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STRUCTURES AND FUNCTIONS OF OLIGOSACCHARINS: THE ROLE OF *ENDO*GLYCANASES

Final Technical Report

I. Interactions of *Endo*polygalacturonases and Polygalacturonase-Inhibiting Proteins

Introduction.

The enzymes produced by fungi that degrade plant cell wall pectin play crucial roles in both agriculture and industry. Crop loss due to phytopathogenic fungi is a worldwide problem. We are studying the chemistry and biology of protein/protein, protein/carbohydrate, and protein/protein/carbohydrate interactions. Our goal is to understand the physical and biochemical properties of these specific molecular combinations in determining the outcome of host-pathogen interactions.

One plant-pathogen interaction we are studying is that of *Botrytis cinerea* and how it infects the aerial portions of hundreds of plant species, particularly when tissues are senescing. *B. cinerea* causes gray mold on many economically important crops; pre- and post-harvest rotting of fruit and vegetables and blight on leaves and flowers by *B. cinerea* results in significant losses of commodities [1]. No durable genetic resistance to *B. cinerea* has been identified. On the other hand, the pectin degrading enzymes (PDEs) produced by fungi, including *Botrytis*, are widely used in the production of juices, jams, jellies and purees as well as in the isolation of essential oils and pigments from citrus, and represent a multi-billion dollar industry [2-5]. In addition, *Botrytis* can play a beneficial role in the production of sweet wines (hence its name "Noble Rot") [6]. Further, fungal degradation of fruit is an important mechanism of seed dispersal.

Investigating *B. cinerea* infection of fruit adds important information to the field of plant-pathogen interactions. *B. cinerea* causes disease in many tissues under diverse conditions, thus it has multiple strategies for infection. Therefore, plants likely use several strategies to limit infections. Not all plant tissues are equally susceptible to infections by *B. cinerea*. Unripe fruit are largely resistant to rotting by *B. cinerea,* but ripe fruit are particularly susceptible [7]. *B. cinerea* uses cell wall degrading proteins (CWDPs) as virulence factors to macerate host tissues both in the establishment and expansion phases of infection [8-12]. Thus, knowing what the target substrates of *B. cinerea* CWDPs in the plant cell wall are and which proteins are secreted by the fungus during infection is key to understanding the interactions of this pathogen with plants. Many fungi use *endo*polygalacturonases (EPGs) to hydrolyze the cell wall polysaccharide homogalacturonan (HG) as one of the first steps in invasion [13]. A variety of plant defense mechanisms have evolved, some of which are directed toward EPGs. During pathogenesis, interactions between fungal EPGs and plant-derived polygalacturonase-inhibiting proteins (PGIPs) may alter the hydrolytic activity of the EPGs and thus the contribution of EPGs to pathogenesis [14].

Until recently, little was known about the mechanism of action of EPGs, despite their widespread industrial and agricultural economic significance. There has been an equal lack of information regarding the mechanism of interaction between EPGs and PGIPs during attempted infection. In the last few years, work by our lab and others have begun to unravel this interaction [15-23]. We are currently in the unique position of having accumulated a variety of samples and developed and optimized techniques in order to attempt a systematic study of the mechanism and specificity of EPG-PGIP interactions. For our studies of EPG, PGIP and pectin substrate interaction, our toolkit contains mass spectrometric methods for the analysis of N- and O-linked glycosylation and proteomics, as well as surface plasmon resonance spectrometry (SPR) protocols to assess the thermodynamics and kinetics of protein-protein and protein-carbohydrate interactions. We, along with our collaborators, have the ability to express EPGs and EPG mutants produced by the fungi *B. cinerea* and *Aspergillus niger* (an industrially important fungal species),

as well as PGIPs from bean, tomato, pear, and grape, and have access to plants with enhanced or diminished levels of PGIPs.

In previous studies, we used mass spectrometry, SPR, and molecular modeling to study the binding site of EPG for its HG substrate and the mechanism of substrate cleavage. HG is the simplest, non-esterified, linear form of pectin. We have now used these same techniques to investigate the binding of EPG to PGIP and the interaction between these two glycoproteins, and, in addition, to begin studies of the impact of the HG portion of the pectin matrix on binding and inhibition. We have also used mass spectrometry to characterize the N- and O-linked glycosylation on both EPGs and PGIPs as preliminary steps toward fully defining the role played by glycosylation in the interactions of these two classes of glycoproteins. To complement these studies, we have also begun a detailed study of host-pathogen interactions utilizing both fungal and plant mutants through the use of advanced proteomics methodologies.

Results on the work accomplished in studying the chemistry and/or biology of EPGs and PGIPs and their interactions are as follows:

(1) The fungus *Aspergillus niger* (*A. niger*) secretes a wide variety of plant polysaccharide-modifying enzymes which are capable of degrading plant cell wall polysaccharides such as pectin and xylan and are commonly used in the food industry to clarify juice and to isolate the essential oils and pigments from citrus [2,3,5]. *A. niger* is able to utilize pectin as its sole carbon source for growth *in vitro* and produces a broad spectrum of polygalacturonases with a variety of patterns of action on HG. Presently, seven EPG encoding genes (PGI, PGII, PGA, PGB, PGC, PGD and PGE) have been cloned and individually overexpressed [24-28]. PGA is of interest because it and PGB are constitutively expressed by the fungus, while the major expressed EPGs (PGI and PGII) must be induced by the presence of pectin [29]. PGA and PGB are likely required during the early stages of pathogenicity, and it has been suggested that PGA and PGB are scouting enzymes which help the fungus sense the presence of pectin by generating low molecular weight inducers while other EPGs are subsequently expressed. Many of the PDEs produced by fungi have been identified as being glycosylated [30-32]. The glycosylation state of the various PDEs may impact pathogenesis; however, the effects of the carbohydrate side chains on the properties of glycosylated PDEs are not known. A thorough understanding of the glycosylation of each PDE is essential when such enzymes are overexpressed for both industrial and basic research applications. The various conditions and hosts that are chosen for overexpression may induce variation in the post-translational modifications of the recombinant proteins. Therefore, the enzymes must be characterized to ensure the validation of the product of overexpression.

In this project we used a combination of matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS) and liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS) coupled with enzymatic digestion to identify the sites of N- and O-glycosylation in PGA. An N-linked high-mannose carbohydrate structure at Asn214 was identified. We also detected the presence of up to seven O-hexose modifications on the N-terminal peptide. Their identification as single mannose residues was consistent with the identification of individual O-linked mannose residues on the PGC isoform (see below). The locations of O-mannose modifications are of particular interest in understanding the biological role of this enzyme. As mentioned above, enzymes such as PGA are thought to be released by the fungus in the initial phases of pathogenesis in an attempt to degrade the plant cell wall. The plant responds to this by the production of proteinaceous inhibitors known as PGIPs [15] and the mannoses are placed in the region where, according to one model, the PGIP is proposed to interact with the EPG [16].

(2) The structure of the *A. niger* EPG isozyme PGC, was also studied. In a manner similar to that described for PGA, its carbohydrate structures and glycosylation site heterogeneities were analyzed by mass spectrometry. MALDI-MS was used to analyze the molecular mass of the intact protein as well as that of its deglycosylated form. The N-linked glycopeptides were identified by ESI-MS by precursor ion scanning utilizing an orifice potential stepping technique [33-35]. The presence of O-linked glycosylation was detected based on carbohydrate heterogeneity observed by LC-MS. In this study, the sites of O-

linked glycosylation on PGC were identified by a combination of β-elimination with Michael addition (BEMAD) [36] and pseudo-neutral loss scanning on a linear ion-trap mass spectrometer. This is, to the best of our knowledge, the first use of BEMAD to study O-linked mannosylation in any eukaryotic system, and represents a powerful new tool for studying this important post-translational modification. As noted for PGA above, the locations of the O-mannosylation on PGC are of interest, as they are also placed in a location which would impact the binding of the EPG to the PGIP.

(3) Six EPG genes and 13 EPG isoforms have been described in *B. cinerea* [37], although the possible functions for each in pathogenesis have not been fully elucidated. Purification and isolation of these isozymes is not possible because the *B. cinerea* PGs (BcPGs) are refractory and have low levels of expression; therefore, the yeast *Pichia pastoris* has been used as a heterologous host by our collaborators to produce the BcPG proteins [38]. The presence of multiple N-linked glycosylation sites in the six EPGs (*Bc*PG1-6) is predicted by their deduced amino acid sequences. In this study, the glycosylation sites and the attached oligosaccharide structures on *Bc*PG6 were analyzed. The molecular mass of the intact glycoprotein was determined by MALDI-TOF MS analysis. *Bc*PG6 contains seven potential N-linked glycosylation sites. Occupancy of these glycosylation sites and the attached carbohydrate structures were analyzed by tryptic digestion, followed by liquid chromatography-mass spectrometry (LC-MS) using a stepped orifice voltage approach. Five out of seven potential N-linked sites present in *Bc*PG6 were determined to be occupied by high-mannose type oligosaccharides. Four of them were readily determined to be at Asn^{58} (T3 peptide), Asn^{198} (T7 peptide), Asn^{237} (T9 peptide) and Asn^{256} (T11 peptide), respectively. Another high-mannose type oligosaccharide was located on the T8 peptide, which contained two potential N-linked sites, Asn^{224} and Asn^{227} (SNN$\underline{N}^{224}$VT$\underline{N}^{227}$ITFK). LC-MS/MS of an N-glycanase treated sample placed the oligosaccharide in this peptide at Asn^{224} rather than at Asn^{227}. The potential glycosylation site on Asn^{146} (T6 peptide) was not glycosylated. In addition, two disulfide bonds were observed, linking the Cys residues within the T13 and T16 peptides.

(4) The glycosylation patterns on EPGs and PGIPs have been implicated in the interactions between these two classes of proteins. In this study, the BcPG3 from *Botrytis cinerea* strain B05.10, which was over-expressed in *Pichia,* was analyzed in order to identify and characterize glycosylation sites as well as the structures of the attached oligosaccharides. As was done for BcPG6 (see 3 above), the molecular mass of BcPG3 was determined using MALDI-MS together with analyzing the results of the calculated mass from the amino acid sequence. The mass difference was assumed to be the result of post-transcriptional modifications (glycosylation). The BcPG3 protein was then digested with Endoglycosidase H (Endo H), which cleaves N-linked oligosaccharide side chains, and re-analyzed using MALDI-MS. The change in mass between the predicted parent EPG and that following digestion with Endo H was assumed to be due to O-linked glycosylation. As in section 2 above, the O-linked sites were determined by β-elimination followed by Michael addition with DTT (BEMAD) and LC-MS. The N-linked glycosylation sites and structures were identified using a combination of MALDI-MS and Q-TOF mass spectrometry. In this project we obtained the first evidence for extended O-mannosylation in a polygalacturonase. This may be a result of the use of *Pichia* for overexpression of the enzyme, thus this knowledge is of primary importance if the enzymes are used in biochemical assays, as the extended glycosylation may result in altered activities or other properties as compared to the native enzyme.

(5) PGIPs, found in plant cell walls, exhibit exquisite specificity with respect to the fungal EPGs that they bind to and inhibit *in vitro*. The PGIPs of a single species may be present as a set of isoforms, each existing as a series of glycoforms. Protein glycosylation has proven to be important in maintaining protein structure and function and can play a key role in protein-protein interactions [39,40]. This structural variability provides the potential for a wide range of specificity of EPG-PGIP interactions within any plant–pathogen pairing. As discussed above, the mode of action of a particular fungal EPG and its inhibition by PGIPs may be one of the critical factors in determining whether the fungus is a viable pathogen. In this project we obtained the first full characterization of the carbohydrates present on a PGIP. An earlier study using MALDI-TOF MS presented a partial characterization of the carbohydrate

structures present on the two N-linked sites in *Phaseolus vulgaris* (bean) PGIP [41]. The present study looked at the seven sites of N-glycosylation found on the PGIP from *Pyrus communis* (pear), and demonstrated the presence of both fucosylated and non-fucosylated structures at the same Asn. We then used modeling to locate the carbohydrate structures on the PGIP. The localization of these oligosaccharide side chains, using methods similar to what we have done for the EPGs above, will help in understanding what role these carbohydrate structures play in the modulating the interactions of EPGs with PGIPs.

(6) PGIPs belong to the class of proteins known as leucine rich repeat (LRR) proteins, which is a class of defense proteins known to exist in animals, plants, and bacteria [42-45]. The LRR structure is tailored for efficient binding to target proteins. The mode of action of a particular fungal EPG and its inhibition by PGIPs may be critical factors in determining whether the fungus is a viable pathogen. In this study, the kinetics of the interaction of different fungal EPGs with *Phaseolus vulgaris* (bean) PGIP2 have been studied using SPR and analyzed using various physical models. The results, consistent with previous studies, show that the interaction of EPGs and PGIP2 can be described using a 1:1 stoichiometric (Langmuir) model. Further, the presence of de-esterified HG has a moderate to strong effect on the EPG/PGIP2 interaction and the strength of the effect is dependent on the exact EPG/PGIP2 pairing. These results indicate a three-component complex is involved in these interactions, similar to that observed for the heparin-ATIII-thrombin, the FGF-FGFR-heparin, or the hedgehog-interference hedgehog-heparan complexes. This data points to an architecture in which the inhibitor binds at a location distant from the substrate binding site on EPG. We applied differential proteolysis mass spectrometry (DPMS) to study the location of the binding site between EPG and PGIP2. DPMS studies indicate that PGIP2 does not bind directly over the active site, but instead binds on the face opposite to the active site, a finding that is consistent with the SPR results.

(7) PGIPs selectively inhibit PGs secreted by invading plant pathogenic fungi. PGIPs display differential inhibition towards PGs from different fungi and also towards different isoforms of PGs originating from a specific pathogen. A PGIP-encoding gene from *Vitis vinifera* (*Vvpgip1*) was isolated and characterized. PGIP purified from grapevine was shown to inhibit crude polygalacturonase extracts from *Botrytis cinerea*, but this inhibitory activity has not yet been linked conclusively to the activity of the *Vvpgip1* gene product. In this study we used a transgenic over-expression approach to show that the PGIP encoded by the *Vvpgip1* gene is active against PGs of *B. cinerea* and that over-expression of this gene in transgenic tobacco confers a reduced susceptibility to infection by this pathogen. A calculated reduction in disease susceptibility of 47–69% was observed for a homogeneous group of transgenic grapevine lines that was statistically clearly separated from untransformed control plants following infection with *Botrytis* over a 15-day period. VvPGIP1 was subsequently purified from transgenic tobacco and used to study the specific inhibition profile of individual PGs from *Botrytis* and *Aspergillus*. The heterologously expressed and purified VvPGIP1 selectively inhibited PGs from both *A. niger* and *B. cinerea*, including BcPG1, a PG from *B. cinerea* that has previously been shown to be essential for virulence and symptom development. Altogether, our data confirm the antifungal nature of the VvPGIP1, and the *in vitro* inhibition data suggest, at least in part, that the VvPGIP1 contributed to the observed reduction in disease symptoms by inhibiting the macerating action of certain *Botrytis* PGs *in planta*. The ability to correlate inhibition profiles to individual PGs provides a more comprehensive analysis of PGIPs as antifungal genes with biotechnological potential, and adds to our understanding of the importance of PGIP:PG interactions during disease and symptom development in plants.

(8) To further study the role of EPGs in Botrytis virulence, six EPGs (BcPG1 to BcPG6) as well as site-specific mutant, inactive forms of BcPG1 and BcPG2 were transiently expressed in leaves of *Nicotiana benthamiana* using agroinfiltration. Expression of BcPG1, BcPG2, BcPG4, BcPG5 and mutant BcPG1-D203A caused symptoms, whereas BcPG3, BcPG6 and mutant BcPG2-D192A caused no symptoms. Expression of BcPG2 caused the most severe symptoms, including wilting and necrosis. BcPG2 has previously been shown to be essential for *B. cinerea* virulence. The *in vivo* effect of this

enzyme and the potential inhibition by a PGIP was examined by co-expressing *Bcpg2* and the *Vvpgip1* genes from *Vitis vinifera* in *N. benthamiana*. Co-infiltration resulted in substantial reduction of symptoms inflicted by the activity of BcPG2 *in planta*, as evidenced by quantifying the variable chlorophyll fluorescence yield. However, no interaction between pure VvPGIP1 and pure BcPG2 was detected *in vitro*. Specifically, VvPGIP1 neither inhibited BcPG2 activity nor altered the degradation profile of HG by BcPG2. Furthermore, using SPR, no physical interaction between VvPGIP1 and BcPG2 was detected *in vitro* (experiments performed in buffers). The data suggest that the *in planta* environment provided a context to support the interaction between BcPG2 and VvPGIP1, leading to a reduction in symptom development, whereas neither of the *in vitro* assays detected any interaction between these proteins. This also supports our SPR studies that demonstrated that deesterified HG can have a significant impact on EPG-PGIP interactions (section 6 above), and leads us to continue these studies on the effects of esterified pectin.

(9) As mentioned previously, *Botrytis cinerea* secretes a number of enzymes, including EPGs, that are involved in the early stages of the blight of the host plant. The successful invasion of the plant by the fungus depends on the effectiveness of these secreted enzymes in softening of the plant cell wall. Thus, there is a need to understand the diversity of the secreted enzymes in different plant environments. We have begun a broad proteomic study of the interactions of tomato (*Lycopersicum*) with *Botrytis*. In the initial phase of this study, *Botrytis* was grown on pectin, sucrose or polygalacturonic acid as the carbon source in liquid culture. The secreted proteins were isolated, concentrated, trypsin digested, and analyzed by LC-MS/MS using an ion-trap mass spectrometer. Spectral counts were used to quantitate the differences in identified proteins secreted by the fungi grown on the various carbon sources. We have been able to identify over 90 proteins present when the fungus is grown in the presence of pectin, and have found a unique expression pattern of secreted proteins from the fungus in each liquid medium.

(10) *B. cinerea* is a necrotrophic plant pathogen that secretes a number of enzymes to facilitate its penetration into the plant by degradation of the plant cell wall. The plant cell wall is a complex structure of polysaccharides, proteins and phenolics and is a primary barrier of defense against pathogens. The polysaccharide network within the wall is an interlaced network composed of pectin, cellulose and hemicellulose. Unripe or green tomato fruit is more resistant to infection by *B. cinerea* than is red ripe tomato fruit. The mechanism(s) responsible for this increase in susceptibility to *B. cinerea* upon ripening are unknown. The major change during the ripening process is softening of the fruit. The loss of firmness is primarily the result of a change in the structure and composition of the wall. Plants also possess natural defenses against pathogenic infection including the production of phytoalexins, cell wall modifications, and the induction of plant defense proteins. Inhibiting proteins, redox proteins and other pathogenesis related proteins (PR proteins) are known to be produced by the plant during infection. Thus, susceptibility or resistance of fruit may be associated with differences in timing, magnitude and production of different sets of proteins.

B. cinerea secretes cell wall degrading and other enzymes in both initial and late stages of pathogenesis. In our studies (see section #9 above), we identified those proteins secreted when growing the fungus in liquid culture, and our results indicated that the secretome of *B. cinerea* was dependent on the carbon source. Thus, the changes in susceptibility of the green and red tomato fruit may also be a consequence of differences in the secretome due to differences in the composition and the structure of the plant cell wall. In this study we used proteomics to examine both the defensive proteins produced by the fruit in response to infection by *B. cinerea* as well as the secretome of the fungus. These studies were performed when green, red or mutant *rin* tomatoes were infected with *B. cinerea*. This is a first study of proteins involved in a complex plant-fungal interaction.

In summary, we have determined the glycosylated structures of several EPGs and a highly glycosylated PGIP; we have explored the roles of PGIPs in susceptibility to infection in grapevines; and we have begun a series of studies to examine the role of the matrix polysaccharides, particularly HG, in EPG-PGIP interactions. We have also begun a series of proteomic studies on *Botrytis*-tomato

interactions. In doing this work, we have effectively used analytical methodologies developed at the CCRC.

II. The Secretome of *Magnaporthe oryzae*: Requirement for Pathogenicity

In response to various growth conditions, fungi secrete a large number of extracellular proteins (ECPs) that are thought to play roles in diverse biological activities such as breakdown of host cell walls, nutrition uptake, growth, cell-cell communication, and molecular interactions between the pathogen and its host [46-48]. For example, *Magnaporthe oryzae* (formerly *Magnaporthe grisea*), the destructive rice blast fungus [49], secrete various isoforms of *endo*-β-1,4-xylanases [49-53] that degrade arabinoxylans, the quantitatively major hemicellulose in the cell walls of Poaceae [54,55]. Some of the arabinoxylan-degrading enzymes are probably pathogenicity factors that provide ingress to the cell walls, facilitate biotrophic growth, generate metabolizable nutrients, and release oligosaccharins that elicit host defense responses [53]. Early work in our laboratory provided evidence that *M. oryzae* secretes an *endo*-β-1,4-D-xylanase and an α-L-arabinofuranosidase that generate oligosaccharin-active arabinoxylan fragments from rice cell walls [56]. Another example is the ethylene-inducing xylanase (EIX) from *Trichoderma viride*, where a glycopeptide rather than the enzyme activity acts as an elicitor [57]. Furthermore, a gene encoding an *endo*polygalacturonase (EPG) in *Botrytis cinerea* and a gene that regulates expression of cell wall degrading enzymes in *Cochliobolus carbonum* have been shown to be required for pathogenicity [10,58]. Our main accomplishments to functionally dissect the roles of *endo*xylanases, as well as other ECPs, in the *M. grisea*-rice pathosystem are summarized below.

(1) The genome of *Magnaporthe oryzae* [55; http://www.mgosdb.org] encodes at least 21 putative *endo*-β-1,4-D-xylanases belonging to three glycoside hydrolase (GH) families. These genes are expressed and their enzyme products secreted differentially under various growth conditions [50-53]. For example, while most xylanase genes are expressed both in infected rice leaves and in culture three days post-inoculation/infection, *XYL-3* transcripts were detected at a very early stage (24 h post-inoculation) during infection, and *XYL-4*, as well as *XYL-5*, are exclusively expressed in infected host tissues. Interestingly, shotgun proteomic detection did not find any GH11 xylanases in either the culture or infected host tissues, even though some GH11 transcripts (e.g. *XYL-3*) are abundantly present in either growth conditions. Gene knockout mutants were generated for nine xylanases. Infection assays showed significant reduction in pathogenicity for each of the Δ*xyl-3*, Δ*xyl-4* and Δ*xyl-5* mutants. In contrast, the Δ*xyl-2* mutant appears to have gained in virulence. To unequivocally determine that XYL-2, XYL-3, XYL-4 and XYL-5 are pathogenicity effectors, we have transformed each of the native genes back into their respective knockout mutants. The resulting "revertants" are being analyzed to determine whether each of the native genes restores the wildtype *M. oryzae* phenotype.

(2) In *Aspergillus niger*, an industrial fungus, production of the cell wall-degrading enzymes is regulated by a transcription factor, XlnR, which has two structurally similar sequences (*MgXR1* and *MgXR2*) in the *M. grisea* genome [49,59]. In an attempt to determine their biological function, Δ*mgxr1* and Δ*mgxr2* mutants were created. In either mutant the transcript level was suppressed for some xylanase genes, but enhanced for others. The secreted xylanase activity was about 90% reduced as compared to the wild-type. Surprisingly, neither mutant exhibits any reduction in growth and virulence, indicating either *MgXR1* or *MgXR2* is dispensable for the viability and pathogenicity of *M. oryzae*. However, since attempts to generate a Δ*mgxr1*-Δ*mgxr2* double mutant were unsuccessful, we are not sure whether both genes are dispensable. This finding is consistent with what was observed in *Fusarium oxysporum* [59].

(3) In order to effectively produce and purify *M. grisea* xylanases for biochemical and biological studies, we chose to express the proteins in the heterologous host *Pichia pastoris* using either a commercial vector, pPic3.5k, or a modified vector, pPicH, that includes DNA sequences encoding a c-myc epitope and a $(His)_6$ tag [52]. With the exception of XYL-5, all the genes attempted resulted in secretion of the corresponding protein into the *Pichia* culture media. However, the purified XYL-7 and XYL-8 do not exhibit xylanase activity [unpublished results].

These enzymes (XYL-1, XYL-2, XYL-3, XYL-4, XYL-6), as well as GH43 enzymes, are being studied for their biochemical properties e.g. substrate-specificity [54,55]. Fractionation of limited hydrolytic products (oligosaccharides) from a commercial insoluble xylan indicated some differences among XYL-3, XYL-4 and XYL-6 [unpublished results]. Structural makeup of the hydrolytic products specific to each xylanase may imply biological significance, and applications in research and to the food, paper and biofuel industries.

These purified enzymes were also screened for possible elicitor activity by spotting them onto detached rice leaf segments for induction of necrosis, indicative of induced plant defense responses. Preliminary results indicate that XYL2 and XYL3 were strong necrosis-inducers, and XYL-1, XYL-4, XYL-6 and AFS-1 were moderate inducers. We also tested some of the enzymes' ability to induce alkalinization of cultured rice cells, another symptom of plant defense response. Data indicated that XYL-2 at the concentration of ~10 nM were able to trigger alkalinization within 10 min of application. Further experiments are required to confirm that these xylanases, or their hydrolytic products of oligoxylan fragments, are effectors of host defense responses.

Interestingly, the XYL-4 expressed in *Pichia* is heavily glycosylated, and after the glycan has been removed by digestion with the glycanase EndoH, the "native" enzyme appears to be a much stronger necrosis-inducer on either rice leaf segments or attached tobacco leaves.

Inactive forms of XYL-2 and XYL-3 or XYL-4 had also been created using site-directed mutagenesis to replace the active site of Glu-128 with Asp-128 on XYL-2 and Glu-86 with Asp-86 on XYL-3 or XYL-4, respectively [53,55]. These mutant genes are now ready to be introduced back into the *M. oryzae* mutants (Δ*xyl-2* and Δ*xyl-3* or Δ*xyl-4*) to test whether the inactive form of a xylanase restores the wild-type phenotype in its corresponding knockout mutant [50-52]. For example, if *M. grisea* transformants of $XYL\text{-}3_{\text{inactive}}$ fully restore the ability of the Δ*xyl-3* mutant to infect its host, then the XYL-3 protein molecule itself is the pathogenicity factor. If $XYL\text{-}3_{\text{inactive}}$ does not, but $XYL\text{-}3_{\text{active}}$ does fully restore the virulence of the mutant strain, the enzyme activity of XYL3 is likely the pathogenicity factor.

(4) Xylanases are only a small fraction of the 1000 ECPs that make up the secretome of *M. oryzae* [48,49,52]. To profile the secretion of ECPs in response to culture and infection growth conditions, high-throughput shotgun proteomics technologies, such as multidimensional liquid chromatography-mass spectrometry (MDLC/MS), were employed to identify over 100 *M. oryzae* ECPs [48]. About half of the ECPs identified are putatively associated with a known function such as glycoside hydrolases and proteases. Several of the putative GH10 and GH43 xylanases were detected either in culture or in infected rice seedlings. A number of the unknown ECPs have intriguing structural features such as cysteine repeats, a membrane-bound GIP anchor, or domains related to elicitor activity. Several of the ECPs have been expressed in *Pichia* for further studies.

References Cited

[1] Elad, Y., B. Williamson, P. Tudzynski, and N. Delen. 2004. *Botrytis* spp. and diseases they cause in agricultural systems – an introduction. In: *Botrytis: Biology, Pathology and Control* (Y. Elad *et al.*, eds.), pp. 1-8. Kluwer Academic Publishers.

[2] Grassin, C. and P. Fauquembergue. 1996. Application of pectinases in beverages. In: *Pectins and Pectinases* (J. Visser and A.G.J. Voragen, eds.), pp. 453-462. Elsevier Science B.V.

[3] Heldt-Hansen, H.P., L.V. Kofod, G. Budolfsen, P.M. Nielsen, S. Hüttel, and T. Bladt. 1996. Application of tailormade pectinases. In: *Pectins and Pectinases* (J. Visser and A.G.J. Voragen, eds.), pp. 463-474. Elsevier Science B.V.

[4] Thakur, B.R., R.K. Singh, and A.K. Handa. 1997. Chemistry and uses of pectin – A review. *Crit. Rev. Food Sci. Nutri.* **37:** 47-73.

[5] Barense, R.I., M.A. dos S.C. Chellengatti, M.J.V. Fonseca, and S. Said. 2001. Partial purification and characterization of exopolygalacturonase II and III of *Penicillium frequentans*. *Braz. J. Microbiol.* **32:** 327-330.

[6] Nelson, K.E. and M.A. Amerine. 1956. Use of *Botrytis cinerea* for the production of sweet table wines. *Am. J. Enol. Viticul.* **7:** 131-136.

[7] Thompson, D.S. 2008. Space and time in the plant cell wall: relationships between cell type, cell wall rheology and cell function. *Ann. Bot.* **101:** 203-211.

[8] Kars, I., M. McCalman, L.I.A. Wagemakers, and J.A.L. Van Kan. 2005. Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of sstrain B05.10. *Mol. Plant Pathol.* **6:** 641-652.

[9] ten Have, A., W.O. Breuil, J.P. Wubben, J. Visser, and J.A.L. van Kan. 2001. *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. *Fungal Genet. Biol.* **33:** 97-105.

[10] ten Have, A., W. Mulder, J. Visser, and J.A.L. van Kan. 1998. The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* **11:** 1009-1016.

[11] Gillaspy, G., H. Ben-David, and W. Gruissem. 1993. Fruits: A developmental perspective. *Plant Cell* **5:** 1439-1451.

[12] Giovannoni, J. 2001. Molecular biology of fruit maturation and ripening. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **52:** 725-749.

[13] Jones, T.M., A.J. Anderson, and P. Albersheim. 1972. Host-pathogen interactions IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f.sp. lycopersici. *Physiol. Plant Pathol.* **2:** 153-166.

[14] Cervone, F., G. De Lorenzo, B. Aracri, D. Bellincampi, C. Caprari, A. Devoto, F. Leckie, B. Mattei, L. Nuss, and G. Salvi. 1996. The PGIP (polygalacturonase-inhibiting protein) family: Extracellular proteins specialized for recognition. In: *Biology of Plant-Microbe Interactions* (G. Stacey, B. Mullin, and P.M. Gresshoff, eds.), pp. 93-98. International Society for Molecular Plant-Microbe Interactions, St. Paul, MN.

[15] Kemp, G., L. Stanton, C.W. Bergmann, R.P. Clay, A. Darvill, and P. Albersheim. 2004. Polygalacturonase-inhibiting proteins can function as activators of polygalacturonase. *Mol. Plant-Microbe Interact.* **17:** 888-894.

[16] King, D., C. Bergmann, R. Orlando, J.A.E. Benen, H.C.M. Kester, and J. Visser. 2002. Use of amide exchange mass spectrometry to study conformational changes within the endopolygalacturonase II-homogalacturonan-polygalacturonase inhibiting protein system. *Biochemistry* **41:** 10225-10233.

[17] Di Matteo, A., L. Federici, B. Mattei, G. Salvi, K.A. Johnson, C. Savino, G. De Lorenzo, D. Tsernoglou, and F. Cervone. 2003. The crystal structure of polygalacturonase-inhibiting protein (PGIP), a leucine-rich repeat protein involved in plant defense. *Proc. Natl. Acad. Sci. USA* **100:** 10124-10128.

[18] Federici, L., C. Caprari, B. Mattei, C. Savino, A. Di Matteo, G. De Lorenzo, F. Cervone, and D. Tsernoglou. 2001. Structural requirements of *endo*polygalacturonase for the interaction with PGIP (polygalacturonase-inhibiting protein). *Proc. Natl. Acad. Sci. USA* **98:** 13425-13430.

[19] Stotz, H.U., J. Bishop, C.W. Bergmann, M. Koch, P. Albersheim, A.G. Darvill, and J.M. Labavitch. 1999. Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors. *Physiol. Mol. Plant Pathol.* **56:** 117-130.

[20] Leckie, F., B. Mattei, C. Capodicasa, A. Hemmings, L. Nuss, B. Aracri, G. De Lorenzo, and F. Cervone. 1999. The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed β-strand/ β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. *EMOB J.* **18:** 2352-2363.

[21] Bishop, J.G. 2005. Directed mutagenesis confirms the functional importance of positively selected sites in polygalacturonase inhibitor protein. *Mol. Biol. Evol.* **22:** 1531-1534.

[22] Sella, L., C. Castiglionic, S. Roberti, R. D'Ovidio, and F. Favaron. 2004. An endo-polygalacturonase (PG) of *Fusarium moniliforme* escaping inhibition by plant polygalacturonase-inhibiting proteins (PGIPs) provides new insights into the PG-PGIP interaction. *FEMS Microbiol. Lett.* **240:** 117-124.

[23] Sicilia, F., J. Fernandez-Recio, C. Caprari, G. De Lorenzo, D. Tsernoglou, F. Cervone, and L. Federici. 2005. The polygalacturonase-inhibiting protein PGIP2 of *Phaseolus vulgaris* has evolved a mixed mode of inhibition of endopolygalacturonase PG1 of *Botrytis cinerea*. *Plant Physiol* **139:** 1380-1388.

[24] de Vries, R.P. and J. Visser. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *MIcrobiol. Mol. Biol. Rev.* **65:** 497-522.

[25] Benen, J.A.E., H.C.M. Kester, and J. Visser. 1999. Kinetic characterization of *Aspergillus niger* N400 endopolygalacturonases I, II, and C. *Eur. J. Biochem.* **259:** 577-585.

[26] Parenicová, L., J.A.E. Benen, H.C.M. Kester, and J. Visser. 1998. pgaE encodes a fourth member of the endopolygalacturonase gene family from *Aspergillus niger*. *Eur. J. Biochem.* **251:** 72-80.

[27] Benen, J.A.E., H.C.M. Kester, L. Parenicová, and J. Visser. 1996. Kinetics and mode of action of *Aspergillus niger* polygalacturonases. In: *Pectins and Pectinases* (J. Visser and A.G.J. Voragen, eds.), pp. 221-230. Elsevier Science B.V.

[28] Parenicová, L., H.C.M. Kester, J.A.E. Benen, and J. Visser. 2000. Characterization of a novel endopolygalacturonase from *Aspergillus niger* with unique kinetic properties. *FEBS Lett.* **467:** 333-336.

[29] Parenicová, L., J.A. Benen, H.C.M. Kester, and J. Visser. 2000. *pga*A and *pga*B encode two constitutively expressed endopolygalacturonases of *Aspergillus niger*. *Biochem. J.* **345:** 637-644.

[30] Yang, Y., C. Bergmann, J. Benen, and R. Orlando. 1997. Site-specific structural characterization of the glycans of recombinant *endo*polygalacturonase II (EPG-II) by mass spectrometry. *Rapid Commun. Mass Spectrom.* **11:** 1257-1262.

[31] Colangelo, J., V. Licon, J. Benen, J. Visser, C. Bergmann, and R. Orlando. 1999. Characterization of the glycosylation of recombinant *endo*polygalacturonase I from *Aspergillus niger*. *Rapid Commun. Mass Spectrom.* **13:** 1448-1453.

[32] Colangelo, J. V. Licon, J. Benen, J. Visser, C. Bergmann, and R. Orlando 1999. Characterization of the *N*-linked glycosylation site of recombinant pectate lyase. *Rapid Commun. Mass Spectrom.* **13:** 2382-2387.

[33] Huddleston, M.J., M.F. Bean, and S.A. Carr. 1993. Collisional fragmentation of glycopeptides by electrospray ionization LC/MS and LC/MS/MS: methods for selective detection of glycopeptides in protein digests. *Anal. Chem.* **65:** 877-884.

[34] Ritchie, M.A., A.C. Gill, M.J. Deery, and K. Lilley. 2002. Precursor ion scanning for detection and structural characterization of heterogeneous glycopeptides mixtures. *J. Am. Soc. Mass Spectrom.* **13:** 1052-1064.

[35] Hunter, A.P. and D.E. Games. 1995. Evaluation of glycosylation site heterogeneity and selective identification of glycopeptides in proteolytic digests of bovine alpha 1-acid glycoprotein by mass spectrometry. *Rapid Commun. Mass Spectrom.* **9:** 42-56.

[36] Wells, L., K. Vosseller, R.N. Cole, J.M. Cronshaw, M.J. Matunis, and G.W. Hart. 2002. Mapping sites of O-GlcNAc modification using affinity tags for serine and threonine post-translational modifications. *Mol. Cell Proteomics* **1:** 791-804.

[37] van der Cruyssen, G., E. de Meester, and O. Kamoen. 1994. Expression of polygalacturonases of *Botrytis cinerea in vitro* and *in vivo*. *Med. Fac. Ladbouwwet. Rijksuniv. Gent.* **59:** 895-905.

[38] Kars, I., G.H. Krooshof, L. Wagemakers, R. Joosten, J.A. Benen, and J.A. van Kan. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *Plant J.* **43:** 213-225.

[39] Haltiwanger, R.S. and J.B. Lowe. 2004. Role of glycosylation in development. *Ann. Rev. Biochem.* **73:** 491-537.

[40] Martin, P.T. 2003. Dystroglycan glycosylation and its role in matrix binding in skeletal muscle. *Glycobiology* **13:** 55R-66R.

[41] Mattei, B., M.S. Bernalda, L. Federici, P. Roepstorff, F. Cervone, and A. Boffi. 2001. Secondary structure and post-translational modifications of the leucine-rich repeat protein PGIP (polygalacturonase-inhibiting protein) from *Phaseolus vulgaris*. *Biochemistry* **40:** 569-576.

[42] Liu, J., X. Liu, L. Dai, and G. Wang. 2007. Recent progress in elucidating the structure, function and evolution of disease resistance genes in plants. *J. Genet. Genomics* **34:** 765-776.

[43] Wilmanski, J.M., T. Petnicki-Ocwieja, and K.S. Kobayashi. 2008. NLR proteins: integral members of innate immunity and mediators of inflammatory diseases. *J. Leukoc. Biol.* **83:** 13-30.

[44] Altenbach, D. and S. Robatzek. 2007. Pattern recognition receptors: from the cell surface to intracellular dynamics. *Mol. Plant-Microbe Interact.* **20:** 1031-1039.

[45] Kaparakis, M., D.J. Philpott, and R.L. Ferrero. 2007. Mammalian NLR proteins: discriminating foe from friend. *Immunol. Cell Biol.* **85:** 495-502.

[46] Xu, J.R., Y.L. Peng, M.B. Dickman, and A. Sharon. 2006. The dawn of fungal pathogen genomics. *Annu. Rev. Phytopathol.* **44:** 337-366.

[47] Bouws, H., A. Wattenberg, and H. Zorn. 2008. Fungal secretomes--nature's toolbox for white biotechnology. *Appl. Microbiol. Biotechnol.* **80:** 381-388.

[48] Wu, S.-C., J. Johnson, A.G. Darvill, P. Albersheim, and R. Orlando. 2004. Proteomics of *Magnaporthe grisea*: Liquid chromatography mass spectrometry for the identification of extracellular proteins. In: *Rice Blast: Interaction with Rice and Control* (S. Kawasaki, ed.), pp. 39-46. Kluwer Press, Dordrecht, The Netherlands.

[49] Dean, R., N.J. Talbton, D.J. Ebbole, M.L. Farman *et al*. 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* **434:** 980-986.

[50] Wu, S.-C., S. Kauffmann, A.G. Darvill, and P. Albersheim. 1995. Purification, cloning and characterization of two endo-β-1,4-xylanases from *Magnaporthe grisea*, the rice blast fungus. *Mol. Plant-Microbe Interac.* **8:** 506-514.

[51] Wu, S.-C., K.-S. Ham, A. G. Darvill, and P. Albersheim. 1997. Deletion of two endo-β-1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. *Mol. Plant-Microbe Interac.* **10:** 700-708.

[52] Wu, S.-C., J.E. Halley, C. Luttig, L. Fernekes, G. Gutiérrez-Sanchez, A.G. Darvill, and P. Albersheim. 2006. Identification of a *Magnaporthe grisea* endo-β-1,4-D-xylanase by gene knockout analysis, protein purification and heterologous expression. *Appl. Environ. Microbiol.* **72:** 986–993.

[53] Beliën T., S. van Campenhout, J. Robben, and G. Volckaert. 2006. Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? *Mol. Plant-Microbe Interac.* **19:** 1072–1081.

[54] Carpita, N.C. and M. McCann. 2000. The cell wall. In: *Biochemistry and Molecular Biology of Plants*, Chapter 2 (B.B. Buchanan, W. Gruissem, and R.J. Jones, eds), pp. 25–109. American Society of Plant Biologists, Rockville, Massachusetts.

[55] Liab, K., P. Azadi, R. Collins, J. Tolan, J. S. Kim, and K. L. Eriksson. 2000. Relationships between activities of xylanases and xylan structures. *Enzyme Microbe Technol.* **27:** 89-94.

[56] Côté, F., K.-S. Ham, M.G. Hahn and C.W. Bergmann. 1998. Oligosaccharide elicitors in host-pathogen interactions: Generation, perception and signal transduction. In: *Plant-Microbe Interactions* (B.B. Biswas and H. Das, eds.), pp. 385-432. Plenum Press, New York.

[57] Ron, M. and A. Avni. 2004. The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell* **16:** 1604-1615.

[58] Tonukari, N.J., J.S. Scott-Craig, and J.D. Walton. 2000. The *Cochliobolus carbonum* SNF1 gene is required for cell wall-degrading enzyme expression and virulence on maize. *Plant Cell* **12:** 237-247.

[59] Calero-Nieto, F., A. Di Pietro, M.I. Roncero, and C. Hera. 2007. Role of the transcriptional activator xlnR of *Fusarium oxysporum* in regulation of xylanase genes and virulence. *Mol. Plant-Microbe Interact.* **20:** 977-985.