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Molecular Genetics of Non-processive Glycosyltransferases

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Introduction

Although *Arabidopsis* has become the model for plant genomic and developmental research, it remains behind the rest of the field with respect to biochemical and structural analyses. However, the availability of the genome sequence provides an alternative and probable first approach towards identification, by homology, of potentially interesting genes. Subsequently, such a genetic approach has been useful in identifying a class of enzymes involved in cell wall biosynthesis, the polysaccharide glycosyltransferases.

Most enzymes that are involved in glycan biosynthesis are glycosyltransferases. These enzymes act mainly by adding monosaccharides (donor molecules) one at a time to specific positions on acceptor molecules and thus assemble monosaccharides into linear and branched sugar chains. It has been estimated that more than 100 distinct glycosidic linkages are present in the glycoconjugate repertoire of any given multicellular organism. Because each linkage is usually the product of a different glycosyltransferase, it appears that a large number of distinct glycosyltransferases is present in higher eukaryotes. Many, but not all, of these enzymes are found within the ER-Golgi pathway where the synthesis of many glycoconjugates occurs. Glycosyltransferases may act as either processive or non-processive enzymes. Processive glycosyltransferases (often referred to as polysaccharide synthases) add sugar moieties to the growing end of a linear polysaccharide without releasing the acceptor substrate. One classic example is cellulose synthase. Non-processive glycosyltransferases add single monosaccharides to specific positions of an acceptor molecule, then move on to other acceptor sites. These enzymes are usually type II membrane proteins possessing a single hydrophobic segment spanning the Golgi membrane, which functions as a signal-anchor sequence. A short amino-terminal portion faces the cytosol and the carboxy-terminal catalytic

domain is positioned within the lumen of the Golgi apparatus (Figure 1).

Many glycosyltransferases have been identified and extensively studied in animal, fungal, and bacterial systems; these are classified into different families, based on the type of sugar that they transfer and the type of linkage that they establish. For instance, an enzyme transferring a galactose residue from UDP-D-galactose onto the 2-hydroxyl group of another sugar moiety would be considered a (1,2)-galactosyltransferase. The sugar moiety may be added in either alpha or beta configuration adding further complexity to the types of linkages that can be formed. If in the above example the newly attached galactose residue is in the beta configuration, the enzyme would be classified as a β (1,2)-galactosyltransferase. Since the sugar moiety in UDP-D-galactose exists in the alpha configuration, β -galactosyltransferases act with inversion of configuration whereas α -galactosyltransferases act with retention of configuration.

Like other eukaryotic organisms, plants have glycoproteins and glycolipids; however, a major feature distinguishing plants from animals is the presence of a complex wall surrounding every cell. These walls play a crucial role in development, signal transduction, and response to environmental factors, including microbial pathogens and insects. Plant cell walls are mainly composed of cellulose microfibrils and matrix polysaccharides that encompass hemicelluloses and pectins. The synthesis of polysaccharides can be divided into two main stages: synthesis of the backbone, performed by polysaccharide synthases, and addition of side-chain residues mediated by glycosyltransferases. The structural complexity of many of these polysaccharides is attributed to numerous modifications that involve additions of various sugars by glycosyltransferases followed by acetylations, methylations, and addition of phenolics. Although biochemical analyses have demon-

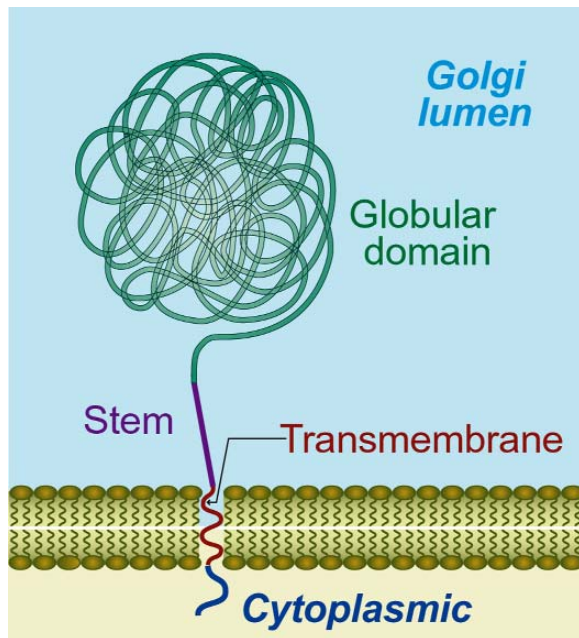


Figure 1. Schematic diagram of most Golgi-resident glycosyltransferases. They are characterized by a short NH₂-terminus oriented toward the cytoplasmic side of the Golgi membrane, a single membrane spanning region, and a variable length stem connecting the globular domain, located in the lumen of the Golgi.

strated the existence of many glycosyltransferases in plants (White et al., 1993; Keegstra & Raikhel, 2001 and references therein), to date only three genes encoding non-processive glycosyltransferases in cell wall polysaccharide biosynthesis have been cloned, and several genes that are involved in glycoprotein biosynthesis have been identified as well. Low amino acid sequence similarity between families of glycosyltransferases and the difficulties of obtaining acceptor substrates contribute to the lack of success in identifying plant cell wall glycosyltransferases. In this chapter we will highlight some of the advances made in this area over the past few years.

Xyloglucan Fucosyltransferase

Xyloglucan (XG) is the most abundant and best characterized hemicellulose of primary plant cell walls. An important function of XG is to bind and cross-link cellulose microfibrils and thereby regulate cell growth. The XGs are linear chains consisting of a $\beta(1,4)$ -linked glucan backbone with three-fourths of the glucosyl residues substituted with

xylosyl residues in a regular repeating pattern. Some of the xylosyl residues are decorated by galactosyl and fucosyl units to form disaccharide and trisaccharide side-chains (Figure 2). XG fucosylation is thought to straighten the glucan backbone, which may facilitate the binding of XG to cellulose, thereby contributing to cell wall strength (Levy et al., 1997). However, the storage XGs in tamarind or nasturtium seeds lack fucosyl residues and can serve as exogenous acceptors to assay for fucosyltransferase (FUTase) activities present in the Golgi membranes of rapidly growing plant tissues such as pea epicotyls (Maclachlan et al., 1992).

Partial amino acid sequence data of XG-specific FUTase from pea epicotyls were obtained following purification of the enzyme. The information deduced from these sequences was then used to isolate a cDNA clone (*AtFUT1*) from *Arabidopsis* (Perrin et al., 1999) and later from pea (*PsFUT1*; Faik et al., 2000). The identity of *AtFUT1* as encoding a XG-specific fucosyltransferase was shown by analyzing its activity when expressed in a heterologous system and by immunoprecipitating endogenous activity using antibodies against *E. coli*-expressed *AtFUT1* (Perrin et al., 1999). Data from the *Arabidopsis* Genome Sequencing Initiative showed that *AtFUT1* is located on chromosome 2. As shown recently, this protein contains a missense mutation in *mur2* (Vanzin et al., 2002), a fucose-deficient mutant identified by Reiter et al. (1997). This study demonstrated that a single point mutation in *AtFUT1* was sufficient to virtually eliminate XG fucosylation in all major organs of the plant, indicating that *AtFUT1* accounts for all of the XG fucosyltransferase activity in *Arabidopsis*. This conclusion is supported by the preliminary analysis of an *AtFUT1* knockout mutant that is entirely devoid of fucose in its XG (Tanya Wagner, Rodrigo Sarria, Kenneth Keegstra, and Natasha Raikhel, unpublished observation).

The *PsFUT1* enzyme has been biochemically analyzed and shown to be XG-specific (Faik et al., 2000). An evaluation of the amino acid sequences of *AtFUT1* and *PsFUT1*, which are 62.3% identical, indicates that these plant fucosyltransferases show very limited sequence similarity to fucosyltransferases from other organisms. This explains why it has not been possible to use animal or fungal genes as heterologous probes to isolate genes for plant cell wall biosynthetic enzymes, despite several attempts by various groups.

Identification of *AtFUT1* made it possible to use bioinformatic approaches to identify nine additional *Arabidopsis* genes related to *AtFUT1* in the *Arabidopsis* genome (Sarria et al., 2001). All these genes, named *AtFUT2-10* are located on chromosome 1 or 2 and clustered in four BAC clones. *AtFUT* family members contain motifs that are present in $\alpha(1,6)$ - and $\alpha(1,2)$ -fucosyltransferases (Oriol et al., 1999), and seven of the new *AtFUT* proteins contain

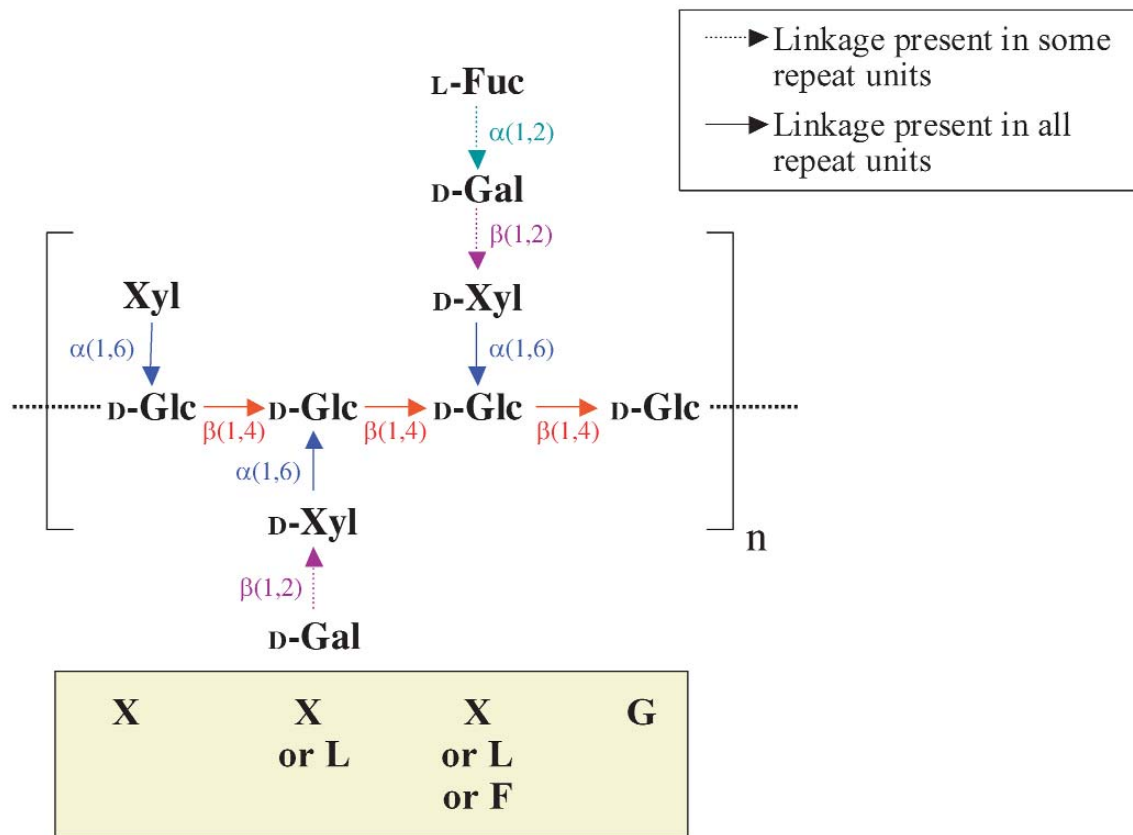


Figure 2. Acceptor structure of XG backbone decorated with galactosyl and fucosyl residues. This structure of XG is typical of that found in many dicots. This representation simplifies XG structure and is not intended to convey all the topological nuances.

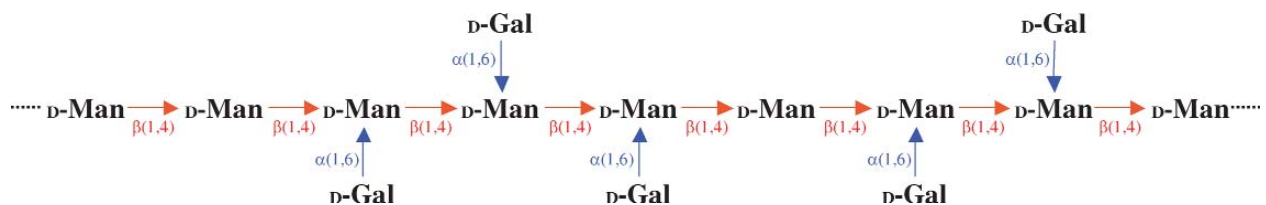


Figure 3. An overview of the branching pattern of the less abundant noncellulosic polysaccharide galactomannans; a backbone composed exclusively of (1,4)-D-mannose decorated with α -D-galactosyl units added at the O-6 positions.

a motif that is proposed to bind GDP-fucose (Takahashi et al., 2000). AtFUT1-10 and PsFUT1 have been assigned to glycosyltransferase family 37 that is distinct from the fucosyltransferases that have been identified from fungi, animals, and bacteria (Coutinho and Henrissat, 1999). Pairwise comparison of the amino acid identity/similarity along the entire coding sequence among the family members shows that AtFUT1 shares 37.7-54.4% identity with family members.

AtFUT3, *AtFUT4*, and *AtFUT5* were expressed in tobacco cell lines to produce protein for biochemical assays. These proteins did not fucosylate XG *in vitro* (Sarría et al., 2001), which is consistent with the absence of XG fucosylation in *AtFUT1* mutants.

In summary, the analysis of *AtFUT3*, *-4*, and *-5* suggest that these AtFUT proteins are not functionally redundant with *AtFUT1* and may have unique functions in the fucosylation of AGPs and pectic components. Extensive genetic

Table 1.

***Arabidopsis* homologs to α -galactosyltransferase from fenugreek**

<u>Putative AtXTs</u>	Genbank accession	Length (amino acids)	Sequence identity to fenugreek GalT	Chromosomal location	Number of ESTs
AtXT ₁	CAB80743	459	44%	Chr. IV	1
AtXT ₂	CAB83122	460	42%	Chr. III	2
AtXT ₃	BAB11451	457	46%	Chr. V	1
AtXT ₄	AAF27110	513	42%	Chr. I	8
AtXT ₅	AAG5237	457	44%	Chr. I	3
AtXT ₆	AAC32437	449	55%	Chr. II	6
AtXT ₇	CAB38308	432	55%	Chr. IV	4

analysis of the *AtFUT* genes in various combinations may be the best approach to address the biological roles of these genes.

Galactomannan α (1,6)-galactosyltransferase

Several legumes such as guar, locust bean, and fenugreek deposit galactomannans as storage polysaccharides in the walls of their seed endosperms. The galactomannans contain a backbone of α (1,4)-linked D-mannose residues substituted at the 6-position by α -D-galactose moieties (Figure 3). Accordingly, these galactomannans are structurally similar to XGs with mannose replacing glucose and galactose replacing xylose. Since the degree of galactose substitution in seed storage galactomannans is of some relevance to the food industry, Edwards et al. (1999) used a biochemical approach to clone α (1,6)-galactosyltransferase from fenugreek (*Trigonella foenum-graecum* L.). Membrane fractions from developing fenugreek endosperm were used to establish a correlation between galactomannan galT activity and a 51 kDa protein. Determination of partial amino acid sequences from the protein permitted the design of degenerate oligonucleotide primers to eventually clone a full-length cDNA via RT-PCR and RACE protocols. This cDNA was predicted to encode a type II membrane protein of 51,282 kDa and an isoelectric point of 6.65 closely matching the results obtained during partial protein purification (molecular mass of 51 kDa and pI of 6.5). Expression of the cDNA in the methy-

lotrophic yeast *Pichia pastoris* yielded substantial amounts of galactomannan α -galT activity providing conclusive evidence that the desired coding region had been cloned.

A search of *Arabidopsis* databases for homologs to the fenugreek galT revealed seven putative type-II membrane proteins with substantial sequence similarity to the fenugreek enzyme, in particular within the predicted catalytic domain (Perrin et al., 2001; see Table 1). The *Arabidopsis* coding regions fall into three phylogenetic groups, one of which shows sequence identities of 55% between the fenugreek and *Arabidopsis* enzymes (encompassing GenBank accessions AAC32437 and CAB38308). Neither the fenugreek galT nor its *Arabidopsis* homologs are closely related to known galactosyltransferases presumably because seed storage type galactomannans are unique to higher plants, and glycosyltransferases acting on unrelated acceptor substrates tend to share very little sequence similarity to each other. Although the *Arabidopsis* genes were annotated as α -galactosyltransferases (family 34 at <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>), one of them has recently been shown to encode XG xylosyltransferase (Faik et al., 2002) suggesting that at least some of these coding regions play a role in XG xylosylation.

Xyloglucan Xylosyltransferase

To try and clone genes involved in the xylosylation of the XG backbone, we established a biochemical assay for the XG- α (1,6)-xylosyltransferase. Using pea microsomes

that are capable of XG biosynthesis (White et al., 1993), we solubilized an α -(1,6)-xylosyltransferase that catalyzes the transfer of xylose from UDP-[^{14}C]xylose onto β -(1,4)-linked glucan oligosaccharides. When cellopentaose was used as acceptor, product analysis revealed that the xylose was present in an α -(1,6)-linkage to a glucosyl residue, as expected for an enzyme involved in XG biosynthesis. Preliminary characterization of the pea xylosyltransferase activity together with the identification of the seven *Arabidopsis* genes, led to the hypothesis that one or more of these genes may encode an α -xylosyltransferase. To test this, full-length cDNA clones of six of the putative xylosyltransferase genes (*AtXT*) from *Arabidopsis* were expressed in the yeast *Pichia pastoris*, and the expression products were tested using the biochemical assay described above. The product of one of the candidate genes showed cello-oligosaccharide-dependent xylosyltransferase activity, producing a product similar or identical to that generated by the pea enzyme. Thus, we conclude that *AtXT1* encodes xylosyltransferase involved in XG biosynthesis (Faik et al., 2002).

While xylosyltransferase activity was not observed with the other putative *AtXTs* when they were tested in this assay, it is possible that they require acceptors that already contain a xylosyl residue and are involved in adding other xylosyl residues to the XG backbone. It is known that glycosyltransferases have restricted specificity, suggesting that XG biosynthesis may require more than one xylosyltransferase. In addition, even though the structure and function of a protein can be intrinsically related to its primary structure, sequence-related proteins do not

necessarily share the same mechanisms of activity. For example, preliminary attempts to measure xylosyltransferase and galactosyltransferase activities for *AtXT2*, which is 85% identical to *AtXT1*, were unsuccessful. One hypothesis is that this gene may encode a xylosyltransferase which cannot function independently but requires cooperative action from glucan synthase (Faik et al., 2002). We will continue genetic and biochemical analysis of these putative *AtXTs* genes and their products in order to address their biological function.

Complex *N*-glycans on Plant Glycoproteins are Unique

Like other eukaryotes, plants possess both *N*- and *O*-linked glycans, and the basic biosynthetic pathway toward the synthesis of *N*-linked glycans is highly conserved among all eukaryotes. *N*-glycans are attached to asparagine residues and *O*-glycans are attached to hydroxyproline, serine, or threonine residues. Several types of *N*-glycans are known including high mannose glycans with the composition $\text{Man}_5\text{GlcNAc}_2$ and complex glycans which carry fewer mannose residues but are decorated with other sugars such as Fuc, Xyl, GlcNAc and Gal (Figure 4). The biosynthesis of high mannose glycans in plants is similar to that in fungi and animals. However, complex plant *N*-glycans are rather small and lack sialic acid but contain β (1,2)-xylose and α (1,3)-fucose residues, which are not found in mammalian glycoproteins, but are present in molluscs, insects, and spiders (Oriol et al., 1999). Plant glycoproteins are often highly immunogenic when used for production of antibodies, and this immunogenicity could come from the α (1,3)-fucose and β (1,2) xylose residues. It is also often speculated that plant complex glycans are the cause of allergic symptoms in mammals.

Several genes that encode glycosyltransferases responsible for these modifications have been isolated in order to gain a better understanding of the unique features of plant glycans. Using degenerate primers representing highly conserved regions of known GlcNAc-transferases from animals, a cDNA coding for GlcNAc-transferase was isolated from a *Nicotiana tabacum* library. The identity of the GlcNAc-transferase was confirmed by heterologous expression of the predicted catalytic domain from the putative enzyme: Man_5 - or $\text{Man}_3\text{GlcNAc}_2$ are acceptor substrates for this enzyme (Strasser et al., 1999). The plant GlcNAc-transferase possesses 41% identity to known mammalian enzymes with the same specificity. The

Plant *N*-linked complex glycan

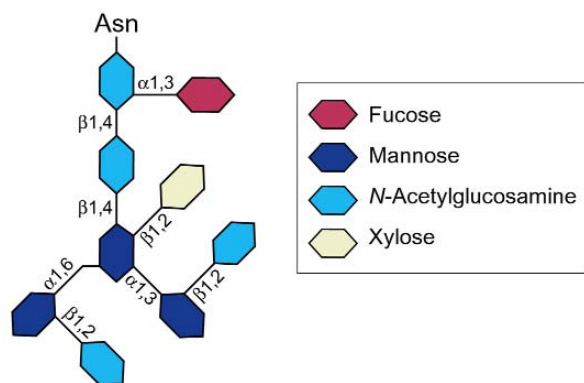


Figure 4. Schematic illustration of the plant glycoprotein motifs of *N*-linked oligosaccharides. The different sugars are shown in different shapes. No attempt is made to depict the position or anomeric configuration of the glycosidic linkages. Asn, asparagine.

$\alpha(1,3)$ -fucosyltransferase catalyzing the transfer of fucose into a 1,3-linkage to the innermost GlcNAc-residue of an *N*-glycan was purified from mung beans to homogeneity, sequence information was obtained, and the corresponding cDNA was cloned (Leiter et al., 1999). The recombinant fucosyltransferase was expressed in a heterologous system and the predicted activity of this enzyme was demonstrated. The plant $\alpha(1,3)$ -fucosyltransferase has a relatively low but significant level of identity (28%) to other known enzymes that belong to the same family. An evaluation of the *Arabidopsis* genome sequence enabled Wilson et al. (2001) to identify two close *Arabidopsis* homologs (FucTA and FucTB) to the mung bean enzyme, and to express the corresponding cDNAs in *Pichia pastoris*. The expected $\alpha(1,3)$ -fucosyltransferase activity could be demonstrated for FucTA but not FucTB. Curiously, most *FucTB* cDNAs contained a four-nucleotide insertion due to the use of an alternate splice site. Translation of these cDNAs would lead to the formation of a truncated protein lacking most of the catalytic domain. Using bioinformatics approaches, Wilson et al. (2001) identified a third putative glycoprotein fucosyltransferase gene (*FucTC*) within the *Arabidopsis* genome, and expressed the corresponding cDNA in *P. pastoris*. FucTC shows approximately 35% amino acid sequence identity to FucTA and FucTB over a stretch of 115 amino acids at its C-terminus. Furthermore, it displays significant sequence similarities to a murine fucosyltransferase involved in the creation of so-called "Lewis epitopes." These *N*-linked glycans represent important blood group antigens in mammals, and have also been found in a variety of plant species with the notable exception of *Brassicaceae* including *Arabidopsis* (Fitchette et al., 1999). Using a variety of acceptor substrates, no fucosyltransferase activity could be demonstrated for the *FucTC* gene product raising the possibility that no functional enzyme is produced even though the gene is transcribed.

Using amino acid sequences derived from soybean Xyl-transferase, the *Arabidopsis* Xyl-transferase gene was isolated. Recombinant protein was produced that exhibits Xyl-transferase activity *in vivo* (Strasser et al., 2000). Using computer searches with dbEST as the database, homologs of *Arabidopsis* $\beta(1,2)$ -xylosyltransferase could be found in a variety of plant species including cotton, alfalfa, tomato, rice, and aspen; however, no close homologs were discovered in animals, fungi or prokaryotes which is in line with the observation that xylosylation of the second "core" GlcNAc residue is unique to plants and a few species of invertebrates.

All three genes encoding glycosyltransferases that mediate decoration of complex glycans in plants are predicted to be type II membrane proteins and to be localized to the Golgi, however, this has not been demonstrated yet. Availability of these glycosyltransferases could be very

useful in studying an unusual immunogenicity and allergenicity of plant glycoproteins.

Conclusions

Our continuing interest in cell wall biosynthesis is critical to our understanding of plant development. This current interest is expanding because of the increasing evidence that the properties of the cell wall mediate cellular interactions during growth, development and differentiation. Emphasis placed on the identification of glycosyltransferases, due to their obvious importance in polysaccharide synthesis, will increase our understanding of the requirements for biosynthesis of the cell wall. Genomic strategies have begun to unveil genes encoding enzymes involved in polysaccharide biosynthesis, allowing the identification of hundreds of glycosyltransferases. The paucity of demonstrated glycosyl-transferases from the plant kingdom could be related to the unique structure of plant polysaccharides, which makes it difficult to identify coding regions within plant genomes that are highly homologous to glycosyltransferases from animals or prokaryotes. The classification of glycosyltransferases into 50 families (so far) is testimony to their diversity. Such diversity is presumably related to the plethora of combinations of substrates, linkages, and recognition of the acceptors.

To date, the genes for only three plant glycosyltransferases that are involved in cell wall biosynthesis have been cloned and convincingly characterized; these enzymes were first purified using a biochemical approach. However, the biochemical intractability of many enzymes involved in plant cell wall biosynthesis has led to a growing realization that novel methods will be necessary to make progress in this field. With the identification of additional genes for each of the glycosyltransferases discussed above, we should keep in mind that some may encode proteins that are redundant, while others may transfer the monosaccharide to an alternative acceptor substrate in the cell. Characterization into these genes has begun and will be reported on as the biological function of each is obtained. It is also clear that in numerous cases, the biochemical approach is extremely time-consuming and often unproductive. Availability of genomic and genetic tools certainly will accelerate the progress on this, one of the most important areas of plant biology. Ultimately, chemical and biochemical tools will have to be employed in order to elucidate specific exogenous acceptor substrates or conditions for solubilization of active enzymes. Only then will we be able to unequivocally demonstrate a particular enzymatic function.

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