European Journal of Plant Pathology **103:** 1–14, 1997.

Mini review

Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi

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Accepted 6 September 1996

Key words: cellulase, cutinase, pathogenicity, pectinase, xylanase

Abbreviations: CWDE – cell wall-degrading enzymes; PG – polygalacturonase; PL – pectate lyase; PNL – pectin lyase; PME – pectin methylesterase; RHG – rhamnogalacturonase.

Introduction

Both saprophytic and plant parasitic fungi produce extracellular enzymes which can degrade the cell wall components of plants. These fungi not only digest plant cell wall polymers to obtain an important nutrient source but also degrade the cell wall to aid in penetrating cells and spreading through plant tissue. DeBary (1886) was the first to suggest that extracellular enzymes may be involved in the infection process of plant pathogenic fungi. Since then, much research has been focused on trying to determine the role and importance of extracellular cell wall-degrading enzymes (CWDE) to the virulence of plant pathogenic fungi. Traditionally this has been done by purifying and characterizing CWDE and examining the effect of purified or partially purified enzymes on plant cells. Despite considerable progress, this has not resulted in any definitive conclusions on the importance of CWDE to plant pathogenic fungi. However, in recent years, a new approach using recombinant DNA techniques has been employed to try to provide more conclusive evidence concerning the role of CWDE in plant pathogenesis. This review will examine recent developments in the molecular biology of CWDE of plant pathogenic fungi.

Of the numerous CWDE produced by plant pathogenic fungi, most research has concentrated on the pectin degrading enzymes. This is because the pectinases are typically produced first, in the largest amounts, and are the only CWDE capable of macerating plant tissue and killing plant cells on their own (Cooper, 1983). The pectin matrix of plants is found throughout the primary cell wall but is most concentrated in the middle lamella between cells (Carpita and Gibeaut, 1993). The pectin matrix is thought to stabilize cellulose microfibrils, other neutral sugar polymers and proteins in the primary cell wall (Carpita and Gibeaut, 1993). The pectin matrix consists of homogalacturonan and rhamnogalacturonan with various degrees of methyl esterification of the carboxyl group of the galacturonate residues (McNeil et al., 1984). Both polymers have side chains of arabinans, xylans and/or arabinogalactans (McNeil et al., 1984).

Fungi produce different types of pectinases that are classified by their substrates, type of lysis and mode of action on the pectin polymer. Unesterified pectate polymers can be degraded by polygalacturonase (PG), which uses hydrolytic cleavage, and pectate lyase (PL), which uses β-elimination cleavage and the formation of a double bond in one of the resulting galacturonate residues (Rexová-Benková and Markovič, 1976). Esterified pectin polymers are attacked by pectin lyase (PNL) or polymethylgalacturonase (Rexová-Benková and Markovič, 1976). Rhamnogalacturonase (RHG) cleaves the bond between the alternating galactose and rhamnose residues in rhamnogalacturonan (Suykerbuyk et al., 1995). Two types of pectinases have been differentiated by their cleavage pattern: an endo form

that cleaves internal regions of pectin chains randomly and an exo form that removes terminal residues (Cooper, 1983). Pectin methylesterase (PME), which is also produced by fungi, removes the methyl group from esterified galacturonic acid residues in pectin chains and thereby decreases the steric hinderance to other pectinases allowing them to attack the backbone of the pectin chain (Keon et al., 1987; Rexová-Benková and Markovič, 1976). Many fungi have been found to produce only one type of each pectinase (González-Candelas and Kolattukudy, 1992; Scott-Craig et al., 1990), however for each type of pectinase, as well as many other CWDE, most fungi appear to produce multiple isozymes that differ in isoelectric point, molecular weight and often in their regulation (Keon et al., 1987). This multiplicity of isozymes may give flexibility to a pathogen, with each enzyme having its own unique properties that contribute to the performance of all of the enzymes (Keon et al., 1987).

Other fungal CWDE may also be important in the breakdown of plant cell walls and colonization of plant tissue (Carpita and Gibeaut, 1993; Cooper, 1983). Carpita and Gibeaut (1993) have proposed that xyloglucans are interlaced with cellulose microfibrils and act as a network providing tensile strength to the cell wall. Breakdown of the xyloglucan spanning the space between cellulose microfibrils by fungal cellulases and xylosidases could weaken the wall and provide increased access for fungal cellulases to degrade the cellulose microfibrils (Carpita and Gibeaut, 1993). Cellulose microfibrils are considered to provide the structure and support for all the other components of plant cell walls (Carpita and Gibeaut, 1993), and their degradation by fungal cellulases may contribute to the weakening of the cell wall (Cooper, 1983). Enzymes involved in cellulose degradation include endo-1,4-β-glucanase which cleaves internal bonds, and cellobiohydrolase which cleaves the disaccharide cellobiose from the end of the polymer (Sheppard et al., 1994).

Polysaccharides also provide inter-connections between the main components of the cell wall (Carpita and Gibeaut, 1993). Xylans in dicot cell walls provide interconnections between rhamnogalacturonan and other cell wall components (Keon et al., 1987; McNeil et al., 1984). Arabinogalactans and arabinans are probably attached to many of the rhamnosyl residues on rhamnogalacturonan and therefore have a role in determining the spacing between pectin polymers and limiting the size of pores in the pectin matrix (Carpita and Gibeaut, 1993). Fungal arabinases, arabinogalactanases, xylanases, and glycosidases may act in concert to increase access to the main polymers by degrading their respective substrates.

Many polymers, such as xyloglucans and rhamnogalacturonan, have side chains consisting of single or short chains of xylose, fructose, galactose and other saccharides (McNeil et al., 1984). Fungal glycosidases such as β-galactosidase, xylosidase and arabinosidase may release sugar moieties that can be used as a nutritional source for the fungus during its growth through plant tissue. Glycosidases also may have a role in decreasing the steric hinderance for other CWDE by removing protruding side chains from the backbone of polymers and providing increased access for endo forms of CWDE (Keon et al., 1987).

The outer surface of the plant epidermis and the substomatal cavity are covered with a cuticle layer composed of wax and cutin (Kolattukudy, 1992; Kolattukudy et al., 1995). The major structural component of the cuticle is cutin, which is a polymer of C_{16} and C_{18} fatty acids. Cutinases, along with mechanical force, have been proposed to be important in the penetration of intact plant surfaces by the infection peg of phytopathogenic fungi (Kolattukudy, 1992; Kolattukudy et al., 1995).

Genes encoding fungal pectinases

Genes encoding PGs, PNLs and PLs have been isolated from fungi, bacteria and plants. Commercial preparations of pectinases produced by saprophytic fungi are used in the food industry and the PG, PNL and PL genes produced by some industrially important fungi have been studied (Bussink et al., 1992; Dean and Timberlake 1989; Kusters-van Someren et al., 1992). Pectinases also have been implicated in the development of many diseases caused by phytopathogenic fungi, and a few of these fungi have been examined for the involvement of PG, PNL and PL genes in disease development (e.g. Bowen et al., 1995; Gao et al., 1996; Guo et al., 1995a, 1995b; Scott-Craig et al., 1990). Pectinases also are involved in tissue maceration during infection of plants by soft rot bacteria and other phytopathogenic bacteria, and PG, PNL and PL have been studied as possible pathogenicity or virulence factors for these bacteria (Barras et al., 1994; Collmer and Keen, 1986; Kotoujansky, 1987). In plants, pectinases have been postulated to be involved in a variety of functions, and PG genes have been cloned that are highly expressed during pollen tube growth, leaf and flower abscission,

and fruit ripening (Allen and Lonsdale, 1993; Bird et al., 1988; Kalaitzis et al., 1995; Robert et al., 1993).

Several PG genes have been cloned from saprophytic and phytopathogenic fungi (Table 1). All of the fungal PG genes cloned thus far are between 1100 and 1350 bp long and most of the genes have one to four introns of 50 to 81 bp with at least one intron approximately 55 bp in length, except for a *Sclerotinia sclerotiorum* gene which has no introns (Fraissinet-Tachet et al., 1995). Comparisons of DNA sequences of PG genes between different fungal species have not revealed significant homology within the coding regions except for some of the genes of the *Aspergillus* species. An *A. flavus* PG gene was reported to be 99% homologous to the gene from *A. oryzae*, and the *PGII* gene from *A. tubigensis* was 90% similar to *PGII* from *A. niger* and was identical to a PG gene cloned from *A. niger* by Ruttkowski et al. (1991) (Bussink et al., 1990, 1991a; Whitehead et al., 1995). The conservation of the intron positions of the PG genes of *A. niger*, *A. flavus* and *A. parasticus* indicates that these genes may have a common ancestor (Whitehead et al., 1995). The *A. niger*, *A. oryzae*, *A. tubigenesis*, *S. sclerotiorum*, and *Fusarium moniliforme* PG genes have similar putative TATA boxes, CAAT sequences and pyrimidine-rich regions upstream from the transcription start site and similar polyadenylation and transcription termination sites downstream from the coding region (Bussink et al., 1992; Caprari et al., 1993a; Frassinet-Tachet et al., 1995; Kitamoto et al., 1993; Reymond et al., 1994).

At the amino acid level, fungal PG genes are approximately 60 to 65% similar to each other, except for the *F. moniliforme* gene which is approximately 40% similar to the *S. sclerotiorum pg1* and the *Colletotrichum lindemuthianum Clpg1* genes, and the fungal PG genes are approximately only 20% similar to PG genes from bacteria and plants (Bussink et al., 1991a, 1992; Centis et al., 1996; Kitamoto et al., 1993; Reymond et al., 1994). The sequence of the N-terminal end is very similar for the predicted peptides of the PG genes from *A. niger*, *A. tubigensis*, *A. oryzae*, and *Cochliobolus carbonum*, and the sequenced N-terminal peptides of *C. lindemuthianum* and *S. sclerotiorum* (Bussink et al., 1992; Keon and Waksman, 1990). A region of approximately 80 residues in PGs from fungi, bacteria and plants has many highly conserved amino acids and may contain the active site and/or be involved in binding of the substrate (Bussink et al., 1991a; Caprari et al., 1993a; Kitamoto et al., 1993; Reymond et al., 1994). Biochemical studies have found a histidine residue and a carboxylate group are essential to PG activity (Rexová-Benková and Mracková, 1978) and a histidine residue and some of the serine and aspartic acid residues flanking it are highly conserved in the amino acid region common to all PGs (Bussink et al., 1991a; Caprari et al., 1993a; Kitamoto et al., 1993). Based on the DNA sequences, all of the PG proteins have a signal peptide 20 to 40 amino acids long, mature proteins of 360 to 380 amino acids, and calculated sizes between 33 and 38 kDa. However, because so many of the fungal PGs that have been cloned thus far are from *Aspergillus* species, the range of variation in PG genes may be underestimated.

One RHG gene has been cloned from *A. aculeatus* (Table 1). It is 1320 bp long with three introns of 64, 64 and 66 bp, and has a signal peptide (Suykerbuyk et al., 1995). The predicted gene product is 46 kDa, and there is only 10.3% amino acid homology with any of the cloned PG genes of *A. niger*. However, there is amino acid homology in the regions predicted to be important for PG activity.

Genes encoding PNL and PL have been cloned from several fungi (Table 1). Three genes for PNL have been cloned from *A. niger*, but the presence of six PNL genes was suggested by DNA hybridization studies (Harmsen et al., 1990). The three cloned *A. niger* PNL genes have little homology in their promoters, have four introns of 56 to 65 bp, two of which are shared among the three genes, and have 70% DNA homology in their coding regions (Kusters-van Someren et al., 1991, 1992). The PNL genes from *A. niger* are 60 to 65% similar in their deduced amino acid sequences, but have only small regions of similarity to PNL cloned from bacteria (Gysler et al., 1990; Kusters-van Someren et al., 1991, 1992). *Glomerella cingulata* has at least four pectinase genes in total; three of these are PNL genes, one of which has been cloned, and the fourth is a PL gene (Templeton et al., 1994). The cloned PNL gene from *G. cingulata* has six introns which are in different locations than those of *A. niger*. It has 55 to 62% amino acid homology to the PNL genes from *A. niger* and 21 to 26% homology to the PNL genes from *Erwinia* spp. and tobacco. The PL gene of *G. cingulata* has homology to a PL gene from tobacco and the *pelA* gene from *Erwinia carotovora* (Templeton et al., 1994). Three PL genes have been cloned from *N. haematococca*; the *pelA* gene has 65% amino acid identity with the *pelB* gene and 51% amino acid identity with the *pelC* gene (González-Candelas and Kolattukudy, 1992; Guo et al., 1995a, 1995b). These genes have very low homology to pectinases

Table 1. Genes encoding pectinases cloned from filamentous fungi

Substrate	Enzyme	Gene(s)[1]	Fungus	Reference(s)
Pectin/pectate	Polygalacturonase	*pg1, PG2, PG3*	*Sclerotinia sclerotiorum*	Fraissinet-Tachet et al., 1995; Reymond et al., 1994
		PGN1	*Cochliobolus carbonum*	Scott-Craig et al., 1990
		PG[2]	*Fusarium moniliforme*	Caprari et al., 1993a
		Clpg1	*Colletotrichum lindemuthianum*	Centis et al., 1996
		enpg1	*Cryphonectria parasitica*	Gao et al., 1996
		pgaI, pgaII, pgC, PG[2]	*Aspergillus niger*	Bussink, et al., 1990, 1991a, 1991b, 1992; Ruttkowski et al.,1991
		pecA, pecB	*Aspergillus flavus*	Whitehead et al., 1995
		PG[2]	*Aspergillus oryzae*	Kitamoto et al., 1993
		pecA	*Aspergillus parasiticus*	Cary et al., 1995
		pgaII	*Aspergillus tubigensis*	Bussink et al., 1991a
	Rhamnogalacturonase	*rhgA*	*Aspergillus aculeatus*	Suykerbuyk et al., 1995
	Pectin lyase	*pnlA, pnlB*[3], *pnlC*[3]	*Glomerella cingulata*	Templeton et al., 1994
		pelA, pelB, pnlD	*Aspergillus niger*	Harmsen et al., 1990; Gysler et al., 1990; Kusters-van Someren et al., 1991, 1992
	Pectate lyase	*pelA*[3]	*Glomerella cingulata*	Templeton et al., 1994
		pelA, pelB, pelC	*Nectria haematococca*	González-Candelas and Kolattukudy, 1992; Guo et al., 1995a, 1995b
		pelA	*Aspergillus nidulans*	Dean and Timberlake, 1989; Ho et al., 1995
	Pectin methylesterase	*pmeA*	*Aspergillus niger*	Khanh et al., 1991

[1] Names of genes cloned.
[2] No name has been designated for the cloned polygalacturonase (PG) gene.
[3] Portion of gene cloned using the polymerase chain reaction.

of other fungi, and the highest homology is only 20 to 25% between the *pelA* gene of *N. haematococca* and the PNL genes of *A. niger* (González-Candelas and Kolattukudy, 1992). The location of two introns in the *pelA* and *pelB* genes are conserved indicating that they may have both been derived from the same gene (Guo et al., 1995b). The *pelA* and *pelB* genes are also similar in that they lack the three highly conserved consensus sequences found in pectate lyases, and these two genes may form a new group of PL genes (Guo et al., 1995b). *A. nidulans* has one gene for PL that has two short introns and less than 30% amino acid similarity to other PL genes (Dean and Timberlake, 1989; Ho et al., 1995). It is more closely related to PL genes of plants than those of fungi.

PME activity is produced by many saprophytic and plant pathogenic fungi (Cooper, 1983) and one gene encoding this enzyme was cloned from *A. niger* (Table 1). The *A. niger* PME gene has 6 introns of 50 to 60 bp, and translation initiation sites similar to those found for other fungal genes (Khanh et al., 1991). The gene encodes a protein that is 314 amino acids and has 25 to 30% homology to tomato and *E. chrysanthemi* PMEs.

Studies with antibodies specific for pectinase isozymes reveal some pectinase proteins have some similarity in their tertiary structure, even though their primary structure is not homologous. Polyclonal antibodies raised to purified *A. niger* proteins encoded by the *PGI* and *PGII* genes cross-reacted with *A. niger* PGC protein, and the antibody to PGII cross-reacted to PG proteins produced by *S. sclerotiorum*, *F. moniliforme*, *F. oxysporum*, *Rhizoctonia fragariae*, *R. solani*, and *Sclerotium rolfsii* (Bussink et al., 1992; De Lorenzo et al., 1988). The reaction with *F. moniliforme* PG is particularly surprising since the PG from this fungus has little DNA or amino acid homology to the *A. niger* PGs (Caprari et al., 1993a). The *pelA* gene product of *N. haematococca* is immunologically related to the *pelB* and *pelC* gene products (Guo et

al., 1995a, 1995b). However, antibodies made to one PG each of *A. niger*, *S. sclerotiorum*, and *C. lindemuthianum* did not cross-react with PGs from other fungi (Keon and Waksman, 1990). Keon and Waksman (1990) suggested these proteins have unique peptide sequences, but the peptide sequence of their N-terminals is highly homologous.

Although relatively few PG, PNL and PL genes have been examined, it is interesting to note that several fungi may have families of PG, PNL and PL genes. *A. niger* has numerous PG and PNL genes which appear to be divergent copies of one gene, and in the case of the *PGC* gene, it appears this gene is a hybrid of the *PGI* and *PGII* genes (Bussink et al., 1992; Kusters-van Someren et al., 1991). *S. sclerotiorum* has at least three related PG genes, *G. cingulata* has possibly three related PNL genes and *N. haematococca* has at least three related PL genes, and these families of genes also may be divergent copies of a single gene (Fraissinet-Tachet et al., 1995; Guo et al., 1995a, 1995b; Reymond et al., 1994; Templeton et al., 1994).

The existence of families of pectinase genes has also been found in some bacterial plant pathogens and may provide a useful comparison. For example, multiple genes for endo-PL are present in the soft rot bacterium, *E. chrysanthemi*, and are arranged in two clusters, one containing the *pelA*, *pelD*, and *pelE* genes and the other containing the *pelB* and *pelC* genes (Kotoujansky, 1987; Tamaki et al., 1988). The paired genes of *pelB*/*pelC* and *pelD*/*pelE* have high homology in their promoter and coding regions, and each pair were thought to be diverged duplications of single genes (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1992; Tamaki et al., 1988). The *pelA* gene has some homology to *pelE*, but it encodes a protein with a much lower pI than the other PLs and does not cross-react to antibodies specific to the other PL proteins (Kotoujansky, 1987). There are at least four or five additional PL genes produced by *E. chrysanthemi* that appear to be only induced with plant tissue (Kelemu and Collmer, 1993). One of these genes, *pelL*, which has been shown to be expressed in culture, was cloned, and it encodes a protein with no homology to the other endo-PL genes but homology to *pelX*, an exo-PL of *E. chrysanthemi* (Łojkowska et al., 1995). Another soft rot bacterium, *E. carotovora* subsp. *carotovora*, also appears to have multiple pectinase genes with four genes for PL and one for PNL (Kotoujansky, 1987).

Relationship between pectinase isozymes and their genes

The multiple isozymes of PG, PNL and PL found in many fungi may be encoded by multiple genes and/or result from post-translational modifications of one or a few proteins. The calculated molecular weight for *PGI* and *PGC* from *A. niger*, the PG from *F. moniliforme*, and the PME from *A. niger* are lower than that found with denaturing gel electrophoresis indicating that post-translational modifications probably occurs (Bussink et al., 1991b, 1992; Caprari et al., 1993b; Khanh et al., 1991). Many PGs, PNLs and PLs have been found to be glycoproteins (Cervone et al., 1986; Keon et al., 1987), and most of the deduced amino acid sequences of fungal PLs, PNLs and PGs have numerous possible N-glycosylation sites. In *F. moniliforme*, multiple glycosylation of a single PG protein encoded by one gene results in four isozymes with molecular weights ranging from 38 to 48.5 kDa (Caprari et al., 1993a; Caprari et al., 1993b). Many of the PNL and the PME enzymes from *A. niger* are glycoproteins with high mannose content (Gysler et al., 1990; Khanh et al., 1991), however glycosylation alone cannot explain the large discrepancy between the calculated molecular weights of 35 kDa and 36.2 kDa for the *A. niger PGI* and *PGC* proteins, respectively, and their sizes of 55 and 61 kDa as determined by gel electrophoresis (Bussink et al., 1991b, 1992). The multiple isozymes and types of PG, PNL and PL found in fungi may be due to both multiple genes and post-translational modifications of the proteins (Bussink et al., 1992; Kusters-van Someren et al., 1992; Templeton et al., 1994). It has been suggested that multiple forms of an enzyme can allow an organism greater flexibility in its pathogenicity (Keon et al., 1987). Studies of the promoters of *A. niger* PG and PNL genes suggested there might be differences in the regulation of these related genes (Bussink et al., 1991a, 1992; Kusters-van Someren et al., 1991). The multiple isozymes of PG observed in many fungi leads one to believe that they also may have multiple genes encoding PG.

Genes encoding other fungal CWDE cloned from phytopathogenic fungi

Fungi produce many other plant CWDE, such as β-1,3-glucanases, various glycosidases, cellulases, xylanases and cutinases, and genes for these CWDE have been cloned from several plant pathogenic fungi

(Table 2). For instance, a fungal gene encoding an exo-β-1,3-glucanase which cleaves callose, a plant polysaccharide associated with defense responses, was cloned from *C. carbonum*, and genes encoding β-galactosidase and β-glucosidase genes were cloned from *S. sclerotiorum* (Schaeffer et al., 1994; Waksman, 1988; 1989).

Numerous cellulase genes have been cloned from soil fungi, particularly *Trichoderma reesei* (Barnett et al., 1991), wood rotting fungi (Covert et al., 1992), rumen inhabiting fungi (Xue et al., 1992; Zhou et al., 1994), and an increasing number of plant pathogenic fungi (Table 2). Five cellulase genes were cloned from *F. oxysporum* that encode proteins belonging to four different families of cellulases including endo-1,4-β-glucanases and cellobiohydrolases (Sheppard et al., 1994). Four of these had 56% to 63% amino acid identity to different cellulases of *Humicola insolens*, and one has 63% amino acid identity to a cellobiohydrolase of *T. reesi*. Two cellulase genes encoding endo-1,4-β-glucanase have also been cloned from *Macrophomina phaseolina* (Wang and Jones 1995a, 1995b). One of these cellulase genes had 72% amino acid identity to an endo-1,4-β-glucanase of *T. reesei* and is typical of the cellulases of saprophytic fungi (Wang and Jones, 1995a). The other cloned cellulase, however, differs from the cellulases of saprophytic fungi but has 48% amino acid identity to the endo-1,4-β-glucanase of the plant pathogenic bacterium, *Pseudomonas solonacearum*. The substrate requirement of four contiguous β-1,4 linkages for activity of this cellulase, rather than three linkages as for cellulases of saprophytes, is the same as that of plant cellulases, and may permit the fungus to loosen rather than extensively hydrolyse cell walls thus allowing the fungus to spread in the plant with less response from the host (Wang and Jones 1995b). It was proposed that *M. phaseolina* utilizes one form of endo-1,4-β-glucanase for pathogenicity and another for saprotrophy (Wang and Jones 1995b). A cellulase gene has also been cloned from *C. carbonum* which encodes a protein with 70% amino acid similarity to a cellulase of *T. reesi* (Sposato et al., 1995). Both the *M. phaseolina* and *C. carbonum* cellulase genes are somewhat unusual in that, unlike the vast majority of cellulases of saprophytic fungi, they encode enzymes that lack a cellulose binding domain.

One xylanase gene was cloned from the corn pathogen, *C. carbonum*, which encodes a xylanase with strong amino acid similarity to seven prokaryotic xylanases but not to 11 other prokaryotic xylanases or the xylanase from *Cryptococcus albidus* (Apel et al., 1993). Two xylanases have also been cloned from the rice pathogen, *Magnaporthe grisea*, and although these xylanases are not similar to each other in amino acid sequence, one is similar to family F and the other is similar to family G xylanases produced by saprophytic fungi (Wu et al., 1995). Xylanase family G also includes xylanases from *C. carbonum* and *Gaeumannomyces graminis*.

Several genes encoding cutinase have been cloned (Table 2). Most fungi examined thus far appear to have only one copy of a cutinase gene, but *Alternaria brassicicola* and some isolates of *N. haematococca* have multiple copies (Koller et al., 1995; Soliday et al., 1989). Cutinase genes appear to be highly homologous, since the cutinase gene from *M. grisea* is approximately 75% homologous to the genes from *Colletotrichum* spp. and *N. haematococca* (Sweigard et al., 1992a). In *A. brassicicola*, a cutinase gene was cloned which had 69 to 74% amino acid similarity to the cutinases of *Colletotrichum*, *Nectria* and *Magnaporthe* (Yao and Koller, 1994). However, two additional enzymes with cutinase activity were observed after the cloned cutinase gene was disrupted, and these additional cutinases appear to have little homology to the cloned gene (Koller et al., 1995). The similar methods employed to clone cutinase genes thus far may be selecting for cutinases with high homology to each other and may be underestimating the diversity among cutinases.

The number and diversity of CWDE cloned from phytopathogenic fungi has greatly increased in recent years and this should continue in the near future, since the techniques for manipulating fungal DNA have improved and methods for cloning many CWDE from fungi have been developed (Walton, 1994).

Regulation of CWDE genes

The molecular basis for the regulation of the pectinase genes produced by phytopathogenic fungi is not well understood. The mRNA and enzyme activity of PG, PNL and PL genes cloned from *Aspergillus* spp., *Fusarium* spp. and *C. carbonum* are induced by polygalacturonic acid and repressed by glucose (Bussink et al., 1992; Dean and Timberlake, 1989; De Lorenzo et al., 1987; González-Candelas and Kolattukudy, 1992; Kusters-van Someren et al., 1992; Scott-Craig et al., 1990). This suggests that these PG, PNL and PL genes are regulated at the transcriptional level. Transformants

Table 2. Genes encoding selected cell wall-degrading enzymes cloned from phytopathogenic fungi

Substrate	Enzyme	Gene(s)[1]	Fungus	Reference(s)
Cellulose	Endo-1,4-β-glucanase	*egl1, egl2*	*Macrophomina phaseolina*	Wang and Jones, 1995a, 1995b
		Kfam1	*Fusarium oxysporum*	Sheppard et al., 1994
	Cellobiohydrolase	*Bfam1*	*Fusarium oxysporum*	Sheppard et al., 1994
	Endo-1,4-β-glucanase and/or cellobiohydrolase	*Cfam1, Cfam2, Ffam1*	*Fusarium oxysporum*	Sheppard et al., 1994
		CEL1	*Cochliobolus carbonum*	Sposata et al., 1995
Xylan	Xylanase	*XYL1*	*Cochliobolus carbonum*	Apel et al, 1993
		xyn22, xyn33	*Magnaporthe grisea*	Wu et al., 1995
Cutin	Cutinase	cutinase[2]	*Nectria haematococca*	Soliday et al., 1989
		CUT1	*Magnaporthe grisea*	Sweigard et al., 1992a
		CUTAB1	*Alternaria brassicicola*	Yao and Koller, 1994
		cutinase[2]	*Colletotrichum capsici*	Ettinger et al., 1987
		cutinase[2]	*Colletotrichum gloeosporioides*	Ettinger et al., 1987
Callose	Exo-β1,3-glucanase	*EXG1*	*Cochliobolus carbonum*	Schaeffer et al., 1994
Disaccharides	β-glucosidase	β-glucosidase[2]	*Sclerotinia sclerotiorum*	Waksman, 1989
	β-galactosidase	β-galactosidase[2]	*Sclerotinia sclerotiorum*	Waksman, 1988

[1] Name of genes cloned.
[2] No name has been designated for the cloned gene.

of *A. nidulans* containing individual PG and PNL genes from *A. niger* produced higher levels of activity than the corresponding *A. niger* transformants, and some of the *A. nidulans* PG transformants were not repressed by glucose (Bussink et al., 1992; Kusters-van Someren et al., 1992). This suggests that there is a mechanism causing catabolite repression of these genes, possibly a *trans* regulator, which operates in *A. niger* but is missing in *A. nidulans*.

Constitutive expression of pectinase genes also occurs in fungi. Gene fusions with the *pelA* and *pelB* genes of *N. haematococca* encoding PL show that only *pelA* gene expression is induced by pectin and repressed by glucose; *pelB* expression is constitutive (Guo et al., 1995b).

In *A. nidulans*, transcription of its PL gene is catabolite repressed by glucose, and using a gel mobility shift assay, the DNA binding protein CREA was found to bind to a region of the PL gene promoter (Ho et al., 1995). CREA has been shown to have a critical role in carbon catabolite repression for genes involved in ethanol utilization, and it may also be involved in PL regulation in *A. nidulans*. However, it is difficult to assess the role of CREA because there are multiple *cre* genes in *A. nidulans* and disruption of the gene encoding CREA is lethal. The regulation of PG, PNL and PL produced by phytopathogenic fungi will be clarified with the analysis of these and other potential regulatory genes and by deletion and mutational analysis of the promoters of the genes.

The pectinase genes of phytopathogenic bacteria, such as *E. chrysanthemi*, are better studied at a molecular level and can provide insights into the potential complexity of negative and positive regulation of pectinases (Barras et al., 1994; Kotoujansky 1987). The patterns of regulation indicate that the PL genes of *E. chrysanthemi* are regulated either as groups, according to the homology of their promoter regions, or individually depending upon the induction and repression conditions (Bourson et al., 1993; Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1992). Regulation of PL genes is based not only the amount of enzyme substrate and product, but also on a variety of environmental signals associated with infection. For example, several PL genes are positively controlled by a gene which also negatively regulates iron transport in the bacterium, thus potentially coordinating pectinase production with the low iron conditions found in the apoplast of plants

(Sauvage and Expert, 1994). The pectinase enzyme genes can also be coregulated with other genes, such as those involved in pectin catabolism and the secretion pathway of PL and other extracellular enzymes, thus providing coordination between PL synthesis, PL secretion and pectin catabolism and other CWDE (Barras et al., 1994; Condemine and Robert-Baudouy, 1995). Regulation of PL gene expression of *E. chrysanthemi* is also affected by host factors. Several PL genes have been found to be expressed at higher levels in plant tissues than in culture (Łojkowska et al., 1993), and differences in the level of expression of individual PL genes were observed when different plant extracts are used as inducers (Bourson et al., 1993).

Regulation of the cellulase and xylanase genes of phytopathogenic fungi has not been extensively studied. The mRNAs of cellulase and xylanase genes cloned from *C. carbonum* and *M. grisea* are induced by extracted host cell walls or the enzyme substrates and are repressed by sucrose or glucose (Apel et al., 1993; Sposato et al., 1995; Wang and Jones 1995a, 1995b; Wu et al., 1995). Wu et al. (1995) found 10-fold higher levels of mRNA for one xylanase gene of *M. grisea* compared to another xylanase gene suggesting that these genes are regulated independently.

The regulation of fungal cutinases involves specific promoter regions interacting with DNA binding proteins, and activation/deactivation of these proteins by kinases and possibly phosphatases (Kolattukudy, 1992; Kolattukudy et al., 1995). Cutinase gene expression in *N. haematococca* is suppressed by glucose and induced by unique hydroxy fatty acids that are monomers of cutin released from the plant by cutinases attached to the fungal spore surface (Kolattukudy et al., 1995). A 360 bp region in the 5′-flanking region of a cutinase gene was found to contain the promoter elements responsible for the induction and repression of cutinase expression (Kämper et al., 1994). Four promoter elements were found: a positive SP1-like element, a silencer region which maintains a low basal level of expression, a G-C rich palindrome which is needed for induction by the hydroxy fatty acids, and a basal transcription region in the first 141 bp which is essential for expression of the gene (Kämper et al., 1994). Two cutinase transcription factor have been identified; one binds to the basal transcription region and the other binds to the palindrome. The genes for both transcription factors were isolated. The transcription factor (CFT2) which binds to the basal transcription regions appears to be a general transcription factor (Kolattukudy et al., 1995). The other transcription factor (CFT1) is a 50 kDa protein that has several potential phosphorylation sites, a nuclear localization signal and a zinc finger motif similar to the zinc finger DNA-binding domains of transcription factors of other fungi and mammals (Li and Kolattukudy, 1995).

The transcription factor, CFT1, is phosphorylated in the presence of the hydroxy fatty acid monomers. The phosphorylated factor then binds to the palindromic region of the cutinase gene, probably as a dimer, to activate gene expression (Kolattukudy et al., 1995). Cyclic AMP-dependent phosphorylation may also be involved in the repression of cutinase expression by glucose (Kolattukudy et al., 1995). Several protein kinases genes have been identified in *N. haematococca*, but their role in phosphorylation of the transcription factors has yet to be determined (Kolattukudy et al., 1995).

Role of CWDE in pathogenicity

The importance of CWDE to the pathogenicity (the ability to cause disease) or virulence (the level of disease induced) of phytopathogenic fungi is not clear. Studies attempting to correlate CWDE production to virulence or pathogenicity in phytopathogenic fungi have been complicated by the multiple isozymes of each CWDE produced by most fungi. Different isozymes of each CWDE may have different functions, including providing nutrients during saprophytic growth, and may be produced at different stages during infection of plant tissue (Cooper, 1983; Keon et al., 1987). Both classical genetic studies utilizing mutants and much more precise molecular genetic techniques have been employed to address this question.

Mutants of the wilt pathogens *Fusarium oxysporum* f.sp. *lycopersici*, *Verticillium albo-atrum*, and the brown rot fungus *Sclerotinia fructigena* that have reduced PG and PME activities were created by treatment with irradiation or mutagenic chemicals, and these mutants were reported to have similar virulence or slightly less virulence than wild types on their host plants (Durrands and Cooper, 1988a; 1988b; Mann, 1962; Puhalla and Howell, 1975). This suggests that these enzymes may be minor virulence factors but are not pathogenicity determinants. However, these studies had at least one of the following deficiencies; the mutants still produced pectinase enzymes, the assays used to screen for PG activity could not measure low levels of endo or exo-activity, and the mutants were wound inoculated into the plants so any

role of the enzymes in initial infection could not be determined. Most significantly, many of the mutants used in these studies also had abnormalities in growth or production of other enzymes demonstrating the imprecision of using mutagenic agents which can disrupt many genes in addition to the ones of interest.

A non-pathogenic mutant of *Colletotrichum magna* was produced by UV mutagenesis which grew endophytically in its host without producing disease symptoms (Freeman and Rodriguez, 1993). Unlike the wild type, this mutant lacked extracellular PG or PL activity in culture, even though it did show mRNA expression of PL (Wattad et al., 1995). The PL activity accumulated in the hyphae and was not exported. Therefore, it appears that a disruption in the extracellular secretion of PL, and perhaps PG, may be responsible for the lack of pathogenicity. Although this is good evidence for a role of CWDE in pathogenicity, the problem of multiple gene disruptions is still a concern because the original mutant was created by UV treatment.

Disruption of individual genes coding for a single CWDE can provide more precise evidence for the importance of an enzyme in pathogenicity or virulence of a phytopathogenic fungus. A series of mutants of *C. carbonum* have been made that were disrupted in a single copy of the genes for endo-PG, xylanase I, cellulase or β-1,3-glucanase, and each of these mutants has the same virulence to corn as the wild type (Apel et al., 1993; Scott-Craig et al., 1990; Schaeffer et al., 1994; Sposato et al., 1995). Although this indicates that PG, xylanase, cellulase and β-1,3-glucanase activity are not required for virulence or pathogenicity, none of these mutants is completely deficient in its ability to degrade its respective substrate. The PG, xylanase and β-1,3-glucanase mutants still produce 25 to 35%, 5 to 15%, and 44%, respectively, of the activity produced by the wild type, and all mutants except the β-1,3-glucanase mutant can still grow on their respective substrates as sole carbon sources in culture (Apel et al., 1993; Scott-Craig et al., 1990; Schaeffer et al., 1994). There is also no significant reduction in growth on cellulose by the cellulase mutant (Sposato et al., 1995). *C. carbonum* also produces an exo-PG, xylanase III, two other forms of β1,3-glucanase and multiple cellulases, and these may be important for infection of corn tissue. There is also the possibility that other endo-PGs, xylanases, cellulases and β-1,3-glucanases may be produced by the fungus only during growth in the plant, as has been found for CWDE of the bacterial pathogen, *E. chrysanthemi* (Kelemu and Collmer, 1993).

Disruption of a PL gene of *N. haematococca* and a PNL gene of *G. cingulata* also did not result in reduced virulence to their respective hosts (Bowen et al., 1995; Guo et al., 1995a). However, another PL gene has been identified in *N. haematococca*, and its role in virulence remains to be determined (Guo et al., 1995a). Similarly, additional PNL and PL genes have been identified in *G. cingulata* which may be important in virulence (Templeton et al., 1994). In *C. parasitica*, no reduction in virulence was observed when a PG gene was disrupted (Gao et al., 1996). However, it was found that this gene was expressed at very low levels during infection, even though it is highly expressed in culture. Two additional PG isozymes, which are not detectable in culture, appear to be the major forms produced in cankers.

Cutinase may be a pathogenicity factor for the penetration of fungi into plant tissue (Kolattukudy et al., 1989). The insertion of a cutinase gene from *N. haematococca* into a *Mycosphaerella* spp., which is unable to penetrate intact cuticles and only infects through wounds, causes the fungus to produce a cutinase identical to that of *N. haematococca* and increases its ability to cause disease in plants with intact cuticles (Dickman et al., 1989). However, cutinase deficient mutants of *M. grisea* and *N. haematococca*, which were created by the disruption of single copy cutinase genes, have the same virulence on host plants as the wild types (Stahl and Schäfer, 1992; Sweigard et al., 1992b). Therefore in at least two phytopathogenic fungi, cutinase was shown not to be essential for pathogenicity (Sweigard et al., 1992b; Stahl and Schäfer, 1992). It was proposed that cutinase was actually important in saprotrophy since the only effect of cutinase gene disruption is a reduction in saprophytic growth when cutin is the carbon source (Stahl and Schäfer, 1992). This theory is supported by the cloning of a lipolytic enzyme gene from the saprophytic fungus, *A. oryzae*, that is found to encode a protein with very high amino acid similarity to the cutinases of phytopathogenic fungi (Ohnishi et al., 1995). However, Rogers et al. (1994) showed that the cutinase-disrupted mutant of *N. haematococca* is less virulent than the wild type and that most of the lesions of the mutant developed where the fungus penetrated through stomata, thus bypassing the cuticle. Stahl et al. (1994), however, concluded that stomatal infection is of minor importance, and both the wild-type and cutinase disrupted mutant of *N. haematococca* could directly penetrate epidermal cells. Cutinase may also have other roles than penetration. Cutinase has been

implicated in the adhesion of uredospores to the host cuticle (Deising et al., 1992).

The cutinase gene cloned from *A. brassicicola* encodes an enzyme with high amino acid homology to other cutinases, and disruption of this gene does not affect pathogenicity (Koller et al., 1995). The only difference between the mutant and the wild-type is a decrease in saprophytic growth on cutin. However, this cutinase gene is not expressed on leaf surfaces in the wild-type which would explain why the gene disruption did not affect pathogenicity. Two other enzymes with cutinase activity are still produced in the cutinase-disrupted mutant and wild-type, and these enzymes are expressed during infection of the leaves. The role of these enzymes in pathogenicity remains to be determined. *A. brassicicola* may have different forms of cutinase for saprotrophy and pathogenicity, as has also been proposed for the cellulases of *M. phaseolina* (Wang and Jones, 1995b).

In phytopathogenic bacteria, there is evidence both for and against CWDE being required for pathogenicity or virulence depending upon the bacteria and its host. Mutation of the single PG gene of *A. tumefaciens* Biovar 3, *E. carotovora* subsp. *carotovora*, or *P. viridiflava* decreases the virulence of these mutants to plant tissue (Liao et al., 1988; Rodriguez-Palenzuela et al., 1991; Saarilahti et al., 1992). In contrast, a mutant of *X. campestris* pv. *vesicatoria* with a disrupted PL gene produces the same symptoms on plants as the wild type (Beaulieu et al., 1991). Studies with mutants of *E. chrysanthemi* in which single PL genes were disrupted, found that the disruption of *pelA*, *pelD*, *pelE* and the gene for PME reduces the virulence of the mutants (Beaulieu et al., 1993; Boccara et al., 1988). A mutant of *E. chrysanthemi* in which all the *pelA,B,C,D,E* genes and the genes for exo-PL and PME are deleted is still able to macerate plant tissue but at a greatly reduced level (Kelemu and Collmer, 1993). However, this mutatnt was found to produce a second set of plant-inducible PL isozymes, and disruption of the gene, *pelL*, encoding one of these, resulted in reduced virulence (Kelemu and Collmer, 1993; Łojkowska et al., 1995). The *pelL* protein is capable of macerating plant tissue, even though the secondary PLs have a much lower enzymatic activity compared to the PLs produced by *pelA,B,C,D,E* (Łojkowska et al., 1995). Finally, the disruption of an endoglucanase gene in *P. solanacearum* caused a reduction in virulence, but this enzyme is probably not required for pathogenesis (Roberts et al., 1988).

The role of CWDE in the virulence and pathogenicity of phytopathogenic fungi and bacteria has been difficult to determine even with the application of molecular genetic techniques. For both bacteria and fungi, disruption of export can result in a simultaneous loss of CWDE secretion and a reduction in virulence or loss of pathogenicity (Kotoujansky, 1987; Wattad et al., 1995). However, disruption of individual CWDE genes has almost always failed to show that they are essential for pathogenicity, and many of these genes appear to be unrelated to virulence. If there is only a single copy of a gene for a particular degradative ability then the disruption of the gene could determine the importance of that particular enzyme in pathogenicity. Unfortunately for the scientist, most fungi and bacteria produce multiple related and/or unrelated forms of several CWDE and regulate their production differently making it difficult to assess their importance or even readily determine the number of CWDE produced. As a result, the mutation of a single CWDE often does not eliminate the ability of the microbe to degrade a polymer. Examination of CWDE of plant pathogens by molecular techniques is showing that determining the roles of these enzymes is much more complicated than expected. Plant pathologists may have underestimated the importance of these enzymes in the saprophytic growth of plant pathogens and failed to appreciate how well adapted pathogens are to environmental stimuli. Pathogenic fungi and bacteria have a wide variety of highly developed infection strategies, and the importance of CWDE to pathogenicity and/or virulence may be different for each plant-pathogen interaction.

Considerations in future research

Although the study of the molecular biology of CWDE of plant pathogenic fungi is relatively recent, certain recurring themes seem apparent. Cloning and gene disruption remain the most powerful tools for studying these genes, but the success of any study will also depend on understanding the expression of the genes. Disrupting genes which are weakly or not expressed during infection provides little insight into the role of a CWDE in pathogenicity. One way to avoid this potential problem is to directly clone genes expressed during the infection process, such as by creating a cDNA library from infected material, and then screening this library for genes encoding CWDE. However, if the

gene is cloned based on a cDNA library or an isozyme obtained from culture, then it is important to first examine expression of the cloned genes during infection. Also, because of the widespread redundancy of CWDE genes, it is important to know the contribution that a particular gene makes to the total level of activity of that CWDE during infection. Even if the level of CWDE activity is significantly reduced in a mutant, it may be sufficient to cause disease under laboratory conditions. Finally, a careful examination of the plant material inoculated with the CWDE-disrupted mutants is advisable. Changes due to the disruption of a particular CWDE may be partially compensated by the pathogen or may cause subtle changes to the interaction which would be missed if only one macroscopic factor, such as lesion length, is measured. Microscopic and biochemical analyses, as well as a variety of inoculation procedures, may be necessary to observe changes in spore adhesion, penetration, infection, and other infection processes. Many stages occur from fungal germination to reproduction during infection, and a particular gene may only play a role in one or only a few of these stages. It remains premature to draw any generalizations about the importance of fungal CWDE in plant pathogenesis, but molecular techniques, such as gene disruption, are powerful tools to address this question if carefully applied.

Acknowledgements

Supported by the Natural Sciences and Engineering Research Council of Canada.

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