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Abstract

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The plant cell wall is a complex and integral component of the cell mediating the cellular response to both external and internal stimuli. As the first physical barrier to the environment external to the cell, the cell wall must be able to withstand pressures or attacks while also sensing more subtle environmental perturbations. As the physical support structure and shaper of the cell, the wall must be able to dynamically adapt to necessary growth or developmental conditions as the cell matures, including adapting to stress conditions. Recent transcriptomic and targeted proteomic and metabolomic studies have implicated cell wall-related biosynthetic and modification genes and enzymes in biotic and abiotic stress responses. Targeted manipulation of expression of cell wall-modifying enzymes confers either stress tolerance or susceptibility to transgenic plants. Research coupling the cell wall-related transcriptomic response to chemical wall analyses in field-grown *Sorghum bicolor* exposed to drought stress shows that while a large and consistent transcriptomic response occurs, large compositional shifts in the cell wall do not occur, the only exception being in the younger leaves of a drought-tolerant genotype, which had increases in matrix monosaccharides. Further, the cell wall response to drought stresses in these *S. bicolor* leaves did not negatively impact sugar yield, in one genotype increasing sugar yield from non-structural polysaccharides like starch.

Changes in the cell wall-related transcriptomes and cell wall compositions of field-grown, drought stressed *S. bicolor* leaves and roots frequently implicated changes in pectin biosynthesis and modification. This is consistent with drought and in fact several biotic and abiotic stress studies in both eudicotyledons and commelinid grasses. As a heterogeneous polysaccharide containing domains with distinct branching, architecture, and chemistry, pectin mediates cell wall hydration status, extensibility of the expanding or maturing wall, and even acts as a signaling molecule. Recent studies have demonstrated that targeted pectin modification through reverse genetics confers either tolerance or susceptibility to a variety of biotic and abiotic stresses. Despite its significance in the wall, pectin biosynthesis is still poorly understood, with less than twenty-five of the more than sixty putative transferases necessary for its unique linkages described. One of the most heterogeneous domains of pectin, rhamnogalacturonan I (RG-I), is defined by its repeating -(1,2)-α-L-Rha*p*-(1,4)-β-D-Gal*p*A- backbone. This backbone can be substituted on the rhamnosyl residues at the O3 or O4 positions with four potential side chains, consisting of either linear arabinan, linear galactan, type I arabinogalactans, or type II arabinogalactans. However, structures other than these four canonical side chains have been described previously. Though galactans comprise the bulk of RG-I, the full biosynthetic pathway is still unknown. To date, two different RG-I galactosyltransferases have been described, both involved in linear (1,4)-β-D-galactan elongation. An additional RG-I galactosyltransferase,

GT47F, has been identified that is crucial to normal development in *Nicotiana benthamiana* and embryo and vegetative development in *Arabidopsis thaliana*. The silencing of *GT47F* results in dwarfed *N. benthamiana* and *A. thaliana*, with misshapen cells and mildly chlorotic leaves in *N. benthamiana*. RG-I composition from silenced *N. benthamiana* plants demonstrated a smaller RG-I with less galactan per RG-I moiety. Although monoclonal antibody binding to isolated pectin from these lines did not demonstrate a difference in linear (1,4)-β-D-galactan or the unsubstituted RG-I backbone, an increase in branched (1,6)-β-galactans was observed in silenced *N. benthamiana* lines. T-DNA lines in which the third exon is interrupted, and which likely abolish all activity, are homozygous embryo-lethal in *A. thaliana*. *N. benthamiana* microsomes over-expressing a YFP-tagged *GT47F* demonstrated binding of UDP-galactose in microscale thermophoresis experiments. Taken together, *GT47F* likely encodes a developmentally-required galactosyltransferase that contributes to RG-I galactosylation. Absence of this galactosylation results in increased branched galactan epitopes and smaller molecular weight of RG-I.

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The Plant Cell Wall and Drought Stress Response

Plant cell walls have three critical roles: structural support, mediating cell expansion, and functioning as a barrier to the environment. The components of the plant cell wall and their architectural organization contribute to the physical properties of the wall (1-6). Although cell wall composition varies between both primary and secondary cell walls and between commelinids and eudicots, there exist several components shared across all vascular plants (2, 6-8). Generally speaking, primary cell walls consist of the polysaccharides cellulose, hemicellulose, and pectin, in addition to wall-associated proteins such as arabinogalactan proteins (AGPs), wall associated peroxidases, and other wall-modifying enzymes. Cellulose microfibrils are thought to be the main load-bearing structure of the cell wall and their deposition pattern strongly influences anisotropic cell expansion (9-11). Current wall models attribute cellulose microfibril tethering to hemicelluloses, while typically describing pectin as a matrix filler in the primary cell wall (2, 6, 8), although recent studies have proposed a more prominent role for pectin in influencing cell wall physical properties (12-17) in addition to providing evidence of cellulose-pectin interactions (15, 18). Similarly, a greater focus on wall-associated proteins such as expansins, AGPS, and wall-associated receptor-like kinases (RLKs), reveals significant roles in mediating environmental signals and development (19-24). Secondary cell walls are deposited after primary cell wall deposition in specialized cell types that are dead at functional maturity. Secondary wall components consist of cellulose, hemicellulose, wall-associated proteins and lignin, hydrophobic aromatic polymers that contribute to cell wall stiffening (25-27).

Although commelinids and eudicots share basic cell wall components, their relative abundance and structures vary. Eudicot primary cell walls are largely composed of cellulose, the hemicellulose xyloglucan, and pectin (2, 6, 8). In contrast, commelinid primary cell walls largely contain cellulose, the hemicellulose xylan, and a (1,3;1,4)-β-mixed linkage glucan (MLG) unique to commelinids (6-8, 28). The structural features and arrangement of these polysaccharides within the cell wall confers unique physical properties, ranging from increased extensibility to increased stiffness, and can be tuned to developmental regimens and environmental stresses (2, 23, 29, 30). Because of the structural and compositional variety of cell walls found in different plant lineages, it is important to note that these plants may employ different cell wall-related responses during environmental stresses.

Despite differences in cell wall composition, many vascular plants have a broadly shared response to drought stress. Under water deficit, plants typically share a common strategy: slow or halted growth until drought stress is alleviated. When water is limiting, plants face two major challenges. The first challenge is the balance between conservation of water and carbon fixation. Under drought stress, many plants close their stomata to prevent evaporative water loss. Depending on the duration of drought stress, plants may also decrease their photosynthetic output as a means to conserve water (31-35). During severe or sustained drought stress, photosynthesis may be more or less halted altogether, resulting in arrested growth, most especially in the shoot (31, 32, 35, 36). The only continued growth in plants under severe drought stress is at the root apex, a strategy employed to find deeper reserves of groundwater (31, 32, 35-38). The maintenance of root apex extensibility while carbon fixation decreases or halts is intriguing, as extensibility relies on the continued deposition and rearrangement of cell wall materials, an energetically costly process.

The second challenge that plants face under drought stress is preventing damage from the loss of turgor pressure and the dehydration of cells. Excessive negative pressure (tension) in the vasculature of plants prevents cavitation, a vapor-cavity formation within tracheary elements that impedes water transport. Additionally, turgor pressure, along with cell wall extensibility and deposition, is required for cell expansion and growth. In shoots, reduced turgor pressure is mitigated by the cessation of growth and by the stiffening of cell walls (39-41). In addition to maintenance of turgor pressure, plants must also avoid damage from dehydration of tissues. Dehydration can destabilize protein folding, impact cell pH and intracellular ion reserves, and collapse and damage the cell wall. Plants employ a variety of responses to mitigate these issues. The most prominently studied responses are the production of osmoprotectants to maintain turgor pressure, prevent unregulated ion flux, and help stabilize protein folding. Common osmoprotectants produced under drought stress are glycine betaines, proline, and mannitol, although some plants are not capable of producing all of these osmoprotectants (31, 32, 42).

Given that both major challenges that plants face under drought stress are related to cell wall function (cell expansion and structural support), it is a logical assumption that cell wall modifications are an integral component of the plant drought stress response. Additionally, because the shoot response to drought stress is to halt growth and the root response to drought stress is to maintain apex extensibility, it is likely that cell wall modifications are starkly different across these tissue types. In shoots, the cessation of growth and stiffening of cell walls is reflected in microarray and transcriptomic studies that show a down-regulation of cell wall polysaccharide biosynthetic and modifying enzymes and an up-regulation of lignin biosynthetic enzymes and cell wall-bound peroxidases (39-41, 43, 44). The down-regulation of cell wall polysaccharide biosynthetic enzymes correlate to a halt in growth – no new polysaccharides are deposited, thus cells are not growing and expanding. The up-regulation of lignin biosynthetic enzymes and cell wall-bound peroxidases correlates to increased lignification and phenolic cross-linking of cell wall polysaccharides (43, 45-48). These modifications are consistent with the stiffening of the shoot during periods of prolonged or severe drought stress, as lignin is a wall stiffening agent and phenolic cross-linking reduces wall flexibility (49, 50).

In roots, the response to drought stress is dependent on zones of development. The elongation zone in drought stressed maize roots is decreased in length relative to well-watered plants (1, 37, 51). Root tissues basal to the elongation zone have down-regulated cell wall associated enzymatic expression. Conversely, root tissues encompassing the apex and elongation zone maintain extensibility and have increased cell wall modifying enzymatic activity. The most commonly up-regulated wall modifying enzymes in the root apex are expansins, xyloglucan endotransglycosylases/hydrolases (XTHs), and endo-(1,3;1,4)-β-D-glucanases (1, 39-41, 52-55). Expansins have been demonstrated to increase cell wall extensibility through pH-dependent rearrangement of cell wall polysaccharides, although the mechanism of action is still unknown (56). XTHs cleave and rearrange xyloglucan polysaccharides in the cell wall and also demonstrate increased extensibility of cell walls when active (57). The phytohormone abscissic acid (ABA) regulates expansin and XTH activity under drought stress, indicating that these cell wall modifying enzymes participate in the ABA-dependent drought response pathway (20, 57). Additionally, constitutive overexpression of expansins in *Arabidopsis thaliana* and *Nicotiana benthamiana* confers an additional measure of drought or osmotic tolerance, indicating that extensibility of root apices is required for drought stress survival (19).

Studies using reverse genetics have demonstrated that constitutive overexpression of expansins and XTHs confers drought tolerance (19, 58) in *A. thaliana*. Constitutive overexpression of a pepper pectin methylesterase inhibitor conferred drought tolerance in *A. thaliana* (58), while constitutive overexpression of a rice polygalacturonase made *A. thaliana* more susceptible to drought (14). The *irregular xylem* mutants in *A. thaliana*, often encoding crucial cell wall biosynthetic enzymes required for cellulose, xylan, and lignin biosynthesis, often have improved drought stress and freezing survival rates compared to control plants, although this increased tolerance is complicated by reduced stature and an already collapsed vasculature primed for stress response (59-63). Additionally, drought survival assays comparing cell wall engineered lines with control lines in *A. thaliana* demonstrate that lines engineered for low xylan content, low lignin content, and low xylan acetylation have greater drought tolerance than control lines. Importantly, this study shows that reductions in xylan, lignin, and acetylation contribute to increased drought tolerance in *A. thaliana* independent of the *irregular xylem* phenotype (64). All these studies involving alterations in cell wall modifying and biosynthetic gene expression underscore the crucial role the cell wall plays in mediating drought stress.

Although a great deal of enzymatic activity (48, 65, 66), proteomic (54, 67-70), and transcriptomic research (47, 51, 55, 71-78) demonstrates the modification of cell walls in response to drought stress, there is relatively little data on cell wall compositional and architectural changes in response to drought. Early studies examining impacts on cell wall composition under drought stress in wheat and *N. benthamiana* have the disadvantage of occurring under conditions unlikely to be encountered outside of a lab, testing mainly seedlings or suspension cultures grown with polyethylene glycol (PEG), creating an osmotic stress imitating drought stress (79-83). Wheat studies consistently demonstrate changes in arabinoxylan, MLG, and to a lesser degree, pectin content, although the compositional changes vary between studies (79, 80, 82, 83). Changes are likely due to differences in tissues analyzed and in application of drought or osmotic stress. More recent studies using bioenergy crops such as *Sorghum bicolor, Panicum virgatum, Miscanthus* spp., and other commelinids have demonstrated contrasting results concerning cell wall composition under drought stress (84-87). While these studies typically demonstrated a decrease in structural sugars, encompassing cellulose and hemicelluloses, in addition to either unaffected or slightly improved sugar yield from biomass, results were not always uniform. Additionally, many of these studies did not differentiate compositional changes beyond cellulose, hemicellulose, and sugar yield. Further, none of these studies coupled compositional analysis of the cell wall with transcriptional analysis. All these factors – differences in plants and tissues analyzed, unrealistic or short drought induction conditions, shallow compositional analysis, and de-coupling of transcriptional and cell wall compositional analysis – make it difficult to predict how the plant cell wall physically responds and changes under drought stress.

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Cell Wall Compositions of *Sorghum bicolor* Leaves and Roots Remain Relatively Constant Under Drought Conditions

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Abstract

Background: Renewable fuels are needed to replace fossil fuels in the immediate future. Lignocellulosic bioenergy crops provide a renewable alternative that sequester carbon from the atmosphere. To prevent the displacement of food crops, it would be advantageous to grow biofuel crops on marginal lands. These lands will likely face more frequent and extreme drought conditions than conventional agricultural land, so it is crucial to see how proposed bioenergy crops fare under these conditions and how that may affect lignocellulosic biomass composition and saccharification properties.

Results: We found that while drought impacts the plant cell wall of *Sorghum bicolor* differently according to tissue and timing of drought induction, drought-induced cell wall compositional modifications are relatively minor and produce no negative effect on biomass conversion. This contrasts with the cell wall-related transcriptome, which had a varied range of highly variable genes (HVGs) within four cell wall-related GO categories, depending on the tissues surveyed and time of drought induction. Further, many HVGs had expression changes in which putative impacts were not seen in the physical cell wall or which were in direct opposition to their putative impacts. Interestingly, most pre-flowering drought-induced cell wall changes occurred in the leaf, with matrix and lignin compositional changes that did not persist after recovery from drought. Most measurable physical post-flowering cell wall changes occurred in the root, affecting mainly polysaccharide composition and cross-linking.

Conclusions: This study couples transcriptomics to cell wall chemical analyses of a C4 grass experiencing progressive and differing drought stresses in the field. As such, we can analyze the cell wall-specific response to agriculturally-relevant drought stresses on the transcriptomic level and see whether those changes translate to compositional or biomass conversion differences. Our results bolster the conclusion that drought stress does not significantly affect the cell wall composition of specific aerial and subterranean biomass nor impede enzymatic hydrolysis of leaf biomass, a positive result for biorefinery processes. Coupled with previously reported results on the root microbiome and rhizosphere and whole transcriptome analyses of this study, we can formulate and test hypotheses on individual gene candidates function in mediating drought stress in the grass cell wall, as demonstrated in sorghum.

Keywords: *Sorghum bicolor*, drought, cell wall, biomass conversion

Background

As fossil fuel resources decline and usage continues to have adverse effects on global climate, it is crucial to develop alternative fuel sources to supply energy demand. Lignocellulosic bioenergy crops have the advantage of being renewable energy resources with reduced carbon emissions compared to petroleum-based fuel. Recent studies have demonstrated that the use of lignocellulosic biofuels has the potential to sequester carbon from the atmosphere by increasing soil organic carbon, which may help mitigate increased carbon release to the atmosphere (88-90). Despite these advantages, lignocellulosic biofuels have several requirements to meet before they can be considered competitive with petroleum-based fuels. The foremost requirement is cost-competitiveness, which is primarily dependent on the recalcitrance of the cell wall and the ability to valorize waste products from biofuel production. Cell wall recalcitrance can often be simplified to several key factors: the amount and complexity of lignin, the amount and crystallinity of cellulose, the presence of biomass conversion inhibitors such as acetate, and the ratio of C5:C6 sugars, with a low ratio tending to favor saccharification efficiency and conversion into fuels (91-95). In addition to biomass conversion efficiency, lignocellulosic feedstocks should ideally be grown on land that is not suitable for food crops to avoid displacing food crops. A major factor that makes cropland marginal is low rainfall and/or increased drought. With irrigation water being a severely limited, expensive, and energetically intensive resource, lignocellulosic feedstocks must be robust enough to generate high biomass yields even under adverse conditions like drought. In addition to high biomass yields, the lignocellulosic properties of the feedstocks would ideally not be altered by drought (90, 91, 95).

The impact of drought on the plant cell wall-related genes has been investigated in transcriptomic and proteomic studies (39-41, 47, 48, 51, 54, 96). Over the last several years, researchers have also performed basic chemical analyses of plant cell walls produced under drought conditions. However, these studies are limited in several ways. First, most transcriptomic studies lacked cell wall analysis to verify that transcriptomic changes actually translated into chemical changes in the wall. Secondly, much of the chemical cell wall analyses that were performed were either on specialized tissue types, from multiple plant lineages, or were under conditions unlikely to be encountered outside of a laboratory (79-83, 97). Thirdly, more recent studies analyzing compositional and sugar yield differences use techniques that do not distinguish between different polysaccharides nor are they coupled to transcriptomic analyses (84-87). These variables can make it incredibly difficult to draw anything other than very broad conclusions for a given plant on how the cell wall will respond to drought.

Several grasses have been proposed as lignocellulosic feedstocks including: *Sorghum bicolor, Panicum virgatum*, and *Miscanthus* spp (95, 98). These grasses have all been selected for their high vegetative biomass yields and for their increased tolerance to prolonged drought and nutrient scarcity (95, 98). *S. bicolor* has several advantages as a crop and as an experimental system including: high yield under water and nitrogen-deficient conditions, a sequenced genome, genetic tractability, and demonstrated genetic modification techniques (95, 98-103). Furthermore, *S. bicolor* has the advantage of having different either pre-flowering drought-tolerant or post-flowering drought-tolerant cultivars (104, 105). For this study, we used RTx430, a pre-flowering drought-tolerant line, and BTx642, a post-flowering drought-tolerant line (104, 105), to explore how some different drought-tolerance strategies affect cell wall composition under both pre-flowering and post-flowering drought stress.

Results

Transcriptome & Cell Wall-Related Highly Variable Genes

Drought stress had different impacts on the transcriptome relating to cell wall-specific genes depending on the tissue type and the developmental time of drought induction (details on how we defined cell wall-specific genes are described in Methods). The cell wall-specific transcriptome was modified under all conditions relative to the well-watered control: pre-flowering drought induction, recovery after pre-flowering drought induction, and post-flowering drought induction. Within the cell wall-related transcriptome, we focused our analysis on highly variable genes (HVGs), those genes in the top 10% of differentially expressed genes when referenced against the watered control ranked by log-fold change. Leaves had fewer cell wall HVGs, with 15-55 highly variable cell wall-related genes across treatments (Figure 1). Notably, leaves experiencing a pre-flowering drought from both genotypes had only 15 highly variable cell wall-related genes. Of these genes, most appeared to encode cell wall modifying enzymes such as expansins, xyloglucan endotransglucosylase/hydrolases (XTHs), peroxidases, and pectin-modifying enzymes (Figure 1 & Figure S1). In contrast, roots experienced a greater number of cell wall-related highly variable genes, with 40-117 highly variable cell wall-related genes across treatments (Figure 1) in which HVGs with putative modification functions also predominated (Figure S2). A recent estimate of genes encoding enzymes related to cell wall biosynthesis and modification in sorghum is 520, largely comprised of 160 glycosyltransferases, 201 glycosyl hydrolases, and 83 expansins (106). However, this estimate only included some well-characterized gene families, and in particular, the number of cell wall-related glycosyltransferases is certainly much higher. In fact, a broader survey of cell wall-related genes in sorghum found approximately 1,200 genes related to the cell wall, including substrate synthesis, membrane trafficking, post-depositional modification, and signaling in addition to polysaccharide biosynthesis (107).

Generally speaking, the root cell wall-related transcriptome followed broad patterns across treatments. Both pre-flowering and post-flowering drought induction across genotypes resulted in a decrease in highly variable cell wall-related gene expression (Figure S3). However, the *S. bicolor* RTx430 genotype experiencing a pre-flowering drought induction had an increase in highly variable cell wall-related gene expression upon re-watering; this effect was not seen as dramatically in the BTx642 cultivar (Figure S3). Effected highly variable genes predominantly encoded putative cell wall-modifying and biosynthetic enzymes, with a decrease in expression of glycosyltransferases, acetyl- and methyltransferases (Figure S4), peroxidases, pectin modifiers

(esterases, lyases, etc.), glycosyl hydrolases, and expansins and XTHs (Figure S5). Based on these results, we hypothesized that drought induction resulted in the decrease in cell wall deposition and modification in basal roots, with altered polysaccharide-lignin cross-linking and potentially altered pectin structure. It is interesting that pectin-related genes were so highly represented in tissues that should be predominantly secondary cell walls in a species with reportedly low pectin content (7). We also hypothesize that re-watering after a pre-flowering drought induction results in increased cell wall deposition and expansion in the RTx430 genotype relative to the well-watered control and in comparison to the recovery of the BTx642 genotype.

By contrast, the leaf cell wall-related transcriptome did not have universal patterns across drought treatments. Aside from the pre-flowering drought induction treatment, which did not have many appreciable cell wall-related transcriptomic changes in either genotype, the genotypes had exaggerated differential expression for recovery and post-flowering drought (Figure S3). The two genotypes diverged during the recovery period, with BTx642 having a greater number of glycosyltransferases (GTs) among the HVGs with increased expression relative to the watered control when compared to RTx430 lines (Figure S4). Conversely, the RTx430 genotype experiencing post-flowering drought stress had a greater number of GTs with higher expression relative to the watered control when compared to BTx642 (Figure S4). It is possible that this higher GT activity in older leaves, related to xylan, mixed-linkage glucan, and pectin biosynthesis, has a negative relationship with drought tolerance, as increased expression of these GTs occurred in the genotypes more susceptible to the drought stress or less efficient in recovery. Additionally, the RTx430 genotype experienced greater increase in expression of acetyl- and methyltransferases than BTx642 during post-flowering drought, but this dynamic was reversed during recovery from pre-flowering drought stress (Figure S4). Thus, leaf cell wall-related transcriptomic changes appear to implicate different changes in polysaccharide abundance and structure. Sorghum from both genotypes that were re-watered after experiencing a pre-flowering drought stress had an increase in expression of putative wall expanding genes, such as expansins and XTHs (Figure S5). Additionally, the same sorghum plants also had an increase in expression of putative secondary cell wall cellulose synthases and xylan backbone-synthesizing genes (Figure S6). Taken together, the cell wall-related transcriptome of re-watered sorghum leaves after a pre-flowering drought induction suggests that these cell walls experience an increase in secondary cell wall deposition and expansion, with potentially modified polysaccharide structure in xylan and pectin. For sorghum experiencing a post-flowering drought stress, there was an observed increase in expression of putative secondary cell wall cellulose synthases and acetyl- and methyltransferases (Figures S4 & S6). Interestingly, expression of expansins, XTHs, and pectin modifying enzymes (lyases and pectin methylesterases) appeared to decrease while expression of some glycosyl hydrolases increased (Figure S5). This suggests that post-flowering drought induction may result in increased deposition of the secondary cell wall in mature leaves with potentially affected pectin structure and esterification. Again, genes encoding putative pectin biosynthetic and modifying enzymes appear to be unusually highly represented, given that pectin is a very small portion of commelinid cell walls.

Based on the expression pattern of the majority of cell wall related HVGs over the course of the experiment (Supplementary File 1), we selected tissues from weeks seven and fourteen for cell wall chemical analysis. During these weeks, plants have been subjected to four weeks of either pre-flowering drought stress, recovery, or post-flowering drought stress alongside the watered

controls. Based on the differential expression analysis of cell wall-related HVGs, this should give the plant ample time to implement the downstream changes resulting from differential expression while also not occurring after the differential expression ceases.

Cell Wall Composition Analysis

Plants exposed to a pre-flowering drought stress experienced the greatest deal of compositional change in cell wall monosaccharide composition relative to the well-watered condition in RTx430 (Figure 2). Young leaves had an increase in matrix monosaccharides, including rhamnose, arabinose, glucose, and galacturonic acid (62, 33, 62, 37%, respectively, Figure 2A). BTx642 leaves experiencing pre-flowering drought stress also had an increase in matrix glucose, but the significance of the increase was lost when correcting for multiple comparisons (Figure 2B). Basal roots of BTx642 also experienced an increase in matrix glucose (76%, Figure 2D) but demonstrated no additional monosaccharide compositional changes either in the matrix nor in the cellulosic sugars during pre-flowering drought stress.

Plants from either genotype recovering from a pre-flowering drought stress experienced no cell wall matrix or cellulosic compositional changes (Figure 2, 3).

Plants exposed to a post-flowering drought stress had few cell wall compositional changes compared to plants exposed to pre-flowering drought stress (Figure 2, 3), with only BTx642 showing significant changes in the leaves and root. Leaves of BTx642 showed an increase in cellulose-associated galacturonic acid (38%, Figure 3B), while roots experienced an increase in matrix arabinose and galactose (81% and 130%, Figure 2D).

Lignin

RTx430 plants experienced no change in total lignin content across leaves and roots across the different irrigation conditions. However, RTx430 roots experiencing a pre-flowering drought stress showed a decrease in H units relative to total monolignol content, implying that the lignin in these roots may be more complex and resistant to degradation during a pre-flowering drought (Figure 4). RTx430 plants recovering from a pre-flowering drought showed a decrease in *p*-coumarate in the leaves and an increase in *trans*-ferulate in the roots, suggesting an alteration in cross-linking in the more extensible leaves and in the rigidifying roots.

BTx642 plants exposed to a post-flowering drought stress had an increase in total lignin in older leaves (Figure 4) but otherwise BTx642 plants experienced no change in lignin or hydroxycinnamate content during drought conditions.

Saccharification Efficiency

Drought stress had no negative implications on saccharification efficiency of leaves from either genotype, whether drought stress was induced pre- or post-flowering (Figure 5). Interestingly, when assaying the saccharification efficiency of leaves with starch content intact, an increase in the efficiency was observed under all drought conditions of RTx430, with up to 66, 39, and 36% increases for leaves experiencing pre-flowering drought, recovery, and post-flowering drought (Figure 5B). These increases in saccharification efficiency were lost when analyzing leaves with starch content enzymatically removed (Figure 5A).

Discussion

Transcriptome to Cell Wall

One of the biggest questions answered by this study is whether the transcriptome of droughted plants, when compared to well-watered plants, can accurately predict chemical changes within the plant cell wall. Concerning the cell wall-related HVGs, the transcriptome does not necessarily translate into measurable chemical wall changes. While some transcriptional changes resulted in expected cell wall changes, many were either correlated with no compositional changes or even the opposite of the expected changes. While many reported effects of drought stress on the plant cell wall are derived from transcriptomic studies that are rarely experimentally validated (40, 41, 72, 78), this study demonstrates the need to validate the chemical and physical changes in the cell wall, especially as the tissues and conditions with the highest number of HVGs had the fewest changes in the wall, while the leaves experiencing pre-flowering drought induction had the fewest number of HVGs but the greatest compositional changes in the cell wall. These results support a growing body of literature reporting contrasting transcriptome results concerning drought stress, with *Brachypodium distachyon* demonstrating a wall-related transcriptome that did not always align with the metabolome in response to drought stress (47).

Despite significant differences in expression of genes with cell wall-related GO terms, we detected few changes in the composition and saccharification efficiency of the cell walls of both *S. bicolor* cultivars in response to drought stress. This disconnect between transcriptome and wall composition can be explained by several different scenarios. The first scenario being that biosynthesis and modification is occurring through the enzymatic activity of the differentially expressed gene products, but this action is highly specialized, either in expression pattern or in activity pattern. For example, many GT61 family genes show differential expression in the roots of both cultivars, but there is no corresponding significant change in arabinose or xylose content (108). This could be due to a specific action of potential xylan arabinosyl- or xylosyltransferases on particular residues of the xylan backbone. Differential and specific substitution patterns on xylan with resulting impacts on wall ultrastructure has been demonstrated in eudicotyledons and gymnosperms (109-111), but have not been determined in grasses. A previous study in maize leaves indicated that plants experiencing drought stress had decreased levels of caffeoyl-O-methyltransferase, but showed no reduction in lignin, instead impacting the amount of free monolignols for future incorporation into lignin (48). It is possible that the HVGs encode enzymes that are minor contributors to wall biosynthesis and modification, with more active members of the gene families remaining constant under drought stress. Alternatively, there could be a large differential expression in these genes that is specific to a cell or tissue type, but the resulting change in composition is masked by that of other cell types. Another scenario could be that the enzymatic action is again occurring, but compositional changes are masked by the flux of carbon into and out of the cell wall. For example, it is possible that a great deal of new wall material is being deposited and/or modified, but either this new material or old material is being exuded from the wall for signaling and/or symbiotic processes with the rhizosphere (12, 112, 113). An additional scenario is that differentially expressed genes may not result in significantly different amounts of active enzymes or may not result in higher enzymatic activity, potentially due to both post-transcriptional and/or post-translational modifications (114). Assuming that transcription does result in increased active enzyme levels, it is possible that the activity levels of the enzymes encoded by these HVGs are very low, or that the actual enzymatic activity is different from its putative function, as most of these encoded enzymes have not had any demonstrated activity in sorghum. A last scenario is one in which differential expression of cell

wall-related genes, particularly those involved in biosynthesis and modification, could simply be the result of cells and their walls either ceasing to expand and grow, a known and visually obvious phenotype of progressive drought in leaves, or continuing to maintain extensibility in the roots despite reduced soil moisture availability. The ability to pinpoint the most likely set of scenarios will rely on further experiments exploring the composition and architecture of specific polysaccharides (xylan and pectin branching), exploring root sugar exudates, and exploring composition in a tissue and/or cell-specific context. Further useful experiments would also explore the cell wall-related proteome of these plants to understand whether differential expression also leads to differential enzyme accumulation.

Of the compositional changes that were observed, the most prominent changes happened to be in the matrix – namely, an increase in pectic rhamnose and galacturonic acid, arabinose, and glucose in the young leaves of RTx430 plants experiencing a pre-flowering drought stress and an increase in arabinose and galactose in the roots of BTx642 plants experiencing post-flowering drought stress. The changes in young leaves likely indicate an increase in pectin, arabinosylated xylan, and mixed-linkage glucan, although changes in abundance of arabinogalactan proteins could also be possible. Because these changes occurred in the pre-flowering drought-tolerant RTx430, but not BTx642, it is possible that these pectic and hemicellulosic differences contribute to pre-flowering drought tolerance. Root changes likely indicate an increased in arabinosylated xylan and either pectic galactan or arabinogalactan proteins. Despite not seeing an increase in any GALS1, PAGR, or GT31 homolog expression (115-117), we still observed an increase in galactose in the roots of BTx642. This could be due to an entirely different and unidentified galactosyltransferase, or alternatively it could be that known galactosyltransferase activity is regulated on the enzymatic level and thus is not observed in the transcriptome. In the same vein, we also did not observe any differential expression of GAUT or RRT1 homologs (118-120) during pre-flowering drought stress in RTx430 plants to account for changes in rhamnose and galacturonic acid. Pectin has been implicated in the plant drought response in the past (14, 16, 17, 121), and pectic galactan and arabinan from rhamnogalacturonan I have been proposed to behave as “plasticizers” in drought and desiccation conditions for several different plants (53, 67). However, this focus on pectin’s role in the plant drought response has primarily been relegated to eudicotyledons, in which pectins comprise a much greater percentage of the plant primary cell wall. These results coupled with that in the literature indicate that even in plant lineages where pectin is a very small portion of the plant primary and secondary cell wall, this polysaccharide may still play an outsize role in responding to and/or mitigating plant stress. Moreover, the over-representation of genes encoding pectin modifying enzymes amongst the HVGs in this study is striking, given that there are relatively few in sorghum, with only 16 pectic lyases and 35 carbohydrate esterases (including pectin methylesterases) (106). More work looking into pectic side chain composition, pectin esterification, and cleavage of pectic oligosaccharides in response to drought stress can help clarify the role of pectin in these processes.

An increase in matrix glucose was observed which often, but not always, correlated with a similar transcriptomic response in the CSLF gene family, which is known to encode mixed-linkage glucan synthases (122). It is possible that this increase in glucose is in part derived from another cell wall polysaccharide, most likely amorphous cellulose that is released during hydrolysis with trifluoroacetic acid. As with the pectin study, it is possible that either an unidentified glucosyltransferase is responsible for this increase in glucose or that the known

glucosyltransferases are regulated tightly at the enzymatic level and the transcriptomic regulation does not reflect that. However, this is in contrast to previous literature in which mixed-linkage glucan decreased in the aerial biomass of other grasses, with a mixed response in the roots (79, 80, 82, 83, 97). MLG is deposited in the expanding cell wall and is correlated with increased wall extensibility (122, 123).

Secondary Cell Wall Deposition

Transcriptomic changes in other putative cell wall biosynthetic genes such as cellulose synthases, the GT61 family responsible for glycosylating the xylan backbone (108), and lignin biosynthetic enzymes (124) were not reflected in cell wall compositional analyses. These upregulated genes could simply be a proxy for continued growth, including cell expansion and secondary cell wall deposition. Secondary cell wall CESAs were upregulated in older leaves of both cultivars in response to both post-flowering drought stress and pre-flowering drought stress recovery. This suggests an increase in secondary cell wall deposition in these older leaves as they either recover from drought stress or experience drought stress post-anthesis, likely from continued cell expansion and/or increased maturation. However, it does not suggest that the total secondary cell walls are more enriched in cellulose or lignin, which is borne out in the compositional wall analyses.

Cell Wall Modification

Increased extensibility of root cell walls in response to low water potential linked to expansin abundance and activity has been explored and verified in the roots of maize seedlings exposed to drought or osmotic stress (51, 65). Our own transcriptomic results align well with transcriptomic surveys of other grasses exposed to drought stress in relation to wall expanding genes such as expansins and XTHs (41, 47, 72, 78). Expansin activity during drought stress results in more highly expansible cell walls, as has been shown in the apical domain of the maize root (65), and overexpression of a rose expansin in *Arabidopsis thaliana* has previously conferred drought tolerance (19). Interestingly, expansin expression decreased with distance from the root apex, implying that expansin activity during drought was focused to provide increased extensibility to the root apex, the portion of the root still expanding for deeper water reserves (51). Our data shows that expansin activity in the basal root is reduced during drought conditions, suggesting that in both genotypes, the basal root has decreased extensibility in response to drought stress. Interestingly, re-watered roots of the RTx430 pre-flowering drought tolerant genotype had increased expansin and XTH expression levels during the pre-flowering drought recovery period. As indicated by (112), RTx430 appeared to have a faster recovery from pre-flowering drought stress, both in biomass recovery, but also in root microbiome recovery. This may be another indicator of pre-flowering drought tolerance, in which RTx430 is primed to resume cell division and expansion more rapidly after re-watering, which in turn gives a performance boost to the plant and its associated microbial communities via increased carbon flux.

Biomass Conversion Efficiency

On a more practical level, this study indicates that drought stress does not impact saccharification efficiency of cell wall sugars in either cultivar and in fact can contribute to saccharification efficiency through starch accumulation in the RTx430 line (an increase of up to 66% in some assayed timepoints). As bioenergy crops like sorghum will likely be exposed to

greater frequencies and durations of drought stress, this is welcome news, as saccharification penalties combined with potential biomass yield penalties would make bioenergy crops even less competitive in the energy market. From this study, combined with previously published data using this field trial (77, 112), we can conclude that the sorghum cultivars RTx430 and BTx642 experience minimal biomass yield penalties and no saccharification penalties under both pre-flowering and post-flowering drought stress. Furthermore, this study suggests that biorefineries do not need to be concerned with significant changes in biomass properties depending on drought patterns.

Conclusions

Transcriptome to Cell Wall

While there have been several studies detailing the transcriptomic differences of drought-stressed grasses (47, 72, 78), and several studies detailing cell wall changes in grasses affected by drought stress (46, 48, 84-87), this is the first study to detail the cell wall-related transcriptomic differences and the cell wall differences in a drought-tolerant C4 grass affected by several different drought stresses in the field. As with previous transcriptomic studies (40, 41, 47, 55, 72, 74, 77, 78), we see that GO terms and categories relating to the cell wall are significantly affected by drought stress, although the transcriptomic response to drought stress is large in sorghum, with more than 40% of expressed genes experiencing an effect on expression patterns relative to well-watered conditions (77). In our own study, we noted a wide range of 15-117 cell wall related HVGs depending on the genotype and drought treatment, out of a total of 520 cell wall biosynthesis and modification genes already described in sorghum (106). Across these studies, genes thought to be involved in cell wall modification, particularly involving the extensibility of the wall, are particularly affected by drought. Conversely, recent studies on field-grown bioenergy grasses, including switchgrass, *Miscanthus*, and corn stover, demonstrate contrasting effects on cell wall composition and saccharification (84-86). Despite the decrease in structural sugars found in these studies, sugar conversion was often unaffected or improved in plants exposed to drought stress, possibly due to a less recalcitrant cell wall. Our results, coupled with findings from these studies, indicate that drought stress does not result in biomass conversion penalties when using enzymatic hydrolysis, indicating that the cell wall is as or more accessible to enzymatic hydrolysis. This does not rule out the presence of microbial inhibitors. Our findings suggest that large compositional changes in the cell wall do not occur during drought stress, but this does not rule out structural changes in the cell wall and amongst its components. Importantly, drought, while predicted to have an increase in rigidification of tissues used for biomass processing, does not seem to affect the recalcitrance of the wall in the drought-tolerant *S. bicolor* RTx430 and BTx642 genotypes.

Methods

Sorghum Growth Conditions and Drought Induction

This work was done in collaboration with the DOE BER-funded project EPICON (Epigenetic Control of Drought Response in *Sorghum bicolor*; Grant DE-SC0014081) and utilized the same plant material that has previously been published (77, 112). Sorghum planting, field conditions, and irrigation can all be found here (77, 112). Briefly, sorghum plants were grown in Parlier, CA (36.6008°N, 119.5109°W) for seventeen weeks, with week 0 starting on June 1 with seedling

emergence. Plants were subjected to three different growth conditions: an irrigated control condition with weekly drip irrigation consisting of 80% calculated evapotranspiration five days prior to the weekly sampling date for the duration of the experiment, a pre-flowering drought condition with no watering (weeks 3-8) followed by weekly watering and recovery during post-anthesis, and a post-flowering drought condition with no water (weeks 10-17) during post-anthesis. Eighteen plots of randomly assigned irrigation conditions and genotypes were planted with three replicates per condition per genotype. The third and fourth fully emerged leaf from the primary tiller was collected each week from ten plants per plot and pooled into a single sample for each plot resulting in three pooled samples for each genotype for each condition, bagged in foil and flash-frozen in liquid nitrogen. Additionally, the roots of ten plants per plot (about 30 cm in depth from topsoil) were also collected each week and pooled into a single sample as described for the leaves, vortexed for two minutes in epiphyte removal buffer (0.633% $NaH_2PO_4 \cdot H2O$, 1.65% $NaH_2PO_4 \cdot 7H20$, 200 µl Silwet-77/L), washed twice in root washing buffer (0.633% $NaH_2PO_4 \cdot H_2O$, 1.65% $NaH_2PO_4 \cdot 7H_20$), gently dried, bagged in foil, and flash-frozen in liquid nitrogen. All tissues were collected the same day of the week at the same time of day (10am to 1pm). We studied two *S. bicolor* lines in this study; the pre-flowering "early senescing" drought-tolerant RTx430 and the post-flowering "stay green" drought-tolerant BTx642 to better understand how lines that may fall under either drought tolerance strategy fare.

Computational Methods

We downloaded transcriptomic data and preliminary statistical analysis from samples obtained from the same field experiment. The transcriptomic data consists of almost 400 samples of leaf and root tissues under well-watered conditions, pre- and post-flowering drought stress, and a pre-flowering drought recovery period, for two both RTx430 and BTx642.

The full computational pipeline is described in (77). Briefly, raw data was checked for quality analysis and one sample of low-quality was removed, resulting in 198 samples for leaves and 198 samples for roots. We then filtered low expressed genes and applied upper-quartile normalization on the remaining set of genes using EDA-seq (125, 126). Differential expression analysis was performed using a method akin to EDGE (127): We modeled the gene expression data as a smooth function over time, with a different functional form estimated for each watering condition and genotype, and identified genes differentially expressed between the control condition and the two drought conditions over time for both genotypes. We also identified genes differentially expressed between the two genotypes. The data and results of these analyses are available on https://www.stat.berkeley.edu/~epicon/publications/rnaseq/.

Identifying "Highly-Variable" Genes

We then identified highly variable genes for leaf and root samples as follows. First, for each sample and drought condition, we selected genes differentially expressed across both genotypes by combining p-values obtained as described above using Fisher's method (128). We thus obtained for each gene, each drought condition, and each sample type a unique p-value assessing whether this gene is affected by drought. We then corrected for multiple tests using Benjamini-Hochberg. Second, we computed the overall log-fold change across the time-course experiment using the method described in (77) by applying the following formula:

$$\mathrm{lfc}_i^C = \mathrm{sign}\left(\frac{1}{T}\sum_{t=1}^{T} L_i^{C,t}\right) \times \left(\frac{1}{T}\sum_{t=1}^{T} L_i^{C,t}\right),$$

where $L_i^{C,t}$ corresponds to the log-fold change of gene i at time t and for each drought condition C. The vector $L_i^C \in \mathbb{R}^T$ is estimated by limma (129).

For each drought condition and each genotype, we ranked genes found differentially expressed (adjusted p-value < 0.05) and labeled the top 5% and bottom 5% as highly variable, obtaining four distinct lists (RTx430 – Pre-flowering drought, RTx430 – Post-flowering, BTx642 – Pre-flowering, and BTx642 – Post-flowering). We then applied a similar procedure on Pre-flowering conditions, considering only time-points during drought and time-points during recovery, thus obtaining an additional four lists. We then merged the 8 lists of "highly-variable" genes to obtain a set of genes highly affected by drought in leaves and in roots.

Highly variable genes were categorized via assigned gene ontology (GO) terms related to cell wall-specific components or processes outlined in Table 1 and were analyzed further (Tables 2-5). An additional set of hand-selected putative cell wall-related HVGs were also compiled based on preliminary analysis of leaf and root tissues at week 13 (data not shown). All putative glycosyltransferases and glycosyl hydrolases were referenced against a recent phylogenetic analysis of *S. bicolor* Carbohydrate Active enZyme (CAZy) families (106). Cell wall-related HVGs were split into biosynthesis, modification, and signaling categories and main trends in differential expression between the three different conditions when compared to well-watered plants were assigned (increased, decreased, or no change in expression during the bulk of the condition).

Cell Wall Extraction & Isolation

The alcohol insoluble residue (AIR) was extracted from frozen, ground plant tissue as described by (130) with modifications. Briefly, samples were boiled and successively washed in 100% ethanol until no pigment remained. Pellets were then washed in 70% ethanol followed by 100% acetone and dried in a 50°C oven overnight. AIR extracts were then subjected to starch removal as described by (130) with modifications. AIR samples were subjected to a two-step enzymatic degradation with an initial ten minute thermostable α-amylase (Megazyme, E-BLAAM) digestion at 85°C followed by a two-hour amyloglucosidase (Megazyme, E-AMGDF) and pullulanase (Megazyme, E-PULBL) digestion at 50°C. Samples were washed twice with 70% ethanol after enzymatic digestion before drying the pellet via a solvent concentrator.

Cell Wall Hydrolysis

The hemicellulosic and pectic matrix of 0.5-2 mg de-starched AIR samples were hydrolyzed in 1 ml 2 M trifluoroacetic acid at 120°C for one hour. TFA was removed via a solvent concentrator and the pellet was resuspended in 1 ml of nanopure water. Samples were then filtered through a 0.45 µm centrifugal filter and taken for analysis of released matrix monosaccharides.

The pellets of TFA-hydrolyzed AIR samples were washed twice in isopropanol to remove all hydrolyzed sugars and leave the cellulosic components of the AIR samples. Cellulosic sugars were released by Saeman's hydrolysis (131) by incubating in 63 µl of 72% (v/v) sulfuric acid at room temperature for one hour and subsequently diluting the samples in water to a final

concentration of 1 M sulfuric acid and incubating for an additional three hours at 100°C. Sulfuric acid was neutralized and precipitated using solid barium carbonate and removed by centrifugation. Samples were dried via a solvent concentrator before re-suspending in 250 µl of nanopure water for cellulosic monosaccharide composition analysis.

Monosaccharide Composition Analysis

Matrix and cellulosic acid-hydrolyzed AIR samples were detected and quantified using High Performance Anion Exchange Chromatography with Pulsed-Amperometric Detection (HPAEC-PAD) as described by (132) with some modifications using a Thermo Scientific Dionex ICS-5000 system. Neutral sugars were separated over a 4 mM – 1 mM sodium hydroxide 0.4 ml/min gradient over 23 minutes before separating the uronic acids using 450 mM sodium hydroxide at 0.4 ml/min over 18 minutes using a Dionex CarboPac PA20 column (3 x 30 mm, 060144). Amounts were quantified using a range of monosaccharide standards (2.5-200 µM).

Ferulic Acid Content Determination

Ferulic acid was extracted from de-starched AIR samples as described by (133). Briefly, hydroxycinnamates were extracted from 5 - 10 mg de-starched AIR samples in 2 M sodium hydroxide at 30°C overnight with light shaking (300rpm) before acidification with hydrochloric acid. Ferulic acid and other hydroxycinnamates were isolated via three successive ethyl acetate extractions. Extracted hydroxycinnamates were dried and resuspended in 50% methanol before quantification via High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) using the Agilent 1200 Series system. Hydroxycinnamates were eluted on a 10 mM ammonium acetate flow with a gradient acetonitrile mobile phase over 15.6 minutes using the Agilent Eclipse Plus Phenyl-Hexyl column (4.6 mm x 250 mm, 959990-912). Acetonitrile mobile phase gradients were 27% from 0-12 min, 72% from 12-12.1 min, 90% from 12.1-12.8 min, and 27% from 12.8-15.6 min.

Lignin Quantification

Soluble lignin content was determined and calculated using the acetyl bromide method as described by (134). De-starched AIR samples (5 mg) were hydrolyzed in 25% (v/v) acetyl bromide in glacial acetic acid in a 50°C water bath for three hours with occasional mixing. Samples were then treated with 25 mM hydroxylamine hydrochloride to produce a change in optical density and their absorbance was subsequently measured using a quartz cuvette at 280 nm. Lignin content was quantified as described by (134) using an extinction coefficient average (18.19509) of known commelinid extinction coefficients via (135).

Saccharification Efficiency Assay

Saccharification efficiency was determined on de-starched AIR samples and AIR samples with intact starch. A hot water pre-treatment was administered by autoclaving 2 – 10 mg of AIR samples in 340 µl water at 121°C for one hour. A 0.01% (v/v) Cellic CTec3 cellulase cocktail (Novozymes, Bagsværd, Denmark) in 50mM citrate buffer pH 5.0 (650 µl) was added to each sample and incubated shaking at 800 rpm at 50°C for 72 h. Sugar content was assayed prior to incubation (T0) and every subsequent 24 h for 72 h using a 3,5-dinitrosalicylic acid colorimetric assay (136). Supernatant from each sample was incubated with 3,5-dinitrosalicylic acid at 95°C

for 10 min. Absorbance from each sample was measured at 540 nm and samples were quantified against a linear range of glucose standards (0-2 mg).

Statistics

Statistics of all chemical cell wall analyses were performed by performing the Student's t Test comparing the droughted treatment to its watered control for the specified week sampled. The resulting *p* values were corrected for multiple comparisons concerning False Discovery Rate (FDR) using the Benjamini-Hochberg correction (137) to determine the statistical significance of *p* values < 0.05. FDRs were assumed to be the conservative 0.25 and samples were compared within genotypes and within tissue types to see differences pertaining to genotypes and tissue types.

List of Abbreviations

Highly variable genes (HVGs)

Gene ontology (GO)

Glycosyltransferase (GT)

Glycosyl hydrolase (GH)

Xyloglucan endo-transglucosylase/hydrolase (XTH)

Trifluoroacetic acid (TFA)

Cellulose synthases (CESAs)

Alcohol insoluble residue (AIR)

Carbohydrate active enzymes (CAZy)

False Discovery Rate (FDR)

Declarations

Ethics Approval & Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data & Materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

MM, JV, JD, RH, DCD, PL, and EP designed the EpiCon field trial. NV & EP designed the transcriptomic experiments. NV generated and analyzed transcriptomic data. TSD & HVS designed the cell wall experiments. JO and MB generated lignin monomer composition data. TSD generated and analyzed cell wall data. TSD and HVS wrote the manuscript. All authors approved the final manuscript.

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Table 1. Sources of cell-wall related HVGs analyzed in this study.

Source	**GO Term**	**GO Number**
Curated List – Based on preliminary chemical analysis of RTx430 from week 14	N/A	N/A
GO Association	Plant-type cell wall biogenesis	0009832
GO Association	Cell wall organization	0071555
GO Association	Cell wall organization or biogenesis	0071554
GO Association	Cell wall biogenesis	0042546

Table 2. List of cell wall-related HVGs in RTx430 leaves. Indicates sorghum gene IDs, changes in expression during drought treatments relative to control plants, the category of putative cell wall effect, and the putative affected wall component.

Gene ID (SOBIC.)	Pre-Flowering	Recovery	Post-Flowering	Putative Functions/ Arabidopsis homologs	Category	Component
001G224300	No Change	Increase	Increase	CESA4	Biosynthesis	Cellulose
002G205500	No Change	Increase	Increase	CESA7	Biosynthesis	Cellulose
003G296400	No Change	No Change	Increase	CESA8	Biosynthesis	Cellulose
003G337400	No Change	No Change	Increase	C4H	Biosynthesis	Lignin
003G431100	No Change	Decrease	Increase	GT61	Biosynthesis	Xylan
010G135300	Increase	No Change	Increase	DUF23	Biosynthesis	Galactan/Pectin
002G118700	No Change	Increase	No Change	CESA6	Biosynthesis	Cellulose
002G222800	No Change	Decrease	No Change	GALS	Biosynthesis	Galactan/Pectin
004G320600	No Change	Decrease	No Change	GT61	Biosynthesis	Xylan
001G455700	No Change	No Change	Increase	MAP70	Biosynthesis	Cellulose/Other

002G368300	No Change	No Change	Increase	COBL4	Biosynthesis	Cellulose/Other
002G368600	Increase	Increase	Increase	RGP1	Biosynthesis	
003G251800	No Change	No Change	Increase	NAC073	Signaling	
004G124800	No Change	Increase	Decrease	FUT1	Biosynthesis	
008G054100	No Change	No Change	Decrease	FUT1	Biosynthesis	
001G038200	No Change	No Change	Increase	TBL33	Biosynthesis	Acetylation/ Methylation
001G409100	No Change	No Change	Increase	IRX9	Biosynthesis	Xylan
006G242100	No Change	No Change	Increase	IRX14L	Biosynthesis	Xylan
007G132600	No Change	No Change	Increase	MYB20	Signaling	
002G427400	Decrease	Decrease	Decrease	COBL7	Biosynthesis	Cellulose/Other
007G166900	No Change	Decrease	Decrease	WAT1	Modification	
003G410800	No Change	Increase	No Change	GUT1/2/IRX10	Biosynthesis	Xylan
009G241600	No Change	Decrease	No Change	Zinc Finger CCCH Domain	Signaling	
001G525000	Increase	Increase	Increase	Pectin Lyase	Modification	Pectin
003G321200	Decrease	No Change	Decrease	PME	Modification	Pectin
003G338801	No Change	Increase	Decrease	EXP1	Modification	
004G121900	No Change	No Change	Decrease	EXP11	Modification	
006G217900	Decrease	No Change	Decrease	FLS2	Signaling	
007G014200	No Change	No Change	Decrease	Peroxidase	Modification	
009G173700	No Change	Increase	Decrease	EXP1	Modification	
010G128700	Decrease	No Change	Decrease	Peroxidase	Modification	
010G246600	No Change	Increase	Decrease	XTH22	Modification	

003G153200	No Change	No Change	Increase	Pectin Lyase	Modification	Pectin
004G127200	No Change	Decrease	Increase	XTH26	Modification	
004G197600	No Change	No Change	Increase	GPDL2/MRH5/SHV3 PLC-phosphodiesterase	Modification	
007G094900	No Change	No Change	Increase	XTH22	Modification	
010G255500	No Change	No Change	Increase	EPC1/GIPC mannosylation	Biosynthesis	Glycosylinositolphosphoceramides
003G223100	No Change	No Change	Decrease	Pectin Lyase	Modification	Pectin
004G113900	No Change	No Change	Decrease	Pectin Lyase	Modification	Pectin
001G012300	No Change	Increase	No Change	CYP79B2	Biosynthesis	Lignin
001G309000	No Change	Increase	No Change	XTR8/XTH31	Modification	
002G302000	No Change	Increase	No Change	XTH32	Modification	
004G028700	No Change	Increase	No Change	Pectin Lyase	Modification	Pectin
005G140001	No Change	Increase	No Change	EXGTA4/XTH5	Modification	
006G031900	No Change	Increase	No Change	EXP11	Modification	
007G146200	No Change	Increase	No Change	PMEI	Modification	Pectin
009G243500	No Change	Increase	No Change	Pectin Lyase	Modification	Pectin
010G232500	No Change	Increase	No Change	RCI3/Peroxidase	Modification	
010G246500	No Change	Increase	No Change	XTH22	Modification	
010G246700	No Change	Increase	No Change	XTH25	Modification	
003G442500	No Change	Decrease	No Change	CSLE	Biosynthesis	
004G237800	No Change	Decrease	No Change	GAUT8/QUA1	Biosynthesis	Pectin

003G436800	No Change	Decrease	No Change	Peroxidase	Modification	
009G216100	No Change	Decrease	No Change	Pectin Lyase	Modification	Pectin
006G191700	Increase	No Change	No Change	EXP13	Modification	
006G205600	Decrease	No Change	No Change	XTH24	Modification	
010G246400	Decrease	No Change	No Change	XTH22	Modification	
003G232600	Increase	No Change	No Change	Pectin Lyase	Modification	Pectin
003G127100	Decrease	No Change	No Change	Peroxidase	Modification	
001G027800	No Change	Decrease	Increase	GH/mannosidase	Modification	Mannan
001G406700	No Change	No Change	Increase	ESK1/TBL29	Modification	Acetylation/ Methylation
006G157700	No Change	No Change	Increase	GH3/xylosidase	Modification	Xylan
006G235600	No Change	Decrease	No Change	BXL4/b-d-xylosidase	Modification	Xylan
003G321200	Decrease	No Change	Decrease	PME	Modification	Pectin
004G208700	No Change	No Change	Decrease	CHITIV	Modification	Chitin
006G132300	No Change	No Change	Decrease	CHITIV	Modification	Chitin
006G132400	No Change	Decrease	No Change	CHITIV	Modification	Chitin
006G132700	No Change	Decrease	No Change	CHITIV	Modification	Chitin
003G085900	No Change	No Change	Increase	XYL1/a-xylosidase	Modification	Xylan
003G240100	No Change	Increase	Increase	TBL21	Modification	Acetylation/ Methylation
005G034100	No Change	No Change	Increase	TBL10	Modification	Acetylation/ Methylation
005G110460	No Change	No Change	Increase	GH3 Xylosidase	Modification	Xylan
007G132600	No Change	No Change	Increase	MYB20	Signaling	
008G005100	No Change	No Change	Increase	PMR5	Modification	Acetylation/ Methylation

010G273600	Decrease	No Change	Decrease	Chitinase	Modification	Chitin
K029900	Decrease	No Change	Decrease	Chitinase	Modification	Chitin
003G293800	No Change	Increase	No Change	GH/Mannosidase	Modification	Mannan
006G132500	No Change	Decrease	No Change	Chitinase	Modification	Chitin
001G516000	No Change	Increase	No Change	Chitinase	Modification	Chitin
006G132200	No Change	Increase	No Change	Chitinase	Modification	Chitin

Table 3. List of cell wall-related HVGs in RTx430 leaves. Indicates sorghum gene IDs, changes in expression during drought treatments relative to control plants, the category of putative cell wall effect, and the putative affected wall component.

Gene ID (SOBIC.)	Pre-Flowering	Recovery	Post-Flowering	Putative Function/Arabidopsis homolog	Category	Component
001G224300	No Change	No Change	Decrease	CESA4	Biosynthesis	Cellulose
001G263300	No Change	Increase	No Change	GT61	Biosynthesis	Xylan
002G205500	No Change	Increase	Decrease	CESA7	Biosynthesis	Cellulose
002G333900	Decrease	Decrease	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
002G334000	No Change	No Change	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
002G334100	No Change	Increase	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
002G334200	Decrease	Increase	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
003G095600	Decrease	Increase	No Change	GT61	Biosynthesis	Xylan
003G296400	No Change	No Change	Decrease	CESA8	Biosynthesis	Cellulose
004G141200	Decrease	No Change	No Change	C4H	Biosynthesis	Lignin

008G125700	No Change	No Change	Decrease	CSLD5	Biosynthesis	
010G152400	No Change	Increase	Decrease	GT61	Biosynthesis	Xylan
010G152500	No Change	Increase	No Change	GT61	Biosynthesis	Xylan
003G095100	No Change	Decrease	No Change	GT61	Biosynthesis	Xylan
010G196400	No Change	Increase	Decrease	DUF23/GT92	Biosynthesis	Galactan/Pectin
001G283400	No Change	Increase	No Change	CSLD3	Biosynthesis	
003G094700	Increase	Increase	No Change	GT61	Biosynthesis	Xylan
003G087700	No Change	Increase	No Change	GT61	Biosynthesis	Xylan
003G337400	No Change	Increase	No Change	C4H	Biosynthesis	Lignin
004G320600	Decrease	Increase	No Change	GT61	Biosynthesis	Xylan
002G222800	Decrease	No Change	No Change	GALS	Biosynthesis	Galactan/Pectin
010G030900	Decrease	No Change	No Change	GT61	Biosynthesis	Xylan
003G095200	Decrease	No Change	No Change	GT61	Biosynthesis	Xylan
003G431100	Decrease	No Change	No Change	GT61	Biosynthesis	Xylan
007G132600	No Change	No Change	Increase	MYB20	Signaling	
001G086000	No Change	No Change	Decrease	COBL	Biosynthesis	Cellulose/Other
002G368600	No Change	No Change	Decrease	RGP1	Biosynthesis	
003G236701	No Change	No Change	Decrease	Zinc Finger CCCH Domain	Signaling	
003G251800	No Change	No Change	Decrease	NAC073	Signaling	
004G124800	Decrease	No Change	Decrease	FUT1	Biosynthesis	
006G160900	No Change	Increase	Decrease	NAC007	Signaling	
007G018100	No Change	No Change	Decrease	NAC043	Signaling	

007G039100	No Change	No Change	Decrease	MYB103	Signaling	
007G166900	No Change	Decrease	No Change	WAT1	Modification	
008G054100	Decrease	Decrease	Decrease	FUT1	Biosynthesis	
008G112200	No Change	No Change	Decrease	MYB46	Signaling	
009G241600	No Change	No Change	Decrease	Zinc Finger CCCH Domain	Signaling	
002G368300	No Change	No Change	Decrease	COBL4	Biosynthesis	Cellulose/Other
005G034500	No Change	No Change	Decrease	LRRK	Signaling	
009G200200	No Change	No Change	Decrease	TPS1	Modification	
007G177100	Decrease	No Change	No Change	MYB32	Signaling	
004G125100	Decrease	Decrease	No Change	FUT1	Biosynthesis	
004G308600	Decrease	Decrease	No Change	FUT1	Biosynthesis	
001G336700	No Change	Increase	No Change	COBL4	Biosynthesis	Cellulose/Other
001G455700	No Change	Increase	No Change	MAP70	Biosynthesis	Cellulose/Other
004G231300	No Change	Increase	No Change	QUA2	Biosynthesis	Pectin
010G082000	No Change	Increase	No Change	FUT1	Biosynthesis	
010G155100	No Change	Increase	No Change	NAC033	Signaling	
002G427400	Decrease	Decrease	No Change	COBL7	Biosynthesis	Cellulose/Other
001G012300	Increase	Increase	No Change	CYP79B2	Biosynthesis	Lignin
004G028700	No Change	Increase	No Change	Pectin Lyase	Modification	Pectin
005G169500	Increase	No Change	Increase	GAUT6/4	Biosynthesis	Pectin
007G094900	Increase	Increase	Increase	XTH22	Modification	
009G032800	Increase	Increase	No Change	RCI3/Peroxidase	Modification	

009G216100	No Change	Increase	No Change	Pectin Lyase	Modification	Pectin
001G045800	Decrease	Increase	No Change	PGAZAT/polygalacturonase abscission	Modification	Pectin
001G189000	No Change	Increase	No Change	Peroxidase	Modification	
001G237900	No Change	No Change	Decrease	EXP11	Modification	
001G314000	No Change	Increase	No Change	RCI3/Peroxidase	Modification	
001G499900	No Change	Increase	No Change	EXP11	Modification	
002G003200	No Change	Increase	Decrease	Peroxidase	Modification	
002G003700	No Change	Increase	No Change	RCI3/Peroxidase	Modification	
002G391300	No Change	Increase	Decrease	Peroxidase	Modification	
002G391400	Decrease	No Change	Decrease	Peroxidase	Modification	
002G391900	Decrease	Increase	Decrease	Peroxidase	Modification	
002G392000	No Change	Increase	Decrease	Peroxidase	Modification	
002G392100	Decrease	Increase	Decrease	Peroxidase	Modification	
003G050300	Decrease	No Change	No Change	Peroxidase	Modification	
003G140600	Decrease	Increase	Decrease	Peroxidase	Modification	
003G140700	Decrease	Increase	Decrease	Peroxidase	Modification	
003G148300	Decrease	Increase	No Change	PMEI	Modification	Pectin
003G152000	Decrease	No Change	Decrease	Peroxidase	Modification	
003G152200	Decrease	No Change	Decrease	Peroxidase	Modification	
003G167300	Decrease	No Change	Decrease	Peroxidase	Modification	
003G232600	No Change	Increase	No Change	Pectin Lyase	Modification	Pectin

003G338801	No Change	No Change	Decrease	EXP1	Modification
004G013500	No Change	Increase	Decrease	UGP2 (UDP-glucose pyrophosphorylase)	Biosynthesis
004G273900	Decrease	No Change	Decrease	RCI3/Peroxidase	Modification
005G051500	Decrease	No Change	No Change	Peroxidase	Modification
005G140001	No Change	Increase	Decrease	XTH5	Modification
006G031900	No Change	Increase	Decrease	EXP11	Modification
006G116000	No Change	No Change	Decrease	SVL1	Modification
006G205700	No Change	Increase	Decrease	XTH16	Modification
006G217900	Decrease	No Change	Decrease	FLS2/LRRK	Signaling
006G224500	No Change	No Change	Decrease	Peroxidase	Modification
006G277500	Decrease	No Change	Decrease	Peroxidase	Modification
006G277700	No Change	Increase	No Change	Peroxidase	Modification
006G277800	Decrease	No Change	Decrease	Peroxidase	Modification
007G014200	Decrease	No Change	Decrease	Peroxidase	Modification
007G018000	Decrease	No Change	No Change	EXP25	Modification
007G086300	No Change	No Change	Decrease	XTH22	Modification
008G125700	No Change	No Change	Decrease	CSLD5	Biosynthesis
009G033500	Decrease	Decrease	Decrease	Peroxidase	Modification
009G186500	No Change	Increase	Decrease	RCI3/Peroxidase	Modification
010G128700	No Change	Increase	Decrease	Peroxidase	Modification
010G194500	No Change	No Change	Decrease	EXP20	Modification

K026900	Decrease	Increase	Decrease	Peroxidase	Modification	
010G246600	No Change	No Change	Increase	XTH22	Modification	
001G238200	Decrease	No Change	Decrease	EXP11	Modification	
002G302000	No Change	Increase	Decrease	XTH32	Modification	
002G391200	No Change	No Change	Decrease	Peroxidase	Modification	
002G416000	No Change	No Change	Decrease	PME2	Modification	Pectin
002G420100	No Change	No Change	Decrease	GAUT7	Biosynthesis	Pectin
003G148400	No Change	Increase	Decrease	PME2	Modification	Pectin
003G223100	No Change	No Change	Decrease	Pectin Lyase	Modification	Pectin
006G106900	Decrease	Increase	Decrease	UPF0497	Modification	
007G075600	No Change	No Change	Decrease	Pectin Lyase	Modification	Pectin
007G146200	Decrease	No Change	Decrease	PMEI	Modification	Pectin
008G114700	No Change	No Change	Decrease	RCI3/Peroxidase	Modification	
009G055300	No Change	Increase	Decrease	RCI3/Peroxidase	Modification	
003G050100	Increase	No Change	No Change	Pectin Lyase	Modification	Pectin
003G153100	Increase	Increase	No Change	Pectin Lyase	Modification	Pectin
004G315000	No Change	Increase	No Change	TBR/DUF828	Biosynthesis	Acetylation/ Methylation
007G090436	No Change	Increase	No Change	XTH22	Modification	
009G111000	Decrease	No Change	No Change	PMEI	Modification	Pectin
001G085200	No Change	Increase	No Change	PDCB3/plasmodesmata callose-binding protein	Modification	
001G238000	No Change	Increase	No Change	EXP11	Modification	

001G360400	Decrease	Increase	No Change	Peroxidase	Modification	
001G525000	No Change	Increase	No Change	Pectin Lyase	Modification	Pectin
003G096300	No Change	Increase	No Change	HDG1/homeodomain GLABROUS	Signaling	
003G153200	Increase	Increase	No Change	Pectin Lyase	Modification	Pectin
003G436800	Decrease	Increase	No Change	Peroxidase	Modification	
004G126700	No Change	Increase	No Change	XTH12	Modification	
004G313900	Increase	Increase	No Change	HDG11/homeodomain GLABROUS	Signaling	
007G090460	No Change	Increase	No Change	XTH13	Modification	
009G033400	No Change	Increase	No Change	PA2/peroxidase	Modification	
009G243500	No Change	Increase	No Change	Pectin Lyase	Modification	Pectin
010G232500	No Change	Increase	No Change	RCI3/Peroxidase	Modification	
001G284600	No Change	Decrease	No Change	XTH27	Modification	
001G309000	Decrease	Decrease	No Change	XTH31/XTR8	Modification	
002G237900	Decrease	Decrease	No Change	CSLE1	Biosynthesis	
003G178000	No Change	Decrease	No Change	Pectin Lyase	Modification	Pectin
009G033300	Decrease	Decrease	No Change	Peroxidase	Modification	
001G499800	Increase	No Change	No Change	EXP11	Modification	
006G172000	Increase	No Change	No Change	Pectin Lyase	Modification	Pectin
001G238300	Decrease	No Change	No Change	EXP11	Modification	
003G321200	Decrease	No Change	No Change	PME	Modification	Pectin

007G085600	Decrease	No Change	No Change	XTH15	Modification	
009G055100	Decrease	No Change	No Change	RCI3/Peroxidase	Modification	
003G320800	Decrease	No Change	No Change	RCI3/Peroxidase	Modification	
003G442500	Decrease	No Change	No Change	CSLE	Biosynthesis	
001G027800	No Change	Increase	Increase	GH	Modification	
001G372500	No Change	Increase	Increase	UGE2/UDPgluc-UDPgal4epimerase	Biosynthesis	
006G132100	No Change	No Change	Increase	CHITIV	Modification	Chitin
006G132300	Increase	No Change	Increase	CHITIV	Modification	Chitin
010G273600	No Change	Increase	Increase	CHIB/chitinase	Modification	Chitin
K029900	No Change	Increase	Increase	CHIB/chitinase	Modification	Chitin
001G038300	No Change	No Change	Decrease	TBL33	Biosynthesis	Acetylation/ Methylation
001G038400	No Change	No Change	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
001G066900	No Change	Increase	No Change	GH/mannosidase	Modification	Mannan
001G242100	Decrease	Increase	Decrease	TBL19	Biosynthesis	Acetylation/ Methylation
001G406700	No Change	No Change	Decrease	ESK1/TBL29	Biosynthesis	Acetylation/ Methylation
001G406901	No Change	No Change	Decrease	TBL3	Biosynthesis	Acetylation/ Methylation
001G407000	Decrease	No Change	No Change	PMR5/TBL44	Biosynthesis	Acetylation/ Methylation
002G391400	Decrease	No Change	Decrease	Peroxidase	Modification	
002G399400	Decrease	No Change	No Change	TBL10	Biosynthesis	Acetylation/ Methylation
003G219300	No Change	Increase	No Change	TBR/DUF828	Biosynthesis	Acetylation/ Methylation
003G239700	No Change	No Change	Decrease	TBL38	Biosynthesis	Acetylation/ Methylation

004G047600	No Change	No Change	Decrease	DUF579	Signaling	
005G004900	No Change	Increase	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
005G005100	Decrease	Increase	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
005G211500	Decrease	Decrease	No Change	BXL2/b-xylosidase	Modification	Xylan
006G235600	No Change	No Change	Decrease	BXL4	Modification	Xylan
008G005100	No Change	Increase	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
008G005200	No Change	Increase	No Change	TBL34	Biosynthesis	Acetylation/ Methylation
009G012100	Decrease	Decrease	No Change	CHIB/chitinase	Modification	Chitin
009G035900	Decrease	Increase	No Change	Chitinase	Modification	Chitin
009G106500	No Change	Increase	Decrease	TBL33	Biosynthesis	Acetylation/ Methylation
010G096700	No Change	Increase	No Change	TBL27	Biosynthesis	Acetylation/ Methylation
010G194500	No Change	No Change	Decrease	TBL38	Biosynthesis	Acetylation/ Methylation
006G132400	No Change	No Change	Increase	CHITIV	Modification	Chitin
006G132500	Decrease	No Change	Increase	CHITIV	Modification	Chitin
005G229100	No Change	Increase	Decrease	TBL27	Biosynthesis	Acetylation/ Methylation
006G132700	Decrease	No Change	No Change	CHITIV	Modification	Chitin
K022500	Decrease	No Change	No Change	GH	Modification	
	Increase	No Change	No Change	ARAF1/arabinofuranosidase	Modification	
001G516000	Decrease	Decrease	No Change	CHIB/chitinase	Modification	Chitin
004G251800	No Change	Decrease	No Change	TBL21	Biosynthesis	Acetylation/ Methylation
006G186200	No Change	Decrease	No Change	GT18 XyG Gal	Biosynthesis	Xyloglucan
003G148700	Decrease	No Change	No Change	TBL38	Biosynthesis	Acetylation/ Methylation

004G208700	Decrease	No Change	No Change	CHITIV	Modification	Chitin
009G130100	Decrease	No Change	No Change	CHIB/chitinase	Modification	Chitin

Table 4. List of cell wall-related HVGs in BTx642 leaves. Indicates sorghum gene IDs, changes in expression during drought treatments relative to control plants, the category of putative cell wall effect, and the putative affected wall component.

Gene ID (SOBIC.)	Pre-Flowering	Recovery	Post-Flowering	Putative Function/Arabidopsis homolog	Category	Component
001G224300	No Change	No Change	Increase	CESA4	Biosynthesis	Cellulose
002G205500	No Change	Increase	Increase	CESA7	Biosynthesis	Cellulose
003G296400	No Change	No Change	Increase	CESA8	Biosynthesis	Cellulose
003G337400	No Change	No Change	Increase	C4H	Biosynthesis	Lignin
003G431100	No Change	Decrease	No Change	GT61	Biosynthesis	Xylan
002G094600	No Change	Increase	No Change	CESA6	Biosynthesis	Cellulose
002G118700	No Change	Increase	No Change	CESA6?	Biosynthesis	Cellulose
002G171200	No Change	Increase	No Change	CSLF	Biosynthesis	Mixed-Linkage Glucan
003G094600	No Change	Increase	No Change	GT61	Biosynthesis	Xylan
007G050600	No Change	Increase	No Change	CSLF	Biosynthesis	Mixed-Linkage Glucan
010G230300	No Change	Increase	No Change	GT31 (AGP GalT)	Biosynthesis	Arabinogalactan Proteins
002G222800	No Change	Decrease	No Change	GT31 (AGP GalT)	Biosynthesis	Arabinogalactan Proteins
004G320600	No Change	Decrease	No Change	GT61	Biosynthesis	Xylan
010G008600	Decrease	No Change	No Change	CSLD3	Biosynthesis	

010G030900	Decrease	No Change	No Change	GT61	Biosynthesis	Xylan
001G455700	No Change	Increase	Increase	MAP70	Biosynthesis	Cellulose/Other
002G368300	Increase	No Change	Increase	COBL4	Biosynthesis	Cellulose/Other
002G368600	Increase	No Change	Increase	RGP1	Biosynthesis	
003G251800	No Change	No Change	Increase	NAC073	Signaling	
004G124800	No Change	No Change	Decrease	FUT1	Biosynthesis	
008G054100	No Change	No Change	Decrease	FUT1	Biosynthesis	
007G132600	No Change	Decrease	No Change	MYB20	Signaling	
007G166900	No Change	Decrease	No Change	WAT1	Modification	
001G399900	No Change	Increase	No Change	COBL7	Biosynthesis	Cellulose/Other
003G250700	No Change	Increase	No Change	FLA11	Modification	
007G018100	No Change	Increase	No Change	NAC043	Signaling	
009G026101	No Change	Increase	No Change	IRX9	Biosynthesis	Xylan
001G525000	No Change	Increase	Increase	Pectin lyase	Modification	Pectin
008G022500	No Change	Increase	Increase	GAUT7	Biosynthesis	Pectin
003G321200	No Change	No Change	Decrease	PME	Modification	Pectin
003G338801	No Change	Increase	Decrease	EXP1	Modification	
004G121900	No Change	No Change	Decrease	EXP11	Modification	
006G116000	No Change	No Change	Decrease	SVL1	Modification	
006G217900	Decrease	Increase	Decrease	FLS2	Signaling	
007G014200	No Change	Decrease	Decrease	Peroxidase	Modification	
009G111000	No Change	Decrease	Decrease	PMEI	Modification	Pectin

009G173700	No Change	No Change	Decrease	EXP1	Modification	
010G005000	No Change	No Change	Decrease	Pectin lyase	Modification	Pectin
010G128700	Decrease	No Change	Decrease	Peroxidase	Modification	Pectin
010G246600	No Change	Increase	Decrease	XTH22	Modification	
004G127200	No Change	Decrease	No Change	XTH26	Modification	
007G094900	No Change	Increase	No Change	XTH22	Modification	
010G255500	No Change	Decrease	No Change	EPC1 (nucleotide sugarT)	Biosynthesis	
003G223100	No Change	Increase	No Change	Pectin lyase	Modification	Pectin
001G012300	No Change	Increase	No Change	CYP79B2	Biosynthesis	Lignin
001G309000	No Change	Increase	No Change	XTH31	Modification	
001G399900	No Change	Increase	No Change	COBL7	Biosynthesis	Cellulose/Other
002G302000	No Change	Increase	No Change	XTH32	Modification	
003G282600	No Change	Increase	No Change	GAUT15	Biosynthesis	Pectin
004G028700	No Change	Increase	No Change	Pectin lyase	Modification	Pectin
005G140001	No Change	Increase	No Change	XTH5	Modification	
006G031900	No Change	Increase	No Change	EXP11	Modification	
007G146200	No Change	Increase	No Change	PMEI	Modification	Pectin
009G032600	No Change	Increase	No Change	Peroxidase	Modification	
009G243500	No Change	Increase	No Change	Pectin lyase	Modification	Pectin
010G232500	No Change	Increase	No Change	Peroxidase	Modification	

010G246500	No Change	Increase	No Change	XTH22	Modification	
010G246700	No Change	Increase	No Change	XTH25	Modification	
003G442500	No Change	Decrease	No Change	CSLE	Biosynthesis	
004G237800	No Change	Decrease	No Change	GAUT8	Biosynthesis	Pectin
006G191700	Increase	No Change	No Change	EXP13	Modification	
002G324100	Decrease	No Change	No Change	XTH8	Modification	
006G205600	Decrease	No Change	No Change	XTH24	Modification	
010G008600	Decrease	No Change	No Change	CSLD3	Biosynthesis	
010G246400	Decrease	No Change	No Change	XTH22	Modification	
001G027800	No Change	Decrease	Increase	GH	Modification	
001G406700	No Change	No Change	Increase	TBL29/ESK1	Biosynthesis	Acetylation/ Methylation
004G013800	No Change	Increase	Increase	TBL12	Biosynthesis	Acetylation/ Methylation
006G157700	No Change	No Change	Increase	GH	Modification	
006G235600	No Change	Increase	Increase	XYL4	Modification	Xylan
004G208700	No Change	No Change	Decrease	Chitinase	Modification	Chitin
004G233700	No Change	No Change	Decrease	GH	Modification	
006G132300	No Change	No Change	Decrease	Chitinase	Modification	Chitin
006G132400	Decrease	Decrease	Decrease	Chitinase	Modification	Chitin
006G132700	No Change	Decrease	Decrease	Chitinase	Modification	Chitin
004G127200	No Change	Decrease	No Change	XTH26	Modification	
005G034100	No Change	Increase	No Change	TBL10	Biosynthesis	Acetylation/ Methylation
008G005100	No Change	Increase	No Change	TBL34	Biosynthesis	Acetylation/ Methylation

010G273600	Decrease	No Change	Decrease	Chitinase	Modification	Chitin
K029900	Decrease	No Change	No Change	Chitinase	Modification	Chitin
003G293800	No Change	Increase	No Change	GH	Modification	
010G078300	No Change	Increase	No Change	TBR	Biosynthesis	Acetylation/ Methylation
006G132100	No Change	Decrease	No Change	Chitinase	Modification	Chitin
006G132500	No Change	Decrease	No Change	Chitinase	Modification	Chitin
006G132200	Decrease	No Change	No Change	Chitinase	Modification	Chitin

Table 5. List of cell wall-related HVGs in BTx642 roots. Indicates sorghum gene IDs, changes in expression during drought treatments relative to control plants, the category of putative cell wall effect, and the putative affected wall component.

Gene ID (SOBIC.)	Pre-Flowering	Recovery	Post-Flowering	Putative Function/Arabidopsis homolog	Category	Component
001G224300	No Change	No Change	Decrease	CESA4	Biosynthesis	Cellulose
001G263300	No Change	No Change	Decrease	GT61	Biosynthesis	Xylan
002G205500	No Change	No Change	Decrease	CESA7	Biosynthesis	Cellulose
002G333900	Decrease	No Change	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
002G334000	No Change	No Change	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
002G334100	Decrease	No Change	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
002G334200	Decrease	No Change	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
003G095600	Decrease	No Change	Decrease	GT61	Biosynthesis	Xylan
003G095700	No Change	No Change	Decrease	GT61	Biosynthesis	Xylan

003G296400	No Change	No Change	Decrease	CESA8	Biosynthesis	Cellulose
004G141200	Decrease	No Change	Decrease	C4H	Biosynthesis	Lignin
008G125700	No Change	No Change	Decrease	CSLF	Biosynthesis	Mixed-Linkage Glucan
010G152400	No Change	No Change	Decrease	GT61	Biosynthesis	Xylan
010G152500	No Change	No Change	Decrease	GT61	Biosynthesis	Xylan
003G095100	No Change	Decrease	No Change	GT61	Biosynthesis	Xylan
001G283400	No Change	Increase	No Change	CSLF	Biosynthesis	Mixed-Linkage Glucan
003G094700	Increase	Increase	No Change	GT61	Biosynthesis	Xylan
003G095500	No Change	Increase	No Change	GT61	Biosynthesis	Xylan
010G256400	Increase	Decrease	No Change	GT61	Biosynthesis	Xylan
003G087700	Increase	No Change	No Change	GT61	Biosynthesis	Xylan
003G108500	Increase	No Change	No Change	GT61	Biosynthesis	Xylan
002G222800	Decrease	No Change	No Change	GT31/ AGP GalT	Biosynthesis	Arabinogalactan Proteins
003G363100	Decrease	No Change	No Change	GT29A	Biosynthesis	Arabinogalactan Proteins
010G008600	Decrease	No Change	No Change	CSLF	Biosynthesis	Mixed-Linkage Glucan
010G030900	Decrease	No Change	No Change	GT61	Biosynthesis	Xylan
007G132600	No Change	No Change	Increase	MYB20	Signaling	
001G086000	No Change	No Change	Decrease	COB	Biosynthesis	Cellulose/Other
002G368600	No Change	No Change	Decrease	RGP1	Biosynthesis	
003G236701	Decrease	No Change	Decrease	Zinc Finger CCCH Domain	Signaling	

003G251800	No Change	No Change	Decrease	NAC073	Signaling	
004G124800	Decrease	No Change	Decrease	FUT1	Biosynthesis	
006G160900	No Change	No Change	Decrease	NAC007	Signaling	
007G018100	No Change	No Change	Decrease	NAC043	Signaling	
007G039100	No Change	No Change	Decrease	MYB103	Signaling	
007G166900	No Change	Decrease	Decrease	WAT1	Modification	
008G054100	Decrease	No Change	Decrease	FUT1	Biosynthesis	
008G112200	No Change	No Change	Decrease	MYB46	Signaling	
009G241600	No Change	No Change	Decrease	Zinc Finger CCCH Domain	Signaling	
010G082400	No Change	No Change	Decrease	FUT1	Biosynthesis	
007G177100	Decrease	Increase	No Change	MYB32	Signaling	
001G479800	No Change	Decrease	No Change	GUX2	Biosynthesis	Xylan
004G125100	Decrease	Decrease	No Change	FUT1	Biosynthesis	
004G308600	Decrease	Decrease	No Change	FUT1	Biosynthesis	
002G427400	Decrease	No Change	No Change	COBL7	Biosynthesis	Cellulose/Other
003G304600	Increase	No Change	No Change	RGP1	Biosynthesis	
003G250700	Decrease	No Change	No Change	FLA11	Signaling	
001G012300	Increase	Increase	Increase	CYP79B2	Biosynthesis	
004G028700	No Change	No Change	Increase	Pectin lyase	Modification	Pectin
005G169500	Increase	No Change	Increase	GAUT6/4	Biosynthesis	Pectin
007G094900	Increase	Increase	Increase	XTH22	Modification	
009G032800	No Change	Increase	Increase	Peroxidase	Modification	

009G216100	No Change	Increase	Increase	Pectin lyase	Modification	Pectin
001G045800	Decrease	No Change	Decrease	PGAZAT	Modification	Pectin
001G189000	No Change	No Change	Decrease	Peroxidase	Modification	
001G189200	No Change	No Change	Decrease	Peroxidase	Modification	
001G237900	Decrease	No Change	Decrease	EXP11	Modification	
001G238400	No Change	No Change	Decrease	EXP11	Modification	
001G314000	No Change	No Change	Decrease	Peroxidase	Modification	
001G345300	No Change	No Change	Decrease	PMEI	Modification	Pectin
001G499900	No Change	No Change	Decrease	EXP11	Modification	
002G003200	No Change	No Change	Decrease	Peroxidase	Modification	
002G003700	No Change	No Change	Decrease	Peroxidase	Modification	
002G391300	No Change	No Change	Decrease	Peroxidase	Modification	
002G391400	No Change	No Change	Decrease	Peroxidase	Modification	
002G391900	No Change	No Change	Decrease	Peroxidase	Modification	
002G392000	Decrease	No Change	Decrease	Peroxidase	Modification	
002G392100	Decrease	No Change	Decrease	Peroxidase	Modification	
003G050300	Decrease	No Change	Decrease	Peroxidase	Modification	
003G140600	Decrease	No Change	Decrease	Peroxidase	Modification	
003G140700	Decrease	No Change	Decrease	Peroxidase	Modification	
003G148300	Decrease	No Change	Decrease	PMEI	Modification	Pectin
003G152000	No Change	No Change	Decrease	Peroxidase	Modification	
003G152200	Decrease	No Change	Decrease	Peroxidase	Modification	

003G167300	No Change	No Change	Decrease	Peroxidase	Modification	
003G232600	No Change	No Change	Decrease	Pectin lyase	Modification	Pectin
003G292100	No Change	Decrease	Decrease	Pectin lyase	Modification	Pectin
003G338801	No Change	No Change	Decrease	EXP1	Modification	
004G013500	No Change	No Change	Decrease	UGP2	Biosynthesis	
004G113900	No Change	No Change	Decrease	Pectin lyase	Modification	Pectin
004G273900	Decrease	No Change	Decrease	Peroxidase	Modification	
005G051500	Decrease	No Change	Decrease	Peroxidase	Modification	
005G140001	No Change	No Change	Decrease	XTH5	Modification	
006G031900	No Change	No Change	Decrease	EXP11	Modification	
006G116000	No Change	No Change	Decrease	SVL1	Modification	
006G205500	No Change	Increase	Decrease	XTH25	Modification	
006G205700	No Change	No Change	Decrease	XTH16	Modification	
006G217900	Decrease	No Change	Decrease	FLS2	Signaling	
006G224500	No Change	No Change	Decrease	Peroxidase	Modification	
006G273300	No Change	No Change	Decrease	UPF0497	Modification	
006G277500	Decrease	No Change	Decrease	Peroxidase	Modification	
006G277550	Decrease	Increase	Decrease	Peroxidase	Modification	
006G277600	No Change	No Change	Decrease	Peroxidase	Modification	
006G277700	No Change	No Change	Decrease	Peroxidase	Modification	
006G277800	No Change	No Change	Decrease	Peroxidase	Modification	
007G014200	Decrease	No Change	Decrease	Peroxidase	Modification	

007G018000	Decrease	No Change	Decrease	EXP25	Modification	
007G086300	No Change	No Change	Decrease	XTH22	Modification	
008G125700	No Change	No Change	Decrease	CSLD5	Biosynthesis	
009G033500	Decrease	No Change	Decrease	Peroxidase	Modification	
009G186500	No Change	No Change	Decrease	Peroxidase	Modification	
009G203200	No Change	No Change	Decrease	Pectin lyase	Modification	Pectin
009G250800	No Change	No Change	Decrease	Pectin lyase	Modification	Pectin
010G128700	No Change	No Change	Decrease	Peroxidase	Modification	
010G194500	No Change	No Change	Decrease	EXP20	Modification	
K026900	Decrease	No Change	Decrease	Peroxidase	Modification	
001G238200	Decrease	No Change	No Change	EXP11	Modification	
003G223100	No Change	Decrease	No Change	Pectin lyase	Modification	Pectin
007G146200	Decrease	No Change	No Change	PMEI	Modification	Pectin
009G055300	No Change	Increase	No Change	Peroxidase	Modification	
001G283400	No Change	Increase	No Change	CSLD5	Biosynthesis	
002G370300	No Change	Increase	No Change	Pectin lyase/QRT1	Modification	Pectin
003G050100	No Change	Increase	No Change	Pectin lyase	Modification	Pectin
003G141800	No Change	Increase	No Change	Pectin lyase	Modification	Pectin
003G153100	Increase	Increase	No Change	Pectin lyase	Modification	Pectin
003G437400	No Change	Increase	No Change	Peroxidase	Modification	
004G315000	No Change	Increase	No Change	TBR	Biosynthesis	Acetylation/ Methylation
007G090436	No Change	Increase	No Change	XTH22	Modification	

009G111000	No Change	Increase	No Change	PMEI	Modification	Pectin
003G096300	Increase	No Change	No Change	HDG1	Signaling	
003G153200	Increase	No Change	No Change	Pectin lyase	Modification	Pectin
003G436800	Decrease	No Change	No Change	Peroxidase	Modification	
002G237900	Decrease	No Change	No Change	CSLE1	Biosynthesis	
003G178000	Decrease	No Change	No Change	Pectin lyase	Modification	Pectin
009G033300	Decrease	No Change	No Change	Peroxidase	Modification	
001G499800	Increase	No Change	No Change	EXP11	Modification	
006G172000	Increase	No Change	No Change	Pectin lyase	Modification	Pectin
001G238300	Decrease	No Change	No Change	EXP11	Modification	
003G321200	Decrease	No Change	No Change	PME	Modification	Pectin
004G237800	Decrease	No Change	No Change	GAUT8	Biosynthesis	Pectin
007G085600	Decrease	No Change	No Change	XTH15	Modification	
009G055100	Decrease	No Change	No Change	Peroxidase	Modification	
010G008600	Decrease	No Change	No Change	CSLD3	Biosynthesis	
010G017600	Decrease	No Change	No Change	PMEI	Modification	Pectin
001G027800	No Change	Increase	Increase	GH	Modification	
001G372500	No Change	Increase	Increase	UGE2	Biosynthesis	
006G132100	No Change	No Change	Increase	Chitinase	Modification	Chitin
006G132300	Increase	No Change	Increase	Chitinase	Modification	Chitin
010G273600	No Change	No Change	Increase	Chitinase	Modification	Chitin
K029900	No Change	No Change	Increase	Chitinase	Modification	Chitin

001G038300	No Change	No Change	Decrease	TBL33	Biosynthesis	Acetylation/ Methylation
001G038400	No Change	No Change	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
001G066900	No Change	Increase	Decrease	GH	Modification	
001G242100	Decrease	No Change	Decrease	TBL19	Biosynthesis	Acetylation/ Methylation
001G406700	No Change	No Change	Decrease	TBL29/ESK1	Biosynthesis	Acetylation/ Methylation
001G406901	Decrease	No Change	Decrease	TBL3	Biosynthesis	Acetylation/ Methylation
001G407000	No Change	Decrease	Decrease	TBL44/PMR5	Biosynthesis	Acetylation/ Methylation
002G399400	No Change	No Change	Decrease	TBL10	Biosynthesis	Acetylation/ Methylation
003G142100	No Change	No Change	Decrease	BXL2	Modification	Xylan
003G219300	No Change	No Change	Decrease	TBR	Biosynthesis	Acetylation/ Methylation
003G239700	No Change	Decrease	Decrease	TBL38	Biosynthesis	Acetylation/ Methylation
004G047600	Decrease	No Change	Decrease	DUF579	Signaling	
005G004900	Decrease	No Change	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
005G005100	Decrease	No Change	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
005G211500	Decrease	No Change	Decrease	BXL2	Modification	Xylan
006G235600	Decrease	No Change	Decrease	BXL4	Modification	Xylan
008G005100	Decrease	No Change	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
008G005200	Decrease	No Change	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
008G005800	No Change	No Change	Decrease	TBL34	Biosynthesis	Acetylation/ Methylation
009G012100	Decrease	No Change	Decrease	Chitinase	Modification	Chitin
009G035900	No Change	No Change	Decrease	Chitinase	Modification	Chitin
009G106500	No Change	No Change	Decrease	TBL33	Biosynthesis	Acetylation/ Methylation

010G096700	No Change	No Change	Decrease	TBL27	Biosynthesis	Acetylation/ Methylation
010G155800	No Change	No Change	Decrease	TBL38	Biosynthesis	Acetylation/ Methylation
006G132500	No Change	Increase	No Change	Chitinase	Modification	Chitin
005G229100	No Change	Increase	No Change	TBL27	Biosynthesis	Acetylation/ Methylation
005G110457	No Change	Increase	No Change	GH	Modification	
006G132700	No Change	Increase	No Change	Chitinase	Modification	Chitin
K022500	No Change	Increase	No Change	GH	Modification	
010G096400	No Change	Increase	No Change	TBL22	Biosynthesis	Acetylation/ Methylation
003G240400	No Change	Decrease	No Change	TBL21	Biosynthesis	Acetylation/ Methylation
004G238801	Increase	No Change	No Change	EXP1	Modification	
003G148700	Decrease	No Change	No Change	TBL38	Biosynthesis	Acetylation/ Methylation
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009G130100	Decrease	No Change	No Change	Chitinase	Modification	Chitin

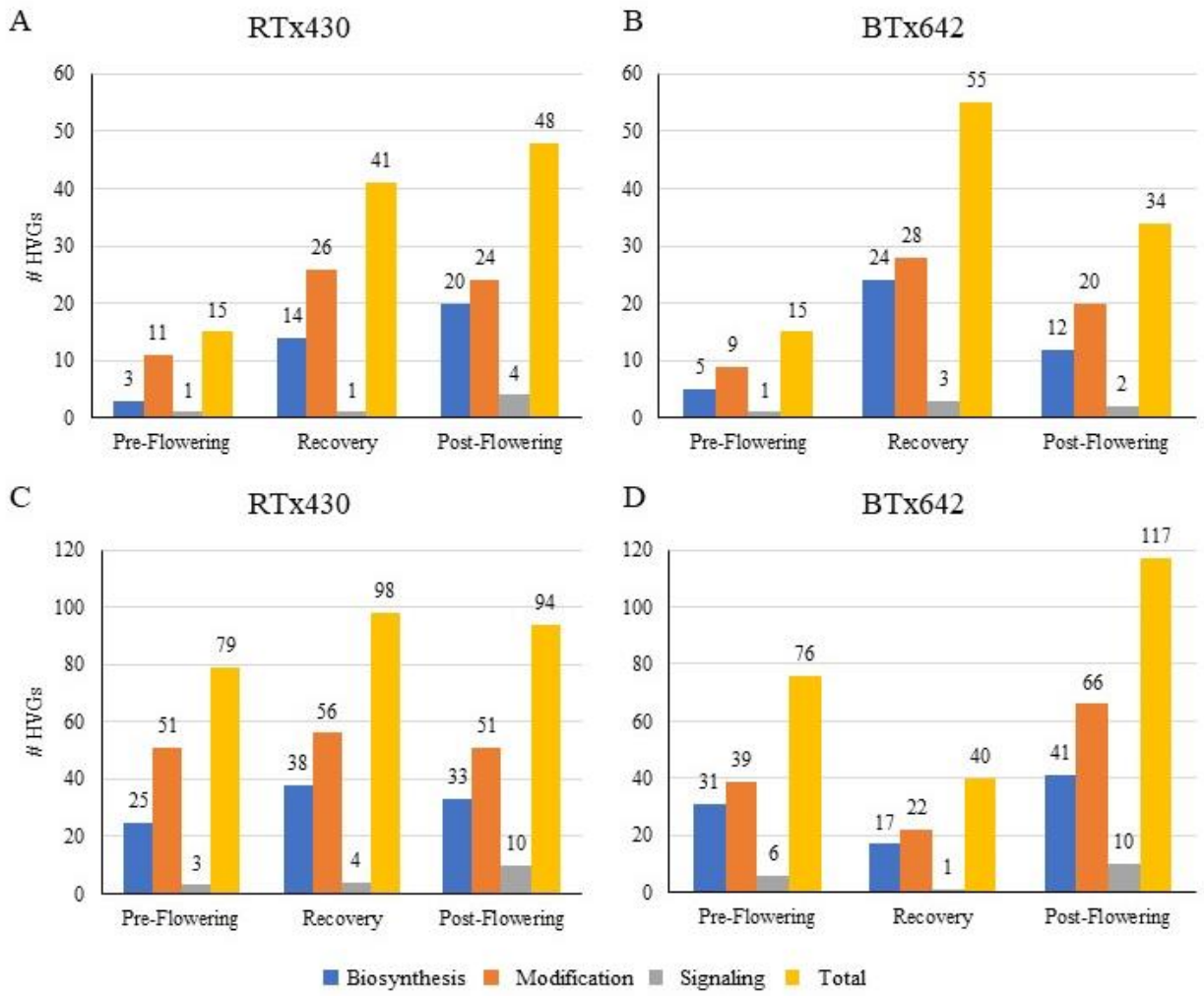

Figure 1. Total number of highly variable genes (HVGs) with cell wall-related gene ontology (GO) terms in leaves (A & B) and roots (C &D). Highly variable genes are those genes in the top 10% of differentially expressed genes in response to drought conditions, of the HVGs, those genes with cell wall-related GO terms were analyzed and classed via putative functions related to biosynthesis, modification, and signaling of the cell wall.

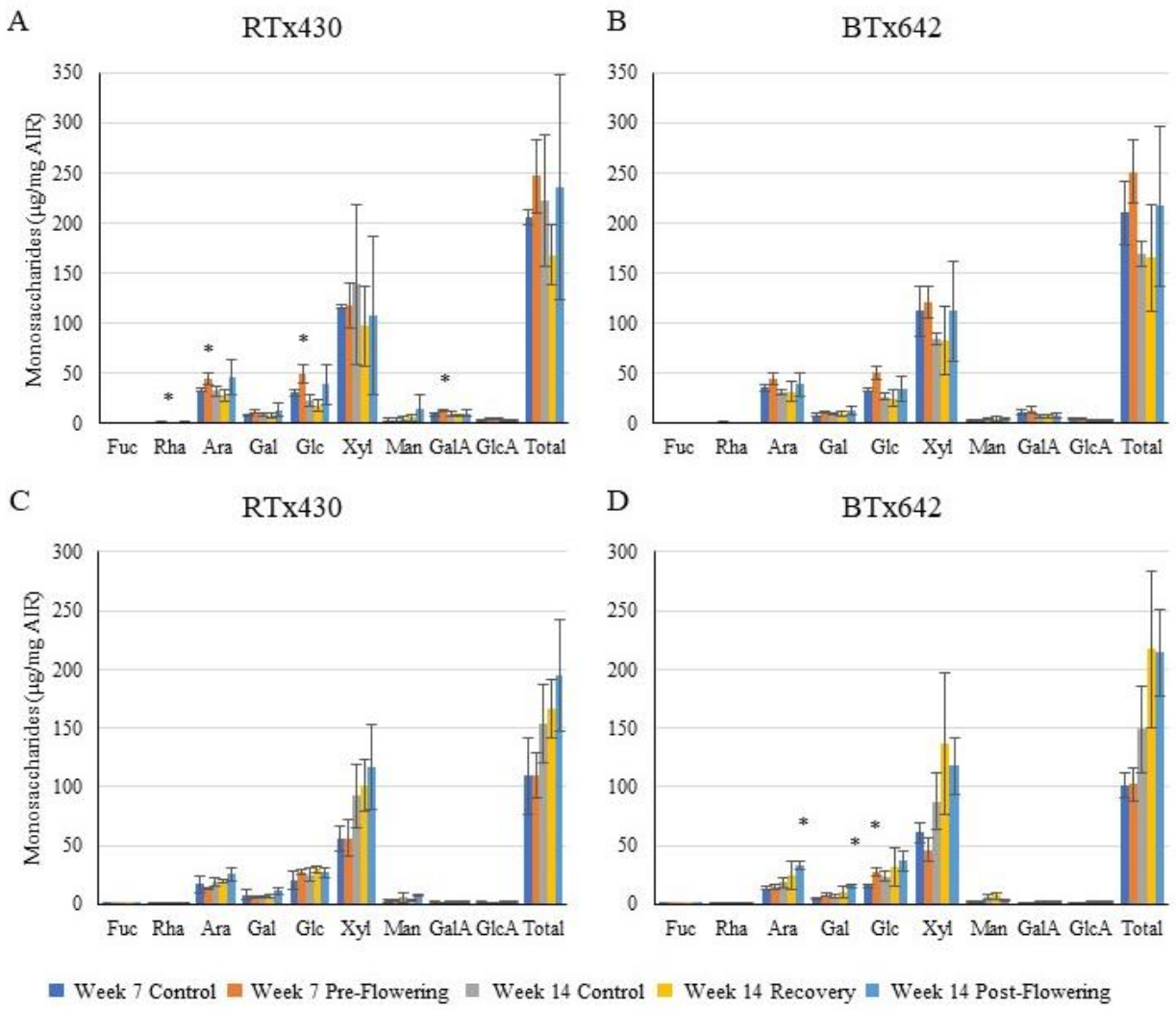

Figure 2. Matrix monosaccharide composition analysis. Monosaccharides released from a trifluoroacetic acid (TFA) hydrolysis of the alcohol insoluble residue (AIR) from leaves (A & B) and roots (C & D). Data represents mean ± SD of three biological replicates per condition per genotype where biological replicates themselves consist of ten pooled plants from the same planting block. Asterisks indicate $p < 0.05$ determined by Student's t-test with the Benjamini-Hochberg correction.

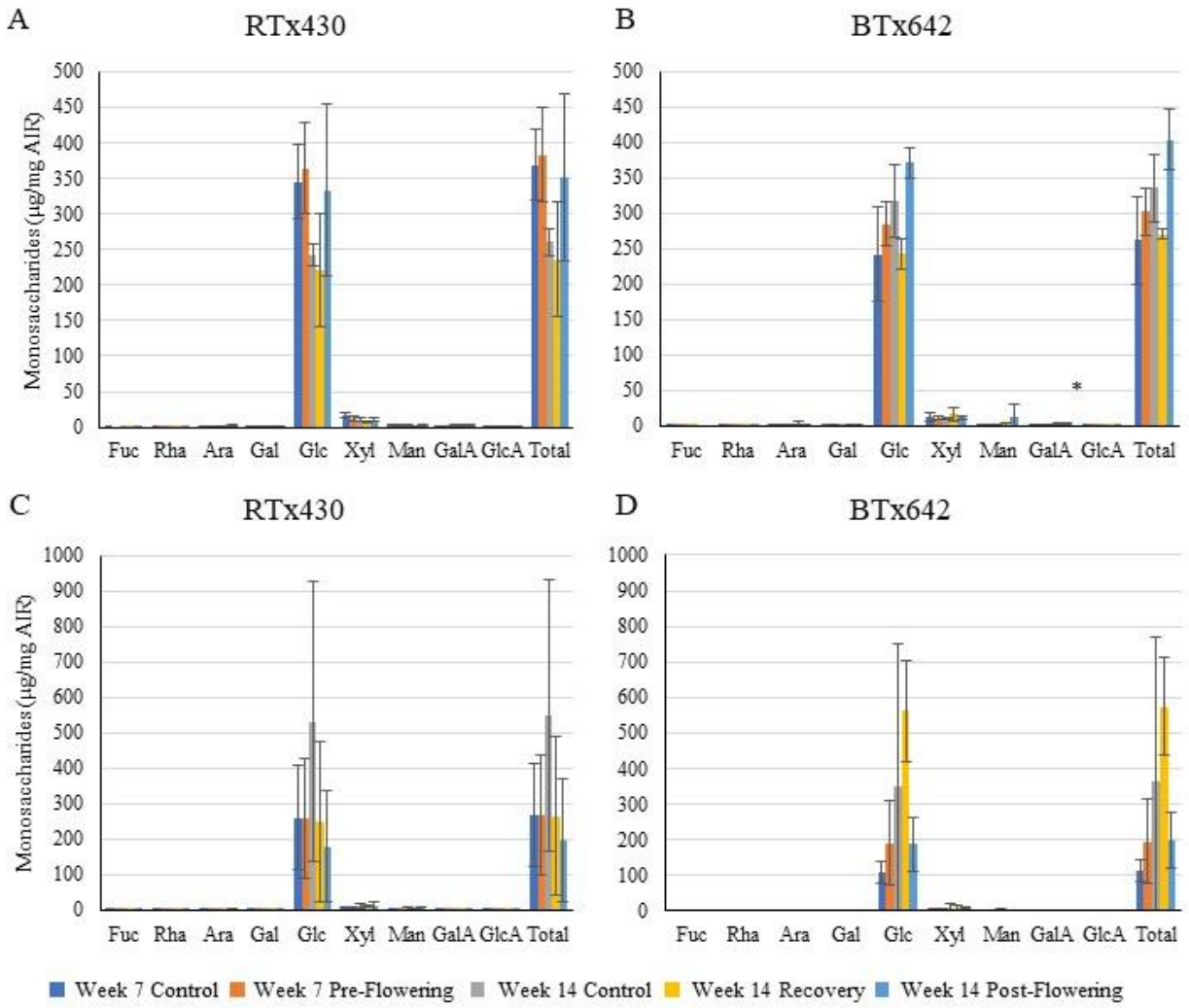

Figure 3. Cellulosic monosaccharide composition analysis. Monosaccharides released from a sulfuric acid hydrolysis of AIR already treated with TFA (matrix monosaccharides removed) from leaves (A & B) and roots (C & D). Data represents mean ± SD of three biological replicates per condition per genotype where biological replicates themselves consist of ten pooled plants from the same planting block. Asterisks indicate $p < 0.05$ determined by Student's t-test with the Benjamini-Hochberg correction.

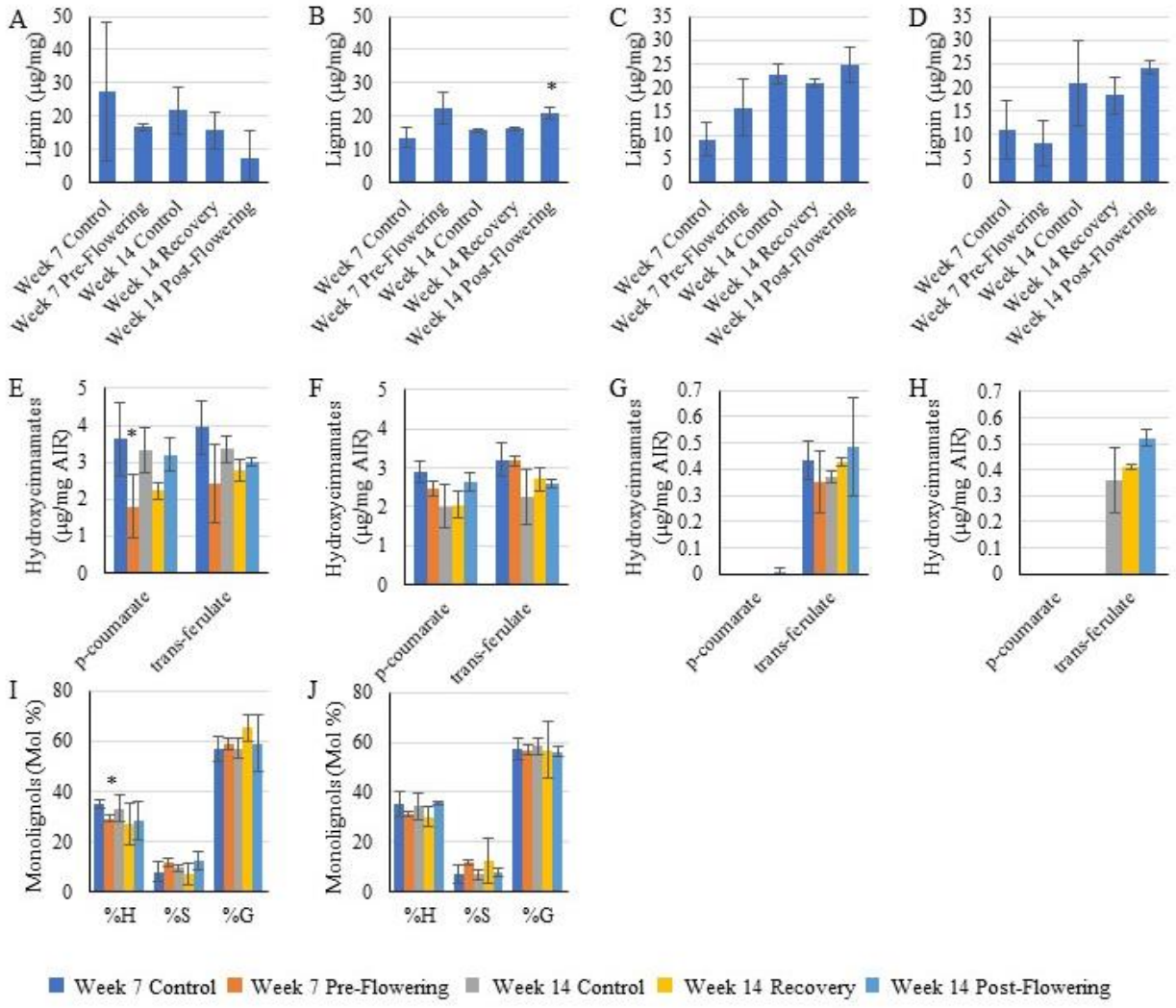

Figure 4. Lignin analysis of RTx430 (A, C, E, G, I) and BTx642 (B, D, F, H, J) leaves and roots. Released lignin from acetyl bromide treatment of leaves (A & B) and roots (C & D). Data represents mean ± SD of three biological replicates per condition per genotype where biological replicates themselves consist of ten pooled plants from the same planting block. Some replicates have been removed in this analysis, leaving two biological replicates rather than three, due to being extreme outliers. Hydroxycinnamate content of leaf samples (E & F) and root samples (G & H), where data represents mean ± SD of three biological replicates per condition per genotype where biological replicates themselves consist of ten pooled plants from the same planting block. Several replicates had to be removed from the root samples. Some replicates have been removed in this analysis, leaving two biological replicates rather than three, due to being extreme outliers. Monolignol relative composition of roots (I & J). Data represents mean ± SD of three biological replicates per condition per genotype where biological replicates themselves consist of ten pooled plants from the same planting block. Standard deviation error bars, with data points without error bars representing points in which only one sample had detectable data. Asterisks indicate $p < 0.05$ from Student's t-tests with the Benjamini-Hochman correction.

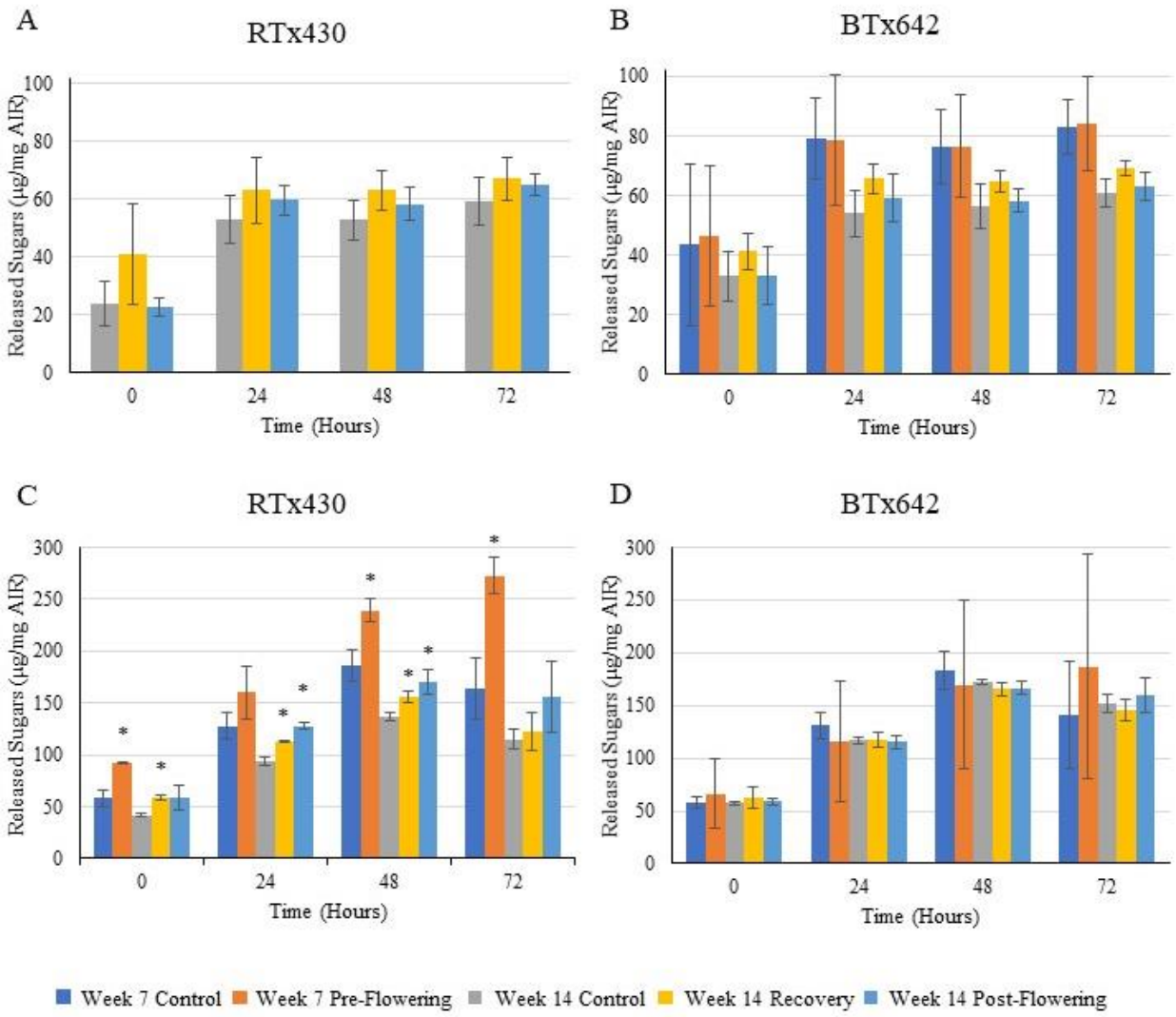

Figure 5. Saccharification efficiency with a hot-water pre-treatment of leaf samples. Samples were pre-treated using hot water before a 72-h digestion with CTec3. Reducing sugars were assayed via 3,5-dinitrosalicylic acid. A & B refer to de-starched samples, C & D refer to AIR with starch intact. The data represents mean ± SD of three biological replicates per genotype per condition with samples themselves consisting of leaves pooled from ten plants within the same planting block. Standard deviation error bars, with asterisks indicate $p < 0.05$ using Student's t-test with the Benjamini-Hochberg correction.

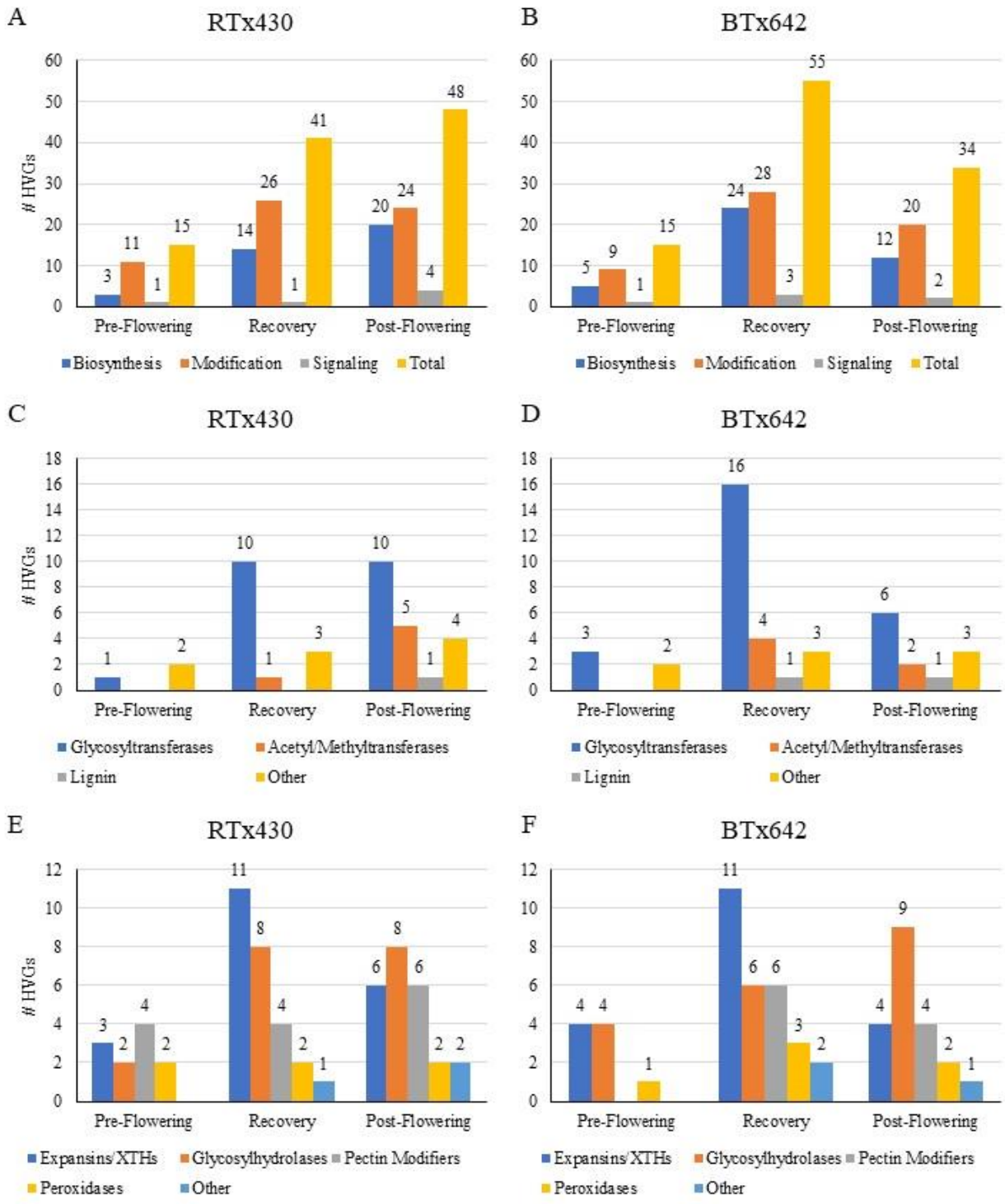

Figure S1. Cell wall-related transcriptome analysis of cell wall-related highly variable genes (HVGs) in leaves. Charts indicate the number of cell wall-related HVGs classified by putative function (A & B), with more resolution of the biosynthesis (C & D) and modification (E & F) categories.

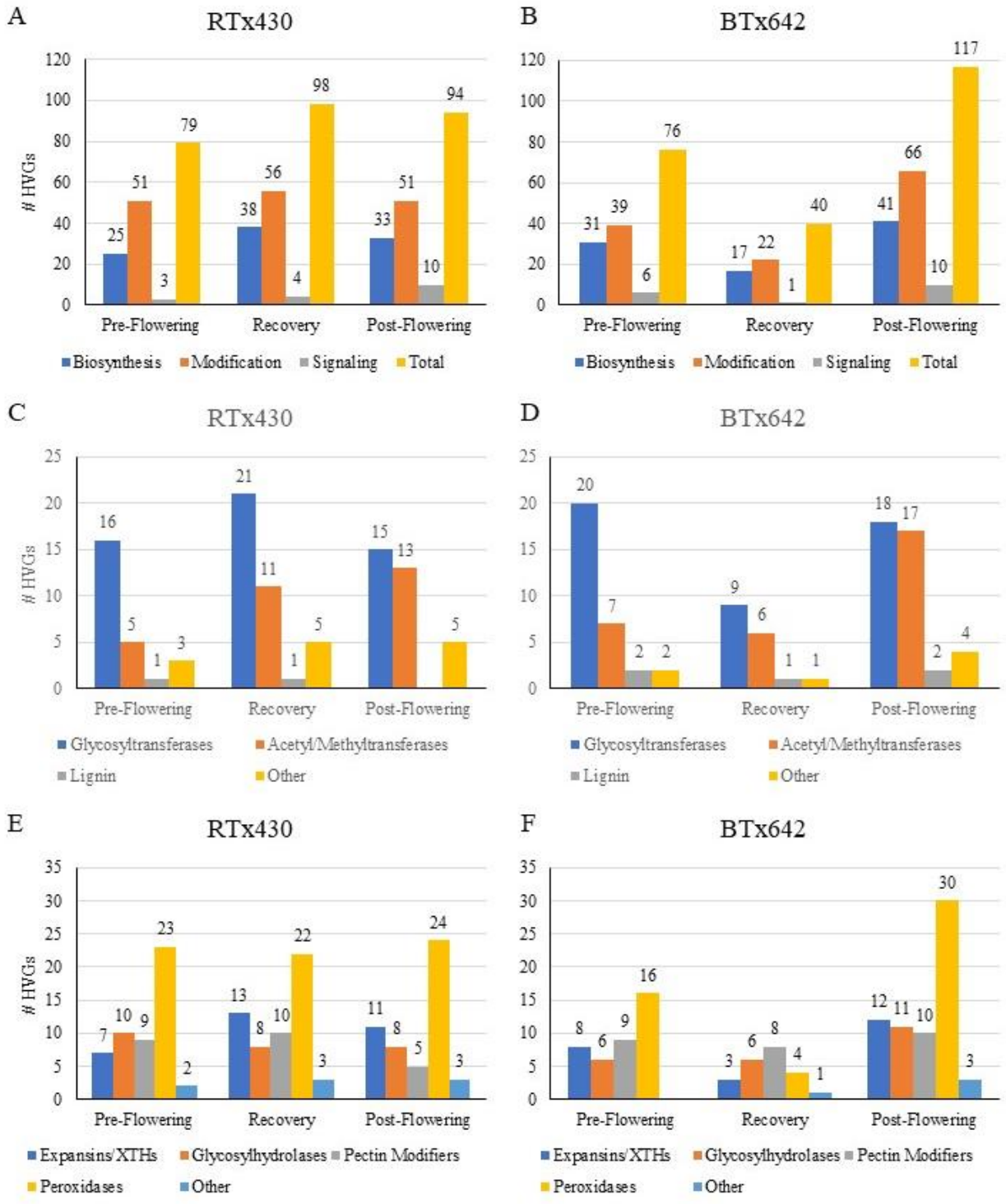

Figure S2. Cell wall-related transcriptome analysis of cell wall-related HVGs in roots. Charts indicate the number of cell wall-related HVGs classified by putative function (A & B), with more resolution of the biosynthesis (C & D) and modification (E & F) categories.

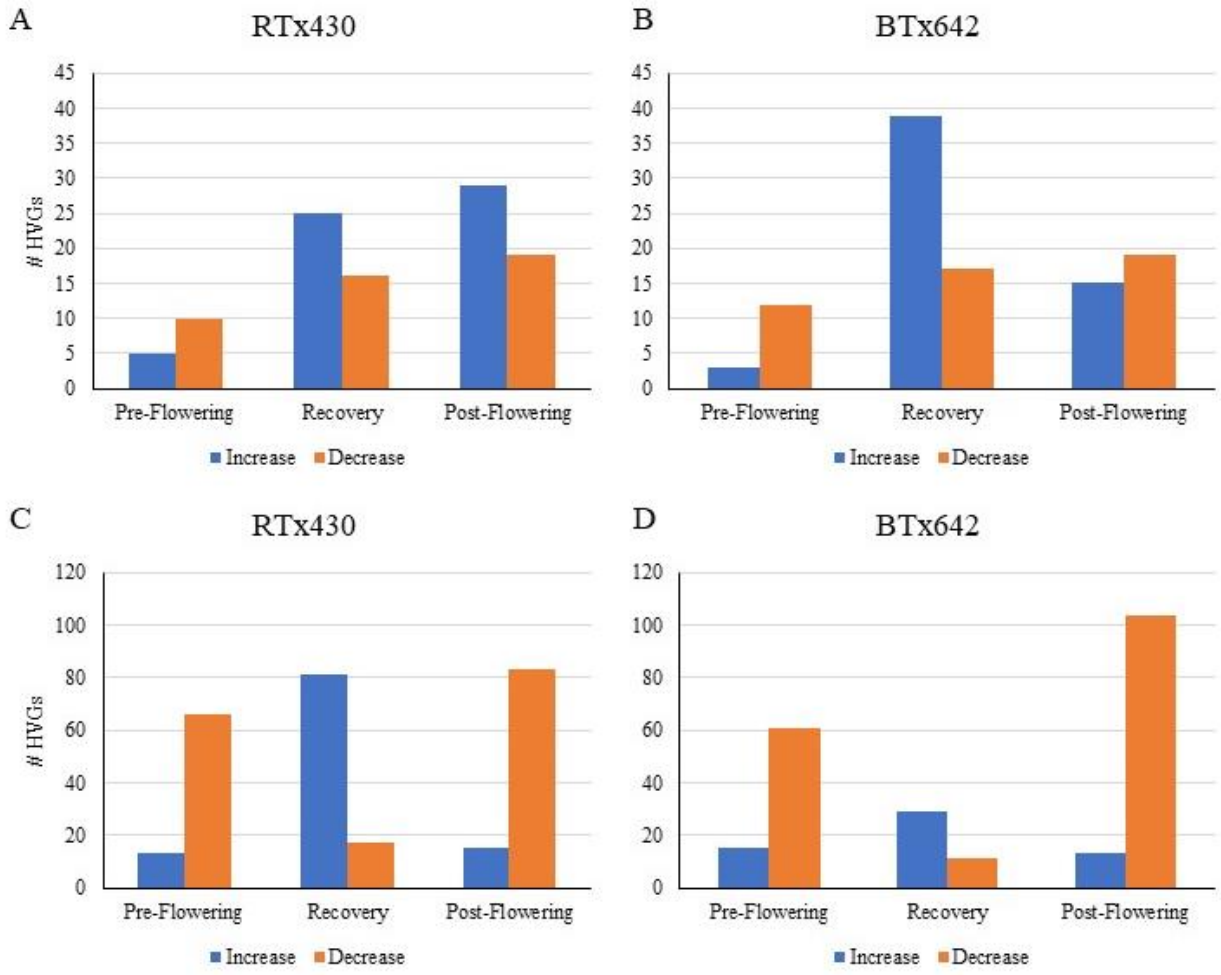

Figure S3. Number of cell wall-related HVGs with increased and decreased expression relative to the watered control in leaves (A, B) and roots (C, D).

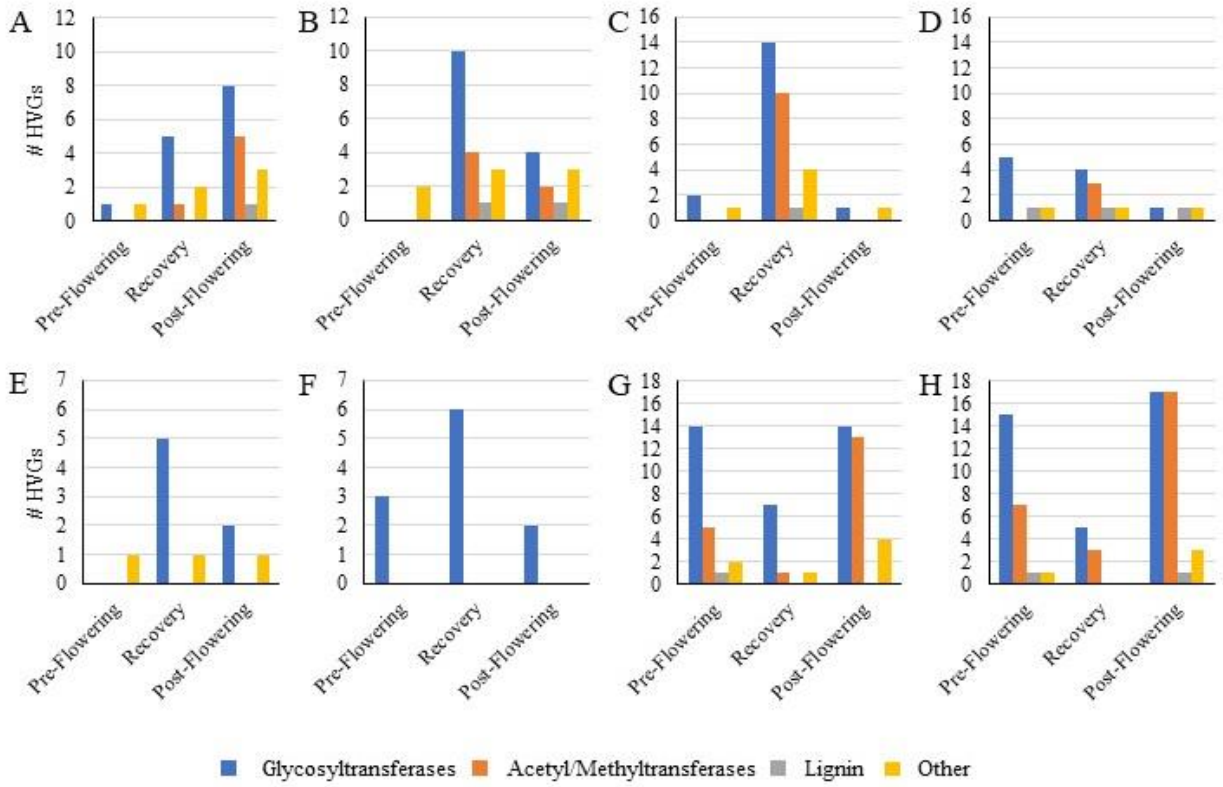

Figure S4. Biosynthesis category of HVGs in RTx430 (A, C, E, G) and BTx642 (B, D, F, H). Increased differential expression (A – D), decreased differential expression (E – H). Total number of HVGs pertaining to cell wall biosynthesis in leaves (A, B, E, F) and roots (C, D, G, H).

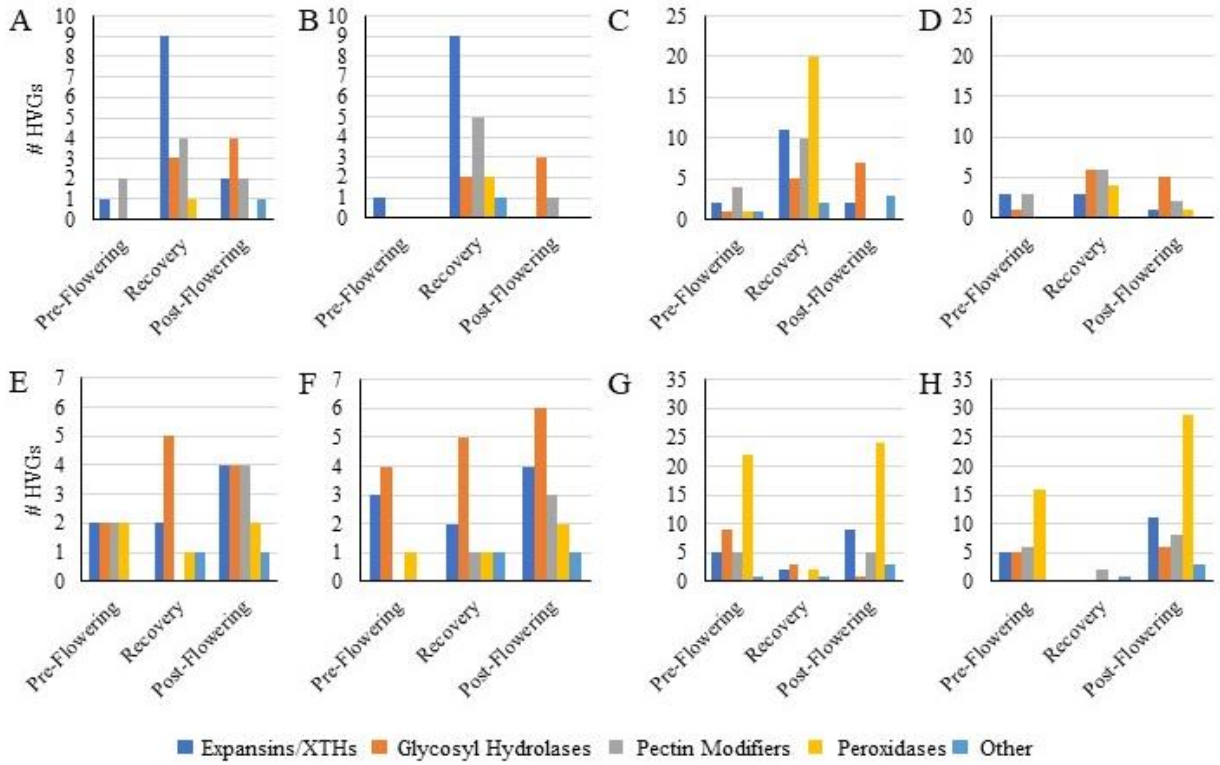

Figure S5. Modifications category of HVGs in leaves (A, B, E, F) and roots (C, D, G, H). RTx430 (A, C, E, G) and BTx642 (B, D, F, H) samples. Increased differential expression (A – D) and decreased differential expression (E – H).

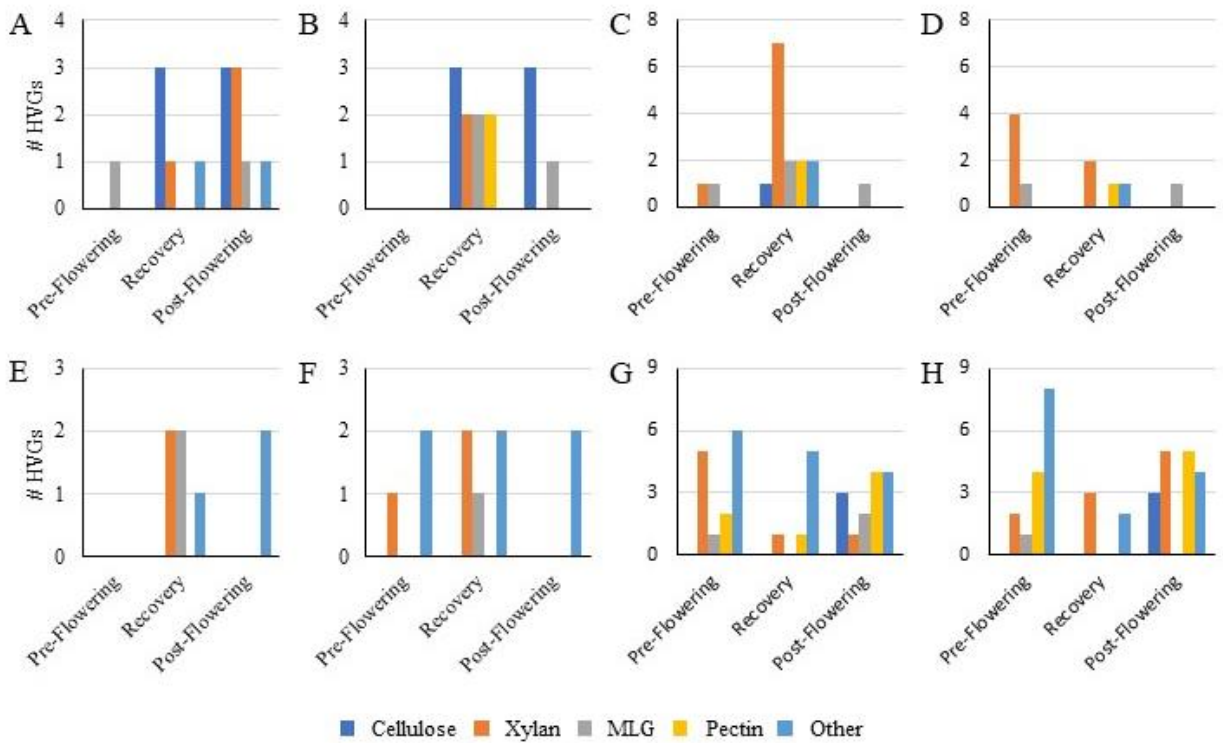

Figure S6. Glycosyltransferase category of HVGs in leaves (A, B, E, F) and root (C, D, G, H). RTx430 (A, C, E, G) and BTx642 (B, D, F, H) samples. Increased differential expression (A – D) and decreased differential expression (E – H).

Pectin: Structure, Function, and Biosynthesis

Pectin is an integral component of the plant primary cell wall, involved in wall hydration, viscoelasticity, signaling, and mediating the plant stress response (12, 16, 113, 138). In recent models, primary plant cell walls are described as load-bearing cellulose microfibrils embedded in a hemicellulosic and pectic network of crosslinking, glycosidic linkages, and covalent linkages to other pectic domains and other wall components (15, 139-141). A growing body of literature implicates pectin as a necessary component for mediating plant growth conditions under a range of conditions, from typical development to abiotic and biotic stresses (12, 13, 16, 142, 143). To better understand pectin's role in mediating and facilitating plant growth, it is important to first grasp the structure of pectin and how it influences pectic properties within the wall.

Structure

Pectin is a heteropolymeric polysaccharide typically consisting of four different domains, homogalacturonan (HG), xylogalacturonan (XG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (Figure 1). While HG predominates the pectic composition of many primary cell walls, the other pectic domains have more specific distributions both within the cell wall and across cell types (138, 139, 144). Further, experiments demonstrate that rather than behaving as extensive side chains attached to an HG backbone, the pectic domains are continuous with HG (145, 146). Although these four domains are typical of most primary vascular plant cell walls, there are other, less abundant pectic domains unique to different plants (147, 148).

Homogalacturonan is the most abundant pectic domain and comprises a linear chain of α-(1, 4)-D-galacturonic acid (Gal*p*A). The Gal*p*A residues can either be methylated at C6, acetylated at O2 or O3, or remain unesterified. Unesterified Gal*p*A chains consisting of at least 9-10 non-methylesterified GalA residues are then available to form ionic cross-links between the charged C6 in Gal*p*A residues of parallel HG chains using apoplastic Ca^{2+} (17, 138, 139, 142, 144). Studies have also demonstrated that pectin is covalently cross-linked to RG-I and RG-II, indicating that the pectic backbones are continuous within a pectin polymer (145, 146). There is also evidence suggesting that HG can be covalently bonded to xyloglucan (XyG) (149, 150) and arabinogalactan proteins (AGPs) (151). HG can have a degree of polymerization (DP) up to 150 residues, based on endogenous acceptor assays in *Nicotiana benthamiana* (152), but preparations of HG from apples, beets, and citrus peels have demonstrated DPs ranging from 72-100 residues (153). Xylogalacturonan comprises a HG backbone with xylose substitutions on O3, either as single residue substitutions or as linear β-(1, 4)-xylose chains (154). HG can also be substituted with apiose residues at the O2 or O3 position, although documentation of this occurrence is limited to aquatic plants (147, 148).

Rhamnogalacturonan II is a minor but crucial component of the plant primary cell wall. Consisting of a HG backbone, RG-II has a highly conserved set of six side chains attached via the O2 or O3 involving twelve different sugars and 22 unique linkages (155). RG-II domains are able to dimerize via borate-mediated cross-linking, a configuration necessary for cell wall integrity (139, 143, 144, 156, 157).

Rhamnogalacturonan I is the second most abundant pectic domain in the primary cell wall and boasts remarkable diversity in side chains. Unlike the other pectic domains, RG-I has a backbone

consisting of repeating diglycosyl α-(1,2)-L-Rha*p*-α-(1,4)-D-Gal*p*A units. This backbone can be unsubstituted, as in mucilage (158-161), or rhamnose residues can be substituted with a variety of side chains at C4, including linear and branched galactans, arabinans, and Type I and II arabinogalactans (AGs) (138, 139, 144, 162, 163). Linear galactans consisting of β-(1,4)-D-galactose linkages can be up to 47 residues in length (146). However, galactans can also be branched and incorporate arabinose residues at the O3 position, as in Type I AGs. Alternatively, β-(1,3)-D-galactan can be branched at O6 with galactan or arabinogalactan to form Type II AGs. Linear α-(1,5)-L-arabinan can be branched at O2 or O3 with arabinose residues or arabinan chains (139, 144, 162, 164, 165). Even this does not cover the complexity and diversity of RG-I side chains not only through plant development, but across plant tissues and species (163, 164, 166-171).

Function

Pectin plays a critical role in modulating cell wall expansion and rigidity through the abundance and modifications of its domains. Non-methyl esterified chains of HG can form cross-links via Ca^{2+} in the apoplast, creating eggbox structures that contribute to increased rigidity of the cell wall (172). Studies overexpressing different polygalacturonases in apple, tobacco, and *A. thaliana* targeting non-methyl esterified HG have defects in wall expansion, wall strength, and plant height (173, 174). Modulating esterification of HG is a common strategy employed from the level of cell division and expansion to plant tissue and organ development, including organ initiation, hypocotyl elongation, pollen tube growth, and seed development (17, 171, 175-183). Additionally, esterification levels of HG have been shown to mediate biotic and abiotic stresses. A pectin methyl esterase inhibitor (PMEI) in pepper prevents de-methyl-esterification and is required for basal disease resistance, anti-fungal activity, and osmotic and oxidative stress tolerance (121). Pectin methyl esterases (PMEs) remove methyl esters from the HG backbone, promoting calcium-mediated cross-linking, and their activity is required for heat and cold tolerance in rice and *A. thaliana* (184-186). Several studies have detailed the impact that HG methyl-esterification and acetylation have on disease resistance, demonstrating the crucial role HG physicochemical properties play in mediating defense against pathogens (187-196). Xylosylation of HG, creating XG, appears to be correlated with cell detachment. XG is predominantly localized to reproductive tissues, but is also secreted at the root cap, allowing for border cell detachment from the root surface (144, 197-199).

RG-II is a critical pectic domain necessary for normal plant development. Due to its ability to form borate diesters, RG-II contributes to wall strength and integrity. The dimerization of RG-II likely plays a role in borate as an essential micronutrient (143, 156, 200-202). Recent research has suggested that the disruption of borate cross-linking of RG-II contributes to hypersensitivity to salt stress in wall integrity-impaired *A. thaliana* mutants, implicating RG-II in the cell wall integrity system (203).

As with HG, RG-I has broad functions in the cell wall. Decreases in both HG esterification and RG-I side chains are correlated with increased rigidity in the cell wall (171). RG-I side chains are correlated with increased flexibility of the wall and are involved in increased wall expansibility and plasticity. In guard cells, arabinan was demonstrated to be crucial to stomatal opening and closure in fusicoccin- and ABA-treated guard cells treated with arabinases. Normal stomatal closure was rescued when treated with PGs and PMEs or with calcium chelators, implying that arabinan chains act as physical barriers preventing HG cross-linking and providing greater wall

elasticity (204). Arabinan is also necessary for salt stress tolerance, as demonstrated by the rescued phenotype of dwarfed root growth of the *Atmur4* mutant grown under high salt concentrations (205). Additionally, arabinan has been hypothesized to act as a plasticizer in desiccation response of plants, preventing the wall from becoming rigid and breaking during extreme desiccation conditions (53). Similarly, studies show that a drought-tolerant wheat cultivar has more pectic arabinose and galactose in roots than a drought-sensitive cultivar and these neutral chains may be maintaining greater extensibility in the root apical region in the tolerant cultivar (79, 82). Structural analysis of apple pectins before and after cold storage demonstrated a decrease in branched arabinan and arabinosylated linear galactan chains (206). An abundance of branched galactan side chains consisting of β-(1,6)-D-galactosylation of linear β-(1,4)-D-galactan side chains at least three residues in length was detected in the phloem sieve-tube elements in *A. thaliana*, *Miscanthus x giganteus*, and beet roots, and has been correlated with increased elasticity in *Miscanthus* phloem sieve-tube elements relative to that of companion cells abundant in linear β-(1,4)-galactan (170). Experiments compressing early and late stage pea cotyledons correlate linear RG-I galactan with increased firmness (207). These experiments taken together suggest that RG-I side chains increase flexibility by preventing HG packing and cross-linking and that branched side chains increase flexibility much more relative to linear side chains. Additionally, they indicate that RG-I side chain structural modifications are a common response to a range of abiotic stresses.

Finally, pectin is also involved in cell wall integrity sensing not only under biotic stresses, but also under abiotic stresses such as salt stress. This is through pectin polysaccharide or oligosaccharide detection via wall-associated kinases (WAKs) or receptor-like kinases (RLKs) in the cell wall. Pathogen detection through WAKs is well documented and occurs via WAK1 and/or WAK2 binding of oligogalacturonides released via pathogen polygalacturonases. WAKs have also been shown to bind to HG and other pectic domains, suggesting a role for intact pectin structure in cell wall integrity sensing (208, 209). Additionally, the RLK Feronia in *A. thaliana* has been implicated in pectin binding when mediating salt stress acclimation. Experiments using *fer* mutants demonstrated that the salt hypersensitive response in the roots could be rescued by addition of both calcium and boron, implicating both HG and RG-II in maintaining cell wall integrity during salt stress acclimation (203).

Biosynthesis

Pectin biosynthesis is still poorly understood, with about twenty enzymes described for the more than 60 known unique linkages in pectin (12, 139, 144, 162). Pectin biosynthesis is thought to occur in the Golgi, where an influx of nucleotide sugar substrates come from the cytosol for glycosyltransferase (GT) use (165, 210). Two enzymes have been demonstrated to synthesize the HG backbone in the lumen of the Golgi, the interacting galacturonosyltransferases GAUTs 1 and 7, with GAUT1 having demonstrated catalytic activity and GAUT7 acting as a Golgi membrane anchor (118). More recently, the homologs GAUT 4 and GAUT11 have also been shown to synthesize HG *in vitro* (211). Insertional mutants of GAUT8 in *A. thaliana*, termed *qua1-1* and *qua1-2* demonstrate decreased homogalacturonan in the vasculature and subepidermal cells of the stems and a much greater reduction in the roots of the mutants (119, 212). The other QUASIMODO mutant, *qua2*, is a likely pectin methyltransferase. The *qua2-1* insertional mutant in *A. thaliana* has a 50% reduction in HG, with no change in methyl-esterification of the remaining HG. This is hypothesized to be because HG polymerization and methyl-esterification

are inter-dependent (213). It is also possible that non-methyl-esterified HG is more susceptible to apoplastic PGs. Insertional mutant *qua2-1* displays cell adhesion defects resulting in non-coordinated, tumor-like growth (214). Similarly, cotton Golgi-related (CGR) proteins 2 and 3 are implicated in pectin methyl esterification. Double knock-out mutants of *cgr2/3* in *A. thaliana* have a reduction in pectin methyl esterification and decreased methyltransferase activity in isolated microsomes while over-expressors demonstrate the opposite cell wall and microsome activity phenotypes (215). Additionally, a pectin acetyltransferase has recently been described, with demonstrated acetyltransferase activity on a range of oligogalacturonides (196). Finally, HG xylosylation to form XG has been demonstrated through the enzymatic xylosyltransferase activity of GT family 47 Xylogalacturonan Deficient 1 (XGD1) in *A. thaliana* (216).

For RG-II, several enzymes have been linked to biosynthesis. RGXT1 and 2 in *A. thaliana* have demonstrated xylosyltransferase activity and catalyze the addition of xylose to a fucosyl residue in the A chain of RG-II (217). Two sialyltransferases in *A. thaliana* have been proposed to be involved in the transfer of the rare Dha and/or Kdo residues onto RG-II side chains and display incredibly aberrant growth phenotypes in the pollen grains of *A. thaliana* mutants and no homozygous progeny, however there is no evidence of this transferase activity (218).

RG-I biosynthesis is incredibly complex, with several enzymes demonstrated or implicated. Both GAUT11 and the GAUT-like GATL5 *A. thaliana* insertional mutants have decreases in seed coat mucilage rhamnose and galacturonic acid content (219, 220). This suggests that GATL5 may be involved in catalyzing the transfer of galacturonic acid onto the RG-I backbone in mucilage. However, GAUT11 has been shown to have HG synthase activity and hence it is unlikely to directly synthesize the RG-I backbone (211). A recent study has determined the rhamnosyltransferase responsible in part for extending the RG-I backbone, termed RRT1 from the previously uncharacterized GT family 106, in *A. thaliana* seed coat mucilage (120). RG-I side chain biosynthesis is performed in part by two non-redundant GT family 47 arabinosyltransferases, ARAD1 and 2, with steep RG-I arabinose content reductions in *A. thaliana* insertional mutants and distinct immunolabeling patterns when using a JIM13, a monoclonal antibody specific to long, unbranched arabinan (130, 221). RG-I galactan side chain biosynthesis includes in part the galactan synthase (GALS) enzymes from GT family 92. The three GALS enzymes in *A. thaliana* are all demonstrated galactosyltransferases capable of elongating a linear β-(1,4)-gal*p* chain of at least four residues or longer. Triple knock-out mutants display an absence of the linear β-(1,4)-gal*p* epitope, however linkage analysis does not show a change in rhamnosyl substitution or RG-I arabinan content, suggesting that these enzymes are not necessary for the first 1-3 galactose linkages on the RG-I backbone (115, 222). Interestingly, GALS1 is a bifunctional enzyme with an additional role in arabinosylating linear β-(1,4)-galactan, preventing further glycosylation of galactan chains, although its affinity for UDP-galactose is much higher than for UDP-arabinopyranose (223). RG-I arabinogalactan biosynthesis is influenced by PAGR, a member of the DUF-246 family. In *Nicotiana benthamiana*, knockdown *pagr* lines (via virus-induced gene silencing or VIGS) have a decrease in galactose, a reduction in Type I AG epitopes and reduced RG-I backbone substitution. Curiously, *A. thaliana* lines over-expressing *AtPAGR* displayed in increase in Type II AGs, suggesting that this enzyme may have different effects in the two plants (116). Finally, a putative acetyltransferase, trichome birefringence-like (TBL) 10, has been demonstrated to be involved in acetylation of the RG-I backbone. Knock-down mutants *tbl10-1* and *tbl10-2* have a reduction in wall-bound acetate of pectic fractions, specifically in RG-I-enriched fractions (224).

Conclusion

Pectin mediates many developmental and environmental processes and stresses as a complex, dynamic polysaccharide in the cell wall. Possessing a heteropolymeric organization of specialized domains with diverse architecture that can shift depending on a variety of cues throughout the life of the plant, pectin presents a unique challenge for researchers. Due to the heterogeneity of the polysaccharide, the variety of known side chains, and the reorganization upon signaling cues, it is difficult to provide an exact understanding of the structure of pectin in all its forms or even of the conformation and distribution of its component domains in the plant. Additionally, the critical function of some pectin structures can lead to homozygous-lethal phenotypes or functionally redundant enzymes, both of which hinder elucidation of the biosynthetic machinery. Lastly, many pectin structures are complex and not commercially available, making enzymatic activity assays tricky or impossible. Despite these drawbacks, advances have been made in understanding pectin structure, distribution, and biosynthesis with the use of co-expression networks, glycome profiling, enzymatic fingerprinting, immunolabeling using cell wall monoclonal antibodies, and novel acceptor-synthesizing techniques. By better understanding the structure and biosynthesis of pectin, it is possible to engineer pectic epitopes to test the effects on physical wall properties and hypothesize pectic structures that would better withstand stress conditions and confer and adaptive advantage to the plant.

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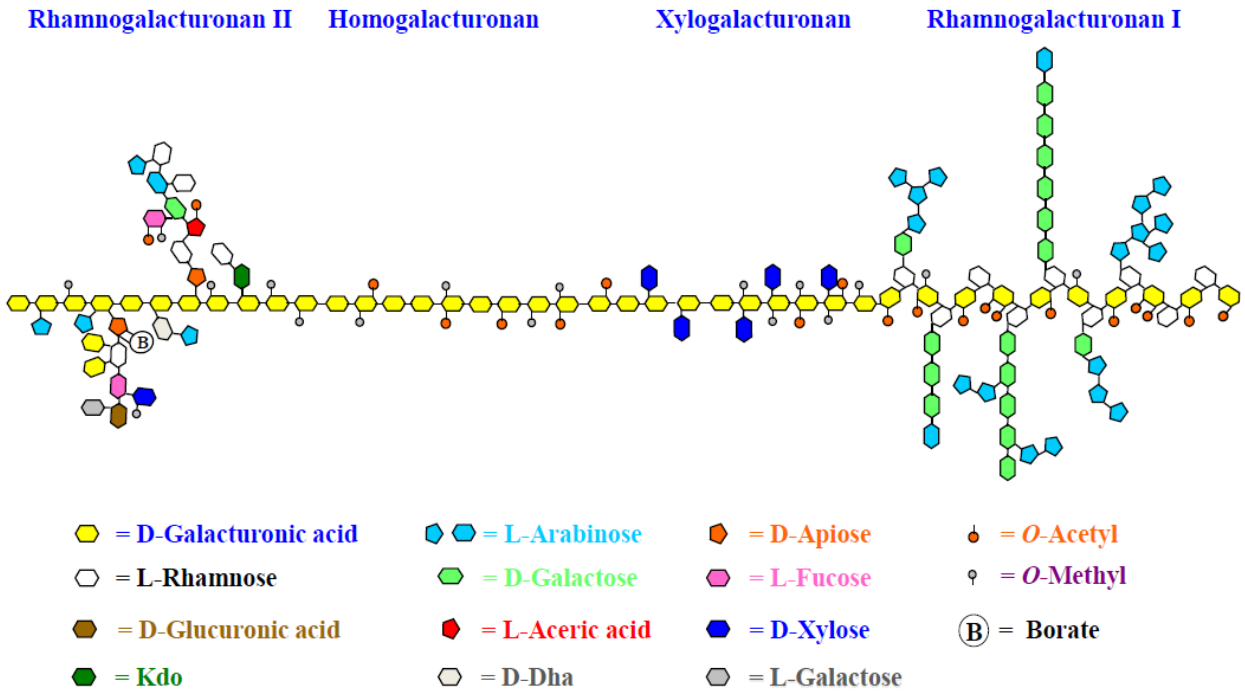

Figure 1. A schematic of pectin as a heteropolymer containing its most common domains and the monosaccharide constituents.

Characterization of an Essential GT47 Glycosyltransferase Required for Galactan Biosynthesis

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Abstract

Background: Pectin is an essential component of the plant cell wall, mediating cell wall deposition, cell expansion, and responses to biotic and abiotic stresses. Despite its crucial role in plant development and environmental response mediation, pectin biosynthesis is still poorly understood. Particularly in complex and heterogeneous pectic domains such as rhamnogalacturonan I (RG-I), the enzymes involved in glycan chain elongation and branching are minimally described, with only three classes of glycosyltransferases described in RG-I side chain biosynthesis, including two gene families involved in RG-I galactan biosynthesis, GT92 and DUF-246.

Results: A GT47 glycosyltransferase, termed GT47F, is required for normal development in *Nicotiana benthamiana* and *Arabidopsis thaliana*, with knock-down phenotypes including dwarfism, defects in cell shape and organization, and homozygous embryo lethality in *A. thaliana* insertional mutants. Cell wall defects in *N. benthamiana* include a decrease in galactose and concomitant increase in galacturonic acid in whole cell wall preparations. Isolation of *N. benthamiana* virus-induced gene-silenced (VIGS) RG-I demonstrated galactan loss in this polysaccharide, with a smaller molecular weight than control plants. Immunoblots of whole cell wall preparations and pectin-enriched fractions indicated an increase in less common pectic epitopes, such as branched galactan, xylogalacturonan and/or xyloglucan, and unsubstituted RG-I, and a slight decrease in linear β-(1,4)-galactan. Pectinolytic digests of the RG-I backbone of *N. benthamiana* VIGS lines had altered patterns, with a higher preponderance of smaller fragments, indicating less RG-I backbone branching. *A. thaliana* plants over-expressing *AtGT47F* showed an increase in galactose in whole cell wall preparations. Microscale thermophoresis experiments using *N. benthamiana* microsomes over-expressing a YFP-tagged *GT47F* demonstrated binding of UDP-galactose, suggesting that *NbGT47F* encodes a galactosyltransferase involved in RG-I biosynthesis.

Conclusions: GT47F is a probable galactosyltransferase required for normal development in *N. benthamiana* and *A. thaliana*. Though many effects are seen in the walls of VIGS lines, the most prominent, repeatable change is a decrease in galactan attributable to compositional changes in RG-I. Further, increases in galactose in *A. thaliana* over-expressors and microscale thermophoresis experiments using *N. benthamiana* over-expressor microsomes all provide supporting evidence of GT47F being a galactosyltransferase likely acting on RG-I.

Keywords: Rhamnogalacturonan I, GT47, *Nicotiana benthamiana*, *Arabidopsis thaliana*

Background

Plant cell walls play an essential role in mediating plant growth and development and in plant response to environmental perturbations (40, 140, 225). Functioning as structural support, a defensive barrier, and as a sensor of the external environment, the plant cell wall is a complex and dynamic component of the cell. Generally speaking, embryophytes today share a primary cell wall composed of load-bearing cellulose microfibrils embedded in a hemicellulose-pectin matrix and a secondary cell wall composed of cellulose microfibrils embedded in a hemicellulose-lignin matrix (2). While wall compositions and hemicellulosic constituents vary between monocots and eudicots in the angiosperms, this general compositional trend holds true (7, 95). In secondary cell walls, which are dead at functional maturity, the hemicellulose-lignin matrix properties confer strength, rigidity, and hydrophobicity of the cell walls, necessary characteristics for plant vasculature (91, 94). In primary cell walls, the hemicellulose-pectin matrix properties allow for deposition and expansion of growing cells, requiring both strength and flexibility. Pectin, with its heterogeneous structure, is crucial in modulating wall properties (12, 16, 139).

Pectin is a heteropolymeric polysaccharide mainly consisting of three common domains, homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II), although other structures have been described in the past. HG and RG-II both have an α-(1, 4)-D-galacturonic acid (Gal*p*A) backbone (138, 139, 226). HG can be methyl esterified and acetylated along its backbone, influencing its physical properties within the cell wall (16, 17, 184, 196). HG can also be glycosylated along its backbone, with xylosylation resulting in xylogalacturonan (XG), a structure found in small amounts in the cell wall and which is correlated with cell detachment (197-199). Additionally, apiose residues along the HG backbone result in apiogalacturonan, a pectic domain found in two aquatic plant species (147, 148). RG-II, in contrast, consists of a highly conserved structure of six side chains attached to the Gal*p*A backbone, with twelve different monosaccharides and 22 unique linkages (143, 155, 200). RG-II is able to dimerize via borate esters and this is critical to normal plant development, cell adhesion, and salt acclimatization (143, 156, 157, 203, 218). The other pectic domain, RG-I is unique in that it has a repeating diglycosyl α-(1,2)-L-Rha*p*-β-(1,4)-D-Gal*p*A backbone. The RG-I backbone can then be branched with arabinan, galactan, and arabinogalactan side chains, of which there is great structural diversity (139, 162, 163, 227).

Pectin structural properties can translate to major effects in the plant cell wall. The degree of methyl-esterification of the HG backbone relates to the stiffness or flexibility that it can impart to the wall through Ca^{2+} cross-linking of non-esterified HG chains (17, 176, 177, 180-182, 186, 190). Oligogalacturonides released from HG via pathogen-derived polygalacturonases (PGs) act as signaling molecules in the plant defense response (208, 209, 228). RG-II confers structural stability and cell wall integrity via its borate-ester dimerization (143, 156, 203). Finally, RG-I arabinan and galactan side chains appear to confer greater flexibility in the cell wall and act as plasticizers during development or during stress conditions (53, 79, 82, 163, 170, 171, 204, 205, 207).

Despite the functional importance of pectin, and perhaps due in part to its structural complexity, few of the glycosyltransferases are known for the more than 65 estimated unique linkages found in pectin. To date, several GAUT enzymes from GT8 family have demonstrated galacturonosyltransferase activity or have been implicated in HG, RG-II, or RG-I backbone biosynthesis (118, 119, 211, 219, 220, 229). Additionally, a recent study has determined a

GT106 rhamnosyltransferase involved in RG-I biosynthesis in *A. thaliana* seed coat mucilage (120). Further, a GT47 xylogalacturonan xylosyltransferase involved in XG biosynthesis in addition to a rhamnogalacturonan xylosyltransferase involved in A chain biosynthesis in RG-II have been previously described (216, 217). Interestingly, several enzymes have been described that have been demonstrated to be involved in RG-I side chain biosynthesis, including two non-redundant GT47 arabinosyltransferases responsible for arabinan side chain biosynthesis (130, 221), three GT92 galactosyltransferases involved in linear β-(1,4)-galactan side chain biosynthesis (115, 222, 223), and a putative glycosyltransferase-like DUF-246 family enzyme involved in arabinogalactan biosynthesis (116). Despite these studies, many pectin biosynthetic components are still unknown. This holds true for RG-I side chains in particular, with unknown enzymes catalyzing Type I and II AGs, branched galactans, and even the initial 1-3 Gal*p* residues of β-(1,4)-galactan (162, 163, 222).

Though it can be difficult to study pectin biosynthesis due to redundancy of genes (222), severity of phenotype (156, 230, 231), or the complexity or commercial rarity of the polysaccharide (139, 144, 162), several studies have demonstrated means to elucidate pectin biosynthetic components. *A. thaliana* gene co-expression networks, compiled by ATTED-II and available to the public (232), have been used to find candidate GTs for pectin biosynthesis by using known pectin GTs as bait for potential interacting partners (217, 233). GTs that exhibit severe or lethal phenotypes in *A. thaliana* insertional mutants have been characterized instead in *N. benthamiana* plants using virus-induced gene silencing (VIGS), or via RNA-interference (RNAi) in *A. thaliana* (116, 234). Finally, commercial unavailability of pectic oligosaccharides or domains impeding GT characterization has been circumvented through diagnostic pectinolytic digests, immunolabeling of tissues or immunoblotting of cell wall extracts, or enzymatic digestion of pectin to derive pectic oligosaccharides for enzymatic activity assays (116, 120, 155, 222, 223). Here, we use a combination of these techniques in addition to a novel ligand binding assay (235) to identify and characterize a putative GT47 galactosyltransferase involved in RG-I galactosylation.

Results

AtGT47F *Phylogenetic & Co-Expression Map Analysis*

At1g21480, termed *AtGT47F*, is a member of the GT47 family with an exostosin domain. Publicly available co-expression maps of nine different plant species, including *A. thaliana*, are compiled and curated by ATTED-II and based on microarray and RNA-Seq data (232). The co-expression map for genes connected with *AtGT47F* (http://atted.jp/cgi-bin/locus.cgi?loc=At1g21480) via microarray data is enriched in genes encoding putative or known pectin biosynthetic enzymes, including one member of GT106 (At4g24530), GAUT8, a pectin lyase-like superfamily member (At1g80170), five putative methyltransferases (At2G39750, At1G77260, At5G04060, At1G29470, At1G19430), and the 3-KDS reductase required for Kdo biosynthesis (At3G06060), a sugar used exclusively in pectin biosynthesis. Previously published phylogenetic analysis of the GT47 family , including a focus on GT47 in *A. thaliana*, depicts *AtGT47F* as forming its own, separate clade within the GT47 family (236), with homologs in land plants ranging from a moss, *Physcomitrella patens* (PHYPA_124241), to the C4 grass *Sorghum bicolor* (SOBIC_003G360300). Given its unique reported phylogeny and co-expression network in *A. thaliana* and the presence of homologs across land plants, we selected *AtGT47F* as a candidate GT for pectin biosynthesis.

GT47F Insertional Mutants are Homozygous Lethal

T-DNA insertional mutants interrupting the fourth intron of *AtGT47F* result in heterozygous plants with no detectable phenotype or cell wall differences (data not shown). Heterozygous T-DNA insertional mutant (CS811400) lines have a seed abortion rate of 0.23:0.77, or nearly 1:3, indicating an embryo lethal phenotype (Figure 1, Table 1). Similarly, attempts to generate knock-out mutants of the *P. patens* homolog using recombinant cloning techniques resulted in no surviving *P. patens* colonies after selection, suggesting a lethal phenotype in regenerating protoplasts.

As with the heterozygous insertional T-DNA lines, *35S:HA-AtGT47F* plants have no visible growth defects. However, these plants do have an increase in whole cell wall galactose (Figure 1).

GT47F *Silenced Lines Have Growth Defects*

NbGT47F-VIGS plants have reduced stature and compact internodes compared to control plants (Figure 2). Leaves appear to have reduced chlorophyll content, although this has not been quantitatively assessed. Additionally, *NbGT47F*-VIGS plants display altered cell shape and patterning, appearing much more disorganized and prone to tearing in cross-sections (Figure 2). Expression analysis demonstrates a reduction in mRNA transcript abundance in VIGS plants, indicating that these phenotypes are due to reduced expression of *NbGT47F* (Figure 2).

AtGT47F-RNAi T0 plants also have reduced stature compared to control *GUS*-RNAi plants. Rosette radius is also reduced in the T0 plants, although this has not been quantified (Figure 3).

NbGT47F-*VIGS Plants Have Reductions in RG-I Galactan*

Whole cell wall extracts from leaves and the first three internodes encompassing the shoot apex of *NbGT47F*-VIGS plants displayed altered matrix monosaccharide composition. Although the most prominent, recurring shift was a decrease in the relative amount of galactose (74% of control) when compared to total monosaccharide content (mol %), other sugars were often affected, including an increase in GalA (113% of control) (Figure 4). Pectin-enriched cell wall fractions also displayed a decrease in galactose (62-68% of control) with an increase in GalA (111-127% of control) in addition to decreases in fucose (86% of control) and mannose (55-59% of control) (Figure 4). Although changes in galactan content were not seen when comparing the mol % of RG-I monosaccharides, we noticed differences in the retention times of RG-I isolated from VIGS and control *N. benthamiana* plants (Figure 5). This difference in retention times corresponded to a reduction in molecular mass of VIGS plants compared to control plants in both the leaves and stems (81% and 77%, respectively). Interestingly, it appears that RG-I in *N. benthamiana* has different molecular weights depending on its origin, with differences between the stems and leaves in both the VIGS and control lines (Figure 5). Given the difference in molecular weights, we sought to determine the mass contributions of each monosaccharide to RG-I, as has been previously described (116) and discovered a reduction in the mass contribution of galactose to RG-I isolated from VIGS plants (71-74% of control) (Figure 4).

NbGT47F-*VIGS Plants Have Altered Pectin Structure*

Interestingly, RG-II composition was also affected in VIGS plants relative to control plants. Decreases in relative amounts of fucose (74-78% of control), arabinose (57-68% of control), and

GalA (74-79% of control) were observed in all tissues (Figure 4). The molecular weights of RG-II did not differ between VIGS and control plants (Figure 5).

Digestion of isolated RG-I from *NbGT47F*-VIGS and control plants demonstrates a difference in digestion patterns between the two plant populations. Using rhamnogalacturonan hydrolase A (RG-hydrolase) coupled with polysaccharide analysis using carbohydrate gel electrophoresis (PACE), it is clear that the VIGS plants digest into smaller fragments more readily than control plants (Figure 5). Although higher level bands corresponding to larger oligomers appear in the VIGS samples, they are much fainter than in the control samples. This indicates that RG-I in the VIGS line has a potentially less substituted RG-I backbone or smaller side chains. Similarly, *N. benthamiana* VIGS and control RG-I samples digested with RG-hydrolase were analyzed via high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) and differences in chromatograms were evident. In particular, differences in oligosaccharides eluting between 18-23 minutes are evident, with VIGS digests resembling digested *A. thaliana* mucilage more than control digests (Figure 5).

Immunoblots of both whole cell wall extracts and pectin-enriched extracts from *N. benthamiana* VIGS and control plants showed a difference in labeling of monoclonal antibodies specific to pectic epitopes (Figure 6). In particular, an increase in labeling of a branched β-(1,6)-galactan epitope was observed. Decreases in labeling of the unsubstituted RG-I backbone occurred in VIGS samples, while slight decreases in β-(1,4)-galactan epitope labeling were observed. Interestingly, VIGS samples also had reduced labeling of non-methylesterified HG epitopes and an increase in labeling of xylosylated epitopes related to XG and xyloglucan (XyG).

35S:YFP-NbGT47F *Localizes to the Golgi and Preferentially Binds UDP-Gal*

N. benthamiana plants co-infiltrated with *35S:YFP-GT47F* and *35S:mCherry-mannosidase* demonstrate a co-localization of these expressed fluorescently-tagged constructs to the Golgi, where the majority of GTs are localized (Figure 7). Additionally, *N. benthamiana* microsomes expressing *35S:YFP-GT47F* preferentially bind UDP-Gal over other UDP-sugars, such as UDP-Xylose (UDP-Xyl) and UDP-Glucose (UDP-Glc), with a K_d two orders of magnitude lower (16.7 μM, 4 mM, 7 mM, respectively) (Figure 7, Table 2).

Discussion

Based on the reported analyses, we conclude that GT47F is a probable galactosyltransferase involved in RG-I galactosylation. Decreases in galactose in both whole cell wall and pectin-enriched extracts in addition to isolated RG-I in *N. benthamiana* VIGS plants indicate that GT47F is required for normal RG-I galactosylation. Additionally, increases in galactose in *A. thaliana* over-expressing plants bolsters the hypothesis that GT47F is involved in RG-I galactosylation. While analysis of the *N. benthamiana* VIGS plants has shed light on a greater range of pectic alterations, a greater characterization of the pectic and galactan changes in both the *A. thaliana* over-expressing and RNAi lines is needed, including quantitative expression analysis confirming expression changes and RG-I characterization as described above.

Microscale thermophoresis experiments demonstrated a preferential binding for UDP-Gal over UDP-Xyl and UDP-Glc. Microscale thermophoresis (MST) takes advantage of the molecular movement of the protein over a temperature gradient, which is influenced by the protein's physicochemical properties, including size, charge, and conformation, to elucidate binding

interactions with other molecules. Using an infrared laser to induce a precise temperature gradient, fluorescently-coupled proteins can be assayed for binding affinity to different ligands. Thus, the change in fluorescence intensity acts as a direct measure of molecular movement in response to thermophoresis and differences in fluorescence intensity signal differences in binding affinity (235). Although our MST results indicate that UDP-Xyl and UDP-Glc have binding-induced fluorescence intensity changes, the K_d of these UDP sugars is at least two orders of magnitude higher than that of UDP-Gal, indicating a strong preferential binding of UDP-Gal. However, more UDP sugars remain to be tested, as a previous study demonstrated that an RG-I galactosyltransferase had bifunctional capabilities, also functioning as an arabinosyltransferase when concentrations of available UDP-Ara were increased (223).

Structural differences in RG-I in the *N. benthamiana* VIGS plants suggest a plant with decreased RG-I side chain length. Enzymatic digests using RG-hydrolase coupled with HPAEC-PAD and PACE show a decrease in higher molecular weight RG-I oligomers and a possible reduction of long, neutral oligosaccharides. HPAEC-PAD analysis shows an absence of peaks during minutes 18-23 of elution. Oligosaccharide separation and retention time via HPAEC-PAD is influenced by several factors. The size, degree of branching, and charge associated with oligosaccharides all contribute to their retention times, with larger, more branched, and more negatively charged oligosaccharides all having longer retention times. Interestingly, terminal galactose residues in oligosaccharides also increase retention time (237). Based on the results of the chromatogram, it is tempting to speculate that a decrease in large, neutral pectin side chains is occurring in *NbGT47F*-VIGS RG-I. RG-hydrolase has demonstrated hydrolytic activity cleaving the RG-I backbone, releasing tetra- and hexasaccharide backbone fragments with side chains intact (238). Interestingly, both PACE and HPAEC-PAD results demonstrated a closer resemblance of the *NbGT47F*-VIGS RG-I digestion pattern to *A. thaliana* mucilage than control *N. benthamiana* RG-I. *A. thaliana* mucilage contains RG-I that is very lowly substituted (132, 160, 239), thus, this indicates that the *N. benthamiana* VIGS plants have reduced RG-I backbone substitution or alternatively, a reduction in the size of the side chains. Further clarification of RG-I side chain substitution patterns could be pursued through additional diagnostic enzymatic digestion assays, including the use of galactanases to determine the quantity of linear and branched galactan (115, 222, 240).

Immunoblots demonstrate a decrease in labeling of the CCRC-M35 epitope, consisting of at least two consecutive unbranched disaccharides in the RG-I backbone (241), demonstrating an increase in RG-I backbone substitution. While this may appear to contrast with the RG-hydrolase digests, it is possible that the RG-I backbone from VIGS plants is more substituted, however, the side chains are much smaller than those of control plant RG-I. This interpretation is complicated by the increase in LM26 labeling and the slight decrease in LM5 labeling. Both monoclonal antibodies label pectic galactan epitopes, with LM26 recognizing specifically the β-(1,6)-galactose branched off linear β-(1,4)-galactan and LM5 recognizing linear β-(1,4)-galactan at least four residues in length (170, 242). LM5 has also been recently demonstrated to only bind at the reducing end of the galactan chain, not internally (243). Thus, it is possible that linear β-(1,4)-galactan chains in the VIGS plants are reduced in length, although not necessarily shorter than four galactose residues. However, this scenario is puzzling also, given that the GALS GTs have already been shown to elongate β-(1,4)-galactan oligomers at least four residues in length (222). Given the severity of the GT47F silencing phenotype and lack of developmental phenotype in even the GALS triple knock out, it is unlikely that GT47F is elongating linear

galactan chains of four residues or more. It is possible that a reduction in *NbGT47F* expression in VIGS plants reduces the abundance of acceptors for GALS enzymes, catalyzing one or most of the initial four β-(1,4)-galactan residues attached to the RG-I backbone. However, it is unlikely that GT47F is catalyzing the first galactosyl substitution, given the reduction in CCRC-M35 labeling. The abundance of GTs that appear to be involved in RG-I galactosylation illustrates the complexity of this pectic domain. Again, galactanase digestion assays would be critical to determine this phenotype. Alternatively, it is possible that VIGS plants have altered arabinogalactan side chain composition, which could be validating by profiling with monoclonal antibodies specific to pectic arabinogalactan epitopes (116, 244). Glycosidic linkage analysis of the isolated RG-I would also help to clarify the biochemical phenotype.

In addition to altered RG-I structure and composition, *N. benthamiana* plants also demonstrate altered pectin and hemicellulosic structures. Decreases in LM19 labeling and increases in LM23 labeling in *N. benthamiana* VIGS whole cell wall extracts indicate changes in pectin and potentially xyloglucan. LM19 recognizes the non-methylesterified HG as an epitope (245), while LM23 recognizes xylosyl residues from both xylogalacturonan and xylan (246, 247). These results may be pleiotropic, but they may also be compensatory. A decrease in non-methylesterified HG epitope labeling indicates that the HG backbone has a higher degree of esterification in the VIGS plants. This may be a result of attempting to increase elasticity of the cell walls due to the decrease in RG-I side chain composition and size. Changes in LM23 labeling could indicate either an increase in XG or xylan. XG and the LM23 epitope specifically have been implicated in cell detachment (197-199), so an increase in this epitope may relate to the cellular disorganization seen in VIGS cross-sections. Additionally, pectin has been demonstrated to be covalently linked to xylan and arabinogalactan proteins, so it is possible that an alteration in RG-I leads to alterations in xylan-pectin cross-linking, with either pleiotropic or compensatory effects, and increased LM23 labeling is attributable to changes in xylan (151). Further characterization of cell wall extracts using xylan-specific monoclonal antibodies could determine if there is a change in xylan structure or abundance in VIGS plants (244). Lastly, pectin and xylan immunoblot data coupled with compositional, but not molecular mass, changes in the RG-II of VIGS plants indicate that GT47F is catalyzing the transfer of a glycosidic linkage required for normal cell wall organization and composition. As pectic side chains and RG-I have been demonstrated to bind to cellulose, xylan, xyloglucan, and arabinogalactan proteins (15, 18, 150, 151), it is a rational conclusion that a defect in these side chains could have widespread cell wall effects. However, studies demonstrating defects in RG-I side chain biosynthesis have not shown changes in other cell wall components. Given the severity of the *NbGT47F*-VIGS and *A. thaliana* insertional phenotypes, this is perhaps not surprising.

The severe phenotypes in silenced *N. benthamiana* and *A. thaliana* plants indicate that GT47F is required for normal development in these plants. Additionally, the embryo lethal phenotype in *A. thaliana* insertional mutants and the lethal phenotype in *P. patens* homologous recombination mutants indicate that at least some basal level of GT47F is required for embryo maturation and protoplast regeneration. Severe phenotypes in GT mutants are not uncommon, with cellulose, xylan, lignin, and pectin mutants all having documented phenotypes severely affecting plant growth and development. As cellulose and lignin confer structural strength to the cell wall and plant, dwarfism and embolism in these lines are not unexpected (4, 124). Similarly, with a documented role in wall strengthening and cellulose binding (109, 248), xylan mutants also exhibit the expected dwarfism and embolism phenotypes (63, 249, 250). Pectin mutants also

display dwarfism, in addition to cell adhesion defects, deformed organs, stress hypersensitivity, and cell death (116, 203, 230, 231). Most severe pectin mutants occur in lines with affected RG-II, particularly interruptions in its dimerization via borate cross-linking (231, 251-253). RG-I mutants have not typically exhibited any phenotypes under optimal lab conditions (120, 130, 222), with the exception of a member of GT 106 involved in RG-I side chain biosynthesis (116). Thus, severe phenotypes in plants with reduced or no *GT47F* expression represent another example of disrupted RG-I side chain biosynthesis having large developmental effects.

Conclusion

Pectin is a structurally diverse polysaccharide with a range of functions in the plant cell wall including providing strength and/or flexibility to the cell wall, cell wall integrity sensing, and stress mediation (41, 53, 139, 225, 254). RG-I, a complex pectic domain with a great deal of reported variety in structure, has specifically been implicated in increasing wall flexibility and mediating stress responses (18, 53, 79, 82, 163, 204). Despite its importance, RG-I biosynthesis is still poorly understood. Here, we present a novel putative galactosyltransferase involved in RG-I biosynthesis. Although our results suggest that *GT47F* is galactosylating RG-I in some way, further experiments characterizing the RG-I side chains in *N. benthamiana* and *A. thaliana* silenced lines and *A. thaliana* over-expressors must be performed. In particular, enzymatic characterization of galactan and arabinogalactan side chains coupled with more in-depth glycome profiling could provide greater clarity (116, 222, 244). Additionally, greater characterization of the RG-I in these plants could lead to better hypotheses about the exact glycosyl linkage that this GT is making. These hypotheses could in turn be tested via enzymatic activity assays using synthesized or derived acceptors (120, 222, 243, 255).

Methods

Plant Growth Conditions

A. thaliana plants were grown under a ten-hour photoperiod at 22°C, 60% humidity with 90 μmol/m^2s illumination during the day. Flowering was induced after four weeks by transferring plants to a 16-hour photoperiod. Both the wild-type Columbia-0 accession and *A. thaliana* T-DNA line CS811400 from the Arabidopsis Biological Resource Center (ABRC, Ohio State University) were grown. *N. benthamiana* plants were grown under 16-hour photoperiod at 25 °C, 60% humidity with 200 μmol/m^2s illumination during the day.

Virus-Induced Gene Silencing (VIGS)

NbGT47F (Niben101Scf02218g01008) was identified in the Sol Genomics Network Database as a reciprocal best BLAST hit for *AtGT47F* in the *N. benthamiana* genome (256). Alignment of the NbGT47F and AtGT47F predicted amino acid sequences indicated that NbGT47F was a likely homolog (Appendix 1). A *NbGT47F* sequence was amplified from *N. benthamiana* cDNA using primers 5'- TTACTCGAGGCCTGGCTCCACGTGGTGAAT-3' and 5'-TTACTCGAGGCAGAGGCATGGGTGGTTTCCA-3' for subsequent VIGS cloning. Using a tobacco rattle virus-based (TRV) VIGS cloning system, this fragment was cloned into the Xho1 site in *pYL156*/*pTRV2* to generate *pYL156-NbGT47F* (257). As a control, *pYL156* with a fragment of the GUS gene was used (*pYC1*) as previously described (116, 258). Both constructs were independently transformed into *Agrobacterium tumefaciens* strain GV3101 as previously described (259). Virus-induced gene silencing was induced in 2–3 week old *N. benthamiana*

plants as previously described (257). Tissue was collected 14 days post-infection for analysis. Tissues collected include the entire portion of shoot grown post-infection, the third youngest leaf, and the first three internodes of the shoot apex. *N. benthamiana* plants were also grown for 4 weeks post-infection to analyze height differences during flowering.

GT47F Overexpression and Localization

The coding sequence of At1g21480 (*GT47F*) was amplified from *A. thaliana* cDNA using Phusion High-Fidelity DNA polymerase (New England Biolabs) and the primers 5'-CACCATGGCGAGCTTAACTAGTAAT-3' and 5'- AGACCTCCTATGCCTCTACCT-3'. The PCR product was cloned into pENTR-D-Topo (Invitrogen) according to the manufacturer's protocol. A Gateway LR recombination reaction was performed according to the manufacturer's protocol (Invitrogen) to transfer the coding sequence of *GT47F* into *pEarleygate 201* to produce *35S::GT47F-YFP-HA* for production of *N. benthamiana* microsomes (260). Additionally, *35S::HA-GT47F* was constructed in the same way using *pEarleygate201* to produce transgenic *A. thaliana* plants (260). These constructs were transformed into *A. tumefaciens* strain GV3101 and *A. thaliana* Col-0 plants were transformed via the floral dip method as previously described (261). For localization studies, the coding sequence of *GT47F* was recombined into *pGWB44* to produce *35S::GT47F-CFP*. *35S::GT47F-CFP* was transiently co-expressed with a Golgi-localized α-mannosidase (262) in four week old *N. benthamiana* leaves as previously described (263), with a modified infiltration medium consisting of 100 mM 2-(N-morpholino)-ethanesulfonic acid, 100 mM MgCl2, and 10 μM acetosyringone. Expression in *N. benthamiana* epidermal cells was imaged using a Zeiss 710 confocal laser-scanning microscope (Carl Zeiss).

RNA Interference (RNAi)

A 400 base pair portion of *AtGT47F* was amplified from *A. thaliana* genomic DNA using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and the primers GT47FRNAiF1 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGAACTAGTAATAAGCCTAGAAATTT-3' and GT47FRNAiR1 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGAAGATCTACGTTTATGATGAGAAT-3'. The PCR product was cloned directly into *pDONR223* (Invitrogen) according to the manufacturer's instructions. A Gateway LR recombination reaction (Invitrogen) was performed according to the manufacturer's instructions to transfer the 400 base pairs of *AtGT47F* coding sequence to *pHELLSGATE12*, a gene-silencing vector used to create hairpin DNA for RNAi, resulting in *GT47FpHG12* (264). This construct was cloned in *A. tumefasciens* strain GV3101 as previously described (259). *A. thaliana* Col-0 plants were transformed via floral dip as previously described (261).

Quantitative RT-PCR

RNA was extracted from *N. benthamiana* tissues using the RNEasy plant mini kit (Qiagen) according to the manufacturer's instructions. After RNA extraction, cDNA was synthesized using Superscript-III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative RT-PCR was performed on a StepOne-Plus Real-Time PCR system (Applied BioSystems) using Syber-Select Real-Time PCR reagents (Invitrogen) according to the manufacturer's instruction. *NbGT47F* was probed using the primers At1g21480Q1F 5'-

TGCCTCGCTCCTCGTGGGGAA-3' and At1g21480Q1R 5'- AGAGGCATAGGAGGTCTC-3'. For quantitative real-time RT-PCR analysis of *NbGT47F* silencing, data were analyzed using *Elongation Factor 1α* as a reference gene (265-267). For *NbGT47F*, primers St575Q1_F 5'-GGAAAGGTGGGTCGCCTTCAGC-3' and St575Q1_R 5'- TCACCACGTGGAGCCAGGCA-3' were used in addition to *Elongation Factor 1α* primers NbEF1qF 5'-AGGGTCCAACCCTCCTTGAGGC-3' and NbEF1qR 5'-GCCCCTTTGGCTGGGTCGTC-3'.

Cell Wall Extraction & Isolation

The alcohol insoluble residue (AIR) was extracted from fresh plant tissue as described by (130) with modifications. Briefly, samples were boiled in 100% ethanol for 30 minutes before grinding in a ball mill. Samples were successively washed in 100% ethanol until no pigment remained. Pellets were then washed in 70% ethanol followed by 100% acetone and dried in a 50°C oven overnight. AIR extracts were then subjected to starch removal as described by (130) with modifications. AIR samples were subjected to a two-step enzymatic degradation with an initial ten minute 1U thermostable α-amylase (Megazyme, E-BLAAM) digestion at 85°C followed by a two hour 1U amyloglucosidase (Megazyme, E-AMGDF) and 1U pullulanase (Megazyme, E-PULBL) digestion at 50°C. Samples were washed twice with 70% ethanol after enzymatic digestion before drying the pellet via a solvent concentrator.

Pectin Enrichment & Rhamnogalacturonan I & II Isolation

RG-I was isolated as described by (268). Briefly, 5-10 mg of AIR was digested overnight with 3U of pectin methyl-esterase (Novoshape Pure PME, Novozymes, 232-807-0) and 20U of endo-polygalacturonanase M2 (Megazyme, E-PGALUSP) at 37 °C. The hydrolysate was filtered through a 0.2 μm centrifugal filter to produce the pectin-enriched fraction. From this, RG-I and RG-II were enriched using a 10 kDa Molecular Weight Cutoff spin filter (Amicon) with nanopure water. Pectic domains were separated by size-exclusion chromatography (SEC) in 50 mM ammonium formate (pH5.0) on a Superdex 200 10/300GL column (GE Healthcare BioSciences, P/N 17-5175-01) at a flow rate of 0.5 ml/min. Polysaccharide elution from the column was monitored with a Shodex RI-101 refractive index detector (Shodex, http://www.shodex.com). Fractions were collected manually and lyophilized. Estimates of the molecular weights of RG-I were determined using Dextran molecular weight standards (Sigma-Aldrich). The mass contribution of each monosaccharide was determined as previously described (116). The monosaccharide mass ratio was determined by dividing the product of the mol % and the molar mass of each monosaccharide by the sum of the products of the molar mass and mol % of each monosaccharide. The monosaccharide mass ratios were then multiplied by the estimated molecular weight of the RG-I fraction to get the mass contribution.

Rhamnogalacturonan I Digestion & Analysis

RG-I isolated from *NbGT47F*-VIGS, control *N. benthamiana*, and *A. thaliana* mucilage was digested using 10U of an *Aspergillus aculeatus* rhamnogalacturonan hydrolase A (Novozymes, BE-2001-00107) for one hour at 55°C and subsequently dried via a solvent concentrator. The oligosaccharides were analyzed via High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) as previously described (237), using a Thermo Scientific Dionex ICS-3000 system. Oligosaccharides were separated over a 50-500 mM sodium acetate gradient in 100 mM sodium hydroxide at a flow rate of 1 ml/minute

over 60 minutes using a Dionex CarboPac PA100 column (4 x 250 mm, 043055) to determine the digestion pattern. The oligosaccharides were also alternatively derivatized using the fluorescent probe 8-aminonapthalene-1,3,6-trisulfonic acid (ANTS) prior to analysis using polysaccharide analysis using carbohydrate gel electrophoresis (PACE) as previously described (222, 223).

Cell Wall Hydrolysis

The hemicellulosic and pectic matrix of 0.5-2 mg de-starched AIR samples, enriched pectin samples, and isolated RG-I and RG-II samples were hydrolyzed in 1 ml 2 M trifluoroacetic acid (TFA) at 120°C for one hour. TFA was removed via a solvent concentrator and the pellet was resuspended in 1 ml of nanopure water. Samples were then filtered through a 0.45 µm centrifugal filter and taken for analysis of released matrix monosaccharides.

Monosaccharide Composition Analysis

Monosaccharides from TFA-hydrolyzed samples were detected and quantified using High Performance Anion Exchange Chromatography with Pulsed-Amperometric Detection (HPAEC-PAD) as described by (132) with some modifications using a Thermo Scientific Dionex ICS-5000 system. Neutral sugars were separated over a 4 mM – 1 mM sodium hydroxide 0.4 ml/min gradient over 23 minutes before separating the uronic acids using 450 mM sodium hydroxide at 0.4 ml/min over 18 minutes using a Dionex CarboPac PA20 column (3 x 30 mm, 060144). Amounts were quantified using a range of monosaccharide standards (2.5-200 µM).

Immunoblotting

Immunoblotting of *N. benthamiana* cell wall extracts and pectin-enriched fractions was performed as previously described (115, 222). 1-5 mg of AIR from the leaves and stems of *NbGT47F*-VIGS and control plants with corresponding controls were extracted by ball milling in 4 M KOH with 0.1% $NaBH_4$ and subsequently neutralized with HCl. The samples were diluted and 1ml of each of the dilutions was spotted onto a nitrocellulose membrane. Alternatively, pectin-enriched fractions as described above were re-suspended in nanopore water (normalized to the dry weight of the whole cell wall extract) and spotted with corresponding controls onto a nitrocellulose membrane. Dry membranes were washed with phosphate-buffered saline (PBS), and blocked with PBS with 5% non-fat milk. Membranes were probed with a 1:300 dilution of the primary cell wall monoclonal antibody for 1.5 hours, followed by a 1:5,000 dilution of goat anti-rat IgG conjugated to horseradish peroxidase (Sigma-Aldrich, P/N A9037) for one hour, before applying ECL detection reagent (Clarity Western ECL Substrate, BioRad, P/N 1705060). Membranes were imaged using the Amersham Imager 600 (GE Healthcare).

Microscale Thermophoresis

2.5 µg of microsomal preparations from *N. benthamiana* leaves infiltrated with *35S::GT47F-YFP-HA* or a *p19* control were incubated in varying concentrations of UDP sugars (Table 2) in 40 mM MES with 10 µM $MnCl_2$ and 1% Triton X-100 at 30°C for one hour (255). Microscale thermophoresis was performed using the Monolith NT.115 system (Nanotemper) according to the manufacturer's instructions (269).

Statistics

Statistics of all chemical cell wall analyses were performed by performing the Student's t Test comparing the droughted treatment to its watered control for the specified week sampled. The resulting *p* values were corrected for multiple comparisons concerning False Discovery Rate (FDR) using the Benjamini-Hochberg correction (137) to determine the statistical significance of *p* values < 0.05. FDRs were assumed to be the conservative 0.25. Lines were compared within genotypes and within tissue types to see differences pertaining to genotypes and tissue types.

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Table 1. Number of mature and aborted embryos in *A. thaliana* heterozygous CS411800 plants.

Embryo Phenotype	Mature	Aborted
Silique 1	21	6
Silique 2	26	5
Silique 3	15	2
Silique 4	13	7
Silique 5	16	7
Silique 6	19	9
Silique 7	23	5
Silique 8	18	4
Silique 9	20	7
Total	171	52
Proportion	0.7668161435	0.2331838565

Table 2. A summary of the microscale thermophoresis experiments performed using *N. benthamiana* microsomes infiltrated with *35S::GT47F-YFP-HA* on the Monolith MT.115, including experimental parameters and results.

Experiment Name	GT47F + UDP-Xyl	GT47F + UDP-Glc	GT47F + UDP-Gal	p19 + UDP-Gal
Target	GT47F	GT47F	GT47F	p19
Target Concentration	20 nM	20 nM	20 nM	20 nM
Ligand	UDP-Xylose	UDP-Glucose	UDP-Galactose	UDP-Galactose
Ligand Concentration	$7.63e^{-5}$ mM – 2.5 mM	$7.63e^{-5}$ mM – 2.5 mM	$7.63e^{-5}$ mM – 1.25 mM	$7.63e^{-5}$ mM – 2.5 mM
n	3	3	3	3
Excitation Power	20%	20%	20%	20%
MST Power	40%	40%	40%	40%
Temperature	22°C	22°C	22°C	22°C
K_d	0.0044366	0.0070097	$1.6746e^{-5}$	0.00049687
K_d Confidence	± 0.0053908		± $2.8306e^{-5}$	
Response Amplitude	55.899819	64.336654	9.128078	358641.82
Standard Error of Regression	2.5405803	2.7191696	1.8555279	1.5763997
Signal to Noise	23.634773	26.453137	5.5498281	254360.46

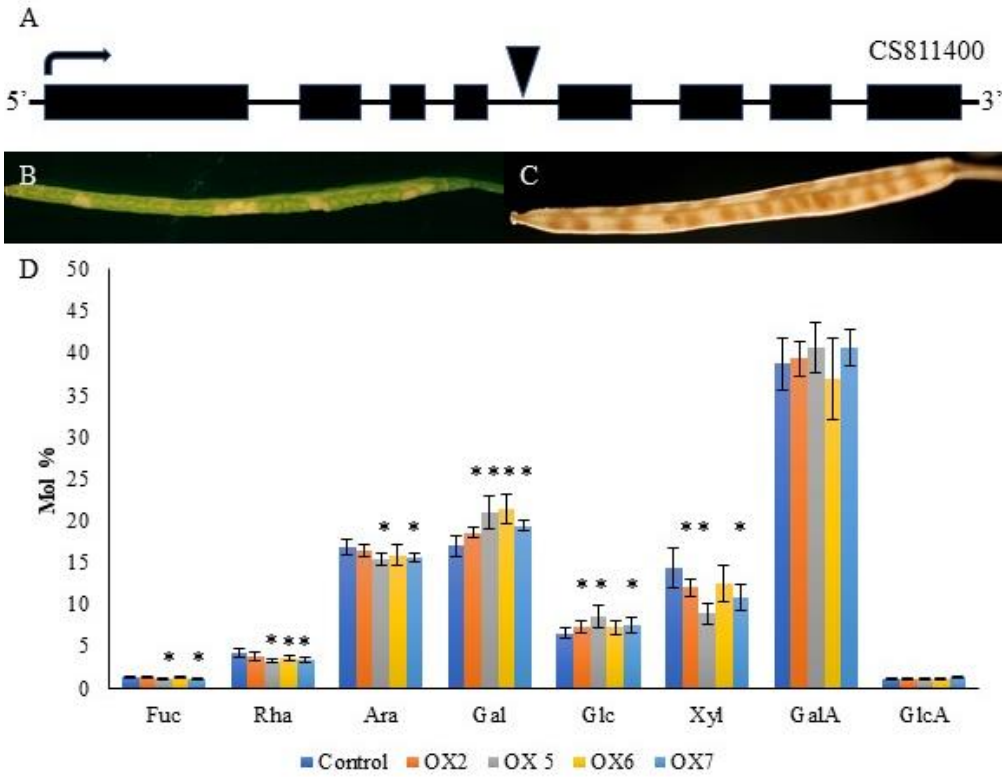

Figure 1. Analysis of *AtGT47F* insertional mutant and over-expressing lines. A. A diagram of the T-DNA insertion in *AtGT47F*(CS811400). Boxes represent exons, lines represent introns. The inverted triangle represents the T-DNA insertion. B. Live and C. dried siliques from heterozygous *AtGT47F* T-DNA mutants. D. Monosaccharides released from a trifluoroacetic acid (TFA) hydrolysis of the alcohol insoluble residue (AIR) from leaves of *35S::HA-GT47F* plants. Data represents mean ± SD of 4-8 biological replicates per genotype. Asterisks indicate $p < 0.05$ determined by Student's t-test with the Benjamini-Hochberg correction.

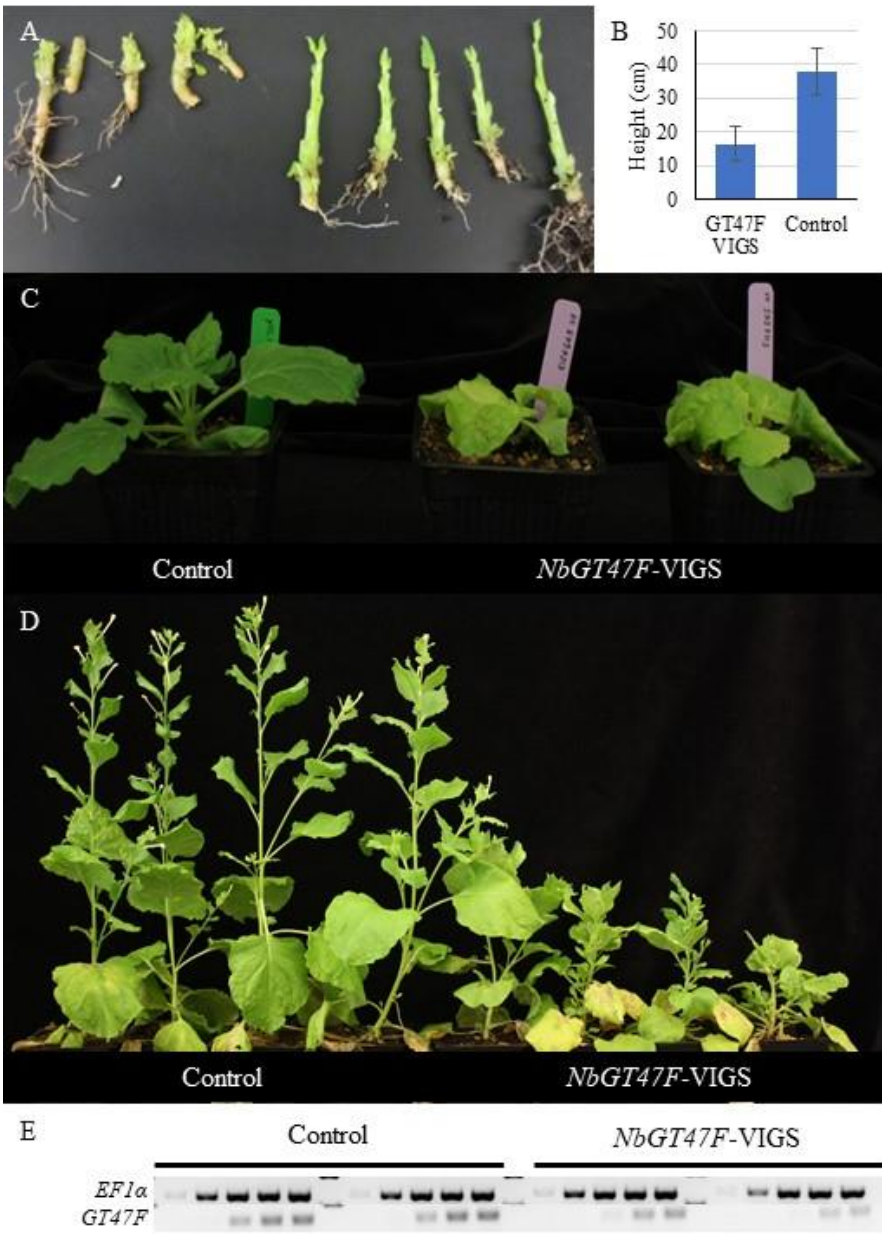

Figure 2. Developmental analysis of *NbGT47F*-VIGS plants. A. A comparison of internodes between *NbGT47F*-VIGS (left) and control (right) plants two weeks post-infection. Data represents mean ± SD of 16 biological replicates per genotype with $p < 0.05$ determined by Student's t-test. B. A comparison of the flowering height of shoots between *NbGT47F*-VIGS and control plants eight weeks post-infection. C. *NbGT47F*-VIGS and control plants two weeks post-infection. D. *NbGT47F*-VIGS and control plants eight weeks post-infection. E. *NbGT47F*-VIGS quantitative PCR analysis of leaves from *NbGT47F*-VIGS and control plants two weeks post-infection using two biological replicates per genotype. *Elongation Factor 1α* (*EF1α*) was used as a reference gene to determine the degree of silencing in VIGS plants.

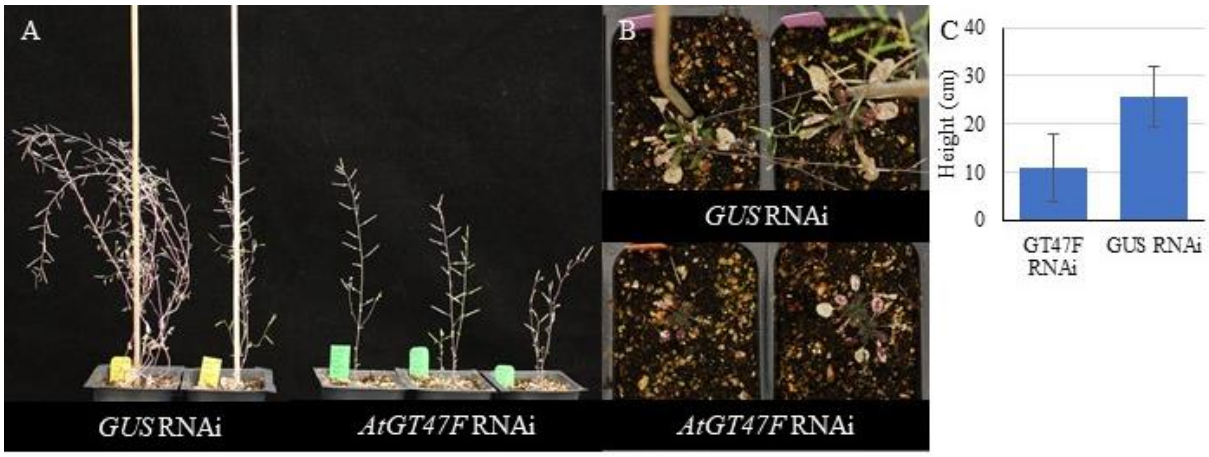

Figure 3. Developmental analysis of *AtGT47F* RNAi plants. A. A side-by-side comparison of mature *AtGT47F* RNAi and *GUS* RNAi plants. B. A comparison of rosette size between *AtGT47F* RNAi and *GUS* RNAi plants. C. A comparison of the main inflorescence heights of *AtGT47F* RNAi and *GUS* RNAi plants. Data represents mean ± SD of 14-26 biological replicates per genotype with $p < 0.05$ determined by Student's t-test.

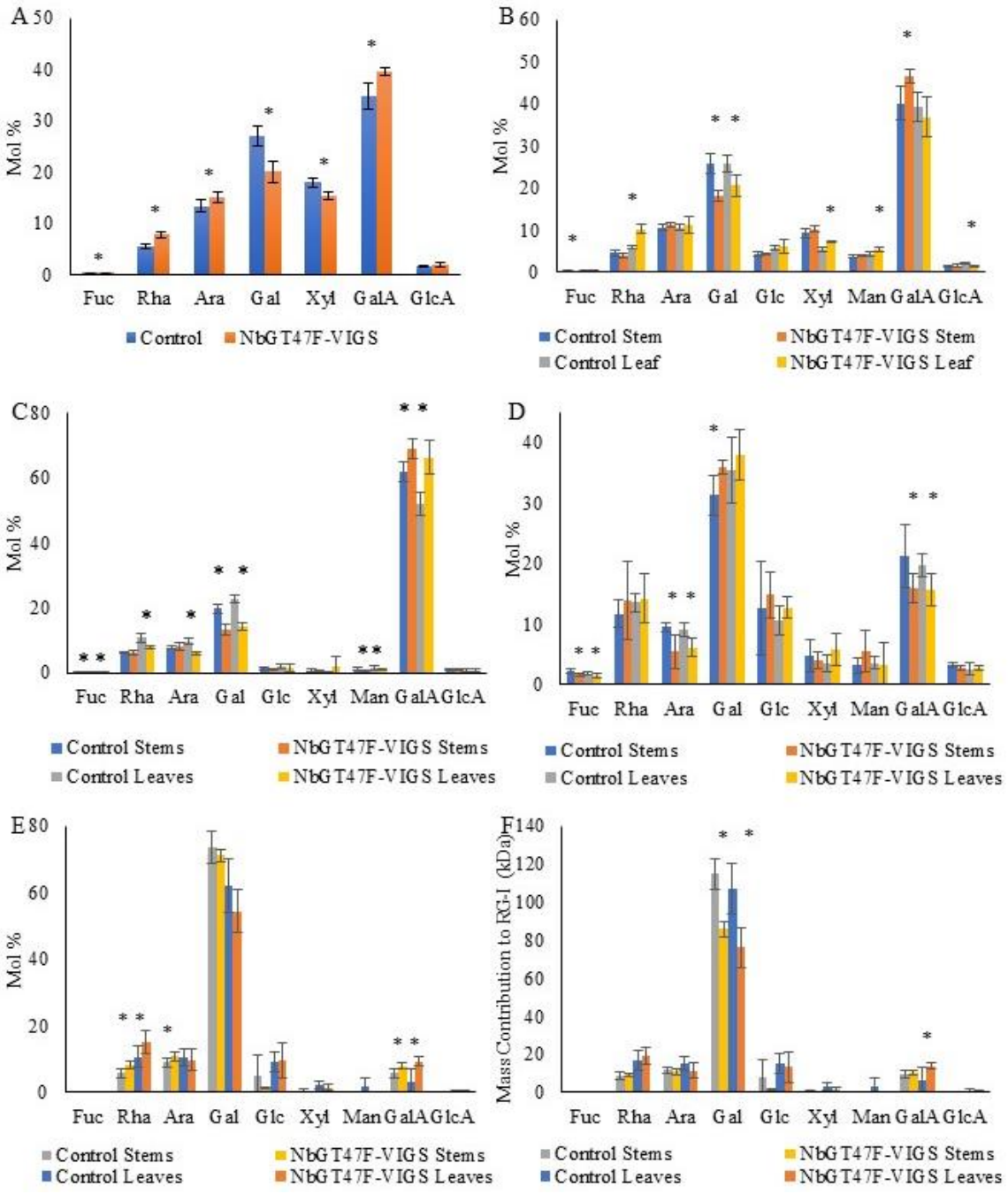

Figure 4. Cell wall analysis of *NbGT47F*-VIGS plants. Monosaccharides released from a trifluoroacetic acid (TFA) hydrolysis of cell wall extracts from *NbGT47F*-VIGS and control plants two weeks post-infection. Whole cell wall monosaccharide analysis of the alcohol insoluble residue (AIR) from whole shoots (A) and the third youngest leaf and first three internodes of the shoot apex (B). Monosaccharide analysis of pectin-enriched extracts (C), isolated RG-II (D), and isolated RG-I (E, F). Data represents mean ± SD of 4-5 biological replicates per genotype. Asterisks indicate $p < 0.05$ determined by Student's t-test with the Benjamini-Hochberg correction.

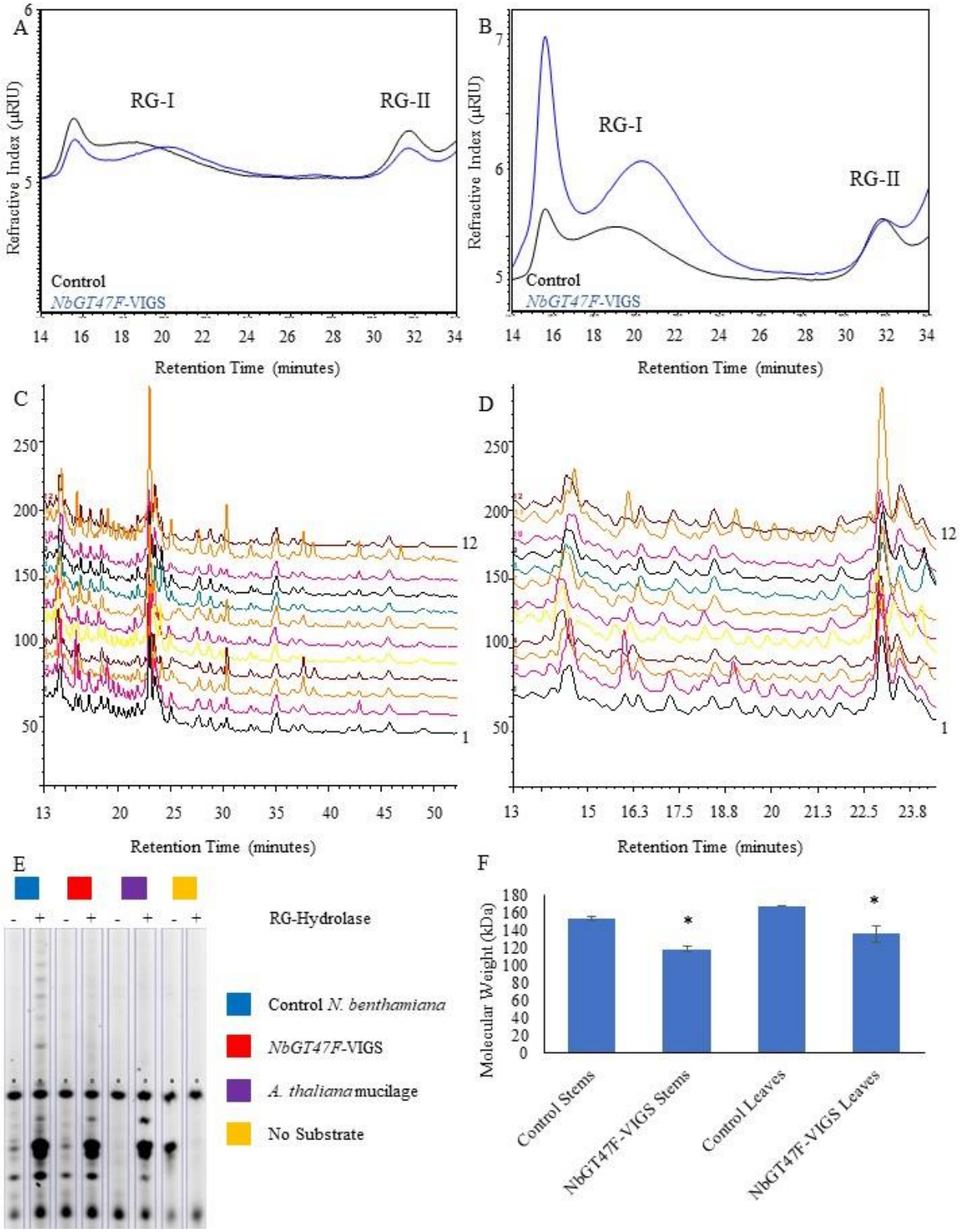

Figure 5. Analysis of RG-I structure in *NbGT47F*-VIGS plants. Chromatograms of *NbGT47F*-VIGS and control plant RG-I and RG-II isolated from stems (A) and leaves (B) of plants two weeks post-infection. Pectic domains were separated via size-exclusion chromatography (SEC)

and collected for further analysis. C. Oligosaccharides released from an RG-I digestion via a rhamnogalacturonan I hydrolase (RG-hydrolase) of *NbGT47F*-VIGS and control plants two weeks post-infection and analyzed via high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Samples 1-5 consist of control plant RG-I digests, samples 6-10 consist of *NbGT47F*-VIGS RG-I digests, sample 11 is an uninfected *N. benthamiana* RG-I digestion and sample 12 is an *A. thaliana* mucilage digestion. D. A closer look at the oligosaccharides with a retention time between 13-24 minutes using HPAEC-PAD analysis. Polysaccharide analysis using carbohydrate gel electrophoresis (PACE) analysis of oligosaccharides released from an RG-I digestion via RG-hydrolase of *NbGT47F*-VIGS and control plants two weeks post-infection. F. A comparison of RG-I molecular weights of leaves and stems from *NbGT47F*-VIGS and control plants two weeks post-infection. RG-I was isolated via SEC and the molecular weights were determined using Dextran molecular weight standards. Data represents mean ± SD of 6 biological replicates per genotype with asterisks indicating a $p < 0.05$ determined by Student's t-test for both leaves and stems.

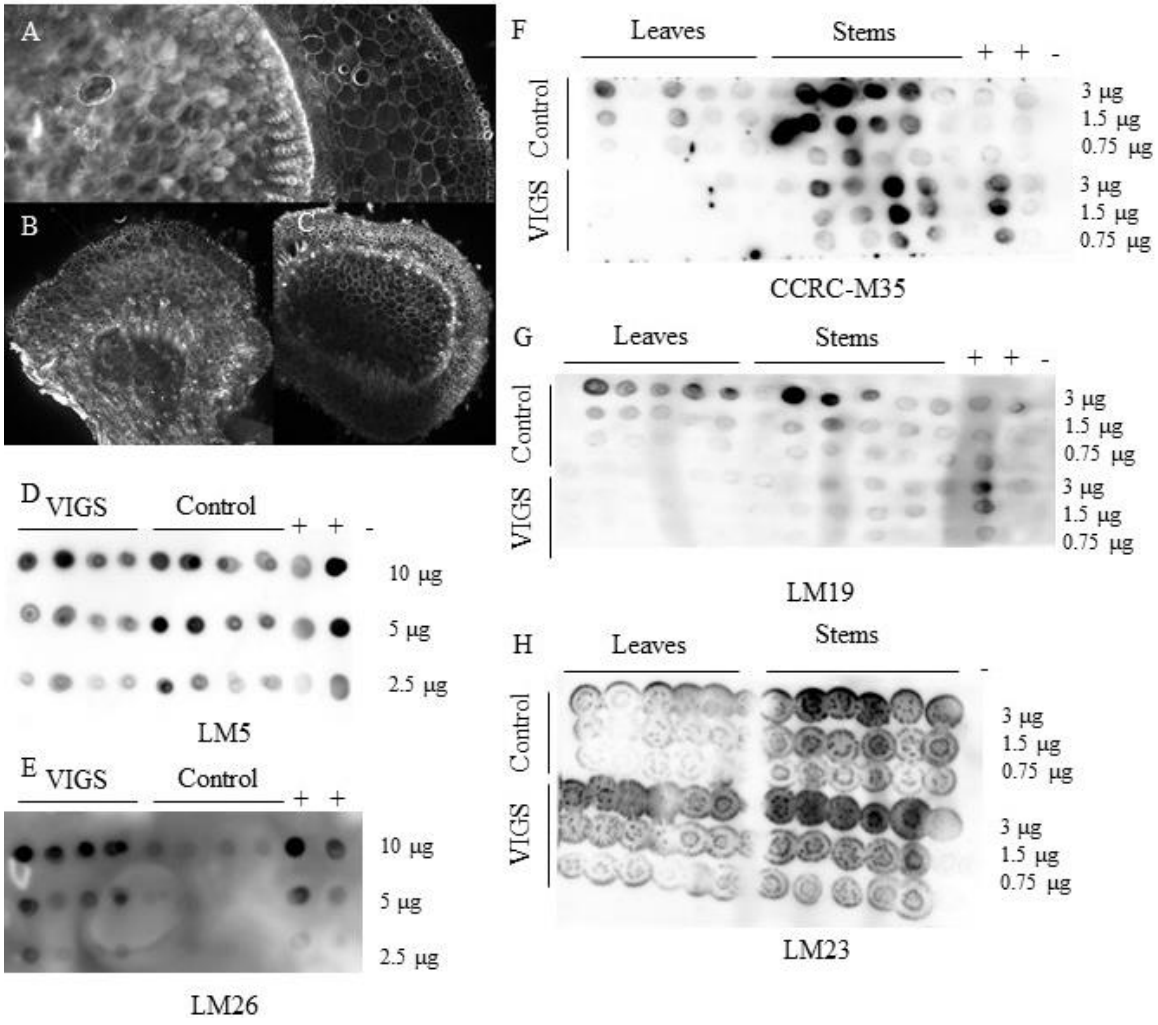

Figure 6. Immunolabeling analysis of *NbGT47F*-VIGS and control plants two weeks post-infection using monoclonal cell wall antibodies. 60 µm-thick sections of agarose-embedded *N. benthamiana* control plant internodes 5-6 (A), *NbGT47F*-VIGS plant internodes 1-3 (B), and control plant internodes 1-3 (C) starting from the shoot apex were probed with the LM5 antibody and a fluorescent secondary antibody. Immunoblot assays using pectin-enriched fractions of alcohol insoluble reside (AIR) isolated from the first three internodes of the shoot apices of *NbGT47F*-VIGS and control plants were probed using the LM5 (D) and LM26 (E) antibodies and a peroxidase-conjugated secondary antibody. Positive controls consist of citrus peel pectin and potato galactan while the negative control is water. Immunoblot assays using whole cell wall AIR isolated from both the third youngest leaves and the first three internodes of the shoot apices of *NbGT47F*-VIGS and control plants were probed using the CCRC-M35 (F), LM19 (G), and LM23 (H) antibodies and a peroxidase-conjugated secondary antibody. Positive controls consist of citrus peel pectin and beet pectin while the negative control is the KOH extraction buffer used to prepare the AIR.

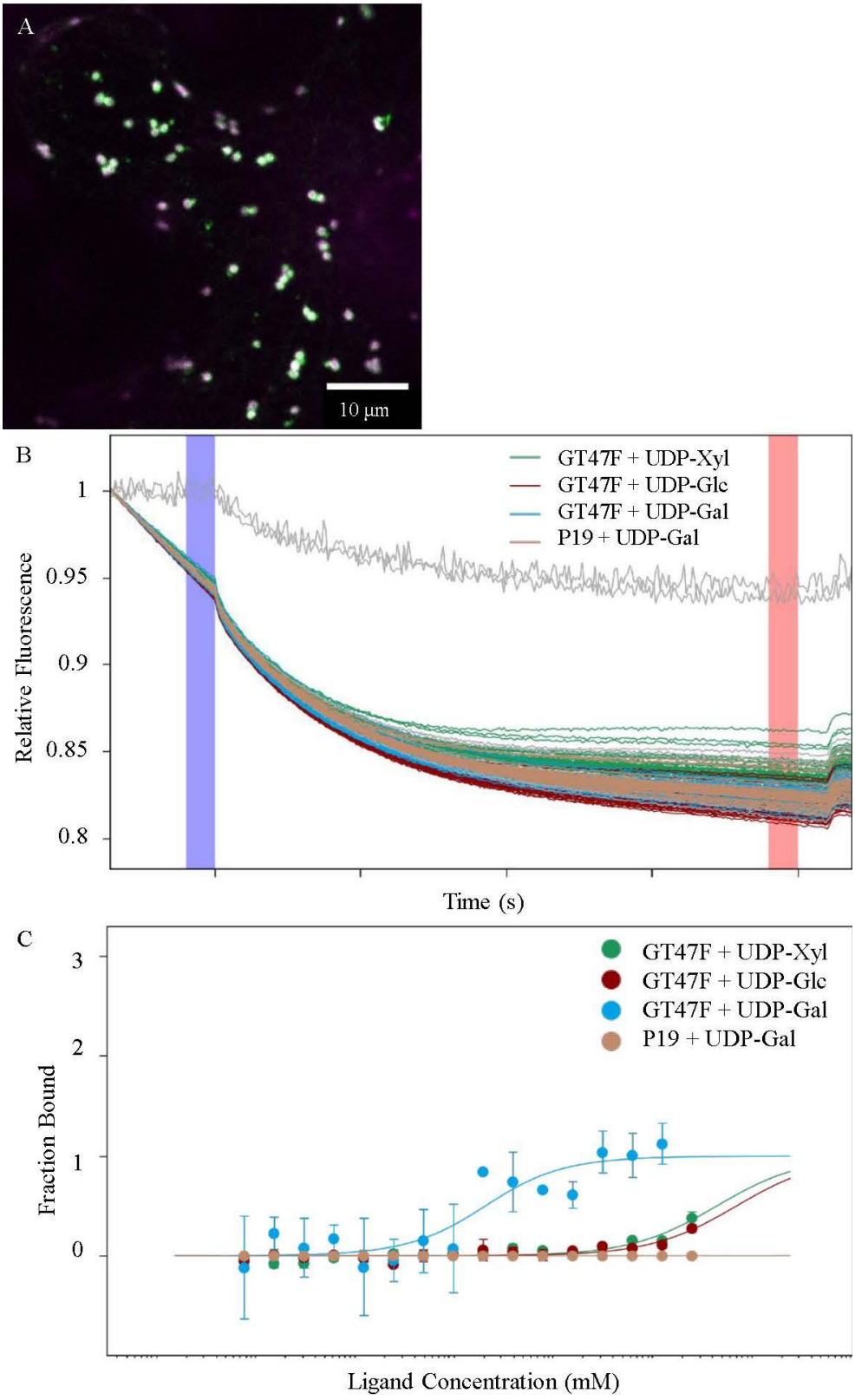

Figure 7. Analysis of GT47F localization and activity in *N. benthamiana*. A. *35S::GT47F-YFP-HA* was co-expressed with a CFP-tagged α-mannosidase in *N. benthamiana* leaves and epidermal

cells were imaged. Analysis of GT47F-YFP-HA interactions with UDP sugars using microscale thermophoresis (MST), with relative fluorescence measured over time (B) and ligand binding interactions determined (C). Data represents mean ± SD of 3 experimental replicates per UDP sugar.

New Directions for Cell Wall Dynamics and Biosynthesis

The plant cell wall is an integral component of the cell and plant, responding dynamically to developmental, environmental, and biotic cues for the benefit of the entire organism. The plant cell wall is a complex network of polysaccharides, proteins, and, in secondary cell walls, lignin, interconnected through hydrogen bonding and ionic and covalent linkages (2, 5, 270). Cell wall polysaccharide biosynthesis is performed through the actions of a suite of glycosyltransferases (GTs), methyl- and acetyltransferases, glycosyl hydrolases (GHs), esterases, and other modifying enzymes (8, 141, 162, 271). The research presented here demonstrates that the cell wall of *Sorghum bicolor* undergoes changes at the transcriptomic and compositional level in response to drought stress, although those compositional changes are relatively minor. Changes in *S. bicolor* cell wall composition and transcriptome in response to drought stress often implicated the polysaccharide pectin. Further, *Nicotiana benthamiana* and *Arabidopsis thaliana* GT47F mutants demonstrated defects in pectin biosynthesis and normal plant development, lending credence to the crucial role of pectin in mediating developmental programs. Although research, including that which is presented here, has demonstrated transcriptomic, proteomic, and even compositional changes in the plant cell wall in response to development and biotic and abiotic stresses (39-41, 140, 272), there remains much to be explored concerning the link between the plant cell wall and how the plant grows and responds to its surroundings. In particular, cell wall architecture, including the structure and arrangements of its component polysaccharides, and cell wall biosynthesis present challenges to a deeper understanding of how the plant cell wall mediates the development and environmental responses of the plant.

Historically, studying cell wall architectural dynamics has been incredibly difficult, with impediments including utilizing dead or harvested tissue, using bulk plant tissue, and using insufficient tools for analysis (12, 273). By using dead or harvested tissue, it is impossible to study dynamic changes as they happen. In using bulk plant tissue for analysis, specific local compositions and structures can be masked. Additionally, using tools such as molecular probes with poorly characterized epitopes on laboriously prepared sections of harvested material presents a temporal and interpretable bottleneck for cell wall structural characterization (12, 273). Recent developments in cell wall visualization techniques offer potential solutions to these impediments. Click-chemistry, a technique in which azido or alkynyl sugar analogs are incorporated into the cell wall and conjugated using copper with a fluorophore to study live polysaccharide deposition, has shown the dynamic incorporation of fucosylated pectin in *A. thaliana* roots, giving temporal and spatial resolution at both the tissue and cellular levels (274, 275). Cellular resolution of polysaccharide architecture is still currently resolved via molecular probes. Though many of these probes, consisting mainly of cell wall monoclonal antibodies and carbohydrate binding modules (CBMs), do not have well-characterized epitopes, recent work has sought to change this, including the synthesis of oligosaccharides and carbohydrate arrays to characterize the epitopes of cell wall monoclonal antibodies (276-280).

Not being able to fully characterize the diversity of cell wall structures and organization in turn leads to a difficulty in elucidating cell wall biosynthetic pathways. Due to the complexity of many polysaccharides, particularly pectin, a heteropolymeric structure composed of more than 65 unique linkages (12, 144, 162), it can be difficult to determine GT function for more subtle and rare architectures using traditional means such as compositional analyses. Whilst immunolabeling of sectioned tissues provides resolution at the cellular level that can aid in

discovering GT function, this procedure is not high through-put. An alternative is to screen a wide array of probes using extracted cell walls, as in glycome profiling (244), before a more targeted visualization of sectioned tissues. Additionally, the complexity of plant cell wall polysaccharides can prevent the generation of adequate oligosaccharide acceptors for confirming enzymatic activity of GTs (12, 162). Recently developed techniques, such as microscale thermophoresis (MST) and the previously mentioned carbohydrate acceptor arrays, allow for the elucidation of UDP sugar donors and oligosaccharide acceptors to assay GT enzymatic activity (235, 269, 277-283). Coupled with conventional cell wall analyses, new cell wall visualization techniques have the potential to provide a deeper level of understanding of cell wall dynamics and biosynthesis during plant development and mediation of biotic and abiotic stresses.

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Appendix 1. Alignment of *Arabidopsis thaliana* and *Nicotiana benthamiana* GT47F predicted amino acid sequences using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), where asterisks denote conserved residues, colons denote conservation of strongly similar properties (charge, hydrophobicity, etc.), and periods denote conservation of weakly similar properties (size, general spatial configuration, etc.).

```
AtGT47F MASLTSNKPRNFGAYSHYATPCTRTHQIGALFLVVSTFFVTRLFDQWFSESNSVTPVIDL 60
NbGT47F ------------------------------------------------------------ 0


AtGT47F RRTSSSYGIKTDNGIIRWPERGYGSHLSLKIYVYDENEIDGLKELLYGRDGSVKTTACLK 120
NbGT47F ------------------------------------------------------------ 0


AtGT47F GQWGSQVKIHKLLLESKFRTIKKDEADLFFVPAYVKCVRMLGGLNDKEINQTYVKVLSQM 180
NbGT47F -----------MLLQSRLRTRKKEEADLFFVPAYPKCVRVMGGLNDKEINQTYVQVLSQM 49
                  :**:*::** **:********** ****::**************:*****

AtGT47F PYFRRSGGRDHIFVFPSGAGAHLFRSWSTFINRSIILTPEADRTDKKDTTAFNSWKDIII 240
NbGT47F PYFRLSGGRNHIFVFPSGAGAHLFKSWVTYLNRSIILTPEGDRTDKRDTSAFNTWKDIII 109
        **** ****:***************:** *::**********.*****:**:***:******

AtGT47F PGNVDDAMTKNGQPDVQPLPLSKRKYLANYLGRAQGKAGRLKLIDLSKQFPDKLECPDLK 300
NbGT47F PGNIDDGMTTHGARLVEPLPLSKRKHLANYLGRAQGKVGRLQLIDLAKQFPDKLECPKLK 169
        ***:**.**.:*   *:********:***********.***:****:**********.**

AtGT47F FSGTEKFGRTTYFEHLRNAKFCLAPRGESSWTLRFYESFFVECVPVLLSDHAELPFQNVI 360
NbGT47F FSGPDKLGRREYFEHLRNAKFCLAPRGESSWTLRFYESFFVECVPVILSDQAELPFQNVI 229
        *** :*:**  **********************************:***:*********

AtGT47F DYAQVSIKWPSTRIGSEFLDYLASISDRDIEGMIARGRKIRCLFVYGPDSAPCSAVKGIL 420
NbGT47F DYTQISIKWPSTHIGTALLDYLESIPDKDIDEMIARGRKIRCLFAYTPESDSCSAFNAIM 289
        **:*:*******:**: :**** ** *:**: ************.* *:*  ***.:.*:

AtGT47F WELQRKVRHFQQSTETFWLHNGSFVNRELVQFSSWRPPMPLP    462
NbGT47F WELQRKVRQFHQSSETFWLHNRTIVNRDLVEFSKWKPPMPLP    331
        ********:*:**:******* ::***:**:**.*:******
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