Final Report:

Grant No. DE-SC0008376 (DE-FG02-12ER16326)

Recipient: University of Georgia, Athens

Project Title: Identification and characterization of glycosyltransferases involved in the synthesis of the side chains of the cell wall pectic polysaccharide rhamnogalacturonan II

PI: Malcolm A O'Neill

CoPIs: Maor Bar-Peled and Michael G Hahn

Executive Summary

Our goal was to gain insight into the genes and proteins involved in the biosynthesis of rhamnogalacturonan II (RG-II), a borate cross-linked and structurally conserved pectic polysaccharide present in the primary cell walls of all vascular plants. The research conducted during the funding period established that (*i*) Avascular plants have the ability to synthesize UDP-apiose but lack the glycosyltransferase machinery required to synthesize RG-II or other apiose-containing cell wall glycans. (*ii*) RG-II structure is highly conserved in the Lemnaceae (duckweeds and relatives). However, the structures of other wall pectins and hemicellulose have changed substantial during the diversification of the Lemnaceae. This supports the notion that a precise structure of RG-II must be maintained to allow borate cross-linking to occur in a controlled manner. (*iii*) Enzymes involved in the conversion of UDP-GlcA to UDP-Api, UDP-Xyl, and UDP-Ara may have an important role in controlling the composition of duckweed cell walls. (*iv*) RG-II exists as the borate ester cross-linked dimer in the cell walls of soybean root hairs and roots. Thus, RG-II is present in the walls of plants cells that grow by tip or by expansive growth. (*v*) A reduction in RG-II cross-linking in the maize *tls1* mutant, which lacks a borate channel protein, suggests that the growth defects observed in the mutant are, at least in part, due to defects in the cell wall.

Background

RG-II is a structurally conserved, complex pectic polysaccharide present in the primary cell walls of all vascular plants, where it exists as a borate ester cross-linked dimer. Mutations that result in altered RG-II structure or decreased borate cross-linking of RG-II are lethal or severely impair plant growth. Nevertheless, few of the genes and proteins involved in RG-II synthesis have been identified and functionally characterized. This has limited our ability to understand how RG-II structure and cross-linking contributes to the formation of a functional cell wall that is required for normal plant growth and development.

UDP-Apiose synthases in avascular plants

In our original proposal we hypothesized that UDP-apiose (UDP-Api) is a key factor in controlling RG-II synthesis and its availability may also affect the amounts of RG-II formed by a plant. Apiose is a key component of RG-II as it links two of the four oligosaccharide side chains to the homogalacturonan backbone and is involved in borate cross-linking of two RG-II molecules.

RG-II is present in the cell walls of all vascular plants analyzed to date. However, its occurrence in the walls of avascular plants (mosses, liverworts and hornworts) remains a subject of debate. We hypothesized that the appearance of functional UDP-Api synthase (UAS) was one of the critical events in the appearance of RG-II and the evolution of vascular plants. To obtain experimental data to substantiate or negate this hypothesis we used a combination of comparative genomics, molecular biology, and biochemical methods to determine if avascular plants contain homologs of UAS and if they can synthesize UDP-Api.

UAS-like homologs with >70% amino-acid sequence identity to Arabidopsis UAS1 were identified in mosses, liverworts, hornworts using publically available data from the 1,000 Plants Project and the Phytozome genomics portal. We generated recombinant versions of the proteins by expressing synthetic versions of the avascular plant genes and the native gene from the duckweed *Spirodela polyrhiza* in *Escherichia coli.* The recombinant proteins were shown by ESI mass spectrometry and ^{1}H-NMR spectroscopy to form UDP-Api from UDP-GlcA, albeit in different amounts. Thus, we have demonstrated that avascular plants do indeed have the ability to produce UDP-Api. No discernible amounts of apiose or other RG-II-specific glycoses were present in the cell walls of these plants or in the moss *Physcomitrella patens* that had been engineered to over-express Spirodela UAS. However, we did detect apiose in aqueous methanol extracts of growing tissues from the avascular plants and increased amounts of apiose in the transgenic *P. patens*. We conclude that avascular plants likely synthesize apiose-containing secondary metabolites but lack the glycosyltransferase machinery required to synthesize apiose-containing cell wall glycans

including RG-II. The results of this study have now been accepted for publication (Smith et al., 2016).

RG-II and Apiogalacturonans in the cell walls of the Lemnaceae

A goal of the original proposal was to generate oligosaccharides that can serve as acceptors for potential glycosyltransferases involved in RG-II synthesis. Specifically, we wished to target the rhamnosyltransferase(s) that catalyzes the formation of the Rha*p*-(1,3')-Api*f* linkage in RG-II. The Api*f* is itself linked to *O*-2 of the galacturonan backbone.

The cell walls of *S. polyrhiza* and *Lemna minor* (duckweeds) have been shown to contain relatively large amounts of apiogalacturonan - the Api*f* is linked to *O*-2 of the galacturonan backbone. To determine if these or other members of the duckweed family produce apiogalacturonans suitable for the generation of rhamnosyltransferase acceptor molecules, we analyzed the cell walls of selected members of the Lemnaceae (duckweeds and their relatives). The plants analyzed included members of the sub-family Lemnoideae (*S. polyrhiza, Landoltia punctata, L. minor, Lemna gibba, Lemna obscura, Lemna aequinoctialis, Lemna tenera, Lemna minuta, and Lemna yungensis*) and the sub-family Wolffioideae (*Wolffiella lingulata*, *Wolffiella repanda, Wolffiella welwitschii, Wolffia borealis, Wolffia arrhiza, Wolffia globosa, and Wolffia brasiliensis*). These studies indicate that Lemnoid apiogalacturonans provide a potential source of acceptor substrates. However, many challenges still remain in the heterologous expression of functional rhamnosyltransferases and other glycosyltransferase involved RG-II synthesis.

Somewhat unexpectedly, our glycosyl residue composition analyses revealed that Lemnoideae walls contain three to six-fold more apiose than the walls of the Wolffioideae. Nevertheless, RG-II structure is highly conserved in the duckweeds where it exists as the borate cross-linked dimer. RG-II is readily released by endopolygalacturonase (EPG)-treatment of the oxalate extracts of Wolffioids walls. EPG treatment of Wollfia walls also released fragments of xylogalacturonan in addition to oligogalacturonides. By contrast, EPG treatment released little RG-II from the comparable extracts of Lemnoid walls. Rather, the sugars characteristic of RG-II were present mainly in a high-molecular weight, apiose-rich EPG-resistant material. Such data suggest that RG-II is linked to an EPG-resistant apiose-rich pectin in the walls of the Lemnoids but is linked to an EPG-susceptible pectin in the walls of the Wolffioids.

We found that Lemnoid and Wolffioid walls contain similar amounts of fucose, rhamnose, galactose and galacturonic acid whereas Wolffioid walls contain somewhat more arabinose and xylose than their Lemnoideae counterparts. Thus it is likely that enzymes that convert UDP-GlcA to UDP-Api, UDP-Xyl, and UDP-Ara, which are sugar donors used for the biosynthesis of diverse

cell wall polysaccharides including RG-II, have an important role in determining the types of polysaccharides present in a plants primary cell wall.

In a separately funded study (Grant No. DE-FG02-12ER16324, 2014 project report), we have identified substantial differences in the structures of the xyloglucan and 4-*O*-methyl glucuronoxylan, which are two hemicellulosic polysaccharides present in the primary walls of the Lemnoids and Wolffioids. Many of the structural differences in pectin and hemicellulose are correlated with the phylogeny of the Lemnaceae. Thus, this small family of aquatic monocots provide a unique opportunity to study the molecular and cellular factors that control changes in cell wall structure as vascular plants evolved. Manuscripts describing our structural studies of duckweed pectin and hemicellulose are now in preparation.

A fraction enriched in RG-II oxidizes 3,3'5,5',tetramethylbenzidine

Somewhat unexpectedly we found that RG-II (or a molecule that co-elutes with RG-II during size-exclusion chromatography) in the presence of hydrogen peroxide oxidizes the colorless 3,3'5,5',tetramethylbenzidine (TMB) to its blue-colored derivative. We also observed this reaction with RG-II isolated from red wine, from the walls of suspension-cultured sycamore and tobacco cells, from Arabidopsis leaves and an RG-II-containing fraction from the duckweed *Spirodella*. To address the possibility that RG-II is associated either specifically or non-specifically with a cell wall peroxidase and that the two molecules co-elute on the sizing column, RG-II was chromatographed on anion and cation-exchange columns. The RG-II and oxidation of TMB co-chromatographed on the cation exchange column. However, RG-II and the material responsible for the oxidation of TMB did not co-elute when fractionated on an anion-exchange column showing that the oxidative activity is not due to RG-II itself. Nevertheless, the possibility still exist that RG-II is closely associated with a wall-localized peroxidase.

RG-II in soybean root and root hair cell walls

In collaboration with Russel Carlson at the CCRC and Gary Stacey at the University of Missouri we determined if there are differences in the cell wall compositions of soybean (*Glycine max*) root hairs and soybean roots stripped of root hairs.

Three major peaks corresponding to RG-I, RG-II, and oligogalacturonides were obtained by fractionating the material released by endopolygalacturonase treatment of root hair and root cell walls on a Superdex SD-75 SEC. At least 95% of the RG-II existed as the borate ester cross-linked dimer irrespective of tissue source. Moreover, the glycosyl residue compositions of the RG-II from roots and hairs were indistinguishable. By contrast, the RG-I-enriched fraction from root

hair and roots did not have identical glycosyl residue compositions. The ratios of bvackbone sugars (Rha + GalA) to sidechain sugars (Ara + Gal) suggests that root hair wall RG-I contains more neutral sidechains than the corresponding polysaccharide from root cell walls.

No differences were found in the structures RG-I and RG-II from root hair and root cell walls of soybean plants grown in the presence and absence of its nitrogen fixing symbiont *Bradyrhizobium japonicum*. It is known that only a small fraction of root hairs respond to *B. japonicum* or deform in response to lipo-oligosaccharide nodulation factors and that mechanisms have evolved in legumes to limit the number of nodules formed. Thus, we suspect that any changes in wall composition that occur as a consequence of the root and bacterium interaction are likely to have been diluted to below detectable levels by the presence of non-responding tissue. Nevertheless, our study is the first to provide and in-depth comparison of the types of pectic and hemicellulosic polysacchairides in root and root hair cell walls. The hemicellulosic components of these walls are described in the 2014 project report Grant No. DE-FG02-12ER16324. The cumulative results of these studies have been published (Muszinski et al., 2015).

Borate cross-linking in the *tassel-less1* (*tsl1*) maize mutant

We collaborated with Paula McSteen at the University of Missouri who is studying the maize *tassel-less1* (*tls1*) mutant. Positional cloning established that *tls1* encodes a protein in the aquaporin family and is co-orthologous to the Arabidopsis borate channel protein AtNIP5;1. The *tls1* mutant has defects in inflorescence and vegetative development that are similar to the growth defects observed when maize is grown with insufficient boron.

To determine if the *tls1* mutation affects borate cross-linking of RG-II, cell walls were isolated from the leaves and tassels of wild type and *tls1* plants. The de-starched walls were then treated with EPG and the material solubilized then analyzed by size-exclusion chromatography on a Superdex-75 column. Approximately 65% of the RG-II was cross-linked in the immature wild type tassels, whereas only 38% of the RG-II was cross-linked in the tassels of the *tls1* mutant. The ~42% reduction in cross-linking is comparable to the reduction in B concentration in immature *tls1* tassels compared to wild type. The reduction of RG-II dimers in *tls1* mutant tassels suggests that the defects observed in mutants are, at least in part, due to defects in the cell wall. At least 90% of the RG-II existed as the dimer in the leaves of wild type and *tls1* plants. As RGII dimerization is known to occur when sufficient B is present, it is likely that mutant leaves contained sufficient amounts of B for near complete cross-linking of RGII to occur. By contrast the more

severe B deficit in mutant tassels likely prevented complete RG-II dimerization. The results of this study have been published (Durback et al., 2014).

Publications resulting from funding

Durbak AR, Phillips KA, Pike S, O'Neill MA, Mares J, Gallavotti A, Simon T. Malcomber ST, Gassmann W, McSteen P. (2014) Transport of Boron by the tassel-less1 Aquaporin Is Critical for Vegetative and Reproductive Development in Maize. *Plant Cell* **26**:2978.

Muszyński A, O'Neill MA, Ramasamy E, Pattathil S, Avci U, Peña MJ, Libault M, Shakhawat Hossain MD, Brechenmacher L, York WS, Barbosa RM, Hahn MG, Stacey G, Carlson RW (2015) Xyloglucan, galactomannan, glucuronoxylan, and rhamnogalacturonan I do not have identical structures in soybean root and root hair cell walls. *Planta* **242**: 1123.

Smith JA, Yang Y, Levy S, Adelusi OO, Hahn MG, O'Neill MA, Bar-Peled M (2016) Functional characterization of UDP-apiose synthases from avascular plants and green algae provides insight into the appearance of apiose-containing glycans. *J Biol Chem*. Accepted for publication.