

Elucidating the Enzymatic Framework for the Initiation of Pectic Galactan

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Article

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Title: Elucidating the Enzymatic Framework for the Initiation of Pectic Galactan.

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Running Title: Unravelling Galactan Initiation

Abstract

Pectins are a major class of plant cell wall polysaccharide crucial for plant survival and adaptation. Despite their importance, the biosynthesis of pectin remains poorly understood due to its exceptional structural and functional diversity. Functional interrogation of pectin biosynthetic enzymes *in planta* is often limited by severe developmental or difficult to interpret carbohydrate phenotypes resulting from genetic disruption. Using a direct biochemical approach, we report the identification and functional characterization of a Galactan Initiation (GalTi) enzyme from the aquatic monocot *Spirodela polyrhiza*, a member of CAZy glycosyltransferase family 47, that initiates galactan side-chain biosynthesis on rhamnogalacturonan-I. We further show that orthologs from *Arabidopsis thaliana* exhibit identical catalytic activity, revealing conservation of this biosynthetic initiation mechanism across monocots and dicots. Comprehensive carbohydrate structural analyses demonstrate that GalTi enzymes specifically catalyze the transfer of single β -D-galactopyranosyl residues to the O-4 position of backbone rhamnose residues, establishing their direct role in galactan side-chain biosynthesis. Co-synthesis experiments show 3-O-acetylation of backbone rhamnose residues inhibits SpGalTi-mediated galactosylation, suggesting a potential regulatory mechanism by which plants use to modulate side-chain patterning *in vivo*. Together, these findings provide a new framework for RG-I galactan assembly, advancing our mechanistic understanding of plant cell wall biosynthesis.

Keywords: Rhamnogalacturonan-I, Galactan Biosynthesis, Galactosyltransferase, GT47, Pectin Biosynthesis, Plant Cell Wall

Introduction

Plants are the master architects of carbohydrate complexity, synthesizing the array of polysaccharides that form the foundation of the plant cell wall. These complex saccharide structures are categorized into different functional classes including cellulose, hemicelluloses, pectins, and structural glycoproteins. Unlike nucleic acids and proteins, whose synthesis are template-driven, polysaccharides are assembled and modified through the coordinated activities of carbohydrate-active enzymes (CAZymes; <http://www.cazy.org/>) (Drula et al., 2022), presenting a significant challenge in elucidating biochemical pathways. In this regard, enzymes that act on pectins present one of the most outstanding challenges due to their immense structural diversity.

Pectins are a major class of matrix polysaccharides characterized by the presence of 1,4-linked α -D-galacturonic acid (GalA) residues. They are abundant in primary cell walls and the middle lamella, where they contribute to cell adhesion, wall porosity, hydration, and mechanical properties (Obomighie et al., 2025). Rhamnogalacturonan-I (RG-I) is the most structurally heterogeneous pectic domain, with a backbone composed of a rhamnose (Rha) and GalA repeating disaccharide $\rightarrow 2)-\alpha\text{-L-Rha}p-(1\text{-}4)-\alpha\text{-D-GalpA}-(1\text{-}$. This backbone can be extensively branched at *O*-4 of $\alpha\text{-L-Rha}$ residues with $\beta\text{-D-Galp}$, $\beta\text{-D-glucuronosyl}$ (Glc p A), $\beta\text{-1,4-D-galactans}$, arabinans, and arabinogalactans (Kaczmarska et al., 2022; Zhang et al., 2025) (Figure 1A). In addition to saccharide substituents, other modifications such as *O*-acetyl esterification are important structural features of RG-I (Shahin et al., 2023). The structure and quantity of individual substituents can vary depending on the species, cell type, and developmental state of the plant (Atmodjo et al., 2013).

Despite its abundance in primary cell walls, the biosynthetic machinery responsible for RG-I assembly remains only partially characterized. It is well established that RG-I backbone synthesis requires two distinct glycosyltransferase (GT) activities, RG-I Rhamnosyltransferase (RRT; GT106) (Takenaka et al., 2018) and RG-I Galacturonosyltransferase (RGGAT; GT116), also known as MUCILAGE-RELATED 70 (MUCI70) (Amos et al., 2022; Voiniciuc et al., 2018). These enzymes are responsible for incorporating L-Rha and D-GalA, respectively, to synthesize the backbone. Unlike backbone elongation, pathways for the synthesis of RG-I sidechains remain less clear. More than

20 years ago, it was hypothesized that three types of galactosyltransferases would be required for synthesizing β -1,4 galactan sidechains (Ridley et al., 2001). In this model, galactan synthesis is initiated by Type I GalTs, which we refer to here as Galactan Initiation (GalTi) enzymes, that catalyze the transfer of β -D-Galp to backbone Rha residues. Extension by a second Gal residue, forming the first Gal- β -(1,4)-Gal bond, is thought to be mediated by Type II GalTs, which we refer to as Galactan Extension (GalTe) enzymes. Finally, Galactan Synthases (GalS) from the GT92 family (Type III GalTs) polymerize the galactan sidechain. GalS enzymes elongate β -1,4-galacto-oligosaccharides with a degree of polymerization (DP) of at least three (Laursen et al., 2018) or four (Ebert et al., 2018; Prabhakar et al., 2023), with longer acceptors being more efficient substrates. GalS enzymes play an additional role in terminating chain synthesis with an α -1,4-Arap residue (Prabhakar et al., 2023). Given that biochemically characterized GalS enzymes require oligosaccharide acceptors, there is a precedent for the existence of functionally distinct GTs responsible for initiating and extending sidechain formation (Laursen et al., 2018). However, the identities of the Type I and II GalTs involved in this process have remained enigmatic.

Here, we developed an acceptor-based biochemical screen for the direct functional characterization of plant glycosyltransferases (Figure S2). Interrogation of members of CAZy glycosyltransferase family 47 (GT47) enzymes from the aquatic monocot *Spirodela polyrhiza* (GT47) (Figure 1C) resulted in the identification of enzymes that catalyze the transfer of β -D-Galp to backbone Rha residues. Detailed analysis of saccharide reaction products functionally confirms GalTi catalyzes the transfer of single β -D-Galp substituents to O-4 of backbone Rha residues of RG-I (Figure 1B), forming the foundation for galactan sidechain synthesis. We additionally show that an ortholog from *Arabidopsis thaliana* possesses an identical function, showcasing the potential conservation of this initiation pathway in both monocots and dicots. Together, these findings underscore the complexity of RG-I biosynthesis and highlight the need for continued identification and characterization of the GTs and other modifying enzymes involved in its assembly.

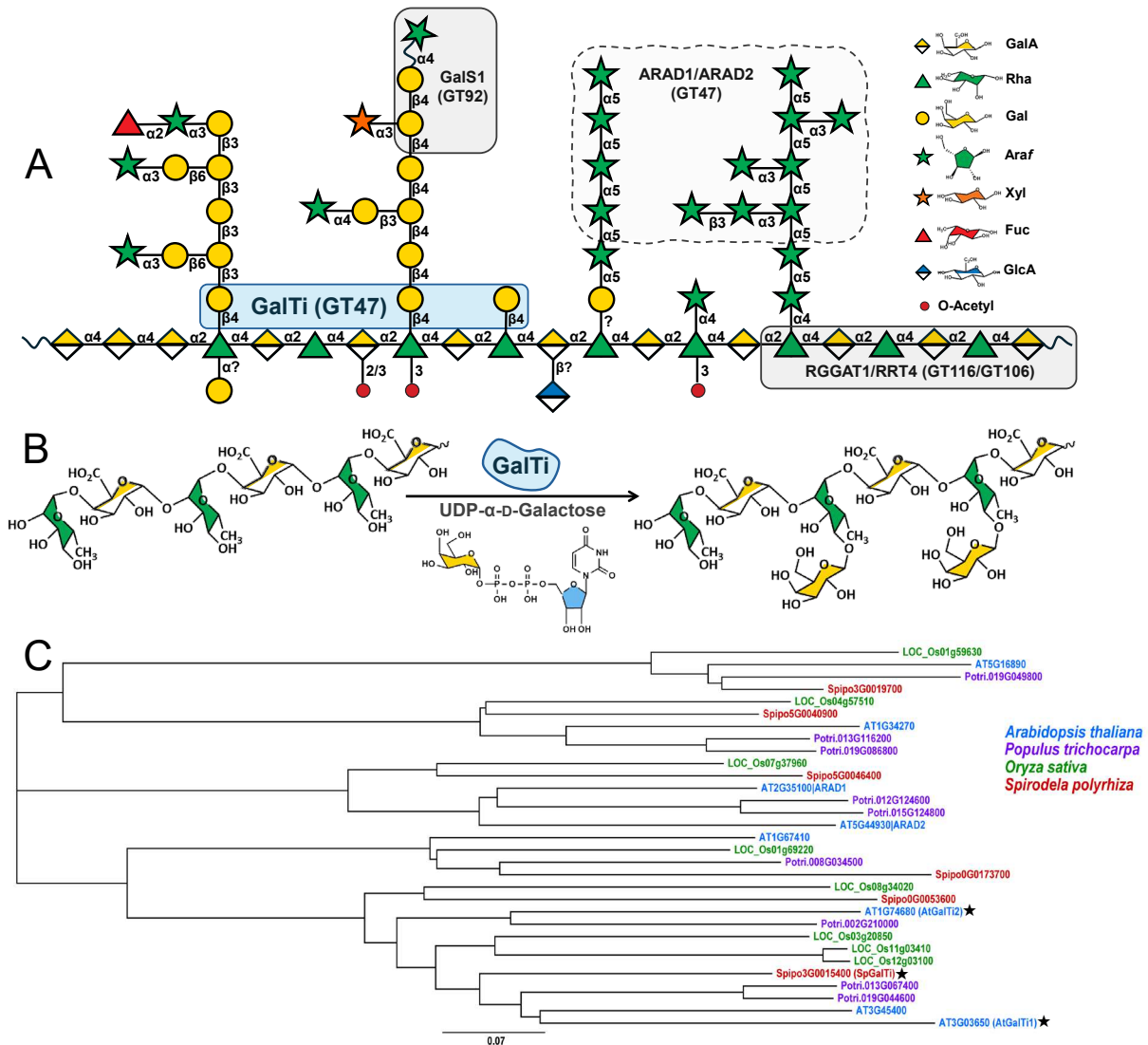


Figure 1: Structure and biosynthesis of RG-I.

(A) Schematic of RG-I structure including representations of galactan, arabinan, and arabinogalactan sidechains (accurate sidechain spacing is not depicted) (Kaczmarska et al., 2022; Renard et al., 1999; Shahin et al., 2023; Zhang et al., 2025) along with known enzymes involved in synthesis (Amos et al., 2022; Ebert et al., 2018; Harholt et al., 2006; Harholt et al., 2012; Laursen et al., 2018; Liwanag et al., 2012; Prabhakar et al., 2023). (B) Representative GalTi reaction depicting the transfer of β -D-Galp from UDP- α -D-Gal to O-4 of backbone Rha residues. (C) Phylogenetic tree showcasing the GT47-B clade constructed using amino acid sequences from *A. thaliana* (blue), *Populus trichocarpa* (purple), *Oryza sativa* (green), and *Spirodela polyrhiza* (red). Enzymes denoted with a star represent the GalTis examined in this study. The Symbol Nomenclature for Glycans (SNFG) (Varki et al., 2015) was used for the graphical representation of glycan structures.

Results

Heterologous Expression of GalTi Enzymes

The full-length coding sequences of SpGalTi (Spipo3G0015400), AtGalTi1 (At3G03650, *EMBRYO SAC DEVELOPMENT ARREST 5, EDA5*), AtGalTi2 (At1G74680), and At3g45400 were derived from the *S. polyrhiza* (v2) and *A. thaliana* genomes (TAIR11) via the Phytozome 13 database. The expressed fusion proteins consisted of a signal sequence, an 8X-His tag, AviTag, “superfolder” GFP, the 7-amino acid recognition sequence of the tobacco etch virus (TEV) protease followed by the indicated catalytic domain regions of SpGalTi (amino acids 37-465), AtGalTi1 (amino acids 42-499), AtGalTi2 (amino acids 52-461), or At3g45400 (amino acids 61-475). Each enzyme was expressed as a soluble secreted fusion protein by transient transfection of suspension culture HEK293-F cells and purified using immobilized metal affinity chromatography (IMAC) as described previously for plant GTs (Prabhakar et al., 2020). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicated that the purified proteins are the expected size (Figure S1). We were unable to recombinantly express At3g45400 for functional analysis.

Identification of a Galactosyltransferase Catalyzing RG-I Galactan Sidechain Initiation (GalTi)

Plant GTs involved in cell wall polysaccharide synthesis catalyze the transfer of sugar moieties from activated nucleotide sugar donors to specific acceptor molecules, forming glycosidic bonds. The products of these bi-substrate reactions are glycosylated molecules and free nucleotide-phosphate leaving groups from the activated donor. In order to identify and assign the specific roles of GTs in glycan synthetic pathways, three types of information are required: donor selectivity, acceptor substrate specificity, and the structure of the resultant saccharide product.

The identification of GalTi began with determining the acceptor substrate selectivity for a functionally undefined GT encoded by the *S. polyrhiza* gene Spipo3G0015400, denoted herein as Sp154-B. To achieve this, Sp154-B was screened for transferase activity using our “acceptor first” HTP assay (Figure S2). An equimolar mixture of UDP-sugars (UDP-Gal, UDP-Glc, UDP-GalA, UDP-GlcA, UDP-Arap, UDP-Araf, UDP-Xyl) was used as a universal donor substrate to screen a library of plant specific saccharide acceptor substrates by quantification of the UDP by-product using the UDP-Glo™ Glycosyltransferase Assay. Acceptor screening revealed that Sp154-B exhibited

significant transferase activity with pectin-rich substrates, including seed mucilage isolated from *A. thaliana*, potato derived RG-I, and sugar beet pectic arabinan (Figure 2A), suggesting that this enzyme plays a role in pectin biosynthesis. Next, the donor selectivity of Sp154-B was determined by screening the enzyme against a library of individual UDP-sugar donors using *A. thaliana* seed mucilage, our top hit from the acceptor screen, as a model acceptor. We found that Sp154-B showed the highest transferase activity with UDP- α -D-Gal (UDP-Gal) and a much lower, but measurable amount of UDP production, with UDP-Arap (Figure 2B). After obtaining promising acceptor/donor candidates, Sp154-B was additionally screened for transferase activity at various pH conditions, and it was found to have an optimal activity at a pH of 7 (Figure S3A). Substrate binding affinity was investigated by microscale thermophoresis (MST), showing that Sp154-B exhibited a lower dissociation constant (K_d) for the UDP-Gal donor compared to the acceptor (Figure S4). Given that all Sp154-B exhibited transferase activity with RG-I derived acceptors, with *A. thaliana* seed mucilage (consisting of unsubstituted RG-I backbone) as our top hit, we hypothesized that Sp154-B likely catalyzes the addition of galactose (Gal) to the RG-I backbone. To support this hypothesis, we additionally screened Sp154-B against an equimolar mixture of RG-I and arabinan with similar degrees of polymerization (DP) using the donors UDP-Gal, -Arap, and -Araf. This was carried out to determine if Sp154-B exhibited any arabinan selectivity, given that pectic arabinan was also identified as a potential acceptor candidate. As expected, the only significant activity observed was with the RG-I acceptor in combination with the UDP-Gal donor, suggesting no arabinan selectivity (Figure S5).

After identifying the likely acceptor and donor for Sp154-B, we set out to determine the structure of the resultant saccharide product(s). To achieve this, we prepared model RG-I oligosaccharide acceptor substrates and incubated them with Sp154-B alongside UDP-Gal. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the reaction products showed mass increments (162 Da) indicative of the addition of up to three galactosyl residues to our RG-I acceptors (Figure 2C). Given that the number of hexose modifications increased concomitantly with the acceptor DP, we speculated that Sp154-B was adding single Gal additions to every free Rha of the backbone and not participating in galactan extension. To confirm this, we performed MS/MS on reaction products to determine Gal patterning. The

resultant fragmentation pattern indicated a product structure with single Gal substitutions at every Rha residue apart from the free-end Rha (at the nonreducing-end of the structure), suggested by a fragment with a m/z value of 1185.723 (Figure S6A). This was also supported by reaction products produced from a DP 5 RG-I acceptor, lacking a free-end Rha residue, where all Rha residues were galactosylated (Figure S6B). Finally, to fully confirm that Sp154-B was only participating in the addition of single Gal substitutions to RG-I backbone Rha residues, we incubated galactosylated RG-I products with a RG-I lyase (BT4175), fragmenting the backbone into repeating disaccharide units. After digestion, the resultant disaccharide products had mass shifts consistent with the addition of single Gal substitutions (Figure S7), showing that Sp154-B was only synthesizing single Gal additions per Rha residue. Although Sp154-B did not appear to have activity in galactan extension, there is the possibility that the enzyme may have a secondary function in the extension of other sidechain structures after transferring the initial Gal to the backbone. To examine this possibility, we compared the transferase activity of Sp154-B between unsubstituted and previously galactosylated RG-I polymer with the donors UDP-Gal, -Arap, and -Araf. Transferase activity was significantly diminished with Gal-substituted acceptors, even with the UDP-Arap and UDP-Araf donors, implying that Sp154-B was not exhibiting activity outside of adding the first Gal to the RG-I backbone (Figure 2E).

The data suggests that Sp154-B is a GalTi, catalyzing the addition of the first galactosyl residue to the RG-I backbone. Considering galactan is canonically β -1,4 linked to the Rha residues of RG-I, we set out to determine the Gal linkage of the products. To accomplish this, we incubated enzymatically galactosylated RG-I with an α -1,4 (BT4157) or β -1,4 (BT4151) galactosidase (Luis et al., 2018). The resulting data showed that only products incubated with the β -galactosidase displayed Gal removal (Figure 2D), providing evidence for Sp154-B generating β -linked Gal backbone substitutions, consistent with its classification as an inverting GT.

To conclude our structural characterization of the galactosylated products generated by Sp154-B, we conducted a comprehensive structural investigation via nuclear magnetic resonance (NMR). To achieve this, scaled-up reactions with RG-I polymer were set up to achieve maximum product output. Galactosylated product was subsequently digested into oligomers with a RG-I hydrolase, purified, and examined with ^1H NMR spectroscopy (Figure S9). The spectra of the galactosylated

186 oligomers and unmodified substrates were then compared (Figure 2F: 1-2). The ^1H NMR signals
187 of the product were assigned on the basis of 1D and 2D NMR spectroscopy (Figure S9 and S10).
188 A full analysis of the galactosylated oligomers revealed a new anomeric proton signal for the
189 incorporated Gal (Gal-H1) at δ 4.65 ppm (Figure 2F: 2), and the large coupling constant for this
190 signal ($J = 7.81$ Hz) confirmed its β -anomeric configuration (Fontana and Widmalm, 2023). The
191 specific site of Gal attachment was then determined by 1D and 2D NMR. A clear inter-residue 1D
192 ROESY (Rotating-frame Overhauser Effect Spectroscopy) correlation was observed between the
193 anomeric proton of Gal (Gal-H1) and the H4 proton (δ 3.66 ppm) of a Rha residue (Figure 2F: 4-
194 5). This linkage was further confirmed by 2D Heteronuclear Multiple-Bond Correlation (HMBC)
195 (Figure S10B) between the Gal anomeric carbon (Gal-C1, δ 103.3 ppm) and the same Rha H4
196 proton (δ 3.66 ppm). Together, these ROESY and HMBC correlations established the formation of
197 the β -D-Gal-(1-4)- α -L-Rha linkage, confirming the expected product structure. Concluding our
198 initial findings, we have determined that Sp154-B is a galactosyltransferase involved in the
199 initiation of galactan synthesis on RG-I, specifically adding D-Gal to the Rha residues of the RG-I
200 backbone via a β -(1-4) linkage. With this in mind, we will henceforth refer to Sp154-B as SpGalTi.

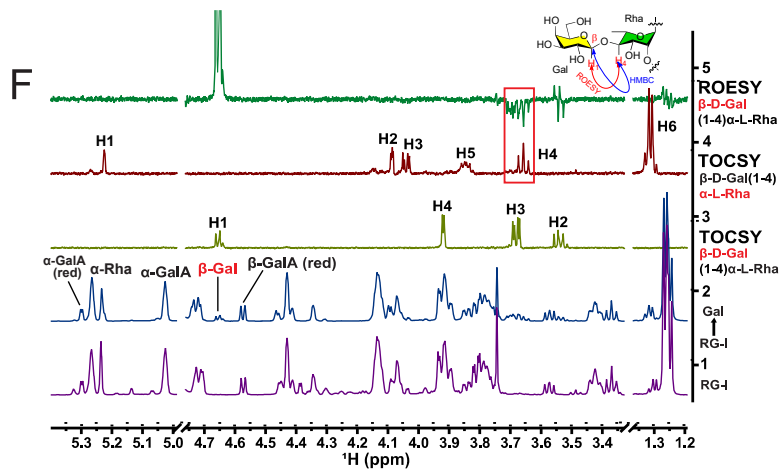
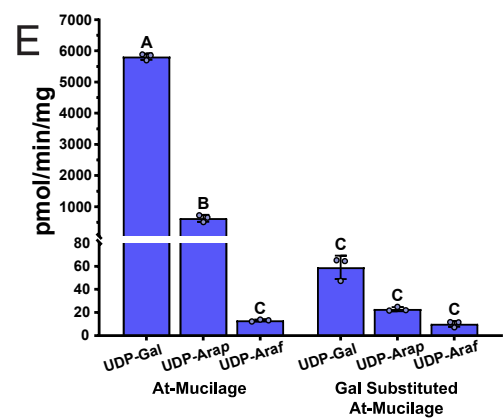
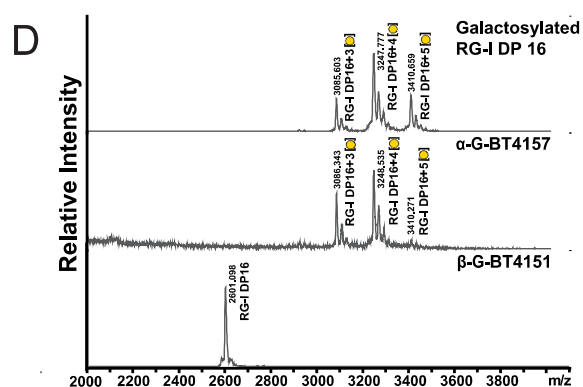
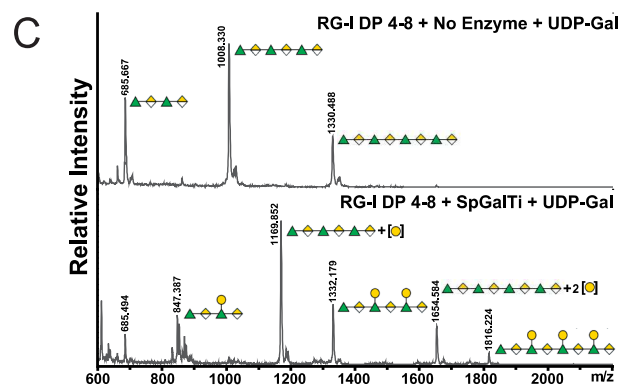
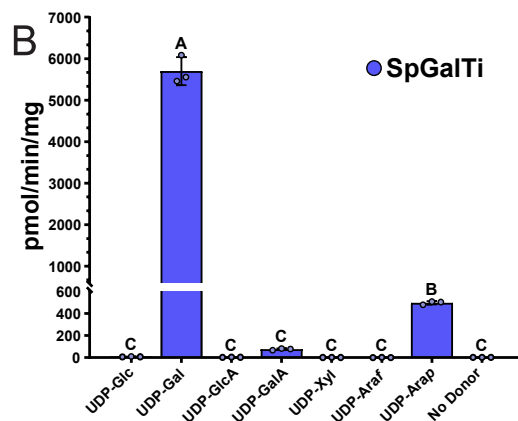
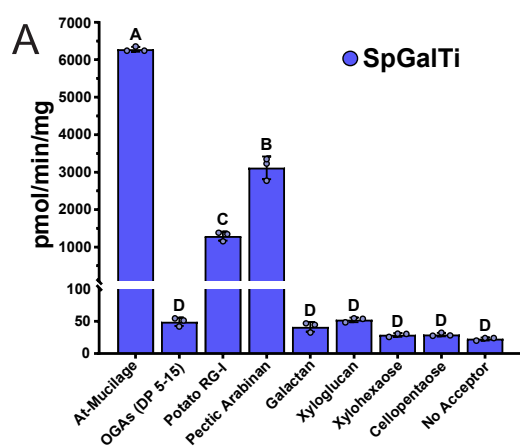


Figure 2: SpGalTi is a β -1,4 galactosyltransferase specific to RG-I.

(A) SpGalTi transferase activity across a series of diverse saccharide acceptors using a UDP-sugar mix as a general donor substrate. Quantification of enzyme activity was accomplished by measuring the UDP biproduct after the transfer of a UDP-sugar donor to an acceptor substrate. (B) Screening SpGalTi for donor substrate selectivity using a library of UDP-sugar donors and *A. thaliana* seed mucilage as an acceptor substrate. (C) MALDI-TOF MS analysis of SpGalTi reaction products with RG-I DP 4-8 oligosaccharides in the presence of a UDP-Gal donor. Hypothetical oligosaccharide structures are shown for reaction products. Masses corresponding to an increase of 162 Da represent an addition of one hexose (Gal). (D) MALDI-TOF MS analysis of SpGalTi-galactosylated RG-I products treated with an α or β galactosidase. (E) Examining SpGalTi donor selectivity for UDP-Gal, UDP-Arap, or UDP-Araf with unmodified *A. thaliana* seed mucilage or Gal-substituted *A. thaliana* seed mucilage acceptors. (F) 1D NMR spectrum and schematic of RG-I oligosaccharides galactosylated by SpGalTi. **1:** ^1H NMR spectrum of the un-substituted RG-I acceptor substrate. **2:** ^1H NMR spectrum of the galactosylated product, showing the new anomeric proton signal (Gal-H1) at δ 4.66 ppm. **3:** 1-D selective gradient TOCSY spectrum with irradiation at δ 4.66 ppm (Gal-H1), which was used to identify the spin system of the newly attached galactose residue. **4:** 1-D selective gradient TOCSY spectrum with irradiation at δ 1.31 ppm, confirming the rhamnose spin system. **5:** 1-D selective gradient ROESY spectrum with irradiation of the Gal-H1 proton (δ 4.66 ppm), which reveals the inter-residue correlation to the rhamnose ring and confirms the glycosidic linkage. Error bars indicate Mean $\pm$ SD with an *n* of 3 biological replicates. Letters above indicate statistical significance accomplished by a one-way ANOVA (A-B) or two-way ANOVA (E) followed by a Tukey's multiple comparison test ($P \leq 0.01$).

SpGalTi Substrate Specificity

Concluding the functional identification of SpGalTi, we found it relevant to investigate whether certain structural aspects of the RG-I acceptor influenced enzyme activity. Depending on the method of RG-I digestion, the subsequent oligosaccharides can take on distinct structural features. Lyase digested oligosaccharides possess an unsaturated GalA residue at the non-reducing end of the structure, allowing for UV detection and facile purification of precise length oligomers (Zhang et al., 2006). Utilizing this strategy, we generated and purified RG-I oligosaccharides of distinct lengths in order to assess the impact of acceptor length on enzyme activity. To determine this, RG-I acceptors of differing lengths (DP 6, DP 12, DP 16) were incubated with SpGalTi. We found that the transferase activity was significantly influenced by acceptor DP, with the highest activity observed with DP 16 acceptors (Figure 3A). The reaction products from each acceptor were additionally examined via MALDI-TOF MS. As expected, the extent of galactosylation correlated linearly with the number of available Rha residues composing the acceptor backbone (Figure 3B-D).

239 Aside from acceptor length, we also sought to determine if specific structural changes to the
240 acceptor influenced activity. Specifically, acetylation is a common modification found on RG-I
241 known to impact plant growth, disease resistance, and stress response (Nafisi et al., 2015). RG-I
242 is known to be acetylated at the *O*-2 and *O*-3 of GalA and at the *O*-3 of Rha backbone residues
243 (Shahin *et al.*, 2023). Given that SpGalTi galactosylates RG-I at the *O*-4 position of Rha residues,
244 we wanted to determine if Rha acetylation at *O*-3 influences SpGalTi in its ability to galactosylate
245 RG-I. To test this, we examined the transferase activity of SpGalTi with acetylated and non-
246 acetylated RG-I DP 6 substrates. When comparing the transferase activity of SpGalTi with both
247 substrates, there was a slight reduction in activity observed with the acetylated RG-I acceptors
248 (Figure 3E). The reaction products generated with the acetylated substrates were also examined
249 via MALDI-TOF MS. RG-I DP 6 acceptors containing one acetyl group had a similar galactosylation
250 pattern to that of their non-acetylated counterpart, with one or two Gal additions to the
251 backbone. However, acceptors containing two acetyl groups had mass shifts indicating only single
252 Gal additions (Figure 3F). Taken together, these data imply that the acetylation of Rha on RG-I
253 inhibits SpGalTi-mediated galactosylation, which we hypothesize may be a mechanism plants use
254 to influence and/or control sidechain patterning *in vivo*.

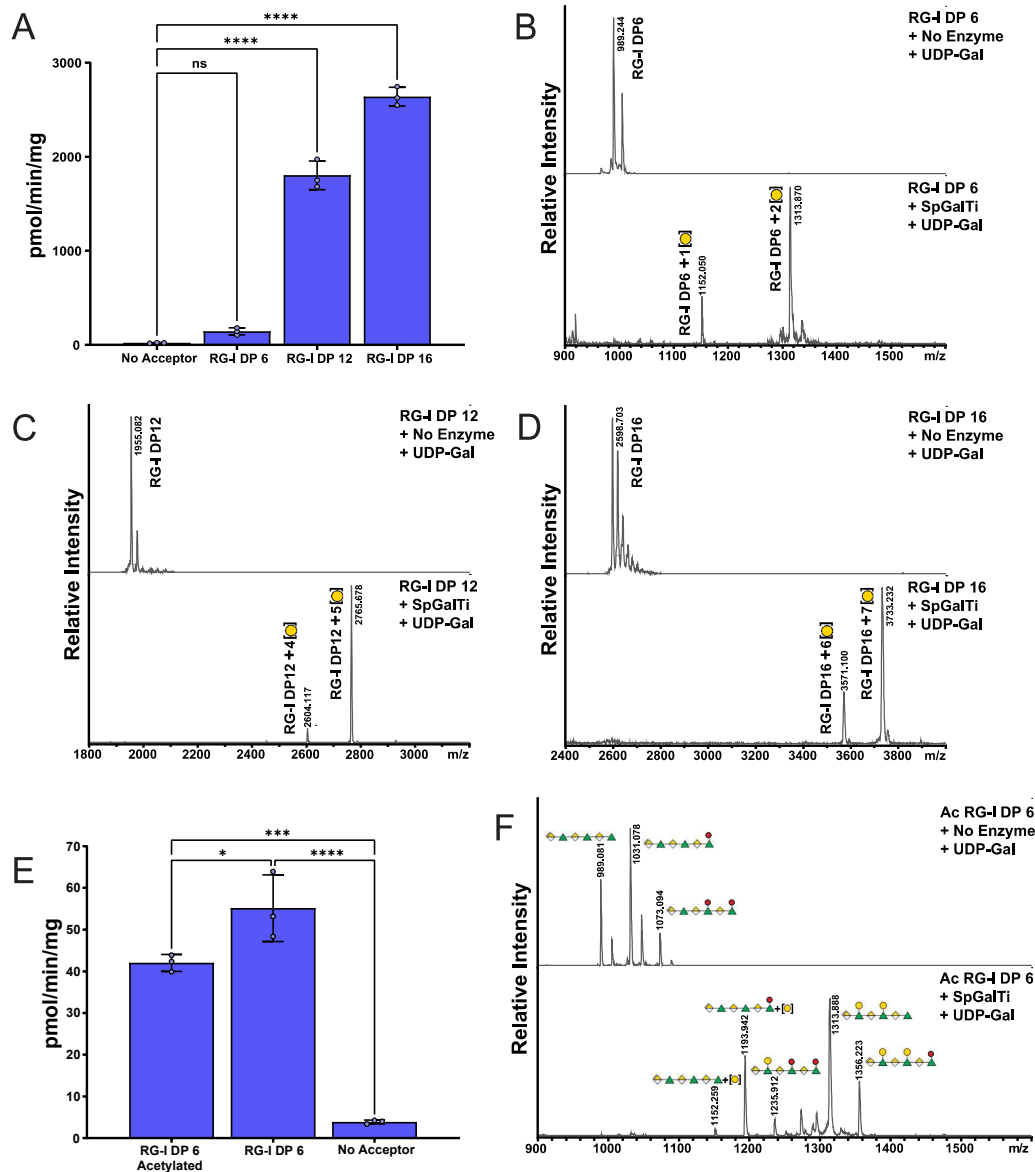


Figure 3: SpGalTi substrate selectivity and product analysis with structurally distinct RG-I acceptors.

(A) Examining SpGalTi transferase activity with RG-I oligosaccharides of differing length (DP 6, DP 12, or DP 16). (B-D) MALDI-TOF MS analysis of SpGalTi reaction products with lyase generated RG-I oligosaccharides ((B) DP 6, (C) DP 12, and (D) DP 16) in the presence of a UDP-Gal donor. (E) Examining SpGalTi transferase activity with either unsubstituted or acetylated RG-I DP 6 oligosaccharides. (F) MALDI-TOF MS analysis of SpGalTi reaction products with acetylated RG-I DP 6 oligosaccharides using a UDP-Gal donor. Hypothetical oligosaccharide structures are shown for reaction products. Masses corresponding to an increase of 162 Da represent an addition of one hexose (Gal). Error bars indicate Mean ± SD with an n of 3 biological replicates. Letters above indicate statistical significance accomplished by a one-way ANOVA (A and E) followed by a Tukey's multiple comparison test ($P \leq 0.01$).

Identification of GalTi Orthologs in Arabidopsis

With the identification of SpGalTi shown to directly initiate galactan sidechain synthesis, we set out to determine the existence of functionally equivalent enzymes outside of *S. polyrhiza*. With this in mind, we chose to identify potential orthologs in *A. thaliana* due to its status as a model dicot for plant cell wall research. We successfully expressed two potential SpGalTi orthologs from *A. thaliana* and showed both enzymes exhibited transferase activity with *A. thaliana* seed mucilage and UDP-Gal (Figure 4A-D), implicating them as potential SpGalTi orthologs. Given this, both enzymes were denoted as AtGalTi1 and AtGalTi2. Since AtGalTi2 exhibited poor stability and transferase activity (Figure S11), detailed biochemical examination was limited to AtGalTi1. In a similar manner to SpGalTi, AtGalTi1 was screened against RG-I oligos of differing lengths (DP 6, DP 12, DP 16) to assess DP preference. Much like its duckweed counterpart, AtGalTi1 preferred higher DP acceptors (Figure 4E), although its overall activity was less than that of SpGalTi. To further investigate the difference in activity between SpGalTi and AtGalTi, DP 16 RG-I reaction products from both enzymes were compared to determine the extent of galactosylation by each enzyme. With equivalent reaction conditions, AtGalTi1 catalyzed the addition of up to three Gal residues to the acceptor, while SpGalTi added up to five (Figure 4F). AtGalTi1 was additionally screened to determine its potential involvement in either galactan or arabinan extension. After incubation with galactosylated *A. thaliana* seed mucilage along with the donors UDP-Gal, -Arap, and -Araf, there was no indication of galactan or arabinan extension by AtGalTi1 (Figure 4G). AtGalTi1 was also examined to determine whether it was capable of catalyzing the addition of more than one Gal substitution per Rha backbone residue. After lyase digestion of AtGalTi1 treated substrates, the disaccharide products displayed mass shifts consistent with the addition of only single Gal substitutions (Figure S7). Finally, we examined the Gal-specific linkage of the products synthesized by AtGalTi1 using the α -1,4 and β -1,4 galactosidases, as shown in Figure 3F. As observed with SpGalTi, only AtGalTi1 reaction products incubated with the β -galactosidase showed Gal removal (Figure 4H). Taken together, SpGalTi and AtGalTi1 appear to be functionally identical in producing β -linked single Gal substitutions to RG-I Rha backbone residues.

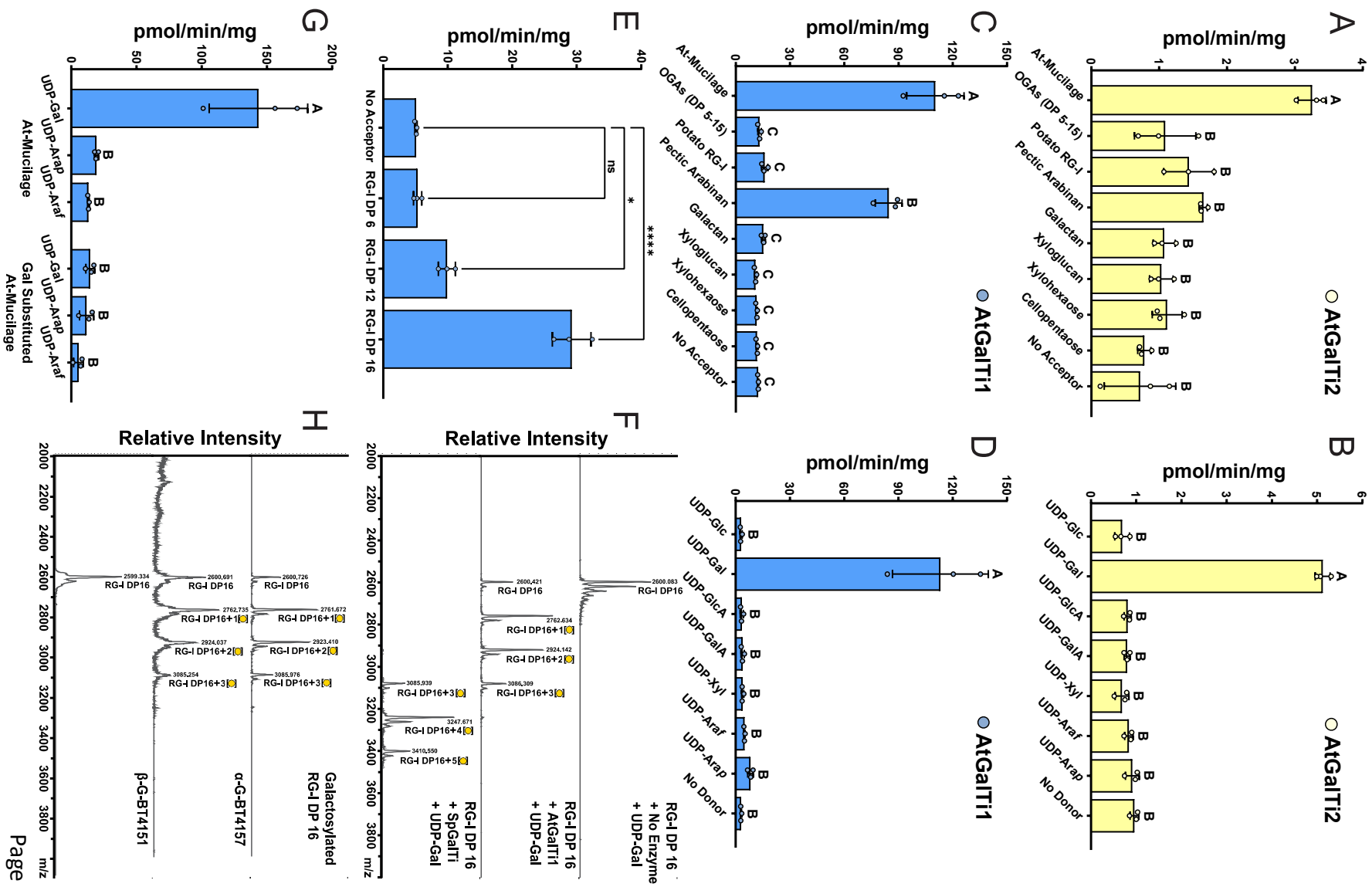


Figure 4: Identification of GalTi enzymes in *A. thaliana*.

(A and C) Screening AtGalTi2 (A) and AtGalTi1 (C) for transferase activity across a series of diverse saccharide acceptors using a universal UDP-sugar mix as a general donor substrate. Quantification of enzyme activity was accomplished by measuring the UDP biproduct after the transfer of a UDP-sugar donor to an acceptor substrate. (B and D) Screening AtGalTi2 (B) and AtGalTi1 (D) for donor substrate selectivity using a library of UDP-sugar donors and *A. thaliana* seed mucilage as an acceptor substrate. (E) Examining AtGalTi1 transferase activity with RG-I oligosaccharides of differing length (DP 6, DP 12, or DP 16). (F) MALDI-TOF MS analysis of AtGalTi1 and SpGalTi reaction products with RG-I DP 16 oligosaccharides in the presence of a UDP-Gal donor. (G) Examining AtGalTi1 activity with the donors UDP-Gal, UDP-Arap, or UDP-Araf with unmodified *A. thaliana* seed mucilage or galactose-substituted *A. thaliana* seed mucilage acceptors. (H) MALDI-TOF MS analysis of AtGalTi1 galactosylated RG-I products treated with an α or β galactosidase. Masses corresponding to an increase of 162 Da represent an addition of one hexose (Gal). Error bars indicate Mean $\pm$ SD with an *n* of 3 biological replicates. Letters above indicate statistical significance accomplished by a one-way ANOVA (A-E) or two-way ANOVA (G) followed by a Tukey's multiple comparison test ($P \leq 0.01$).

A Missing Link for Galactan Elongation

With the existence of this conserved mechanism for galactan initiation, it is relevant to speculate on the existence of Type II GalTs and whether they are required for galactan synthesis. It has already been shown that Type III GalTs, in the form of GalS enzymes from the GT92 family, participate in galactan polymerization from pre-existing galactan sidechain structures with a DP of 3-4 (Ebert *et al.*, 2018; Liwanag *et al.*, 2012; Prabhakar *et al.*, 2023). In the interest of determining if complete galactan synthesis can take place with just Type I and Type III GalTs, we evaluated the ability of three GalS enzymes (PtGalS1, AtGalS1, and AtGalS2) to utilize either SpGalTi-galactosylated RG-I polymer or unsubstituted RG-I polymer as an acceptor substrate. We found that AtGalS1 and AtGalS2 showed minimal transferase activity with all acceptors, while PtGalS1 showed activity with only the galactan acceptor included as a positive control (Figure 5A-C). To verify this, we performed an additional set of experiments with purified RG-I DP 16 acceptors appended with 3–5 Gal residues. Examination of the products via MALDI-TOF MS showed that no mass shifts were observed on the starting substrate upon incubation with any of the GalS enzymes (Figure 5D). The absence of transferase activity suggests that none of the GalS enzymes evaluated herein were able to catalyze galactan synthesis using RG-I modified by single Gal substituents. This was expected given that all biochemically characterized GalS enzymes to

date require an acceptor substrate of three or more Gal residues for synthesis to occur (Laursen *et al.*, 2018; Prabhakar *et al.*, 2023).

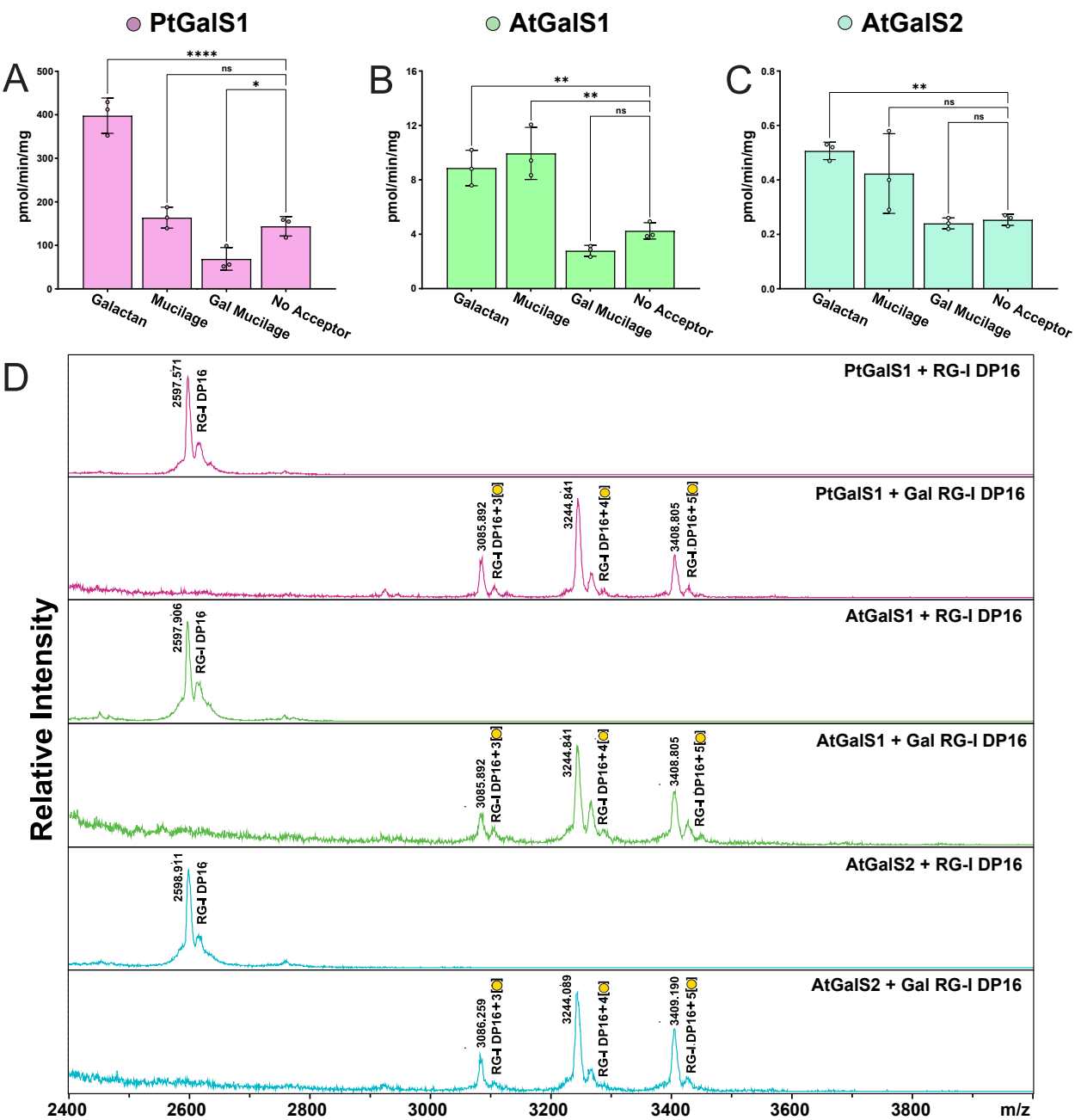


Figure 5: Examining transferase activity and product formation of GalS enzymes in the presence of SpGalTi-galactosylated RG-I.

(A-C) screening PtGalS1 (A), AtGalS1 (B), and AtGalS2 (C) for transferase activity against galactan, unsubstituted RG-I, and SpGalTi-galactosylated RG-I using UDP-Gal as a donor substrate. Quantification of enzyme activity was accomplished by measuring the UDP biproduct after the transfer of a UDP-sugar donor to an acceptor substrate. (D) MALDI-TOF MS analysis of PtGalS1 (Pink), AtGalS1 (Green), and AtGalS2 (Blue) reaction products with SpGalTi-galactosylated RG-I DP 16 oligosaccharides in the presence of a UDP-Gal donor. Error bars indicate Mean $\pm$ SD with an n of 3 biological replicates. Letters above indicate statistical significance accomplished by a one-way ANOVA (A-C) followed by a Tukey's multiple comparison test ($P \leq 0.01$).

The characterization of these novel enzymes has also allowed us to investigate certain structural characteristics via predictive modeling. Utilizing AlphaFold 3, we have modeled both the donor (UDP-Gal) and the acceptor (RG-I DP 6) with SpGalTi, highlighting potential enzyme-substrate residue interactions (Figure S12A). The donor and acceptor appear to be positioned within the C-terminal and N-terminal domains respectively, a canonical orientation of acceptors and donors within GT-B fold enzymes. Among potential residue interactions, an aspartate (ASP315) can be observed in proximity to both the donor and acceptor, potentially acting as the catalytic base associated with SN2-type inverting mechanisms (Figure S12B). The electrostatic surface of SpGalTi reveals additional insights into substrate binding. The negatively charged acceptor is fit within a positively charged pocket where residue interactions with the reducing end GalA position the adjacent Rha in proximity to UDP-Gal for transfer (Figure S12C-D).

Discussion

Understanding the biosynthesis of plant cell wall polysaccharides is a major challenge at the forefront of plant research, with pectin biosynthesis being of particular interest given its structural and functional diversity. Identification of RG-I biosynthetic enzymes remains challenging due to the extensive structural complexity of the polymer, complicating analytical approaches, while genetic disruption of pectin-associated enzymes frequently results in severe developmental phenotypes that limit functional interrogation. For example, the characterization of GalTi's *in planta* may be difficult due to T-DNA insertion lines of AtGalTi1 (At3G03650, *EMBRYO SAC DEVELOPMENT ARREST 5*, *EDA5*) being reported to arrest embryonic development (Pagnussat et

al., 2005). Consequently, we adopted a direct biochemical strategy to bypass these constraints and directly interrogate enzyme function.

Prior speculation on galactan synthesis has proposed a model requiring three distinct GalTs (Ridley *et al.*, 2001). In this study, we have biochemically characterized the Type I GalTs from a monocot and a dicot, which we refer to here as GalTi enzymes from subclade B of the GT47 family. Structural analyses of the reaction products show they catalyze the addition of single β -1,4 Gal residues to O-4 of unsubstituted Rha residues of the RG-I backbone. Taken together, our findings are consistent with a model requiring at least three enzymes. Initially, the first sugar of the galactan sidechain is added by GalTi. This is followed by the incorporation of up to three galactosyl residues by GalTe enzymes; however, whether the second, third, and fourth sugars are added by a single processive enzyme or by multiple distinct extension enzymes remains unresolved. Once the β -1,4-galactan chain has reached a DP of ≥ 3 -4, the dual function GalS enzymes polymerize and then terminate sidechain synthesis by the addition of multiple β -1,4-Gal residues before terminating the chain with an α -1,4-Arap residue (Prabhakar *et al.*, 2023). Given that acetylation of Rha prevents GalTi from galactosylating the same backbone residue, we hypothesize that Rha acetylation functions as a key element in the regulation of sidechain spacing along the backbone.

It is also important to consider the implications of GalTi in the synthesis of other pectic sidechains such as arabinans and arabinogalactans. The abundance and patterning of RG-I sidechains may also be partially influenced by the activity of GalTi given that the Gal additions synthesized at the O-4 of Rha directly occupy positions where other sidechains, such as arabinans, commonly attach to the backbone. Additionally, GalTi may potentially play a direct role in the synthesis of these sidechains, as it has been reported that arabinans may be linked to the RG-I backbone through a short galactan anchor (Øbro *et al.*, 2004). Overexpression of GalTi could yield some insight into the validity of this assumption. There is also the case of ARABINAN DEFICIENT 1 (ARAD1) and ARABINAN DEFICIENT 2 (ARAD2), two GT47s found within the same clade as GalTi, which have been shown to have a chemotype with decreased arabinose in *A. thaliana* mutants (Harholt *et al.*, 2006; Harholt *et al.*, 2012). These two genes may harbor similar functions to GalTi in the initiation of pectic arabinan through the direct transfer of arabinan substituents to the backbone. More interestingly, it may also be possible that ARAD1 and ARAD2 act as Type I GalTs, synthesizing

galactan anchors for subsequent arabinan synthesis. Despite numerous attempts by our team and others to generate recombinant versions of these enzymes, we have so far been unsuccessful and can only speculate on their true functions.

Methods

GalTi Identification, Expression, and Purification

The DNA of the catalytic domain of SpGalTi (37-465 residues), AtGalTi1 (42-499 residues), and AtGalTi2 (52-461 residues) without the transmembrane domain, as predicted by DeepTMHMM - 1.0 (Hallgren et al., 2022) were generated via gene synthesis (Twist Biosciences) in the pTwist Gateway ENTR Kozak vector and transferred into the mammalian expression vector pGen2-DEST (Moremen et al., 2018) by GatewayTM recombination. Sp154-B was expressed as a soluble secreted fusion protein by transient transfection of suspension culture HEK293-F cells (FreeStyleTM 293-F cells, Thermo Fisher Scientific, Waltham, MA). Protein production was carried out for 5 days after transfection before harvesting. Cultures were then clarified by sequential centrifugation at 1200 rpm for 10 min followed by 3500 rpm for 15 min at 4°C, the supernatant was passed through a 5 µM filter (Millipore, Billerica, MA). The proteins were purified by immobilized metal affinity chromatography (IMAC) with HisTrap FF prepacked columns (Cytiva) using an AKTA 25L system (Cytiva) at 4°C (Moremen *et al.*, 2018; Prabhakar *et al.*, 2020). Purified proteins were stored at 4°C for short-term or -80°C for long-term storage. Purified proteins were loaded on the SDS-PAGE to confirm the expected molecular weight.

Glycosyltransferase Activity Assays

The UDP-GloTM Glycosyltransferase Assay (Promega) was used to determine the relative activity of GTs through quantifying UDP (a byproduct produced when a GT transfers a sugar from its UDP-bound donor state to an acceptor substrate) according to the manufacturer's instructions. GT reactions (10 µL) consisted of 250 mM Tris+MES+MOPS pH 6.5, 0.1 mM UDP-sugar donor/s, 0.2 mg/mL acceptor, and 0.1 µM enzyme. Reactions were carried out at room temperature for either 30 minutes or 1 hour. 5 µL of each reaction was mixed with 5 µL of UDP-GloTM Detection Reagent in white polystyrene, low-volume, 384-well assay plates (Corning Incorporated) and incubated for 60 min at room temperature. Luminescence was recorded with a multifunctional microplate

reader BioTek Synergy LX Multi-Mode Reader (Agilent). A standard curve was generated using UDP dilutions with a detection limit ranging from 0.122 pmol to 125 pmol. All UDP-sugar donors used in reactions were CIAP-treated (Calf Intestinal Alkaline Phosphatase) in order to achieve low UDP background (Sheikh et al., 2017).

Substrates

UDP-Glc, UDP-Gal, and UDP-GalA were purchased from Ambeed (USA). UDP-GlcA was purchased from Biosynth (USA). UDP-Xyl and UDP-Arap were obtained from Carbosource Services (USA). UDP-Araf was purchased from Peptide Institute Inc. (Japan). OligoGalA's (OGA's) were generated in house (Bellincampi et al., 2000). Xyloglucan was generated at the CCRC from tamarind seed flower as described previously (Tuomivaara et al., 2015). Potato RG-I, pectic arabinan, galactan, xylohexaose, and cellopentaose were all obtained from Megazyme. *A. thaliana* seed mucilage and RG-I oligosaccharides were generated as described below.

Extraction of Seed Mucilage

A. thaliana seed mucilage was generated by obtaining ~1 gram of *A. thaliana* seeds. Seeds were placed in 200 mL of water overnight with constant mixing. Supernatant containing non-adherent mucilage was collected the next day and filtered via vacuum filtration through a nylon mesh membrane. The filtered supernatant was then dialyzed in 4 L of water with a 3500 Dalton molecular weight cut-off (MWCO) dialysis membrane before being lyophilized and stored for later usage.

RG-I Oligosaccharide Generation (Hydrolase Digestion)

5 mg of *A. thaliana* seed mucilage was suspended in 50 mL of sodium acetate (50 mM) pH 5 and mixed until mucilage was solubilized. RG-hydrolase (0.4 mg) from *Aspergillus aculeatus* obtained from Novozymes (Denmark) was added to the mixture and incubated at 40°C for 5 minutes or overnight (with constant mixing) before being heat deactivated. The mixture was then dialyzed twice in 2 L of water with Spectro/Por 1000 Dalton molecular weight cut-off (MWCO) dialysis membrane (Spectrum Labs) before being centrifuged at 15,000 RPM for 30 minutes. The supernatant was collected and spun through a 10,000 MWCO centrifugal filter (Genesee

Scientific) to remove protein and undigested RG-I polymer. Finally, the flow-through was collected and lyophilized.

RG-I Oligosaccharide Generation (Lyase Digestion)

RG-I substrates were generated and purified by digesting RG-I polymer (*A. thaliana* seed mucilage) with recombinantly expressed and purified *Bacteroides thetaiotaomicron* (BT) RG-I lyase (BT4175). Separation of oligomers was carried out on a Dionex™ CarboPac™ PA1 semi-preparative column under a linear gradient from 5% (v/v) to 100% (v/v) of reagent B (Ammonium formate [1M]) at a flow rate of 2 ml min⁻¹. Fractions containing homogeneous pectic oligomers were pooled, lyophilized, and analyzed using MALDI-TOF MS.

Acetylation of RG-I Oligosaccharides

RG-I oligomers were acetylated with Trichome Birefringence (TBR) enzyme as described in (Marina Leal Gavarrón and Lubana Shahin, 2024). Briefly, *in vitro* enzymatic assays (100 µL) were performed and consisted of 5 µM TBR enzyme, 1 mM 4-Methylumbelliferyl acetate (4MU-Ac; Sigma Aldrich) pseudo acetyl donor, and RG-I DP 6 acceptor substrates (0.25 mM) in MES (50 mM) at pH 5 at 30°C overnight. Reactions were analyzed using MALDI-TOF MS for confirmation of products. The acetylated RG-I oligomers were then dialyzed twice in 2 L of water with a Spectro/Por 1000 Dalton MWCO dialysis membrane (Spectrum Labs) and lyophilized.

MALDI-TOF MS

For MALDI-TOF MS analysis, reactions consisting of 100 mM MES pH 6.5, 1 mM UDP-Gal, 0.2 mM or 0.4 mM RG-I acceptor, and 1 µM enzyme were incubated at room temperature for 8 hours or at specified time points. Reactions were then dialyzed in water with Spectro/Por 1000 Dalton MWCO dialysis membrane (Spectrum Labs). Nafion 117 (MilliporeSigma) was applied on a target plate and left to dry. Afterwards, 1 µL of the dialyzed reactions were mixed with a matrix solution (2 µL; 20 mg mL⁻¹ 2,5-dihydroxybenzoic acid (DHB) in 50% methanol) on the target plate and left to dry. Samples were then analyzed with a smartfleX spectrometer (Bruker) in positive ion mode. Fragmentation of reaction products was analyzed using a rapifleX spectrometer (Bruker), following the same protocol described above.

Nuclear Magnetic Resonance

A 5 mL reaction (containing 100 mM MES pH 6.5, 5 mg of *A. thaliana* seed mucilage, 2 mM UDP-Gal, and 1 μ M enzyme) was split into 25 separate 200 μ L reactions and left to incubate overnight at room temperature. After incubation, reactions were combined and 1.6 mg of RG-hydrolase (from *Aspergillus aculeatus* obtained from Novozymes (Denmark)) was added to the mixture. The reaction was left to incubate at 40°C with constant mixing overnight before being heat deactivated. The solution was then centrifuged at 15,000 RPM for 30 minutes before collecting the supernatant. The supernatant was spun through a 10,000 MWCO polyethersulfone (PES) concentrator (Thermo Scientific) to remove protein and undigested RG-I polymer. The flow-through was then dialyzed twice in 4 L of water with Spectro/Por 1000 Dalton MWCO dialysis membrane (Spectrum Labs). After dialysis, the solution was lyophilized and resolubilized in 500 μ L D₂O before being lyophilized once again.

Samples for NMR spectroscopy were prepared by dissolving the material in 600 μ L of D₂O (99.8% D, Thermo Scientific) and transferring the solution into a 5 mm NMR tube. All NMR data were acquired at 25 °C on a Bruker 600 MHz spectrometer (operating at 600.13 MHz for ¹H) equipped with a TCI cryoprobe. Standard pulse programs from the spectrometer library were used to acquire 1D ¹H, ¹H,¹H-TOCSY ¹H,¹H-ROESY and 2D ¹H,¹H-COSY, ¹H,¹³C-HSQC and ¹H,¹³C-HMBC experiments. NMR data were processed using MestReNova v12.0 (Mestrelab Research). ¹H chemical shifts were referenced to the residual HDO signal at δ 4.79 ppm.

Phylogenetic Analysis

Sequences were downloaded from Phytozome 14 using the databases: Arabidopsis thaliana Araport 11, Oryza sativa v7.0, Populus trichocarpa v4.1, Spirodela polyrhiza v2. Protein sequence alignment was performed in Geneious Prime (version 2025.0.3) using MUSCLE with default settings. A phylogenetic tree was constructed using the Neighbor-Joining method, also with default settings. Finally, a Neighbor-Joining tree including only the proteins from clade B of the GT47 family was generated using the same default parameters.

Statistical Analysis

Quantifiable data is represented as a mean $\pm$ standard deviation (SD) of three biological replicates. Statistical significance was accomplished by either employing a one-way or two-way ANOVA followed by a Tukey's multiple comparison test ($P \leq 0.01$) using Prism 10 software, and is indicated in the figure legend.

Data Availability

All the relevant data can be found within the manuscript and its supporting information and/or are available from the corresponding author upon reasonable request. NMR data will be deposited into the NSF Network for Advanced NMR resource connector.

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Author Contributions

Conceptualization, BRU; methodology, CJC, LN, SJ, LZ, LS, DC, TJ, KWM, PKP, and BRU; formal analysis, CJC, SJ, TJ, and LN; investigation, CJC, LN, SC, SJ, LZ, LS, DJQ, DC, TJ, PKP, and BRU; writing – original draft, CJC, and BRU; visualization, CJC, LN, and SJ; writing – review & editing, CJC, PKP, SJ, LZ, LS, DC, TJ, DJQ, LN, SC, KWM, and BRU; PKP, BRU, and KWM, supervision; project administration, BRU; funding acquisition, PKP, KWM, and BRU.

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Declaration of Interests

The authors declare no competing interests.

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