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Biochemical control of xylan biosynthesis — which end is up?

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Xylans are major components of land plant secondary cell walls and are required for normal plant growth and development. Secondary walls also account for the bulk of lignocellulosic biomass, a potential feedstock for large-scale production of biofuels. Glucuronoxylan and arabinoxylan affect the conversion of lignocellulosic biomass to fermentable sugar, a crucial and expensive step in biofuel production. Thus, knowledge of xylan biosynthesis may provide tools to modify secondary cell wall structure and thereby improve the bioprocessing characteristics of biomass. Recent studies have shown that glucuronoxylan structure and biosynthesis are far more complex than previously appreciated and the number of glycosyltransferases implicated in this process continues to increase. New hypotheses regarding the mechanisms of glucuronoxylan biosynthesis challenge some widely held views.

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Introduction

Xylans are a family of structurally diverse plant polysaccharides with a backbone composed of 1,4-linked β-D-xylopyranosyl residues (Figure 1). True xylans are rare and in almost all cases the backbone is substituted, to varying degrees, with monosaccharide or disaccharide side chains. Backbones may be substituted with glucuronic acid and 4-*O*-methyl glucuronic acid (glucuronoxylan, GX), arabinose (arabinoxylan), or a combination of acidic and neutral sugars (glucuronoarabinoxylan). Glucuronoxylans are major components of the secondary walls of dicots. Arabinoxylans, and to a lesser extent, glucuronoarabinoxylans are present in the walls of grasses. Glucuronoarabinoxylans are minor components of the secondary walls of soft woods [1•]. Xylans with a high degree of backbone substitution occur as exudate gums in many plant species [1•]. Immunocytochemical studies suggest that arabinoxylans are also present in the walls of hornworts but not other bryophytes [2] and, if confirmed, would add support to molecular data indicating a sister relationship of the hornworts with vascular plants (see Popper, this issue).

Xylan-containing lignocellulosic secondary cell walls are the most abundant repository of biomass on earth. This biomass is a renewable, carbon-neutral energy source that has considerable potential as a feedstock for the large-scale production of liquid fuels [3,4]. The widespread use of biofuels will require dedicated bioenergy crops to be grown on a vast scale [5,6] and a reduction in the cost of converting biomass to biofuels [7•]. One major barrier to cost reduction is the resistance of biomass to conversion to fermentable sugar [7•,8]. Developing sustainable bioenergy crops with walls that have improved and cost-effective bioprocessing characteristics requires understanding xylan biosynthesis at the molecular level and knowledge of the mechanisms that incorporate these polysaccharides into a functional wall.

Recent reviews have described wall biosynthesis in general [9•] and some of the factors controlling wall biosynthesis at the level of gene expression [10•]. Here, we summarize recent studies and hypotheses on the structure and biosynthesis of xylans and briefly describe the progress in understanding some of the biochemical factors that control their formation.

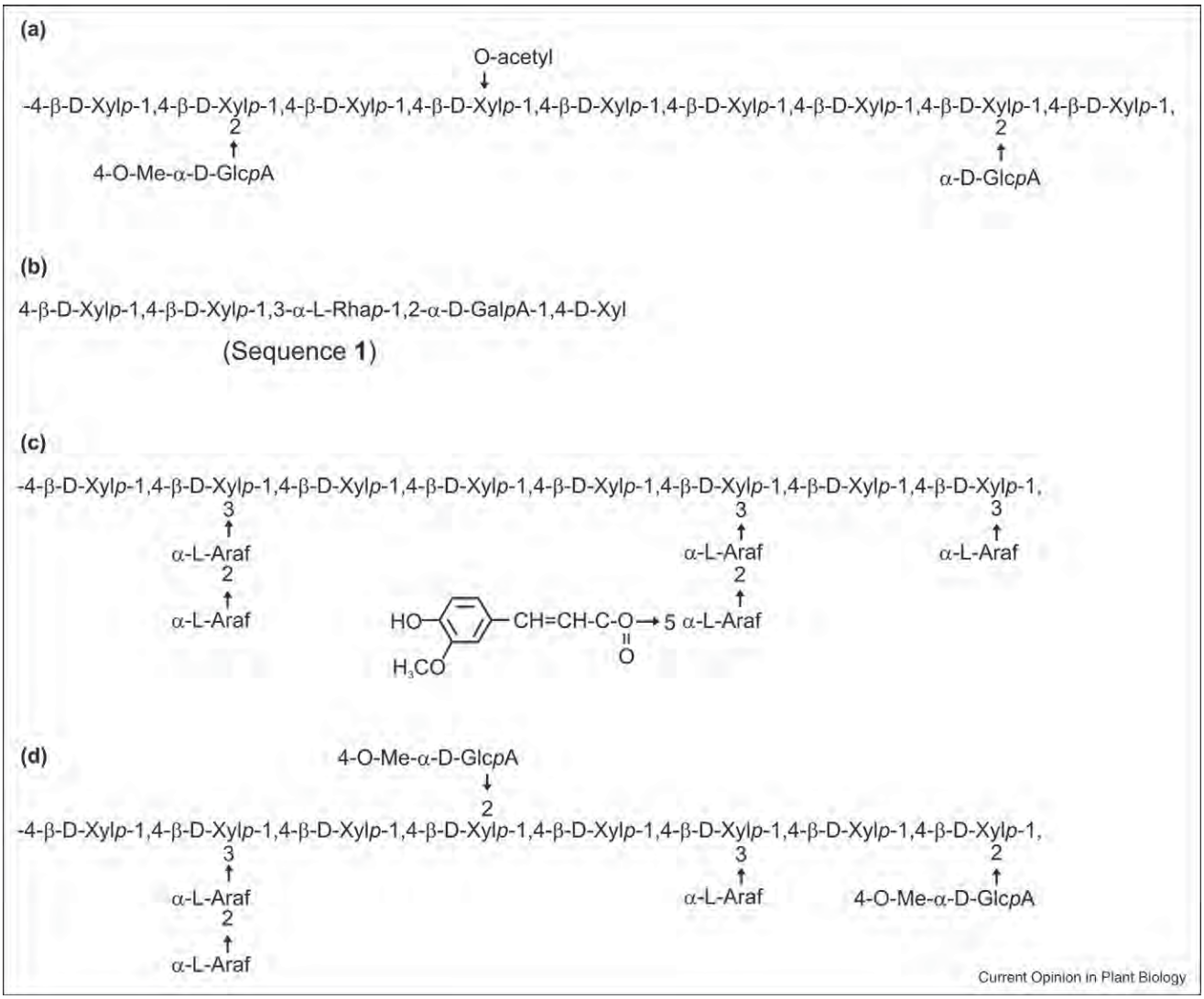
Glucuronoxylans have a unique sequence of glycosyl residues at their reducing ends

Early studies [11–13] established that dicot and gymnosperm GXs have a unique sequence of glycosyl residues at their reducing ends (Figure 1b). This work was largely overlooked until Peña *et al.* [14••] rediscovered this structure and demonstrated that this glycosyl sequence is required for normal xylan synthesis in secondary walls of dicots. It is not known if the arabinoxylans of grasses have unique glycosyl sequences at their reducing ends.

Glucuronoxylan and arabinoxylan biosynthesis

Several papers published between 2004 and 2007 provide evidence that CAZy family GT2 glycansynthases, encoded by members of the cellulose-synthase-like (*CSL*) family of genes, catalyze the biosynthesis of mannan (*CSLA*), galactomannan (*CSLA*), glucomannan (*CSLA*), β-glucan (*CSLF*), and xyloglucan (*CSLC*) back-

Figure 1



Diversity of xylan structures in land plants. **(a)** The glucuronoxylans produced by dicots and gymnosperms. **(b)** Sequence **1** present at the reducing end of dicot and gymnosperm glucuronoxylan. **(c)** Feruloylated arabinoxylans produced by grasses. **(d)** Glucuronoarabinoxylans present in the secondary walls of soft woods.

bones [9•,15–17]. Two non-*CSL* genes (*IRX9* and *IRX14*) have been implicated in xylan backbone biosynthesis [14••,18•,19,20]. Nevertheless, the possibility that *CSL* genes also have a direct role in this process cannot be discounted.

Glucuronoxylan has typically been viewed as a polysaccharide whose synthesis would require a xylan synthase for backbone formation and one or two glycosyltransferases for the addition of GlcA and 4-*O*-Me GlcA side chains. This perspective changed when Peña *et al.* [14••] demonstrated that Arabidopsis GX contains → 4)-β-D-Xyl*p*-(1 → 3)-α-L-Rha*p*-(1 → 2)-α-D-Gal*p*A-(1 → 4)-D-Xyl*p* (sequence **1**, see Figure 1b) at its reducing end and that this sequence is required for normal GX synthesis in secondary walls. At least three glycosyltransferases are required to form this sequence. At least one more glycosyltransferase is required if this sequence links xylan to another polymer [14••].

To date five genes (*FRA8* [At2g28110], *IRX8* [At5g54690], *IRX9* [At2g37090], PARVUS [At1g19300], and IRX14 [At4g36890]) that are likely to have a role in GX synthesis have been identified in Arabidopsis [14••,19, 21–23]. These genes encode putative glycosyltransferases that may have a role in forming reducing end sequence **1** (FRA8, family GT47; IRX8, family GT8; PARVUS, family GT8) and in xylan backbone synthesis (IRX9 and IRX14, family GT43).

Experimental proof is required to validate these hypotheses because none of these genes have been shown to encode functional glycosyltransferases.

The suggestion that IRX9 is involved in xylan backbone elongation [14••] is supported by the demonstration that microsomal fractions from *irx9* mutant plants do not elongate xylo-oligosaccharides as effectively as wild-type microsomes [20]. Similar results have been obtained with microsomal preparations from *irx14* mutant plants [19], suggesting that IRX14 is also involved in elongation of the xylan backbone. It is possible that IRX9 and IRX14 are components of a xylansynthase complex that is functionally impaired if either one of them is absent [19] because *IRX9* and *IRX14* do not complement one another.

Plants carrying mutations in *FRA8* (also known as *IRX7*), *IRX8*, *PARVUS*, *IRX9*, and *IRX14* exhibit reduced growth and have abnormal vascular tissues. Their stem cell walls have reduced amounts of GX that contains more 4-*O*-Me-GlcA and less GlcA than wild-type [14••,19,24]. These mutations also affect the number of GX chains formed, their degree of polymerization (DP), and size heterogeneity [14••]. For example, wild-type GX is homodisperse, with a DP of ~100, while GX produced by *fra8* and *irx8* mutants is heterodisperse [14••]. More than 78% of the GX chains in these mutants lack sequence 1, suggesting that this sequence is involved in controlling chain length. Arabidopsis *qua1* and *atclsd5* mutants have abnormal vascular tissues and reduced xylan and homogalacturonan synthase activities [25,26]. Although *ATCSLD5* and *QUA1* may have a role in xylan synthesis the authors conclude that it is more likely that the reduced GX content of stems results from abnormal cell wall formation at a stage of cellular development before xylem maturation.

No genes encoding GTs that add GlcA or 4-*O*-Me GlcA to the backbone of dicot xylans or genes encoding xylan-specific *O*-acetyl transferases have been identified. Radiolabeled GlcA has been reported to be transferred from UDP-^{14}C-GlcA to xylo-oligosaccharides in the presence of Arabidopsis microsomes [20]. However, the products formed were not structurally characterized and the possibility cannot be discounted that Xyl as well as GlcA was transferred to the exogenous acceptors. Several isoforms of Arabidopsis UDP-GlcA decarboxylase, the enzyme that catalyzes the formation of UDP-Xyl from UDP-GlcA, are believed to be membrane bound [27] and the activity of these UDP-xylose-forming enzymes may be high in tissues producing large amounts of xylan.

Although arabinoxylans are major components of grass cell walls little is known about their biosynthesis. Xylosyltransferase and arabinosyl transferase activities have been detected in microsomal fractions isolated from wheat and barley [28–31]. Several candidate rice genes have been identified using bioinformatics [32] but none have been functionally shown to encode GTs involved in arabinoxylan synthesis.

Mechanisms of xylan biosynthesis

Organisms have developed diverse mechanisms to synthesize complex carbohydrates. Glycans may be assembled by the direct transfer of a glycose from a nucleotide sugar or the glycose itself may first be transferred to a lipid intermediate. Oligosaccharides may be assembled on a lipid intermediate and then transferred to the growing glycan. Glycan chains may be elongated by addition of glycoses to their terminal nonreducing end or to their reducing ends. These two processes have fundamentally different mechanisms. Growth from the nonreducing ends involves the activated sugar as a donor and the growing chain as the acceptor. These roles are reversed when a polysaccharide is extended from its reducing end. There is increasing evidence for biological control of the initiation and termination of glycan synthesis and the final size of a glycan.

Our current rudimentary knowledge of the mechanisms of plant polysaccharide biosynthesis necessarily limits us to informed and modest conjecture. In the following, we discuss several explicit models for xylan biosynthesis that may lead to widely different conclusions about the roles of donor and acceptor molecules in hemicellulose biosynthesis and how biosynthesis may be initiated and terminated.

Several different models for xylan biosynthesis are consistent with the chemotypes of the various xylan-deficient mutants described above. Although few aspects of these models have been experimentally validated, they are useful tools for designing experimental protocols to study this complex process. The different models are not mutually exclusive, as the precise mechanisms may be species-specific, or even tissue-specific and cell-specific. For example, xylan backbone biosynthesis may proceed by completely different mechanisms in grasses, which produce arabinoxylans, than in dicots, which produce GXs. Rather than presenting a single model, we now describe specific aspects of the various models, which may be combined to form many different overall models.

The involvement of protein complexes in xylan biosynthesis

It is likely that protein complexes, rather than autonomously acting enzymes, catalyze the biosynthesis of hemicelluloses. This notion is supported by increasing evidence for hemicellulose synthase activity of *CSL* genes, and the observation that cellulose synthases themselves function as part of large protein complexes [33]. The synthesis of polymeric GX and xyloglucan by microsomes is most efficient when donor substrates for the backbone and side-chain residues are both included in

the reaction, consistent with the existence of protein complexes that utilize both substrates [34,35].

Interaction of enzymes in a polysaccharide synthase complex

Some hemicellulose synthases may consist of protein complexes that include CSL proteins along with other enzymes. For example, the decreased capacity of microsomes from *irx9* and *irx14* plants to elongate exogenous oligoxylosyl substrates *in vitro* [19,20] suggests that IRX9 and IRX14 are required components of a complex xylan synthase and have a direct role in GX chain elongation. As such, they could catalyze the transfer of xylosyl residues directly to the nascent GX. A fully competent xylan synthase complex may contain several different proteins with xylosyl transferase activity. For example, IRX9, IRX14 and one or more CSL proteins may all be xylosyl transferases that combine to form a complex in which each protein catalyzes a different step required for elongation of the xylan backbone.

Glycosyl intermediates and the implications of catalytic mechanism

The anomeric configuration of the product of a GT-catalyzed reaction is determined by its catalytic mechanism (inverting or retaining) and the anomeric configuration of the donor substrate. Typical GT donor substrates include nucleotide diphosphate (NDP) sugars and lipid-linked glycosides. Although no lipid-linked intermediates have been shown to be GT donor substrates for the biosynthesis of plant polysaccharides, it has been suggested that sitosterol β-glycosides are primers for cellulose synthesis [36]. The anomeric configuration of a lipid-linked donor substrate is often the opposite to that of its NDP precursor. For example, dolichol-phosphate-mannosyl transferase (an inverting GT2 enzyme, related to CSA and CSL proteins and to hyaluronan synthase) transfers mannose from GDP-α-D-mannose to form dolichyl-phosphate-β-D-mannose [37], which itself is the donor substrate of dolichyl-phosphate-mannose-glycolipid α-mannosyltransferase (an inverting GT58 enzyme) involved in *N*-glycan biosynthesis [38]. By this mechanism, two inverting enzymes generate an α-linkage, even though the initial donor substrate is an NDP sugar with an α-linkage. Thus, the specific role of a GT (such as an IRX protein) cannot be deduced solely from its catalytic mechanism.

Single or multiple active sites that catalyze elongation of xylan chains

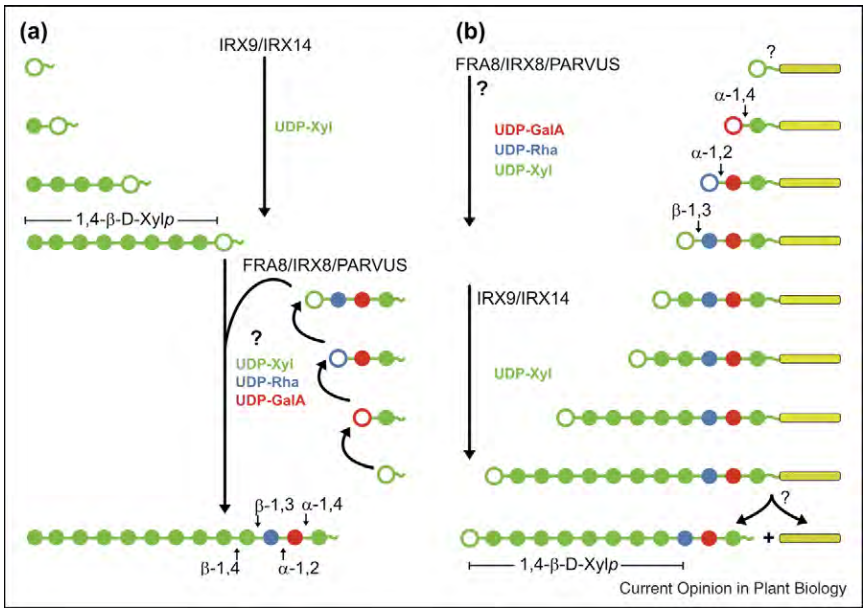
The elongation of each GX chain may be catalyzed by a single active site or by the cooperative action of two active sites, which alternately add glycosyl residues or oligosaccharide blocks to the polymer. Hyaluronan synthases (HASs) are family GT2 glycosyl transferases (related to CSL proteins) that provide a precedent for the cooperative action of two separate active sites. HASs catalyze the formation of two different glycosidic linkages between two different glycoses during the synthesis of hyaluronic acid [39••], which consists of the repeating disaccharide[-4)-β-D-GlcA-(1-3)-β-D-GlcNAc-(1-]. CSL glycan synthases in plants also appear to contravene the one-enzyme, one-linkage paradigm. For example, transgenic Arabidopsis containing a rice *CSLF* gene has been reported to synthesize, albeit in small amounts, a polysaccharide composed of β-1,4 glucosyl and β-1,3 glucosyl residues [40••]. The mixed linkage β-glucan is not synthesized by wild-type Arabidopsis. Nevertheless the possibility that the formation of the 1,4-linked and 1,3-linked glucosyl bonds requires the activity of the rice CSLF protein in combination with an Arabidopsis protein cannot be discounted. The product of a single gene (*CSLA*) is believed to be capable of using GDP-mannose and GDP-glucose to synthesize glucomannan, a polysaccharide composed of β-1,4-linked glucosyl and mannosyl residues [41]. In the case of mixed linkage β-glucan synthesis, it would be interesting to know whether the donor substrate (e.g. UDP-Glc versus GDP-Glc) affects the outcome of the glycosyl transfer (formation of a 1,3-linkage or 1,4-linkage). It is possible that the ability of CSL proteins to generate more than one type of linkage is their interaction as subunits of a multienzyme complex.

Control of chain length — primers or terminators

Regulation of GX chain length is disrupted in *fra8* and *irx8* plants [14••] and in *parvus* plants (Hahn, Peña, O'Neill, unpublished results). These mutations also lead to a decrease in the amount of sequence **1** (Figure 1b) at the reducing end of the GX and an increase in the proportion of GX molecules that lack this sequence [14••], suggesting that sequence **1** has a key role in regulating GX chain length. Hydrolysis of the connection between GX and sequence **1** is unlikely to be the dominant mechanism for release of GX from the synthase complex as virtually all wild-type GX chains have sequence **1** at their reducing end [14••]. However, less than *22%* of GX chains in *fra8* and *irx8* plants have sequence **1** [14••], suggesting that either first, hydrolytic release dominates when sufficient amounts of sequence **1** are not available or second, an alternative release mechanism that does not involve sequence **1** dominates in *fra8* and *irx8* plants.

A model with sequence **1** acting as a chain terminator (Figure 2a) can account for the observed effects of *fra8* and *irx8* on GX chain length. GX biosynthesis may occur by a mechanism in which the backbone is elongated by addition of xylosyl residues to the reducing end, and the nascent GX is then displaced from the xylan synthase by transfer to sequence **1** (Figure 2a). This model correctly predicts the presence of sequence **1** at the reducing end of nearly all GX chains produced by wild-type plants and the accumulation of heterodisperse GX chains in mutant

Figure 2



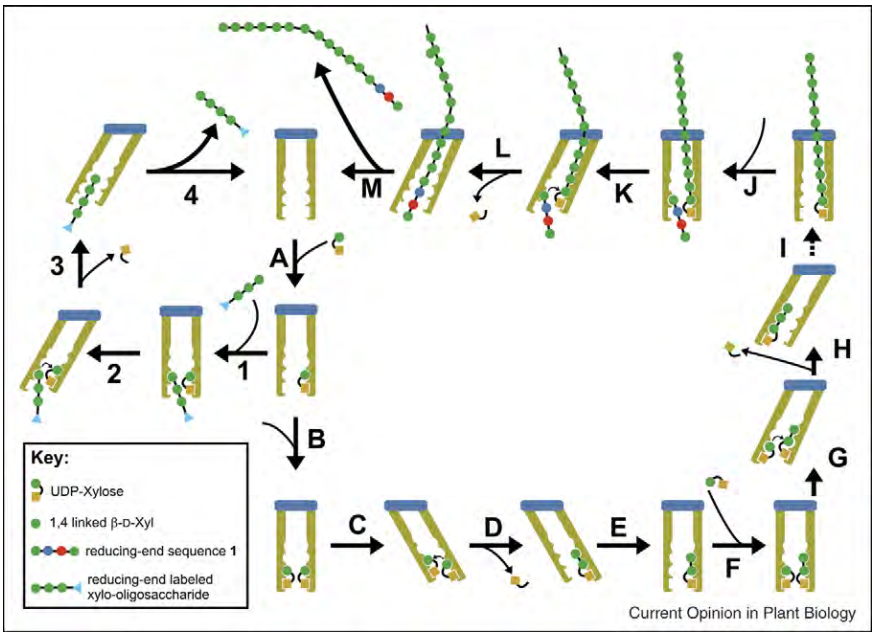
Two general models for GX biosynthesis. Gene products that may catalyze the various steps are indicated, but the specific reaction catalyzed by each of these gene products has not been experimentally established. The most recently added glycosyl residues are represented by open circles. In model **(a)**, GX is synthesized by transfer of xylosyl residues to the reducing end of the chain. The elongation process is terminated by transfer of the nascent chain to sequence **1** (see Figures 1b and 3). In model **(b)**, sequence **1** (see Figure 1b) acts as a primer, and xylosyl residues are sequentially added to the nonreducing end. The elongated yellow box represents a putative moiety (protein, lipid, or other polysaccharide) to which sequence **1** may be covalently linked at the time of its biosynthesis. Many animal proteoglycans are composed of a polysaccharide covalently linked to protein by a linker oligosaccharide [46]. Other have suggested that glucuronxylan is linked to protein [47] or pectin [23]. Additional studies are required to substantiate this claim and to determine whether sequence **1** is a linker or a primer.

plants, which have lower levels of the chain-terminating sequence **1**. Transfer of monosaccharides or oligosaccharides to the reducing end is a common mechanism for lipopolysaccharide *O*-antigen and Group I capsular polysaccharide (CPS) synthesis in bacteria [42]. Termination of LPS *O*-antigen chain elongation likely occurs when the *O*-antigen is transferred to the lipid A core. Termination of CPS polymerization has been reported to involve tyrosine autokinases (Wzc), though the mechanism is not well understood [42].

Various authors [19,24] have referred to sequence **1** as a GX 'primer', assuming that GX chain elongation occurs by sequential addition of xylosyl residues to this sequence (Figure 2b). However, it is difficult to reconcile such a mechanism with the apparent role of sequence **1** in controlling GX chain length. It is conceivable that GX chain length is controlled by a 'molecular timer' (see, e.g. Lu *et al.* [43]) that determines the half-life for the association of the nascent GX chain with the xylan synthase. In this scenario, reducing the concentration of the primer sequence **1** would decrease the number of protein complexes that are actively synthesizing xylan and reduce the competition for donor substrates. As a result, the elongation rate for individual GX chains could increase, allowing them to grow abnormally long before the timed chain termination event occurs. Alternatively, UDP-Xyl, UDP-GlcA, UDP, and other metabolites could act as effectors that promote or inhibit chain termination. Reducing the number of catalytically active xylan synthase complexes could affect the concentrations of these metabolites, thereby altering GX chain length. Clearly, altering metabolite levels could have multiple effects and lead to the formation of heterodisperse GX.

The ability of microsomal preparations to elongate fluorescently labeled 1,4-linked β-D-xylo-oligosaccharides [20] has been taken as an evidence that sequence **1** acts as a GX primer rather than as a terminator [24]. In this system, addition of xylosyl residues can only occur at the nonreducing end, as the reducing end is blocked by the fluorescent label. However, it has been suggested [44•] that addition of residues to the nonreducing end of artificial acceptor substrates (such as xylo-oligosaccharides) does not necessarily reflect the mechanism that occurs *in vivo*. As illustrated in Figure 3 (steps A–M), enzyme-bound NDP glycosides may act as acceptor substrates when elongation occurs at the reducing end of the chain [45••]. However, these same NDP-glycosides could act as glycosyl donors in the presence of high concentrations of the artificial acceptor (Figure 3 steps 1–4). Under these conditions, glycosyl residues would be

Figure 3



Mechanistic model for GX elongation by transfer of xylosyl residues to the reducing end of the chain. This testable model is based on P.H. Weigel's pendulum hypothesis for hyaluronan synthesis (Glycoforum: Hyaluronan Today, URL: http://www.glycoforum.gr.jp/science/hyaluronan/HA06a/HA06aE.htm) [45••]. Each step in the normal catalytic cycle is labeled with an uppercase letter (A–M). Xylan chains are initiated by the binding of UDP-Xyl molecules to two active sites of the xylan synthase (A and B). Movement of the catalytic sites (C) positions O4 of one of the UDP-Xyl molecules for nucleophilic attack at C1 of the other UDP-Xyl (D), leading to transfer of the Xyl residue and release of UDP. Reorientation of the catalytic sites (E) leads to binding of another UDP-Xyl (F), and the cycle is repeated (G–I) until polymeric xylan is generated. This corresponds to processive addition of xylose to the reducing end of the growing chain. Sequence **1** then binds to the complex (J). Reorientation of the active sites (K) positions O4 of the β-Xyl residue at the non-reducing terminus of sequence **1** for nucleophilic attack at C1 of the Xyl residue linked to UDP at the reducing end of the polymer (L). The polymeric product (bearing sequence **1** at its reducing end) is released from the xylan synthase complex (M), which is now available for another round of xylan synthesis. In the presence of high concentrations of artificial acceptor substrates (e.g. β-1,4-linked xylo-oligosaccharides), an alternate mechanism may dominate, resulting in the addition of xylosyl residues to the non-reducing termini of the artificial substrates (1–4). One of these acceptor substrates binds to the singly charged complex (1) in a manner similar to the binding of sequence **1** (J). Reorientation of the active sites (2) positions O4 at the nonreducing end of the xylo-oligosaccharide for nucleophilic attack at C1 of the UDP-Xyl (3), transferring a single Xyl to the nonreducing end of the artificial substrate. UDP and the extended substrate are released (4) and the xylan synthase complex is ready to bind another UDP-Xyl molecule (A). Consistent with the observed results, this mechanism is not processive. Note that the xylo-oligosaccharide could also bind to a xylan synthase complex bearing a polymeric or oligomeric xylan chain, mimicking step (J) and terminating chain elongation even in the absence of sequence **1**.

added to the nonreducing end of the artificial substrate, interfering with the normal transfer of the reducing end of the nascent polymer from one active site to another in the enzyme complex.

Care must be taken when interpreting the results of experiments that use artificial acceptor substrates at high concentration to determine the mechanism of chain elongation. Pulse-chase experiments with ^{3}H-labeled, ^{14}C-labeled, and ^{31}P-labeled donor substrates performed in the absence of exogenously added acceptor substrates have been key to understanding the catalytic mechanisms of HAS [45••]. Partial hydrolysis of the resulting polymeric products using exo-glycanses that release monosaccharides from the nonreducing end allows the location of the most recently added monosaccharide residues to be inferred. For example, streptococcal HAS-catalyzed reactions were pulsed with UDP-[^{14}C]GlcA and chased with unlabeled UDP-GlcA. The initial rate of release of radiolabeled monosaccharides by β-glucuronidase was higher than in experiments where the reaction was pulsed with unlabeled UDP-GlcA and chased with UDP-[^{14}C]GlcA [45••]. That is, the most recently added GlcA residues were the last to be released by β-glucuronidase, which acts at the nonreducing end, indicating that elongation occurred at the reducing end of the chain. Similar experiments might be used to establish the molecular mechanisms of xylan biosynthesis *in vivo*.

Conclusion

It is likely that the development of new, more efficient bioenergy crops will benefit from better knowledge of the mechanisms by which lignocellulosic biomass is synthesized in the plant, as major components of lignocellu-

losic biomass, hemicelluloses, especially xylans, have a considerable effect on the two main steps in bioconversion of biomass to biofuels — the recalcitrance of biomass to saccharification and the bioconversion of the released monosaccharides to liquid fuels. Although recent experimental results indicate that xylan biosynthesis is more complex previously appreciated, they have also inspired new ways of thinking about this process. New models for xylan biosynthesis remain speculative, but provide a theoretical framework within which specific experiments can be designed to investigate the mechanistic details of xylan biogenesis. Understanding these mechanistic details may lead to technologies that allow us not only to control the amount of xylan that is produced during biomass formation, but also to modify the structural features of the xylan, including its chain length and side-chain substitution pattern. Such technologies are likely to be crucial for the development of new bioenergy crops that retain important horticultural traits, including drought and pest resistance, while producing biomass that is efficiently converted into biofuels.

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