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


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

Fusarium oxysporum es capaz de colonizar a sus plantas huéspedes penetrando a través de las raíces e invadiendo su sistema vascular. Esta especie produce una 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 proteínas (extensinas). Nuestro grupo ha purificado y caracterizado bioquímicamente varias enzimas líticas, una endopoligalacturonasa mayoritaria (PG1), dos exopoligalacturonasas (PG2 y PG3), una endoxilana nasa (XYL1), y una endopectato-lyasa (PL1). Recientemente hemos aislado los genes responsables de algunas de estas enzimas hidrolíticas: pg1, pgx4, pg5, xyI2, xyI3, prP1 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 especiales de F. oxysporum mediante análisis Southern y PCR. Todos los genes estudiados se expresan "in planta" en distintos estados 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 homologa. 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

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 endopoligalacturonase (PG1), two exopoligalacturonases (PG2 and PG3), an endoxylanase (XYL1) and an endo pectate-lyase (PL1). We have isolated the following CWDE-encoding genes: pg1, pgx4, pg5, xyI2, xyI3, prP1 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.

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

©1999 Revista Iberoamericana de Micología
Aptdo. 699, E-48080 Bilbao (Spain).
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.

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

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Encoded enzyme type</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg1</td>
<td>Endopolygalacturonase</td>
</tr>
<tr>
<td>pgx4</td>
<td>Exopolygalacturonase</td>
</tr>
<tr>
<td>pg5</td>
<td>Endopolygalacturonase</td>
</tr>
<tr>
<td>pl1</td>
<td>Endo-pectate lyase</td>
</tr>
<tr>
<td>xyl2</td>
<td>Endo-xylanase, family F</td>
</tr>
<tr>
<td>xyl3</td>
<td>Endo-xylanase, family F</td>
</tr>
<tr>
<td>xyl4</td>
<td>Endo-xylanase, family G</td>
</tr>
<tr>
<td>prt1</td>
<td>Serine protease</td>
</tr>
</tbody>
</table>

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]).
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 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* pg1 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* PG, 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 extracellular 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 pg/l transcript. When this isolate was transformed with a vector carrying a functional copy of pg/l, it secreted amounts of the enzyme comparable to the original PG1 producing f.spp. 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 pg/l promoter. Transformation experiments with pg/l constructs bearing different promoter deletions is currently carried out in our laboratory to identify S-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.spp. 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 plnA 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.spp. 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.spp. 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.spp. 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. carbo- num 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 xyl 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 conside-

rably 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. carbo-num 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
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 *Metarrhizium 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 specialae 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-isozymes secreted in culture may not at all be produced in planta, or that new sets of CWDE-isozymes 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 *pl1* 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 and stained for pectinolytic activity. Activity bands corresponding to PL1 and PG1, respectively, were readily detected in roots and stems, by the pathogen in planta. Activity bands with pI 7.0 and 9.0 correspond to PG1 and PL1, respectively.

![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.](image)

![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.](image)

![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.](image)
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 a transformation system for hygromycin resistance. Preliminary analyses indicate that GFP expression in the transformants under different culture conditions reflects that of the resident *pgl* 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 *pgl*. 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].

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.
Bibliografia


31. Reddy PV, Lam CK, Belanger FC.Mutualistic fungal endophytes express a protease that is homologous to proteases suspected to be important in fungal pathogenicity. Plant Physiol 1996; 111:1209-1218.


