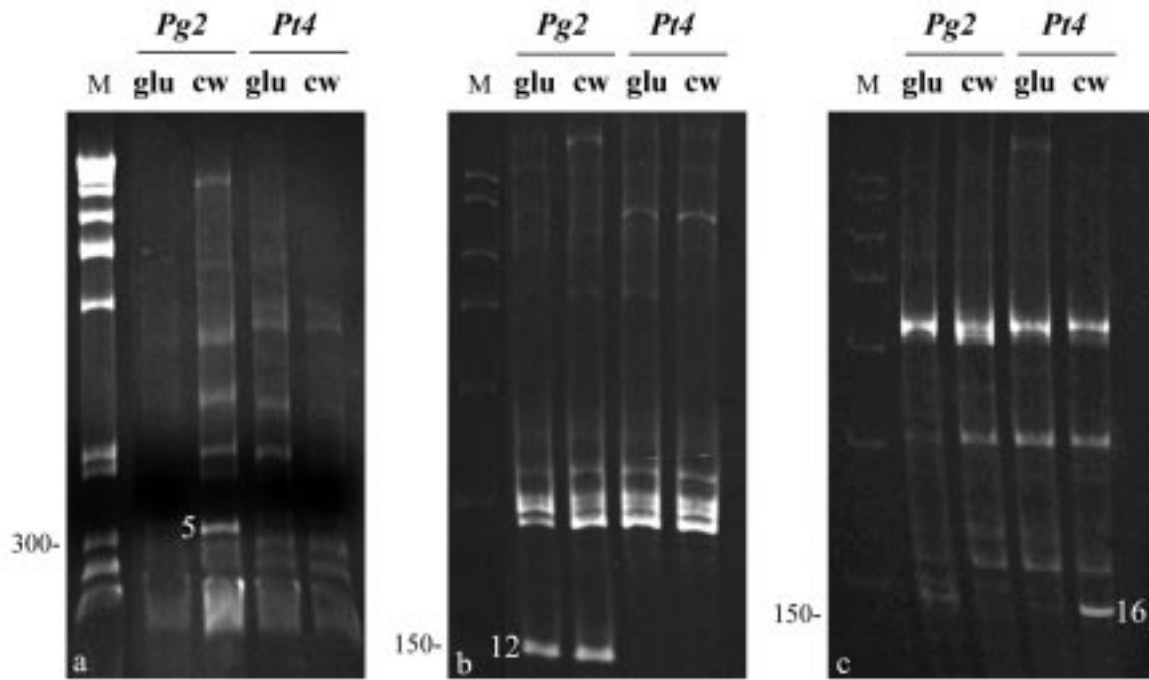


Fig. 1. Differential display patterns. Polyacrylamide gel electrophoresis of the DDRT-PCR products of *Pyrenophora* spp. relative to combinations of one anchored oligodT (T_{11} -C) with 3 arbitrary primers (a: 150, b: 152, c: 149). RNA samples were derived from Pg2 and Pt4 mycelia, supplied by glucose (glu) or induced by host cell walls (cw). Selected differential bands: 5, 12 and 16. M: molecular weight markers (a: GIBCO-BRL 1 Kb Ladder; b and c: Sigma PCR Marker).

checked by a FASTA3 search program against the fungi nucleic acid database (Pearson, 1990).

RESULTS

Differential display. In order to search for differential transcripts in the two *Pyrenophora* species, total RNAs were isolated from *P. graminea* and *P. teres* mycelium (Pg2 and Pt4) that was induced *in vitro* by barley cell walls or grown in standard conditions (glucose as a carbon supply) as a control. All RNAs were submitted to a non-radioactive DDRT-PCR protocol.

With a combination of five random primers and one anchored oligo-dT (T_{11} -C), twenty-two major differential bands were identified, all shown to be reproducible, which fell within two classes in relation to their putative biological significance. A first group represents species-specific constitutive transcripts, present in *P. graminea* (4) or in *P. teres* (2); a second group includes species-specific transcripts differentially induced by host cell walls in *P. graminea* (11) or in *P. teres* (5). Among the most abundant transcripts, standing for higher specific expression, three cDNAs resulted representative of both classes, being one constitutively expressed in *P. graminea* and absent in *P. teres* (band 12, about 150 bp) and two specifically induced by barley leaf cell walls either in *P. graminea* (band 5, about 330 bp) or in *P. teres* (band 16, about 150 bp). Therefore they were selected for further analysis (Fig. 1).

Cloning, expression analysis and sequencing. The three selected differential transcripts (12, 5 and 16) were re-amplified and cloned in a proper vector (pGEM-T Easy): respectively clones DD12, DD5 and DD16.

In order to check whether clones, mainly the constitutive transcript DD12 in *P. graminea*, were species-specific at genomic level as well, they were matched to DNAs extracted from *P. graminea* and *P. teres* in DNA slot blot experiments (Fig. 2A). No differential recognition was displayed for any clone, implying for all cDNAs a specificity limited to transcriptional level.

When tested in RNA slot blot analysis on control samples (glucose supplied) or induced by host cell walls, the three clones confirmed their differential expression (Fig. 2B). Therefore they were submitted to automatic sequencing. Sequence data were deposited at the EMBL databank with accession numbers: AJ512138 for clone DD5, AJ512139 for clone DD12 and AJ512140 for clone DD16. Their nucleotide sequences are displayed in Fig. 3.

Sequence analysis. The three cDNAs were processed in a Fasta3 similarity analysis against a fungi nucleic acid database, with default parameters except open gap penalties -2 . All of them displayed significant homology with known fungal genes. The best alignment of each clone is reported in the first line of Table 1; besides it, additional alignments with scores likewise significant (even with higher ungapped identities) have been pointed out, with phytopathogenic fungi sequences being putatively consistent with our experimental system.

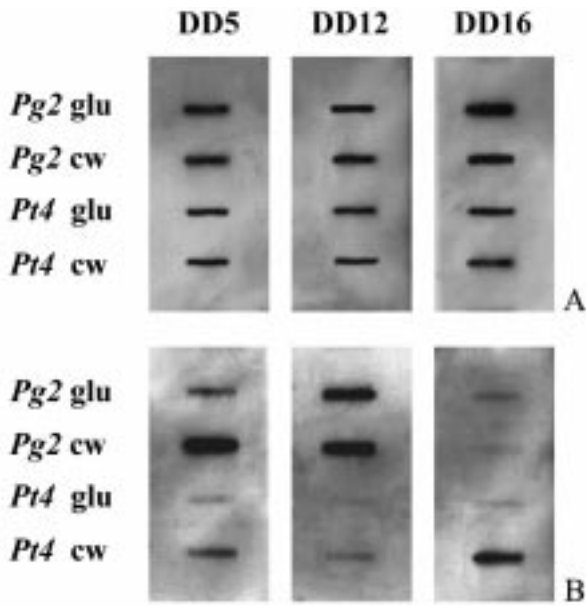


Fig. 2. A) DNA slot blot. Genomic DNAs (100 ng) derived from Pg2 and Pt4 grown as in Fig. 1, hybridised to DIG-labelled DD5, DD12 and DD16 clones and detected by chemiluminescence. B) RNA slot blot. Total RNAs (2 µg each) isolated from the same samples, induced (cw) or control (glu), hybridised and detected as described in A.

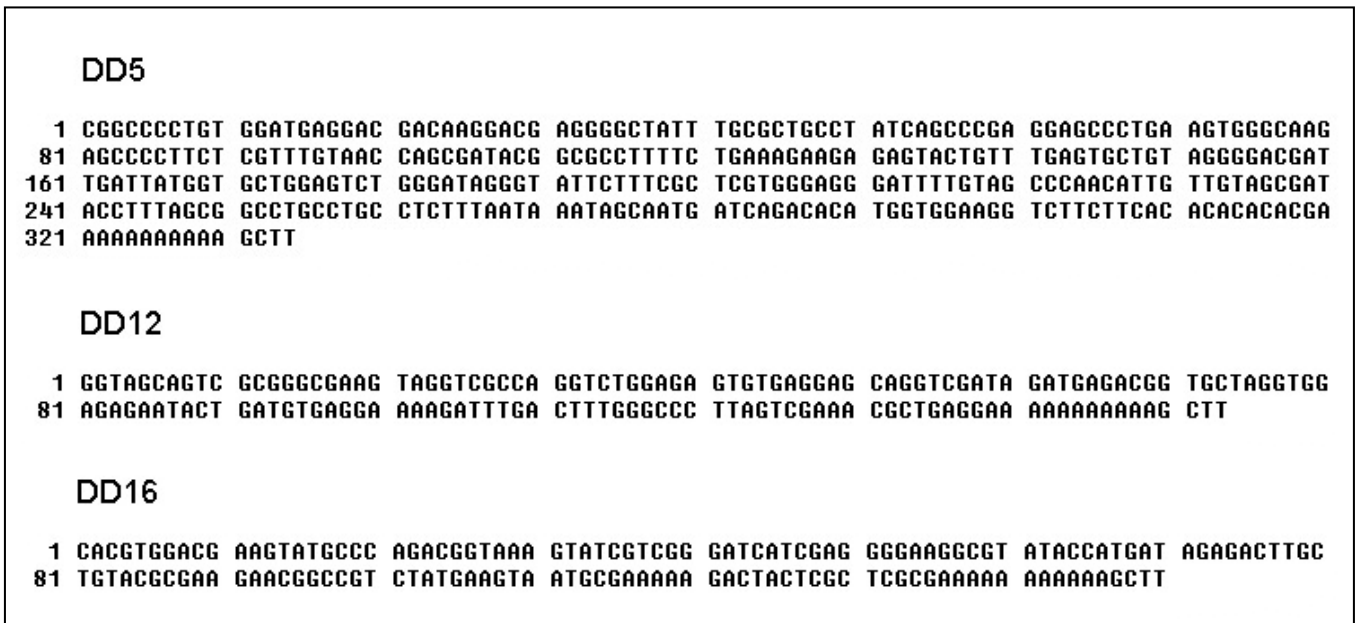


Fig. 3. Nucleotide sequences of clones DD5, DD12 and DD16.

The transcript DD12, 153 bp long, expressed constitutively in *P. graminea* in both of the conditions tested and absent in *P. teres* transcripts, displayed homology with heterogeneous genes, the best one being with the *Phaffia rhodozyma* glyceraldehyde-3-phosphate dehydrogenase gene, *GAPDH*. Two more alignments are shown in Table 1, with a *Nectria haematococca* pathogenicity cluster gene, *PEP2* (Han *et al.*, 2001), and with an *Alternaria alternata* gene controlling AK-toxin biosynthesis and pathogenicity, *AktR-1* (Tanaka and Tsuge, 2000). It is worth noting that clone DD12 recognises the end of *PEP2* coding sequence, both poly-A tails being overlapped.

The transcript DD5, 334 bp long, induced by host cell walls specifically in *P. graminea*, displayed high homology with several cell wall degrading enzymes

(CWDE) fungal genes, although the best alignment came out to be with *Cryptococcus neoformans* *Myo2* gene coding for a myosin V which orientates the mitotic spindle in yeast (Yin *et al.*, 2000). Most alignments were found with pectinases, such as five polygalacturonases in the same genic region: *Botryotinia fuckeliana* *Bcpg1* endopolygalacturonase (Ten Have *et al.*, 1998), *Penicillium olsonii* *pg2* (Wagner *et al.*, 2000) and three *Sclerotinia sclerotiorum* endopolygalacturonases with accession numbers L29041, L12023 and L29040 (Fraissinet-Tachet *et al.*, 1995). A high degree of homology was also shown by clone DD5 with a *Penicillium purpurogenum* endo-xylanase A gene, *XynA* (Chavez *et al.*, 2001), an *Aspergillus aculeatus* rhamnosidase b precursor, *rhaB* (Manzanares *et al.*, 2001) and a *Talaromyces emersonii* endo b 1,4-glucanase gene, *eg1* (accession number

Table 1. FASTA3 analysis of clones. Similarity results of FASTA3 analysis against fungi genome database. Homologous genes with relative accession numbers and recognition quantitative data are displayed. Identity is referred to total nucleotides (nt) stretch in target gene; ungapped identity when gaps are not considered. *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *AktR-1*: AK-toxin R-1; *PEP2*: pea pathogenicity cluster gene 2; *MYO2*: myosin V; *BcpG1*: endopolygalacturonase 1; *XynA*: endoxylanase A; *PET494*: translational activator; *TB3*: serine/threonine kinase.

Clone	Gene homology	Accession number	Z-scores	Bits	Identity	Ungapped identity
DD12 153 bp	<i>Phaffia rhodozyma</i> <i>GAPDH</i>	AF006483	722	144	62% in 178 nt	84% in 178 nt
	<i>Alternaria alternata</i> <i>AktR-1</i>	AB035491	693	139	57% in 195 nt	92% in 195 nt
	<i>Nectria haematococca</i> <i>PEP2</i>	AF315805	678	134	59% in 181 nt	90% in 181 nt
DD5 334 bp	<i>Cryptococcus neoformans</i> <i>MYO2</i>	AF542529.1	1381	272	55% in 409 nt	83% in 409 nt
	<i>Botrytis cinerea</i> <i>BcpG1</i>	U68715	1352	261	55% in 412 nt	84% in 412 nt
	<i>Penicillium purpurogenum</i> <i>XynA</i>	AF249328	1294	251	55% in 391 nt	86% in 391 nt
D16 150 bp	<i>Saccharomyces bayanus</i> <i>PET494</i>	AF056620	732	145	60% in 180 nt	88% in 180 nt
	<i>Colletotrichum trifolii</i> <i>TB3</i>	U14989	686	137	60% in 179 nt	85% in 179 nt

AF440013). With respect to CWDE genes, only two similarity results are displayed in Table 1: the best among endopolygalacturonases and the endo-xylanase because of xylan abundance in monocots cell walls.

Finally the transcript DD16, 150 bp long, induced by host cell walls only in *P. teres*, recognises transcriptional regulators, such as activators, phosphatase and more frequently serine/threonine kinase genes. The highest homology concerned the *Saccharomyces bayanus* *PET494* gene coding for a mitochondrial mRNA-specific translational activator (Costanzo *et al.*, 2000), while among kinases the best alignment was found with the coding sequence of the *Colletotrichum trifolii* *TB3* kinase gene, involved in hyphal elongation and branching (Buhr *et al.*, 1996; Chen and Dickman, 2002).

DISCUSSION

Establishment of the infection results from complex interactions between host and pathogen, involving pe-

culiar processes both in the fungus, such as activation of mechanisms for penetration and colonization, and in the plant, such as host defence responses. Expression of several fungal genes is specifically induced or increased during interaction with the plant host. Thus one would expect factors essential for pathogenesis to be identifiable among such genes.

P. graminea and *P. teres* are closely related phylogenetically although they differ with respect to several aspects, such as symptomatology, systemic or local infection and ability to produce successful secondary infections on barley leaves. Therefore we believe that analysis of differences at a molecular level in the host-pathogen interaction may provide information on functions involved in their pathogenicity. Thus, we looked for differences in the production of cell wall degrading enzymes or molecules induced by plant signals; comparing by mRNA differential display (DDRT-PCR) transcript populations in *P. graminea* versus *P. teres* in response to barley cell walls. In fact, this inductive medium has been already exploited to study regulative aspects of plant-pathogen interactions

(Favaron and Marciano, 1992; Tonukari *et al.*, 2000).

A non-radioactive DDRT-PCR protocol (Boschi and Vergara, 1998) was applied, which is more convenient both for gel visualization and for manipulation of rescued PCR products. Results show the potential of this technique for studying variability at transcription level in the fungi analysed. It is noteworthy that our analysis covers only one third of the total transcript population, as all cDNAs should be theoretically recognized by using all three different one-base anchored oligos-dT (dT-C, dT-A and dT-G). Despite this, we obtained two species-specific transcript classes with different putative biological significance: constitutive and host leaf cell wall-inducible transcripts. The first class may be related to basic molecular differences between the two species; while the second one allows study of molecular interactions with the host cell wall. Based on their putative biological role, three cDNA clones were selected, whose expression could be associated to species-specific constitutive functions (clone DD12 from *P. graminea*) or to specific interaction with the host cell wall (clones DD5 from *P. graminea* and DD16 from *P. teres*). Remarkably all three recognised gene sequences related to pathogenicity whose functions could be correlated to the selected biological system.

In particular, the sequence corresponding to the DD12 transcript, constitutively present in *P. graminea*, aligns with pathogenesis related gene sequences, as a high degree of homology was found with the polyA region in *PEP2* gene of *Nectria haematococca* and with *AktR-1* gene of *Alternaria alternata*. *PEP2* is part of a cluster of six genes (*PEP*), required for pathogenicity on pea, located on a "conditionally dispensable" chromosome (Han *et al.*, 2001). *AktR-1* is contained in a complex region required for AK-toxin production and may itself encode for a protein essential for toxin biosynthesis (Tanaka and Tsuge, 2000). Interestingly these two genes align in their complete coding sequence up to the ends. Furthermore it is worth noting that both gene clusters recognised by clone DD12 are somehow involved in host specificity. As a matter of fact, the host specific AK-toxin is produced by the *Alternaria alternata* Japanese pear pathotype, while isolates of *N. haematococca* without *PEP* gene homologues are not pathogenic on pea, but are virulent on ripe tomato fruits and carrot roots, indicating that *PEP* genes are not required for pathogenicity on these hosts (Hatta *et al.*, 2002; Temporini and VanEtten, 2002). The homologies we found, combined with data reported in the literature, could suggest that DD12 belongs to a genomic region in *P. graminea* in some way similar to portions of gene clusters conferring host specific pathogenicity.

The DD5 transcript, differentially induced in *P. graminea* by barley leaf cell walls, shows similarity with several genes coding for cell wall degrading enzymes, a finding that is consistent with our inductive system. Among these genes, significant alignment was found with *Botrytis cinerea Bcpg1*, encoding for an endopolygalactur-

onase expressed during infection of tomato leaves and required for full virulence (Ten Have *et al.*, 1998) and with *Penicillium purpurogenum* endoxylanase *XynA* (Chavez *et al.*, 2001). Fungal xylanases may be key biochemical components in pathogenesis; in fact, endoxylanase genes have been found to be expressed by *Cochliobolus sativus* in inoculated barley seedlings or by *Helminthosporium turcicum* in maize plants during infection (Degefu *et al.*, 2001; Emami and Hack, 2001). In our system the DD5 transcript, preferentially expressed in *P. graminea* after cell wall induction, seems to belong to a genomic region sharing common traits with different CWDE genes.

The DD16 transcript, specifically induced in *P. teres* by barley leaf cell walls, displays high homology with many genes coding for regulative proteins, such as transcriptional factors, serine/threonine phosphatase and mostly kinase. Therefore it could represent a switch of responses either to host cell walls or to host signals, as phosphatase or kinase enzymes are often involved in signal transduction. In particular this clone showed homology to the *Colletotrichum trifolii TB3* gene coding for a serine/threonine protein kinase involved as a transcriptional factor in regulation of hyphal growth (Chen and Dickman, 2002). The *TB3* gene is known to be homologous to several kinases, such as *Neurospora crassa cot-1* gene (Buhr *et al.*, 1996) also required for hyphal elongation, and *Ustilago maydis ukc1* gene that plays an important role in morphogenesis, pathogenesis and pigmentation (Durrenberger and Kronstad, 1999). This demonstrates the functional conservation of these genes for growth and differentiation among pathogenic and saprophytic fungi. In addition, a mitogen-activated protein kinase gene (*PTK1*) has been recently shown, by RT-PCR (reverse transcription polymerase chain reaction) analysis, to be expressed in *P. teres* during infection of barley leaves. The gene is directly involved in appressoria formation and in the ability of *P. teres* to infect and colonize barley leaves (Ruiz-Roldan *et al.*, 2001). Mitogen-activated protein kinase (MAPK) genes, required for plant infection, have also been found in *Cochliobolus heterostrophus* (*CHK1*) and in *Fusarium graminearum* (*MGV1*) (Lev *et al.*, 1999; Hou *et al.*, 2002). Although no homology was found between *PTK1* and DD16 *P. teres* sequences, another protein belonging to the kinases family could be induced by host leaf cell wall components in our biological system. However, since clone DD16 is specifically induced in *P. teres* and is related to regulative functions, it may be involved in a differential regulation of pathogenicity mechanisms in *P. teres* compared to *P. graminea*.

Additional work is needed to assign a specific biological role to all the differential transcripts described.

In conclusion, we successfully exploited the DDRT-PCR technique to analyse differential expression patterns in *P. graminea* and *P. teres*. We believe it is possible to use specific transcripts as a tool to discriminate at transcriptional level between the two species, in order to investigate about their different pathogenetic mechanisms.

ACKNOWLEDGEMENTS

This research was supported by the MiPA Project "Piano Nazionale Biotecnologie Vegetali", 166-4. M. Vergara is a researcher at Scuola Normale Superiore of Pisa, Italy.

REFERENCES

- Alghisi P., Favaron F., 1995. Pectin-degrading enzymes and plant-parasite interactions. *European Journal of Plant Pathology* **101**: 365-375.
- Annis S.L., Goodwin P.H., 1997. Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *European Journal of Plant Pathology* **103**: 1-14.
- Benito E.P., Prins T., Van Kan J.A.L., 1996. Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction. *Plant Molecular Biology* **32**: 947-957.
- Boschi E., Vergara M., 1998. A protocol for nonradioactive differential display tested on carrot auxin-resistant mutants. *Plant Molecular Biology Reporter* **16**: 88.
- Buhr T.L., Oved S., Truesdell G.M., Huang C., Yarden O., Dickman M.B., 1996. A kinase-encoding gene from *Colletotrichum trifolii* complements a colonial growth mutant of *Neurospora crassa*. *Molecular and General Genetics* **251**: 565-572.
- Chavez R., Almarza C., Schachter K., Peirano A., Bull P., Eyzaguirre J., 2001. Structure analysis of the endoxylanase A gene from *Penicillium purpurogenum*. *Biological Research* **34**: 217-226.
- Chen C., Dickman M.B., 2002. *Colletotrichum trifolii* TB3 Kinase, a *COT1* homolog, is light inducible and becomes localized in the nucleus during hyphal elongation. *Eukaryotic Cell* **1**: 626-633.
- Chomczynski P., Sacchi N., 1987. Single step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**: 156-159.
- Collinge M., Boller T., 2001. Differential induction of two potato genes, *Stprx2* and *StNAC*, in response to infection by *Phytophthora infestans* and wounding. *Plant Molecular Biology* **46**: 521-529.
- Costanzo M.C., Bonnefoy N., Williams E.H., Clark-Walker G.D., Fox T.D., 2000. Highly diverged homologs of *Saccharomyces cerevisiae* mitochondrial mRNA-specific translational activators have orthologous functions in other budding yeasts. *Genetics* **154**: 999-1012.
- Degefu Y., Paulin L., Lubeck P.S., 2001. Cloning sequencing and expression of a xylanase gene from the maize pathogen *Helminthosporium turcicum*. *European Journal of Plant Pathology* **107**: 457-465.
- De Lorenzo G., Castoria R., Bellincampi D., Cervone F., 1997. Fungal invasion enzymes and their inhibition. In: Carroll G.C., Tudzynski P. (eds.). *The Mycota V, Part A, Plant Relationships*, pp. 61-83. Springer-Verlag, Berlin, Germany.
- Durrenberger F., Kronstad J., 1999. The *uck1* gene encodes a protein kinase involved in morphogenesis, pathogenicity and pigment formation in *Ustilago maydis*. *Molecular and General Genetics* **261**: 281-289.
- Favaron F., Marciano P., 1992. Polygalacturonase regulation in *Sclerotinia sclerotiorum*: effect of carbon source on the isoenzymatic pattern. *Rivista di Patologia Vegetale S. V*, **2**: 111-123.
- Emami K., Hack E., 2001. Characterisation of a xylanase gene from *Cochliobolus sativus* and its expression. *Mycological Research* **105**: 352-359.
- Fraissinet-Tachet L., Reymond-Cotton P., Fevre M., 1995. Characterization of a multigene family encoding an endopolygalacturonase in *Sclerotinia sclerotiorum*. *Current Genetics* **29**: 96-99.
- Friis P., Olsen C.E., Moller B.L., 1991. Toxin production in *Pyrenophora teres*, the Ascomycete causing the net-spot blotch disease of barley (*Hordeum vulgare* L.). *Journal of Biological Chemistry* **266**: 13329-13335.
- Haegi A., Porta-Puglia A., 1995. Purification and partial characterization of a toxic compound produced by *Pyrenophora graminea*. *Physiological and Molecular Plant Pathology* **46**: 429-444.
- Han Y., Liu X., Benny U., Corby Kistler H., Van Hetten H., 2001. Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen *Nectria haematococca*. *The Plant Journal* **25**: 305-314.
- Hatta R., Ito K., Hosaki Y., Tanaka T., Tanaka A., Yamamoto M., Akimitsu K., Tsuge T., 2002. A conditionally dispensable chromosome controls host-specific pathogenicity in the fungal plant pathogen *Alternaria alternata*. *Genetics* **161**: 59-70.
- Hou Z., Xue C., Peng Y., Katan T., Kistler C.H., Xu J.R., 2002. A mitogen-activated protein kinase gene (*MGV1*) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *Molecular Plant-Microbe Interactions* **15**: 1119-1127.
- Husted K., 1994. Development of specie-specific probes for identification of *Pyrenophora graminea* and *P. teres* by dot-blot or RFLP. In: Schots A., Dewey F.M., Oliver R. (eds.). *Modern assay for Plant Pathogenic Fungi. Identification, Detection and Quantification*, pp. 191-197. CAB International, Wallingford, UK.
- Kim W.K., Mauthe W., Hausner G., Klassen G.R., 1990. Isolation of high molecular weight DNA and double-stranded RNAs from fungi. *Canadian Journal of Botany* **68**: 1898-1902.
- Lev S., Sharon A., Hadar R., Ma H., Horwitz B.A., 1999. A mitogen-activated protein kinase of the corn leaf pathogen *Cochliobolus heterostrophus* is involved in conidiation, appressorium formation, and pathogenicity: diverse roles for mitogen-activated protein kinase homologs in foliar pathogens. *Proceedings of National Academy of Sciences USA* **96**: 13542-13547.
- Liang P., Averboukh L., Pardee A.B., 1993. Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Research* **21**: 3269-3275.

- Liang P., Pardee A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**: 967-971.
- Liang P., Zhu W., Zhang X., Guo Z., O'Connell R.P., Averboux L., Wang F., Pardee A.B., 1994. Differential display using one-base anchored oligo-dT primers. *Nucleic Acids Research* **22**: 5763-5764.
- Manzanares P., Van Den Broeck H.C., de Graaff L.H., Visser J., 2001. Purification and characterization of two different alpha-L-rhamnosidases, *RbaA* and *RbaB*, from *Aspergillus aculeatus*. *Applied and Environmental Microbiology* **67**: 2230-2234.
- Muñoz C.I., Bailly A.M., 1998. A cutinase-encoding gene from *Phytophthora capsici* isolated by differential-display RT-PCR. *Current Genetics* **33**: 225-230.
- Nevins D.J., English P.D., Albersheim P., 1967. The specific nature of plant cell wall polysaccharides. *Plant Physiology* **42**: 900-906.
- Pearson W.R., 1990. Rapid and Sensitive Sequence Comparison with FASTP and FASTA. *Methods in Enzymology* **183**: 63-98.
- Porta-Puglia A., Delogu G., Vannacci G., Aragona M., Arru L., Bellini L., Cristani C., Faccini N., Haegi A., Montigiani M., Pecchia S., Pecchioni N., Stanca A.M., Valé G., Vergara R., 2000. *Pyrenophora graminea*-barley: a model for biomolecular studies of seed-borne diseases. *Journal of Plant Pathology*, **82**: 82 (Abstract).
- Pringle R.B., Braun A.C., 1957. The isolation of the toxin of *Helminthosporium victoriae*. *Phytopathology* **47**: 369-371.
- Ruiz-Roldan M.C., Maier F.J., Schafer W., 2001. *PTK1*, a mitogen-activated-protein kinase gene, is required for conidiation, appressorium formation, and pathogenicity of *Pyrenophora teres* on barley. *Molecular Plant-Microbe Interactions* **14**: 116-125.
- Sambrook J., Fritsch E.F., Maniatis T., 1989. A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, USA.
- Smedegaard-Petersen V., 1977. Isolation of two toxins produced by *Pyrenophora teres* and their significance in disease development of net-spot blotch of barley. *Physiological Plant Pathology* **10**: 203-211.
- Smedegaard-Petersen V., 1983. Cross fertility and genetic relationship between *Pyrenophora teres* and *P. graminea*. The causes of net blotch and leaf stripe of barley. *Seed Science and Technology* **11**: 673-680.
- Sturtevant J., 2000. Applications of differential-display reverse transcription-PCR to molecular pathogenesis and medical mycology. *Clinical Microbiology Reviews* **13**: 408-427.
- Tanaka A., Tsuge T., 2000. Structural and functional complexity of the genomic region controlling AK-toxin biosynthesis and pathogenicity in the Japanese pear pathotype of *Alternaria alternata*. *Molecular Plant-Microbe Interactions* **13**: 975-986.
- Temporini E.D., VanEtten H.D., 2002. Distribution of pea pathogenicity (*PEP*) genes in the fungus *Nectria haematococca* mating population VI. *Current Genetics* **41**: 107-114.
- 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 Interactions* **11**: 1009-1016.
- Tonukari N.J., Scott-Craig J.S., Walton J.D., 2000. The *Cochliobolus carbonum* *SNF1* gene is required for cell wall-degrading enzyme expression and virulence on maize. *The Plant Cell* **12**: 237-248.
- Wagner F., Kusserow H., Schaefer W., 2000. Cloning of targeted disruption of two polygalacturonase genes in *Penicillium olsonii*. *FEMS Microbiological Letters* **186**: 293-299.
- Yin H., Pruyne D., Huffaker T.C., Bretscher A., 2000. Myosin V orientates the mitotic spindle in yeast. *Nature* **406**: 1013-1015.

Received 16 May 2003

Accepted 25 August 2003