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Bi-functional glycosyltransferases catalyze both extension and termination of pectic galactan oligosaccharides

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Running head: Guiding pectic galactan biosynthesis
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Summary

Pectins are the most complex polysaccharides of the plant cell wall. Based on the number of methylations, acetylations, and glycosidic linkages present in their structures, it is estimated that up to 67 transferase activities are involved in pectin biosynthesis. Pectic galactan constitute a major part of pectin in the form of side chains of rhamnogalacturonan-I. In Arabidopsis, Galactan Synthase 1 (GALS1) catalyzes the addition of galactose units from UDP-Gal to growing β-1,4-galactan chains. However, the mechanisms for obtaining varying degrees of polymerization remain poorly understood. In this study, we show that ArGALS1 is bifunctional, catalyzing both the transfer of galactose from UDP-α-D-Gal and the transfer of an arabinopyranose from UDP-α-L-Ara, to galactan chains. The two substrates share a similar structure, but UDP-α-D-Gal is the preferred substrate, with a tenfold higher affinity. Transfer of Ara to galactan prevents further addition of galactose residues, resulting in a lower degree of polymerization. We show that this dual activity occurs both in vitro and in vivo. The herein described bi-functionality of ArGALS1 may suggest that plants can produce the incredible structural diversity of polysaccharides without a dedicated glycosyltransferase for each glycosidic linkage.

Introduction

The plant cell wall is a highly complex composite of polysaccharides, lignin, and glycoproteins with an estimated 10% of the genome involved in its biosynthesis, secretion, assembly, and remodeling (McCann and Rose 2010). Despite recent advances in our understanding of cell wall structure (Dick-Perez et al. 2011), the identification and characterization of the enzymes involved in its biosynthesis remain limited (Atmodjo et al. 2013). In order to engineer the cell walls of crop plants to obtain increased nutritional value or produce biofuel and plant derived chemicals, we need to both expand our knowledge of how the plant cell wall is biosynthesized and elucidate the mechanisms governing its regulation.

Pectin, the most complex class of polysaccharides in nature, comprises up to 35% of the primary cell wall of dicots. Pectin is composed of four major domains: homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and xylogalacturonan (XG) (Harholt et al. 2010). Based on the types of methylations, acetylations, and glycosidic linkages found in pectin, it has been estimated that around 67 different transferases are required for its biosynthesis (Mohnen 2008, Caffall and Mohnen 2009). RG-I consists of alternating α-L-rhamnose and α-D-galacturonic.
acid residues in a backbone of the alternating disaccharide repeat of \[\text{-}(4\rightarrow3)\text{-GalA}\text{-}(1\rightarrow2)\text{-}\alpha\text{-L-Rha}\text{-}1\] moieties substituted with galactan, arabinan and arabinogalactan side chains (Harholt et al. 2010). Pectic galactan is mainly composed of linear \(\beta\text{-}(1\rightarrow4)\) linked galactose units (Figure 1) although Type II arabinogalactans with \(\beta\text{-}(1\rightarrow3)\) and \(\beta\text{-}(1\rightarrow6)\) linked galactose may also be present. Some \(\beta\text{-}(1\rightarrow4)\) galactan side chains of the RG-I backbone have been shown to contain, in addition to galactose residues, an occasional internal arabinofuranose (Ara\(_f\)) residue, and the galactan sidechains may also be branched with Ara\(_f\) or terminated with arabinopyranose (Ara\(_p\)) residues (Huisman et al. 2001) (Figure 1). Galactose is a hexose amenable for fermentation, hence, biosynthesis of galactan has been a target for optimizing feedstocks for biofuel production (Gondolf et al. 2014). Terminal Ara\(_p\) has been suggested to cap and terminate the extension of galactan (Ishii et al. 2005). Therefore, in order to increase the amount of galactan and reduce the amount of arabinose, it is essential to understand the biological function of the different galactan structures and to identify the enzymes involved in their biosynthesis and regulation.

Biosynthesis of pectin takes place in the Golgi and therefore depends on the availability of substrates. Nucleotide sugars, produced through de novo and salvage pathways, are interconverted by the action of enzymes primarily located in the cytosol (Bar-Peled and O'Neill 2011). Nucleotide sugars are then transported into the Golgi by highly specific nucleotide sugar transporters. In the Golgi lumen, glycosyltransferases catalyze the transfer of sequential sugar moieties to the growing pectic polysaccharides. Biosynthesis of galactan and arabinan with exogenous oligosaccharide substrates was characterized using hypocotyl microsomal fractions from etiolated mung bean (Vigna radiata) seedlings. These studies characterized the sequential transfer of Ara\(_f\) (Konishi et al. 2006) and single addition of Ara\(_p\) to \(\alpha\text{-L}(1\rightarrow5)\)-arabinooligosaccharide substrates (Nunan and Scheller 2003, Ishii et al. 2005), as well as sequential transfer of Gal (Ishii et al. 2004) and single addition of Ara\(_p\) to \(\beta\text{-D}(1\rightarrow4)\)-galactooligosaccharide substrates (Ishii et al. 2005). More recently, enzymes capable of galactan biosynthesis were characterized in Arabidopsis thaliana. UDP-Gal is transported into the Golgi lumen by the UDP-Rha/UDP-Gal Transporters (URGTs) (Rautengarten et al. 2014) where extension of \(\beta\text{-}(1\rightarrow4)\) linked galactan is catalyzed by Galactan Synthase 1 (GALS1) (Liwanag et al. 2012). The biosynthetic enzymes catalyzing transfer of Ara to galactan have not been identified. Arabinose is mainly found in plant glycans in the furanose ring configuration (Ara\(_f\)) derived from the pyranose precursor (Ara\(_p\)). Hence, most arabinosyltransferases are expected to catalyze transfer of Ara\(_f\). ARAD proteins (ARAD1 and 2) form a multiprotein complex (Harholt et al. 2012). These are the only transferases currently known to be involved in arabinan biosynthesis (Harholt et al. 2006). However, their biochemical activities have not been demonstrated in vitro.

Pectic polysaccharides are deposited early in the primary cell wall of growing and dividing cells, leading to a middle lamella that is rich in pectin (Keegstra 2010). Although the exact mechanism remains elusive, pectin plays a role in regulating cell growth and extension (Derbyshire et al. 2007).
Pectin was initially thought to provide a soft matrix in which cellulose fibers were embedded, however, increasing data suggest direct interactions to cellulose (Wang et al. 2015) and xyloglucan (Popper and Fry 2008). To date, most data indicate the importance of HG for coordination between pectin and cellulose (Chebli and Geitmann 2017). Methylation of HG changes the cross-linking between polymers and thereby the accessibility for polygalacturonases to cleave pectin in response to expansion (Xiao et al. 2014). Furthermore, neutral sugar side chains such as galactan and arabinan have been shown to non-covalently bind to cellulose microfibrils, and play a role in cross-linking the microfibrils of primary cell walls (Zykwinska et al. 2005, Zykwinska et al. 2007). The arabinan chain length of RG-I, but not galactan, is involved in pollen cell wall development (Cankar et al. 2014). Thus, reorganization of the primary cell wall during plant growth requires modification of pectin. However, the role of galactan and its degree of polymerization (DP) both remain unknown.

In this study, we show that ArGALS1 is bi-functional, catalyzing the extension of β-(1,4) galactan as well as the termination of extension by addition of Araβp. These counter-acting activities were affected by the availability of nucleotide sugar donors and hence provide a mechanism to alter the formation of specific galactan oligosaccharides. Furthermore, the bi-functionality of GALS1 challenges the paradigm that each glycosidic linkage requires a dedicated transferase. In the case of GALS1, the two substrates are quite similar (Figure S1). We propose that complex cell wall polysaccharides may be biosynthesized by fewer enzymes than initially anticipated (Mohnen 2008, Caffall and Mohnen 2009) and that the structure of wall polysaccharides may be influenced by the availability of substrates.

Results

Establishing a method for Polysaccharide Analysis using Carbohydrate gel Electrophoresis (PACE)

In order to study the biosynthesis of the different β-(1,4) galactan structures (Figure 1) we labeled chemically synthesized galactooligosaccharide substrates with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) at the reducing end (Figure 2A). Initially, we employed the well-characterized GALS1 from Arabidopsis thaliana (Liwanag et al. 2012) to validate whether labeled galactan substrates (Gal3, Gal4, Gal5, Gal6, Gal7 and Gal6Ara1) were appropriate substrates for activity studies. Crude microsomes from Nicotiana benthamiana leaves transiently expressing ArGALS1 with an N-terminal FLAG peptide were used as a source of GALS activity. Immunoblot analysis using anti-FLAG antibody verified the expression of ArGALS1 as compared to control plants expressing p19 alone (Figure 2B). Subsequently, we established the method for Polysaccharide Analysis using Carbohydrate Electrophoresis (PACE) to monitor the activity of ArGALS1, which allowed for the separation of products based on charge and degree of polymerization (DP) (Goubet et al. 2002, Mortimer et al. 2015). Administration of the different galactan substrates with and without UDP-Gal
as the donor sugar revealed a preferential activity of *ArGALS1* towards longer substrates (Gal$_{4–7}$), generating products with a maximum detected DP of approximately 11 (Figure 2C). It is likely that galactans of DP greater than 11 may be synthesized at higher enzyme concentrations and/or substrate concentration or longer reaction times, although this remains to be determined. Branched galactan substituted with an α-(1,6) linked Ara$_p$ at the fourth Gal-position (Gal$_5$Ara$_4$) did not serve as substrate for *ArGALS1* (Figure 2C).

**Screening of nucleotide sugar donors using mung bean microsomes as model system**

We utilized the PACE assay to study the biosynthesis of β-(1,4) galactan structures (Figure 1) using ANTS labeled galactan substrates (Figure 2A). Previous arabinan and galactan activity studies have been performed using microsomes from etiolated mung bean seedlings. Therefore, we initially verified previously detected activities from mung bean microsomes using Gal$_5$ as acceptor substrate and UDP-Gal, UDP-Ara$_p$, or UDP-Ara$_p$, as donor sugars. Under the conditions tested, we detected a ladder of products upon addition of UDP-Gal with a maximum oligosaccharide of DP 11 (Figure 3A). Administration of UDP-Ara$_p$ yielded a single product slightly below Gal$_6$ (Figure 3A). However, administration of UDP-Ara$_p$ did not result in considerable product formation; hence we were unable to detect the formation of galactan products corresponding to internal or branched Ara$_p$ as previously characterized in soybean pectin (Huisman et al. 2001) (Figure 1, structures b and c). Terminal Ara$_p$ has been suggested to cap and terminate the extension of galactan (Ishii et al. 2005) (Figure 1, structure d). Therefore, we performed a sequential incubation with UDP-Ara$_p$, followed by the addition of UDP-Gal or UDP-Ara$_p$ (Figure 3B). After the two hour initial incubation we supplemented UDP-Gal or UDP-Ara$_p$ to the reactions. Galactan products with the Ara$_p$ did not serve as substrate when UDP-Gal was added as donor sugar, as illustrated by the accumulation of Gal$_5$Ara$_1$. The formation of extended galactan oligosaccharides was derived from un-metabolized Gal$_5$ substrate. Extension of the Gal$_5$Ara$_1$ products would have resulted in products with a slightly altered retention compared to Gal$_{10}$ products due to the presence of Ara$_p$. Furthermore, no decrease in the initially-formed Gal$_5$Ara$_1$ was observed during the subsequent incubation with UDP-Gal. This suggests a fundamental mechanism to affect the DP of galactan by addition of Ara$_p$ to terminate extension (Figure 3B).

*ArGALS1* is bi-functional catalyzing reactions with both UDP-Gal and UDP-Ara$_p$

As the only characterized glycosyltransferase involved in galactan biosynthesis (Liwanag et al. 2012), we tested the ability of *ArGALS1* to catalyze the transfer of Gal$_p$, Ara$_p$ and Ara$_p$ to galactan substrates. As previously shown (Figure 2C), addition of UDP-Gal onto a galactooligosaccharide acceptors of DP ≥ 4 resulted in formation of a ladder of products with a maximum oligosaccharide product DP of 11 (Figure 3C). Interestingly, we consistently observed a predominant formation of Gal$_5$ products. Upon administration of UDP-Ara$_p$, *ArGALS1* catalyzed the formation of galactan with a single Ara$_p$ residue (Figure 3C). UDP-Ara$_p$ did not serve as donor sugar when Gal$_5$ was used as acceptor...
substrate. ArGALS1 displayed a similar specificity for long galactan substrates with UDP-Ara, as donor substrate (Figure S2) compared to UDP-Gal (Figure 2C). In order to validate the biological significance of the UDP-Ara, activity of ArGALS1 we determined the kinetic parameters for both UDP-Gal and UDP-Ara, using Gal₅ as substrate. Both substrates followed sigmoidal kinetics, which were best fitted to the Hill equation (Figure 3D,E) compared to a hyperbolic fit for enzymes following Michaelis-Menten kinetics based on the Chi²-test (Figure S3). Sigmoidal kinetics suggests cooperativity related to multiple binding sites or formation of enzyme oligomers (Porter and Miller 2012). The activity for both UDP-Gal and UDP-Ara, yielded comparable Vmax values of 656 and 454 pmol min⁻¹ mg⁻¹ microsomal protein, respectively. For sigmoidal fits the apparent substrate affinity is described by the substrate concentration at half-maximum velocity (Kₐ₅), which was determined to be 142 and 1057 μM for UDP-Gal and UDP-Ara, respectively (Figure 3D,E). Furthermore, the cooperativity of the two donor sugars was evaluated based on the Hill coefficient (n), which describes cooperativity for n > 1 (Weiss 1997). The cooperativity for UDP-Gal showed n = 1.92 ± 0.28 indicating two binding sites for UDP-Gal. For UDP-Ara, n = 1.19 ± 0.18 indicating a single binding site for UDP-Ara, and no significant cooperativity even though the sigmoidal fit was better than the hyperbolic fit. Since the sequence of ArGALS1 does not indicate the presence of multiple binding sites, the sigmoidal kinetics more likely indicates a multimeric enzyme complex. Both catalytic activities were dependent on divalent cations as tested by the addition or exclusion of Mn²⁺ (Figure 3F).

**Nucleotide sugar availability affects galactan degree of polymerization**

Based on the in vitro experiments described above, we hypothesized that GALS1 is bi-functional, catalyzing the addition of both Gal and Ara, to growing galactan chains with DP > 3 (Figure S2), and that these counter-acting activities are favored by the available pool of donor sugars. We predicted that alteration of the concentration of UDP-Ara, and UDP-Gal in vivo would alter the frequency at which GALS integrates Ara, into pectic galactans. In order to study the physiological role of GALS1 on pectic galactan DP, we established an in planta setup for altering the intracellular nucleotide sugar levels. In plants, nucleotide sugars are typically derived from UDP-Glc generated through photosynthesis and the biosynthesis involves a complex grid of interconversions (Bar-Peled and O’Neill 2011). However, salvage pathways allow uptake of specific sugars including D-galactose and L-arabinose, which are directly converted into UDP-Gal and UDP-Ara, respectively (Bar-Peled and O’Neill 2011). It has previously been shown that growth of Arabidopsis seedlings on media supplemented with arabinose as a carbon source affects the intracellular concentration of UDP-Ara (Behmuller et al. 2016). To examine the effects of UDP-Ara and UDP-Gal concentration in the nucleotide sugar pool on the biosynthesis of galactans, we grew Arabidopsis seedlings in the dark on media supplemented with sucrose, D-galactose or L-arabinose as the sole carbon source. To test whether possible effects were due to the activity of GALS1 we compared Arabidopsis seedlings of...
wild type (Col-0) and GALS1 overexpresser (OE) plants. Seedlings grown with sucrose as the carbon source served as a control for further analyses (Figure 4A). Seedlings grown on D-galactose plates displayed similar growth with a slight decrease in height for GALS1 OE seedlings compared to Col-0. Both Col-0 and GALS1 OE displayed stunted root growth on D-galactose plates as compared to growth on sucrose. Likewise, seedlings grown on L-arabinose plates displayed stunted root growth and with a significant decrease in seedling height for GALS1 OE (Figure 4B). The intracellular nucleotide sugar composition of GALS1 OE seedlings grown on sucrose, D-galactose or L-arabinose was quantified by LC-MS/MS and normalized to the seedling fresh weight (Figure 4C). The UDP-Gal/UDP-Glc ratio in seedlings increased from approximately 0.25 when grown on sucrose to 1.95 when grown on D-galactose (Table 1). In contrast, UDP-Gal/UDP-Glc remained unaffected for seedlings grown on L-arabinose plates compared to sucrose plates. However, the UDP-Ara_p/UDP-Glc ratio increased from 0.06 to 0.09 and 0.38 for seedlings grown on sucrose, D-galactose and L-arabinose, respectively (Table 1). These effects correlated with the dramatic increase in intracellular UDP-Gal when seedlings were grown on D-galactose plates and UDP-Ara_p when seedlings were grown on L-arabinose plates. Hence, these sugars were efficiently taken up by the seedlings and converted into activated nucleotide sugars by the salvage pathways (Bar-Peled and O’Neill 2011).

**Nucleotidie sugar levels control galactan polymerization mediated by bi-functional GALS**

We initially validated the effects of altered intracellular nucleotide sugar concentration (Figure 4C) on cell wall polysaccharide composition by measuring the total monosaccharide composition of alcohol insoluble residue (AIR) from Col-0 and GALS1 OE. Increasing the intracellular concentration of UDP-Gal and UDP-Ara_p resulted in an increase in total galactose and arabinose content, respectively (Figure S4). We proceeded to analyze the effects on the monosaccharide composition of pectic galactan in both Col-0 and GALS1 OE Arabidopsis seedlings. Galactan was digested by treating the AIR material with endo-β-(1,4) galactanase. GALS1 OE seedlings grown on D-galactose plates displayed a significant increase in galactose released by the galactanase treatment, compared to Col-0 and to growth on sucrose media (Figure 5). In contrast, GALS1 OE seedlings grown on L-arabinose plates displayed an increase in solubilized arabinose and a significant decrease in galactose, corresponding to a reduction in galactan production. This supports the hypothesis that addition of Ara_p to galactan serves to terminate extension of the galactan resulting in reduced DP at high concentrations of UDP-Ara_p. We found similar trends for Col-0 seedlings, although the changes in galactan content were not statistically significant. In order to further study galactan DP, we performed glycosyl linkage analysis on isolated RG-I from GALS1 OE seedlings grown on sucrose, D-galactose and L-arabinose (Table 2). DP was evaluated based on the amounts of 1,4-linked galactose (4-Gal), terminal galactose (t-Gal), 1,2-linked rhamnose (2-Rha) and 1,2,4-linked rhamnose (2,4-Rha). We were unable to quantify terminal arabinopyranose. Therefore, based on this study we cannot conclude that the Ara_p is added to the terminal galactose unit of galactans. The ratio 4-Gal/t-Gal represents an
estimate of galactan DP containing a terminal galactose unit. Since galactan is a substitution of 2,4-Rha, the ratio 4-Gal/2,4-Rha represents an estimate of the average galactan DP. We want to emphasize that these calculations make several assumptions and are meant for comparison rather than accurate estimates of DP. Seedlings grown on D-galactose plates displayed a dramatic increase in galactan DP with 11.8 4-Gal/t-Gal and 36.1 4-Gal/2,4-Rha compared to 3.0 and 4.6, respectively, for seedlings grown on sucrose (Table 2). In contrast, seedlings grown on L-arabinose plates displayed a reduction in galactan DP with 0.5 4-Gal/t-Gal and 2.0 4-Gal/2,4-Rha (Table 2). This further supports that the specific activity of GALS1 depends on substrate availability and is capable of utilizing both UDP-Gal and UDP-Ara_p under physiological conditions in vivo.

Discussion

Bi-functional galactosyltransferases as a mechanism to obtain galactan diversity

Based on the number of different methylations, acetylations, and glycosidic linkages, biosynthesis of pectin is thought to involve more than 67 transferase activities (Mohnen 2008, Caffall and Mohnen 2009). This notion is based on the paradigm that each glycosidic linkage requires a dedicated transferase enzyme, but in reality, few of the enzymes have been characterized and it is not clear how broad their specificities are. Here, we present evidence that the structural diversity may be facilitated by glycosyltransferases that can utilize more than one donor nucleotide sugar. Our in vitro data provides strong evidence for the bi-functionality of GALS1. GALS1 catalyzes the extension of growing galactan chains, however, in the presence of UDP-Ara_p, GALS1 transfers a single Ara_p residue (Figure 3C), which serves to terminate elongation of the galactan chain (Figure 3B). In vivo, this arabinosyl-transferase activity results in decreased galactose content in pectin (Figure 5) with reduced DP (Table 2). A previous study supported that GALS1 acts to elongate the growing galactan chains rather than adding the initial galactose substitution on RG-I (Liwanag et al. 2012). This is further reinforced by the preference of GALS1 for galactan substrates with a minimum DP of four Gal units (Figure 2C). Therefore, the number of galactan substitutions on the RG-I backbone is likely unaffected by GALS and the effects observed in this study may be directly linked to the DP.

Bi-functional galactan synthases have previously been characterized from bacteria catalyzing the formation of galactan with alternating Gal\(\beta\)-(1,5)-Gal and Gal\(\beta\)-(1,6)-Gal linkages through a single substrate binding pocket (May et al. 2009, Levengood et al. 2011, May et al. 2012). This demonstrates the ability of glycosyltransferases to catalyze the transfer of individual monosaccharides with two distinct glycosidic linkages. In contrast, GALS1 catalyzes the transfer of two distinct monosaccharides, namely Gal and Ara_p to pectic galactan. Furthermore, GALS1 displayed sigmoidal kinetics for both UDP-Gal and UDP-Ara_p (Figure 3D), which suggest allosteric binding or formation of multienzyme complexes (Porter and Miller 2012). The existence of multiple substrate binding sites...
could provide a mechanism for adding sugar moieties in blocks of two or more in contrast to sequential transfers and explain the predominant formation of Gal₈ oligomers (Figure 3). This is supported by the lower cooperativity for the single transfer of UDP-Arap. As a general approach in plants, multifunctional glycosyltransferases could significantly reduce the number of enzymes required for obtaining the impressive diversity of glycosidic linkages in the plant cell wall, giving rise to the diverse functional roles. In the specific case of GALS1, it should be emphasized that UDP-β-D-Gal and UDP-β-L-Arap are relatively similar substrates (Figure S1) and the enzyme is able to accommodate both substrates in the donor site, albeit with a preference for UDP-Gal. In contrast, the acceptor site does not have this flexibility since the Arap containing product cannot serve as substrate for further extension with galactose units (Figure 3B). We should point out that it has not been demonstrated in this study that Arap is (1,4)-linked to the penultimate Gal, although that would seem most likely.

**Galactan composition is modified by the availability of UDP-Gal₆ and UDP-Arap₆.**

Modulation of cell wall glycan biosynthesis may be achieved in many ways. In this study we showed that availability of UDP-sugars changed the galactan composition facilitated by bi-functional GALS1 (Figure 5). The low affinity for UDP-Arap (Figure 3E) combined with the lower intracellular concentration of UDP-Arap when seedlings were grown on sucrose (Table 1) suggests that elongation of galactan is the dominant catalytic reaction. The intracellular concentration of nucleotide sugars may be altered through salvage pathways by growing seedlings on MS plates supplemented with specific sugars (Figure 4), which altered the specific activity of GALS. In planta, other mechanisms are thought to control the substrate availability, which includes nucleotide sugar mutases and specific Golgi-localized nucleotide sugar transporters. Furthermore, cell wall biosynthesis has been proposed to involve formation of Golgi-localized enzyme complexes, which may have a role in channeling substrates to specific products (Oikawa et al. 2013, Rautengarten et al. 2014). In agreement with this notion, we have observed that knock-out and overexpression of URGT1 affected β-(1,4) galactan content without affecting other galactose-containing glycans (Rautengarten et al. 2014). Alternatively, modulating the nucleotide sugar concentrations may provide a key mechanism to alter the composition of the cell wall polysaccharides. In other biosynthesis pathways, dynamic enzyme complexes, metabolons, are known to allow for enzymes to interact transiently and facilitate substrate channeling by increasing the local substrate concentration and direct feeding between sequential enzymes (Laursen et al. 2015, Laursen et al. 2016). It is interesting to speculate that such dynamic complexes could also be involved in generating plasticity in cell wall polysaccharide structures.

In this study, we have demonstrated that growing Arabidopsis seedlings on D-galactose and L-arabinose reduced the intracellular ratio of UDP-Gal/UDP-Arap from 21 to 0.8, respectively (Table 1). The relative increase in intracellular UDP-Arap resulted in a dramatic decrease in the amount of galactan due to the addition of Arap galactan chains (Figure 5). This enables the plant to alter the

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composition of pectin in response to environmental requirements by modulating the nucleotide sugar concentrations in the Golgi lumen. To date, a specific UDP-Ara\textsubscript{p} transporter has not been reported, however, a UDP-GlcA/UDP-GalA transporter (UUAT1) was recently shown to also transport UDP-Ara\textsubscript{p}, albeit with lower efficiency (Saez-Aguayo et al. 2017). Accumulation of intracellular UDP-Ara\textsubscript{p} correlates with increased Ara in galactan (Figure 5) and therefore suggests a route for UDP-Ara\textsubscript{p} to enter the Golgi lumen, possibly through the UUAT1 transporter. Curiously, we did not observe an increase in UDP-Xyl or UDP-Ara\textsubscript{f} as would be expected to occur through interconversion of UDP-Ara\textsubscript{p} (Bar-Peled and O’Neill 2011).

Reduction in galactan DP correlated with stunted seedling growth (Figure 4B). However, since GALS mutants with decreased galactan content and decreased DP show no growth phenotype (Liwanag et al. 2012), the observed effect on seedling growth is likely not a direct result of the changes in galactan structure.

**Biotechnological applications for optimizing the biosynthesis of linear galactan**

In order to optimize the production of galactan for improving feedstocks for biofuel production, we propose an approach to minimize the effect of Ara\textsubscript{p} to terminate the extension of linear galactan chains. This may be achieved by modifying the binding site for UDP-Ara\textsubscript{p} of GALS1 or alternatively by reducing the Golgi luminal concentration of Ara\textsubscript{p}. UDP-xylose-4-epimerase in the Golgi lumen is the key enzyme for producing UDP-Ara\textsubscript{p}, but targeting this enzyme may result in decrease in UDP-Ara\textsubscript{p}, which is required for synthesis of many different glycans. Instead, increasing UDP-Ara\textsubscript{p} transport out of the Golgi lumen or conversion of UDP-Ara\textsubscript{p} to UDP-Ara\textsubscript{f} could possibly result in a preferential formation of linear β-(1,4) galactan. This study demonstrates the importance of characterizing all enzymes involved in cell wall biosynthesis and the pitfalls in assuming that glycosyltransferases have a very narrow specificity.

**Experimental procedures**

**Chemicals**

All chemicals were of analytical grade and purchased from Sigma-Aldrich unless otherwise stated. UDP- -L-Ara\textsubscript{p} and UDP- -L-Ara\textsubscript{f} were purchased from CarboSource Service (Complex Carbohydrate Research Center, Athens, GA) and Peptides International (Louisville, KY), respectively.

**GALS-FLAG expression in Nicotiana benthamiana**

*Arabidopsis thaliana* Galactan Synthase 1 (GALS1) constructs were cloned into pEarleyGate 202 (Earley et al. 2006) containing an N-terminal FLAG tag under the constitutive 35S promoter as described elsewhere (Liwanag et al. 2012). The resulting 35Spro:FLAG-GALS1 construct was
transformed into *Agrobacterium tumefaciens* strains GV3101 and used for transient expression in *Nicotiana benthamiana*. Leaves of 4-week-old plants were infiltrated with an *Agrobacterium* strain carrying 35Spro:FLAG-GALS1 (OD<sub>600</sub> = 0.2) and co-infiltrated with a strain carrying the p19 gene of tomato bushy stunt virus (OD<sub>600</sub> = 0.2), as described elsewhere (Sparkes et al. 2006). The *N. benthamiana* leaves transiently expressing FLAG-GALS1 were harvested 4 d after infiltration.

**Growth of etiolated mung bean seedlings**

Mung bean (*Vigna radiata*) seeds were soaked overnight in deionized water at room temperature in the dark and spread on moist glass wool before being grown for four days at 28 °C in the dark. The hypocotyls (2 cm in length from the cotyledons) were harvested with a razorblade and used for preparation of microsomal membranes.

**Preparation of microsomal membranes**

Microsomal membranes were prepared according to the protocol described elsewhere (Nunan and Scheller 2003). In short, plant tissue was ground in microsomal extraction buffer (50 mM HEPES-KOH pH 7.0, 400 mM sucrose, 20 mM sodium ascorbate, 1 mM PMSF, 1% w/v PVPP). The suspension was filtered through Mira cloth to remove cell debris followed by centrifugation (3,000 g for 10 min). The supernatant was transferred to a new tube and membranes were isolated by centrifugation (100,000 g for 1 h). The microsomal pellet was resuspended in buffer (50 mM HEPES-KOH pH 7.0, 400 mM sucrose) using a brush and homogenized with a Potter Elvehjem homogenizer (Sigma-Aldrich). Microsomes were flash frozen and stored at -80 °C until use.

**ANTS-labeling and analysis of labeled galactan substrates**

Linear galactan substrates Gal<sub>n</sub> (n=3-7) and branched Gal<sub>6</sub>-Ara<sub>1</sub> were chemically synthesized as described elsewhere (Andersen et al. 2016). Galactan substrates, 200 μg of each oligosaccharide dissolved in H2O, were reductively aminated with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS; Invitrogen) as follows: Oligosaccharides were dried on a speedvac and resuspended in 5 μl of 0.2 M ANTS solution (dissolved in 17:3, water:acetic acid) and 5 μl of 0.2 M 2-picoline borane (resuspended in DMSO) was added to each tube, as described in (Mortimer et al. 2015). Samples were dried and dissolved in 100 μL mQ water. Excess fluorophore was removed using GlykoClean S Cartridges (Prozyme). Labeled oligosaccharides were dried and resuspended to reach 1 μg/μL and stored at -80 °C until use.

**Polysaccharide analysis using carbohydrate gel electrophoresis (PACE)**

All reactions were performed in a total of 25 μL containing MnCl<sub>2</sub> (10 mM), Triton X-100 (1% v/v) in buffer (50 mM MES, pH 6.5). Screening of Gal<sub>n</sub> substrates included 2 μg Gal substrate, UDP-Sugar (200 μM) and microsomal membranes (50 μg total protein). Reactions were incubated for 2 h at 30 °C with agitation (800 rpm). Reactions were terminated by heating (100 °C, 3 min) and...
precipitated protein and lipids were sedimented by centrifugation (10,000 g for 10 min). Supernatants (15 μL) were mixed with 15 μL urea (3 M) and the samples (5 μl) were analyzed by separation on large format Tris-borate acrylamide gel prepared as described elsewhere (Goubet et al. 2002) and electrophoresed at 200V for 30 min followed by 1000V for 1.5 h. The PACE gels were visualized with a G-box (Syngene, www.syngene.com) equipped with a UV detection filter and long-wave UV tubes (365 nm emission).

Kinetics of UDP-Galp and UDP-Arap

Kinetic measurements of GALS1 transiently expressed in *Nicotiana benthamiana* were performed on microsomal membranes. All measurements were performed in the linear range to avoid misinterpretations due to depletion of substrate. Reactions were performed in a total volume of 25 μL containing Gal₅ (50 μM) MnCl₂ (10 mM), Triton X-100 (1% v/v) in buffer (50 mM MES, pH 6.5). When UDP-Galₕ was administered as donor sugar reactions were incubated for 15 min under agitation (800 rpm) and contained 1 μg/μL total microsomal protein and UDP-Gal in concentrations: 0, 10, 20, 30, 50, 75, 100, 150, 200, 300 and 500 μM. When UDP-Araₕ was administered as donor sugar reactions were incubated for 60 min under agitation (800 rpm) and contained 2 μg μL⁻¹ total microsomal protein and UDP-Gal in concentrations: 0, 10, 25, 50, 75, 100, 150, 250, 500, 750, 1000, 2000 and 3000 μM.

Products were separated by PACE and quantified using ImageJ software. Data were plotted in Igor Pro (Wavemetrics) and fitted to hyperbolic or sigmoidal equations using built-in fitting parameters and evaluated based on Chi-Square test.

Growth of etiolated *Arabidopsis* seedlings on sugar plates

*Arabidopsis* seedlings, Col-0 and GALS1 over expression line (OE) were grown on ½ Murashige and Skoog (MS) medium plates with 0.7 % agar containing 1 % sucrose, 1 % D-galactose or 1 % L-arabinose as the sole sugar source. Seeds were sterilized by incubation in 50% Clorox® Concentrated Regular Bleach, 1% Tween 20 for 5 min and washed with sterile water prior to plating. Plates were placed at 4 °C for 3 days for stratification and grown at 22 °C for 4 days in the dark. Etiolated seedlings were harvested and used for further analyses as described later.

Monosaccharide analysis and galactanase treatment

Alcohol-insoluble residue (AIR) was prepared as previously described (Harholt et al. 2006), hydrolyzed in 2 M trifluoroacetic acid for 1 h at 120 °C, and analyzed by high-performance anion exchange chromatography (HPAEC) as described elsewhere (Øbro et al. 2004).

Isolation of galactan fractions from cell walls of *Arabidopsis* seedlings grown on sugar plates were prepared by digestion with endo-β-1,4-galactanase (Megazyme, product code E-EGALN) as previously described (Stonebloom et al, 2016). After digestions, the supernatant and pellet were separated by centrifugation and analyzed by hydrolysis in TFA and HPAEC as described above.

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Extraction and purification of nucleotide sugars

Extraction of nucleotide sugars from *Arabidopsis* seedlings were performed according to previously published protocol (Arrivault et al. 2009). Seedlings were flash frozen and ground to a powder in a pre-cooled mortar. Powder (30-40 mg) was transferred to a pre-cooled 2 mL Safe-Lock microfuge tube and extracted with 250 μL cold CHCL₃/CH₃OH (3:7 v/v). Samples were spiked with 1 pmol TDP-Glc mg⁻¹ powder as an internal standard and incubated for 2 h at -20 °C. H₂O (200 μL) was then added and the aqueous upper phase containing soluble nucleotide sugars was collected and transferred to a new tube following centrifugation (420 g for 4 min). Extraction was repeated twice and the combined aqueous phase containing water-soluble metabolites was dried in a speedvac and resuspended in 1 mL buffer (10 mM ammonium bicarbonate). Nucleotide sugars were purified on reverse phase SPE columns (ENVI-CARB) according to protocol described elsewhere (Rabina et al. 2001). In short, columns were conditioned prior to sample load by addition of 3 mL 60% acetonitrile, 0.3% ammonium formate (pH 9.0) followed by flushing with 3 mL H₂O. Samples were added followed by wash with 3 mL H₂O and 1 mL 60% acetonitrile. Samples were eluted with 3 mL 60% acetonitrile, 0.3% ammonium formate (pH 9.0).

Chromatographic separation and detection of nucleotide sugars

Separation of nucleotide sugars was performed by liquid chromatography (LC) on a 1260 Infinity series HPLC system (Agilent Technologies) with porous graphitic carbon as the stationary phase according to a previously established protocol (Rautengarten et al. 2014) with important modifications. A Hypercarb column (Thermo Scientific) (150 mm x 1 mm, 5 μm) with a Hypercarb guard cartridge (1 mm x 2.1 mm, 5 μm) was used at a flow rate of 100 μL min⁻¹ with solvents (A) (water with 0.3% formic acid (pH 9.0) with ammonia, 5% acetonitrile) and (B) (100% acetonitrile). Separation conditions were 100% (vol/vol) A for 5 min, followed by a gradient to 75% (A) in 30 min and then 50% (vol/vol) (A) in 5 min, which was held for a further 5 min before a return to 100% (vol/vol) (A) in 5 min and held for 40 min for reconditioning of the column. The system was run in Micro flow mode with a mix rate of 200 μL min⁻¹, and the column compartment was set to 50 °C with samples kept at 10 °C. A total of 5 μL of extracted nucleotide sugars spiked with 1 pmol TDP-Glc pr mg seedling fresh weight was loaded.

The separated nucleotide sugars were detected using a 4000 QTRAP LC-tandem MS (MS/MS) system (AB Sciex) equipped with a TurboIonSpray (AB Sciex) ion source, essentially as previously reported (Rautengarten et al. 2014). The mass spectrometry parameters are shown in Table S1.

Isolation of RG-I fractions

RG-I fractions were isolated by enzymatic digestion and size-exclusion chromatography as described elsewhere (Stonebloom et al. 2016).
Glycosyl linkage analysis

Samples were permethylated, depolymerized, reduced, and acetylated. The resultant partially methylated alditol acetates (PMAAs) were analyzed by gas chromatography-mass spectrometry (GC-MS) as described elsewhere (Heiss et al. 2009). We detected some glucose linkages, which were assigned to contamination from the dextran-agarose column used for isolation of RG-I and consequently omitted in the data analysis.

About 1.0 mg of the sample was used for linkage analysis. The sample was suspended in 200 μL of dimethyl sulfoxide and left to stir for 3 days. Permethylation was performed by two rounds of treatment with sodium hydroxide (15 min) and iodomethane (45 min). After addition of 2 mL water, residual iodomethane was removed by sparging with nitrogen. The permethylated carbohydrate was extracted with dichloromethane, which was then washed twice with water and evaporated. The permethylated material was hydrolyzed using 2 M TFA (2 h in sealed tube at 121 °C), and the acid was removed by repeated evaporation with 2-propanol. The sample was reduced with NaBD₄ in 1 M ammonium hydroxide for 1 h at room temperature. Borate was removed by repeated evaporation with 9:1 methanol-acetic acid and a final evaporation with methanol. The sample was then acetylated using acetic anhydride (250 μL) and TFA (230 μL). After quenching the reaction by addition of 2 mL 0.2 M sodium carbonate, the PMAAs were extracted with 2 mL dichloromethane and analyzed on an Agilent 7890A GC interfaced to a 5975C MSD (mass selective detector, electron impact ionization mode). A 30 m Supelco SP-2331 bonded phase fused silica capillary column was used for separation of the PMAAs.

Acknowledgements

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Author contributions

T.L., S.H.S. and H.V.S designed the research project. M.H.C. chemically synthesized galactan substrates. T.L., V.R.P. and J.C.M. performed in vitro enzyme characterization using the PACE assay.

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D.S.B. and S.H.S. performed monosaccharide analysis. T.L. and S.H.S. performed nucleotide sugar analysis. T.L., S.H.S. and H.V.S. wrote the manuscript. All authors approved the final manuscript.

Short Supporting Information Legends

Figure S1. Haworth projections of -D-Gal, -L-Araβ, and -L-Araγ.

Figure S2. Bi-functional GALS1 display preference for long galactan substrates.

Figure S3. AtGALS1 kinetics fitted to a hyperbolic curve.

Figure S4. Monosaccharide composition of total AIR preparations from Col-0 and GALS1 OE.

Table S1. LC-MS/MS parameters for nucleotide sugar quantification.

References


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Tables

Table 1. Nucleotide sugar concentration in GALS1 OE seedlings grown on sugar plates.

Seedlings were grown in the dark on plates supplemented 1% sucrose, D-galactose or L-arabinose as sole carbon source. Samples were spiked with TDP-Glc corresponding to 1 pmol mg⁻¹ fresh weight sample. Values are given in pmol mg⁻¹ fresh weight and represent three technical replicates ±SD

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Table 2. Linkage analysis of RG-I isolated from etiolated GALS1 OE seedlings.

RG-I was isolated from etiolated GALS1 OE Arabidopsis seedlings grown on plates supplemented with 1% sucrose, 1% D-galactose or 1% L-arabinose as carbon source. Values are averages of two independent replicates and represented as peak area %; sugars not detected in the analysis are marked with n.d. Ratios indicate the length of galactan chains for seedlings grown on various carbon sources.

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Figures legends

**Figure 1.** RG-I backbone substituted with diverse β-1,4-galactan-rich oligosaccharide side chains.

RG-I is composed of an alternating backbone of galacturonic acid and rhamnose, which can be substituted with galactan, arabinan and arabinogalactan. Galactan is composed of β-(1,4)-linked galactose units (a) and may contain internal arabinofuranose units (b), branched arabinofuranose units (c) or terminal arabinopyranose units (d).

**Figure 2.** Polysaccharide Analysis using Carbohydrate gel Electrophoresis (PACE).

A) Several synthetic galactan substrates were reductively labeled with the fluorescent probe ANTS for PACE assays. B) The expression of AtGALS1, transiently expressed in *Nicotiana benthamiana*, in the microsomal fraction was analyzed by immunoblot using Anti-FLAG antibodies, which displayed a distinct band at approximately 70 kDa compared to the p19 background. C) PACE assay using AtGALS1 microsomes and ANTS labeled substrates in the presence and absence of UDP-Gal as donor sugar displayed a preference for longer galactan substrates (Gal$_{4-7}$). No conversion was observed in the absence of UDP-Gal.

**Figure 3.** GALS1 is bi-functional catalyzing addition of Gal and Ara$_p$ to galactan substrates.
A) PACE assay was employed to screen for galactan biosynthetic activities in microsomes prepared from etiolated mung bean hypocotyls using Gal₃ as substrate and UDP-Gal, UDP-Araᵧ or UDP-Araᵧ as donor sugars. Administration of UDP-Gal resulted in the formation of a ladder of products, whereas only a single product was formed with UDP-Araᵧ. UDP-Araᵧ did not serve as donor sugar in this assay. B) Sequential activity assay with different donor sugars (listed in the top) supplemented for 2 h followed by administration of a second donor sugar in the same reaction for 1 h (listed below). Sequential addition of UDP-Araᵧ followed by administration of UDP-Gal revealed that formation of galactan with a Araᵧ served to terminate the growing galactan chain. C) AtGALS1 expressed in Nicotiana benthamiana is bi-functional catalyzing the extension of galactan (Gal₃) upon addition of the donor UDP-Gal to the reaction and a single Araᵧ extension product upon addition of the donor UDP-Araᵧ. UDP-Araᵧ did not serve as donor sugar. D,E) AtGALS1 displayed sigmoidal kinetics for both UDP-Gal (D) and UDP-Araᵧ (E) as donor sugars using Gal₃ as substrate. Data were fitted to the Hill equation and evaluated based on the Chi² Test, K₀.₅ represents apparent affinity and n describes the cooperativity for n>1. F) Both activities were dependent on divalent cations as demonstrated by supplementation of Mn²⁺. Values are average of three technical replicates ±SD.

Figure 4. Modifying the intracellular nucleotide sugar concentrations.

A) Arabidopsis seedlings of wild type (Col-0) and GALS1 overexpressor (GALS1 OE) were grown in the dark on plates supplemented with 1% sucrose, 1% D-galactose or 1% L-arabinose. B) Col-0 displayed similar seedling height on all sugar plates. GALS1 OE seedlings were slightly shorter when grown on galactose and significantly shorter when grown on arabinose plates compared to seedlings grown on sucrose plates. C) Nucleotide sugar analysis of GALS1 OE grown on the different sugar plates revealed an increase in UDP-Gal when grown on galactose plates and an increase in UDP-Araᵧ when grown on arabinose plates. This effect is attributed to the salvage pathways for both galactose and arabinose. Values are average of three technical replicates ±SD.

Figure 5. The effect on pectin of seedlings with modified nucleotide sugar levels.

Monosaccharide analysis of the galactan fraction isolated from etiolated Arabidopsis seedlings (Col-0 and GALS1 OE) grown on plates supplemented with sucrose, D-galactose or L-arabinose. Values are average of three technical replicates ±SD.