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Chapter 5
Cell Wall Polysaccharide Synthesis

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5.1 Introduction

One of the many challenges associated with trying to understand the synthesis, structure, and function of higher plant cell walls is the obvious chemical diversity between tissue types within a plant and between plant species. For instance, an analysis of the cell wall sugar composition of different tissues in Arabidopsis revealed that each of the tissue types analyzed had a strikingly different composition (1). In spite of this diversity, there is substantial evidence in support of the hypothesis that most cells of all higher plants have the same six major types of polysaccharides: cellulose, xyloglucan, xylan, homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (2). According to this idea, variation in wall composition arises from variation in the amounts of the various polysaccharides and in variations of the structures of the various polysaccharides. Thus, in order to understand how cell walls are synthesized, much of the task may be condensed into understanding how these classes of polysaccharides are synthesized and deposited in walls. Additionally, several other polysaccharides are found in some species. These include xylogalacturonan, mannan, and mixed-linkage glucans. Lignin associated with secondary cell walls represents the other major component. Although cell wall proteoglycans are a quantitatively minor component, they may be relevant for understanding certain aspects of assembly. Similarly, callose plays an important role in synthesis of de novo cell walls or in specialized cell walls such as pollen tubes but is not usually a quantitatively significant component of most types of cell walls.

Plant walls are divided into two basic types, the primary and secondary wall (Figure 5.1). The primary wall is the first wall laid down in dividing and growing plant cells and it is the terminal wall in many cells in the soft parts of the plant, including the palisade cells in leaves and parenchyma cells present throughout the plant. The primary wall contains 80–90% polysaccharide and 10–20% protein. Cellulose, hemicellulose, and pectin are the main polysaccharide components in the primary wall. The most abundant hemicellulose in most primary walls is xyloglucan. However, in grasses and other commelinoid monocots glucuronoarabinoxylan is the major hemicellulose and during cell expansion in grasses, β-1,3;β1,4-mixed linkage glucans are prevalent in the primary wall. Secondary walls are produced by specialized cells that serve a structural role such as fibers and xylem cells in vascular bundles. Secondary walls generally have less pectin, contain more cellulose and more of the hemicellulose β-1,4-xylan (often as glucuronoxylan), and are often rigidified

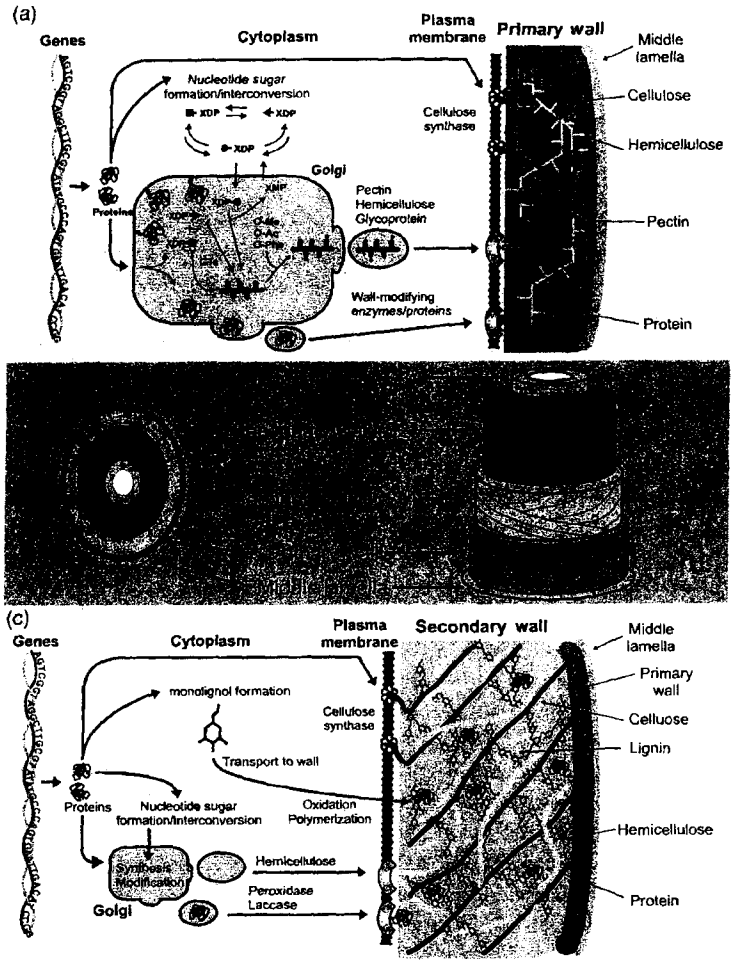


Figure 5.1 Model of plant primary and secondary walls and their synthesis. (a) Primary wall polysaccharides are synthesized at the plasma membrane (cellulose) and in the Golgi (pectin and hemicellulose) by the action of glycosyltransferases that use nucleotide-sugar substrates. (b) Some cells (e.g., xylem and fiber cells) form secondary walls internal to the primary wall. Secondary walls have increased amounts of cellulose and hemicellulose, less pectin, and are often rigidified with lignin. Secondary walls in wood tissue consist of three layers (S1, S2, and S3) that differ in cellulose microfibril orientation and chemical composition. (c) Secondary wall synthesis includes formation of cellulose microfibrils at the plasma membrane, hemicellulose within the Golgi followed by deposition in the wall, and lignin polymerization from monolignols within the wall matrix. (Figure with permission by Malcolm O'Neill, CCRC, University of Georgia)

by lignification. The synthesis of both the primary and secondary walls utilizes nucleotide-sugar substrates that are synthesized in the cytosol or in the lumen of the endoplasmic reticulum and the Golgi.

In the following overview, we have summarized the current status of knowledge about how the major classes of cell wall polymers are synthesized, modified, secreted, and assembled.

Because of the importance of Arabidopsis as an experimental model, much of the recent progress has been obtained using Arabidopsis. However, information from other species is included where it is available. The following description of cellulose synthesis is an update of a recent review (3).

5.2 Cellulose

Cellulose microfibrils are insoluble cable-like structures that are typically composed of about 36 hydrogen-bonded glucan chains each of which contains between 500 and 14 000 β-1,4-linked glucose molecules. Cellulose microfibrils comprise the core component of the cell walls that surround each cell. Studies from mutants deficient in secondary cell wall cellulose show very irregular deposition of non-cellulosic polysaccharides and lignin (4). Thus, it is apparent that cellulose is a central scaffold of cell walls.

The cellulose chains in microfibrils are parallel, and successive glucose residues are rotated 180°, forming a flat ribbon in which cellobiose is the repeating unit. The parallel chains are compatible with evidence that the chains in a microfibril are made simultaneously (3). The cellulose chains are held in a crystalline structure by hydrogen bonds and Van der Waals forces to form microfibrils. It is not yet known to what extent the "crystallization" of the nascent glucan chains into cellulose microfibrils is facilitated by proteins other than the catalytic enzyme. Jarvis (5) has shown that the two main forms of cellulose (i.e., cellulose Iα and Iβ) can be interconverted by bending. He suggested that the sharp bend that is thought to take place when cellulose emerges from the rosette and becomes appressed to the overlying cell wall may be sufficient to induce the interconversion. Additional forms, which are primarily of interest in the context of industrial uses of cellulose, can be produced from natural cellulose by extractive treatments. For instance, in cellulose II, the chains are antiparallel – something that is unlikely to occur in native cellulose. Cellulose I is converted to cellulose II by extraction under strongly alkaline conditions.

The molecular weight of the individual glucan chains that comprise cellulose microfibrils has been difficult to determine because the extraction may lead to degradation. Analyses of secondary wall cellulose in cotton suggest a degree of polymerization (DP) of 14 000–15 000 (6). Primary wall cellulose appears to have lower molecular weight. Brown (7) reports a DP of 8000 for primary wall cellulose. However, Brett (6) reported a low molecular weight fraction of ~500 DP and a fraction with a DP of 2000–4000. Brett (6) suggested that the low molecular weight fraction may be chains at the surface of microfibrils whereas the high DP fraction may be chains in the microfibril interior. Since a DP of 2000 corresponds to about 1 μm of length, the implication is that the primary wall cellulose fibrils, which are frequently observed to be much longer than 1 μm, must be composed of chains with breaks at various locations along the fibrils. As noted below, this is compatible with genetic evidence that a cellulase is required for cellulose synthesis in both plants and bacteria (8, 9). Whatever the exact length, it is apparent that in some cells the fibrils can be extremely long relative to other types of biological macromolecules.

Based on electron micrographs, the width of cellulose fibrils varies from about 25 to 30 nm in Valonia and other green algae, to about 5–10 nm in most plants (10). The variation in size may indicate that cellulose microfibrils from different sources contain different numbers of chains, and it may reflect variation in the kind or amount of hemicellulose coating on the fibrils. In a study of onion primary wall by solid state NMR (10), the spectral interpretation

was consistent with the idea that the 8 nm wide microfibrils were composed of six 2-nm fibrils, each containing about 10 chains. Herth (11) estimated by electron microscopy that the microfibrils of Spirogyra contained 36 glucan chains. Thus, the measurements are generally consistent with the idea that each of the six globules in a rosette is composed of a number of subunits that synthesize 6–10 chains that hydrogen bond to form the 2 nm fibrils. Six of these 2 nm fibrils then bond to form the microfibrils.

The analyses of cellulose structure indicate that cellulose synthase is a highly processive enzyme, that it has many active sites that coordinately catalyze glucan polymerization, that alternating glucan units are flipped 180°, and that interspecies variation exists in the number of glucan chains per fibril, or possibly in the kind or amount of hemicellulose. What is not clear is whether the enzyme participates in facilitating the hydrogen bonding of the glucan chains or whether proximity of the glucan chains as they emerge from the enzyme is sufficient to cause formation of the highly ordered microfibrils.

Cellulose synthase can be visualized by freeze fracture of plasma membranes in vascular plants as symmetrical rosettes of six globular complexes approximately 25–30 nm in diameter. The rosettes have been shown to be cellulose synthase by immunological methods (12). The only known components of cellulose synthase in higher plants are the CESA proteins. The completion of the Arabidopsis genome sequence revealed that Arabidopsis has ten *CESA* genes that encode proteins with 64% average sequence identity (13, 14) and other species have been found to have similar numbers of CESA proteins (3). The proteins range from 985 to 1088 amino acids in length and have eight putative transmembrane (TM) domains. Two of the TM domains are near the amino terminus and the other six are clustered near the carboxyl terminus. The N-terminal region of each protein has a cysteine-rich domain with a motif that is a good fit to the consensus for a RING type zinc-finger. RING fingers have been implicated in mediating a wide variety of protein–protein interactions in complexes (15). Otherwise, the N-terminal domain is structurally heterogenous among the ten CESAs in Arabidopsis. The average overall sequence identity of the amino terminal domains is 40% compared with an average overall identity of 64%.

A large "central domain" of approximately 530 amino acids lies between the two regions of transmembrane domains and is thought to be cytoplasmic. Using this feature to anchor the topology of the protein indicates that the N-terminal domain is also cytoplasmic. The central domain is highly conserved among all the CESA proteins except for an approximately 64–91 residue region of unknown significance where there is weak sequence identity. The domain contains a motif (Q/RXXRW) that is associated with bacterial cellulose synthases and other processive glycosyltransferases (16), such as chitin and hyaluronan synthases, and glucosylceramide synthase (17). Additionally, a DXXD motif and two other aspartate residues have been associated with this class of enzymes and are referred to collectively as the D,D,D,Q/RXXRW motif. Site-directed mutagenesis experiments of the chitin synthase 2 of yeast showed that the conserved aspartic acid residues and the conserved residues in the QXXRW motif are required for chitin synthase activity (18). Similarly, Saxena *et al.* (19) replaced the aspartate residues in the *A. xylinum* cellulose synthase and found that they were required for catalytic activity.

Analysis of mutants with defects in secondary wall cellulose has revealed that three separate CESA proteins are required in the same cell at the same time (20) and that the proteins physically interact (21). Thus, it appears that within a cell type there is a single type of complex containing three types of CESA subunits. A detailed summary of the properties of mutations that alter cellulose accumulation has been published recently (3). In brief, null

mutations in several of the primary wall CESA proteins are lethals, whereas others are not, presumably because of redundancy. Mutations that eliminate secondary cell wall cellulose are not lethal but impair the structural integrity of vascular cells. In addition to mutations in CESA genes, a number of other proteins have been implicated in the overall process but the role of these proteins is not understood.

5.2.1 Enzymology

Attempts to measure cellulose synthase activity in vitro have been problematic (3). Steady progress has been made in defining the conditions for assay and solubilization of cellulose synthase activity, although rates are still rather low (22–24). A recent study, that was carried out with large volumes of detergent-solubilized membranes from suspension cultures of *Rubus fruticosus* that facilitated structural analysis of the products (24), provided compelling evidence for synthesis of high molecular weight crystalline cellulose from UDP-glucose in vitro. The cellulose was visualized by electron microscopy and the properties characterized by linkage analysis and X-ray diffraction, leaving no doubt as to the identity of the in vitro product. The recent recovery of high levels of cellulose synthase activity from detergent extracts of membrane preparations from stationary phase suspension cultures of hybrid aspen (25) offers a new system for biomolecular studies of cellulose synthase activity.

Kudlicka and Brown (23) examined cellulose synthesized in vitro by electron microscopy and observed globular particles that have the same appearance as rosettes attached to the ends of the cellulose microfibrils. Similar structures were observed by Lai-Kee-Him and coworkers (24) who localized them at the non-reducing ends of the nascent cellulose fibrils. This result is in keeping with the work of Koyama and coworkers (26), where the addition of glucose units on the cellulose microfibrils from *Acetobacter aceti* was observed at the non-reducing ends of the growing ribbons. The question of the direction of chain growth remains controversial, however, since cellulose chains from *A. xylinum* were described by Han and Robyt (27) to elongate from the reducing ends. In view of evidence that β-chitin, starch, and glycogen, are polymerized from their non-reducing ends (28), and in view of the evidence from Lai-Kee and coworkers (24) it seems likely that polymerization of cellulose occurs from the non-reducing ends.

The issue of a metal requirement for catalytic activity has not yet been completely resolved by the in vitro studies. The addition of Mg^{++} was necessary for maximal rates of cellulose synthesis from *R. fruticosus* extracts that were solubilized with the detergent Brij 58, but inhibited activity of extracts solubilized with taurocholate (24). Activity in the absence of a divalent metal would distinguish cellulose synthase from the SGC domain proteins in which a divalent metal must bind anew at each catalytic cycle to form the nucleotide sugar-binding domain (29). Because the metal is transiently bound in SGC domain proteins, trace amounts in the assay would not be expected to support significant rates of activity. Thus, we infer that cellulose synthase is not a member of the SGC domain proteins.

The mechanism of cellulose synthesis is poorly understood. One of the persistent issues about mechanism concerns the fact that adjacent sugar residues have opposed orientations. It has been proposed that cellulose synthase has two active sites, one for each orientation

in order to facilitate the simultaneous polymerization and extrusion of the linear polymer (26, 30). The same situation applies to the processive glycosyltransferases that make chitin, hyaluronan, and heparin. Recently the first test of the two site model was reported for chitin synthase (31). These authors reasoned that if there are two UDP-GlcNAc-binding sites in close proximity, then dimeric nucleoside inhibitors should be more potent inhibitors of catalysis than the corresponding monomers. Potential bivalent inhibitors were synthesized by linking together 5′deoxy-5′-aminouridine residues connected by ethylene glycol linkers of various lengths. Certain dimers were an order of magnitude more potent than monomeric derivatives, supporting the idea of a two-site mechanism. Conversely, UDP-chitobiose was not a substrate for chin synthesis, mediating against the idea that an accessory protein might first condense two molecules of UDP-GlcNAc as a substrate for the synthase (32). Although these results suggest a two-site model, the CESA proteins contain only one QXXRW motif, suggesting that if two sites exist, they have distinct structural features (19).

A potentially important observation was made by Peng and coworkers (33) following inhibition of cellulose synthesis in cotton with the inhibitor CGA 325′615. They treated the resulting cell walls with cellulase with the intention of releasing cellulose synthase from nascent cellulose microfibrils. They observed that a tryptic peptide corresponding to residues 388–413 of Arabidopsis CESA1 was modified by mass amounts equivalent to the addition of 2–6 glucose residues. This seems to imply that a covalent attachment of glucan to the protein is involved in cellulose synthesis. Retaining glycosyltransferases may contain a transient covalent linkage between an Asp of the enzyme and the reducing end of the growing glycan chain (29). However, CESA proteins are considered to be members of family 2 glycosyltransferases and are proposed to function as inverting enzymes (34), which do not have such a predicted intermediate. Peng and coworkers (33) suggest that CGA 325′615 may have caused some abnormal linkage to be created.

There has been persistent interest in the concept that cellulose synthesis is initiated from a primer. Studies of the matter using bacterial synthase are controversial (9). Delmer and colleagues have suggested that sterol glucoside is a primer for cellulose synthesis (35). One line of evidence is that expression of cotton CESA1 in yeast caused formation of sterol cellodextrin from exogenously supplied sterol glucoside. Although this is intriguing, the ability to modify sterol glucoside at extremely low rates under highly artificial conditions does not mean that a primer is involved in vivo; many enzymes are assayed with artificial substrates that bear limited structural similarity to in vivo substrates. A second line of evidence is that treatment of cotton fibers with DCB reduces incorporation of radioactive glucose into sterol glucoside. Since the mode of action of DCB is not known, it could be that DCB acts by inhibiting formation of UDP-glucose or through some other indirect effect. Indeed, evidence was presented that exogenous addition of sterol glucoside could overcome the effects of DCB on in vivo cellulose synthesis in cotton fibers. Although the results are interesting, the demonstrations of in vitro cellulose synthesis did not require addition of any primer. The hypothesis should be tested by analysis of mutants of Arabidopsis with defects in the synthesis of sterol glucosides, as suggested by Peng and coworkers (35). Unfortunately, analysis of an Arabidopsis mutant with TDNA insertions in genes for the two known sterol glycosyltransferases indicated only a 40-fold reduction in sterol glucosides (Scheible and coworkers, unpublished results), which renders the absence of any apparent effect on cellulose synthesis ambiguous.

Ihara and coworkers (36) expressed the central domain of GhCESA2 in *Pichia pastoris* and found that it was soluble. It catalyzed incorporation of glucose into a product in the presence of an extract from cotton ovules but the product was not β-1,4-glucan.

5.2.2 Cellulose deposition

A distinguishing feature of plant cells is the presence of cortical microtubules adjacent to the plasma membrane (37). It has been noted since the discovery of cortical microtubules that the orientation of cortical microtubules in expanding cells is similar to that of cellulose microfibrils (38). This led to the hypothesis that the deposition of cellulose is oriented by an interaction between cellulose synthase and the microtubules, an idea that was reinforced by many observations of correlations between microtubule and microfibril organization which have been comprehensively and critically reviewed by Baskin (39). In the model of Giddings and Staehelin (40), as recast in an influential textbook (41), the movement of cellulose synthase is constrained by a close association between cortical microtubules and the plasma membrane, much like a bumper car bouncing along between rails of cortical tubulin. It is generally assumed that the energy of polymerization provides the motive force that moves the cellulose synthase complex through the membrane.

However, as noted in a recent critique of the model, there is no direct evidence for involvement of microtubules in microfibril orientation and many inconsistencies mediate against the idea (42). For instance, short treatment of Arabidopsis with the microtubule destabilizing drug oryzalin or the microtubule stabilizing drug taxol caused no apparent change to the orientation of cellulose microfibrils in cells that expanded during the treatment, as visualized by field emission scanning electron microscopy (43, 44). Long treatments caused changes in cellulose orientation but these may have been due to effects on the orientation of cell division. Similarly, when microtubule polymerization was impaired by shifting the temperature-sensitive *mor1-1* mutant to non-permissive temperature, cellulose microfibrils exhibited a similar pattern of deposition as in controls (45, 46).

Recently, Paredez and coworkers (47) produced a functional N-terminal YFP fusion to CESA6 that complemented the corresponding mutant in Arabidopsis. When expressed under the native promoter, a substantial amount of the fusion protein accumulates in the Golgi apparatus where it assembles into distinct particles that can be seen to move to the plasma membrane. This is compatible with previous evidence from electron microscopy indicating that cellulose synthase rosettes assemble in the Golgi (48). Within less than a minute of arriving in the plasma membrane, the cellulose synthase particles begin moving in linear paths at a constant rate of about 300 nm min^{-1}, somewhat slower than the rate observed by Hirai and coworkers (49) on tobacco membrane sheets. This is reminiscent of yeast chitin synthase III, in which activity is regulated by a specialized mechanism of vesicle sorting coupled with endocytic recycling (50). In this model, chitin synthase is maintained inside specialized vesicles called chitosomes (TGN/early endosome vesicles) and is transported to the specific sites of function where it becomes activated. Inactivation occurs via endocytosis. Because plant Golgi do not synthesize cellulose, it is apparent that the cellulose synthase complexes observed there are not active but that they become activated upon arrival at the plasma membrane. Rosettes have also been estimated to have only a 20 minutes lifetime in moss (51), which may suggest that they are also dissociated or endocytosed.

When viewed in cells in which the microtubules are labeled with CFP, the YFP-labeled cellulose synthase particles can be seen to move along the microtubules. Importantly, inhibition of tubulin polymerization with oryzalin rapidly leads to strong disruptions of the normal patterns of movement of the cellulose synthase particles that aggregate in patterns resembling meandering streams. Similarly, treatment of seedlings with Morlin, a novel inhibitor of microtubule treadmilling and membrane attachment, caused stalling of the cellulose synthase complexes (52). Thus, from live cell imaging it is readily apparent that microtubules exert a strong effect on the orientation of cellulose synthase movement (which presumably reflects cellulose synthesis) (47). However, Paredez and coworkers (47) observed that after relatively long periods of oryzalin treatment, when most or all of the cortical microtubules have depolymerized, the cellulose synthase particles resume movement in relatively straight parallel paths. The rigidity of cellulose probably explains why no guidance is necessary to ensure that cellulose synthase moves in relatively straight lines. It is not clear what orients the pattern of deposition in these cells but models for the formation of oriented patterns of cellulose based on geometric considerations have been proposed (53) and may be testable in these experimental materials. These observations suggest that both sides of the microtubule–microfibril alignment debate are correct and that the discrepancies and inconsistencies between experiments reflect the limitations of using static imaging methods and different treatment times and conditions. The availability of the new imaging tools outlined here should facilitate a resolution of the matter.

Alignment of GFP-labeled cellulose synthase with microtubules was previously reported by Gardiner and coworkers (54), who used an N-terminal fusion of GFP to the xylem-specific CESA7 (irx3) protein. Because of difficulties viewing the vascular tissues by confocal microscopy, the images of this GFP:CESA7 construct are difficult to discern. However, it appears that the distribution of fluorescence is not uniform and there are bands of fluorescence that are perpendicular to the long axis of the cells. Attempts to colocalize tubulin with CESA7 using immunofluorescence methods (54) indicate a similar pattern. However, the resolution of the images was not high enough to provide a critical analysis. Treatment with the microtubule assembly inhibitor, oryzalin, rapidly reduced the banding pattern. Given the technical limitations of working with xylem-localized markers, the observations of Gardiner and coworkers (54) appear to be entirely consistent with the more recent work of Paredez and coworkers (47).

A surprising twist to the microtubule–cellulose synthase story was the observation that in tobacco protoplasts, inhibition of cellulose synthase activity prevented the development of oriented microtubule arrays (55). These data are consistent with the hypothesis that cellulose microfibrils or cellulose synthase, directly or indirectly, provide spatial cues for cortical microtubule organization. Similarly, microtubule organization in spruce pollen tubes was altered by isoxaben (56), and the orientation of microtubules in Arabidopsis root epidermal cells was disrupted by DCB (46).

5.2.3 Regulation of cellulose synthesis

In bacteria, cellulose synthase appears to be constitutively produced and is activated by the regulatory molecule Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (9, 57). C-di-GMP has not been found in plants but cotton fibers were reported to have a binding

protein (58). However, comparison of the sequence of the apparent binding protein with the Arabidopsis proteome indicates that the putative binding protein is α-tubulin or something that copurified with it.

As noted above, recent results suggest that plant cellulose synthase is activated by a process associated with secretion. In principle, a plasma membrane-localized kinase or phosphatase could alter the activation state of cellulose synthase following transfer from the Golgi, providing a mechanism for keeping it inactive in the Golgi but rapidly activating it upon arrival in the plasma membrane. A proteomics survey of plasma membrane phosphoproteins revealed that CESA1, CESA3, and CESA5 proteins were phosphorylated at a number of sites, and several of the peptides had more than one residue phosphorylated (59). The sites were clustered in the N-terminal domain and in the hypervariable region of the central domain (3). Analysis of the CESA7 protein by mass spectrometry showed that two serine residues in the hypervariable region are phosphorylated (60). Cell extracts catalyzed phosphorylation of these residues and the phosphorylated polypeptide region was rapidly degraded by a proteosome-dependent pathway, leading to the suggestion that phosphorylation may regulate protein turnover. The KOR protein, which is required for cellulose synthase activity in vivo, also had at least two phosphorylated peptides.

During cell expansion, cellulose synthesis is a major consumer of fixed carbon. Thus, it seems likely that whatever regulates cellulose synthesis is coordinated with other aspects of primary carbon metabolism. In plants, UDP-glucose is thought to be largely synthesized by sucrose synthase (SuSy) (61). Amor and coworkers (62) observed a form of SuSy that was associated with the plasma membranes. They also observed that sucrose supported much higher rates of cellulose synthesis by extracts from developing cotton fibers than UDP-glucose and that sucrose synthase is very strongly upregulated in cotton fibers at the onset of fiber elongation. Haigler and coworkers (61) have presented an extensive review of the hypothesis that SuSy might channel UDP-glucose to cellulose synthesis. This is an attractive idea but direct evidence is lacking. Arabidopsis has six SuSy genes but no two isoforms have the same pattern of expression (63). Mutant plants lacking individual isoforms, or double mutants of closely related isoforms, had no alteration in cellulose content. Thus, these studies did not provide support for the idea that SuSy is an important factor in controlling cellulose synthesis. By contrast, transgenic suppression of several SuSy genes in developing cotton fibers prevented formation of fiber cells (64). The effect was more profound than could be attributed solely to an inhibition of cellulose synthesis, obscuring a mechanistic interpretation of the effects. Increased expression of various forms of SuSy in transgenic tobacco plants did not result in increased cellulose per cell, suggesting that UDP-glucose is not the limiting factor in cellulose accumulation in that system (65).

Analysis of the steady-state level of mRNA in major tissues of Arabidopsis with gene chips showed that the *CESA1, 2, 3, 5,* and *6* genes are expressed in all tissues at moderately high levels that differ by about fourfold at most (66). Similar results can be compiled from the large number of public microarray datasets that are now available for Arabidopsis from sites such as Genevestigator (67). As noted below, CESA1, 2, 3, and 6 have been implicated in primary wall synthesis by mutant analysis. Analyses of expression of *CESA* genes in Arabidopsis embryos revealed that *CESA1, 2, 3,* and *9* are the only *CESAs* expressed there (68). Thus, following the nomenclature of Burton and coworkers (69) *CESA1, 2, 3, 5, 6, 9* are probably involved in primary wall synthesis and are referred to at Group-1 CESAs. By contrast *CESA4, 7, 8* are mostly or only expressed in tissues such as stems where secondary cell walls are found

and are designated Group-II (21, 66). *CESA4* promoter:GUS expression studies confirmed that the *CESA4* gene was mostly or only expressed in the vascular tissues (13). Similarly, immunological staining of tissue prints with antibodies against CESA7 and CESA8 showed that the corresponding genes were only expressed in the xylem and interfascicular region (70).

Maize has at least 12 *CESA* genes (71). PCR analysis of transcript levels of six of the genes in various tissues indicated that all of the genes were expressed in all of the tissues examined (13). Analysis of eight of the maize genes by massively parallel signature sequencing indicated that the levels of several of the *CESA* genes varied from one tissue type to another, but no conclusions were reached concerning functional specialization (72). A subsequent analysis that included three additional genes resulted in the identification of three genes that were specifically associated with secondary cell wall formation (71). Thus, maize also shows evidence for specialization of primary and secondary cell wall synthases.

Quantitative information about the relative levels of expression of the Arabidopsis *CESA* genes is lacking because the gene chips used for most studies have not been calibrated for the various *CESA* genes. By contrast, Burton and coworkers (69) used quantitative PCR to measure the expression of the eight known barley *CESA* genes. They observed that the *CESA* genes could be grouped into two expression patterns (i.e., Group I and II) that were generally consistent with roles in primary and secondary wall synthesis. Additionally, they observed that there were large differences in the relative abundance of transcripts for the various members of a CESA group. If the *CESA* genes are translated with similar efficiency, this observation would suggest that the various CESA proteins are not present in identical amounts in the CESA complexes.

Consistent with genetic evidence that at least three CESA proteins are required to produce a functional cellulose synthase complex, correlation analysis of public and private DNA chip datasets revealed that expression of the Arabidopsis *CESA4, 7, 8* gene were indeed very highly correlated (73, 74). The expression of a number of other genes was also very highly correlated with these genes and insertion mutations in several of these genes resulted in cellulose deficient phenotypes. Mutations in some highly correlated genes did not result in obvious effects on cellulose synthases but resulted in other defects in secondary wall synthesis. Thus, the evidence is compatible with the idea that the *CESA* genes that participate in secondary wall synthesis are under developmental control along with other genes required for secondary wall synthesis. The *CESA* genes implicated in primary wall synthesis were less highly correlated. This is consistent with the observation that there are more than three *CESA* genes associated with primary wall synthesis. This presumably indicates that some of the Group-I CESAs are functionally redundant and, therefore, their expression may vary from one tissue to another for unknown reasons. For instance, as noted above, *CESA9* appears to be specifically expressed in embryos.

There is sparse evidence suggesting that cellulose synthesis may be regulated in response to stimuli other than developmental programs. Transgenic trees in which 4-coumarate:coenzyme A ligase expression was reduced by expression of an antisense gene exhibited up to a 45% reduction of lignin and a 15% increase in cellulose (75). However, the apparent increase in cellulose my have been due to a decrease in total mass caused by the reduced lignin content. Conversely, antisense-mediated reduction in expression of an α-expansin in petunia caused a significant reduction in cellulose accumulation in petals (76). According to current theories of expansin action (77) this presumably reflects an

indirect effect from a defect in cell expansion. The properties of this mutant raise the possibility that many or all mutants with defects in cell expansion may have reduced cellulose content due to some form of feedback regulation of cellulose synthesis.

5.3 Hemicellulose

Hemicellulose is an operational term that refers to polysaccharides that are extracted from cell walls by dilute alkali. Xylans are typically extracted by 4% KOH whereas xyloglucans may require 24% KOH. The extraction conditions are thought to dissociate hydrogen bonding between hemicellulose and cellulose. By this definition, hemicelluloses constitute between a quarter and a third of the mass of many types of cell walls. The composition of the hemicellulose fraction varies from one cell type to another and among plant species. The four types of polymers that comprise most types of hemicellulose are xyloglucan, xylans, mixed-linkage glucans, and mannans such as glucomannan, galactomannan, or galactoglucomannan. Xyloglucan is defined by a β-1,4-glucan backbone that is highly substituted with xylose-containing side chains that may also contain galactose, arabinose, or fucose. Xylans contain a β-1,4-xylose backbone which may contain arabinan and glucuronic acid side chains. Mixed linkage glucans are composed of glucose residues linked both β-1,3 and β-1,4. Mannans have a β-1,4-linked mannose backbone or, in the case of glucomannans, may contain both glucose and mannose linked β-1,4. Konjac glucomannan, which is an important dietary fiber, contains glucose:mannose in a ratio of 1:1.6, indicating that the residues do not simply alternate. By contrast, galactomannans have a β-1,4-mannose backbones with α-1,6-galactose branches. The ratio of mannose:galactose varies from about 1:1 in fenugreek gum to about 4:1 in locust bean gum.

Several of the proteins involved in the synthesis of the various hemicelluloses polymers have recently been identified. Somewhat interestingly, it appears that the backbones of several of the hemicellulose polysaccharides are synthesized by a family of related enzymes that were originally termed as "cellulose synthase like" (CSL) because of weak sequence similarity to the CESA proteins that comprise cellulose synthase.

5.3.1 Mannan

Polysaccharides with β-1,4-mannan and β-1,4-glucomannan backbones are abundant constituents of the wood of gymnosperms (78, 79) the cell walls of certain algae (80), and are present in lower amounts in many other species (81). Mannans also serve as carbohydrate reserves in a variety of plant species (78, 82). Several groups have biochemically characterized glucomannan synthase activities from a variety of plant species (78, 79, 82, 83). The enzymes were shown to use GDP-mannose and GDP-glucose as substrates and to produce polymers with varying ratios of the two sugars, depending on the ratio of the sugar nucleotides (83) Recently, transglycosylase enzymes that modify the architecture of mannan polysaccharides in plant cell walls have also been discovered (84).

Mannan synthase is encoded by genes of the CSLA gene family (85). Expression of a cDNA from guar in soybean cells led to the synthesis of a β-1,4-mannan. Similarly, expression of three Arabidopsis CSLA proteins in Drosophila cells resulted in proteins that catalyzed synthesis of mannan from GDP-mannose (86). Similarly, several poplar orthologs expressed in Drosophila cells exhibited glucomannan synthase activity (87). Functional analysis of

CSLA genes from diverse species is consistent with the hypothesis that the function of the CSLA genes is conserved in all plants (81).

As noted previously in studies of impure enzymes (83), when provided with both GDP-glucose and GDP-mannose, the enzymes produce mixed linkage mannans. One of the proteins produced glucan when provided only with GDP-glucose. These studies suggest that no primer is required to initiate mannan synthesis. The ability of the CSLA enzymes to accept GDP-glucose or GDP-mannose is compatible with earlier suggestions that the ratio of mannose:glucose in glucomannans may be controlled by regulating the availability of the two sugar nucleotides. The observation that GDP-glucose is a substrate also may explain old observations from in vitro polysaccharide synthesis experiments that had initially been interpreted as evidence that GDP-glucose was the substrate for cellulose synthesis.

Genes encoding the galactomannan galactosyltransferase responsible for attaching galactan side chains have also been identified following purification of the enzyme from fenugreek (88). The α-1,6GalT galactosyltransferase cDNA encodes a 51282 Da protein, with a single transmembrane alpha helix near the N terminus. The protein has been functionally expressed in the yeast *Pichia pastoris* and is active when the membrane-spanning domain is removed. Thus, presumably the membrane-spanning domain is required only to localize the protein to the Golgi apparatus where mannan is thought to be synthesized. The degree of substitution when UDP-galactose is available is variable and appears to be a stochastic process controlled both by enzyme specificity and the levels of α1,6GalT activity (89). Eight Arabidopsis gene sequences are very similar to the α-GalT from fenugreek (88).

In Arabidopsis, mannans have been localized not only in thickened secondary cell walls of xylem elements, xylem parenchyma, and interfascicular fibers, but also in the thickened walls of the epidermal cell of leaves and stems and, to a lesser extent, in most other cell types (90). Analyses of Arabidopsis mutants containing a transposon insertion in exon seven of the *CslA7* gene showed that disruption of this gene results in defective pollen tube growth and disruption of embryonic development (91). Mutants (called rat4) containing a T-DNA insertion in the 3′ untranslated region of the AtCslA9 gene display resistance to Agrobacterium tumefaciens transformation, apparently caused by decreased binding of bacterial cells to roots. AtCslA9 promotor-GUS fusions indicated that this gene is expressed in a variety of Arabidopsis tissues, including lateral roots and the elongation zone, where the root is most susceptible to Agrobacterium transformation (92). In both mutant studies, the authors suggested that the mutant phenotypes resulted from alterations in polysaccharide content; however, in neither case was such a defect demonstrated.

A GDP-mannose transporter that is localized to the Golgi has been characterized (93).

5.3.2 Xyloglucan

Xyloglucans exist as cell wall components in most species and as storage polymers in seeds of some species (94). Xyloglucan (XG) comprises 20–25% of primary walls of dicots but graminaceous monocots typically contain much less. XG is defined by a β-1,4-glucan backbone in which many glucosyl residues contain α-1,6-linked xylose branches. Xyloglucan from pea had an average molecular mass of 330 kDa representing a backbone of about 1100 glucose residues of about 500 nm in length (95). In many species the xylose residues are further substituted with β1,2-linked galactose which may in turn be linked at the 2-position

Table 5.1 Common elements of single letter code for xyloglucan structure

Code	Structure represented
G	β-D-Glc*p**-[a]
X	α-D-Xyl*p*-(1→6)-β-D-Glc*p**-
L	β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p**-
F	α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p**-

[a]*- D-Glucose in chain or at reducing terminus. See Fry and coworkers (97) for details.

to α-L-fucose or arabinose (94). X-ray fiber diffraction studies of tamarind XG indicated a twofold helix similar to cellulose (96). A single letter code has been developed to describe the structure of xyloglucans (Table 5.1) (97).

Most species have an XXXG type of XG. However, members of *Poacea* and *Solanaceae* have an XXGG type in which a pair of arabinose residues replace fucose (98, 99). In most monocots XG contains less xylose and galactose and does not contain terminal fucose. The structure and molecular distribution of the side chains varies in different plant tissues and species (100–102).

XG may be extensively acetylated (103). In Sycamore cells, the O-2-linked-β-D-galactosyl residue of the nonasaccharide was found to be the dominant site of O-acetyl substitution in XG. Both mono-O-acetylated and di-O-acetylated β-D-galactosyl residues were detected. The degree of O-acetylation of the β-D-galactosyl residue was estimated to be 55–60% at O-6, 15–20% at O-4, and 20–25% at O-3. Approximately, 50% of the β-D-galactosyl residues were mono-O-acetylated, 25–30% were di-O-acetylated, and 20% were not acetylated. In tomato (*Lycopersicon esculentum*), O-acetyl substituents were located at O-6 of the unbranched backbone β-D-Glcp residues, O-6 of the terminal β-D-Galp residue, and/or at O-5 of the terminal α-L-Arap residues (104). Acetylation of XG does not affect the degree to which XG hydrogen bonds to cellulose in vitro (100) and the role of acetylation is unknown. Similarly, the enzymes that acetylate XG are unknown. O-acetylation of galactose residues was considerably reduced in Fuc-deficient mutants (*atfut1*, *mur1*, and *mur2*) that synthesize XG containing little or no Fuc (105). These results suggest that fucosylated XG is a suitable substrate for at least one O-acetyltransferase in Arabidopsis.

Immunoelectron microscopy using antibodies against XG indicates that XG is localized to the cellulose-containing region of the cell wall (106). Hayashi (94) proposed that XG does not have covalent cross-links to other components or if there are links they must be alkali-labile linkages such as O-esters. However, Thompson and Fry (107) have observed cross-links between XG and pectins in Rose cells. Brett and coworkers (108) have also observed such cross-links and found that they form in the Golgi. Feruloyl esters of XG have also been observed in maize cell cultures (109).

Pure XG binds to cellulose in vitro in a pH-dependent manner (110). Levy *et al.* (111) have presented evidence that the structure of the XG side branches may facilitate the binding of XG to cellulose. Native XG-cellulose complexes contain higher ratios than can be obtained in vitro, suggesting that XG may be intercalated into the cellulose microfibrils (110). Also, mild alkali does not completely dissociate the complex and concentrated alkali (e.g., 4M KOH) is required to completely extract XG. The proposed function of XG binding to cellulose is to prevent aggregation of cellulose fibrils (110) but because single strands of XG may be

hydrogen bonded to different cellulose microfibrils (112–114); it may also provide some degree of crosslinking. Thus, XG hydrolysis may be required for growth. Based on the combined chemical and cytological evidence, Pauly and coworkers (100) have developed a model for the cellulose/XG network that posits that XG can have three configurations; hydrogen bonded to the surface of cellulose, cross-linked, and embedded within the microfibril. They propose that the cross-links are the domain that is accessible to enzymes such as xyloglucan endotransglycosylases that are thought to play a role in cell wall expansion. This is supported by observations of XET-mediated incorporation of fluorescent XG fragments into XG in expanding cell walls (115). Pauly and coworkers (100) also note that it is not clear to what extent the various XG structures participate in determining the nature of the XG-cellulose association.

The first progress in defining the genes involved in XG synthesis was the identification of the fucosyltransferase that adds the terminal fucose to XG side chains. A 60-kDa fucosyltransferase (FTase) that adds this residue was purified from pea epicotyls (116). Peptide sequence information from the pea FTase allowed the cloning of a homologous gene, AtFUT1, from Arabidopsis. AtFUT1 expressed in mammalian COS cells resulted in the presence of XG FTase activity in these cells. AtFUT1 shows very little identity with FucTs from other organisms. AtFUT1 and PsFUT1 (the pea XyG FucT homologue) are 62.3% identical (117). Both enzymes contain motifs that had been identified in other FucTs but combine these motifs in a unique manner (117). Three motifs had been identified in (1→2)α- and (1→6)α-FucTs. Motifs I and II had been present in both (1→2)α- and (1→6)α-enzymes, but a particular version of motif III had appeared to be characteristic of each group. AtFUT1 and PsFT1, however, contain a hybrid motif III that has features of both the (1→2)α- and (1→6)α-versions. There are ten genes in Arabidopsis with identity of encoded amino acid sequences to AtFUT1 ranging from 35 to 73.8% (118). The AtFUT1 gene was found to correspond to the fucose-deficient *mur2* mutant of Arabidopsis (119).

The galactosyltransferase that contributes to the synthesis of XG side chains was identified by map-based cloning of the *MUR3* gene of Arabidopsis, which had previously been identified based on a screen for variation in cell wall polysaccharide composition (120). MUR3 belongs to a large family of Type II membrane proteins that is evolutionarily conserved among higher plants. The enzyme shows sequence similarities to the glucuronosyl transferase domain of exostosins, a class of animal glycosyltransferases that catalyze the synthesis of heparan sulfate, a glycosaminoglycan with numerous roles in cell differentiation and development. Arabidopsis has ten genes encoding proteins with significant sequence similarity to the MUR3 xyloglucan GalT (121).

One of the XG xylosyltransferases (XT1) was identified from Arabidopsis based on sequence similarity to the fenugreek mannan α-1,6-galactosyltransferase (122). Expression of the gene in *Pichia pastoris* resulted in a protein with cello-oligosaccharide-dependent xylosyltransferase activity. Characterization of the products obtained with cellopentaose as acceptor indicated that the pea and the Arabidopsis enzymes transfer xylose mainly to the second glucose residue from the non-reducing end in an α(1,6)-linkage to the glucan chain. Arabidopsis has seven related genes, some of which may catalyze addition of xylose to other positions in the repeating unit of XG.

In vitro assays of the glucan synthase involved in synthesis of the XG backbone exhibit maximal activity only if both UDP-glucose and UDP-xylose are present, suggesting that the glucan synthase acts in concert with a xylosyltransferase that adds side chains (94, 123).

The glucan synthase extends existing XG by addition to the non-reducing end but cannot be primed with exogenous primers (94). Also the enzyme does not add xylose to preformed glucans.

Recently, Cocuron and coworkers (124) have obtained evidence that proteins of the cellulose synthase-like 4 (CSLC4) family catalyze synthesis of the XG backbone (99). They expressed *CSLC4* genes from Arabidopsis and tamarind along with the Arabidopsis XT1 gene in *Pichia pastoris* and observed the formation of β-1,4-linked glucan. However, they were unable to detect XG synthase activity in extracts from the cells. Mutations in the Arabidopsis *CSLC4* gene are deficient in xyloglucan, supporting the proposed role in xyloglucan synthesis (Milne and Somerville, unpublished). If substantiated by further work, the work of Cocuron and coworkers (124) appears to represent a long-awaited breakthrough in understanding the synthesis of XG (99). The identification of the genes involved in XG synthesis should pave the way for an analysis of how the amount of XG is regulated and what the consequences are to plant growth and development and the properties of cell walls of genetic variation in the amount of XG.

Some information about genetic variation in XG is available from analysis of Arabidopsis mutants that were recovered by screening for alterations in total cell wall sugar composition (125, 126). Comparison of the mechanical responses of *mur2* (AtFUT1) and *mur3* (XG galactosyltransferase), indicated that galactose-containing side chains of xyloglucan make a major contribution to overall wall strength, whereas xyloglucan fucosylation plays a comparatively minor role (127). Thus, it seems unlikely that it will be possible to develop biomass feedstock crops with significant alterations in the structure of XG without also making compensating changes in another cell wall component. Because Arabidopsis has a number of CSLC genes, it has not yet been possible to develop mutant plants with major reductions in the amount of XG to assess the phenotypic consequences of such alterations.

5.3.3 Xylan

Xylan, a polymer of β-(1-4)-linked D-xylose is one of the main components of woody plants. Xylans are usually substituted by side chains of arabinose or glucuronic acid and may be acetylated. Thus, glucuronoxylan (GX) is composed of a linear backbone of β-(1-4)-linked D-xylosyl (Xyl) residues, some of which bear a single α-D-glucuronic acid (GlcA) or 4-O-methyl-α-D-glucuronic acid (MeGlcA) residue at O-2. The Xyl residues can also be substituted with arabinosyl and acetyl residues (128). Xylosyltransferase and glucuronyltransferase activities have been detected in numerous plants (129). However, none of the genes encoding these enzymes has been identified, nor have any of the enzymes been purified to homogeneity and biochemically characterized.

Mutations in three glycosyltransferases, *FRAGILE FIBER8* (*FRA8*), *IRREGULAR XYLEM8* (IRX8), and *IRX9*, have been shown to be required for normal vessel morphology and wall thickness and for normal amounts of xylose and cellulose in cell walls (73, 74, 130, 131). These genes are specifically expressed in cells undergoing secondary wall thickening. Plants carrying mutations in these genes have reduced amounts of wall GX and a decreased ratio of GlcA to MeGlcA residues in the GX (129, 130, 132).

IRX8, *IRX9*, and *FRA8* are specifically expressed in fibers and vessels and their encoded proteins are localized in the Golgi (129, 130, 132). Thus, they have the properties expected of enzymes involved in glucuronoxylan synthesis. However, it has not been possible to directly

associate enzyme activity with the proteins. Thus, it is not clear how they participate in the synthesis of xylan. Peña and coworkers (129) showed that the glycosyl sequence **4-β-D-Xylp-(1 → 4)-β-D-Xylp-(1 →3)-α-L-Rhap-(1 →2)-α-D-GalpA-(1 →4)-D-Xylp** was present at the reducing end of Arabidopsis GX, as previously noted for birch (*Betula verrucosa*) and spruce (*Picea abies*) wood. They further noted that mutations in *IRX8* and *IRX9*, and by inference FRA8, lead to reductions in the amount of the GX reducing end sequence suggesting that these genes participate in the synthesis of the GX reducing end sequence and suggest that IRX9 has an essential role in the elongation of the xylan backbone.

The fra8 gene encodes a GT47 family enzyme and expression of the poplar (*Populus alba* x *tremula*) *GT47C* gene in *fra8* plants rescues the defects in secondary wall thickness and GX synthesis, suggesting that GT47C is a functional homolog of FRA8 (133). The *FRA8* gene encodes a putative GT in family GT47 (130). This family includes enzymes with an inverting mechanism, which usually leads to β-glycosidic linkages (when typical α-linked donor substrates are used). Thus, if UDP-α-D-Xyl is the donor substrate, it is possible that FRA8 catalyzes the formation of the β-linkage of xylose to either O-3 of the rhamnose or O-4 of the penultimate xylose of the GX reducing end glycosyl sequence (129). However, in plants, the addition of α-Rha residues is catalyzed by inverting GTs that use UDP-β-L-Rha as the donor substrate (134). Therefore, it is also possible that FRA8 catalyzes the addition of rhamnose during the biosynthesis of the GX reducing end sequence.

The *IRX8* (GAUT12) gene encodes a putative GT in family GT8 (73, 74, 129, 132). Several members of the GT8 family catalyze the transfer of uronic acids to glycans. For example, three Arabidopsis GT8 proteins, QUASIMODO1 (QUA1) (135), PARVUS (136), and GALACTURONOSYLTRANSFERASE1 (GAUT1) (137), have been identified and are believed to have a role in pectin biosynthesis. Of these three, only GAUT1 has been biochemically characterized and shown to have galacturonosyltransferase activity (137). Family GT8 enzymes are retaining glycosyl transferases that catalyze the formation of α-glycosidic bonds when using α-linked donor substrates such as UDP-α-D-GalA. Thus, it is possible that *IRX8* catalyzes the addition of an α-D-GalA residue to O-4 of the reducing Xyl residue present in the GX reducing end sequence described above.

Mutation of the *IRX9* gene, which encodes a putative GT in family GT43 (138), was shown to result in plants with decreased amounts of wall GX, suggesting that this gene is required for GX synthesis (131). The poplar (*Populus tremula x tremuloides*) *GT43A* and Ptt *GT43B* genes, which are homologs of *IRX9*, have been shown to be highly expressed during wood formation (139). In addition, a cotton (*Gossypium hirsutum*) gene, which resides in the same phylogenetic subgroup as Ptt *GT43A*, Ptt *GT43B*, and *IRX9*, is highly expressed during cotton fiber development (140). Together, these findings suggest that family 43 GTs have an important role in secondary wall synthesis. Enzymes in this family are distinguished by an inverting mechanism, typically catalyzing the formation of β-glycosidic bonds using α-linked glycosyl donors. Our demonstration that the *irx9* mutation leads to a decrease in the chain length of GX suggests that *IRX9* encodes a xylan synthase responsible for adding β-xylosyl residues to the nascent GX. This hypothesis is consistent with our results indicating that *IRX9* is highly expressed in cells undergoing secondary wall biogenesis and that IRX9 is localized in the Golgi, where GX synthesis occurs (141, 142). However, it is also possible, as we discussed previously (130), that an inverting GT can catalyze the formation of an α-linkage when a β-linked substrate (such as a glycosyl phospholipid) is used as the donor. Such inverting enzymes can also catalyze the formation of high-energy β-linked glycosides (such as glycosyl phospholipids) that are subsequently used as glycosyl donors. Thus, an

alternative interpretation is that IRX9 is directly or indirectly involved in the transfer of α-linked GlcA residues to the GX backbone. Additional studies are required to determine whether IRX9 catalyzes the addition of xylose or GlcA to the GX backbone.

In the lignified walls of the Poaceae, the major non-cellulosic polysaccharides are glucuronoarabinoxylans (GAXs), although the degree of substitution of the xylan main chain is less than in the GAXs of the primary cell walls. In the non-lignified walls of the Poaceae and other species such as pineapple (*Ananas comosus*), ferulic acid is ester-linked to GAXs. These polysaccharides comprise only a minor component of the non-cellulosic polysaccharides of the non-lignified walls of species in the basal Arecales (palms) clade (143), but are a major component of the non-lignified walls of species in the other commelinid clades, particularly the Poales (144–146).

5.3.4 Mixed linkage glucans

Walls of the grasses contain mixed-linkage (1,3;1,4)-β-D-glucans (MLGs) which are not present in walls of dicotyledons or most other monocotyledonous plants (147). Some alga and liverworts may also have MLGs (148). The (1,3;1,4)-β-D-glucans have an unusual structure consisting of an unbranched, unsubstituted glucan chain with two linkages arranged in a non-repeating, but non-random, fashion. The glucan chains consist of primarily cellotriosyl and cellotetraosyl units separated by single (1 → 3)-β-linkages (149). MLGs can be synthesized in vitro from Golgi membrane fractions with UDP-Glc as a substrate (150, 151). The amount of UDP-Glc used in the assay alters the ratio of cellotriosyl and cellotetraosyl units, indicating that this is not a fixed property of the biosynthetic enzyme (152).

Recently, following prescient speculation as to possible structural similarity between cellulose synthase and the MLG synthase (153), it was shown that expression of a rice *CslF* gene in Arabidopsis led to the accumulation of (1,3;1,4)-β-D-glucan biosynthesis in Arabidopsis (154). Thus, it appears that CSLF encodes the mixed glucan synthase. The generally low levels of (1,3;1,4)-β-D-glucan in walls of the transformed Arabidopsis plants is consistent with the concept that limiting levels of other components might be required for high-level synthesis of the polysaccharide or its transfer to the cell wall. Similarly, the preferential deposition of the (1,3;1,4)-β-D-glucan in the epidermal layers of the transgenic Arabidopsis lines, despite the fact that transgene expression was driven by the constitutive 35*S* promoter, could indicate that the epidermal cells contain ancillary factors that are not abundant in other cells of the leaf. The identification of the gene opens up new approaches to understanding the fascinating process by which an enzyme catalyzes substantially different transferase reactions (151, 153).

The role of the MLGs is not clear. The MLGs are synthesized in relatively large amounts during growth and may coat cellulose microfibrils during the synthesis and expansion phase, but they are degraded when elongation ceases (155, 156).

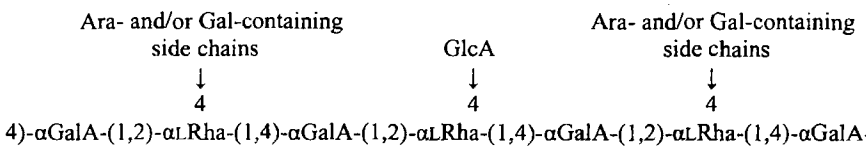
5.4 Pectins

Pectin is likely the most structurally complex family of polysaccharides in nature (Figures 5.2 and 5.3). Pectin is particularly abundant in primary walls, i.e. those walls surrounding

Homogalacturonan

4)αGalA-(1,4)-αGalA-(1,4)-αGalA-(1,4)-αGalA-(1,4)-αGalA-(1,4)-αGalA-(1,4)-αGalA-(1,

Rhamnogalacturonan I



Rhamnogalacturonan II

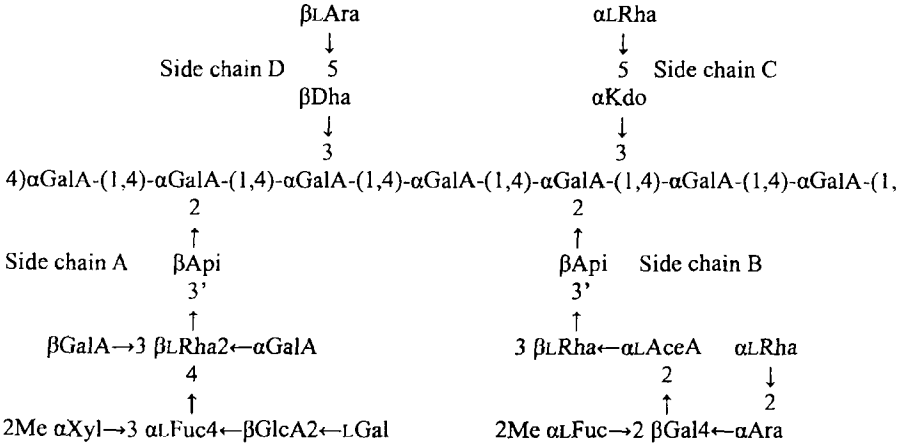


Figure 5.2 Representative structures of the three pectic polysaccharides HG, RG-I, and RG-II.

growing and dividing cells and the terminal wall in many cells of the soft parts of the plant. Pectin is also abundant in the middle lamella which is the junction between adjacent cells. Pectin comprises ~35% of the polysaccharides in dicot and non-graminaceous monocot primary walls, and 2–10% of the wall in the grasses (157, 158). Pectin is also present in the walls of gymnosperms, pteridophytes, and bryophytes as well as Chara, a charophycean alga, which is believed to be the closest extant relative of land plants (159). Although pectin is not a major component of secondary walls, it is present as the outer layer of secondary walls and can represent ~5% of harvested tree wood. Thus, depending on the plant and tissue used, pectin will be present in the biomass used for biofuel production and, since it comprises a complex interconnected matrix in the wall, likely affects the recalcitrance of biomass to deconstruction for biofuel production.

Pectins have multiple roles in plant defense, growth, and development (158, 160). They provide wall structure (161), bind and exchange apoplastic anions and macromolecules (162, 163), influence cell–cell adhesion (164, 165), and are involved in cell signaling (166,

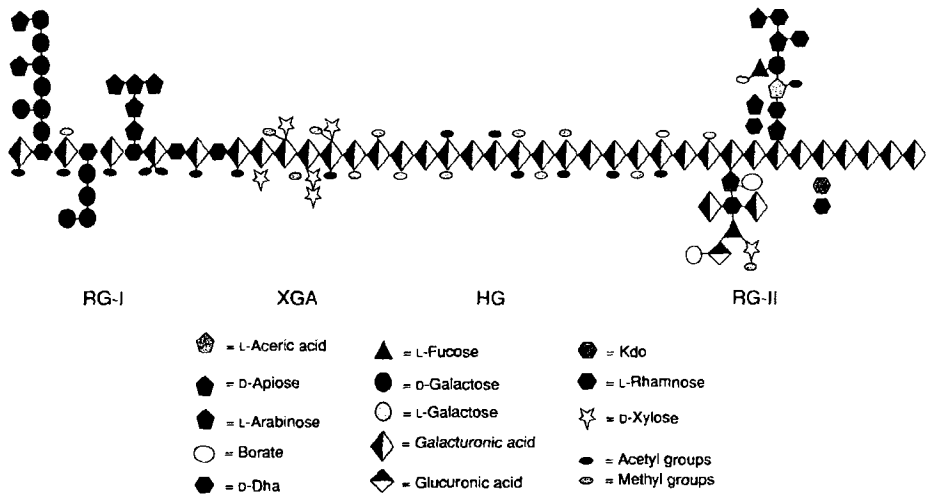


Figure 5.3 Schematic structure of pectin showing the three main pectic polysaccharides homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) linked to each other. A region of substituted galacturonan known as xylogalacturonan is also shown (XGA). The representative pectin structure shown is not quantitatively accurate, HG should be increased 12.5-fold and RG-I increased 2.5-fold to approximate the amounts of these polysaccharides relative to each other plant walls. The monosaccharide symbols used are either from the Symbol and Text Nomenclature for Representation of Glycan Structure. Nomenclature Committee Consortium for Functional Glycomics (http://www.functionalglycomics.org/glycomics/molecule/jsp/carbohydrate/carbMoleculeHome.jsp) or from D. Mohnen. (The figure is modified from http://www.uk.plbio.kvl.dk/plbio/cellwall.htm.) (Reproduced in color as Plate 4.)

167). Pectins have roles in pollen tube growth (168), seed hydration (169–171), leaf abscission (172), guard cell function (173), organ formation (174, 175), fruit development (158), and possibly water movement (176). Pectic oligosaccharides are intercellular signal molecules (177) in plant development (166) and defense responses (178, 179). Mutant plants with altered pectin structure may be dwarfed (161, 180), have brittle leaves (164), reduced numbers of side shoots and flowers (175), and reduced cell–cell adhesion (135, 181).

Pectin is defined as a family of plant cell wall polysaccharides that contain 1,4-linked galacturonic acid (157). Galacturonic acid (GalA) comprises roughly 70% of total cell wall pectin and is a major component of the three major types of pectic polysaccharides: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and the substituted galacturonans for which rhamnogalacturonan II (RG-II) is the most ubiquitous and structurally invariant member (157). In addition, pectin includes the less abundant substituted galacturonan xylogalacturonan (XGA) (182–187) and apiogalacturonan (AG) (158, 188–191). The complex structure of the pectic polysaccharides makes the study of pectin synthesis challenging. It is estimated that at least 58 enzymes are required to synthesize pectins, including methyltransferases, acetyltransferases and numerous glycosyltransferases (192).

HG accounts for ~65% of pectin (193, 194) and is a homopolymer of α-D-1,4-linked GalA residues (Figures 5.2 and 5.3) that is partially methylesterified at the C-6 carboxyl (157, 195), may be O-acetylated at O-2 or O-3 (196–199), and may contain other esters

whose structure remains unclear (200–204). RG-I accounts for 20–35% of pectin (194) and is a family of polysaccharides with an alternating [→4)-α-D-GalA-(1→2)-α-L-Rha-(1→] backbone (Figures 5.2 and 5.3). Between 20 and 80% of the rhamnosyl residues are substituted with side chains composed predominantly of linear and branched α-L-Ara*f* and β-D-Gal*p* (157, 205). The main types of side chains include α-1,5-linked L-arabinan with some 2- and 3-linked arabinose or arabinan branching, β-1,4-linked D-galactans with some 3-linked L-arabinose or arabinan branching and β-1,3-linked D-galactan with β-6-linked galactan or arabinogalactan branching (205). RG-I side branches may also contain α-L-Fuc*p*, β-D-Glc*p*A, and 4-*O*-Me β-D-Glc*p*A residues (206). The composition and length of RG-I side chains varies between cell types and in different plant species (158, 160). RG-II accounts for ~10% of pectin (158, 194) and contains 12 different types of sugars in over 20 different linkages. The HG backbone of RG-II is substituted at O-2 and O-3 with four structurally complex oligosaccharides A–D (159) (Figures 5.2 and 5.3). RG-II in the plant generally occurs as a RG-II dimers crosslinked by borate diesters (159). The 4-linked galacturonans that are substituted at O-3 with D-xylose (the xylogalacturonans, XGA) are often found in reproductive tissues (157, 186, 193, 207) whereas galacturonans substituted at O-2 or O-3 by D-apiofuranose (188, 189) (the apiogalacturonans, AG) are restricted to selected aquatic monocots (e.g., Lemna).

When walls are isolated from the plant, the pectic polysaccharides appear to be covalently cross-linked since harsh chemical treatments or digestion by pectin-degrading enzymes is required to isolate HG, RG-I, and RG-II separately from each other. It is not known, however, how the pectic polysaccharides are covalently linked to each other or to other polymers in the wall. It is also not clear where and how that crosslinking occurs, i.e. via the action of glycosyltransferases in the Golgi or by transglycosylases or other enzymes in the wall. The available data (208) support a model whereby HG, RG-I, and RG-II are linked via their backbones. However, due in part to the uncertainty of how and where the pectic polysaccharides are cross-linked, it is currently not possible to predict the complete repertoire of biosynthetic enzymes that are needed to synthesize pectin. Furthermore, although the general types of pectic polysaccharides are similar in different plant species, there is a growing body of evidence showing that species-, cell-type-, and developmental state-specific differences in pectin structure exist, thereby making it likely that the number and types of enzymes required to synthesize pectin will depend on the plant, tissue and developmental state of the cells of interest.

Finally, a knowledge of the structure of the "mature" polysaccharides in the wall, or at least those that can be isolated from the wall and characterized, does not necessarily reflect the structures as they are synthesized, but rather the structures as they are inserted into the wall and after they have been modified by wall-localized enzyme catalyzed (and chemical) reactions. In the following discussion of pectin, there is no attempt to define species-specific differences in pectin synthesis, since our understanding of the species-specific tailoring of pectin structures and synthesis is only just beginning to be studied. Rather, this review emphasizes our current understanding of biosynthetic enzymes required for the basic pectin structures that appear to be common in all species. Although only few of the genes encoding pectin biosynthetic enzymes have been confirmed by demonstration of enzymatic activity of the encoded proteins, recent progress in identifying genes encoding putative pectin biosynthetic enzymes make it likely that more pectin biosynthetic genes will be functionally identified in the near future. The availability of such genes should facilitate the elucidation

of how diverse pectin biosynthetic enzymes work together, likely within protein complexes (Atmodjo and Mohnen, unpublished results) to synthesize the multifunctional family of pectic polysaccharides.

Several comprehensive reviews on pectin biosynthesis (158, 192, 205, 209), as well as more general reviews on plant wall biosynthesis (72, 99, 126, 155, 210–213, 214) and strategies to identify wall biosynthetic glycosyltransferases (118, 215–218) and regulation of cell wall synthesis (219–221) have previously been published. This review will attempt to merge recent advances in pectin synthesis with the prior studies so as to reflect our current understanding of pectin synthesis.

5.4.1 Location of pectin synthesis

All available evidence, including autoradiographic pulse chase studies using wall biosynthetic precursors (222, 223), immunocytochemical studies using anti-pectin-specific antibodies (224–226), and subcellullar fractionation and topology studies of pectin biosynthetic enzymes (227–231), indicate that pectin is synthesized in the Golgi and transported to the wall in membrane vesicles. Plant cells, unlike animal cells, have multiple Golgi and thus pectin synthesis occurs simultaneously in numerous Golgi stacks in the cell (225, 232). The synthesized pectin and other macromolecules are targeted to the wall by the movement of Golgi vesicles, presumably along actin filaments that have myosin motors (233).

Immunocytochemical studies also indicate that the synthesis of different regions of the pectic polysaccharides occurs in different Golgi cisternae as pectin moves from the cis, through the medial and to the trans-Golgi. For example, the use of antibodies specific to different regions of HG and RG-I suggests that HG and RG-I synthesis begins in the cis-Golgi (225, 234, 235) and continues with more extensive decoration of the backbones as the polymers move through the medial Golgi (224, 225, 235) and into the trans-Golgi cisternae (225, 235). Additional modifications of the pectic glycan structure also appear to proceed in a more-or-less organized manner with HG (236, 237) and RG-I (106, 234, 238, 239) initially synthesized in less modified forms in the cis- and medial-Golgi and becoming more modified (e.g., methylesterified) (236) in the medial- and trans-Golgi (225, 235, 240–242). HG is believed to be transported to the plasma membrane and inserted into the wall as a highly methylesterified polymer (214, 237, 243–245) and once in the wall, HG is deesterified to varying degrees by pectin methylesterases (246) in the wall or at the cell plate (245). The deesterification of HG converts it to a more negatively charged form (240, 247–250) which is then available to bind ions, enzymes, proteins, and other HG molecules through Ca^{++} salt bridges. It is believed that a spatial partitioning of HG esterification and deesterification occurs in the wall based on localization of esterified HG throughout the cell wall (237, 240–242, 243, 245, 250, 251), while relatively unesterified HG is more restricted to the middle lamella. This conclusion is supported by the frequently observed absence of unesterified HG epitopes in the trans-Golgi vesicles. However, since some cell types, such as melon callus cells (240), contain unesterified HG in the trans-Golgi, it is possible that HG may be inserted into the wall in a relatively unesterified form, at least in some cells. Also, since specific pectic epitopes localize to different Golgi compartments in different cell types (5, 225, 234, 237, 244), it is likely that the specific localization of the diverse pectin biosynthetic enzymes may vary in a cell type, species, and development-specific manner (226, 252–255). It must be noted, however, that the interpretation of immunocytochemistry

results can be difficult since the absence of a signal using an epitope-specific antibody may be due to masking of the epitope by additional glycosylation or some other modification (e.g., methylation, acetylation, feruloylation). Thus, to conclude that a particular pectin biosynthetic event does not occur in a cell, the lack of a particular immunocytochemical signal is not sufficient. Information on the presence of the biosynthetic enzyme activity or the actual wall carbohydrate structure itself is required.

5.4.2 Pectin biosynthetic glycosyltransferases

The current view is that the bulk of pectin synthesis is catalyzed by glycosyltransferases (GTs) that transfer a glycosyl residue from an activated form of the sugar, most likely a nucleotide-sugar, to an acceptor. Like other polymer biosynthetic reactions, pectin synthesis is thought to proceed through three stages: initiation, elongation, and termination. There is no detailed understanding of the initiation phase for the synthesis of any of the pectic polysaccharides. It has traditionally been held that the pectic polysaccharides are not synthesized on a protein, thus distinguishing them from the synthesis of animal Golgi-localized proteoglycans (256). More recently, several investigators have presented evidence suggesting that some wall polysaccharide synthesis may occur on protein primers (257–259); however, definitive proof of this hypothesis has yet to be provided. To date all studies of pectin biosynthetic glycosyltransferases have been carried out by assaying the transfer of a glycosyl residue from a radiolabeled nucleotide-sugar substrate (with or without additional unlabeled substrate) onto endogenous acceptors in plant microsomal membranes or onto exogenous acceptors, or alternatively, by transfer of an unlabeled substrate onto fluorescently labeled exogenously added oligosaccharide or polysaccharide acceptors. Most nucleotide-sugars involved in pectin synthesis consist of a nucleoside-diphosphate (NDP) attached to the sugar, and thus the general reaction catalyzed by the GTs is NDP-sugar + acceptor$_{(n)}$ → NDP + acceptor$_{(n+1)}$. The precise number and location of the glycosyl residues in the acceptor that are recognized by a GT will be unique for each GT. However, in this review, for the purposes of calculating a minimal number of GTs required for pectin synthesis, since all pectin biosynthetic GTs characterized to date have been shown to add onto the non-reducing end of the oligosaccharide/polysaccharide acceptor, the assumption has been made that each GT will recognize, as a minimum, the terminal two glycosyl residues at the non-reducing end of the acceptor (i.e., the sugar onto which the transfer takes place, and the adjacent sugar).

Table 5.2 lists the types of glycosyltransferases that are expected to be required for pectin synthesis, based on the premise that a unique glycosyltransferase will be required for the transfer of a unique sugar from a unique nucleotide-sugar onto a unique disaccharide acceptor region at the non-reducing end of the acceptor. In some cases, it is possible that the same enzyme may catalyze the synthesis of a similar region on diverse polysaccharides, (e.g., the same galacturonosyltransferase may catalyze the synthesis of the backbone of HG and the HG region of RG-II. Table 5.2 attempts to list all known or expected GTs that are required for pectin synthesis, and as such, is meant to serve as a reference table. However, in an effort to consider in more depth the synthesis of the different types of pectic polysaccharides, HG, RG-I, RG-II, XGA, and AG; a detailed summary of progress in understanding the synthesis of the specific pectic polysaccharides is described separately.

Table 5.2 List of glycosyltransferases expected to be required for pectin biosynthesis

Type of glycosyl-transferase	Parent polymer[a] (side chain)	Enzyme[b] acceptor substrate *Enzyme activity* (unless noted: enzyme adds to the glycosyl residue on the left*)	Ref.[c] for structure	*Putative* gene Identified (Ref.)[d]	Gene identified (Ref.)[e]
D-GalAT	HG/RG-II	*GalAα1,4-GalA *α1,4-GalAT*	(157)	Put. QUA1-At3g25140 (135, 260)	GAUT1 At3g61130 (137)
D-GalAT	RG-I	L-Rhaα1,4-GalA *α1,2-GalAT*	(157, 261, 262)	–	–
D-GalAT	RG-II (A)	L-Rhaβ1,3′-Api*f* *α1,2-GalAT*	(214, 157)	–	–
D-GalAT	RG-II (A)	L-Rhaβ1,3′-Api*f* *β1,3GalAT*	(214, 157)	–	–
D-GalAT	RG-I/HG	GalAα1,2-L-Rha *α1,4-GalAT*	—	–	–
D-GalAT	HG attached to xylan	*GalAα1,4-GalA *α1,4-GalAT or* Xyl-?.... *α1,4-GalAT* (Xylβ1,4-Xylα1, 3-Rhaα1,2-*GalA*-1,4-Xyl)	(129, 263)	Put. GAUT12 *IRX8* At5g54690 (GT8) (129, 132)	
L-RhaT	RG-I	GalAα1,2-L-Rha *α1,4L-RhaT*	(157, 261, 262)	–	–
L-RhaT	RG-II (A)	Api*f*β1,2-GalA β1, 3′-L-*RhaT*	(214, 157)	–	–
L-RhaT	RG-II ©	Kdo2,3GalA *α1,5-L-RhaT*	(214, 157)	–	–
L-RhaT	RG-II (B)	L-Araα1,4-Gal *α1,2-L-RhaT*	(214, 157)	–	–
L-RhaT	RG-II (B)[f]	L-Araα1,4-Gal β1, 3-L-*RhaT*	(158, 161)		
L-RhaT	HG/RG-I	GalAα1,4-GalA *α1,4-L-RhaT*	—	–	–
D-GalT	RG-I	L-Rhaα1,4-GalA *β1,4-GalT*	(157, 261)	–	
D-GalT	RG-I	Galβ1,4-Rha *β1,4-GalT*	(157, 264)	–	–
D-GalT	RG-I	Galβ1,4-Gal *β1,4-GalT*	(157, 190, 207, 264–267)	–	–
D-GalT	RG-I	Galβ1,4-Gal *β1,6-GalT*	(157, 264)	–	–
D-GalT	RG-I/AGP[g]	Galβ1,3-Gal *β1,3-GalT*	(214)	–	–
D-GalT	RG-I/AGP	Galβ1,3-Gal *β1,6-GalT*	(214)	–	–
D-GalT	RG-I/AGP	Galβ1,6-Gal β1,3-Gal *β1,6-GalT*	(214)	–	–
D-GalT	RG-I	L-Ara*f*-1,4-Gal *1-5-GalT*	(268)		
L-GalT	RG-II (A)	GlcAβ1,4-Fuc *α1,2-L-GalT*	(214, 269)	–	–
D-GalT	RG-II (B)	L-Ace*f*A α1,3-Rha *β1,2GalT*	(214, 269)	–	–
L-AraT	RG-I	Galβ1,4-Rha *α1,3-L-ArafT*	(157, 264)	–	–

L-AraT	*RG-I*	L-Arafα1,3-Gal α*1,2-L-ArafT*	(157, 264)	–	–
L-AraT	RG-I	L-Arafα1,2-Araf 1, 5-L-*ArafT*	(157, 264)	–	–
L-AraT	RG-I	L-Rha α1,4-GalA 1,4-*ArafT*	(264)	–	–
L-AraT	RG-I	L-Arafα1,5-Araf α*1,5-L-ArafT*	(214)	*Put. ARAD1* At2g35100 (270)	–
L-AraT	RG-I	L-Arafα1,5-Araf α*1,2-L-Araf*T	(214)	–	–
L-AraT	RG-I	L-Arafα1,5-Araf α*1,3-L-ArafT*	(214)	–	–
L-AraT	RG-I	L-Arafα1,3-Araf α*1,3-L-Araf*T	(214)	–	–
L-AraT	*RG-I*	Galβ1,4-Gal α*1,3-L-ArafT*	(157, 190, 207, 214, 265, 266)	–	–
L-AraT	RG-I	L-Araf-1,3-Gal 1, 5-L-*ArafT*	(190, 207, 265, 266)	–	–
L-AraT	RG-I/AGP	Galβ1,6-Gal α*1,3-L-ArafT*	(214)	–	–
L-AraT	RG-I/AGP	Galβ1,6-Gal α*1,6-L-ArafT*	(214)	–	–
L-Arap*T*	RG-I/AGP	L-Arafα1,5-Araf β1, 3-L-*ArapT*	(271, 272)	–	–
L-Arap*T*	RG-I	Galβ1,4-Gal α*14-L-ArapT*	(273)	–	–
L-AraT	RG-II (D)	Dhaβ2,3-GalA β1,5-L-*ArafT*	(214, 269)	–	–
L-AraT	RG-II (B)	Galβ1,2-L-Acef*A* α*1,4-L-ArapT*	(214, 269)	–	–
L-AraT	RG-II	L-Rhaα1,2-L-Ara β1, 2-L-*ArafT*	(214, 269)	–	–
L-FucT	RG-I	Galβ1,4-Gal α*1,2-L-FucfT*	(157, 264)	–	–
L-FucT	RG-II (A)	L-Rhaβ1,3′-Api*f* α*1,4-L-FucT*	(214, 269)	–	–
L-FucT	RG-II (B)	Galβ1,2-L-AceAf α*1,2-L-FucT*	(214, 269)	–	–
D-Api*f*T	RG-II (A, B)	GalAα1,4-GalA β1,2-Api*f*T	(214, 269)	–	–
D-XylT	RG-II (A)	L-Fucα1,4-L-Rha α*1,3XylT*	(214, 269)	–	RGXT1 At4g01770, RGXT2 At4g01750, (274)
D-XylT	HG	GalAα1,4-GalA β*1,3 XylT*	(185, 186, 193, 207, 263)	–	–
D-XylT	HG	Xylβ1,3-GalA β*1,4 XylT*	(263)	–	–
D-XylT	HG	Xylβ1,4-Xylβ 1,3-GalA β*1,4 XylT*	(263)	–	–

(*continued*)

Table 5.2 (*continued*)

Type of glycosyl-transferase	Parent polymer[a] (side chain)	Enzyme[b] acceptor substrate *Enzyme activity* (unless noted: enzyme adds to the glycosyl residue on the left*)	Ref.[c] for structure	*Putative* gene Identified (Ref.)[d]	Gene identified (Ref.)[e]
D-XylT	HG	Xylβ1,4-Xyl *β1,4 XylT*	(263)	–	–
D-XylT	RG-I	L-Rhaα1,4-GalA *β1,4 XylT*	(275–277)	–	–
D-XylT	RG-I	Xylβ1,4-L-Rhaα1,4-GalA *β1,4 XylT*	(277)		
D-GlcAT	RG-I	Gal..... *β1,6GlcAT*	(278)	*Put. NpGUT1* (181)	
D-GlcAT	RG-I	Gal..... *β1,4GlcAT*	(278)	–	–
D-GlcAT	RG-II (A)	L-Fucα1,4-L-Rha *β1,4GlcAT*	(214, 269)	–	–
D-KDOT	RG-II (C)	GalAα1,4-GalA *α2,3KdoT*	(159, 214, 269)	–	–
D-DHAT	RG-II (D)	GalAα1,4-GalA *β2,3DhaT*	(214, 269)	–	–
L-Acef A	RG-II (B)	L-Rhaβ1,3′-Api*f* *α1,3AceAfT*	(214, 269)	–	–

[a] HG, homogalacturonan; RG-I, rhamnogalacturonan I; RG-II, rhamnogalacturonan II.
[b] All sugars are D sugars and have pyranose rings unless otherwise indicated.
[c] References for the structure.
[d] Put.: putative, indicates that a gene encoding the putative corresponding GT has been identified, but confirmatory functional enzyme activity of the gene has not yet been provided.
[e] References for gene encoding the GT when available.
[f] Glycosyltransferase activity based on the most extended structure of RG-II. The terminal βRha*p* → 3αAra*p* in side-chain B and the terminal βAra*f* → 2αRha*p* in side-chain B are not present in RG-II from all species (159).
[#] Enzyme activity would also be required to synthesize arabinogalactan proteins (AGPs) [see (279)].

Table 5.3 List of non-glycosyltransferases expected to be required for pectin synthesis

Type of transferase	Parent polymer[a]	Enzyme activity	Enzyme acceptor[b] substrate	Ref.[c]	*Putative* gene identified (Ref.)[d]
MethylT	HG	HG-methyltransferase	GalAα1,4-GalA$_{(n)}$	(227, 281, 286, 287)	Put. At1g78240 (*QUA2*) (287)
AcetylT	HG	HG: GalA 3-*O*-acetyltransferase	GalAα1,4-GalA$_{(n)}$	(196–199)	
AcetylT	RG-I	RG-I: GalA-3-*O*/2-*O*-acetyltransferase	GalAα1,2-L-Rhaα1,4$_{(n)}$	(157, 199, 288–290)	
MethylT	RG-I	RG-I: GlcA-4-*O*-methyltransferase	GlcAβ1,6-Gal	(278)	
MethylT	RG-II	RG-II: xylose-2-*O*-methyltransferase	D-Xylα1,3-L-Fuc	(214, 269)	
MethylT	RG-II	RG-II: fucose-2-*O*-methyltransferase	L-Fucα1,2-D-Gal	(214, 269)	
AcetylT	RG-II	RG-II: fucose-acetyltransferase	L-Fucα1,2-D-Gal	(214, 269)	
AcetylT	RG-II	RG-II: aceric acid 3-*O*-acetyltransferase	L-AcefAβ1,3-L-Rha	(214, 269)	

[a] HG, homogalacturonan; RG-I, rhamnogalacturonan I; RG-II, rhamnogalacturonan II.
[b] All sugars are D sugars and have pyranose rings unless otherwise indicated.
[c] Reference is for the enzyme activity, when available.
[d] Put.: putative, indicates that a possible gene encoding the corresponding GT has been identified, but confirmatory functional enzyme activity of the gene has not yet been provided.

5.4.3 Methyltransferases

Many of the pectin polysaccharides contain sugars that have been modified by the addition of a methyl group. For example, HG has carboxymethyl esters on C-6, RG-I contains a GlcA-4-*O*-methylether, and RG-II contains 2-*O*-methylfucose. Multiple methyltransferases are required to synthesize these polysaccharides, although it is not known if the methyl group is added after the sugar is inserted into the polymer, or rather on the nucleotide-sugar donor. The available evidence suggests that SAM is the methyl donor (227, 280–285). Table 5.3 lists the non-glycosyltransferases "required" for the synthesis of pectin.

5.4.4 Acetyltransferases

Several of the pectic polysaccharides contain sugars modified by acetylation. These include acetylation of the O-3 position of GalA in HG and either O-2 or O-3 acetylation on the GalA in the RG-I backbone. In addition, RG-II contains acetylated Fuc and 3-*O* acetylated aceric acid. The acetyltransferases have not been identified.

5.4.5 Other pectin modifying enzymes

Some plant cell wall polysaccharides contain small amounts of ester-linked hydroxycinnamic acid derivatives such as *p*-courmaric and ferulic acid (291). These ester-linked acid

derivatives can undergo oxidative coupling to form dehydrodimers that may lead to cell wall polysaccharide cross-linking (291, 292) and may also be involved in the formation of polysaccharide–lignin complexes (293, 294). The ester-linked hydroxycinnamic acids are more abundant in the walls of the monocotyledonous group known as the commelinids, including the grass family (295) and such walls are particularly rich in hydroxycinnamic acids linked to the hemicellulose arabinoxylan (291), and to a lesser extent the hemicellulose xyloglucan. However, ester-linked hydroxycinnamic acids have also been shown to be linked to pectins in plants such as spinach and sugar beet (291, 296–298). The types of linkages associated with pectin include the following feruloylated arabinan and (1→4)-linked D-galactosyl oligosaccharides that presumably originate from side chains from RG-I: *O-(6-O-trans-feruloyl)-β-D-galactopyranosyl-(1→4)-D-galactose*, *O-(2-O-trans-feruloyl)-α-L-arabinofuranosyl-(1→5)-L-arabinose*, and *O-α-L-arabinofuranosyl-(1→3)-O-(2-O-trans-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinose* [see (291) and references therein] and *O-[5-O-(feruloyl)-Ara]-(1→5)-[2-O-(feruloyl)-Ara]-(1→5)-Ara* (299).

The mode of synthesis of feruloylated hemicellulose and pectin has received some, albeit, limited study. There is evidence that hemicellulosic arabinoxylan can be feruloylated by both feruloyl-glucose and feruloyl-CoA precursors/substrates, although the precise role of these substrates in the synthesis of feruloylated arabinoxylan within the cell (likely in the Golgi) or in the cell wall remains unclear (300, 301). A Golgi or sub-Golgi fraction from parsley suspension-cultured cells was able to transfer ferulic acid from feruloyl-CoA onto endogenous polysaccharide acceptors. However, since the identity of the polysaccharide(s) that was feruloylated was not determined (302), it is not clear whether the enzyme activity identified was involved in pectin or hemicellulose feruloylation.

Recently, Mitchell and coworkers (303), using a bioinformatics approach to identify genes highly expressed in cereals during the late stages of arabinoxylan synthesis, identified cereal Pfam family PF02458 genes, members of the CoA-acyl transferase superfamily, as candidate feruloyltransferases. However, enzymatic confirmation that these genes are actually involved in feruloylation has not been presented.

5.4.6 Homogalacturonan synthesis

Homogalacturonan (HG) is the most abundant pectic polysaccharide. It is a homopolymer of 1,4-linked α-D-galactosyluronic acid that is partially methylesterified and may be *O*-acetylated at O-2 or O-3. As mentioned above, the distribution of methylesters in HG during synthesis or in the wall is not known (157), although there is evidence that the distribution of non-esterified galacturonosyl residues in the wall is not random (195). The degree of polymerization (DP) of HG, as well as the question of whether it is linear, or may be branched or cross-linked (204) remains a matter of debate. However, the DP has been estimated to range from 72 to 100 (304) or more (214). The synthesis of HG requires at least one HG:α1,4-galacturonosyltransferase (HG-GalAT) (Table 5.2), at least one HG-methyltransferase (HG-MT) (also referred to as pectin methyltransferase), and at least one HG:*O*-acetyltransferase (HG-AT).

5.4.6.1 HG:galacturonosyltransferase (HG:GalAT)

The only HG biosynthetic glycosyltransferase for which enzymatic function of the encoded gene has been established is the HG:GalAT called GAUT1 (Galacturonosyltransferase 1;

At3g61130) (137). The identification of GAUT1 followed an extensive study of HG:α1,4GalAT activity in multiple plant species including in mung bean (305–308), tomato (306), turnip (306), sycamore (309), tobacco suspension (310–312), radish roots (205), pea (230, 313), Azuki bean (*Vigna angularis*) (314), petunia (315), and Arabidopsis (137, 316) (see Table 5.4). This GalAT activity was shown to be particulate (i.e., membrane bound) in all species studied and was measured as the transfer of GalA from UDP-GalA onto endogenous acceptors. The GalAT activity in pea was localized to the Golgi (230) with its catalytic site facing the lumenal side of the Golgi (230), providing the first direct enzymatic evidence that the synthesis of HG occurs in the Golgi.

HG:GalAT activity in microsomal membranes, measured using UDP-[^{14}C]GalA (317, 318), incorporates the GalA moiety onto endogenous acceptors to yield relatively large molecular mass labeled products of ~105 kDa in tobacco microsomal membranes (310) and ≥500 kDa in pea Golgi (230). Cleavage of the radiolabeled product into GalA, digalacturonic acid (diGalA), and trigalacturonic acid (triGalA) by a purified endopolygalacturonase demonstrated that the product was HG (310). In tobacco, the product produced in vitro in microsomes was ~50% esterified (310) while the product produced in pea Golgi was less esterified (230), suggesting that the degree of methyl esterification of newly synthesized HG may be species-specific and that methylesterification may occur after the synthesis of at least a short stretch of HG. GalAT activity can also be studied in detergent-permeabilized microsomes to allow access of the enzymes to exogenous pectic acceptors. For example, detergent-permeabilized microsomes from etiolated azuki bean seedlings transfer [^{14}C]GalA from UDP-[^{14}C]GalA onto acid-soluble polygalacturonate (PGA) exogenous acceptors (314). The azuki bean enzyme exhibited a broad pH range of 6.8–7.8 and a surprisingly high-specific activity of 1300–2000 pmol mg^{-1} min^{-1}, considering the large amount (3.1–4.1 nmol mg^{-1} min^{-1}) of polygalacturonase activity that was also present in the microsomal preparations.

Success in identifying the gene encoding a GalAT required solubilizing GalAT activity from membrane preparations so as to facilitate purification of the enzyme. The first solubilization of an HG:GalAT was achieved with tobacco GalAT (311). Detergent-solubilized GalAT adds GalA onto the non-reducing end (312) of exogenous HG with a preference for HG oligosaccharides (oligogalacturonides; OGAs) of a DP of greater than 9 (311, 315), although OGA acceptors as small as a trimer can be used (315, 319). Although detergent-solubilized GalAT can use polymeric pectin substrates such a polygalacturonic acid and pectin, such polymers are less favorable substrates (314).

Studies carried out under conditions that provide information regarding the mode of elongation of the OGAs by GalAT, i.e., with excess OGA acceptor to UDP-GalA ratios (320), suggest that solubilized tobacco, radish and *Arabidosis* enzymes, and permeabilized pea Golgi galacturonosyltransferase, have a distributive (non-processive) mode of action in vitro (230, 311, 321). Under these conditions, the bulk of the HGA elongated in vitro by solubilized GalAT from tobacco membranes (311), or detergent-permeabilized Golgi from pea (230), is elongated by a single GalA residue. As expected, as the UDP-GalA:OGA ratio is increased, the OGA products become progressively longer (137), but it is important to note that this, in itself, does not denote processivity, it simply means that the enzyme can use the product of a previous catalytic event as a substrate for a subsequent catalytic event. Interestingly, the membrane-permeabilized galacturonosyltransferase activity reported from pumpkin may represent a processive mode of elongation since reactions containing approximately equimolar amounts of UDP-GalA and acceptor yielded a population OGAs elongated by up

Table 5.4 Comparison of catalytic constants and pH optimum of HG-α1,4-GalATs[a,b]

Enzyme[b]	Plant source	Apparent K_m for UDP-GalA (μM)	pH optimum	V_{max} (pmol mg^{-1} min^{-1})	Ref.
GalAT[a]	Mung bean	1.7	6.0	~4700	(307)
GalAT	Mung bean	n.d.[c]	n.d.	n.d.	(322)
GalAT	Pea	n.d.[c]	6.0	n.d.	(313)
GalAT	Pea	n.d.	n.d.	n.d.	(230)
GalAT	Sycamore	770	n.d.	?	(309)
GalAT	Tobacco	8.9	7.8	150	(310)
GalAT (sol)[d]	Tobacco	37	6.3–7.8	290	(311)
GalAT (per)[e]	Azuki bean	140	6.8–7.8	2700	(314)
GalAT (sol)	Petunia	170	7.0	480	(315)
GalAT (per)	Pumpkin	700	6.8–7.3	7000	(319)
GalAT (sol)	Arabidopsis	n.d.	n.d.	n.d.	(316) (137)

[a] Adapted from Refs. (192, 205).
[b] Unless indicated, all enzymes are measured in particulate preparations.
[c] n.d., not determined.
[d] (sol): detergent-solubilized enzyme.
[e] (per): detergent-permeabilized enzyme.

to five galacturonosyl residues (319). However, further information on the size distribution of the OGAs produced in reactions containing excess acceptor will be required to confirm this. Solubilized petunia galacturonosyltransferase, in reactions containing ~60-fold excess UDP-GalA to OGA acceptor, added up to 27 galacturonosyl residues onto the OGA acceptors (315), indicating that the enzyme can elongate OGA products from a previous reaction, but not specifically addressing the mode of elongation of the enzyme (320). The apparent lack of in vitro processivity of the solubilized GalAT suggests either that enzyme does not synthesize HG in a processive manner in vivo, or that the characteristics of HG-GalAT measured in vitro may be an artifact due to the dissociation of a required biosynthetic complex or loss of cofactor(s) or substrate(s) during solubilization of the enzyme. We have been unable to obtain evidence for processive in vitro solubilized GalAT activity (i.e., the production of extensively elongated acceptors under reaction conditions with excess OGA acceptor to UDP-GalA) (Quigley and Mohnen, unpublished results) [see (192)]. We also obtained no evidence that the inclusion of the methyl donor *S*-adenosylmethionine (308, 310, 314) and/or the acetyl donor acetylCoA promote the processivity of GalAT (192) (unpublished results). Clarification of the mode of action of GalAT and the mechanism of HG synthesis should be aided by access to purified or recombinantly expressed enzyme(s), and may require isolation of enzyme complexes (see below).

Efforts to purify GalAT activity from tobacco proved unsuccessful due to loss of activity. Therefore, a partial purification and tandem mass spectrometry approach was used to identify a gene encoding GalAT activity. The detergent-solubilized HG:GalAT from Arabidopsis suspension cells was partially purified by column chromatography to yield an enriched fraction containing approximately 20 protein bands. The proteins were trypsinized, the polypeptides analyzed for amino acid sequence by tandem mass spectrometry (137), and the sequences compared against the Arabidopsis gene database. The partially purified active

GalAT fraction contained two proteins with sequences that identified them as putative glycosyltransferases which were eventually named GAUT1 and GAUT7 (137). Evaluation of their amino acid sequences indicated that both GAUT1 and GAUT7 had characteristics consistent with the biochemical properties of HG:GalAT: a basic PI and an apparent Type II membrane protein topology. The encoded proteins consisted of three domains: a short N-terminal region, a single membrane spanning region, and a larger C-terminal domain. Transient expression of N-terminal truncated forms of the coding regions of GAUT1 and GAUT7 in human HEK293 cells indicated that GAUT1 had GalAT activity. Thus, GAUT1 became the first enzymatically verified HG:galacturonosyltransferase (*GA*lacat*U*ronosyl*T*ransferase 1, GAUT1). The transiently expressed truncated from of GAUT7 did not have GalAT activity.

Sequence comparison of GAUT1 against the Arabidopsis genome identified 24 additional Arabidopsis genes with high sequence similarity to GAUT1. We named this 25-member group of related genes the GAUT1-related gene family (Table 5.5). The GAUT1-related genes represent a subclass of the Arabidopsis CAZy family GT-8 genes. Fifteen of the GAUT1-related genes have high sequence similarity to GAUT1 (37–100% identity/56–100% similarity) and we named these genes GAUT1–GAUT15. The 15 GAUT proteins have predicted masses of 61–78 kDa and most encode proteins with a predicted membrane anchor (GAUTs 1, 6–15) or with a signal peptide (GAUTs 3–5) consistent with a Type II membrane topology or with an intramicrosomal membrane location, respectively. GAUT2 is the only GAUT not predicted to be present in, or pass through, the intracellular membrane transport system (*i.e., the ER/Golgi system*). *The remaining 10 GAUT1-related genes have somewhat lower* sequence similarity to GAUT1 (39–44% identity/43–53% similarity) and we named these the GAUT1-Like (GATL) genes. The GATL genes encode predicted 33–44 kDa proteins with predicted signal peptides. The proven location of GAUT1 and GAUTs 3, 7, 8, and 9 is in the Golgi (323, 324).

Multiple T-DNA insert mutants of many of the GAUT and GATL genes are available (e.g., see http://signal.salk.edu/cgi-bin/tdnaexpress) and several mutants have described phenotypes. The *qua1/gaut8* mutant has ~25% reduced amounts of GalA in the walls of Arabidopsis rosette leaves or total plants (135), ~30% reduced GalA levels in walls of stem (260), and modest reductions in the GalA content of suspension cultured cells (325), suggestive of a role of the mutated gene as a putative GalAT involved in pectin synthesis. However, the mutant walls are also reduced in Xyl (at least stem walls) and protein extracts from mutant stems have both reduced HG:α-1,4-GalAT activity and β-1,4-xylosyltransferase activity (260). The lack of a confirmed enzyme activity of the recombinantly expressed or purified GAUT8 protein, along with the pleiotropic effects of the mutant, have made a conclusive identification of the function of the GAUT8 protein elusive.

The *parvus/glz1/gatl1* mutants (136, 326) also have characteristics consistent with a defect in pectin synthesis. The *parvus/glz1/gatl1* mutants grown under low humidity are semi-sterile dwarfs that have reduced anther dehiscence. The *parvus* mutants also have slightly elevated Rha, Ara, and Gal and reduced Xyl compared to WT (136). These changes in neutral sugar compositions are consistent with a role of the parvus gene in the synthesis of the pectic polysaccharide RG-I. However, since the levels of GalA in the mutant walls were not determined, it is not known if the mutant walls are altered in GalA content.

We PCR-tested 57 Arabidopsis GAUT1-related gene family T-DNA insertion mutant lines from the T-DNA mutant collection (http://signal.salk.edu/cgi-bin/tdnaexpress) and identified 36 homozygous mutant lines (Caffall and Mohnen, unpublished). Glycosyl residue

Table 5.5 The arabidopsis GAUT1-related gene superfamily

Gene[a]	Accession no.[b]	Amino acid identity/ similarity to GAUT1[c]	Clade	Enzyme activity confirmed	Putative enzyme activity	Mutants
GAUT1/JS36/LGT1	At3g61130	100/100	GAUT-A	HG:GalAT		
GAUT2/LGT2	At2g46480	65/78	GAUT-A			
GAUT3	At4g38270	68/84	GAUT-A			
GAUT4/JS36L/LGT3	At5g47780	66/83	GAUT-A			
GAUT5/LGT5	At2g30575	45/67	GAUT-A			
GAUT6	At1g06780	46/64	GAUT-A			
GAUT7/JS33/LGT7	At2g38650	36/59	GAUT-A			
GAUT8/QUA1	At3g25140	58/77	GAUT-B		Put. HG:GalAT and/or Put. β1,4-XylT	*qua1*
GAUT9	At3g02350	57/76	GAUT-B			
GAUT10/LGT4	At2g20810	50/72	GAUT-B			
GAUT11	At1g18580	51/71	GAUT-B			
GAUT12/LGT6/IRX8	At5g54690	40/61	GAUT-C		Put. HG:GalAT or Put. xylan primer GalAT	*irx8*
GAUT13	At3g01040	43/62	GAUT-C			
GAUT14	At5g15470	43/62	GAUT-C			
GAUT15	At3g58790	37/56	GAUT-C			
GATL1/PARVUS/GLZ1	At1g19300	29/49	GATL		*parvus/glz1*	
GATL2	At3g50760	27/52	GATL			
GATL3	At1g13250	23/43	GATL			
GATL4	At3g06260	29/51	GATL			
GATL5	At1g02720	25/44	GATL			
GATL6/LGT10	At4g02130	29/52	GATL			
GATL7	At3g62660	29/51	GATL			
GATL8/LGT9	At1g24170	23/42	GATL			
GATL9/LGT8	At1g70090	27/48	GATL			
GATL10	At3g28340	28/53	GATL			

[a] The name given to each member of the GAUT1-related gene family includes its designation within the LGT family (332) and the names of any characterized Arabidopsis gene mutants (73, 74, 129, 132, 135, 136, 326). The numbering of the GAUT and GATL genes is based on the phylogenetic analysis of the family [see (137)].
[b] From the Arabidopsis Information Resource database or NCBI.
[c] Sequence identity/similarity is compared to 397 amino acids of GAUT1 starting at amino acid position 277.

composition analyses were carried out on amylase-treated walls from 82 unique tissue samples from homozygous mutants of 13 of the 15 GAUT family members by combined gas chromatography/mass spectrometry (GC/MS) of per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides (327). Tissues from mutant lines of 8 of the 13 GAUT1-related family genes tested had ≥15% reduction in the amount of galacturonic acid

in their walls, consistent with a function of the mutated genes as GalATs. However, a detailed biochemical analysis of the enzyme activity of each of the GAUTs will be required to establish gene function. Interestingly, some mutants also accumulated higher amounts of arabinose, rhamnose, fucose, and xylose and reduced amounts of mannose, galactose and/or glucose. The significance of these correlative changes with reduced levels of GalA is not yet clear; however, we speculate that the reduction of one pectic polysaccharide (e.g., HG) may be associated with, or compensated for, by a change in the amount of another polysaccharide (e.g., loss of HG may lead to an increase in RG-I which has Rha, Ara and/or Gal; alternatively, loss of an RG-I biosynthetic GalAT could lead to loss of RG-I and to an increase in HG with a resulting increase in GalA and a reduction in Rha, Ara and and/or Gal). Such a compensation of one wall polymer for another (328, 329) could occur via several mechanisms including sensing of polysaccharide or nucleotide-sugar levels, or the covalent or non-covalent association of one type of polysaccharide with another (e.g., it has been reported that pectin may be covalently linked to the hemicellulose xyloglucan (107).

Although GAUT1 encodes a GalAT, its function in pectin synthesis is far from clear. For example, it is not yet understood at what stage of pectin synthesis, i.e. initiation or elongation, GAUT1 has its primary role. Also, it is not clear whether GAUT1 functions alone or in a complex. Our preliminary results suggest that, at least in vitro, GAUT1 can function in a complex with at least one other GAUT (Atmodjo and Mohnen, unpublished). The enzyme function of the other GAUT and GATL members of the GAUT1-related gene family remains to be determined. QUA1/GAUT8 is a good candidate for a GalAT, but the reduced levels of Xyl and xylosyltransferase activity of the qua1 mutants, at least in stem tissue, raises the question of what the connection is between pectin (e.g., HG) and hemicellulose (i.e., xylan) synthesis). The pectins and hemicelluloses are traditionally considered to be two different classes of wall polysaccharides, although there is some evidence that these two classes may be tightly, and possibly covalently, linked in the wall (258, 332, 333). Thus, the characteristics of the *qua1* mutant raise several questions. Is xylan synthesis dependent upon pectin synthesis? The characteristics of the *parvus* mutant and the GATL proteins are also intriguing. What is the function of the GATLs? The characteristics of the *parvus* mutant are consistent with a function in pectin synthesis; however, these proteins have no apparent membrane spanning domain but rather a signal peptide. If the GATLs are involved in pectin synthesis, do they interact with other lumenal pectin biosynthetic enzymes in the form of Golgi-localized complexes? The identification of GAUT1 as a functional GalAT and of the GAUT1-related gene family provides the gene/protein tools required to address some these questions.

Recently, characterization of mutants of GAUT12/IRX8 have provided evidence supporting a possible role of GAUT12/IRX8 as a HG:GalAT involved in the synthesis of a subfraction of HG to which β-1,4-xylan is attached (132) or as a putative α1,4-GalAT that adds a GalA onto a xylose at the reducing end of a xylan primer (129). Proof of the function of GAUT12, however, requires confirmation of enzyme activity.

5.4.6.2 HG-methyltransferase

HG-methyltransferase (HG-MT) catalyzes the methylesterification of HG at the C-6 carboxyl group of some GalA residues by transfer of a methyl group from *S*-adenosylmethionine to HG. The name HG-MT is preferred, rather than the former name pectin

methyltransferase, to distinguish HG-MT from the enzymes that methylate RG-I or RG-II. HG-MT activity has been identified in microsomal membranes from mung bean (280, 281, 283), flax (227, 282), tobacco (284), and soybean (285). Membrane-bound HG-MTs from flax (333, 334), and tobacco (335) could be solubilized using detergent. HG-MT has been localized to the Golgi (227–229, 336) and its catalytic site has been shown to face the Golgi lumen (229). In vitro biochemical studies showing that UDP-GalA stimulates HG-MT activity in intact membrane vesicles (284, 308) and that polygalacturonic and pectin can function in vitro as acceptors for HG-MT in detergent-permeabilized membranes support a model in which at least a small stretch of HG is synthesized prior to its methylation by HG-MT in the Golgi. The observation that some of the HG-MTs in detergent-permeabilized membranes from flax and soybean show a preference for partially esterified pectin (228, 285, 337) over polygalacturonic acid further suggest that multiple HG-MTs may exist that differ in their specificity for HG of differing degrees of methylation. The question of whether such HG-MTs are preferentially involved in the initial methylation of HG or in the methylation of more highly esterified HG remains to be resolved. The observation that overexpression of an Arabidopsis S-adenosylmethionine (SAM) synthetase gene in flax leads to a concomitant increase in SAM synthetase activity and of pectin methylesterification in the wall, with no increase in HG-MT activity, suggests that the degree of methylesterification of HG may be regulated, at least in part, at the level of substrate (i.e., SAM) concentration (338).

The gene encoding HG-MT has not yet been unambiguously identified. Two apparent HG-MT isozymes, PMT5 and PMT7, were reported from flax (337) and efforts to purify these apparent isozymes resulted in the identification of an additional small polypeptide with HG-MT activity designated PMT18. The definitive identification of one or more of these polypeptides as HG-MT has not yet been reported. Thus, the proposition that the 18-kDa protein is a subunit of the 40- and 110-kDa proteins (337) has not been substantiated.

Recently, Mouille and colleagues (287) have identified a putative methyltransferase as the gene mutated in the Arabidopsis mutant *quasimodo2. Qua2-1* plants are dwarfed and have a 50% reduction in HG. Although confirmation of enzyme activity of QUA2 is required to establish if it indeed encodes an HG-MT, the reduced HG phenotype of *qua2* plants along with the Golgi localization of QUA2–GFP fusions and the putative methyltransferase domain in QUA2 are consistent with a role as an HG-MT. Further work on QUA2 and the 29 QUA2-related proteins in Arabidopsis may shed light on the identity of multiple methyltransferases required for pectin synthesis.

5.4.6.3 HG-acetyltransferase (HG-AT)

HG may be partially *O*-acetylated at C-2 or C-3 of GalA (198, 199). No gene for HG acetyltransferase has been identified; however, *O*-acetyltransferase activity in microsomes from suspension-cultured potato cells (339) has been shown to transfer [^{14}C]acetate from [^{14}C]acetyl-CoA onto endogenous acceptor in the microsomes to yield a salt/ethanol precipitable product from which approximately 8% of the radioactivity could be solubilized by treatment with endopolygalacturonase and pectin methylesterase. Such results could indicate the presence of HG-AT, although the possibility that the radiolabeled acetate was transferred either onto RG-II or RG-I that was solubilized by the glycanase treatments, and thus represents an enzyme that acetylates one of the other pectic polysaccharides that may be covalently linked to HGA, cannot be ruled out.

5.4.7 Xylogalacturonan synthesis

HG may contain regions that are substituted with β-D-xylose linked to C-3 of GalA (185, 186, 193, 267). Such regions of xylosylated HG are referred to as xylogalacturonan (XGA) and have been most frequently identified in reproductive tissues of plants including apple (184, 193, 197), cotton and watermelon (185), and pea (342), but also in carrot (186). However, xylogalactruonan has also been detected in Arabidopsis leaves and stems (187), albeit it in lower levels than in reproductive and storage tissues such as apple and potato. No gene for XGA:xylosyltransferase (XGA:XylT) has been unambiguously identified. XGA:XylT activity was identified in studies of apiogalacturonan synthesis (341, 342). Although the product produced was not characterized in detail, at least some of the radioactive xylose appeared to be incorporated into apiogalacturonan and/or HG.

Interestingly, Nakamura and coworkers (263) has reported that in soybean some XGA may be further elongated with β-1,4-linked xylose residues yielding β-1,4-xylans of up to seven xylosyl residues in length. Such results suggest that HG may, at least in soybean, be a primer or acceptor for a glycosyltransferase or a transglycosylase that establishes a link between pectin and the hemicellulose xylan. Such a linkage would be consistent with the characteristics of the *Qua1* mutant (mutated in GAUT8) (260) and the *irx8* mutant (mutated in GAUT 12) (132).

5.4.8 Apiogalacturonan synthesis

5.4.8.1 Apiogalacturonan-galacturonosyltransferase (AP:GalAT)

It is not known whether apiogalacturonan is synthesized on preexisting HG that is synthesized by GAUT1 or related GalATs, or whether a unique GalAT is responsible for apiogalacturonan synthesis. There have been no reports of efforts specifically targeted at identifying the apiogalacturonan:GalAT.

5.4.8.2 Apiogalacturonan-apiosyltransferase (AP:ApiT)

Apiogalacturonan is a substituted galacturonan that is produced in some aquatic monocotyledonous plants (188, 189) and that consists of HG substituted at O-2 or O-3 with apiose or apiobiose (D-Api*f*-β-1,3-D-apiose) (188, 189). The anomeric configuration of the linkage of apiose to HG may be in the β configuration (189). It is not known whether the same apiogalacturonan:ApiTs synthesize RG-II (see below) and apiogalacturonan. For example, RG-II has two of its four side branches attached to an HG backbone by a β-Api*f* linked to the O-2 of HG (158), and thus, the possibility exists that the β1,2-apiosyltransferase involved in RG-II synthesis may also synthesize apiogalacturonan. In vivo synthesis of apiogalacturonan has been studied in vegetative fronds of *Spirodela polyrrhiza* (343) and D-apiosyltransferase activity has been characterized in cell-free particulate preparations from duckweed (*Lemna minor*) (341). The apiosyltransferase in particulate membrane preparations from *Lemna* transfers [^{14}C]-apiose from UDP-[^{14}C]-apiose onto endogenous acceptors. The enzyme has an apparent K_m for UDP-apiose of 4.9 μM and a pH optimum of 5.7 (341). Since, the rate of apiosyltransferase activity increased twofold when UDP-GalA was added to the reaction

(341) and the product synthesized in the presence of UDP-GalA bound anion exchange resin more tightly than the product synthesized without UDP-GalA (342), it is likely that the apiosyltransferase transfers apiose onto a growing HG chain. The ApiT has not been purified and the gene has not been identified.

5.4.9 Synthesis of rhamnogalacturonan II (RG-II)

RG-II is the most structurally complicated polysaccharide in the cell wall. It is present in the walls of all plants and its structure is highly conserved. RG-II makes up approximately 4% of the cell wall in dicotyledonous plants and less than 1% of the wall in monocots (157). It contains 12 different types of glycosyl residues in at least 20 different linkages (159) including both methyl etherified (e.g., 2-*O* Me-xylose and 2-*O* Me-fucose) and *O*-acetylated glycosyl residues (e.g., 3-*O*-, or 4-*O*-Ac-fucose). RG-II also contains unusual sugars such as aceric acid (3-*C*-carboxy-5-deoxy-L-xylose) (346), KDO (2-keto-3-deoxy-D-*manno*-octulopyranosylonic acid (345), and DHA (3-deoxy-D-*lyxo*-2-heptulopyranosylaric acid) (346). RG-II has a backbone of α-1,4-linked D-galactosyluronic acid with structurally complex side chains attached to C-2 and/or C-3 (2, 157, 159, 269, 345–352). RG-II in the wall exists largely complexed with borate as an RG-II dimer that is cross-linked by a borate diester (159, 352–357). At least 24 transferase activities are expected to be required for RG-II synthesis. There have been very few systematic studies of RG-II synthesis, although progress is beginning to be made on several enzymes.

5.4.9.1 RG-II:galacturonosyltransferase (RG-II:GalAT)

It is not known whether RG-II is synthesized on preexisting HG that is synthesized by GAUT1 or related GalATs, or whether a unique GalAT is responsible for synthesizing the HG backbone of RG-II. It is also not know if the side chains of RG-II are synthesized as individual oligosaccharides and transferred in bulk onto HG, or whether individual glycosyltransferases transfer each distinct glycosyl residue individually onto the growing non-reducing end of the maturing RG-II molecule. The results of Egelund *et al.* (274) on the proposed RG-II:XylTs would be in agreement with the latter model (*see below*).

5.4.9.2 RG-II:apiosyltransferase (RG-II:ApiT)

As mentioned above it is possible that the apiosyltransferase activity identified during the studies of apiogalacturonan synthesis in *Lemna* is involved in RG-II synthesis. However, no gene for RG-II ApiT has been identified. Interestingly, when the gene reported to encode the UDP-apiose or UDP-D-apiose/UDP-D-xylose synthase was downregulated in *Nicotiana benthamiana* by virus-induced gene silencing of *NbAXS1*, the result was a reduction in the amount of RG-II in the walls (358). These results provide evidence that UDP-apiose is the substrate for incorporation of apiose into RG-II and that the UDP-D-apiose/UDP-D-xylose synthase gene encodes the enzyme that synthesizes the required substrate for RG-II synthesis.

5.4.9.3 RG-II:xylosyltransferase (RG-II:XylT)

Recently, work from the Ulvskov and Geshi groups (274) has provided strong evidence that these investigators have identified two *Arabidopsis thaliana* RG-II-α-D-1,3-xylosyltransferases (RG-II-α1,3XylTs) (274). Following the identification of a novel family of 27 putative *Arabidopsis thaliana* glycosyltransferases (215) and through a series of bioinformatic analyses aimed at identifying novel plant cell wall biosynthetic glycosyltransferases with a predicted Type II membrane topology (359), two of these genes, named *RGXT1* (At4g01770) and *RGXT2* (At4g01750) were shown to encode proteins with characteristics consistent with a function as RG-II-α1,3XylTs. *RGXT1* and *RGXT2* encode proteins of 361 and 367 amino acids, respectively, share 90% sequence identity, and are members of GT-family 77 (138) (http://afmb.cnrs-mrs.fr/CAZY/). Two additional Arabidopsis genes, At4g01220 and At1g56550, are 68–75% identical to *RGXT1* and *RGXT2* (274). Expression of truncated soluble forms of RGXT1 and RGXT2 in baculovirus-transfected insect cells and enzyme assays using diverse radiolabeled nucleotide-sugars and free monosaccharide acceptors demonstrated that the expressed proteins catalyze the transfer of Xyl from UDP-α-D-Xyl onto fucose. Biochemical analyses of the synthesized product using specific xylosidases and NMR spectroscopy indicated that the xylose was transferred onto the fucose in an α-1,3-linkage. Based on these results the authors hypothesized that *RGXT1* and *RGXT2* function in the synthesis of the RG-II side chain A that contains 2-*O*-methyl-D-Xyl attached in an α1,3-linkage to α-L-Fuc. Acceptor specificity studies demonstrated that both enzymes preferred L-Fuc with an α-anomeric linkage and disaccharide acceptors with Fuc attached at the position 4, rather than at the 2 or 3 position, to another glycosyl residue; all characteristics consistent with the structure of RG-II (159). Importantly, RG-II isolated from *RGXT1* and *RGXT2* mutant walls, but not RG-II from wild type Arabidopsis walls, served as an acceptor for the enzyme, providing strong evidence that *RGXT1* and *RGXT2* function in RG-II synthesis (274) and providing strong support for the function of *RGXT1* and *RGXT2* as RG-II-α1,3XylTs. The lack of a clear difference in the structure of RG-II isolated from walls of *RGXT1* and *RGXT2* mutants compared to wild type walls, however, is perplexing and leaves open the question of whether there is gene redundancy, thus requiring a double (or more) gene knockout mutant to see a phenotype. Alternatively, the question remains as to whether *RGXT1* and *RGXT2* may have additional or alternative functions in the synthesis of some other, yet to be identified, wall polysaccharide structure. Further studies of *RGXT1* and *RGXT2* and related genes should clarify their role(s) in pectin synthesis.

5.4.9.4 RG-II:glucuronosyltransferase (RG-II:GlcAT)

Studies of the *Nicotiana plumbaginifolia* T-DNA nolac-H18 callus mutant lead to the identification of the mutated gene, *NpGUT1*. *NpGUT1* has 60% sequence homology to animal glucuronosyltransferases that synthesize heparin sulfate. Complementation of the nolac-H18 mutant with the *NpGUT1* gene corrected the non-organogenesis and weak intercellular attachment phenotypes of the mutant. Cell walls of the nolac-H18 mutant contained 86% reduced levels of glucuronic acid, a reduction that was associated with the pectin-enriched fraction of the walls. RG-II from mutant walls was devoid of glucuronic acid, leading the authors to propose that *NpGUT1* encodes RG-II-β-1,2GlcAT that transfers GlcA onto the L-fucose in RG-II side chain A (181). The mutant RG-II in the nolac-H18 showed 82% reduced

RG-II dimer formation, providing further support that RG-II is modified in the mutant. Taken together these results show that *NpGUT1* encodes a putative RG-II:β-1,2GlcAT. Enzymatic confirmation of the activity of the encoded protein has not yet been reported.

5.4.9.5 RG-II:methyltransferase (RG-II:MT)

RG-II contains 2-*O*-methylfucose and 2-*O*-methylxylose. It is not known if the methyl group is added at the stage of the nucleotide-sugar or after the sugar is added to RG-II. The genes encoding the methyltransferases have not been identified.

A pectin methyltransferase activity detergent-solubilized from suspension-cultured flax cells was able to transfer methyl groups from *S*-adenosylmethionine onto RG-II isolated from wine (334). Enzyme reactions containing RG-II had sevenfold great methyltransferase activity than reactions without exogenous acceptor and the radiolabeled product synthesized had a size similar to RG-II monomers and RG-II dimers (158, 334). It was not established where in RG-II the methyl group was added and thus, the methylation may have represented methylesterification of the HG backbone of RG-II, or alternatively, could have been due to methyletherification of RG-II since RG-II contains methyl groups on non-galacturonic glycosyl residues (e.g., 2-*O*-methyl xylose and 2-*O*-methyl fucose (352, 360) of side chain residues. The location of the methylation in RG-II and the identity of the potentially novel enzyme activity that catalyzes its incorporation into RG-II have not been established.

5.4.9.6 RG-II:acetyltransferase (RG-II:AT)

RG-II contains 3-*O*-acetylaceric acid and acetylated methyl fucose. No gene for RG-II acetyltransferase has been identified. However, *O*-acetyltransferase activity in microsomes from suspension-cultured potato cells (339) has been shown to transfer [^{14}C]acetate from [^{14}C]acetyl-CoA onto endogenous acceptors in the microsomes to yield a salt/ethanol precipitable product from which approximately 8% of the radioactivity could be solubilized by treatment with endopolygalacturonase and pectin methylesterase. Thus, it is possible that the radiolabeled acetate solubilized by the glycanase treatments represented acetylated RG-II and that the activity identified was RG-II acetyltransferase.

5.4.9.7 Other RG-II transferases

The glycosyltransferases that insert fucose, KDO, DHA, and aceric acid into RG-II have not been identified. A 10-member Arabidopsis gene family (118) that has 35–73.8% amino acid sequence identify to an Arabidopsis α1,2-fucosyltranferase that fucosylates a side branch in the hemicellulose xyloglucan has been described (116). Whether one or more of these genes encodes fucosyltransferase(s) involved in RG-II synthesis remains to be investigated.

5.4.10 Rhamnogalacturonan I (RG-I) synthesis

RG-I accounts for 7–14% of the primary wall (157) and 20–33% of pectin (194). Unlike HG and RG-II, RG-I has a backbone of up to 100 repeats of the disaccharide [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→] (2, 157, 261, 262, 361, 362). The GalA residues in RG-I

may be *O*-acetylated at C-3 or C-2 (157, 199, 288–290). The average molecular weight of sycamore RG-I has been reported to be 10^5–10^6 Da (157). Between 20 and 80% of the rhamnosyl residues are substituted at C-4, and sometimes at C-3, with side chains composed mostly of arabinosyl and/or galactosyl residues (2, 157, 264), referred to as galactans (157, 264, 278), arabinans (214, 264, 290), and arabinogalactans (2, 157, 190, 214, 264). These side chains may range in size from 1 to 50 or more glycosyl residues (2, 157, 290). A large amount of immunocytochemical evidence based on antibodies against specific RG-I carbohydrate epitopes (160) indicates that the precise structure of the side chains of RG-I varies in a cell type and development-specific manner (214, 363). Representative side chains or portions of side chains that have been identified in RG-I have been previously summarized in (192).

The RG-I galactans may contain only galactosyl residues or may contain other neutral glycosyl residues (157) or acidic residues such as GalA (190), GlcA (157, 190, 278), or 4-*O*-methylGlcA (278). Some galactans also have β-1,6-branching (190). As mentioned above, the size and linkages in the galactan side branches of RG-I vary depending upon the species (157).

RG-I also contains side chains of individual or multiple L-arabinofuranosyl (Ara*f*) residues or chains of 1,5-linked-α-L-Ara*f* substituted at O-3 and occasionally at O-2 with additional Ara*f* residues (190, 290, 364). Such side chains are referred to as arabinans.

Some RG-I side chains contain both arabinosyl and galactosyl residues. These side chains are referred to as arabinogalactans that have been divided into Type I and Type II arabinogalactans. Type I arabinogalactans contain a 1→4-linked β-D-galactan backbone while the Type II arabinogalactans contain a 1→3-linked β-D-galactan backbone and are often highly branched (2, 157, 190, 214). Type II arabinogalactans may be associated with glucuronomannoglycans (190). Some studies suggest that mannose may be a component in some pectins, probably as a side branch to RG-I (190), however, the structural role of mannose in pectin has not been clearly demonstrated and therefore mannose is not discussed as a component in pectin here. Some of the Type II arabinogalactan is associated with arabinogalactan proteins (AGPs) (365–368), hydroxyproline-rich proteins located at the plasma membrane, cell wall, or in media surrounding suspension-cultured cells (366, 367, 369, 370). It is not always clear whether specific Type II arabinogalactan structures isolated from wall extracts are associated with RG-I, AGPs, or both. However, multiple lines of evidence show that some Type II arabinogalactan is associated with RG-I. This includes the cross reactivity of the antibody CCRC-M7 with both RG-I and arabinogalactan proteins (371). CCRC-M7 recognizes a trimer or larger of β-(1,6)-Gal carrying one or more Ara residues (372). Pectic polysaccharides from the *Chenopodiaceae* family including spinach (*Spinacia oleracea*) and sugar beet (*Beta vulgaris*) are esterified with phenolics such as ferulic acid (157, 291, 373), on galactose and arabinose residues that are likely substituents in the side branches of RG-I (157, 291, 292, 374).

5.4.10.1 RG-I:GalAT, RG-I:RhaT, RG-I:GalAT/RhaAT

It is not known whether RG-I is synthesized onto existing HG or rather is synthesized independent of HG. If it is synthesized onto HG, it not known whether GAUT1 is that GalAT responsible for synthesizing the HG backbone region which would serve as a primer for RG-I backbone synthesis, or whether other GAUTs or other enzymes would perform this function.

It is also not known whether the alternating [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→] backbone repeat is synthesized by a single glycosyltransferase containing both RhaT and GalAT activity, or whether, alternatively, the RG-I backbone is synthesized by a protein complex containing both a GalAT and a RhaT. If the backbone is synthesized by a complex, it is also not known whether GAUT1 or one or more of the GAUT1-related gene family members are part of the complex. To date, no RG-I-specific GalAT or RhaT has been reported.

5.4.10.2 RG-I:galactosyltransferase (RG-I:GalT)

RG-I synthesis requires at least eight galactosyltransferase (GalT) activities (Table 5.2). Probable β-1,4-GalT and β-1,3-GalT activities were originally identified in studies of microsomal preparations from mung bean (375, 376) and more recently a mung bean β-1,4-galactosyltranferase activity with a pH optimum of 6.5 was confirmed (377). Multiple galactosyltransferase (GalTs) activities have also been reported in particulate homogenates (378, 379) and solubilized enzyme (380) from flax (*Linum usitatissimum* L.). Detergent-solubilized flax microsomal GalTs transferred [^{3}H]Gal from UDP-[^{3}H]Gal onto exogenous RG-I-enriched and pectic β-1,4-galactan acceptors (381) to yield high molecular mass radiolabeled products. Surprisingly, the pH optimum for transfer onto lupin pectic β1,4-galactan (i.e., pH 6.5) was different than the pH optimum for transfer of Gal onto an endopolygalacturonase-treated RG-I-enriched fractions from flax (i.e., two optima: pH 6.5 and 8.0) (381). Analysis of the products using RG-I-specific enzymes confirmed that the GalTs indeed added Gal onto RG-I (381), and thus, represented RG-I:GalTs. Furthermore, fragmentation of at least part of the product with β-1,4-endogalactanase demonstrated that at least some of the GalT activity represented β-1,4-galactosyltransferase (381). At pH 8, the GalT activity had an apparent K_m of 460 μM for UDP-Gal and characteristics consistent with a function in catalyzing the addition of galactose onto short galactan side branches of RG-I.

Microsomal membranes from potato suspension cultured cells have been shown to contain RG-I:β-1,4-galactosyltransferase activities that both initiate and elongate β-1,4-galactan side chains of RG-I (382). The potato RG-I:β-1,4-GalT activity in microsomal membranes had a pH optimum of 6.0–6.5 and produced a >500 kDa product using UDP-[^{14}C]Gal and endogenous acceptor(s) in microsomal membranes. The product was fragmented by endo-β-1,4-galactanase into [^{14}C]Gal and [^{14}C]galactobiose and into radiolabeled fragments between 50 and 180 kDa in size (382) when treated with the RG-I-specific rhamnogalacturonase A, an endohydrolase that cleaves the glycosydic linkage between the GalA and Rha in the RG-I backbone (383). The GalT activities in the microsomal membranes could be solubilized from the membranes using detergent (382) and the solubilized enzyme fraction was shown to contain at least two distinct GalT activities, one with a pH optimum of 5.6 that preferentially added Gal onto an ~1.2-MDa RG-I acceptor with a mole % Gal/Rha ratio of 0.7; and the other with a pH optimum of 7.5 that preferentially added Gal onto a 21-kDa RG-I acceptor with a mole % Gal/Rha ratio of 1.2. Neither activity could use RG-I acceptor containing lower Gal/Rha ratios, RG-I backbone without side chains, or galactan polymers or oligomers as acceptors, suggesting that the activities identified required recognition of the RG-I backbone and some existing Gal in a side chain (384). Interestingly, only the product synthesized onto the 21-kDa RG-I acceptor was digestible with a

1,4-β-endogalactase, suggesting that either the Gal transferred onto the larger RG-I acceptor was of a linkage other than β,1-4, or that the length of the galactan side chain synthesized was less than three, the minimum size recognized by the 1,4-β-endogalactanase. The RG-I:β-1,4-GalT that elongates the β-1,4-side chains of RG-I was shown, by subcellular organelle fractionation and protease sensitivity experiments, to be a Golgi-resident protein with its catalytic site facing the lumen of the Golgi (385), a location consistent with its role in pectin synthesis. No gene for any RG-I galactosyltransferase has been reported.

More recently, β-1,4-GalT activity in mung bean detergent-treated microsomal membranes was identified that transferred up to eight galactosyl residues in a β-1,4-linkage onto the non-reducing end fluorescently labeled exogenous (1→4)-β-galactooligosaccharide acceptors (386, 387). Of the galactooligosaccharide acceptors used, i.e., degree of polymerization (DP) of 3–7, the galactoheptaose was a most effective acceptor although acceptors of DP 4–6 also functioned. The fluorescently labeled trimer was not active. Interestingly, fluorescently labeled RG-I backbone oligosaccharides of DP 5–7 were also not active, suggesting that the GalT activity identified could not add Gal onto oligosaccharide RG-I backbone regions. The β-1,4-galactan:β-1,4-GalT activity had a pH optimum of 6.5 and apparent K_m of 32 μM for UDP-Gal and 20 μM for the fluorescently labeled galactoheptaose (386).

5.4.10.3 RG-I:arabinosyltransferase (RG-I:AraT)

RG-I contains L-arabinose in multiple linkages (see Table 5.2). Most of the arabinose is in the furanose ring form, although a terminal arabinose exists in the pyranose form in some RG-I side chains (268). AraT activity was originally identified in microsomes from mung bean (*Phaseolus aureus*) (388) and bean (*Phaseolus vulgaris*) (389) although definitive evidence that those AraT activities were involved in pectin synthesis was not demonstrated [see Ref. (205) for review]. The bean AraT activity was primarily associated with enriched Golgi, and to a lesser extent enriched endoplasmic reticulum (390).

The study of AraTs specifically involved in pectin synthesis has been problematic for several reasons. Multiple wall polysaccharides and proteoglycans contain arabinose, including pectin, hemicelluloses (e.g., glucuronoarabinoxylan), and arabinogalactan proteins. Thus, experiments aimed at studying pectin biosynthetic AraTs by incubating microsomal membranes with radiolabeled UDP-Ara have not been very successful. The nucleotide-sugar donor, UDP-Ara, was not available and had to be synthesized (391), although more recently the UDP-β-L-arabinopyranose form has become available through CarbSource (http://www.ccrc.uga.edu/~carbosource/CSS_home.html). However, while most Ara in pectin is in the furanose form, the nucleotide-sugar synthesized by the 4-epimerization of UDP-α-D-Xyl is UDP-β-L-arabinopyranose. Thus, this has been the nucleotide-sugar form most readily available for experimental use. However, there is uncertainty as to the nature of the nucleotide-sugar substrate used for pectin synthesis. Is it UDP-β-L-arabinopyranose (UDP-Ara*p*) or UDP-β-L-arabinofuranose (UDP-Ara*f*)? If it is UDP-β-L-arabinofuranose, how is this synthesized by the plant and, experimentally, what is the most facile way to produce it? Is it synthesized by enzyme-catalyzed ring contraction of UDP-L-arabinopyranose by a mutase (392)? Indeed, recently, Ishii and colleagues (393) identified a UDP-arabinopyranose mutase (UAM) from rice that catalyzes the reversible formation of UDP-Ara*f* from UDP-Ara*p*. Interestingly, UAMs are the same proteins that previously were identified as reversibly glycosylated polypeptides (RGPs), proteins that are reversibly

glycosylated in the presence of UDP-Glc and several other nucleotide-sugars (394–396). The significance of the reverse glycosylation detected *in vitro*, in regards to the role(s) of UAM in wall synthesis, remains to be determined.

Recent efforts to investigate the AraT activity in mung bean confronted some of the above-mentioned problems. Incubation of mung bean microsomal membranes with UDP-β-L-[^{14}C]arabinopyranose (UDP-[^{14}C]Ara) resulted in the incorporation of both [^{14}C]Ara and [^{14}C]Xyl into elongated endogenous acceptors because of the epimerization of some of the UDP-[^{14}C]Ara into UDP-[^{14}C]Xyl by a UDP-Xyl-4-epimerase present in the microsomal fraction (397). Furthermore, digestion of the radiolabeled product synthesized in the microsomes with endo-arabinase yielded very little radiolabeled Ara or arabinose-containing small oligosaccharides, suggesting that the conditions used were not conducive for the synthesis of arabinans. Conversely, digestion of the product with arabinofuranosidase did release some [^{14}C]Ara, indicating that an enzyme activity that could add at least a single Ara residue was present in the microsomes. A breakthrough in identifying pectin biosynthetic AraTs came when detergent-solubilized microsomal membranes and defined arabinooligosaccharides were used as acceptors for study of pectin biosynthetic AraTs (397). The incubation of detergent-solubilized microsomal membranes with (1→5)-α-L-arabinooligosaccharides of DP 5–8 and with UDP-β-L-[^{14}C]arabinopyranose led to the identification of an AraT activity that could transfer a single arabinopyranose residue onto the non-reducing end of α1,5-arabinooligosaccharide acceptors. The enzyme had a pH optimum of 6.5 and was shown to reside predominantly in the Golgi by subcellular fractionation of organelles (397). The anomeric configuration and linkage of the arabinopyranose residue transferred by the mung bean AraT was subsequently shown to be β-(1→3) through the use of fluorescently labeled α-L-arabinooligosaccharide acceptors (271). Thus, mung bean contains an α1,5-arabinan:β-(1→3)arabinopyranose AraT (271).

A second mung bean arabinopyranosetransferase activity was identified (273) that could transfer an individual arabinopyranosyl residue from UDP-β-L-[^{14}C]arabinopyranose onto the non-reducing end of fluorescently labeled 1,4-linked β-D-galactooligosaccharides of DP 3–7, with significantly better activity manifested with 1,4-linked β-D-galactooligosaccharides of DP 5 or greater. The β-1,4-galactan:AraT activity transferred the Ara residue in an α configuration on the O-4 position of the galactooligosaccharides, identifying the AraT as a β-1,4-galactan:α1,4AraT. The enzyme had a pH optimum of 6.0–6.5 and apparent K_m(s) for UDP-β-L-[^{14}C]arabinopyranose and fluorescently labeled galactoheptasaccharide of 330 μM and 45 μM, respectively. Interestingly, the enzyme would not use fluorescently labeled galactooligosaccharides of DP 6–10 that had a single α-L-Ara*p* residue at the non-reducing end as acceptors for the previously described β-1,4-galactan:β-1,4-GalT (386), indicating that the enzyme cannot use mono-arabinosylated galactooligosaccharides as acceptors. The authors propose that the presence of the α-L-arabinopyranosyl residue on the β-1,4-galactan oligosaccharides prevents further galactosylation of the galactooligosaccharides (273).

Recently, a gene encoding a putative arabinan:α-1,5-arabinosyltransferase (*ARAD1*; At2g35100) has been identified in Arabidopsis (270) through analysis of the CAZy GT47 family glycosyltransferase gene At2g35100 (138) (http://afmb.cnrs-mrs.fr/CAZY/) and phenotypic biochemical and immunochemical analyses of the corresponding Arabidopsis T-DNA insert mutant *ARABINAN DEFICIENT 1*. *ARAD1* encodes a protein with a predicted molecular mass of 52.8 kDa and a single transmembrane helix region near the N-terminus.

Although homozygous knockout mutants of *ARAD1* show no visible growth differences from wild type, isolated walls from mutant leaves and stems had 25 and 54%, respectively, reduced levels of Ara compared to wild type walls (270). Transformation of the mutant plant with the *ARAD1* gene complemented the mutant phenotype (i.e. restored the amount of Ara in the wall to wild type levels), thus providing evidence that *ARAD1* affects wall arabinose levels. Immunocytochemical analysis of leaf, inflorescence stem, and stem revealed a reduction in immunolabeling with the anti-α-1,5-arabinan antibody LM6. A lack of difference between the labeling of protein extracts from wild type and mutant with LM6 suggested that the mutant was affected in the synthesis of α-1,5-arabinans, but not in glycoprotein synthesis (270). This observation was confirmed by comparison of RG-I isolated from wild type and *ARAD1* walls. Mutant RG-I had a 68% reduction in Ara content, which linkage data showed was predominantly due to a reduction in 5-linked Ara*f* and also in 2,5 *f*-linked Ara and 2,3,5-linked Ara*f*. These results strongly suggest that *ARAD1* is a putative RG-I arabinan:α-1,5-arabinosyltransferase. Confirmation of this activity will require expression of enzymatically active enzyme expressing α-1,5-arabinosyltransferase activity.

Recently, a novel approach to identify genes involved in cell wall synthesis has been taken (398) and offers promise in leading to the positional cloning for a gene that affects the number of arabinan side chains in RG-I. The method takes advantage of the natural variation that occurs in cell wall synthesis in natural plant populations and of the availability of Arabidopsis recombinant inbred line (RIL) populations which facilitate the identification and cloning of quantitative trait loci (QTLs). Through the use of multiple techniques to analyze cell walls of an RIL population from a cross between Arabidopsis Bay-0 and Shahdara, including global monosaccharide composition and Fourier-transform infrared (FTIR) microspectroscopy, a major QTL was identified that accounted for 51% of the heritable variation observed for the arabinose–rhamnose ratio in the cell walls, a difference that appeared to be due to variation in the amount of RG-linked arabinan. Whether this strategy will lead to the identification of RG-I biosynthetic AraTs remains to be shown.

Two Arabidopsis putative arabinosyltransferases, designated reduced residual arabinose-1 and -2 (RRA1; At1g75120 and RRA2; At1g75110) were recently identified by Egelund *et al.* (399) based on a 20% reduction in the arabinose content in pectin- and xyloglucan-depleted cell wall fractions from meristematic tissue of *rra1* and *rra2* mutant plants. However, whether these genes, which are classified in CAZy family GT77, encode functional arabinosyltransferases, and if so, whether they function in pectin, arabinoxylan, wall structural protein, or other syntheses, remains to be determined.

5.4.10.4 RG-I:methyltransferase (RG-I:MT)

Detergent-solubilized microsomal proteins from flax can use an RG-I-enriched fraction as an exogenous acceptor for methylation in the presence of *S*-adenosylmethione (334). The pectin methyltransferase activity was stimulated by the addition of the enriched RG-I fraction 1.5- to 1.7-fold above levels recovered using endogenous acceptor, and the radiolabeled product had a size similar to RG-I. However, since it was not shown where in RG-I the methylation occurred, it is not clear whether the methylation occurred on GalA in the RG-I backbone or, rather, on possible HG tails that may have been covalently linked to RG-I. Also, it was not established whether some of the methylation may have occurred on

a non-galacturonic substituent such as methylation at the 4-position of glucuronic acid in the side branched of RG-I (278).

5.4.10.5 RG-I:acetyltransferase (RG-I:AT)

GalA in the alternating [→4)-α-D-GalpA-(1→2)- α-L-Rha*p*-(1→] backbone of RG-I may be acetylated on C-2 and/or C-3 (289). Microsomal membranes from suspension-cultured potato cells (339) contain an RG-I acetyltransferase that transfers [^{14}C]acetate from [^{14}C]acetyl-CoA onto endogenous RG-I acceptor to yield a >500-kDa radiolabeled product (339), based on the release of [^{14}C]acetate following incubation of the product with a purified rhamnogalacturonan *O*-acetyl esterase, and fragmentation of the product by rhamnogalacturonan lyase (RGase B) (339). The RG-I acetyltransferase has an apparent K_m for acetyl-CoA of 35 μM and a pH optimum of 7.0.

5.5 The cell biology and compartmentalization of cell wall synthesis

Except for cellulose and callose, all of the other plant polysaccharides appear to be synthesized in the Golgi or to pass through the Golgi en route to the cell wall. The available experimental results do not currently lead to an unambiguous picture of how the diverse wall polysaccharides are synthesized in, and travel through the Golgi to the wall. Immunoelectron microscopy using antibodies to XG, HG, and RGI showed that these polysaccharides are found in Golgi vesicles but not in the ER (106, 234). Different types of Golgi cisternae contain different sets of glycosyltransferases. Thus, the functional organization of the biosynthetic pathways of complex polysaccharides is consistent with these molecules being processed in a cis-to-trans direction like the N-linked glycans. RG-I and HG polysaccharides appear to be synthesized in cis- and medial-cisternae (235). Methylesterification of the carboxyl groups of the galacturonic acid residues in the polygalacturonic acid domains occurs mostly in medial cisternae, and arabinose-containing side chains of the polygalacturonic acid domains are added to the nascent polygalacturonic acid/rhamnogalacturonan-I molecules in the trans-cisternae. In root tip cortical parenchyma cells, anti-RG-I and the anti-XG antibodies are shown to bind to complementary subsets of Golgi cisternae, and several lines of indirect evidence suggest that these complex polysaccharides may also exit from different cisternae (224). On the other hand, xyloglucan and polygalacturonic acid/rhamnogalacturonan-I can be synthesized concomitantly within the same Golgi stack.

O-linked arabinosylation of the hydroxyproline residues of extensin occurs in cis-cisternae, and the glycosylated proteins pass through all cisternae before they are packed into secretory vesicles in the monensin-sensitive, trans-Golgi network (224). The β-1,4-linked D-glucosyl backbone of xyloglucan is synthesized in trans-cisternae, and the terminal fucosyl residues on the trisaccharide side chains of xyloglucan are partly added in the trans-cisternae, and partly in the trans-Golgi network (235). It has been shown by immuno-electron microscopy using anti-α-L-Fuc-(1→2)-D-Gal antibodies, that fucosylated XyG first appears in the lumen of the *trans*-Golgi and *trans*-Golgi network before vesicle mediated secretion

to the cell wall (224, 400). This activity appeared to be spatially distinct from galactosyl- and xylosyltransferase activity (401, 402).

5.6 Nucleotide sugars

The building blocks for polysaccharide synthesis are nucleotide sugars (NDP-sugars). The sugar moieties in NDP-sugars are incorporated into growing polysaccharide polymers by glycosyltransferases (GTs). A major contributor to glycan diversity is the number of NDP-sugars that an organism produces. In the plant kingdom, 30 different NDP-sugars have been identified (403). It is estimated that over 50 enzymes are directly involved in the synthesis of NDP-sugars in plants. To date only 22 NDP-sugar biosynthetic genes have been functionally identified [see (404) and text below] (Table 5.6). While it is widely accepted that NDP-sugars are the precursors for cell wall polysaccharides, glycoproteins, and glycolipids, it should be kept in mind that in addition to NDP-sugars, lipid-bound sugars are also sugar donors for the synthesis of glycans (for example, synthesis of the N-glycan core of glycoproteins in eukaryotes and addition of galacturonic acid to Rhizobium lipopolysaccharides via a prenyl phosphate–galacturonic acid donor substrate) (405, 406). Whether the initiation of plant cell wall polysaccharide synthesis is mediated by a core glycan that requires lipid-bound sugars at the ER, remains to be established.

Relatively few NDP-sugars are made inside the ER and Golgi apparatus where glycans are made. Most NDP-sugars are produced in the cytosol. Thus, specific NDP-sugar transporters exist to facilitate the import of NDP-sugars from the cytosol into the correct lumen of the endomembrane where GTs reside. It is predicted that ~20 NDP-sugar transporters exist in plants of which functionally only six have been characterized (A. Orellana, personal communication). These transporters are localized to the ER and Golgi apparatus (93, 407). While some of the NDP-sugar transporters are specific, the relatively low number of transporters would suggest that some NDP-sugar transporters may accept several NDP-sugars. In addition to the need for GTs, NDP-sugars, and their transporters for wall polysaccharide synthesis, some wall polysaccharides (i.e. pectins and hemicelluloses) are also modified by acetyl and methyl groups. Thus, diverse acetyltransferases and methyltransferases are also required. Little is known about their substrate specificity, as none has been biochemically purified. Basic questions such as what controls the degree and number of methyl modifications on a specific glycan remain elusive. Hence, no wall-related acetyl- or methyltransferases have been functionally cloned. The methyltransferases (MetTs) and acetyltransferases (AceTs) generally utilize *S*-adenosyl-L-methionine (SAM) and acetyl-CoA as methyl and acetyl donors, respectively (229, 285, 335, 337). A recent study in A. Orellana's laboratory led to the biochemical identification of a SAM transporter activity in the Golgi apparatus of pea (A. Orellana, personal communication). Beyond synthesis, plant glycans undergo further modification, including degradation and remodeling by specific glycosidases and esterases. Due to space limitations, transporters and glycan modifying enzymes will not be summarized in this review, rather, the reader is referred to a recent review (246).

Wall biogenesis is a complex cellular event similar to an assembly line. It requires the supply of a wide range of precursors targeted to different subcellular locations for a process that begins in one subcompartment and continues in other subcompartments as the glycans are synthesized and modified. During this process the concerted action of a range of cytosolic,

Table 5.6 NDP-sugar biosynthetic genes

Enzyme	Activity	Syn	*Mutant*, isoforms (putative?), locus	(aa)	Cell location
UDP-sugar PPase	Sugar-1-P + UTP ↔ UDP-sugar + PPi	Sloppy	Sloppy, At5g52560	614	
UDP-Glc PPase	Glc-1-P + UTP ↔ UDP-Glc + PPi	UGlc PPase	UGlcPP#1	470	
			At5g17310	469	
			UGlcPP#2		
			At3g03250		
Sucrose synthase	Sucrose + UDP ↔ UDP-Glc + Frc	SuSy	SuSy1, At5g20830	808	Mito
			SuSy2, At3g43190	807	Cyto
			SuSy3, At5g49190	808	Golgi
			At4g02280	809	
ADP-Glc PPase	Glc-1-P + ATP ↔ ADP-Glc + PPi	AGPase small sub	*ADG1*, Aps1,	520	Chlo
		AGPase large sub	At5g48300	476	Chlo
		LS	Aps2, At1g05610?	521	Chlo
			Apl1, ADG2	523	
			At5g19220	521	
			Apl2, At1g27680	520	
			Apl3, At4g39210		
			Apl4, At2g21590		
GalK	Gal + ATP ↔ Gal-1-P + ADP	Gal1, GalK	GalK, At3g06580	496	
UDP-Glc 4-epimerase	UDP-Glc ↔ UDP-Gal	UGE	UGE1, At1g12780	351	Cyto
			UGE2,At4g23920	350	Cyto
			UGE3, At1g63180	351	Cyto
			Rhd1,UGE4, At1g64440	348	Cyto-Golgi
			UGE5, At1g10960	351	associated
					Cyto
UDP-Rha synthase	UDP-Glc + NAD(P)H →UDP-Rha	URS	Urs1, At1g78570	669?	
		Rhm	At3g14790	667	
			Rhm2, *mum4*, At1g53500		
UDP-Rha epimerase/reductase	UDP-4keto 6deoxyGlc + NAD(P)H →UDP-Rha	NRSer	NRS/er, At1g63000	301	
UDP-Glc dehydrogenase	UDP-Glc + 2NAD →UDP-GlcA + 2NADH	UGlcDH	Ugd1, At5g39320	480	
		UGD	Ugd2, At3g29360?	480	
			Ugd3, At5g15490	480	
			Ugd4, At1g26570	481	

UDP-GlcA 4-epimerase	UDP-GlcA ↔ UDP-GalA	UGlcAE GAE	*UGlcAE1*, *At2g45310* UGlcAE2, Gae6, At3g23820 UGlcAE3, Gae1, At4g30440 UGlcAE4, At4g00110 UGlcAE5, Gae2 At1g02000? UGlcAE6, Gae5, At4g12250?	437 460 429 430 434 436	
UDP-GlcA decarboxylase	UDP-GlcA → UDP-Xyl	UXS	Uxs1, At3g53520 Uxs2, At3g62830 Uxs4, At2g47650 Uxs3, At5g59290 Uxs5, At3g46440 *Uxs6*, *At2g28760*	433 445 443 342 341 343	Membrane Golgi Cytosol
UDP-Api/UDP-Xyl synthase	UDP-GlcA + NAD→ UDP-Api + UDP-Xyl	AXS UAS	AXS1, At1g08200 At2g27860	389 389	Cytosol
AraK	Ara + ATP ↔ Ara-1-P + ADP	Ara1	*Ara1*, At4g16130	1039	
UDP-Xyl 4-epimerase	UDP-D-Xyl ↔ UDP-D-Ara	UXE	*Mur4*, UXE1, At1g30620 UXE2, At2g34850? Uxe3, At3g34850 Uxe4, At5g44480?	419 379 385 436	Golgi
GDP-Man PPase	Man-1-P + GTP ↔ GDP-Man + PPi	GMPPase	*Cyt1*, At2g39770 At4g30570?	361 331	
GDP-Man 4,6-dehydratase	GDP-D-Man → GDP-4keto 6deoxyMan	GMD	GMD1, At5g66280? GMD2, *mur1*, At3g51160	361 373	
GDP-Man 3,5 epimerase/ 4-reducatase	GDP-4keto 6deoxyMan → GDP-L-Fuc	GER	Ger1, At1g73250 Ger2, At1g17890	312 328	
GDP-Man 3′,5′ epimerase	*GDP-D-Man → GDP-L-Gal* GDP-D-Man → GDP-L-Gul	GME	Gme1, At5g28840	377	
KDO-8-P synthase	PEP + D-arabinose 5-phosphate → KDO-8-P	kdsA	kdsA1, At1g79500 kdsA2, At5g09730	290 291	
CMP-KDO synthase	KDO + CTP → CMP-KDO	kdsB	kdsB, At1g53000	290	

ER, and Golgi enzymes, as well as ER, Golgi, vesicular and plasma membrane proteins is required to facilitate the production of one type of glycan. Therefore, knowledge regarding the catalytic topology of each membrane enzyme and the cellular machinery that partitions, regulates, and traffics each protein and the corresponding glycan-intermediates, to their correct subcompartments must be understood to truly comprehend wall assembly and synthesis.

5.6.1 Fermentation and nucleotide-sugars: a long history

It was exactly 101 years ago, while working on fermentation of sugars by yeast, that Harden and Young (408) first reported the chemistry and metabolic roles of sugar-phosphates. Later, different types of sugar kinases from yeast, muscle, and plant sources were identified that were able to convert sugar (monosaccharide) and ATP to the phosphorylated-sugar esters. The seminal work by Cori *et al.* in 1939 (409) proved, for the first time, the role of sugar-phosphates in the synthesis of polysaccharides (i.e., glycogen). Since the discovery of UDP-glucose in yeast (410), which was followed by the isolation of other NDP-sugars in yeast, plants, bacteria, algae, and humans, it has become apparent that NDP-sugars are the prime sugar-substrates used in the biosynthesis of glycans. The biosynthesis of activated-sugars is achieved in three general ways.

5.6.2 Sugar kinase – pyrophosphorylase pathway to synthesize NDP-sugars

Some sugars are first converted directly, or in a series of enzymatic steps, to phospho-1-sugars in the presence of ATP.

$$\text{Sugar} + \text{ATP} \rightarrow \text{Sugar-1-P} + \text{ADP}$$

Following the phosphorylation of the anomeric center, enzymes known as pyrophosphorylases transfer a nucleotide-monophosphate from NTP to the sugar-1-P to form the NDP-sugar. The synthesis of UDP-Glc and GDP-Man are examples of this type of synthesis.

5.6.3 Direct production of NDP-sugars

Some activated sugars such as CMP-KDO are exceptional since the free sugar, synthesized via intermediary metabolism products, is condensed directly with CTP without a prior phosphorylation of C1.

$$\text{Ara-5-P} + \text{PEP} \rightarrow \text{KDO-8-P} \rightarrow \text{KDO} \rightarrow \text{KDO} + \text{CTP} \rightarrow \text{CMP-KDO}$$

5.6.4 NDP-sugar Interconversion Pathway

The interconversion pathway of nucleotide-sugars is a major pathway where specific enzymes convert preexisting NDP-sugars into different stereospecific NDP-sugars (Figure 5.4).

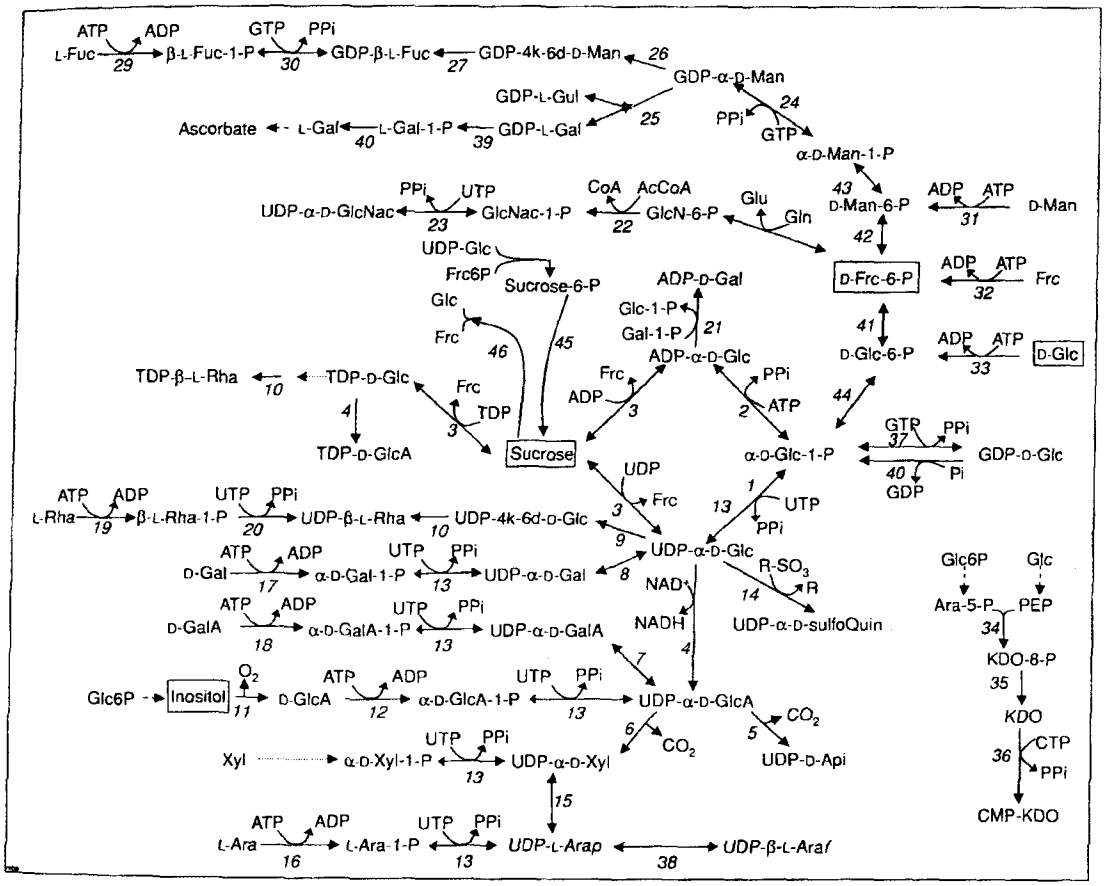


Figure 5.4 The metabolism of NDP-sugars in plants. Glucose, fructose, inositol, and sucrose are major sources of carbon that can be metabolized into NDP-sugars. A salvage pathway is defined as the ability of plant enzymes to recycle sugars that are released from glycoconjugates. The enzyme activity depicted as numbers (italic) are UDP-glucose pyrophosphorylase (1), ADP-glucose pyrophosphorylase (2), sucrose synthase (SuSy, 3), UDP-glucose dehydrogenase (UGD, 4), UDP-apiose/xylose synthase (5), UDP-glucuronic acid decarboxylase (UXS, 6), UDP-glucuronic acid 4-epimerase (UGlcAE, 7), UDP-galactose 4-epimerase (UGE, 8), UDP-glucose 4,6 dehydratase (9), UDP-4-keto-6deoxyglucose-3',5'-epimerase and 4',6'-keto-reductase (NRSER, 10). Reaction 9 and reaction 10 are also carried out by a single polypeptide (URS/MUM/RHM, 9.10), inositol oxygenase (MIOX, 11), glucuronic acid-1-P kinase (12), UDP-sugar pyrophosphorylase (Sloppy, 13), UDP 5'-diphospho-6-sulfoquinovose synthase (14), UDP-xylose epimerase (UXE, 15), UDP-arabinomutase (UAM, RGP, 38), L-arabinose-1-P kinase (Ara1, AraK, 16), D-galactose-1-P kinase (GalK, 17), D-galacturonic acid-1-P kinase (GalAk, 18), L-rhamnose-1-P kinase (RhaK, 19), UDP-rhamnose pyrophosphorylase (20), AMP sugar-1-P transferase or ADP-glucose phosphorylase (21), glucoseamine-6-P acetyltransferase (GNA1, 22), UDP-N-acetylglucoseamine pyrophosphorylase (23), mannose-1-P pyrophosphorylase (24), GDP-Man 3',5' epimerase (GME, 25), GDP-L-galactose phosphorylase (39), L-galactose-1-P phosphatase (40), GDP-mannose 4,6 dehydratase (GMD, 26), GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (GER1, FX, 27), fucose-1-P kinase (29), GDP-fucose pyrophosphorylase (30), mannose-6-P kinase (31), fructose-6-P kinase (32), glucose-6-P kinase (hexokinase, 33), phosphoglucose isomerase (PGI, 41), phosphomannose isomerase (PMI, 42), phosphomannose mutase (PMM, 43), phosphoglucose mutase (PGM, 44), KDO-8-P synthase (34), KDO-8-P phosphatase (35), CMP-KDP synthase (36).

Some of the types of modifications involved in the interconversion pathway are the isomerization of L- and D-sugars, 4-epimerization, specific C-6 oxidation, decarboxylation, and the formation of NDP-deoxy sugar derivatives. Examples of the interconversion pathway are the conversion of UDP-GlcA to UDP-GalA and the conversion of UDP-GlcA to UDP-Xyl. The synthesis of each nucleotide-sugar is described in the following sections.

5.6.5 SLOPPY, a general UDP-sugar pyrophosphorylase

This section on NDP-sugar biosynthesis begins with a description of an enzyme we identified and characterized that does not obey the general rule of enzyme specificity (at least not as we were accustomed to it with other enzymes we had purified in the past). This enzyme, named SLOPPY, is responsible for the synthesis of at least six different UDP-sugars and is unique to the plant kingdom. No other genes with a high sequence similarity to Sloppy have been identified in other organisms to date.

D-Fructose-6-P, a product of photosynthesis, is a central precursor for all monosaccharide residues in plants (403). Using labeling experiments, however, it was also elegantly shown that plant cells can readily take up other free sugars such as Rha, Glc, Gal, Xyl, GalA, GlcA, Ara, and Fuc, and incorporate them into polysaccharides. It was assumed that a free sugar was first phosphorylated with ATP and then uridylated or guanylated with UTP or GTP to form the corresponding NDP-sugar. Indeed, numerous kinase and pyrophosphorylase activities were isolated in the late 1950s and early 1960s that catalyzed such reactions. The kinases were never purified and it was not explicitly clear if different kinases catalyzed the C-1 phosphorylation of each unique sugar or if one kinase phosphorylated several sugars. We discuss the different kinase activities and specificities below. Similarly, it was not clear if the subsequent pyrophosphorylation of each sugar-1-P by pyrophosphorylase was specific or not (411, 412). Three independent research groups identified an enzyme in pea (413) and in Arabidopsis (414, 415) that can pyrophosphorylate various sugar-1-phosphates with UTP. The recombinant Arabidopsis protein (At5g52560), termed SLOPPY, has a higher affinity (e.g., lower K_m) for GlcA-1-P but it also catalyzes the conversion of Glc-1-P, Gal-1-P, Xyl-1-P, Ara-1-P, and GalA-1-P to their respective UDP-sugars (414). The enzyme is very efficient and specific for the production of UDP-sugars and shows no detectable activity when TTP, GTP, ATP, CTP are substituted for UTP. Although Sloppy has broad sugar-1-P specificity, it cannot accept GalNac-1-P.

The existence of a non-specific pyrophosphorylase raises basic questions. What is the source of free sugars in plants? Do the free sugars contribute significantly to the flux of NDP-sugars for wall biosynthesis pathways? Are free sugars generated inside or outside the cells? If they are imported inside, are they derived from long-distances, cell-to-cell transport, or directly from recycled wall? These are central questions that both need to be addressed and obviously raise more questions. If indeed sugars are recycled from glycan degradation, are there sugar-specific transporters? Recently, a plasma membrane sugar transporter (AtPLT5) was functionally identified in Arabidopsis (416). The transporter is a member of a multigene family and seems to be a "non-specific transporter" since competition assays indicate that AtPLT5 can transport Xyl, Rib, Ara, Glc, and myo-inositol, but not sucrose. Unfortunately, the transport of other sugars such as Gal, Rha, and Fuc was not determined for AtPLT5. But it is likely that other sugars are transported either by this, or other, transporters.

The "recycling" of free sugars into the NDP-sugar pool was termed the "salvage pathway" (403). The relative amount of free sugars released from glycolipids, glycoproteins, wall polysaccharides, and glycosides of small metabolites is hard to quantify. Hence, at this time it is not possible to judge the relative contribution of the salvage pathway to the flux of NDP-sugars versus the carbon flux derived from photosynthesis. In the subsequent subsections we will describe the synthesis of specific NDP-sugars.

5.6.6 UDP-α-D-glucose (UDP-Glc)

UDP-Glc, the most abundant NDP-sugar, is the immediate precursor to UDP-Gal, UDP-Rha, and UDP-GlcA. UDP-Glc is produced via three separate metabolic routes. (i) UDP-Glc pyrophosphorylase (UGlcPP) catalyzes the formation of UDP-Glc and PPi from Glc-1-P and UTP in a reversible reaction. (ii) Sucrose synthase (SuSy) transfers the Glc moiety from sucrose onto a UDP acceptor, forming UDP-Glc and Fru. (iii) UDP-D-galactose-4 epimerase (UGE) reversibly epimerases UDP-Gal to UDP-Glc.

5.6.6.1 UDP-Glc Pyrophosphorylase (UGlcPP)

In Arabidopsis, two genes (At5g17310 and At3g03250) encode proteins that share high aa sequence identity to each other (93%) and to the well-characterized potato and barley UDP-Glc PPase (>80%). Recombinant At5g17310 (UGlcPP1) expressed in *E. coli* utilizes only Glc-1-P and UTP to form UDP-Glc. UGlcPP1 is specific for both UTP and Glc1P since TTP, GTP, ATP or other sugar-1-phosphates are not substrates for this enzyme (414). A crystal structure of UGlcPP2 and At3g03250 has been submitted (Wesenberg, G.E., Phillips, G.N., Jr., Bitto, E., Bingman, C.A., Allard, S.T.M.). Early biochemical work established that UDP-Glc PPase is inhibited by UDP-Xyl. If this inhibition occurs in vivo, it would imply that UDP-Xyl, in addition to gene expression, regulates the UDP-glucose pool, and thus, the NDP-sugar pool available for wall synthesis. Recent analysis of rice plants where one of the two rice UDP-Glc PPase genes, *ugp1*, was suppressed, suggests that the production of UDP-Glc during pollen development is critical for callose deposition (417).

5.6.6.2 Sucrose Synthase (SuSy)

Sucrose, a major carbon source for growing cells, is delivered between cells via plasmodesmata (symplastic route) and by long-range transport from source to sink cells via the phloem. SuSy, sucrose synthase (EC 2.4.1.13), catalyzes the reversible conversion of sucrose and UDP into UDP-Glc and fructose. But *in vitro* SuSy also converts sucrose to form TDP-Glc and ADP-Glc from TDP and ADP, respectively (403, 418), as well as GDP-Glc and CDP-Glc (419). SuSy isoforms have been identified in many plant species. In pea, three SuSy isoforms (Sus) were functionally isolated and found to have different kinetic properties. For example, Sus1 was strongly inhibited by Frc (420). In Arabidopsis, six distinct gene members of SuSy are known, and the tissue expression pattern for each SuSy transcript isoform is complex (421) and does not provide clue to their distinct biological functions. Part of the complexity in assigning a biological role for each isoform is the fact that SuSy isoforms differ in protein length and in their amino acid sequence. In addition, cell fractionation studies

and immunogold labeling demonstrate that SuSy isoforms are associated with different subcompartments. For example, distinct SuSy isoforms fractionate with the Golgi apparatus, the tonoplast, and the plasma membrane (422). A recent report also identifies SuSy associated with the actin cytoskeleton (423). Subbaiah and coworkers (424) reported recently that of the three maize SuSy isoforms, SH1 contains a mitochondrial targeting peptide that is required for its intramitochondrial localization. This isoform was proposed to be involved in the regulation of solute fluxes into and out of mitochondria. The association of SuSy with membranes is often observed in growing cells. A specific phosphorylation site on the amino terminal region of SuSy regulates movement of the enzyme between a cytosolic form and a plasma membrane-associated form (425). Biochemical characterization and substrate specificities of each SuSy isoform will be required to elucidate their role in either supporting the flux of carbon to cell wall (i.e., A/T/UDP-Glc) or in carbohydrate storage (ADP-Glc). Lastly, an understanding of the relationship between UDP-Glc PPase isoforms and SuSy isoforms in photosynthetic and non-photosynthetic cells will be needed to understand how the flux of sugars is directed into growth or storage.

5.6.6.3 UDP-D-Galactose-4-epimerase (UGE)

The reversible UDP-Glc/UDP-Gal 4-epimerase (UGE) is a well-studied enzyme in all organisms. Enzyme characterization and crystal structures from numerous organisms revealed that UGE requires NAD^+ as a cofactor for the abstraction of the hydride from C4-OH to yield its 4-keto sugar (C=O) intermediate [for review see (426)]. Some UGEs require exogenous NAD^+ for activity while other types of UGE "tightly hold" the cofactor and require no supply of exogenous NAD^+ in the assay. After comparing UGE crystal structures from different organisms, Thoden and coworkers (427) suggested that the avidity of UGE for NAD^+ depends on the number of hydrogen (H) bonds each UGE has at the NAD-binding pocket. For example, the bacterial UGE "holds-NAD^+" with 19 H-bonds, whereas the human UGE which requires an exogenous supply of NAD^+ for activity, has only 11 H-bonds (427).

In plants, distinct UGE isoforms exist. UGEs were isolated from Arabidopsis (404, 428) and barley (426, 429) and multiple isoforms are found in the rice and maize genome. Recent thorough biochemical, genetic, and molecular studies led by G. Siefert on the five distinct UGE isoforms in Arabidopsis (404, 430) established that (i) isoforms differ in their requirement for exogenous NAD^+, (ii) enzymatic efficiencies vary among the UGE isoforms, (iii) some isoforms likely exist to channel their enzymatic product for the synthesis of a particular glycan, (vi) transcripts of unique isoforms are expressed in the same cell suggesting that each is biologically distinct, and very interestingly (v) some isoforms, for example, UGE1, -2 and -4 are present in the cytoplasm, while UGE4 is enriched close to Golgi stacks. Barber and coworkers (430) suggest that plant UGE isoforms function in different metabolic situations and that enzymatic properties, gene expression patterns, and subcellular localizations contribute to the distinct isoform functions. This suggestion is likely correct, not only for UGE isoforms, but also for other NDP-sugar isoforms involved in the synthesis of specific plant NDP-sugars (431).

The root epidermal bulger 1 (reb1) mutant in *Arabidopsis thaliana* (432) is partially deficient in cell wall arabinogalactan-protein (AGP), indicating a role for REB1 in AGP biosynthesis (433). The REB1 is allelic to ROOT HAIR DEFICIENT 1 (RHD1) (434, 435), one of five ubiquitously expressed UGE genes. The RHD1 isoform is specifically required

for the galactosylation of xyloglucan (XG) and Type II arabinogalactan (AGII), but is not involved in either D-galactose detoxification or in galactolipid biosynthesis. Epidermal cell walls in the root expansion zone lack arabinosylated 1,6-linked-β-D-galactan and galactosylated XG. In cortical cells of *rhd1*, galactosylated XG is absent, but an arabinosylated 1,6-linked-β-D-galactan is present. These results show that the flux of galactose from UDP-D-Gal into different downstream products is compartmentalized at the level of cytosolic UGE isoforms and suggest that substrate channeling plays a role in the regulation of plant cell wall biosynthesis (435).

5.6.7 ADP-α-D-glucose (ADP-Glc)

ADP-Glc is the major precursor for starch synthesis. Starch is a polymer of α-D-Glc consisting of two types of molecules: amylose (linear α-1,4-linked glucose) and amylopectin in which one Glc in every 20 or so residues on an amylose-like structure is branched by an α(1-6)-linkage connected to an α-1,4-linked chain. Although starch synthesis is not related to pectin synthesis, the information available about the regulation of the synthesis of the starch precursor, ADP-Glc, can be used as a paradigm when considering the synthesis and regulation of other NDP-sugars.

Starch, the main carbon storage form in plants, is made in plastids of photosynthetic and non-photosynthetic tissues. Adenosine 5′-diphosphate:glucose pyrophosphorylase (ADPGlc:PPase) catalyzes the first and rate limiting step in starch biosynthesis (i.e., the conversion of Glc-1-P and ATP to ADP-Glc and pyrophosphate (436). In cereal endosperms, two distinct ADPGlc PPases exist, one is found in the cytosol and the other in plastids (437–439). By contrast, ADPGlc PPase is exclusively located in plastids of leaves of both mono- and dicotyledonous plants, as well as in heterotrophic organs of dicotyledonous plants. Plant ADPGlc PPase is composed of two types of subunits (small and large) and is allosterically regulated by 3-phosphoglycerate and phosphate. The Arabidopsis genome consists of six ADPGlc PPase-encoding genes (two small subunits, ApS1 and ApS2; and four large subunits, ApL1–ApL4). Based on recombinant enzyme activities, mRNA expression and the fact that recombinant Aps2 has no ADPGlc PPase activity, it has been proposed (440, 441) that ApS1 is the main catalytic isoform responsible for ADPGlc PPase activity in all tissues of Arabidopsis. The authors suggested that each isoform of the large subunits plays a regulatory role. The large subunit, ApL1 is expressed in source tissues, whereas ApL3 and ApL4 are the main isoforms expressed in sink tissues. Thus, in source tissues, ADPGlc PPase could be regulated by the 3-phosphoglycerate/phosphate ratios, while in sink tissues; the enzyme would be dependent on the availability of substrates for starch synthesis.

In cereal endosperm, on the other hand, a different regulation of starch synthesis may operate. It appears that the transport of ADP-Glc from the cytosol into the plastid is the limiting factor. This became clear during the characterization of a plastidial ADPGlc transporter (HvNst1) barley mutant with low-starch content (442). The mutant accumulates high levels of ADP-Glc in the developing endosperm indicating that cytosolic pool of ADP-Glc is not under metabolic control in this tissue. Lastly, leaves overexpressing SuSy showed a large increase in the levels of both ADP-Glc and starch, compared with WT leaves, while leaves overexpressing antisense SuSy accumulated low amounts of both ADP-Glc and starch (438). The above findings, which originated in the Pozueta-Romero's laboratory, show that in source leaves ADP-Glc produced by SuSy (outside the chloroplast) is directly linked to,

and appears to control starch biosynthesis. This implies that SuSy, but not ADPGlc:PPase, controls the level of ADP-Glc in the cytosol in source leaves (438, 439).

More recently, a new enzyme activity was identified in Arabidopsis that must be considered to better evaluate the metabolic fate of ADP-Glc in the cytosol. Recombinant Arabidopsis At5g18200 has ADP-Glc phosphorylase activity (please note it is not a PPase). The enzyme is capable of transferring AMP from ADP-Glc onto either Pi or Gal-1-P (443) as shown in the scheme below:

$$\text{ADP-Glc} + \text{Pi} \rightarrow \text{Glc-1-P} + \text{ADP}$$
$$\text{ADP-Glc} + \text{Gal-1-P} \rightarrow \text{Glc-1-P} + \text{ADP-Gal}$$

Unlike the human and fungal GalT enzyme, which transfers UMP from UDP-Glc onto Gal-1-P forming Glc-1-P and UDP-Gal, the ADP-Glc phosphorylase cannot utilize UDP-Glc as a donor substrate (443).

We put forward that the above-described distinct regulatory role of ADPGlc PPase and SuSy are examples that highlight the possibility that different isoforms of nucleotide-sugar biosynthetic enzymes may have distinct roles in plants and that different plant species may regulate the same metabolic pathway in different ways.

5.6.8 UDP-α-D-galactose (UDP-Gal)

Galactose is a major constituent of diverse pectic polysaccharides including RG-I. The sugar donor, UDP-Gal, is produced by (i) phosphorylation of C-1 of D-Gal by galactokinase (414) followed by pyrophosphorylase-catalyzed conversion of α-D-Gal-1-P and UTP to UDP-Gal, and by (ii) UDP-Glc-4 epimerase (UGE as described above) that reversibly converts UDP-Glc to UDP-Gal.

1 D-galactokinase activity was isolated by Neufeld and coworkers in 1960 from mung bean (412). The D-GalK is membrane bound and the activity, unlike L-AraK activity, could not be solubilized with digitonin. The galactokinase gene (GalK, Gal1, At3g06580) was cloned from Arabidopsis by functional complementation of yeast (446) and *E. coli* (445) galK mutants that are unable to metabolize galactose. While the Gal1/GalK clone was able to complement the yeast mutant, a definitive substrate specificity of the recombinant Arabidopsis enzyme (GalK) will provide information on whether other "recycled sugars" are substrates. Sequence alignment of various sugar kinase proteins shows that the Arabidopsis GalK shares amino acid sequence similarity (45%) to GalK2, a human kinase with phosphorylation preference to GalNAc (446). A meaningful alignment could not be obtained between the Arabidopsis GalK with the human GalK1 whose true substrate is Gal (447). Whether At3g06580 encodes a Gal-1-P kinase activity and/or GalNac-1-P kinase activity remains to be determined biochemically. The subsequent pyrophosphorylation of Gal-1-P to UDP-Gal is likely mediated via "Sloppy (416)," the non-specific UDP-sugar pyrophosphorylase (At5g52560).
2 The second route to form UDP-Gal is with UDP-Glc-4-epimerase (as described above). In humans and fungi, UDP-Gal is synthesized by uridylyltransferase activity (GalT). GalT transfers UMP from UDP-Glc onto Gal-1P forming Glc-1-P and UDP-Gal. Such activity and corresponding genes have not yet been described in plants.

5.6.9 UDP-L-rhamnose (UDP-Rha)

Rhamnose is a major sugar moiety in pectin and in various glycosides of secondary metabolites. UDP-Rha is the activated sugar for the synthesis of flavonoids (448); however, the activated rhamnose-donor form for pectin synthesis has not been determined. Previously, it was suggested that synthesis of UDP-Rha from UDP-Glc is mediated by three separate enzymes, similar to the conversion of TDP-Glc to TDP-Rha in bacteria (403). UDP-Glc is first modified to the UDP-4-keto-6-deoxyGlc intermediate by UDP-Glc 4,6-dehydratase. The intermediate is modified in the presence of NAD(P)H by a 3,5-epimerase and 4,6-keto-reductase to form UDP-L-β-Rha. A debate in the literature as to whether two or three different enzymes are involved in UDP-Rha came to an end with the functional cloning NRS/er (134) from Arabidopsis (At1g6300). The activity of recombinant NRS/er demonstrates irrefutably that the 3,5-epimerase and 4,6-keto-reductase activities reside in one polypeptide. Interestingly, in vitro NRS/er accepts both TDP- and UDP-4-keto-6-deoxyGlc as substrates to form TDP-Rha and UDP-Rha, respectively. Although TDP-Glc is found in plants (403) and several enzymes can generate TDP-Glc *in vitro*, the physiological significance of the ability of NRS/er to generate TDP-Rha is unclear. Only by isolating a pectin rhamnosyltransferase and characterizing the donor specificity, can the true nature of NDP-Rha form be conclusively determined.

The Arabidopsis genome consists of three genes (At1g78570, At3g14790, At1g53500) each that encodes a large protein (~670 aa) having two domains: an N-terminal domain (~330 aa long) that shares amino acid sequence similarity to 4,6-dehydratase followed by a C-terminal domain (~320 aa long) that shares over 80% sequence identity to NRS/ER. The C-terminal domain of At1g78570 has similar enzyme activity as NRS/ER (134). Mutations in At1g53500, mum4 (449), and rhm2 (450) result in decreasing amounts of Rha and GalA sugar moieties in RG-I structures isolated from seed mucilage. These mutants provided the first genetic evidence for the involvement of these genes (we named URS, UDP-Rha-synthase) in rhamnose synthesis. More recently, when theses genes were recombinantly expressed in yeast, Oka and coworkers (451) reported that all of the Arabidopsis URS genes (also named RHM/MUM) have UDP-rhamnose synthase activity and interestingly, are highly inhibited by UDP-Xylose.

5.6.10 UDP-α-D-glucuronic acid (UDP-GlcA)

In 1952, Dutton and Storey discovered that UDP-GlcA acts as a glucuronosyl donor in the synthesis of glucuronides by liver enzymes. In plants, UDP-GlcA is a key intermediate serving as a branch point between UDP-hexose (six carbons) and UDP-pentose (five carbons) sugars. UDP-GlcA is the precursor for UDP-D-xylose, UDP-L-arabinose, UDP-apiose, and UDP-galacturonic acid that contribute to synthesis of over 40% of cell wall polysaccharides. UDP-GlcA is made by (i) sequential phosphorylation of D-GlcA at C-1 by a membrane bound kinase (411) followed by a pyrophosphorylase activity that converts α-D-GlcA-1-P and UTP to UDP-GlcA, (ii) NAD-dependent oxidation of UDP-Glc to UDP-GlcA by UDP-Glc dehydrogenase (UGD, UDPGDH), and by a controversial pathway (iii) oxidation of UDP-Glc to UDP-GlcA by a bifunctional alcohol dehydrogenase ADH/UDPGDH.

In the 1960s the pathway from myo-inositol to cell wall glycans was proposed as a significant metabolic pathway. Early experiments with ^{3}H-inositol demonstrated that the label

was readily incorporated into cell wall polysaccharides (452). More recently, a labeling experiment with inositol in Arabidopsis showed that radioactivity is found only in the uronic acids, arabinose, and xylose that were released from wall glycans (453). The ability of inositol to drive the synthesis of GlcA was named "the myo-inositol oxidation pathway." We will discuss the myo-inositol and salvage pathways separately.

5.6.10.1 The myo-inositol pathway

In plants, the first step in myo-inositol synthesis is the cyclization of D-Glc-6-P to myo-inositol-1-P (Ino-1P) by 1L-myo-inositol 1-phosphate synthase. In Arabidopsis, two functional isoforms were reported, At4g39800 and At2g22240 (454). The second step involves dephosphorylation of Ino1-P to myo-inositol by myo-inositol monophosphatase (IMPase; EC 3.1.3.25) (455). Distinct multiple but highly conserved IMPase isoforms are found in each plant species. Three IMPases were identified in tomato (456). In Arabidopsis, a conserved IMPase-like-protein (At3g02870) was proposed by Glilaspi to act as IMPase; however, biochemical and genetic data indicate that At3g02870 encodes L-Gal-1-P phosphatase (457, 458). It is possible that other IMPase-like proteins in Arabidopsis (for example, At1g31190, At4g39120) encode the Ino-1P phosphatase activity to form myo-inositol. The identification of the true IMPase gene product is critical to evaluate what controls the pathway to shunt Ino-1P to the myo-inositol oxidation pathway. Free myo-inositol is oxidized by inositol oxygenase (MIOX; E.C. 1.13.99.1) to D-GlcA. Arabidopsis contains four Miox isoforms (453).

It would be interesting to determine if the myo-inositol oxidation pathway operates independently of the pathway leading to synthesis of UDP-GlcA from UDP-Glc. This knowledge could aid in determining which flux of sugars the plant uses to facilitate wall synthesis in specific tissues. For example, myo-inositol in seed is stored as phytic acid (inositol hexaphosphate). During germination, phosphatases provide a rapid source of inositol which is converted, in part, to GlcA. Hence, this would provide a source of UDP-GlcA for wall pectin synthesis. However, during germination, rapid synthesis of L-ascorbate from myo-inositol also occurs. The relationship and coordination of the supply of sugars to wall glycans and to ascorbate synthesis must be better understood at all stages of growth.

5.6.10.2 The salvage pathway

Feeding experiments with free D-GlcA showed that plant cells rapidly incorporate the sugar into pectin (459). Free GlcA, likely released from wall polysaccharides, can be activated to UDP-GlcA, by-passing the need for flux of the sugar from the inositol oxidation pathway. Membrane and soluble protein preparations extracted from a 4-day-old etiolated seedlings of mung bean consist of a kinase activity that specifically phosphorylates GlcA in the presence of ATP and Mg^{2+} to GlcA-1-P. L-Ara and D-Gal are also substrates for this crude kinase preparation. However, GalA is not a substrate for this activity (411). The GlcA-1-P kinase has not been purified and the gene is not yet known. Work in our laboratory (Bar-Peled, 2005) (414) and by Schnurr and coworkers (415) demonstrated that Sloppy (At5g52560) is the true GlcA-1-P pyrophosphorylase and readily converts UTP and GlcA-1-P into UDP-GlcA.

5.6.10.3 UDP-Glc dehydrogenase (UGD)

UDP-Glc dehydrogenase (UDPGDH, UGD) catalyzes the C-6 oxidation of UDP-Glc in the presence of two molecules of NAD^+ to UDP-GlcA and 2NADH. The enzyme is well

characterized from numerous organisms and crystal structures are available. In plants, the gene encoding this activity was first described in soybean (460), and soon after, as genome sequence became available, numerous UDPGDH gene isoforms that share high amino acid sequence similarity to each other were identified in Arabidopsis (461), poplar (462), tobacco (463), maize (464), and Dunaliella (465). In Arabidopsis, four UDPGDH isozymes are present and, likewise, multiple UDPGDH isoforms are found in the genome of every plant species that has been sequenced. The role of multiple UDPGDH isoforms and their specific activities is of great interest since recent work in sugar cane revealed that CTP-glucose and TDP-glucose were oxidized as well, although at low rates compared to UDP-Glc (466). The current knowledge indicates that UDPGDH is cytosolic and its activity is strongly inhibited by a very low level of UDP-Xyl. This suggests that flux of UDP-Glc (hexose) to UDP-pentoses can be feedback-regulated, in part, at the enzyme level.

The source of UDP-GlcA (i.e., from myo-inositol or UDP-Glc) for wall synthesis was elegantly determined by a genetic approach. Two UDP-Glc dehydrogenase mutants were identified in maize, one in isoform A and the other in isoform B of UDPGDH. Polysaccharides isolated from isoform A mutant had lower Ara/Gal and Xyl/Gal ratios when compared with wildtype, indicating the importance of UDPGDH in directly providing major flux of NDP-sugars to wall polysaccharides (464). On the other hand, the lack in "wall alteration" in the isoform B mutant, could suggest either that different UDPGDH isoforms function in a different metabolic pathways, for example in the formation of glycosides, or that isoform B contributes to the synthesis of a glycan structure that is present at such low levels that small alterations in the amount of GlcA was undetected. RG-I, for example, consists of a small amount of GlcA residue (only 1% of total sugars). Similarly, GlcA residues are found in relatively low amounts in RG-II and xylan, representing 3 and 5%, respectively, of total sugars in those polysaccharides.

In 1996, Robertson and coworkers (467) reported that alcohol dehydrogenase (ADH) from *Phaseolus vulgaris* can convert UDP-Glc to UDP-GlcA. This bifunctional activity of ADH to oxidize both ethanol and UDP-Glc opened a debate in the literature. A major difficulty in interpreting this result is that Robertson and coworkers (467) used a spectrophotometer assay which measures NADH formation, not UDP-GlcA directly. Recently, the tobacco ADH homologous genes, cloned and expressed in bacteria, had activity on both ethanol and UDP-Glc. But again, the UDPGDH assay was determined by spectrophotometer assays (463). Whether recombinant ADH catalyzed the formation, UDP-GlcA was not explicitly confirmed. Further kinetic work with pure ADH must be carried out to claim the dual specificity of this enzyme. Only if knockouts of all ADH genes are obtained can it be definitely determined whether ADH is a bifunctional enzyme. Maize mutants lacking ADH1 and ADH2 isoforms, however, had no affect on sugar composition of hemicellulose (464). If only two ADH isoforms exist in maize ADH, this would suggest that ADH is not a bifunctional enzyme.

5.6.10.4 Summary regarding UDP-GlcA formation

What controls the supply and flux of UDP-GlcA in plants is still debatable and it is very likely that different plant species adopt different mechanisms to control the supply of UDP-GlcA. In maize, mutants lacking the activity of one UDPGDH isoform have a reduction in the content of Ara, and Xyl in hemicellulose. This suggests that in maize, UDPGDH-A is a major supplier of the UDP-pentoses and that the myo-inositol oxidation pathway (not

"bifunctional ADH") cannot compensate the flux of "sugar" to the formation of UDP-GlcA. In Arabidopsis mutants lacking MIOX1 and 2 isoform activities, no significant differences in monosaccharide amount or composition were observed in wall polysaccharides when compared to wildtype. Thus, it is likely that the major contributor for flux of NDP-sugars in plants is UDP-Glc. As mentioned above, it is possible that the myo-inositol pathway and the salvage pathway operate in a tissue-specific manner, for example, during pollen tube growth. During pollen tube germination and growth, large amounts of pectin are degraded. It is likely that the free sugars are recycled back by kinases and the activity of Sloppy, to readily form an available pool of NDP-sugars. This pool will provide NDP-sugars for growth of the pollen tube. Pollen tubes are one of the fastest growing cells known (1 cm h^{-1}) and mutants lacking Sloppy have a pollen phenotype (415). A similar regulation scenario likely occurs during seed germination with seeds that store a large amount of phytic acid, as discussed above.

5.6.11 UDP-α-D-galacturonic acid (UDP-GalA)

UDP-GalA, a major sugar donor for pectin synthesis, is made by (i) the salvage pathway, by the phosphorylation of D-GalA to GalA-1-P in the presence of ATP and kinase activity (GalAK). The GalA-1-P pyrophosphorylase catalyzes the conversion of GalA1P and UTP to UDP-GalA. (ii) UDP-GlcA 4-epimerase (UGlcAE), a reversible 4-epimerase that converts UDP-GlcA to UDP-GalA.

1 Feeding experiments with radioactive GalA demonstrated that the label is readily incorporated into pectin (459). Soluble enzyme preparations from a 1-day-old germinating seeds of mung bean have high GalAK activity (468), while no GalAK is observed in a 4-day-old etiolated seedlings (411). The collective biochemical data suggest that the soluble GalA kinase differs from the membrane-associated kinase activity that phosphorylates GlcA. A functional gene encoding GalAK activity was recently discovered in Arabidopsis [Ting and Bar-Pled, 2006, unpublished]. Proton NMR analysis confirm that pure reecombinant GalAK phosphoylates D-GalA in the presence of ATP to GalA-1-P. Currently however, it is difficult to predict the relative amount of GalA that is recycled back to UDP-GalA. Cleaarly, high activities of pectin derading enzymes during pollen germination could provide suffcient substrate for AalAK. Indeed, the non-specific UDP-sugar pyrophosphorlase (fondly named SLOPPY in Arabidopsis (414) and also identified in pea (469)) that converts GalA-1-P with UTP to UDP-GalA is highly expressed during pollen germination. The relative amount of GalA recycled from pectins as free sugars in other plant tissues is unknown, although the "fate" of free GalA could be tissue specific. For example, during strawberry fruit maturation, an increase in GalA-reductase (GalUR) activity was shown to direct GalA, released from pectin, for the synthesis of ascorbic acid (470). Thus, the relative contribution of the GalA-salvage pathway for synthesis of wall polymers remains unclear. Hopefully, current analyses of GalAK mutants in Arabidopsis will shed light on its physiological functioon (i.e. in the wall and in ascorbic acid metabolism).
2 UDP-GlcA 4-epimerase (UGlcAE) is a membrane-bound enzyme that reversibly catalyzes the 4-epimerization of UDP-GlcA to UDP-GalA. In Arabidopsis, six distinct functional genes (UGlcAe) encode isoforms having UDP-GlcA 4-epimerase activity (450, 471, 472). The isoforms can be divided into three evolutionary clades: Type A, B, and C. Members of UGlcAE are predicted to be Type II membrane proteins, suggesting that their catalytic

domain faces the lumen of an endomembrane. The topology of the catalytic domain was not validated experimentally. Mohnen's laboratory has shown that the activity of UGlcAE co-fractionated with the Golgi on sucrose-gradients (230) and expressing UGlcAE1-GFP in plants demonstrates that the chimeric fusion targets UGlcAE1 to the Golgi as well (Gu and Bar-Peled, unpublished data). Multiple UGlcAE isoforms are common and found in other plants, for example, rice and maize. Biochemical analyses of Arabidopsis, rice, and maize UGlcAE isoforms showed that the reversible UGlcAE epimerase has a preference (2:1) to form UDP-GalA and is inhibited by both UDP-Ara and UDP-Xyl. Recent studies (Gu and coworkers, submitted) demonstrated that the maize UGlcAE is especially sensitive to, and strongly inhibited by, UDP-Xyl when compared with Arabidopsis UGlcAE2. The authors suggest that in maize, the relative low amount of pectin (when compared to dicots) could be due to inhibition of UGlcAE by UDP-Xyl. Hence, this may provide an explanation as to why maize has less pectin and more xylan when compared to dicots. The role of multiple UGlcAE isoforms is currently being addressed. One possible explanation is that different isoforms localize to distinct endomembranes, as was suggested by Pattathil and coworkers (473) for the different Uxs isoforms.

5.6.12 UDP-α-D-xylose (UDP-Xyl)

UDP-Xyl is primarily synthesized by UDP-GlcA decarboxylase (UGlcA-DC, also named UDP-Xylose Synthase, Uxs) from UDP-GlcA. The enzyme has a tightly bound NAD^+, which participates first in the oxidation of UDP-GlcA to the UDP-4ketohexose intermediate resulting in decarboxylation (removal of COOH as gas, CO_2) and formation of UDP-4ketopentose. In the second stage, the NADH-bound enzyme reduces the UDP-4ketopentose to UDP-Xyl resulting in the release of NAD-bound enzyme (403). Multiple distinct UXS isoforms encoding this enzyme activity were reported in Arabidopsis (431), rice (474), barley (475), and tobacco (463). Uxs isoforms are very specific enzymes and act only on UDP-GlcA. The 4-epimer of UDP-GlcA, UDP-GalA is not a substrate for Uxs (431). Uxs is active as a dimer and inhibited by UDP-Xyl. In plants, UDP-Xyl is made in the cytosol and in the endomembrane system (473). Phylogeny analysis classified the six Uxs isoforms from Arabidopsis into three distinct clades: Type A (1 isoform, At3g53520); Type B (2 isoforms, At3g62830, At2g47650), and Type C (3 isoforms, at5g59290, At3g46440, At2g28760). Type A and B isoforms have an N-terminal extension (~120 aa long) which encodes longer proteins compared to Type C Uxs isoforms. Type A and B UXS isoforms are predicted Type II membrane proteins with the catalytic domain facing the endomembrane lumen (431). Expression of Type B Uxs isoforms in plants confirmed that Uxs2 is an integral membrane protein. Expressing of a Uxs2-GFP construct in tobacco leaves was shown to localize the chimeric fusion protein in the Golgi apparatus (473). Uxs3 belongs to the Type C clade and the isoform was found in the cytosol as predicted.

5.6.13 UDP-D-apiose (UDP-Api)

UDP-Apiose Synthase (UAS, AXS) converts UDP-GlcA in the presence of NAD^+ to UDP-Api. The enzyme decarboxylates UDP-GlcA to form a UDP-4ketopentose intermediate and the release of CO_2, and then catalyzes rearrangement of the sugar skeleton to form UDP-Api

(476, 477). In vitro, the enzyme forms both UDP-Xyl and UDP-Api (403). We believe that the formation of UDP-Api was not confirmed satisfactorily since the product is readily degraded to cyclic apiose1,2-phosphate. Functional genes encoding AXS were isolated from tobacco (358), Arabidopsis and potato (Guyet and Bar-Peled, unpublished) (478). Two isoforms exist in Arabidopsis (At1g08200 and At2g27860) and are predicted to be in the cytosol. The "dual function" of the enzyme in generating both UDP-Xyl and UDP-Api was assayed by NMR spectroscopy (Guyet and Bar-Peled, unpublished). NMR time course assays for the conversion of UDP-GlcA into UDP-pentose using recombinant potato UAS, confirms explicitly that UDP-Api is made first. The analysis indicates that in vitro, UDP-Xyl synthesis lags behind UDP-Api. Mutation in UDP-Api synthase in *Nicotiana benthamiana* is lethal as a consequence of the lack of RG-II (358). To unambiguously determine if the enzyme is bifunctional and contributes to UDP-Xyl synthesis, a mutation in the three cytosolic UXS (Type C) genes must be carried out.

5.6.14 UDP-L-arabinose pyranose (UDP-Ara)

Arabinose (Ara) is an important sugar in plant walls and with a few exceptions, the predominant form of Ara in plant glycans is the furanose configuration (Ara). However, some polysaccharides, RG-II, for example, carry both forms of the arabinose moiety, i.e. Ara-furanose and Ara-pyranose. UDP-Ara (pyranose form) was identified in all plant extracts and is synthesized by (i) the sequential phosphorylation of L-Ara at its C-1 by a membrane associated L-arabinokinase (412) followed by a pyrophosphorylase that converts L-Ara-1-P and UTP to UDP-Ara*p*, and (ii) a membrane-bound UDP-Xyl-4 epimerase (UXE) that converts UDP-D-Xyl to UDP-L-Ara*p*.

1 Neufeld and coworkers (411, 412, 468) isolated sugar-kinase activities from different sources of tissues. A membrane fraction from bean was shown to catalyze the C-1 phosphorylation of L-Ara to β-L-Ara-1-P. The same membrane preparation phosphorylated D-Gal to α-D-Gal-1-P. However, the GalK kinase and the AraK kinase are different enzymes since AraK requires divalent ion for activity (Mg^{2+}, Mn^{2+}) whereas, the GalK kinase requires no additional divalent ion for activity (412). In addition, treatment of membranes with digitonin solubilizes the L-AraK activity but not the D-GalK activity. The AraK is specific for the L-form since D-Ara (that is common in prokaryotes) is not a substrate.

The *ara1* mutant from Arabidopsis, in the At4g16130 locus, has reduced ability to metabolize arabinose and lacks Ara-1-P kinase activity (479). Bioinformatic analysis suggests that Ara1 belongs to a large family including galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (GHMP kinases). The Ara1 protein is speculated to be a Type Ia membrane protein. If the topology is correct, it would be interesting to know whether the catalytic domain is facing the cytosol or the lumen. Direct biochemical assays and substrate specificity studies of the encoded Ara1 gene were not performed. The subsequent pyrophosphorylation of α-L-Ara-1-P to UDP-Ara can be mediated by "Sloppy," the non-specific UDP-sugar pyrophosphorylase (413).

2 A genetic screen led by Reiter *et al.* (125) identified the *mur4* mutant in Arabidopsis that has a 50% reduction of arabinose in the wall. The encoded recombinant protein (Uxe1,

UDP-Xyl 4-epimerase) was shown to convert reversibly UDP-D-Xyl to UDP-Ara (480). Bioinformatic analysis suggests that Uxe1 is a Type II membrane protein whose catalytic domain is facing the lumen. A Uxe1-GFP chimera was localized to the Golgi apparatus (480). Two isoforms (UXE1, At1g30620 and UXE2, At2g34850) that share 83% aa sequence identity to each other exist in the Arabidopsis genome; two isoforms are in the rice genome and three UXE isoforms were isolated for barley (*Hordeum vulgare*) (Zhang and Fincher, unpublished). Since several GTs were able to transfer the Ara (pyranose) from UDP-Ara into plant glycans (271, 397); it remains a puzzle when the Ara acquires the furanose configuration. One can predict that the Ara*f*-donor has not yet been identified. However, this is unlikely since the *mur4* mutant (involved in the synthesis of UDP-Ara pyranose), lacks glycan consisting of Ara*f*. This could imply that during the arabinosyltransferase catalyzed reaction the Ara*p* is altered to the Ara*f* form. A specific mutase may exist to convert the Ara*p* to Ara*f* on the glycan itself, similar to the conversion of GlcA to IdoA in proteoglycans (481). Alternatively, UDP-Ara (furanose) is made in plants as recently confirmed and described in the following section.

5.6.15 UDP-arabinose furanose (UDP-Araf)

Recent work in Tadashi Ishi's laboratory established a new enzyme activity that was never reported before, a UDP-arabinopyranose mutase (UAM). The enzyme is capable of converting UDP-Ara pyranose to UDP-Ara furanose (UDP-Ara *f*) and it was reported to be a reversible reaction (393). This activity was biochemically purified from rice and the corresponding gene was cloned. In rice, two homologous proteins (UAM1; AK098933, UAM2; AK071012) were identified. The recombinant enzyme at thermodynamic equilibrium produces UDP-Ara in a pyranose:furanose ratio of 90:10. The Arabidopsis homologous proteins were initially named as reversibly glycosylated protein, RGP (396, 484). Several isoforms of RGPs exist in the plant kingdom. The Arabidopsis RGP1 (At3g02230) and RGP2 (At5g15650) proteins were found to localize in the Golgi apparatus (396, 483). However, Sagi and coworkers (484) observed that a chimeric RGP-tagged to green fluorescence protein is localized to both Golgi and plasmodesmata. The specific *in vivo* role of RGP/UAM and the various isoforms in the (i) synthesis of the furanose form of UDP-Ara or as (ii) a reversibly glycosylated protein remains unclear.

5.6.16 GDP-α-D-mannose (GDP-Man)

GDP-Man (485) is a major sugar donor that provides flux of sugar to the synthesis of glycoproteins, polysaccharides, and ascorbic acid in plants. GDP-Man is the precursor for GDP-Fuc and GDP-Gal. Guanosine 5′-diphosphate-mannose pyrophosphorylase (GDP-Man PPase) catalyzes the conversion of α-D-Man-1-P and GTP to GDP-Man and pyrophosphate (486). The enzyme activity requires Mg^{2+} and the enzyme appears to be cytosolic. The pyrophosphorylase is well studied in various organisms and the crystal structure is known. In Arabidopsis, a wall mutant *cyt1* (487), an ozone-sensitive mutant, and ascorbic acid mutant, *vtc1*, identified the same gene product At2g39770 as the locus responsible for the production of GDP-Man (488, 489). The mutant likely survives since another homologous gene, At4g30570, may compensate for its activity. Although the enzymatic activity

of recombinant protein was not described, work in our laboratory confirmed that the encoded gene At2g39770 has GDP-Man PPase activity and is very specific toward Man-1-P as a substrate (Echole and Bar-Peled, unpublished).

5.6.17 GDP-β-L-fucose (GDP-Fuc)

Plant cell wall polysaccharides contain L-fucose (6-deoxy-L-Gal) derived from the sugar-donor, GDP-Fuc. GDP-Fuc synthesis occurs in two enzymatic steps (similar to the synthesis of UDP-Rha from UDP-Glc). These enzymes have been characterized and the corresponding functional genes have been identified in humans, plants, and bacteria. First, GDP-Man 4,6-dehydratase (GMD), converts GDP-Man to a GDP-4keto-6-deoxyMan intermediate. The latter is then converted by GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (GER1, FX) to GDP-Fuc. In Arabidopsis, there are two GMD isoforms (GMD1, At5g66280; GMD2 (mur1) At3g51160) which share 92% aa sequence identity to each other; and two GER isoforms (GER1, At1g73250; GER2, At1g17890) that share high (88%) sequence identity to each other. In some tissues it appeared that GMD isoforms are co-expressed, but in other tissues expression is restricted. For example, GMD2 is expressed in most cell types of the root, but not in the root tip where strong expression of GMD1 is observed (490). Within shoot organs, GMD2 appears to be expressed in most tissues while GMD1 expression is restricted to stipules and pollen grains. The lack of GMD2 above ground (*mur1* mutant) corresponds to an almost complete reduction in Fuc in wall polysaccharides including XG whose Fuc can be substituted by L-Gal presumably as a result of increased GDP-L-Gal availability (491, 492). However, below ground the *mur1* mutation leads to a 40% reduction of Fuc. Some isoforms may have redundant function in a specific cell, but in other tissues of the same plant the existence of isoforms may provide pools of the NDP-sugars to synthesis of different types of glycans.

5.6.18 GDP-β-L-galactose (GDP-Gal), GDP-β-L-gluclose gulose (GDP-Gul)

GDP-Gal is a major precursor for the synthesis of ascorbic acid in plants, and relatively low amounts of L-Gal are found in plant glycans. A GDP-Man 3′,5′ epimerase activity first identified in the Neufeld's laboratory (493) epimerizes GDP-Man into GDP-Gal. Careful biochemical analyses of the specific activity of recombinant Arabidopsis At5g28840 (494) and rice (497) protein demonstrate that the enzyme can convert GDP-Man to both GDP-Gul and GDP-Gal (496). A crystal structure of the enzyme was recently obtained (497).

5.6.19 CMP-β-KDO (CMP-KDO)

The eight-carbon acid sugar KDO, 3-deoxy-D-manno-2-octulosonic acid, is a primary sugar constituent in various types of cell surface extracellular polysaccharides and liposaccharides of Gram-negative bacteria. In plants, KDO is found only in RG-II. Synthesis of CMP-KDO in plants requires the activities of three enzymes.

1 KdsA, KDO-8-P synthase, catalyzes a condensation of PEP, phosphoenolpyruvate, and phosphorylated monosaccharide, D-arabinose 5-phosphate (A5P) in the presence of metal. Functional genes encoding KDO-8-P synthase activity were isolated from various plant species (498–500) and the encoded proteins share ~50% amino acid sequence identity with the bacterial proteins. In Arabidopsis, two gene isoforms (AtkdsA1, At1g79500; and AtkdsA2, At5g09730) were identified. The encoded isoforms share 93% amino acid sequence identity to each other. Interestingly, AtkdsA1 is predominantly expressed in shoots, while AtkdsA2 transcript accumulates to a higher level in roots. The activity of the recombinant plant kdsA toward other phosphorylated-sugars, such as D-erythrose-4-phosphate (E4P) was not tested. Based on bioinformatics, the plant KdsA are predicted to reside in the cytosol.
2 KDO-8-P phosphatase activity removes the phosphate to form KDO. The nature and specificity of this phosphatase is unknown.
3 KdsB, CMP-KDO synthase (CMP-KDOs), catalyzes the transfer of the cytidylyl group (CMP) from CTP to KDO in the production of the unusual nucleotide-sugar, CMP-β-KDO. The resulting activated sugar has a half-life of about 30 minutes in solution. The maize gene homolog was functionally identified (501), and the homologous Arabidopsis protein sequence is encoded by At1g53000. *In vitro*, the recombinant maize CMP-KDO is capable of using both CTP and UTP as nucleotide (Bar-Peled, unpublished). Interestingly, the plant proteins that share 40–50% amino acid sequence identity to the bacterial KdSA proteins have a 50 amino acid N-terminal extension. Bioinformatic analysis suggests that plant CMP-KDO is a Type Ib transmembrane protein with the catalytic domain predicted to face the cytosol. However, the subcellular location of the plant protein is uncertain. Since the function of RGII:2,3KDOT activity was not reported, it remains unclear if CMP or UMP-Kdo are the sugar donors.

5.6.20 Other enzymes involved in NDP-sugar metabolism

Feingold (403) summarized the NDP-sugars identified in plants. Those that were not described above are ADP-L-Ara, GDP-L-Ara; ADP-ribose; GDP-Xyl, ADP-Gal, GDP-D-Gal; ADP-D-Man; UDP-Fructose, ADP-D-Fructose; UDP-D-digitoxose (2,6-dideoxy-D-ribohexose); TDP-GalA; UDP-2-deoxy-2-acetamido-D-Glc, UDP-2-deoxy-2-acetamido-D-Gal; UDP-cellobiose. In addition to these NDP-sugars, pectin consists of other sugar residues: aceric acid and DHA (deoxylyxoheptulopyranosilaric acid). The formation of these NDP-sugars is not well studied.

It is not clear if modifications of sugar residues on pectin (such as methylation or acetylation) occur after transferring the sugar from the respective NDP-sugar. In chloroplasts for example, sulfolipid biosynthesis requires the activated sugar UDP-Glc-6-sulfonate, UDP 5′-diphospho-sulfoquinovose. In this case, the sugar-linked to NDP is modified with a sulfate group prior to the transfer of the sulfoquinovose. Whether such NDP-sugar modifications occur with NDP-sugars required for wall synthesis is unknown. While more is known about synthesis of activated sugars less is known about "catabolism" of NDP-sugars. Recently, work on ascorbic acid metabolism in plants revealed two mutants, *vtc2* and *vtc4*, involved in the degradation of GDP-L-Gal. First, a GDP-L-Gal phosphorylase (*vtc2*, At4g26850) converts GDP-L-Gal and Pi into L-Gal-1-P and GDP (502) and subsequently a dephosphorylase

activity on L-Gal-1-P yields L-Gal (458) (vtc4, At3g02870). Interestingly, the phosphorylase converts GDP-D-Glc to D-Glc-1-P and GDP as well.

5.6.21 Future questions and directions

Currently, little is known about how the synthesis of nucleotide-sugars is controlled in time or space, and how it relates to the glycosyltransferases that actually make the diverse glycan polymers. What is the limiting factor in wall synthesis? Is it supply of NDP-sugars (as is the case for starch) or glycosyltransferases?

We will divide this section into three topics: sugar flux, role of isoforms, topology and protein complexes.

5.6.21.1 Sugar flux

Although a considerable proportion of cellular sugar ends up in wall polysaccharides, some sugar-derivatives are required for glycoprotein, glycolipid, and glycoside synthesis. In addition, significant amounts of sugars are stored either as large glycans such as starch, small-sized glycans (e.g., raffinose, fructan), or as the disaccharide sucrose. We would like to point out two issues related to flux: 1) growth potential of a cell; 2) whether some wall components compensate for the lack, or reduced amount, of other glycans.

1 New meristematic cells need to expand and grow to their prospective developmental tissue (e.g., leaf cells). What determines the growth potential and the cell's final size is unclear. Logically, with limited wall precursors the potential for growth is restricted since wall polymers are not made. The underlying mechanism that controls this complex developmental process is still unknown and poses a fascinating scientific quest. For example, do transcription factors regulate coordinately the expression of "tissue-fate genes" as well as NDP-sugar biosynthetic genes and genes involved in the supply of carbon? If carbon flux is not limited and all NDP-sugar biosynthetic genes are highly expressed – would the cell be larger? Do young, old, or stressed cells sense sugar availability or sugar status for growth and/or for storage in different ways? What are the ultimate determinates for growth; sugars or sugar-phosphates? Several sugar-sensing (signaling) proteins (and corresponding genes) have been isolated. It is assumed that sugar sensing (i.e., the interaction between a sugar molecule and a sensor protein) mediates a signal which initiates signal transduction cascades that result in cellular responses such as altered gene expression and enzymatic activities. Sugars as signaling molecules affect the plants at all stages of growth starting from seed germination to seed development. Sugars, like hormones, can act as primary messengers and regulate signals that control the expression of various genes involved in sugar-phosphates and wall metabolism. But do NDP-sugars function, in part, as signal molecules? In human cells, UDP-GlcNAc serves as a glucose sensor and moves between the cytosol and the nucleus. A cytosolic and nuclear-localized soluble enzyme, known as OGT, catalyzes the O-linked transfer of GlcNAc from UDP-GlcNAc directly to Ser/Thr of target proteins (503). For example, the O-GlcNAcylation, of the transcription factor Sp1 promotes nuclear localization of Sp1 and its ability to transactivate calmodulin (CaM) gene transcription (504). Whether plant cells consist of analogous signals to suppress or activate wall-biosynthetic genes by monitoring levels of NDP-sugars is unknown.

A major task for future research will be to investigate the relationships between isoforms that produce the same nucleotide-sugar, GTs, and sugar-sensing genes. Once the function of wall-related genes becomes known, bioinformatics will be useful in identifying a common set of genes that are coordinately expressed or suppressed to form a specific glycan.

5.6.21.2 Role of isoforms

Of course, the diversity of wall glycans could be more complex than suggested above. It is possible that certain cells will tolerate severe alterations in wall composition or will tolerate the complete lack of one or two types of wall glycans. The cell affected could either "reinforce" its wall with another glycan structure or not. If wall composition is flexible, then why do so many isoforms for the synthesis of the same NDP-sugar exist? One can argue that the existence of multiple NDP-sugar isoforms provides wall flexibility. A hypothetical example follows, reductions in the amounts of wall xylan, whose precursor UDP-xylose is produced by UXS Type A, can be overcome by the overproduction of xyloglucan, whose precursor UDP-Xyl is produced by UXS Type C. Indeed, genetic manipulation of a specific NDP-sugar (UGE) biosynthetic gene altered specific glycan synthesis (404).

One major consequence of the genome-sequencing project is the finding that what differentiates plants from other organisms (human, animal, yeast, or prokaryotes) is the fact that each NDP-sugar is synthesized by multiple isoforms. The current knowledge also raises many questions which were not apparent in the golden days of biochemistry. For example, why have plants evolved to generate so many isoforms for the synthesis of the same NDP-sugar? What is the evolutionary advantage for the synthesis of the same sugar attached to different nucleosides, for example, GDP-, CDP-, ADP-, UTP-, and TDP-glucose? Some species, for example bacteria, have a preference for TDP-, CDP-, and GDP-sugars, while vertebrates have preferences for UDP- and GDP-sugars. Bacteria, for example, produce TDP-Rha, whereas plants produce UDP-Rha. Such an activated sugar is not found in humans. If we keep in mind that all NDP-sugar biosynthetic genes likely evolved from one or several ancestral cells, and that the sequence similarity of all NDP-sugars is so high across species, one can form a testable theory for the rise of synonymous NDP-sugars. We will discuss possible roles of NDP-sugars using an analogy for the evolutionary pressure that presumably lead to the formation of 64 CODONS for the synthesis of 20 amino acids. It is now clear that codon usage is not random and among synonymous codons, some codons are used preferentially. It is even more fascinating that some species, for example Drosophila, have their own particular codon biases, and their usage differs significantly from those preferred by *E. coli*, yeast, or plants. Have NDP-sugars and diverse glycan structures evolved in the same manner? Why, for example, does one species, need GDP-Glc, ADP-Glc, CDP-Glc, and UDP-Glc. After all, it is the same Glc residue that ends up in the glycan (not the nucleoside). Would bacteria infecting humans or plants have an advantage in producing glycans using NDP-sugars not used by the host? After all, NDP-sugars are not imported to, or exported from, the infected cell.

One other possibility for an evolutionary pressure to produce multiple nucleotides bound to Glc, for example, can be explained by the Bar-Peled-Mohnen "Selfish Glycans Theory." The diversity of glycans that each cell produces could not have successfully evolved if different GTs had to compete for the same NDP-sugar. If, for example, the biosynthetic apparatus responsible for the massive synthesis of starch, cellulose, or xyloglucans would all compete for UDP-Glc, the cell would either not grow or not be able to store carbohydrates. The "Selfish Glycan Theory" suggests that glycans are so essential for a living organism (just

like proteins) that each glycan requires its own supply of precursors. Extending this idea can be viewed by the specificity of GlycTs to their donors. It is well established that most, if not all, GlycTs are inhibited by the nucleotide-diphosphate. The data to date suggest that GlycT first binds the nucleotide then the sugar. Collectively, it would make "sense" that specific GlycTs avoid inhibition (by NDP) and compete for the sugar by selective pressure to adopt UDP-Glc rather than ADP-Glc, for example.

To make sure that glycans will succeed, eukaryotic cells also evolved to form these essential glycans in specific subcompartments. Starch, for example, is produced in plastids, whereas xyloglucan is made in the Golgi and cellulose at the plasma membrane. To accommodate synthesis of these glycans, multiple isoforms that synthesize either the same NDP-Glc (UDP-Glc) or different NDP-Glc (ADP-Glc) evolved for each glycan. Of course, it will also make sense that plants would produce the minimum amount of these NDP-sugars inside the cell to avoid metabolic waste and to efficiently incorporate these precursors in the appropriate subcellular sites where each glycan is made.

5.6.21.3 Cellular location and enzyme topology

DNA is a water-soluble molecule, aided by tens of proteins to helping in the packaging and re-packing during RNA and DNA synthesis. Proteins are also soluble and if they are too hydrophobic, either a portion will be embedded in membranes or hydrophobic domains will assemble together with other hydrophobic proteins to maintain their solubility. On the other hand, plant polysaccharides provide us with a challenge in terms of understanding how such massive amounts of insoluble or gel-like structures are made inside the Golgi apparatus without hindering other cellular processes. Bacteria, for example, form insoluble lipo-glycans in stages: UDP-sugars made in the cytosol contribute to synthesis of short side chain glycans attached to the inner membrane. These short glycans are made facing the cytosol. The side chains are then flipped and transferred through the outer membrane to the outside of the cell, where specific enzymes cut and assemble the side chains to form complete glycan structures. This process, by analogy, is very similar to the synthesis of core N-linked glycoproteins. But how are pectins and hemicelluloses made in plants? Are Golgi-synthesized polysaccharides made completely in the Golgi, or are they made in smaller fragments that are assembled together at the wall, as is the case in lipo-glycan synthesis? If the entire pectic polymers are made in the Golgi, then are they sequestered or packed temporarily with or by proteins to help with the challenge posed by their physical properties (i.e., solubility, size, etc.)? Further questions will arise. Is HG made inside the lumen (thus making a jelly-like lumen) or perhaps made in specific Golgi stacks, designated only for the wall (not glycoprotein biosynthesis)? The latter scenario could be attractive in light of the fact that unlike humans and fungi, one plant cell consists of hundreds of Golgi stacks. It is possible that an entire "designated Golgi" is moving with its packed glycan(s) to the wall to "unload" the insoluble matrix.

While many of the putative GTs are Type II membrane proteins (i.e., predicted to have their catalytic domain facing the lumen), some GTs, such as mannan synthase, have multi domains that span the membranes. Where is the catalytic domain of such Golgi-enzymes facing? Furthermore, if a glycan is fully made in the Golgi, is it made in one subcompartment of the Golgi, for example, cis-Golgi, or is it initiated in the cis- and further modified in the medial-Golgi (like glycoproteins)? Certainly, understanding where each wall biosynthetic enzyme functions and where its catalytic domain faces is essential to address the above questions.

In addition to location and topology, it is puzzling how polysaccharides are made in the small Golgi cisternae (estimated to be smaller than 20 × 200 nm). The same cisternae are temporarily packed not only with wall-glycans but also with numerous Golgi-resident proteins and large numbers of secretory proteins. If the average size of the catalytic domain of GTs and membrane-bound NDP-sugar biosynthetic enzymes is 30–40 kDa, they can "touch" each other if inserted opposite to each other. Given the low quantity of these metabolic enzymes and the potential solubility problems of some glycans, one can wonder if synthesis of a specific Golgi-glycan is done in a complex of enzymes, as is the case in the synthesis of cellulose.

5.7 Perspectives

Traditional protein biochemistry and classical purification was instrumental in identifying key wall biosynthetic enzymes, for example, XG:1-2,fucosyltransferse (116, 117) and GM:1-6,galactosyltransferase (88). As most of the glycosyltransferases involved in polysaccharide synthesis are membrane bound and are of low abundance, i.e., estimated 2–6 molecules per cell, it is clear that other methods should be sought to identify wall biosynthetic proteins. Classical genetics has already contributed immensely to the identification of large numbers of genes involved in glycan synthesis such as cellulose synthase and their large gene family (507, 508) and xyloglucan synthesis (120). The genome sequence facilitated the identification of mannan synthase (85) and cellulose-like proteins (154). A combined approach of partial protein purification and proteomics was useful in the identification of a large family associated with the pectin biosynthetic HG:galacturonosyltransferases (137). The classification of GTs in the CAZY database was instrumental in identifying GT gene candidates targeted for a reverse genetic approach using the SALK T-DNA or other mutant library collections. Lastly, microarray analyses of tissues or cell types at different developmental stages were useful in identifying secondary wall synthesis candidates (139, 509). Similar biochemical, genetic, and genomic approaches were successful in identifying biosynthetic genes involved in nucleotide-sugar synthesis and NDP-sugar transporters. The last decade has been a very fruitful and exciting time for the community of wall researchers (finally a crack in the wall).

Can bioinformatics assist in the prediction of GTs? Would the knowledge of UDP-binding sites in several plant GTs be useful to distinguish between GDP-, or ADP-transferases? Would the knowledge of the sugar moiety-binding site be similarly helpful to identify, for example, putative GalTs? Is the binding pocket for UDP-Rha in a flavonoid: RhaT the same as for pectin:RhaT? Clearly, a critical mass of biochemical knowledge is required to start to predict gene function by computer. We are not there yet....

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