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Update on Plant-Microbe Interactions

Deconstructing the Cell Wall[1]

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Any microorganism that attempts to colonize a plant must contend with the cell wall. One of the most conspicuous effects of microorganisms on plant cell walls is enzymic degradation. Our interpretation of the significance of this depends on our concept of the plant cell wall, which, however, is paradoxical. On the one hand, the wall is an inert mechanical support and barrier; on the other hand, the wall is a dynamic, metabolically active organelle (Robinson, 1991, and refs. therein). The wall is a nutritional source for microorganisms and animals, yet it contains noxious peroxidases, phenolics, and activated oxygen (Cooper, 1984). The wall is mainly carbohydrate, yet its proteins are disproportionately studied (Showalter, 1993).

Degradation poses another paradox. On the one hand, plant cell walls are the world's most abundant source of Glc and other sugars; on the other hand, parts of it are resistant to degradation. Since all the polymers of a plant cell wall are eventually degraded by microorganisms, for every type of chemical bond in the wall there must be an enzyme that can cleave it. Some polysaccharidases such as α-amylase are widespread in nature; others, such as ligninase, are very restricted in their distribution.

This review focuses on wall depolymerases produced by plant pathogens and their role in plant diseases. Microbial wall depolymerases were originally and are currently studied for their potential importance in penetration and for the release of nutrients from the wall. Certainly, nutrition is the fundamental basis of pathogenesis as an ecological niche, and ingress is an essential prerequisite for pathogenesis. However, wall depolymerases have also been implicated as signals that trigger various physiological processes, including induction of plant defense responses, the action of plant hormones, and the control of growth and development. These effects will be discussed as they relate to plant disease.

ON THE ATTACK: ENZYMES AS VIRULENCE FACTORS

Extracellular cell-wall-degrading enzymes are ubiquitous among pathogenic and saprophytic microorganisms, including bacteria, nematodes, and fungi. They are made by all groups of pathogens, including obligate parasites and facultative saprophytes such as *Cochliobolus* spp., *Magnaporthe grisea*, and *Cladosporium fulvum* that have highly specific genetic interactions with their hosts. Enzymes that are known from at least one plant pathogen are listed in Table I. Considering the number of additional enzymes known from true saprophytes, many of which are related to pathogens, the list is undoubtedly much longer in reality.

The polymers present in the plant cell wall are insoluble except for certain soluble enzymes and some polymers during incorporation and turnover. Most microbial cell-wall-degrading enzymes are extracellular, i.e. secreted. Enzymes such as β-xylosidase and β-glucosidase, which degrade the soluble products of extracellular enzymes, are either extracellular or intracellular. Microbial wall depolymerases are usually subject to catabolite repression and substrate induction.

In plant pathology, cell-wall-degrading enzymes are considered to be examples of "basic compatibility factors," meaning that they are thought to be required by pathogens but not to be determinants of race or cultivar specificity. There are several ways in which wall depolymerases may be necessary to pathogens. They could be required for penetration, for ramification once a pathogen is inside the plant, and/or for obtaining nutrients from wall polymers. They could also be important to a pathogen if and when it must survive for extended periods in dead tissue or in the absence of a suitable host, i.e. as a free saprophyte. This is a plausible explanation for the existence of cell-wall-degrading enzymes that appear not to be important in pathogenesis (e.g. Scott-Craig et al., 1990). Since so many plant pathogenic microorganisms have saprophytic relatives, it has also been proposed that wall depolymerases might be evolutionary relics of a formerly saprophytic existence and confer no selective advantage to a pathogen qua pathogen.

One problem that has confronted those who study wall depolymerases as virulence factors has been deciding, a priori, which enzymes might be important and thus are worth investigating. The polymers of the cell wall differ considerably in regard to accessibility (do some polymers have to be degraded before others can be attacked?), nutrient suitability (can a pathogen metabolize sugars like Gal or Ara as well as Glc?), and innate resistance to degradation (cellulose and lignin are degraded slowly compared with pectin and xylan). Furthermore, different depolymerases might be equally important, but at different stages of infection. The discovery that some enzymes can mimic the characteristic symptoms of diseases has been the single most important impetus to the field. Histological studies of changes in the walls of infected

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Abbreviations: PG, polygalacturonase; PGIP, PG-inhibiting protein; PL, pectate lyase; PME, pectin methylesterase.

Table I. *Cell-wall-degrading enzymes reported from plant pathogens*

Cellulases
Endo-β-1,4-glucanase (carboxymethylcellulase)
Cellobiohydrolase
β-Glucosidase
Pectinases
EndoPL
ExoPL (exopolygalacturonate lyase)
EndoPG
ExoPG
PME
β-1,4-Xylanase
β-Xylosidase
α-Arabinofuranosidase
Arabinase
α-Glucosidase
β-1,3-Glucanase (laminarinase)
β-1,3-β-1,4-Glucanase (mixed-linked glucanase)
α-Galactosidase
β-Galactosidase
β-1,4-Galactanase
Protease
Phospholipase

plants and the commercial availability of appropriate substrates have also influenced the direction of research in this field.

Most early studies on depolymerases will not be covered in this review. In some cases good correlations were found between the presence and properties of certain enzymes and disease symptoms, tissue susceptibility, and relative virulence. Such studies have become ancillary to those using DNA-mediated transformation to construct specific mutants lacking one or more wall depolymerases. Once a structural gene encoding an enzyme has been cloned, it is possible in both bacteria and fungi to make strains specifically mutated in that gene, and then simply to test the mutant for altered virulence. This has now been done with a number of wall depolymerases, especially bacterial pectinases (Table II). Transformation has also been used to introduce depolymerase genes into pathogens that normally lack them (Dickman et al., 1989).

Pectinase

Research on pectinases (which include endo- and exo-pectate lyase [PL], endo- and exo-polygalacturonase [PG], and pectin methylesterase [PME]) from plant pathogens has a long history, and pectinases have been studied in more pathogens and in more detail than any other wall depolymerase. The original rationale for the study of pectinases was that they are able to cause tissue maceration, the characteristic symptom of soft-rot diseases. Recently, research on pectinases has received new impetus from the demonstration that pectinases and pectic fragments induce numerous physiological effects in plants, as discussed below.

Genes encoding pectinases have been cloned from at least six bacterial and two fungal pathogens. The effects of mutating one or more of these genes on pathogenicity ranges from none to moderate to strong. The most exhaustive line of pectinase research has been on the bacterium *Erwinia chrysanthemi*. When grown on pectin, four or five PL genes are expressed, depending on the strain. When all are mutated by marker exchange-eviction, total pectinase levels decline to less than 0.1% of the wild-type activity, yet *E. chrysanthemi* still grows on pectin as a sole carbon source and can still macerate plant tissue (Ried and Collmer, 1988). Growth on pectin but not tissue maceration can be explained by the presence of an exoPG (exo-acting enzymes, since they remove only one or two sugars at a time from the ends of polymers, do not cause maceration). Another strain mutated in its exoPL and exoPG genes, and all of its endoPL genes, retained almost full pathogenicity on carnation. This surprising result led to the discovery that *E. chrysanthemi* makes an entire new set of pectinases only when growing in planta or in cultures supplemented with plant extracts (Kelemu and Collmer, 1993).

The story of the PL genes of *Erwinia* exemplifies what has become a common theme in wall depolymerase research: that microorganisms rarely make just one enzyme that degrades a particular polymer. When one depolymerase gene is mutated, a new one becomes apparent. However, as discourag-

Table II. *Cell-wall-degrading enzymes that have been tested for a role in pathogenesis by DNA-mediated mutation*

	Bacteria	Fungi
EndoPG	*Erwinia, Agrobacterium, Pseudomonas*	*Cochliobolus*
ExoPG	*Erwinia*	*Cochliobolus* (in progress)
PL	*Erwinia, Pseudomonas*	
PME	*Erwinia*	
Protease	*Xanthomonas, Erwinia*	*Cochliobolus* (in progress)
Cellulase	*Xanthomonas, Pseudomonas*	
Cutinase		*Nectria*
		Magnaporthe
Xylanase		*Cochliobolus, Magnaporthe*
Exo-β-1,3-glucanase		*Cochliobolus*
Mixed-linked glucanase		*Cochliobolus* (in progress)

ing as this effect can be to researchers, it should not be taken to mean that the study of wall depolymerases is intractable. With regard to redundancy, the PL genes of *E. chrysanthemi* are probably an extreme case. For example, *Pseudomonas viridiflava* has only one PL gene whose mutation eliminates the ability to cause soft rotting (Liao et al., 1988). Mutation in the single PG-encoding gene of *Agrobacterium tumefaciens* biovar 3 eliminates PG activity and substantially decreases virulence (Rodriguez-Palenzuela et al., 1991). *Cochliobolus carbonum* makes two enzymes that can degrade β-1,3-glucan, but only one is able to support growth on pure β-1,3-glucan. When the gene for this enzyme is disrupted, growth of the fungus on β-1,3-glucan is severely reduced because the other β-1,3-glucanase (a distinct enzyme) cannot compensate (Schaeffer et al., 1994).

The multiplicity of most pectinase genes, differences between species and strains, and the use of different host plants and inoculation conditions can make it difficult to compare the numerous studies on the role of pectinase in bacterial soft-rot diseases. Recently, Beaulieu et al. (1993) tested a variety of strains of *E. chrysanthemi* mutated in single and multiple PL genes and in PME on four different host plants. Taken together, this and earlier work convincingly show that at least some pectinases, including PME, are, in at least some cases, important virulence factors in diseases caused by bacteria.

Four studies in which pectinase-deficient mutants were produced with UV light or with chemicals all concluded that pectinase contributes little (*Fusarium, Verticillium*) or nothing (*Sclerotinia, Alternaria*) to virulence of filamentous fungi. Although it cannot be excluded that these genetically uncharacterized mutants carry additional mutations that somehow compensate for the loss of pectinase, or that they have additional pectinase genes induced only in planta, the results are consistent with each other and suggest that pectinase is not very important in fungal pathogenesis. Targeted gene disruption of the single endoPG gene of *C. carbonum* likewise had no effect on pathogenicity. Residual pectinase activity in the mutant due to an exoPG supported full growth on pectin and might contribute sufficient pectinase activity for pathogenicity (Scott-Craig et al., 1990). The role of pectin degradation in pathogenicity of *C. carbonum* will not be answerable until a strain unable to grow on pectin can be constructed.

Cutinase

All potential pathogens that penetrate above-ground plant parts directly (as opposed to roots, wounds, hydathodes, or stomata) encounter cutin. Cutinases are produced by both prokaryotes and eukaryotes. The best-studied cutinases are from filamentous fungi.

There is evidence both for and against the importance of cutinase in pathogenesis in different fungal diseases. The best-studied (and most controversial) cutinase is that made by *Nectria haematococca* (*Fusarium solani* f. sp. *pisi*), a pathogen of pea. A genetically engineered cutinase-minus mutant of *N. haematococca* was still fully pathogenic on unwounded pea stems (Stahl and Schäfer, 1992). This contradicted work using anti-cutinase antibodies and esterase inhibitors that concluded that cutinase was an essential pathogenicity trait in this fungus (Kolattukudy, 1985). Because the results supporting the criticality of cutinase relied on biological and chemical reagents whose specificity had not been established, and because targeted gene disruption appears to be a clean and definitive way to make null mutants, it seems that cutinase is actually not required for infection of intact pea stems by *N. haematococca*.

Cutinase does seem to be important in other systems. When the obligate wound pathogen *Mycosphaerella* was transformed with the cutinase gene of *N. haematococca*, it could then penetrate an intact cuticle and cause disease (Dickman et al., 1989). Genetically undefined mutants of *Alternaria* and *Colletotrichum* that are deficient in cutinase production are also nonpathogenic (Tanabe et al., 1988).

Evidence for a different role for cutinase in pathogenesis has recently been presented. Cutinase promotes adhesion of spores to leaves (Deising et al., 1992). Adhesion as opposed to penetration has been proposed as a role for an extracellular proteinase of the mammalian fungal pathogen *Candida albicans* (Cutler, 1991). In adhesion of *Uromyces viciae-fabae* to bean leaves, the presence of active cutinase increased adhesion 2-fold (from 30% of spores adhering to 60%) (Deising et al., 1992). If the more subtle phenomenon of adhesion rather than penetration is the function of cutinase, the apparent importance of cutinase might be sensitive to small alterations in experimental conditions such as spore viability and inoculum density, and plant growth conditions, age, and health. Therefore, one explanation for the disagreement about the role of cutinase in infection of pea by *N. haematococca* is that cutinase might be important under some conditions and not others, even for the same pathogen on the same host.

Cellulase

Cellulases include endo-β-1,4-glucanase, β-glucosidase, and cellobiohydrolase, which cooperate in the complete hydrolysis of cellulose to Glc. Because of the crystalline nature of native cellulose, it is degraded slowly. Plant pathologists have generally felt that cellulases are not particularly important in pathogenesis, since extensive cellulose degradation typically occurs only late in infection, if at all (Cooper, 1984). However, when the major endoglucanase genes of the phytopathogenic bacteria *Pseudomonas solanacearum* and *Xanthomonas campestris* pv *campestris* were disrupted, virulence decreased (Gough et al., 1988; Roberts et al., 1988).

Xylanase

Xylans are major components of the hemicelluloses of land plants, and many microorganisms make endo-β-1,4-xylanase and β-xylosidase. Targeted disruption of the major xylanase gene, *XYL1*, of *C. carbonum* reduced total extracellular xylanase activity by about 90%, but growth on commercial xylan and pathogenicity were unaltered (Apel et al., 1993). Disruption of *XYL1* eliminated two of three xylanase isozymes, the simplest explanation for which is that the two isozymes are products of one gene. DNA blot hybridization under low stringency has revealed two additional xylanase genes in *C. carbonum*. Whether one of these new genes encodes the third

xylanase isozyme is under investigation (P.C. Apel and J.D. Walton, unpublished results). However, disruption of this additional xylanase will probably not completely eliminate xylanase activity in *C. carbonum* for two reasons. First, since commercial xylans used as assay substrates are not pure β-1,4-xylan, any enzymes that can cleave the contaminating linkages will appear in reducing sugar assays as "xylanases." Second, despite the names that scientists give them, wall depolymerases are rarely if ever completely specific. Some xylanases and cellulases, for example, are structurally related and can have reciprocal activity (Henrissat et al., 1989). Hence, it is more difficult to make a definitive conclusion about the pathogenic importance of any particular enzymic activity as opposed to any particular gene.

Protease

Proteases have been extensively studied as potential virulence factors in pathogens of animals, whose intercellular matrix, in contrast to plants, is mainly protein. Proteases have been less well studied in plant pathogens, even though stress-induced wall proteins such as extensins are postulated to have a role in defense (Showalter, 1993).

Protease is a major virulence factor in *X. campestris* pv *campestris* under certain inoculation conditions (Dow et al., 1990). An isolate of *E. chrysanthemi* mutated in its secreted metalloprotease genes was reported to be unaltered in virulence (Dahler et al., 1990), but a recent quantitative analysis found a statistically significant decrease in virulence (G.H. Lacy, personal communication).

Protease has been implicated as a virulence factor for the fungus *Pyrenopeziza brassicae* attacking *Brassica* (Ball et al., 1991). However, a genetically defined protease mutant of *Cochliobolus heterostrophus* is fully pathogenic (Lyngholm, 1993). *C. carbonum* makes a single, extracellular, alkaline protease that is optimally induced by collagen, a Hyp-rich protein analogous to extensin. The N terminus of this protease is highly similar to collagenolytic proteases from other organisms, but not to other known fungal proteases (J.M. Murphy and J.D. Walton, unpublished results). The gene for this protease is being cloned to evaluate its role in pathogenesis.

ON THE DEFENSE: CELL-WALL-DEGRADING ENZYMES AS INDUCERS OF PLANT-RESISTANCE RESPONSES

If successful pathogens need wall-degrading enzymes, there would be a selective advantage to plants to recognize them as signals of incipient attack. Indeed, wall depolymerases from microorganisms are recognized by plants in several different ways.

Wall Polymerases as Elicitors

Treatment of plant tissues or cells with certain enzymes (including PG, PL, and xylanase) elicits putative defense responses, including synthesis of pathogenesis-related proteins, ethylene, proteinase inhibitors, lignin, activated oxygen, and phytoalexins, inhibition of protein synthesis, stimulation of membrane K^+/H^+ exchange, and necrosis. Therefore, microbial wall depolymerases might be as important to the plant as warning signals as they are to a pathogen as virulence factors.

In most cases it appears that plants do not recognize wall depolymerases directly but rather respond to the enzymic products. The term "oligosaccharin" has been coined by Albersheim and co-workers to describe biologically active oligosaccharides that are produced as a result of the action of either endogenous or microbial enzymes on larger, inactive polysaccharides.

Oligogalacturonides have been the most studied as elicitors. Oligogalacturonides induce the production of phytoalexins and activated oxygen (Legendre et al., 1993), influence morphogenesis in tobacco explants (Bellincampi et al., 1993), and induce local (but not systemic) synthesis of proteinase inhibitors in tomato (Ryan, 1988). In most assays, oligogalacturonides with a degree of polymerization between 10 and 15 are the most active.

Plants also respond to other wall depolymerases and oligosaccharides. Characterized oligosaccharins that are active at low concentrations (10^{-9} M) include a hepta-β-glucoside (Cheong et al., 1991) and Fuc-containing oligoxyloglucans (Fry et al., 1993). A variety of other crude and partially purified microbial and plant-derived cell-wall-degrading enzymes and cell-wall preparations elicit plant responses such as ethylene synthesis, phytoalexin accumulation, and necrosis.

Several purified fungal endo-β-1,4-xylanases induce ethylene and pathogenesis-related protein synthesis, electrolyte leakage, necrosis, and phytoalexin accumulation. Fungal xylanases are small proteins (9 kD by gel filtration and 22 kD by SDS-PAGE and gene sequencing [Apel et al., 1993]) and can be transported in the xylem. Like pectinase elicitors, the activity of xylanase as an elicitor is lost by denaturation, but in contrast, xylanase acts directly and not by means of its products (Sharon et al., 1993).

Surprisingly, sensitivity to xylanase as an elicitor is controlled by a single gene in tobacco. Cultivar Xanthi responds but cultivar Hicks does not, and in a cross, xylanase sensitivity is genetically dominant (Bailey et al., 1993). This intriguing result raises the possibility that wall-degrading enzymes might play a role in specific disease resistance, which is often under monogenic control. Does the xylanase-sensitivity gene influence resistance to any pathogen? Do cultivars Xanthi and Hicks respond differently to xylanase-producing microorganisms? If so, what would be the response to a pathogen mutated in its ability to make xylanase?

The major impediment to interpretation of the role of wall-degrading enzymes and their products as elicitors is our poor understanding of elicitation in general and of the importance of the putative defense responses that are being elicited. Wall depolymerases and their products share the property of elicitation with a variety of other biotic and abiotic compounds, including proteins, secondary metabolites, and lipids. Considering the large number and universal presence of elicitor-active substances, it seems unlikely that any one particular elicitor could be a critical determinant of the outcome of an interaction between a plant and a pathogen. Redundancy is a property of both elicitors and cell-wall-degrading enzymes. A major challenge in research on oligosaccharins in particular

will be to determine under what natural conditions they exist and in what natural processes, if any, they participate. Plant mutants with altered cell-wall composition might make a useful contribution to this area (Reiter et al., 1993).

Plant Inhibitors of Wall Depolymerases

Proteinaceous inhibitors of PG, PL, PME, and protease have been described in plants. PGIPs are found in many plants, are constitutive, and are especially abundant in flowers (Salvi et al., 1990). The genes encoding PGIPs have been cloned from bean and pear. Protease inhibitors have been found in stigmas of tobacco (Atkinson et al., 1993).

Because wall depolymerases are important to pathogenesis, endogenous inhibitors of these enzymes could be a defense mechanism. Cervone et al. (1989) have proposed a model in which plant inhibitors of microbial wall depolymerases do not (or do not exclusively) contribute directly to defense, but instead alter the kinetics of depolymerases so that their elicitor activity is intensified. In the absence of PGIP, PG quickly degrades pectin to monomers and oligomers of galacturonic acid that are too small to be active as elicitors. In the presence of PGIP, however, PG action is retarded so that elicitor-active oligogalacturonides (degree of polymerization 10–15) accumulate to higher levels and persist longer than in the absence of PGIP (Cervone et al., 1989).

This attractive hypothesis depends on the relative concentrations of PG, PGIP, and substrate within an incipient lesion, which are not known. The presence of other enzymes that might also be acting as elicitors, either directly (e.g. xylanase) or indirectly (e.g. PL) complicates the actual situation in planta. Transgenic plants with altered expression of PGIP are being made and tested for altered response to PG and to PG-producing pathogens (F. Cervone, personal communication).

In an attempt to understand the functions of plant inhibitors of wall depolymerases, it is important to note that virtually all of the enzymes listed in Table I are also made by plants themselves. Some, such as PG and cellulase, are involved in particular developmental processes such as fruit ripening and abscission. PG and genes that apparently encode PG and PL are expressed in pollen (Pressey and Reger, 1989). The presence of high concentrations of PGIP in flowers raises the possibility that the real target of PGIP is an endogenous rather than a microbial PG (Salvi et al., 1990). Other endogenous depolymerases such as glucanases might be involved in cell-wall turnover during growth (Labrador and Nevins, 1989) and could conceivably be regulated by "inhibiting proteins."

CONCLUSION

Natural selection would favor the evolution of plants that respond defensively to essential pathogenicity determinants. If a pathogen enzyme that is recognized by plants were not required by that pathogen, it could avoid triggering plant defenses by simply evolving to nonproduction. The fact that plants do respond defensively to microbial wall depolymerases is therefore an indirect argument that they are important virulence factors for cellular plant pathogens.

There are two major limitations to further progress on cell-wall depolymerases. The first is our knowledge of the structure of the wall itself. Notwithstanding steady progress on the covalent structure of particular wall polymers, there is much to learn about how these polymers interact both covalently and by ionic and hydrogen bonding. The influence of growth, development, and stress on these interactions, and vice versa, are also still poorly understood. Better knowledge of the interactions of wall polymers and of the relative contribution of these interactions to the properties of the wall might allow more rational predictions of which depolymerases are important to pathogens.

The second limitation is our knowledge of the variety of wall depolymerases made by pathogens. The enzymes that have been studied have been almost exclusively those for which pure substrates are available. It is likely that pathogens make wall depolymerases that are specific for particular uncommon linkages in the native wall, and it is possible that disruption of these linkages is critical for successful pathogen invasion. Such enzymes can be studied only by assaying for the release of certain sugars from complex substrates or by purifying sufficient quantities of particular rare polymers. Availability of a purified fucosyl-xyloglucan oligosaccharin allowed the subsequent study of a novel plant α-fucosidase (Augur et al., 1993). An additional obstacle to the identification of novel wall depolymerases is that the expression of most is under tight regulation, i.e. catabolite repression and substrate induction. This means that finding particular enzymes will require finding the proper conditions for their induction. Also, it will be difficult to characterize any enzymes that are induced only in planta.

One approach being taken in our laboratory to identify enzymes that are biochemically intractable is to make a subtracted cDNA library representing mRNAs that are present when the maize leaf pathogen *C. carbonum* is grown on maize leaf cell walls but not when it is grown on Suc. Differentially expressed genes can be compared with the DNA data bases to identify their possible function, can be analyzed for signal peptide sequences indicative of an extracellular location of the product, and can be expressed in a heterologous system for biochemical characterization. The cDNAs can be used in transformation-mediated gene disruption experiments to create specific mutants, which can then be tested for altered growth and pathogenicity.

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