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Xylan biosynthesis

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Plant cells are surrounded by a rigid wall made up of cellulose microfibrils, pectins, hemicelluloses, and lignin. This cell wall provides structure and protection for plant cells. In grasses and in dicot secondary cell walls, the major hemicellulose is a polymer of β-(1,4)-linked xylose units called xylan. Unlike cellulose — which is synthesized by large complexes at the plasma membrane — xylan is synthesized by enzymes in the Golgi apparatus. Xylan synthesis thus requires the coordinated action and regulation of these synthetic enzymes as well as others that synthesize and transport substrates into the Golgi. Recent research has identified several genes involved in xylan synthesis, some of which have already been used in engineering efforts to create plants that are better suited for biofuel production.

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Introduction

Lignocellulosic biomass is made up largely of the secondary cell walls of plants and is composed primarily of cellulose, lignin, and the hemicellulose xylan. Xylan consists of a backbone of xylose units with various additional substitutions and xylan deposition in the secondary cell wall is required for normal plant growth and development. Xylan also increases cell wall recalcitrance and thereby helps to defend against herbivores and pathogens. Pentoses cannot be fermented by microorganisms such as yeast and therefore xylan represents a large fraction of biomass that cannot be efficiently utilized for fermentation, for example into biofuels. A complete understanding of the genes involved in xylan synthesis, how they are regulated, and how changes to these genes affect plant growth will allow us to design strategies for engineering plants with altered xylan.

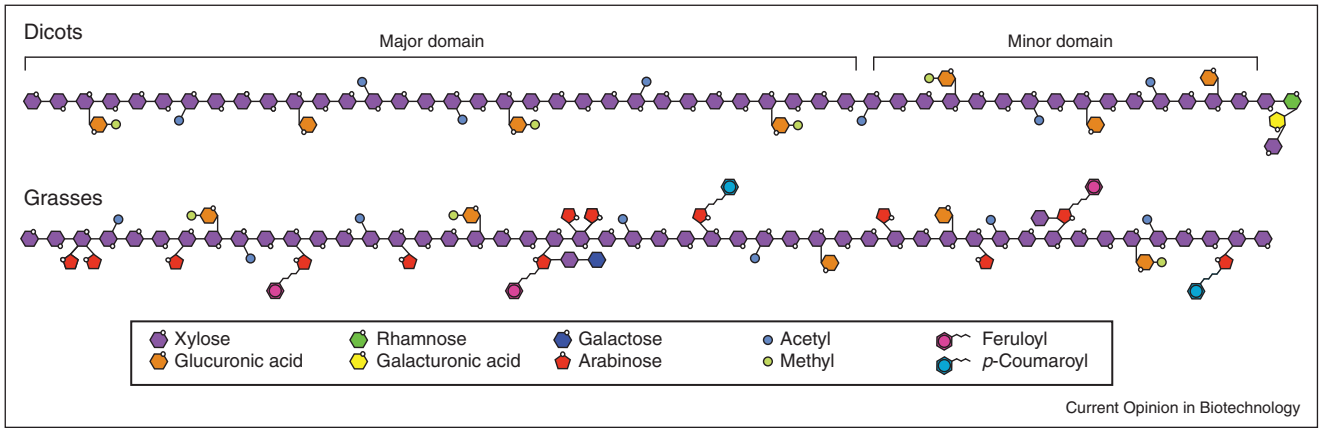
Xylan structure

Xylan consists of a linear polymer of β-(1,4)-linked xylose residues substituted with acetyl, glucuronic acid (GlcA), 4-*O*-methylglucuronic acid (Me-GlcA), and arabinose residues (Figure 1). There is variation in xylan structures between different species and even between different tissues in the same species. In dicots, xylan is the predominant hemicellulose in secondary cell walls, but little is found in primary cell walls. In Arabidopsis, the ratio of Me-GlcA to GlcA substitutions is around two, and (Me-) GlcA substitutions are found, on average, on one out of every eight xylose residues [1••]. No arabinosyl substitutions have been reported in Arabidopsis or poplar, but they are known from other dicots. In contrast, xylans in grasses (Poaceae) contain much more arabinofuranose (Ara*f*) and comparatively little GlcA. Ara*f* may be α-(1,2) or α-(1,3)-linked to the xylan backbone, and the α-(1,3)-linked residues may be further substituted with xylose, coumaric acid, or ferulic acid. Little information is available regarding the frequency and patterns of substitutions in grasses, but in general the frequency of substitutions is lower in older tissues.

Dicot xylan molecules include the tetrasaccharide 4-β-D-Xyl-(1–4)-β-D-Xyl-(1–3)-α-L-Rha-(1–2)-α-D-GalA-(1–4)-D-Xyl at their reducing ends. The function of this tetrasaccharide is not known, although it has been suggested to serve either as an initiator or terminator of xylan backbone synthesis [2]. The structure of xylans is discussed in several recent reviews [3–6].

One elusive question regarding xylan structure is its interactions with other cell wall polymers. Xylan has been proposed to coat cellulose microfibrils and crosslink them with each other or with other polymers via hydrogen bonding, which may be influenced by xylan substitution patterns since these patterns are expected to affect xylan's conformation and solubility [1••]. Xylan may also covalently bind to other polymers: it may be linked to lignin via ester bonds to GlcA and ether bonds to Xyl or Ara [7,8], and the ferulic acid esters in grass xylans can undergo oxidative dimerization to form crosslinks to adjacent xylan chains or to lignin. A recent report provides evidence that in Arabidopsis xylans can be covalently linked to both pectin and arabinogalactan proteins in a large proteoglycan complex called 'Arabinoxylan Pectin Arabinogalactan Protein 1' (APAP1), possibly via the rhamnose backbone residues in rhamnogalacturonan and arabinose residues in arabinogalactan [9••]. The xylan found to be linked to these structures contained arabinose — which has not been observed before in Arabidopsis xylan — suggesting that the xylan in APAP1 is distinct

Figure 1



Generalized structures of xylan. Dicot xylan is substituted with GlcA, Me-GlcA, and acetate. Arabinose substitutions may be present but are frequently not found. The major domain has (Me-) GlcA on evenly spaced residues about eight xylose units apart, while the minor domain has (Me-) GlcA more closely spaced. Rhamnose and galacturonic acid are found at the reducing end. In grasses, xylan may also be substituted with arabinose, xylose, galactose, and ferulic and coumaric acid.

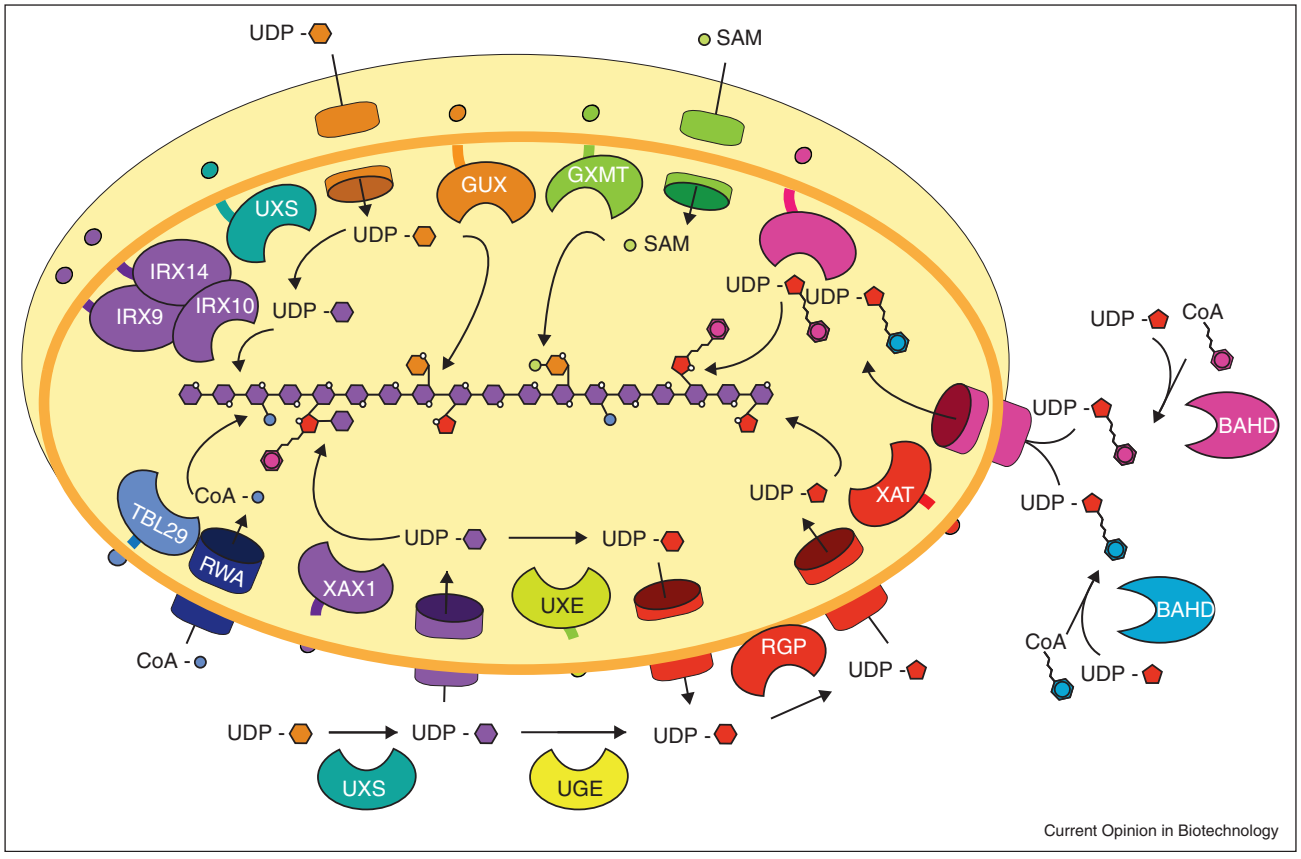
from previously studied Arabidopsis xylans. Very little is known about synthesis of this xylan domain and how it is attached to APAP1. Understanding this process, including how much of xylan is linked to APAP1 and whether linkage formation occurs in the Golgi, as polysaccharides are synthesized, or in the apoplast, will greatly affect our understanding of xylan's biosynthesis and role in the wall.

Glycosyltransferases and other enzymes involved in xylan synthesis

Several enzymes have been implicated in xylan synthesis, many in the last few years. Figure 2 shows a schematic overview of xylan biosynthesis. Two members of Glycosyltransferase Family 43 (GT43), *Irregular Xylem (IRX) 9* and *IRX14*, and one member of GT47, *IRX10*, encode putative xylosyltransferases required for synthesizing the xylan backbone. Mutations in these three genes cause dwarfing and a reduction in xylan content and xylosyltransferase activity [10,11]. In addition, related genes *IRX9-like (IRX9-L)*, *IRX10-L*, and *IRX14-L* appear to encode functionally redundant paralogs [11,12]. Overexpressing IRX9 and IRX14 together in tobacco cell culture results in higher microsomal xylan:xylosyltransferase enzyme activity, leading to the suggestion that these two proteins synthesize the xylan backbone cooperatively [13]. However, overexpression of rice IRX9 in Arabidopsis led to increased xylan synthase activity without the need for simultaneous overexpression of IRX14 [14]. Activity of purified IRX9 or IRX14 has not been shown *in vitro*, and it is not clear whether these proteins and/or IRX10 are catalytically active. A study of wheat xylan synthase showed that homologs of IRX14 and IRX10 could be coimmunoprecipitated, indicating the presence of several glycosyltransferases in the same xylan synthase complex [15•]. The wheat xylan synthase complex also contained UDP-arabinose mutase, which is responsible for converting UDP-arabinopyranose to the UDP-arabinofuranose that is the substrate for xylan arabinosyltransferases [15•,16]. The GlcA substitutions on xylans in the Arabidopsis secondary cell wall are introduced by the action of Glucuronic Acid Substitution of Xylan (GUX) 1 and GUX2, both members of the GT8 family [17,18••,19]. Interestingly, Bromley *et al.* [1••] showed that GUX1 is responsible for adding GlcA to evenly spaced xylose residues ranging from 6 to 26 residues apart, while GUX2 adds GlcA to evenly as well as oddly spaced xylose residues. These two different activities result in synthesis of two distinct xylan domains, most likely separated from each other but coexisting within the same xylan molecule. The functional significance of these different domains is unclear, although they may affect xylan's ability to cross-link cellulose microfibrils [1••].

4-*O*-Methyl groups are transferred from *S*-adenosylmethionine to GlcA residues by GXMT1, a protein containing a Domain of Unknown Function 579 (DUF579) [20••,21••]. Mutations in other DUF579 genes, specifically *IRX15* and *IRX15-L*, have been shown to cause decreases in xylan content in Arabidopsis [22•,23]; however, the biochemical function of IRX15 and IRX15L is unclear, and they are unlikely to methylate glucuronoxylan since the degree of methylation is increased in *irx15* and *irx15-l* mutants [20••]. IRX15 and IRX15-L may instead be noncatalytically active members of a xylan synthesis protein complex, or they may methylate another cell wall polymer, such as pectin, that has an indirect effect on xylan synthesis. Pectin contains

Figure 2



Xylan is synthesized in the Golgi apparatus by Type II membrane proteins anchored by a single N-terminal transmembrane domain and with their catalytic domains in the Golgi lumen. Some proteins, such as IRX10, are predicted to lack a transmembrane domain. Substrates are synthesized in both the cytosol and in the lumen. UDP-GlcA is transported into the Golgi by an unknown transporter and converted into UDP-Xyl by UDP-Xyl Synthase (UXS). Another isoform of UXS is present in the cytosol, and UDP-Xyl synthesized there can also be transported into the Golgi. The relative fluxes through these two separate pathways are unknown. UDP-Xyl is converted to UDP-Ara*p* inside the Golgi by UDP-Xyl Epimerase (UXE) [57] and UDP-Ara*p* is converted to UDP-Ara*f* by the mutase Reversibly Glycosylated Protein (RGP), located on the outer Golgi membrane [16]. Presumably transporters must move UDP-Ara*p* out of and UDP-Ara*f* into the Golgi, but these transporters have not yet been identified. Some isoforms of cytoplasmic UDP-Glc Epimerase (UGE) may also contribute to the conversion of UDP-Xyl to UDP-Ara*p* [58]. BAHD acyltransferases in the cytosol are involved in xylan synthesis and presumably transfer ferulic acid to an intermediate, such as UDP-Ara*f*, which is then transported into the Golgi and transferred onto xylan by unknown proteins. Acetate is likely added to xylan by the DUF231 protein Trichome Birefringence-like 29 (TBL29). Reduced Wall Acetylation (RWA) proteins are also involved in acetylation and may serve as acetyl-CoA transporters. *S*-Adenosylmethionine, the substrate for xylan methylation, is synthesized in the cytosol and must also be transported into the lumen. Not shown are transporters for coproducts such as CoA, and *S*-adenosyl homocysteine, which must be removed from the Golgi and recycled in the cytosol. UMP is removed from the Golgi by the nucleotide sugar transporters, which function as antiporters.

O-methylated fucose and xylose residues [24], and many cell wall mutants show reductions in both pectin and xylan, making it difficult to sort out pleiotropic effects [11,25–27].

A recent paper has identified members of the GT61 family that are likely to add arabinosyl residues to the xylan backbone [28••]. The biochemical function has not been unequivocally demonstrated and it is unclear how many different types of enzymes that are required to add the different O-2 and O-3 linked arabinosyl residues. Grass xylans often have xylose linked to O-2 of feruloylated arabinosyl residues and a recent paper indicated that another GT61 enzyme is a xylosyltransferase responsible for this structure [29••]. Several papers have confirmed that acyltransferases belonging to the BAHD family are involved in the addition of ferulic and coumaric acid esters to xylan [30,31]. BAHD acyltransferases are cytoplasmic as are the hydroxycinnamoyl-CoA substrates and therefore it is surprising that these enzymes can mediate esterification of nascent xylan in the Golgi lumen. Most likely an intermediate acceptor is esterified in the cytoplasm and transported into the Golgi. It has been speculated that

the intermediate could be feruloyl-UDP-arabinofuranose [32], but evidence for this is lacking.

A number of additional glycosyltransferases known as IRX7/FRA8 (and the homolog IRX7L/F8H), IRX8/GAUT12, and PARVUS/GATL1 have been implicated in xylan biosynthesis due to the xylan deficient phenotype of the corresponding loss-of-function mutants [3,4]. These mutants have small amounts of xylan with a high molecular mass and absence of the reducing end oligosaccharide, which has led to the suggestion that they are involved in synthesizing the reducing end and that this structure functions as a type of terminator [2]. However, more recently the moss *Physcomitrella patens*, which does not appear to have the reducing end oligosaccharide, was shown to have likely orthologs of IRX8, IRX7, and PARVUS [33]. The same is true for grasses, even though functional equivalence of the putative orthologs has not been demonstrated. Hence, these glycosyltransferases that affect the reducing end structure in Arabidopsis may not be directly involved in its biosynthesis.

The acetyl esters that are frequently found on O-2 and O-3 of backbone xylose residues are ultimately derived from acetyl-CoA. Proteins belonging to the Reduced Wall Acetylation family are involved and may function at an initial biochemical reaction or as transporters, since none of these proteins are specific for xylan [34,35]. Proteins belonging to the DUF231 family likely operate at a later biochemical reaction and have a restricted specificity. Recently, a DUF231 protein with a specific role in xylan acetylation was identified [36•,37•].

Glycosyl hydrolases involved in xylan synthesis

Since grass xylan in general contains fewer branches in older tissue, one might expect that glycosyl hydrolases are involved in modifying xylan substitutions as the plant matures. Extracts from plants exhibit trans-β-xylanase, xylosidase, and arabinosidase activities, indicating that plants modify their xylan after synthesis [38]. Transcriptional studies in maize have identified several putative xylosidases and arabinosidases that are upregulated during expansion indicating that they might play a role in cell wall loosening or remodeling, possibly mirroring xyloglucan remodeling via xyloglucan endotransglycosylase during expansion in dicots [39]. Glycosyl hydrolases are also implicated in dicot xylan formation: xylanase and xylosidases are thought to be involved in xylan remodeling in Arabidopsis [40,41], and a β-xylosidase has been proposed to play a role in stem bending and tension wood formation in poplar by remodeling xylan [42]. However, it is unclear exactly how these enzymes modify xylan and what purpose these modifications serve. This represents an interesting area for future study, particularly in grasses where xylan substitutions appear to be more plastic throughout development.

Regulation of xylan synthesis

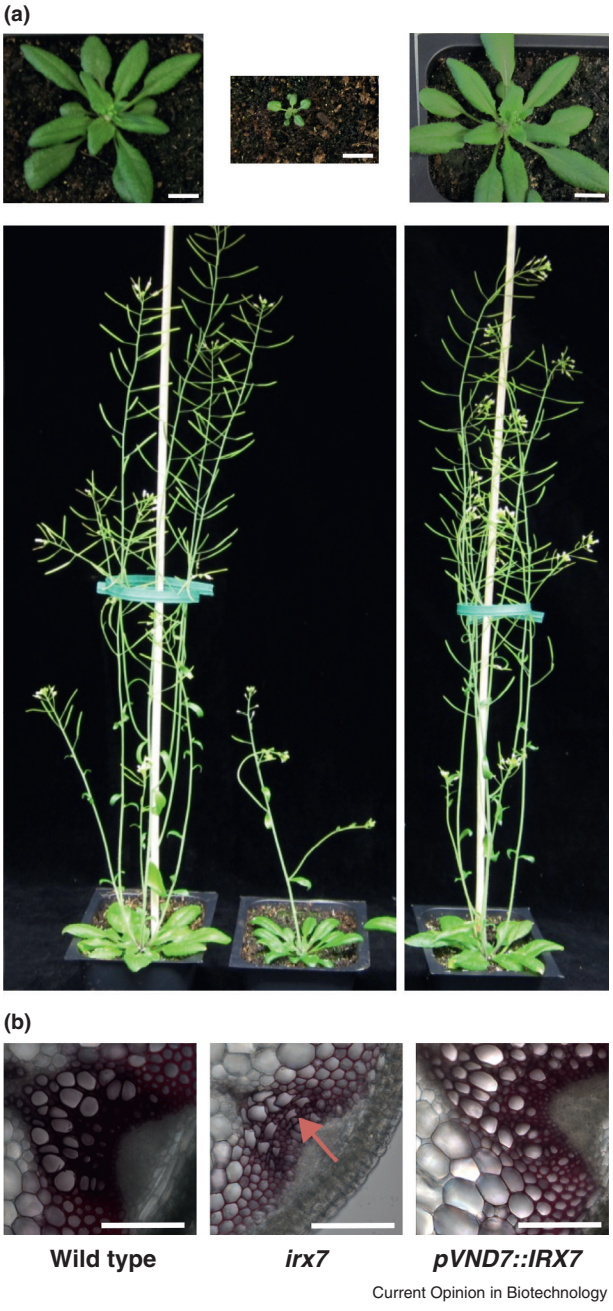
Many transcription factors involved in xylan synthesis in Arabidopsis have been identified, particularly master switches such as Secondary Cell Wall Associated NAC Domain 1 (SND1) and several transcription factors directly downstream, including multiple MYB factors and a KNOTTED1-like homeodomain protein (reviewed in [43–45]). Master regulators are active in separate tissues, such as NAC Secondary Cell Wall Thickening Factor 1 (NST1) which is active in fibers [46] and Vascular-related NAC Domain (VND) 6 and VND7 which are active in vessels [47]. The tissue-specific expression of these genes has made them useful for engineering plant cell walls in cases where it is desirable to target specific cell types [48••,49••]. Identification of downstream transcription factors — including those that directly regulate genes encoding xylan biosynthetic enzymes — may add to the repertoire of promoters available for tailoring expression. Several candidates have been identified by monitoring expression of genes that are induced by known transcription factors [50] and by coexpression analysis [19]. In addition, similar sets of transcription factors appear to be involved in secondary cell wall synthesis in poplar [51], aspen [52], *Brachypodium* [53], and rice [54], indicating that engineering strategies using these genes and their promoters may be broadly applicable for modification of crop plants.

Biotechnology and xylan

Xylan makes up a large fraction of plant biomass, second only to cellulose. However, the pentose sugars derived from xylan are not preferred for fermentation into biofuels and other products, since organisms such as yeast cannot naturally ferment xylose. Therefore, it is desirable to generate plants with a lower content of xylan. Likewise acetate is highly inhibitory for yeast and therefore xylan with a lower acetate content would be advantageous. Ferulic acid esters make xylan more recalcitrant and affect not only xylan hydrolysis but also cellulose hydrolysis. For these reasons there is a significant interest in generating plants with less xylan or altered xylan structure more compatible with downstream processes. In all these cases, simply downregulating the biosynthetic enzymes is not a useful approach, since loss-of-function mutants are severely affected in growth and development (Figure 3).

Ferulic acid content in grasses can be reduced by expressing ferulic acid esterases. This approach has been used by several groups, but at least in wheat the plants were adversely affected [32,55]. An alternative approach has been demonstrated in rice, where overexpression of a putative coumaroyl-transferase leads to increased coumaric acid ester content and decreased ferulic acid ester content, presumably due to substrate competition [30]. Coumaroyl esters do not readily participate in crosslinking and the resulting plants had improved saccharification without

Figure 3



Arabidopsis mutants with less xylan, such as *irx7*, show decreased growth in rosettes and inflorescence stems **(a)** and have collapsed xylem vessels **(arrow, b)**. Xylan biosynthesis can be reintroduced into vessels by complementing *irx7* mutant plants with a functional copy of the *IRX7* gene driven by the vessel-specific promoter pVND7. Xylan biosynthesis in vessels restores normal growth and xylem structure. (a) Bars = 10 mm. (b) Bars = 100 μm. Modified from [48••].

showing apparent adverse effects on growth and development. Like feruloyl esters, acetyl ester groups can be removed by expressing acetyl esterases. Expression of an *Aspergillus* acetylesterase with some specificity for xylan in Arabidopsis and *Brachypodium* led to substantial reduction in cell wall acetylation and improved saccharification [56]. The plants did not display impaired growth, unlike knockout mutants in the xylan acetyltransferase related protein TBL29/ESKIMO1 [36•,37•].

Recently, a novel approach was established where Arabidopsis mutants deficient in xylan biosynthesis were complemented with the functional version of the mutated gene under control of a vessel-specific promoter [48••,49••]. The resulting plants showed growth comparable with the wild type plants and had restored vessel function necessary for efficient water and nutrient transport (Figure 3). However, the plants still had much lower xylan content because of the low content of xylan in the interfascicular fibers. Although the fiber cells had less xylan, some of the plant lines were mechanically as strong as the wild type plants. This approach of using complementation with tissue-specific promoters can likely also be used to improve the performance of mutants in other xylan-related genes.

Conclusions

Recent progress has identified several proteins involved in xylan biosynthesis including biosynthetic enzymes, transporters, and transcription factors. The functions of several individual enzymes have been definitively identified, and groups of proteins such as GT61 and BAHD enzymes are now known to be involved in the introduction of substitutions although the exact functions of different members of these groups still need to be determined. Related proteins may be responsible for different substitution patterns, as is the case in Arabidopsis, and future work may help determine what these patterns are and how they affect xylan structure and interactions with other cell wall polymers. At the same time, several enzymes that have been shown to affect xylan synthesis do not have obvious biosynthetic roles, indicating that there is still much to be learned. Other gaps include our limited knowledge of how linkages between xylan and other polymers are synthesized, the intermediates and transporters that supply substrates for enzymes in the Golgi, and how xylan is processed by glycosyl hydrolases during development. Discoveries in these areas are within reach and may bolster efforts to engineer xylan with improved properties for applications such as biofuel production.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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This paper demonstrates the role of a specific GT61 enzyme in adding xylose residues to arabinofuranose residues on the xylan backbone. The biochemical activity is not shown *in vitro* and the suggested activity is inferred from analysis of the mutant cell walls. Nevertheless, the study points out that GT61 enzymes may be involved in adding both xylose and arabinose to the xylan backbone in grasses. Furthermore, the xax1 mutant is deficient in ferulic acid, and the cell walls more easily saccharified.

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Proteins belonging to the DUF231 family are known to be involved in acetylation of cell wall polysaccharides. In this study the TBL29 (ESKIMO1) protein is shown to be specifically involved in xylan acetylation. The mutant plants are affected in growth and development demonstrating the importance of xylan acetylation. The mechanism of acetylation is not understood and also involves the Reduced Wall Acetylation (RWA) proteins.

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See annotation to [36•].

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Plants with reduced xylan content are normally growth deficient. In this study, plants with reduced xylan and good growth properties were generated. Loss-of-function mutants in xylan biosynthetic genes were complemented with the wild-type version of the affected genes driven by vessel-specific promoters. The results show that xylan is indispensible in the vessels whereas xylan in fiber cells is not essential for growth and development, or even stem mechanical strength.

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This study demonstrates two important approaches for engineering of plant secondary walls. One approach is to complement mutants using vessel-specific promoters and thereby generate plants with reduced lignin while maintaining normal growth and development. The same approach was used in paper [47] for xylan. The other approach is to incorporate an artificial positive feedback loop whereby a specific transcription factor is upregulated specifically in the cell types where it is already expressed. With that approach, plants were engineered to have high cell wall density in fiber cells.

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