Rev Iberoam Micol 2000; 17: S47-S53

Papel de enzimas líticas de la pared celular en la patogenicidad de *Fusarium oxysporum*

M. Isabel G. Roncero, Antonio Di Pietro, M. Carmen Ruiz-Roldán*, M. Dolores Huertas-González, Fe I. Garcia- Maceira, Emese Méglecz, Angeles Jiménez, Zaira Caracuel, Rocío Sancho-Zapatero, Concha Hera, Esperanza Gómez-Gómez, Manuel Ruiz-Rubio, Clara I. González-Verdejo and M. Jose Páez

*Departamento de Genética, Facultad de Ciencias, Universidad de Córdoba, Córdoba, España. * Present address: Center of Applied Molecular Biology of Plants, University of Hamburg, Hamburg, Germany*

Resumen

Fusarium oxysporum es capaz de colonizar a sus plantas huéspedes penetrando a través de las raices e invadiendo su sistema vascular. Esta especie produce gran variedad de enzimas líticas que depolimerizan todos los componentes de las paredes vegetales, como son la celulosa, el xilano, las pectinas, los ácidos poligalacturónicos y las proteinas (extensinas). Nuestro grupo ha purificado y caracterizado bioquímicamente varias enzimas líticas, una endopoligalacturonasa mayoritaria (PG1), dos exopoligalacturonasas (PG2 y PG3), una endoxilanasa (XYL1), y una endopectato-liasa (PL1). Recientemente hemos aislado los genes responsables de algunas de estas enzimas hidrolíticas: *pg1, pgx4, pg5, xyl2, xyl3, prt1 y pl1*. La expresión de los distintos genes se ha determinado mediante análisis Northern del hongo crecido en distintas condiciones de cultivo. Se ha comprobado la existencia de genes homólogos en otras formas speciales de *F. oxysporum* mediante análisis Southern y PCR. Todos los genes estudiados se expresan "in planta" en distintos estadíos del proceso de infección y en diversos tejidos, lo que indica su posible implicación en el proceso de patogénesis. Actualmente se está llevando a cabo la inactivación dirigida de cada uno de estos genes, mediante transformación genética y recombinación homóloga. El análisis patotípico de los mutantes que se obtengan permitirá evaluar la importancia de cada gen y su producto en la patogénesis de *F. oxysporum*.

Pectato liasa, Poligalacturonasa, Proteasa, Xilanasa, "Green fluorescent protein", Tomate

Role of cell wall-degrading enzymes in pathogenicity of *Fusarium oxysporum*

Summary

Fusarium oxysporum invades its host plants through the roots and colonizes the vascular system. It produces a great variety of cell-wall degrading enzymes (CWDE), such as cellulases, xylanases, pectinases and proteases. Our group has purified and characterized an endopolygalacturonase (PG1), two exopolygalacturonases (PG2 and PG3), an endoxylanase (XYL1) and an endo pectate-lyase (PL1). We have isolated the following CWDE-encoding genes: *pg1, pgx4, pg5, xyl2, xyl3, prt1* and *pl1*. Gene expression in different culture conditions has been determined by Northern analysis. The occurrence of these genes in different formae speciales has been analyzed by Southern analysis and PCR. All these genes are expressed during different stages of the interaction with the host plant indicating a possible role in pathogenesis. At present, targeted gene disruption is being carried out, in order to determine the role of each gene in the pathogenicity process

Key words

Pectate lyase, Polygalacturonase, Protease, Xylanase, "Green fluorescent protein", Tomato

Dirección para correspondencia:
Dr. M.I.G. Roncero
Departamento de Genética,
Facultad de Ciencias,
Universidad de Córdoba,
Avda de S. Alberto Magno s/n,
14071 Córdoba, España

1130-1406/99/5.00 Euros

The plant cell wall represents a formidable barrier to microbial invasion that must be overcome by any successful pathogen. The wall provides both an inert mechanical support and a dynamic mosaic with diverse texture and metabolic function. Its main constituents are carbohydrates and different classes of proteins such as extensins [1,2]. Fungal plant pathogens typically secrete an array of cell wall-degrading enzymes (CWDEs) capable of depolymerizing each of these different wall components [3]. CWDEs may serve different purposes for the pathogen during infection, such as penetration and ramification within the host tissue, release of nutrients or interference with the plant defense response. However, most of these enzymes are also produced during the saprophytic life cycle and thus their importance in pathogenicity is uncertain and has been the subject of numerous studies [3].

The soilborne fungus *Fusarium oxysporum* Schlecht., the causal agent of vascular wilt disease, produces economically important losses on a wide variety of crops [4] (Figures 1 and 2). The fungus can survive during extended time periods in the soil in the absence of its host, either in the form of heavily walled chlamydospores or as a saprophyte on organic material. When it encounters an appropriate host plant, the pathogen penetrates the different layers of the root cortex until reaches the vascular system. Once established there, colonization of the plant is rapidly achieved by spreading upward through the xylem vessels, thereby provoking the characteristic wilt symptoms (Figure 3). During the infection process, the fungus encounters diverse structural barriers such as the endodermis, vessel walls, vascular gels and tyloses. It has been claimed that secretion of CWDEs is a major mechanism by which *F. oxysporum* overcomes these barriers [4]. However, in spite of considerable research efforts, the role of these enzymes during pathogenesis remains unclear. We are addressing this question by pursuing the following objectives: 1) Purification and characterization of specific CWDEs (see Table 1), 2) Cloning of CWDE-encoding genes (see Table 2), 3) Study of CWDE expression during plant infection, as compared to saprophytic growth, and 4) Targeted inactivation of specific CWDE genes and determination of the effect on pathogenicity. This approach should allow to answer questions on the role of specific CWDEs in the infection process and to identify general mechanisms that control CWDE expression during pathogenicity.

Table 1. Cell wall-degrading enzymes of *Fusarium oxysporum* f. sp. *lycopersici* purified and characterized in our laboratory.

Name	Enzyme type	MW	pI	pH opt.	Temp.opt.
PG1	Endopolygalacturonase	35,000	7.0	4.0	37°C
PG2	Exopolygalacturonase	74,000	3.5	5.0	55°C
PG3	Exopolygalacturonase	63,000	7.0	4.0-8.0	55°C
PL1	Endo-pectate lyase	25,000	8.7	9.0	42°C
XYL1	Endo-ß-1,4-xylanase	40,000	3.0	4.5	40°C

Table 2. Cell wall-degrading enzyme-encoding genes of *Fusarium oxysporum* f. sp. *lycopersici* cloned in our laboratory

Name	Encoded enzyme type
pg1	Endopolygalacturonase
pgx4	Exopolygalacturonase
pg5	Endopolygalacturonase
pl1	Endo-pectate lyase
xyl2	Endo-xylanase, family F
xyl3	Endo-xylanase, family F
xyl4	Endo-xylanase, family G
prt1	Serine protease

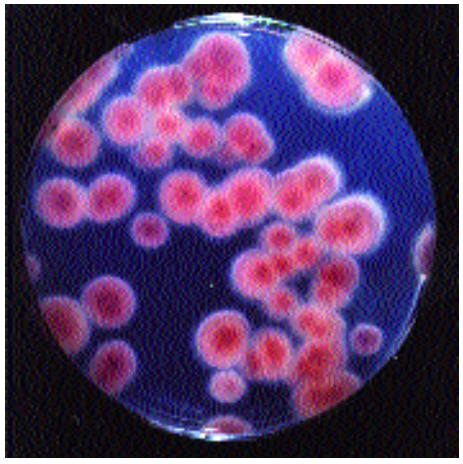

Figure 1. Colonies of *Fusarium oxysporum* growing on minimal medium.

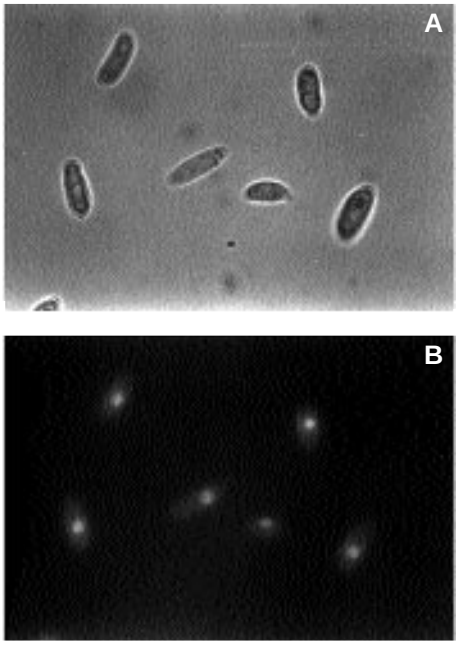

Figure 2. Microscopic analysis of microconidia of *Fusarium oxysporum* **A.** Phase contrast observation **B.** Sample in **A** was stained with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI) and analyzed with epifluorescence to visualize nuclei.

Figure 3. Progressive severity of wilt symptoms in tomato cultivar Moneymaker infected with *Fusarium oxysporum* f. sp. *lycopersici* (reproduced from [38]).

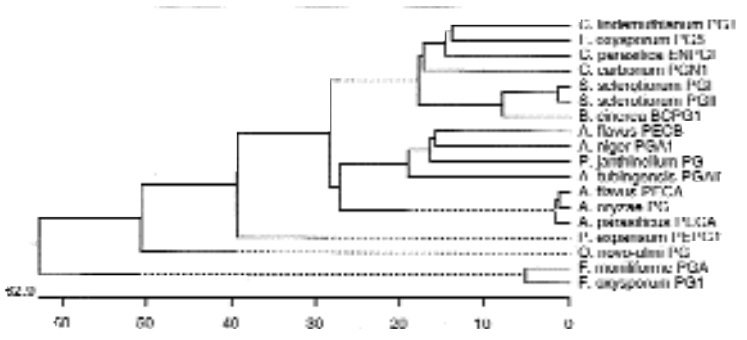

Figure 4. Phylogenetic tree of fungal endopolygalacturonases based on their amino acid sequences. Analysis was performed with the Megalign Lasergene program using the Clustal method. The scale beneath the tree measures the distance between sequences. The Genbank accession numbers of the sequences are as follows: *Colletotrichum lindemuthianum* PG1 (X89370), *Fusarium oxysporum* PG5 (AF078156), *Cryphonectria parasitica* ENPG1 (U49710), *Cochliobolus carbonum* PGN1 (M55979), *Sclerotinia sclerotiorum* PGI (L12013), *S. sclerotiorum* PGII (L29040), *Botrytis cinerea* BCPG1 (U68715), *Aspergillus flavus* PECB (U05020), *A. niger* PGA1 (X58892), *Penicillium janthinellum* PG (D79980), *A. tubingensis* PGAII (X58894), *A. flavus* PECA (U05015), *A. oryzae* PG (D14282), *A parasiticus* PECA (L23523), *P. expansum* PEPG1 (AF047713), *Ophiostoma novo-ulmi* PG (AF052061), *F. moniliforme* PGA (L02239), *F. oxysporum* PG1 (U96456).

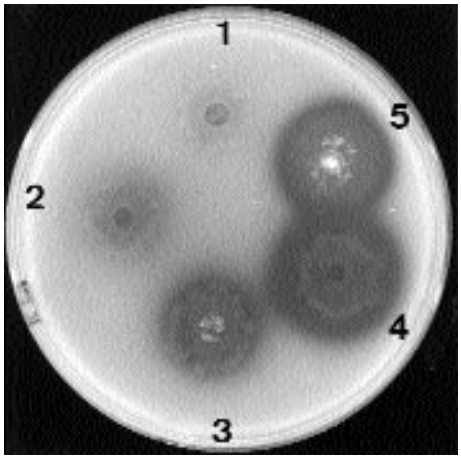

Figure 5. PG1 activity of different *Fusarium oxysporum* strains as determined by the production of clear halos during growth on solid medium containing polygalacturonic acid. The following strains were used: 1, *F. oxysporum* f.sp. *melonis* isolate 18M carrying an inactive allele of the *pg1* gene disrupted by a transposon; 2, transformant CO4 of isolate 18M transformed with an incomplete copy of *pg1*; 3 and 4, transformants CO5 and CO12 of isolate 18M carrying one or multiple functional copies of *pg1*, respectively; 5, *F. oxysporum* f.sp. *lycopersici* isolate 42-87 from which *pg1* was cloned.

Polygalacturonase. Endo- and exopolygalacturonases (endoPGs, exoPGs) have long been proposed to play an important role in fungal pathogenicity to plants by depolymerizing homogalacturonan, a major component of the plant cell wall [3]. In a number of plant pathogenic fungi the role of specific PGs, mainly endoPGs, has been determined by inactivation of the encoding genes, providing contrasting results. No support for an essential role in pathogenicity of the major *in vitro* secreted endoPGs was found in the maize pathogen *Cochliobolus carbonum* and the chestnut blight fungus *Cryphonectria parasitica* [5,6]. In *C. carbonum*, even a double mutant lacking both endo and exoPG was still fully pathogenic [7]. Conversely, endoPG was required for full virulence of *Aspergillus flavus* on cotton bolls and *Botrytis cinerea* on tomato and apple [8,9].

In *F. oxysporum*, a possible involvement of endoPG in development of vascular wilt has been suggested [4]. Nevertheless, information on the number of different PG isoenzymes produced by *F. oxysporum* and their secretion during plant infection was lacking. Using an isoelectric focusing zymogram technique we found that the tomato pathogen *F. oxysporum* f.sp. *lycopersici* produced an array of pectinolytic enzymes during growth on pectic substrates, with isoelectric points (pIs) ranging from 4.0 to 9.0. An endoPG (PG1) and two exoPGs (PG2 and PG3) were purified to apparent homogeneity by preparative isoelectric focusing (Table 1). PG1 was the major endoPG secreted by *F. oxysporum* and hydrolyzed polygalacturonic acid (PGA) simultaneously in an endo- and exomanner, with galacturonic acid as the main final degradation product [10]. PG2 and PG3 acted as typical exoPGs [11,12]. The three PGs were N-glycosylated, as were the other two CWDEs purified so far from *F. oxysporum*. PG1 and PG2 were preferentially induced during growth on pectin whereas PG3 was mainly secreted on PGA.

Different heterologous probes were used to isolate two endoPG genes from a lambda EMBL genomic library of *F. oxysporum* (see Table 2). The gene encoding PG1 was cloned using the endoPG gene of *F. moniliforme* whereas *pg5* was isolated (Garcia Maceira *et al.* unpublished) using the *C. carbonum pgn1* probe. Southern analysis of genomic DNA showed that both endoPG genes were present in single copy in the genome of *F. oxysporum* and did not cross-hybridize with each other. Comparison of the predicted amino acid sequences of *pg1* and *pg5* with the databases confirmed that they were homologous to other fungal endoPGs but belonged to phylogenetically distant subgroups. PG5 is phylogenetically close to endoPGs from other fungal plant pathogens such as *Colletotrichum lindemuthianum*, *C. parasitica* or *C. carbonum*, while PG1, together with *F. moniliforme* PGA, forms a separate subgroup that differs considerably from all the other fungal endoPGs characterized so far (Figure 4).

As in most CWDE encoding genes, expression of *pg1* and *pg5* is strictly regulated by different external stimuli such as carbon source, ambient pH or temperature. Several features make *pg1* particularly useful as a model system for studying the molecular mechanisms regulating CWDE expression. As revealed by Northern analysis, *pg1* mRNA was strongly induced on citrus pectin and during the early growth phase, the PG1 enzyme accounted for almost all the extracelular PG activity [13]. Significantly, *pg1* expression was also induced by tomato vascular tissue, the host tissue preferentially colonized by *F. oxysporum* f.sp. *lycopersici*. It has been suggested previously that activation of CWDE genes functions via generation and uptake of degradation products with a low degree of polymerization, since complex polymers such as pectin cannot enter the fungal cell. In agreement with this hypothesis, we found that the monomer D-galacturonic acid induced *pg1* expression at concentrations up to 0.5%, although at very high concentrations (1%) it acted as a catabolite repressor. Similarly, 1% glucose repressed *pg1* induction by the substrate [13] .

Among 12 *F. oxysporum* isolates belonging to seven different formae speciales, three did not produce significant levels of PG1 activity. Southern hybridization, PCR and Northern analysis revealed that one of these isolates, belonging to forma specialis *melonis*, carried a *pg1* allele that was disrupted by a hAT family transposon [14]

and did not produce a functional *pg1* transcript. When this isolate was transformed with a vector carrying a functional copy of *pg1*, it secreted amounts of the enzyme comparable to the original PG1 producing f.sp. *lycopersici* isolate (Figure 5). Moreover, the introduced gene was regulated in the same way as in the original donor isolate [13] Thus, the deficient isolate provides an excellent system for analysis of the *pg1* promoter. Transformation experiments with *pg1* constructs bearing different promoter deletions is currently carried out in our laboratory to identify 5'-regulatory elements responsible for substrate induction (Zapatero *et al.*, unpublished).

To isolate genes encoding exoPGs, a different approach was followed: degenerate PCR primers were derived from amino acid sequences conserved in the exoPGs from *C. carbonum* and *Aspergillus tubingensis*, the two fungal exoPG genes characterized so far [7]. The primers were used for RT-PCR on mRNA from *F. oxysporum* grown on PGA, leading to the cloning of an exoPG gene. *Pgx4* encodes a 454 amino acid polypeptide with nine potential N-glycosylation sites and a putative 21 amino acid N-terminal signal peptide (García Maceira *et al.*, in press). The deduced protein had 50% identity with the other two fungal exoPGs. *Pgx4* was present in a single copy in different formae speciales of *F. oxysporum* and was expressed during in vitro growth on pectin, PGA and tomato vascular tissue.

Pectate lyase. Pectate lyases (PLs) that catalyze the trans-elimination of pectate, have been shown to be primary virulence factors in bacterial plant pathogens [15]. In fungi, there is contrasting evidence suggesting that PLs may play a role pathogenicity. Polyclonal antibodies against PL of *Fusarium solani* f.sp. *pisi* reduced lesions on pea epicotyl segments but disruption of the *pelA* gene did not affect virulence [16]. Similarly, symptom development by *Colletotrichum gloeosporioides* on avocado fruits was inhibited by anti-PL antibodies but inactivation of the pectin lyase *pnlA* gene did not alter pathogenicity. Thus, the role of PL in plant disease caused by fungi remains to be determined.

In *F. oxysporum*, PLs, have been suggested to be involved in expression of vascular wilt since their application reproduces wilt symptoms in tomato cuttings and their production has been reported in infected host tissue [17]. Using preparative isoelectric focusing, an endo-PL (designated PL1) was purified and characterized from *F. oxysporum* f.sp. *lycopersici* [11]. PL1 had a molecular weight of 25,000 and its N-terminal amino acid sequence was identical to PELA from *F. solani* f.sp. *pisi* [18]. The gene encoding PL1 was cloned using the *pelA* gene as a probe and encodes a 240 amino acid polypeptide with one putative N-glycosylation site and a 15 amino acid N-terminal signal peptide. PL1 showed 89%, 67%, 55% and 56% identity with the products of the *F. solani* f.sp. *pisi* *pelA, pelB, pelC* and *pelD* genes, respectively, but very low homology to other microbial PLs [19]. In contrast to *F. solani* that contains a PL gene family, only a single copy of *pl1* was detected in different formae speciales of *F. oxysporum*, even under low stringency hybridization conditions. The *pl1* transcript was produced during growth on PGA and tomato vascular tissue, but not on pectin or glucose, confirming the results previously obtained in zymogram studies [20] .

Xylanase. Xylan is a heterogeneous carbohydrate composed of β-1,4-D-xylopyranosyl residues and represents a major constituent of plant cell walls. Microbial conversion of xylan to soluble products requires the combined action of several types of enzymes, such as endo-β-1,4-xylanases, β-xylosidases, α-L-arabinofuranosidases and α-glucuronidases [21]. A key group of enzymes are endo-β-1,4-xylanases which are classified into two families, F and G [22]. Members of both families have been identified in a wide variety of bacterial and fungal plant pathogens [3]. Multiple xylanase genes have been cloned and inactivated in two plant pathogenic fungi, *C. carbonum* and *Magnaporthe grisea*, both causing foliar disease in cereals. In both species, the xylanase genes were found to be not essential for pathogenicity, probably due to the presence of additional xylanase genes [23,24]. Nevertheless, a number of studies suggest that xylanases may play an important role in other plant-pathogen interactions [3] .

The presence of endoxylanases in *F. oxysporum* has been described [25], but their production during infection and their role in pathogenicity has not been investigated. We have recently purified an acidic endoxylanase secreted by *F. oxysporum* during growth on larchwood xylan. The enzyme, denominated XYL1, was N-glycosylated and showed typical features of family F xylanases [26]. Screening of the *F. oxysporum* genomic library with the *xylP* gene corresponding to a family F xylanase from *Penicillium chrysogenum* [27] yielded two positive clones, encoding the putative endoxylanases *xyl2* and *xyl3* [28]. While the structure of the *xyl2* product matched that of a typical family F xylanase, XYL3 contained a cellulose-binding domain at the N-terminus, a feature unusual in a fungal xylanase [22]. Both genes showed a high specificity of substrate induction, being expressed during growth on oat spelt xylan and tomato vascular tissue, but not on larchwood xylan, xylose, cellulose or carboxymethyl cellulose. *Xyl2* was widely distributed and structurally conserved among different formae speciales, whereas the restriction fragment hybridizing to *xyl3* was only observed in forma specialis *lycopersici*, although hybridizing fragments of different sizes were also present in other formae speciales [28] .

Besides a number of family F xylanases, *F. oxysporum* also contains at least one family G xylanase [26]. Using the *C. carbonum* family G *xyl1* gene [23], we have recently isolated a gene encoding a *F. oxysporum* family G xylanase which is currently being characterized in our laboratory (Gómez *et al.* unpublished).

Protease. Penetration and colonization of plants by fungal pathogens has traditionally been associated with secretion of different types of carbohydrate-degrading enzymes. Extracellular proteases have received considerably less attention, although it has been claimed that degradation of proteins by pathogens may be a prerequisite for successful colonization of the plant host [3]. Possible targets of these enzymes in the plant include hydroxyproline-rich glycoproteins such as extensins which play a key role in plant cell wall self-assembly and extension [2]. Secretion of proteases may also provide the pathogen with a means to inactivate proteinaceous components of the plant defense response such as chitinases and β-1,3-glucanases. Many plants contain protease inhibitors, further suggesting that these enzymes could be important in pathogenicity [29] .

Proteases have mainly been studied as potential virulence determinants in animal, nematode and insect pathogens or mycoparasites. Although the presence of extracellular proteases has been reported in a number of fungal plant pathogens [3] , their production in planta and their role in pathogenicity have only been studied in a few systems. Targeted disruption of a *C. carbonum* gene encoding the trypsin-like protease ALP1 had no effect on virulence [30]. Recently, a subtilisin-like serine protease from the grass endophyte *Acremonium typhinum* has been

reported to be abundantly produced in infected plants [31].

Production of proteases has been reported previously in the genus *Fusarium* [32]. Our group has isolated a gene, *prt1*, encoding an extracellular serine protease from *F. oxysporum* f.sp. *lycopersici*, based on its chromosomal location close to the pectate lyase *pl1* gene (Huertas unpublished). The deduced product of *prt1* has a primary structure similar to subtilisin-like serine proteases and thus adds to a growing group of subtilisin-family proteases from filamentous fungi. A number of these enzymes, such as Pr1 from the entomopathogen *Metarrhizum anisopliae* or At1 from the endophyte *A. typhinum* have been hypothesized to play a significant role in the interaction of these fungi with their hosts [31, 33]. Southern analysis indicated that *prt1* was widely distributed among different formae speciales of *F. oxysporum*. The expression pattern of *prt1* differed somewhat from that of the other *Fusarium* CWDEs which tend to be strictly substrate-induced and carbon catabolite-repressed. *Prt1* expression was constitutive at basal levels during growth of *F. oxysporum* on a variety of substrates, including either collagen or glucose alone, but strongly induced on collagen plus glucose. This suggests that *prt1* induction in *F. oxysporum* is probably not conditioned by nutrient depletion as in other fungi but rather by an appropriate supply of carbohydrates, as reported for *C. carbonum* [30] .

Expression of CWDEs during the fungus-plant interaction. Previous studies on CWDEs in fungal and bacterial pathogens have shown that the major CWDE-isoforms secreted in culture may not at all be produced in planta, or that new sets of CWDE-isoforms can be induced exclusively during growth in the host tissue [5,34]. Thus, while experiments on production of CWDEs by *F. oxysporum* in culture media has provided extremely useful information on their regulatory mechanisms, it was important to confirm their expression during infection of the host plant. As mentioned above, many of the CWDEs were produced during in vitro growth on tomato vascular tissue, indicating that the plant tissue preferentially colonized by *F. oxysporum* f.sp. *lycopersici* does indeed induce CWDE expression. However, plant compounds may also exert the opposite effect: when *F. oxysporum* f.sp. *lycopersici* was grown on pectin, normally a strong inducer of *pg1* expression, in presence of tissue from the non-host plant melon, *pg1* induction was strongly reduced even though mycelial growth was not affected (Figure 6) [35]. Although the underlying mechanisms remain to be elucidated, these results indicate that plant compounds can have a profound modulating effects on CWDE production in *F. oxysporum*.

Studying CWDE expression during the fungus-plant interaction required moving from in vitro culture systems to in vivo plant infection assays. Extracts from roots and stems of tomato plants inoculated with *F. oxysporum* f.sp. *lycopersici* were analyzed by isoelectric focusing zymograms to identify pectinolytic isozymes secreted by the pathogen in planta. Activity bands corresponding to PL1 and PG1 were readily detected in roots and stems, respectively, suggesting that these two CWDEs are secreted by *F. oxysporum* during the infection process [10]. Nevertheless, due to the limited sensitivity of the zymograms, detection of other CWDEs such as exoPGs, xylanases or proteases was not feasible. Therefore a highly sensitive method, reverse transcriptase-mediated (RT)-PCR with specific primers (Figure 7), was used to detect expression of the cloned CWDE genes in roots and stems of infected tomato plants, at different time points after inoculation. Results summarized in Figure 8 show that

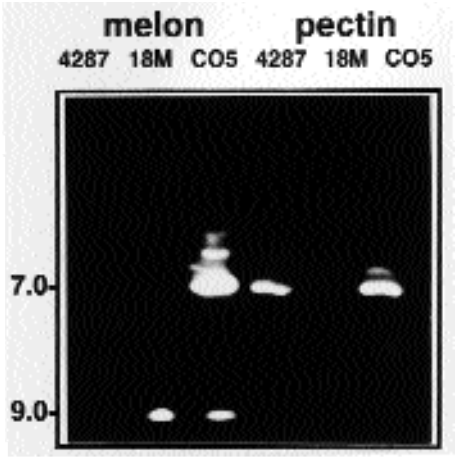

Figure 6. Absence of PG1 production by *Fusarium oxysporum* f.sp. *lycopersici* but not f.sp. *melonis* during saprophytic growth on melon plants. Culture filtrates of strains 42-87 (lycopersici; see Figure 5), 18M and CO5 (melonis; see Figure 5) grown in liquid synthetic medium with pectin or autoclaved melon plants as the sole carbon source were separated by isoelectric focusing and stained for pectinolytic activity. Activity bands with pI 7.0 and 9.0 correspond to PG1 and PL1, respectively.

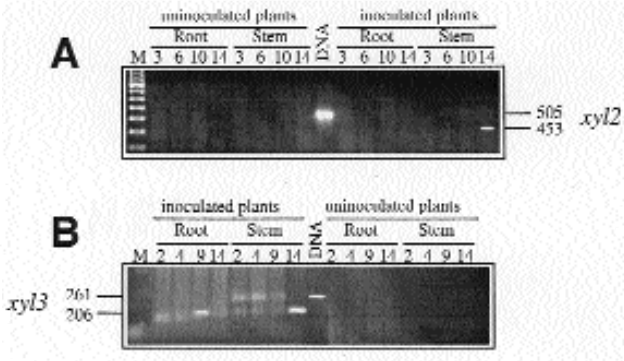

Figure 7. RT-PCR products showing the expression pattern of *xyl2* (A) and *xyl3* (B) during infection of tomato plants by *F. oxysporum* f.sp. *lycopersici*. First-strand cDNAs generated from total RNA isolated at the indicated time points (days after inoculation) from roots and stems of infected or uninfected plants were used as templates for PCR with gene-specific primers. Aliquots of the PCR products were run on a 2% agarose gel with a 100 bp ladder marker. DNA control refers to PCR with genomic DNA as the template.

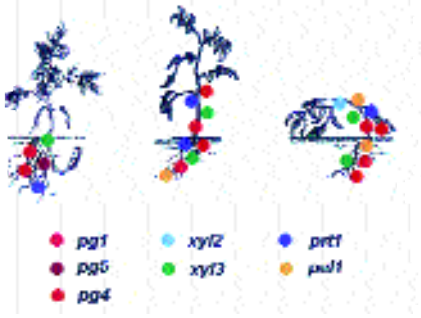

Figure 8. Expression pattern of different CWDE-encoding genes of *Fusarium oxysporum* f.sp. *lycopersici* during development of vascular wilt disease on tomato plants, as determined by RT-PCR with gene-specific primers. Colors referring to specific genes are shown in the legend.

CWDE genes are differentially expressed during the infection process. Most CWDEs (endoPG1, exoPG4, PL1, XYL3 and PRT1) are produced from the early infection stages involving penetration of the root throughout the entire disease cycle, i.e. colonization of the vascular bundles to expression of the characteristic wilt symptoms. Conversely, endoPG5 and XYL2 are secreted at specific infection stages: PG5 during initial penetration of the root and XYL2 during the final phase of disease involving saprophytic growth of *F. oxysporum* on the moribund plant tissue.

Thus, although some CWDEs may perform specific tasks at defined infection stages, most of them are active during the entire disease process. In culture conditions, regulation of CWDE expression tends to be strictly controlled, i.e. is only induced in presence of the substrate and repressed by readily available carbon sources such as glucose. Thus, the fact that CWDEs are expressed during infection strongly suggests that they must somehow contribute to pathogenicity. Unfortunately, the quantitative contribution of each CWDE to pathogenicity cannot be determined by this method, since RT-PCR only detects whether or not a particular gene is transcribed but does not provide quantitative information on gene expression. Determining the exact temporal and spatial pattern of CWDE gene expression in a quantitative manner requires the use of reporter genes such as the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. GFP has been successfully used for observing gene expression in the fungal plant pathogens *Ustilago maydis* which attacks the aerial parts of the plant [36] but not, to our knowledge, in root pathogens. To test the utility of GFP in *F. oxysporum*, we fused the *pg1* promoter sequence to the GFP gene and transformed F. oxysporum f.sp. lycopersici with the construct containing a marker for hygromycin resistance. Preliminary analyses indicate that GFP expression in the transformants under different culture conditions reflects that of the resident *pg1* gene, i.e. strong induction in pectin and repression by glucose (Figure 9) (Méglecz *et al.*, unpublished). Once the feasibility of this approach in *F. oxysporum* has been confirmed, the application of GFP fusions will be extended to studies in planta and to CWDE genes other than *pg1*. This should allow to establish where, when and to what extent the different CWDEs are expressed during pathogenesis.

Role of CWDEs in pathogenicity. Ultimately, the most convincing method yet available to test the importance of a single CWDE in pathogenicity is to construct a mutant specifically lacking the encoding gene [3]. The strategy most frequently used in filamentous fungi consists in replacing the wild type allele, via transformation and a double homologous recombination event, with a modified version of the gene where the entire coding region or part of it has been replaced by a marker gene. Depending on the frequency of homologous integration, which is species-specific, a proportion of the obtained transformants will have an inactive gene copy and thus lack the corresponding CWDE activity. Testing the mutant for altered virulence will then allow to assess the importance of that particular CWDE in pathogenicity. The only major drawback of this approach is that, in the frequent case where a pathogen has multiple genes coding for the same type of CWDE, loss of one CWDE may be compensated by another one with similar characteristics. Thus, even if no change in pathogenicity is observed in the mutants, a contribution of that particular CWDE to pathogenicity in the wild type strain cannot be excluded [3].

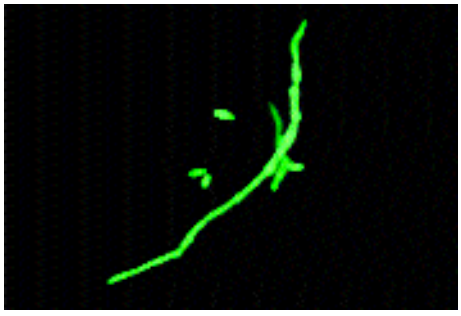

Figure 9. Expression of green fluorescent protein (GFP) in *Fusarium oxysporum*. A transformant carrying the GFP gene under the control of the *pg1* promoter was grown on pectin (inducing conditions) and analyzed microscopically by epifluorescence, using a filter set for fluorescein isothiocyanate. Green fluorescence was not observed under non-inducing conditions (glucose) or in an untransformed isolate (results not shown).

The marker most frequently used for transformation of plant pathogenic fungi is the *hph* gene conferring resistance to the antibiotic hygromycin. In *F. oxysporum*, a transformation system for hygromycin resistance has been established [37]. However, targeted gene inactivation in this species has not been reported so far, although it has been achieved in *F. solani* [16]. We are currently trying to establish this technique in order to perform targeted inactivation of the different CWDE genes cloned in our laboratory. So far we have not been successful since the obtained transformants consistently showed ectopic integration of the constructs [13]. As this technical question will be solved, new insights should be obtained on the long-debated role of CWDEs in vascular wilt disease.

Bibliografía

1. Carpita NC, Gibeaut DM. Structural models of primary cell walls in flowerng plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 1993; 3:1-30.
2. Showalter AM. Structure and function of plant cell wall proteins. Plant Cell 1993; 5:9-23.
3. Walton JD. Deconstructing the cell wall. Plant Physiol 1994; 104:1113-1118.
4. Beckman CH. The nature of wilt diseases of plants. Minnesota, APS Press, 1987.
5. Gao S, Choi GH, Shain L, Nuss DL. Cloning and targeted disruption of *enpg-1*, encoding the major in vitro extracellular endopolygalacturonase of the chestnut blight fungus, *Cryphonectria parasitica*. Appl Environ Microbiol 1996; 62:1984-1990.
6. Scott-Craig JS, Panaccione D, Cervone F, Walton JD. Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell 1990; 2:1191-1200.
7. Scott-Craig JS, Cheng Y-Q, Cervone F, De Lorenzo G, Pitkin JW, Walton JD. Targeted mutants of *Cochliobolus carbonum* lacking the two major extracellular polygalacturonases. Appl Environ Microbiol 1998; 64:1497-1503.
8. Shieh M, Brown RL, Whitehead MP, *et al.* Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl Environ Microbiol 1997; 63:3548-3552.
9. ten Have A, Mulder W, Visser J, van Kan JAL. The endopolygalacturonase gene *Bcpg*1 is required for full virulence of *Botrytis cinerea*. Mol Plant-Microbe Interact 1998; 11:1009-1016.
10. Di Pietro A, Roncero MIG. Endopolygalacturonase from *Fusarium oxysporum* f.sp. *lycopersici*: purification, characterization, and production during infection of tomato plants. Phytopathology 1996; 86:1324-1330.
11. Di Pietro A, Roncero MIG. Purification and characterization of an exo-polygalacturonase from the tomato vascular wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici*. FEMS Microbiol Lett 1996; 145:295-299.
12. Garcia-Maceira FI, Di Pietro A, Roncero MIG. Purification and characterization of a novel exopolygalacturonase from the vascular wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici*. FEMS Microbiol Lett 1997; 154:37-43.
13. Di Pietro A, Roncero MIG. Cloning, expression, and role in pathogenicity of *pg1* encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. Mol Plant-Microbe Interact 1998; 11:91-98.
14. Di Pietro A, García-Macéira FI, Huertas-González MD, *et al.* Endopolygalacturonase PG1 in different formae speciales of *Fusarium oxysporum*. Appl Environ Microbiol 1998; 64:1967-1971.
15. Barras F, Gijsegem FV, Chatterjee AK. Extracellular enzymes and pathogenesis of soft-rot Erwinia. Annu Rev Phytopathol 1994; 32:201-234.
16. Guo W, González-Candelas L, Kolattukudy PE. Identification of a novel *pelD* gene expressed uniquely in planta by *Fusarium solani* f.sp. *pisi* (*Nectria haematococca*, mating type VI) and characterization of its product as an endo-pectate lyase. Arch Biochem Biophys 1996; 332:305-312.
17. Cooper RM, Rankin B. Cell wall-degrading enzymes of vascular wilt fungi. II. Properties and modes of action of polysaccharidases of *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici*. Physiol Plant Pathol 1978; 13:101-134.
18. González-Candelas L, Kolattukudy PE. Isolation and analysis of a novel inducible pectate lyase gene from the phytopathogenic fungus *Fusarium solani* f.sp. *pisi* (*Nectria haematococca*, mating population VI). J Bacteriol 1992; 174:6343-6349.
19. Huertas-González MD, Ruiz-Roldán MC, García-Maceira FI, Roncero MIG, Di Pietro A. Cloning and characterization of *pl1* encoding an in planta secreted pectate lyase of *Fusarium oxysporum*. Curr Genet 1999; 35: 36-40.
20. Di Pietro A, Roncero MIG. Purification and characterization of a pectate lyase from *Fusarium oxysporum* f.sp. *lycopersici* produced on tomato vascular tissue. Physiol Mol Plant Pathol 1996; 49:177-185.
21. Biely P. Microbial xylanolytic systems. Trends Biotechnol 1985; 3:286-290.
22. Gilkes NR, Henrissat B, Kilburn DG, Miller Jr RC, Warren RAJ. Domains in microbial β-1,4-glycanases: sequence conservation, function, and enzyme families. Microbiol Rev 1991; 55:303-315.
23. Apel-Birkhold PC, Walton JD. Cloning, disruption, and expression of two endo-ß-1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl Environ Microbiol 1996; 62:4129-4135.
24. Wu S-C, Kyung-Sik H, Darvill AG, Albersheim P. Deletion of two endo-ß-1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. Mol Plant-Microbe Interact 1997; 10:700-708.
25. Christakopoulos P, Nerinckx W, Samyn B, *et al.* Functional characterization of a cellulose binding xylanase from *Fusarium oxysporum*. Biotechnol Lett 1996; 18:349-354.
26. Ruiz MC, Di Pietro A, Roncero MIG. Purification and characterization of an acidic endo-ß-1,4-xylanase from the tomato vascular pathogen *Fusarium oxysporum* f.sp. *lycopersici*. FEMS Microbiol Lett 1997; 148:75-82.
27. Haas H, Friedlin E, Stöffler G, Redl B. Cloning and structural organization of a xylanase-encoding gene from *Penicillium chrysogenum*. Gene 1993; 126:237-242.
28. Ruiz-Roldán MC, Huertas-González MD, Di Pietro A, Roncero MIG. Two xylanase genes of the vascular wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici* differentially expressed during infection of tomato plants. Mol Gen Genet 1999; 261: 530-536.
29. Ryan CA. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Phytopathol 1990; 28:425-429.
30. Murphy JM, Walton JD. Three extracellular proteases from *Cochliobolus carbonum*: cloning and targeted disruption of *ALP1*. Mol Plant-Microbe Interact 1996; 9:290-297.
31. Reddy PV, Lam CK, Belanger FC. Mutualistic fungal endophytes express a proteinase that is homologous to proteases suspected to be important in fungal pathogenicity. Plant Physiol 1996; 111:1209-1218.
32. Urbanek H, Yirdaw G. Acid proteases produced by *Fusarium* species in cultures and in infected seedlings. Physiol Plant Pathol 1978; 13:81-87.
33. St. Leger RJ, Frank DC, Roberts DW, Staples RC. Molecular cloning and regulatory analysis of the cuticle-degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. Eur J Biochem 1992; 204: 991-1001.
34. Kelemu S, Collmer A. *Erwinia chrysanthemi* EC16 produces a second set of plant-inducible pectate lyase isozymes. Appl Environ Microbiol 1993; 59:1756-1761.
35. Di Pietro A, García FI, Jiménez A, Ruiz MC, Huertas MD, Roncero MIG. Role of polygalacturonase in the saprophytic and parasitic phase of *Fusarium oxysporum*. 4th European Conference on Fungal Genetics, Leon 1998; p. 237 (Abstract).
36. Spellig T, Bottin A, Kahmann R. Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. Mol Gen Genet 1996; 252:503-509.
37. Kistler HC, Benny UK. Genetic transformation of the fungal plant wilt pathogen, *Fusarium oxysporum*. Curr Genet 1988; 13:145-149.
38. Huertas-González MD, Ruiz-Roldán MC, Di Pietro A, Roncero MIG, Cross protection provides evidence for race-specific avirulence factors in *Fusarium oxysporum*. Physiol Mol Plant Pathol 1999; 54: 63-72.