STRUCTURAL STUDIES OF COMPLEX CARBOHYDRATES OF PLANT CELL WALLS

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Final Report

The long term objectives of DOE grant DE-FG02-96ER20220 were to "*develop and apply advanced analytical and immunological techniques to study specific changes in the structures and interactions of hemicellulosic and pectic polysaccharides*." With these techniques, we proposed to "*accurately characterize minute amounts of cell wall polysaccharides, so that subtle changes in structure that occur in individual cell types can be identified and correlated to the physiological and developmental state of the plant*."

Our approach to understanding the role of the cell wall in plant growth and development involved detailed structural analysis of the polysaccharides present in the walls of different tissues and cell types of diverse vascular and avascular plants and in genetically modified Arabidopsis. The results we obtained provided fundamental information required to generate hypotheses concerning the evolution and molecular functions of hemicellulosic polysaccharides.

In the following sections we describe the progress made towards our goals. We emphasize the advances we made in identifying new structural motifs of hemicelluloses and how some of these structures have changed during the course of land plant evolution. We show how we exploited the availability of diverse Arabidopsis mutants together with NMR spectroscopy, mass spectrometry (MS), and immunocytochemistry to identify new cell-specific hemicellulose structures and some of the glycosyltransferases that may be responsible for generating them. Studies with the moss Physcomitrella and genetically modified plants together with bioinformatics allowed us to obtain deeper insight into the structural features of xyloglucan (XyG) and glucuronoxylan that affect plant growth and development. Studies with genetically modified Arabidopsis together with bioinformatics also allowed us to explore the phylogentic relationships between plant cell wall-related fucosyltransferases and the effect of fucosylation in cell wall architecture. We also describe our immunocytochemical studies that advanced Arabidopsis leaf trichomes as a model system for studying cell wall biosynthesis and deposition. Finally, we describe our hypothesis to explain how the recognition of pathogen-derived enzymes that fragment the plant cell wall is involved in the surveillance and response of plants to microbial infection.

1. The hemicellulosic polysaccharides of plant cell walls

Hemicellulosic polysaccharides have numerous important roles in maintaining the structural and biological integrity of the cell walls in land plants [1]. Perhaps the most compelling evidence for this statement is our demonstration that the basic structures of two of the most abundant hemicellulosic polysaccharides, XyG and glucuronoxylan, are largely conserved among the avascular plants (bryophytes) and vascular plants [2, 3]. Nevertheless, there is increasing evidence that plants have the ability to compensate for alterations in their cell walls that result from genetically-induced changes in the amounts or structures of a hemicellulosic polysaccharide. However, such changes may still affect overall plant growth and development. For example, we demonstrated that the Arabidopsis *mur3-3* mutant produces structurally abnormal XyG and is dwarfed [4; see Final Report **Section 3** below]. By contrast, the Arabidopsis double mutant (*xxt1 xxt2*), which produces no detectable amounts of XyG, developed normally under laboratory conditions [5]. Thus, the presence of an abnormal XyG is

more deleterious to plant growth and development than the absence of XyG, a conclusion that is also supported by our studies of other *xxt* double mutants [6,7; see Final Report **Section 4** below]. Such results have led plant scientists to begin to re-evaluate cell wall models that attempt to explain the roles that hemicellulosic polysaccharides play in normal plant growth and development.

2. Moss and liverwort xyloglucans contain galacturonic acid and are structurally distinct from the xyloglucans synthesized by hornworts and vascular plants

Xyloglucan (XyG) is a well-characterized hemicellulosic polysaccharide that is present in the cell walls of all seed-bearing plants [8]. The cell walls of avascular and seedless vascular plants have also been reported to contain XyG [9, 10], but these XyGs have not been structurally characterized. This lack of information is an impediment to understanding XyG function and changes in XyG structure that occurred during land plant evolution. Thus, we isolated and characterized XyGs from diverse plant species [2], including the moss *Physcomitrella patens*, the liverwort *Marchantia polymorpha*, the hornworts *Anthoceros agrestis*, *Phaeoceros sp*. and *Megaceros sp.,* the sporophyte generations of the lycopodiophytes *Lycopodium tristachyum*, *Huperzia lucidulum* and *Selaginella kraussiana*, the gametophyte generation of the monilophyte *Ceratopteris richardii*, and the sporophytes of the monilophytes *Equisetum hyemale*, *Psilotum nudum* and *Platycerium bifurcatum*. Each XyG was fragmented with a XyG-specific endoglucanase (XEG), and the resulting oligosaccharides then structurally characterized using NMR spectroscopy, MALDI-TOF and electrospray MS, together with glycosyl-linkage and glycosyl residue composition analyses. Our data [2] showed that XyG is present in the cell walls of all major divisions of land plants and that these XyGs have several common structural motifs. Our results suggest that hornworts are the only avascular plants that produce XXXG-type XyGs that are structurally similar to the XyG produced by dicots such as Arabidopsis [2] and is consistent with phylogenomic studies that place hornworts as sister to the first vascular plants [11]. In contrast, the moss and liverwort species we studied have XXGGG- and XXGG-type XyGs, respectively, with structural features that had not been previously described. These features included the presence of side chains that contain terminal β-D-Gal*p*A residues and branched (2,4-substituted) α-D-Xyl*p* residues. We also discovered that the XyGs from *P. patens* and several seedless vascular plants have side chains containing α-L-Ara*p* residues, which are structurally homologous to the β-D-Gal*p* residues present in XyGs from a broad range of land plants. Together, our results revealed that the basic XyG structure (i.e., a backbone consisting of 1,4-linked β-D-Glc*p* residues, many of which bear an α-D-Xyl*p* residue at O-6) is conserved among land plants, although the types and distribution of glycoses attached to the Xyl*p* is dependent on the plant species. Such flexibility in side chain structure suggests that differences in XyG structure may have evolved to optimize functional interactions of XyGs with other components of the cell wall [2].

3. Depleting XyG rescues the dwarf phenotype of the Arabidopsis *mur3-3* mutant

The Arabidopsis *MUR3* gene encodes a galactosyltransferase involved in XyG synthesis. MUR3 catalyzes the addition of a β-D-Gal*p* residue to O-2 of the α-D-Xyl*p* residues that are linked to O-6 of a backbone β-D-Glc*p* residue adjacent to an unbranched β-D-Glc*p* residue [12]. This Gal is often fucosylated to form the F side chain: α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*(1→6)-β-D-Glc*p* [8]. The dwarf phenotype of Arabidopsis plants carrying a loss-of-function mutation in *MUR3* has been hypothesized to result from defective endomembrane and actin filament organization rather than abnormal XyG structure [13].

In collaboration with Wolf-Dieter Reiter (University of Connecticut) we tested this hypothesis by characterizing the XyG synthesized by several *mur3* alleles and determined the phenotypic effect of altering XyG structure or depleting XyG to below detectable amounts. Plants carrying

leaky point mutations (*mur3-1* and *mur3-2*) have wild-type phenotypes and synthesize XyG containing discernible amounts of the F side chain. No fucosylated side chains were detected in the XyG synthesized by *mur3-3*, a dwarf loss-of-function mutant. To deplete XyG from the *mur3* background, we crossed *mur3-3* with an Arabidopsis mutant (*xxt1 xxt2*) whose walls contain no discernible amounts of XyG yet which has a wild-type phenotype [5]. The aerial portions of *mur3-3 xxt1 xxt2* and *xxt1 xxt2* were indistinguishable and their cell walls contained no discernible amounts of XyG. By contrast, the dwarf phenotype was retained in a mutant that synthesizes XyG composed only of XXXG subunits. Our results showed that the *mur3-3* phenotype resulted from the synthesis of structurally abnormal XyG rather than from the absence of MUR3 itself and supported the notion that the galactosyl residue added by MUR3 is required for normal XyG function. We also concluded that the presence of an abnormal XyG is more deleterious to plant growth and development than the absence of XyG [4].

4. Analysis of Arabidopsis xyloglucan xylosyltransferase (XXT) multi-mutants

In collaboration with the laboratories of Ken Keegstra (Michigan State University) and Olga Zabotina (Iowa State University) we characterized all the double mutants and the triple mutant of three xylosyltransferase genes (*XXT1*, *XXT2*, and *XXT5*) involved in XyG synthesis [5, 7]. We used immunocytochemistry with 30 XyG-directed monoclonal antibodies together with glycome profiling using our toolkit of cell wall glycan-directed monoclonal antibodies [14] to demonstrate that there is a discernible hierarchy in the roles and importance of each XXT in XyG biosynthesis. Functional XXT1 or XXT2 must be present for any XyG to be synthesized, whereas XXT5 is at least partially functionally redundant with XXT2. The most severe impact on XyG structure resulted from mutations in *XXT5*, either alone or in combination with mutations in either *XXT1* or *XXT2*. Our studies provided additional evidence that the presence of XyG with altered structure has a more severe effect on plant growth and development than does the complete absence of XyG (see Final Report **Section 3**) [6].

5. Arabidopsis root hair cell wall xyloglucan contains β-D-galactosyluronic acid

The cell walls of aerial portions of the dwarf Arabidopsis *mur3-3* mutant contain XyG that lacks α-L-fucose-(1→2)-β-D-galactose-containing side chains, as the plant carries a loss-of-function mutation in the *MUR3* gene that encodes a XyG-specific galactosyltransferase [12]. Somewhat unexpectedly, we found that *mur3-3* root hairs were labeled by monoclonal antibodies (e.g., CCRC-M1) that recognize fucosylated XyG. We then discovered that ~5% of the XyG present in Arabidopsis root cell walls contains at least two previously unidentified side chains: β-D-Gal*p*A-(1→2)-α-D-Xyl*p*-(1→ and α-L-Fuc*p*-(1→2)-β-D-Gal*p*A-(1→2)-α-D-Xyl*p*-(1→. The GalA-containing XyG is likely present only in root-hair cell walls as it was not detected in the root cell walls of Arabidopsis mutants that lack root hairs or in Arabidopsis leaf and stem cell walls. Arabidopsis plants carrying a loss-of-function mutation in a *MUR3* homolog (At1g63450) that is expressed specifically in root hairs synthesize XyG that lacks GalA. The mutant root hairs are ~30% shorter than their wild-type counterparts. However, the density of hairs is comparable on mutant and wild-type roots. Thus, the mutation likely affects root hair elongation rather than root hair initiation. The GalA chemotype of the mutant was complemented by expressing At1g63450 under the control of its native promoter, which suggests that At1g63450 encodes a XyG-specific galacturonosyltransferase (XUT1). The GalA residue is likely fucosylated by the XyG-specific fucosyltransferase, FUT1, as no fucose was detected in the anionic XyG present in the roots of the Arabidopsis *fut1* mutant. In combination with our previous work on Physcomitrella XyG, these data suggest that GalA-containing XyG occurs predominantly in the walls of cells that extend by tip growth and may have a role in cell elongation [4].

6. The glucuronoxylans from the moss *Physcomitrella patens*

The moss *Physcomitrella patens* has numerous characteristics that make it a unique and powerful model system to study the molecular and biochemical mechanisms of plant polysaccharide synthesis and cell wall assembly. For example, Physcomitrella is amenable to targeted genetic manipulation by homologous recombination [15-17]. Moreover, the dominant filamentous and leafy gametophore stages of this moss's life cycle are haploid, making it possible to readily identify phenotypes and chemotypes. Finally, the tissues in both these growth phases are comprised of a limited number of cell types, thereby simplifying the identification and analyses of cell-specific variation in wall composition and structure. Despite these advantages there are very few published studies describing the detailed structures of the polysaccharides present in the cell walls of Physcomitrella. Thus, our initial studies focused on using both structural and immunocytochemical analyses to establish a base-line for future studies.

To this end, we characterized a second hemicellulosic polysaccharide present in the *P. patens* cell wall to advance this moss as a model for cell wall development and biosynthesis. Our studies were the first to provide compelling evidence that mosses synthesize glucuronoxylan (GX), albeit in small amounts and only in specific cell types [3]. We used endoxylanase treatment in combination with ^{1}H-NMR spectroscopy and electrospray-ionization MS to show that the GX isolated from *P. patens* leafy gametophores has a backbone composed of 1,4-linked β-D-xylosyl residues, some of which are substituted at O-2 with GlcA residues but not with 4-*O*-Me-GlcA residues. This distinguishes the *P. patens* GX from the GX present in the secondary cell walls in woody tissues of many hardwoods and herbaceous vascular plants, which typically contain 4-*O*-Me-GlcA as well as GlcA [18]. We obtained evidence that, in *P. patens*, the GX is deposited predominantly in the walls of axillary hair cells suggesting that GX synthesis is cell-specific. We also identified putative *P. patens* orthologs of many of the genes that have been implicated in GX biosynthesis in Arabidopsis [18]. These results, in combination with the sensitive analytical [19] and immunocytochemical techniques developed in our laboratories [14], established the fundamental framework for effective characterization of the molecular mechanisms of hemicellulose biosynthesis in *P. patens* [3].

7. The glucuronoxylans from the lycophyte *Selaginella kraussiana* and the monilophyte *Equisetum hyemale* lack the reducing end sequence present in the xylans of softwoods, hardwoods, and herbaceous dicots

To complement our studies of glucuronoxylan (GX) structure in Physcomitrella [3] and flowering plants [18, 19] we structurally characterized the GX in two seedless vascular plants: the lycopodiophyte *Selaginella kraussiana* and the monilophyte *Equisetum hyemale*. The 1M KOH-soluble GXs were treated with endoxylanase and the oligosaccharides (GXOs) generated then isolated by size-exclusion chromatography. These GXOs were characterized using MALDI-TOF-MS and by 1- and 2-D ^{1}H-NMR spectroscopy. The *S. kraussiana* GXOs are composed of 1,4-linked β-D-Xyl*p* residues, some of which are substituted at O-2 with 4-*O*-Me-α-D-Glc*p*A, as found in many hardwood xylans [1]. No evidence was obtained for the presence of side chains containing α-D-Glc*p*A. The *E. hyemale* GXOs are composed of 1,4-linked β-D-Xyl*p* residues that are substituted at O-2 with α-D-Glc*p*A and with 4-*O*-Me-α-D-Glc*p*A residues, as found in the xylans of herbaceous dicots including Arabidopsis [3, 18].

Herbaceous and woody dicots and gymnosperms synthesize heteroxylans that contain 4-β-D-Xyl*p*-(1,4)-β-D-Xyl*p*-(1,3)-α-L-Rha*p*-(1,2)-α-D-GalpA-(1,4)-D-Xyl*p* at their reducing end. We proposed that this sequence has a role in controlling the degree of polymerization of the xylan polymer [18]. No resonances diagnostic for this glycosyl sequence were detected in the 2-D NMR spectra of the *S. kraussiana* and *E. hyemale* or *P. patens* GXOs. Thus, this glycosyl

sequence was absent, or present in amounts below our detection limits, in the xylans of these plants [3].

8. Labeling of Arabidopsis stem cell walls with 2-aminobenzamide indicates that the 1M KOH-soluble xylan is not linked via its reducing end to other wall components

We obtained evidence that the glycosyl sequence 4-β-D-Xyl*p*-(1,4)-β-D-Xyl*p*-(1,3)-α-L-Rha*p*-(1,2)-α-D-GalpA-(1,4)-D-Xyl*p* (sequence **1**) at the reducing end of the GX synthesized by Arabidopsis is involved in controlling the xylans' degree of polymerization [18]. These studies also led us to hypothesize that this sequence is either a primer or terminator of xylan chain elongation and that, *in muro*, sequence **1** links xylan to another wall component [18, 20]. To test the latter hypothesis, Arabidopsis stem cell walls were reacted with $NaBH_4$ in 250 mM KOH. This procedure converted some of the 4-*O*-Me-GlcA and GlcA residues on the xylan backbone to 4-O-Me-Glc and Glc respectively, suggesting that MeGlcA and GlcA form ester bonds with other wall components (polysaccharide and/or lignin), as $NaBH_4$ reduces activated carboxyl groups but not free carboxyl groups. In addition, the reducing xylose of sequence **1** was converted to xylitol, suggesting that **1** does not link xylan to other wall components. Nevertheless, we could not preclude the possibility that xylan was linked to another wall component via sequence **1** by an alkali-labile bond.

In a second series of experiments, Arabidopsis stem cell walls were reacted at pH 5 with 2-aminobenzamide (2-AB) in the presence of sodium cyanoborohydride to determine if the reducing end of xylan or any other wall component could be reductively aminated with the fluorescent 2-AB. The 2-AB-treated walls were sequentially extracted with ammonium oxalate, 1M and 4M KOH. Only the 1M KOH soluble material contained strongly fluorescent material. The 1M KOH soluble materials were then analyzed by size-exclusion chromatography (SEC) with fluorescence and refractive index detection before and after treatment with endoxylanase. The fluorescent material eluted at the void volume of the SEC column. However, the fluorescent material eluted near the column included volume after endoxylanase treatment. MALDI-TOF-MS and ^{1}H-NMR spectroscopic analyses established that the endoxylanase-generated material was a mixture of fluorescent-labeled and non-fluorescent xylo-oligosaccharides and that at least 85% of sequence **1** present in the fraction was labeled at its former reducing end with 2-AB. Thus, we conclude that little if any of the xylan solubilized by 1M KOH is linked to other polysaccharides via sequence **1**. No comparable 2-AB labeling of wall components was observed when the same reaction was performed with cell walls isolated from the grass *Brachypodium distachyon*. Moreover, we did not detect reducing end sequence **1** in the *B. distachyon* xylan or in the xylans isolated from other grass cell walls. Such results suggest that although the mechanisms of heteroxylan biosynthesis in grasses and in dicots and gymnosperms are likely to be similar, they are not identical.

9. *Arabidopsis thaliana* fucosyltransferases (AtFUT)

We studied the 13 member *Arabidopsis thaliana* fucosyltransferase (*AtFUT*) gene family that encode proteins that transfer the fucosyl residue from GDP-Fuc to diverse cell wall polymers and thus contribute to cell wall formation. To date only three of these genes have been functionally characterized. For example, *AtFUT1* has been shown to encode a fucosyltransferase that catalyzes the formation of the α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→ linkage in the F side chain of XyG [31-33]. Studies with suspension-cultured tobacco cells suggest that *AtFUT4* and *AtFUT6* encode glycosyltransferases that add fucosyl residues to arabinogalactan proteins [21].

We used a molecular approach together with cell wall analyses and bioinformatics to determine the functions of the *AtFUTs*. Our phylogenetic analysis of FUT protein sequences deduced from fully sequenced plant genomes revealed a somewhat unusual clade structure

when compared to the clade structures of other published plant cell wall-related glycosyltransferases [22]. Most *FUT* genes fall into plant-specific clades, with very few *FUTs* being grouped into multi-species clades. For example, AtFUT1 and AtFUT3 cluster with other dicot FUTs, whereas AtFUT2 and AtFUT4-10 cluster together in a distinct clade that is related to AtFUT1. AtFUT3 is most closely related to a group of poplar FUTs and a distinct group of legume (Medicago and soybean) FUTs. Such a clade structure suggests that fucosylation developed independently in each plant. If this unexpected result is confirmed, the value of model organisms for elucidating FUT functions may be of limited value. However, those FUTs that are present in multi-species clades may participate in evolutionarily conserved fucosylation reactions that could be targeted experimentally.

We performed gene expression studies on all *AtFUT* genes in Arabidopsis tissues using RT-PCR and qPCR. Our data revealed both tissue- and cell type-specific expression patterns. These studies were complemented with *promoter::GUS* reporter gene expression studies. For example, *AtFUT6* is highly expressed in roots, emerging lateral root meristems, and root hairs, whereas *AtFUT5* is expressed in all tissues of young Arabidopsis seedlings. *Promoter::GUS* constructs were generated for *AtFUT2, AtFUT3,* and *AtFUT10* and transformed into Arabidopsis in order to study their expression patterns are currently under study. Similar constructs were generated for the remaining members of the AtFUT family. Our data suggested that AtFUT6 is localized to the Golgi complex and that the N-terminal 50 amino acids of the protein were sufficient to localize FUT6 to the Golgi. Similar constructs with full length genes tagged to YFP were made for *AtFUT1, AtFUT3, AtFUT8,* and *AtFUT10.*

We isolated a homozygous mutant plant (*fut6-1*) carrying a T-DNA insert in the Arabidopsis *FUT6* gene (SALK_078357), and compared some of its growth habits and cell wall characteristics with wild-type plants. The *fut6-1* mutant plants have reduced and aberrant root growth when seeds are germinated on solid medium that lacks added sugar. This root phenotype reverts to wild-type when sucrose, glucose, fucose or mannose are added to the growth medium. By contrast, no effect on the root phenotype of the mutant is discernible when arabinose, xylose, or galactose are added. We isolated cell walls from the *fut6* mutant and showed by glycosyl residue composition analyses that the amounts of fucose were reduced in fractions enriched with arabinogalactan and pectic polysaccharides. No differences in glycosyl residue compositions were detected in the fractions enriched in xylan and XyG. However, a much higher concentration of alkali (6M KOH as opposed to 4M KOH for wild-type XyG) was required to solubilize the XyG from the mutant walls. Such a result suggested that the mutation results in XyG being more tightly bound into the wall. All of the wall chemotypes can be complemented by expression of the Arabidopsis *FUT6* gene in the *fut6-1* mutant. Taken together, our data suggested that AtFUT6 is a glycosyltransferase involved in the fucosylation of arabinogalactans and/or pectins and that cell wall organization is altered in plants carrying mutations in *FUT6*.

10. The trichome cell walls of Arabidopsis

Trichomes are small outgrowths on the outer surfaces of plants that vary largely in their appearance and function. Trichomes often provide the plant with protection against pests and herbivores and may also help to reduce water loss [23, 24]. The leaf trichomes of *Arabidopsis thaliana* are typically single epidermal cells that elongate away from the leaf surface to create a stalk, which extends further to form three or four symmetrically arranged branches [24]. These trichomes generally have much thicker cell walls than the primary walls of cells present in other plant organs. The Arabidopsis leaf trichome has become established as a model system to study cell fate and morphogenesis in plants [23]. Indeed, methods for isolating trichomes in quantities sufficient for gene expression profiling studies have been developed [25, 26]. The Arabidopsis trichome may also provide a model system for studying cell-specific wall synthesis

and deposition. However, the amounts of trichome that can be readily obtained are typically not sufficient for detailed cell wall analyses using classical chemical and enzymic methods [26].

To obtain insight into the types of polysaccharides present in trichome cell walls, we undertook a detailed immunohistochemical study of *Arabidopsis* leaf trichomes using our toolkit of cell wall glycan-directed monoclonal antibodies [14; see Figure 1].

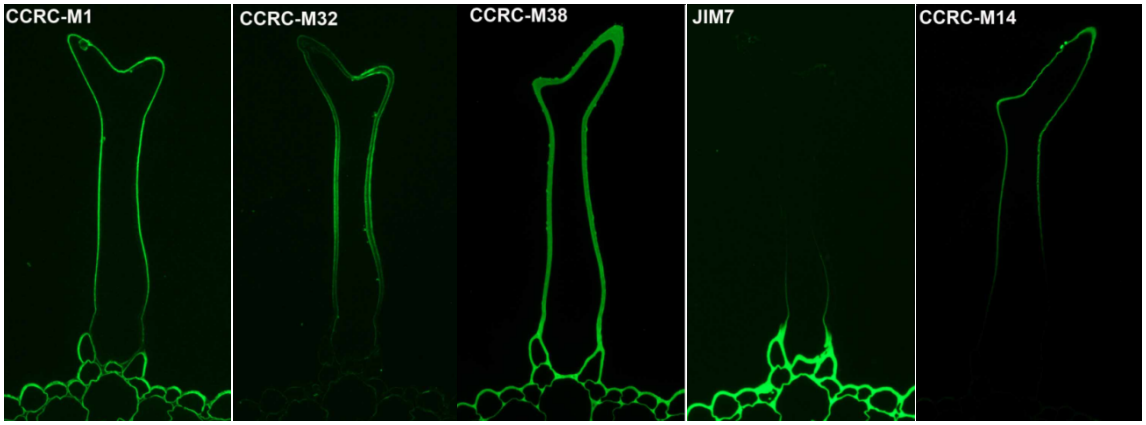

Figure 1. Immunolabeling of Arabidopsis leaf trichomes with glycan-directed monoclonal antibodies. CCRC-M1 recognized fucosylated XyG; CCRC-M32 recognized pectic arabinogalactan; CCRC-M38 recognized de-esterified homogalacturonan; JIM7 recognized highly methyl-esterified homogalacturonan; CCRC-M14 recognized rhamnogalacturonan backbone.

Our studies documented a complex architecture of trichome cell walls, and suggested a correspondingly complex regulatory network that controls the synthesis and assembly of these walls. Specifically, trichome walls are rich in pectic polysaccharides and essentially devoid of xylans. Furthermore, different cell wall polysaccharide epitopes are localized to particular domains in trichome walls. For example, we found that homogalacturonans with a high degree of methyl esterification were present exclusively in an inner wall layer at the base of the trichome, whereas de-esterified homogalacturonans were present throughout the trichome cell wall. By contrast, rhamnogalacturonan I with little if any branching was localized to the trichome branches and upper parts of the trichome stalk. Pectic arabinogalactan epitopes were localized in two distinct layers of trichome walls, while fucosylated XyGs were localized exclusively to the outermost layer of trichome walls. Taken together the results of our immunocytochemical studies suggested that trichomes do indeed provide a valuable experimental system to identify genes involved in cell wall deposition and in pectic polysaccharide biosynthesis in particular.

11. Surveillance and response to microbial infection by recognition of cell wall-degrading enzymes

In collaboration with Robert Thornburg (Iowa State University), we identified and characterized previously unknown downstream effects that occur in tobacco nectar when a fungal xyloglucan-specific endoglucanase (XEG) binds to an XEG inhibitor protein (XEGIP) [27]. The XEGIP family includes NEC4 a tobacco nectar protein and other so-called extracellular dermal glycoproteins (EDGPs) present in diverse plant tissues and cell cultures [28]. These proteins have been implicated in a plant's defense against pathogens, in part due to their location in dermal tissues, which are the initial site of microbial infection [27, 28]. We showed *in vitro* that NEC4 bound to XEG and that the complex formed induced an increase in the glucose oxidase activity of NEC5, a BBE homolog that is abundant in tobacco nectar [29]. This led to a large

increase in the H_2O_2 and gluconic acid content of the nectar. Since gluconic acid binds transition metal ions, it may modulate redox reactions involving these ions. Thus, changes in the concentration of gluconic acid likely affect reactive oxygen species (ROS) metabolism in tobacco nectar.

To gain additional insight into the NEC4-XEG-induced activation of plant defense responses, we used qRT-PCR analysis to show that adding the purified NEC4-XEG complex to suspension-cultured tobacco cells induces a 6-fold increase in the expression of an Avr9/CF-9 rapidly elicited gene (ACRE-189, AY220479). This gene has been shown to be involved in the hypersensitive response to pathogen attack [30]. When XEG alone was added to the cell cultures, the expression of ACRE-189 also increased six fold. We previously showed that formation of the NEC4-XEG complex virtually abolishes the endoglucanase activity of the XEG [27]. In our gene expression experiments, no residual XEG activity was detected after the enzyme was added to the cell cultures, due to the inhibition of XEG by the XEGIP produced by the tobacco cells. Thus, up-regulation of ACRE-189 did not result from XEG-catalyzed fragmentation of XyG present in the cell wall or in the growth media. This data is again consistent with our hypothesis that the interactions between EDGPs (such as XEGIP and NEC4), BBE homologs (such as NEC5) and fungal glycanases (such as XEG) lead to activation of plant defense responses.

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