Update on Xylan Biosynthesis in Grasses

Xylan Biosynthesis: News from the Grass¹

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WHY XYLANS? WHY NOW?

Plant cell wall polysaccharides are critical components of numerous products in our everyday life. Because of this, modification of their constituent components offers unique opportunities for product improvement and economic advancement. Xylans are one of these polysaccharides that have a variety of applications that affect our well-being. For example, xylans are important functional ingredients in baked products. They affect the quality of cereal flours for bread making and the mechanical properties of dough. They also impact brewing properties of grains (Vinkx and Delcour, 1996). Xyl, the main constituent of xylans, can be converted into important value-added products such as xylitol, used as a natural food sweetener, a dental cavity reducer, and a sugar substitute for diabetics; in 1997, its market was estimated to approximately $125 million (Saha and Bothast, 1997). Xylans are also important for the livestock industry, as they are critical factors for silage digestibility (Vinkx and Delcour, 1996). Xylans are major constituents in the nonnutritional constituent of feed (mostly cereal grain by-products) in monogastric animals (poultry; Choc and Annison, 1990). Thus, any small change in the xylan content of grains that go into poultry and swine feed could reflect billions of dollars of savings through an improvement of the food conversion ratio (Donohue and Cunningham, 2009). Given the functional and economical importance of these polymers, their biosynthesis has attracted and puzzled plant cell wall biologists for decades. Only in the last 5 years has significant progress been made in identifying candidates for the glycosyltransferase (GT) genes involved in the biosynthetic process in dicots and monocots. The focus of this Update, which should complement an extensive review on xylan biosynthesis in dicots by York and O’Neill (2008), is the recent progress made on the biosynthesis of xylans in grasses.

Xylans are one of the major hemicelluloses in secondary cell walls of dicots and all walls of grasses. Grasses have typical type II walls that are rich in glucurono(arabino)xylans (GAXs) and β-(1,3/4)glucan (also called mixed-linkage glucan [MLG]; Bacic et al., 1988; Ebringerova et al., 2005). Therefore, grasses have been increasingly used as models to investigate xylans and MLG biosynthesis. Progress has been made in understanding the biosynthesis of many hemicelluloses (including MLG); however, investigating the biosynthetic mechanism of xylans at the biochemical and molecular levels has proven to be more challenging. Now, with the genome sequences of rice (Oryza sativa) and Brachypodium distachyon available, it is an exciting time to work on xylan biosynthesis. Only recently, through an extensive bioinformatics approach (Mitchell et al., 2007), the identity of some GTs has been postulated as candidates for xylan biosynthetic enzymes in grasses. Despite this major advance, the biosynthetic mechanism has yet to be fully characterized. Currently, we cannot delve deeper into the bioinformatics classification and predict the exact biochemical function of putative GTs without isolation of the proteins involved and functional enzymatic assays.

XYLANS FROM GRASSES: REGULAR/REPETITIVE STRUCTURE OR NOT?

Xylans from grasses have the general structure of all xylans (i.e. substitution with α-Araf, GlcA, or MeGlcA residues) but also some unique features (for review, see Ebringerova et al., 2005). The presence of Xyl-arabinofuranosyl side chains (Fig. 1) is considered one of their unique features (Wende and Fry, 1997; Höije et al., 2006; Pastell et al., 2009). Another unique structural feature of grass xylans is the presence of feruloyl groups on the C-5 position of Araf residues (Fig. 1; Bacic et al., 1988; Wende and Fry, 1997). Although the number of substituents and side chains may vary among xylans, two main groups of xylans in grasses can be distinguished: GAX, which makes up 35% of cell walls of vegetative tissues and usually contains both Araf and GlcA/MegkA residues; and AX, which lacks GlcA residues and is found mainly in the starchy endosperm cell walls in cereals (up to 70%; Ebringerova et al., 2005).

The substitution pattern of the xylan backbone is not well known; one outstanding question is whether GAXs from grasses have a regular/repetitive structure. Extensive postsynthesis modifications of these polymers (loss of uronic acid and arabinosyl residues) make a regular structure, originally present in the newly synthesized GAX, difficult to detect. The first suggestion of a repeating structure for GAX came from

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early work carried out by Carpita and Whittern (1986). Using Smith degradation and methylation analysis, they identified a structure for GAX from developing maize (\textit{Zea mays}) coleoptiles and found that the average length of the released oligosaccharides was approximately six residues, but the exact distribution of GlcA and Ara residues was not determined (Carpita and Whittern, 1986). Other structural studies of AXs from barley (\textit{Hordeum vulgare}) and malt showed that substituted Xyl residues were not randomly distributed in the polysaccharide but have a pattern in which up to four isolated unsubstituted residues are separated by one or two substituted residues (Hoffmann et al., 1992; Vietor et al., 1994; Trogh et al., 2004). More recently, Zeng et al. (2008) used a purified endoxylanase from the GH11 family (endoxylanase III) and high-pH anion-exchange chromatography (Dionex) methods to elucidate the structure of the newly synthesized GAX polymer produced by microsomal membranes of wheat (\textit{Triticum aestivum}). Because the endoxylanase III treatment released only three oligosaccharides, the authors concluded that the GAX polymer had a regular structure. Two of the three oligosaccharides released coeluted with authentic AX oligosaccharides having six Xyl and two Ara or five Xyl and two Ara (Zeng et al., 2008). This regular structure was recently confirmed using an affinity-purified GAX synthase activity (A. Faik, unpublished data). Figure 2 illustrates the regular/repetitive structure of the nascent GAX polymer and the possible degradation pattern to explain the formation of the GAX fragments, which are expected to have two unbranched Xyl residues at the reducing end and one or two unbranched Xyl residues at the nonreducing end (due to the mechanism of degradation by endoxylanases from the GH11 family; Gruppen et al.,

![Figure 1. Structural features of the main side chains found in xylans from grasses. The asterisks indicate the position of the attachment of feruloyl groups on the \(\alpha\)-(1,3)-linked Ara residues. Ac, O-acetyl groups; Me, O-methyl groups.](image)

![Figure 2. Proposed structures of the repeating unit in the newly synthesized GAX polymer and the oligosaccharides generated by digestion with a purified endoxylanase III from \textit{Aspergillus niger} (GH11 family). These structures do not take into account the GlcUA residues, as their positions on the xylan backbone are not known. To cleave the xylan backbone, the endoxylanase III requires at least three adjacent and unsubstituted Xyl residues between substituted ones (Davies et al., 1997). The sequence of degradation reactions steps by the endoxylanase is proposed to explain the formation of the final oligosaccharides and monosaccharides detected by high-pH anion-exchange chromatography separation (Zeng et al., 2008). Araf, Arabino-\(\beta\)-furanose residues; Xylp, xylopyranose residues.](image)
This type of regular structure is usually the result of a cooperative mechanism between enzymes, which lends further support to the coordinated action between xylan synthase (XylT), arabinosyltransferase (AraT), and glucuronosyltransferase (GlcAT) activities during GAX biosynthesis, as proposed by Zeng et al. (2008).

### COOPERATIVE ACTION: A COMMON MECHANISM IN XYLAN BIOSYNTHESIS?

Progress in the polysaccharide biosynthesis field has been limited by the lack of enzymological studies, especially considering that most polysaccharides are likely to be the result of well-organized, multienzyme complexes. Xylan biosynthesis is not an exception and appears to be a surprisingly difficult process to study. Although several biochemical studies have investigated xylan biosynthesis in vitro using microsomal membranes from many grass species (Porchia and Scheller, 2000; Kuroyama and Tsumuraya, 2001; Porchia et al., 2002; Urahara et al., 2004), these efforts were not conclusive regarding the biosynthetic mechanism. More recently, Zeng et al. (2008) reported that wheat Golgi-enriched microsomes contained a GAX-GlcAT that was stimulated by the presence of UDP-Xyl in the reaction mixture. There is a striking similarity between this GAX-GlcAT activity in etiolated wheat hypocotyls and the glucuronoxylan (GX)-GlcAT activity in pea (Pisum sativum) epicotyls (Waldron and Brett, 1983; Baydoun et al., 1989). As in wheat, the presence of UDP-Xyl enhanced the capacity of microsomes from pea epicotyls to transfer GlcA from UDP-GlcA into the ethanol-insoluble material. This similarity between wheat and pea GlcAT activities may be the first hint of a similar mechanism for xylan biosynthesis in primary cell walls of both dicots and monocots. Lee et al. (2007a) demonstrated that crude microsomes isolated from Arabidopsis (Arabidopsis thaliana) stems contained GlcAT activity that appeared to slightly stimulate the XylT activity. Furthermore, Zeng et al. (2008) showed that a coordinated action existed between the wheat XylT and AraT; as a result of this cooperative mechanism, the newly synthesized GAX polymer had a regular structure, as represented in Figure 2. The cooperative biosynthetic mechanism and regular structure have also been associated with xyloglucans (XyGs; Ray, 1980; Gordon and Maclachlan, 1989; White et al., 1993) and galactomannans in dicots (Edwards et al., 1999). Thus, plants may have evolved this biosynthetic mechanism to build many of their hemicellulosic polymers, including xylans.

### GLUCURONOARABINOXYLAN BIOSYNTHETIC ENZYMES: DO GRASSES AND DICOTS USE MEMBERS OF THE SAME GT FAMILIES?

According to the structure of xylans from grasses (Fig. 1), at least five GT activities may be involved in the biosynthetic process in addition to ester-forming transferases that add hydroxycinnamonic acids (i.e. ferulic and p-coumaric acids). These GT activities include four inverting enzyme activities, α-(1,2)arabinofuranosyltransferase [α-(1,2)AraT], α-(1,3)arabinofuranosyltransferase [α-(1,3)AraT], β-(1,2)xylosyltransferase [β-(1,2)XylT], and β-(1,4)xylosyl synthase (XylT), and one retaining GT activity, α-(1,2)glucuronolyltransferase (GlcAT). In addition, a mutase activity is required for the conversion of UDP-",arabinopyranose (UDP-"Arap) to UDP-"arabinofuranose (UDP-"Araf). Of particular interest is whether grasses use the same mechanism and members of the same GT families to synthesis AX and GAX as dicot plants (i.e Arabidopsis) use to synthsize GX polymers. To identify candidate GT genes, Mitchell et al. (2007) did an extensive bioinformatics analysis that was based on differential expression of cereal orthologs of the dicot GT genes that might be involved in AX and GAX. Their hypothesis was that the expression of the GT genes involved in these polymers (the most abundant polymers in grass walls) would be higher in cereals as compared with dicots. This analysis revealed that members from the GT43, GT47, and GT61 families (http://afmb.cnrs-mrs.fr/CAZY) were potential candidates for xylan biosynthesis in cereals. The rice genes identified by Mitchell et al. (2007) clustered with IRREGULAR XYLEM14 (IRX14; for the GT43 family) and with IRX10 and IRX10-L (for the GT47 family; Fig. 3); therefore, it is reasonable to assume that the rice genes have functions similar to their Arabidopsis homologs. Thus, bioinformatics analysis in conjunction with the genetic studies carried out in Arabidopsis indicate that members of the GT43 and GT47 families are shared in the biosynthetic process of xylans in both dicots and monocots. The question, then, is what these functions would be.

Compelling data indicate that Arabidopsis members of the GT43 family are XylTs responsible for the synthesis of the xylan backbone: (1) the Arabidopsis mutants irx9 and irx14 showed a drastic reduction in xylan chain length in GX (Brown et al., 2007; Lee et al., 2007a; Pena et al., 2007); and (2) microsomes from these two mutant plants have reduced capacity to transfer Xyl from UDP-Xyl onto xylooligosaccharide acceptors (Brown et al., 2007; Lee et al., 2007a). Of course, the inverting mechanism of the GT43 family is also predicted as a requirement for XylT. The fact that irx9 and irx14 mutants show a similar phenotype may suggest that they perform similar functions and/or complement each other in the same machinery (complex). Actually, a complex of two XylTs (IRX9/IRX14) would work better, because it would eliminate the need for a 180° rotation during Xyl transfer. Confirmation of the XylT activity is still awaiting experimental evidence using isolated proteins. If this function is confirmed, it would completely eliminate the need for any other proteins, such as members of the cellulose synthase-like family, which includes several β-glycan synthases (Dhugga et al., 2004; Liepman et al., 2005; Burton et al., 2006; Cocuron et al., 2007; Doblin et al., 2009).
Prediction of the biochemical function for GT47 members (IRX7/IRX10/IRX10-L) is even more complicated. Possible functions include GlcAT and AraT. Since xylans from both dicots and monocots have GlcA substituents in common and differ largely in the amount of Ara residues, it would be tempting to attribute the GlcAT function to GT47 members. Because the mechanism of this GT47 family is not consistent with the retaining mechanism of GlcAT activity, an intermediate donor substrate containing β-linked GlcA was proposed by Zhong et al. (2005). However, if we assume that the inverting mechanism is valid and no intermediate donor is involved, then some GT47 members could possibly be AraTs. This function could connect the high content in Ara in GAX (compared with GX in dicots) and the high representation of IRX10 and IRX10-L homologs in grasses. Mitchell et al. (2007) reached the same conclusion in their bioinformatics work. The question now is how to explain the involvement of some GT47 members (such as AraTs) in the biosynthesis of a xylan polymer (i.e. GX from Arabidopsis) that presumably does not contain any Ara residues. One possible explanation would be that the nascent GX polymers in dicots contain certain levels of Ara residues, it were removed during the secretion/incorporation of GX into cell walls. In other words, Arabidopsis GX is synthesized as GAX during secondary cell wall deposition and then converted into GX by the action of an α-arabinosidase. This biosynthetic pathway is supported by the observation that the expression of an Arabidopsis α-arabinosidase ARAF1 gene (At3g10740) is well correlated with expression of the FRA8 gene (Chavez Montes et al., 2008; see the ExpressionProfiling tool at http://genecat.mpig.de/cgi-bin/AnInitiator.py) and that GXS from some woody plants have small amounts of Ara residues (Kormelink and Voragen, 1993). The absence of Ara in GXs from the Arabidopsis stem cell wall may explain why Brown et al. (2005) could not detect a decrease in Ara content in stem cell walls from the Arabidopsis irx10 mutant. The removal of Ara residues in GAX during coleoptile growth was also reported in barley (Gibeaut et al., 2005) and maize (Carpita, 1984), presumably due to an α-arabinofuranosidase activity.

The feruloylation of the α(1,3)Ara side chains of AX/GAX polymers (Fig. 1) is unique to grasses and plays an important role in the integrity of type II cell walls (de O Buanafina, 2009). Members of the Pfam family PF02458 (12 members) were suggested as putative AX feruloyltransferases by EST-based differential expression analysis (Mitchell et al., 2007). This conclusion was experimentally supported by recent work by Piston et al. (2010), who showed that down-regulation of four rice genes (Os05g08640, Os06g39470, Os01g09010, and Os06g39390) from this Pfam family resulted in reduced ferulate content in the cell walls of transgenic rice plants. Despite this progress in xylan biosynthesis in grasses, several questions remain unanswered and would require proof of function for certain genes. For example, if some members of the GT47 family are AraTs, what would be the identity of the GlcATs? The GT8 family members could be candidates for GlcATs, as this GT family has a retaining mechanism that fits the GlcAT requirement. But this hypothesis has not been supported by genetic studies, as both irx8 and parvus mutations did not decrease GlcAT activity in their microsomal fractions (Lee et al., 2007a, 2007b). In addition, if members of the GT8 family are GlcAT, why they are not highly expressed in grasses is a puzzle. York and O’Neill (2008) proposed that members of this GT family (IRX8, PARVUS) have a role in generating a unique sequence (called sequence 1) identified at the
reducing ends of GXs from dicots and gymnosperms. This sequence 1 seems to be absent in grass xylans (Fincher, 2009).

Another question is what function the GT61 members would have. Some members of this GT family are highly represented in the cereal EST databases (Mitchell et al., 2007) and were proposed to catalyze the transfer of a β-(1,2)XylT onto the α-(1,3)Araf substituents (Fig. 1). This enzyme activity is still in need of experimental evidence from isolated proteins and the use of well-characterized acceptors. Since the disaccharide Xyl-arabinofuranosyl side chain was found in large amounts in AXs from corn cobs and barley husks (Pastell et al., 2009), it would be possible to develop an enzymatic assay for β-(1,2)XylT activity using microsomes from these plants and to use this assay to screen candidates from the GT61 family.

Despite the fact that Konishi et al. (2007, 2010) provided strong biochemical evidence that members of the GT75 family (also called reversibly glycosylated polypeptides [RGP]) have UDP-AraT mutase activity (presumably necessary for AraT), bioinformatics analysis (Mitchell et al., 2007) did not reveal members of this family as candidates for AX and GAX biosynthesis. Therefore, direct biochemical evidence that links these mutases to AX and GAX biosynthesis is needed. However, in their study of AX biosynthesis in wheat, Porchia et al. (2002) have reported reversible arabinosylation of a 41-kD protein (possibly an RGP).

**CONTROL OF THE XYLAN LENGTH IN GRASSES**

York and O’Neill (2008) proposed a mechanistic model for GX elongation in Arabidopsis in which the xylan backbone could be elongated from its reducing end, but then a mechanism to terminate the xylan backbone is needed (Bodevin-Authelet et al., 2005; Mukerjea and Robyt, 2005; Tlapak-Simmons et al., 2005). The identification of sequence 1 at the reducing ends of GXs from dicots and gymnosperms was proposed to act as a signal to terminate the xylan backbone (Pena et al., 2007; York and O’Neill, 2008) and may suggest an elongation mechanism from the reducing end. The question of interest is whether grasses use a similar mechanism to control the length of GAX polymers during biosynthesis. Zeng et al. (2008) reported that in vitro, wheat microsomes generated only short GAX polymers that eluted on CL-6B columns as a sharp peak around 10 to 15 kD, corresponding to a degree of polymerization (DP) of 50 to 80. Longer reaction incubations and changes in reaction conditions did not produce longer GAX polymers (Zeng et al., 2008). A DP of approximately 50 to 80 is slightly shorter as compared with the size (DP of approximately 80–90) of GX from Arabidopsis (Pena et al., 2007). Thus, it is conceivable that both dicots and grasses evolved mechanisms that terminate xylan backbone elongation after a number of Xyl residues. However, experimental evidence is required to verify this hypothesis using a purified GAX synthase activity and the identification of the sequence terminator. The fact that wheat microsomes could also produce xylan polymers with an apparent molecular mass larger than 500 kD (Porchia and Scheller, 2000) may suggest the existence of several xylan synthase activities in wheat.

One of the most challenging questions concerns the initiation of xylan chain elongation in grasses. For example, several works showed that microsomal membranes or partially purified enzymes make in vitro xylan polymers without the addition of exogenous primers, but they did not eliminate the possibility of the presence of endogenous primers in their enzyme preparations (Baydoun et al., 1989; Porchia and Scheller, 2000; Porchia et al., 2002; Zeng et al., 2008). On the other hand, other research groups demonstrated that xylooligosaccharides added exogenously to permeabilized Golgi membranes act as acceptors (possibly primers), but only a few Xyl residues were added to these oligosaccharides (Kuroyama and Tsumuraya, 2001; Urahara et al., 2004; Brown et al., 2007; Lee et al., 2007a). To accommodate these findings, York and O’Neill (2008) proposed that xylan backbone elongation occurred from its reducing end. In this biosynthetic mechanism, the elongating xylan chain would form a covalent intermediate with XylT or with other proteins associated with XylT. Whether some RGPs could fulfill this function is another challenge for cell wall biology.

**CONCLUSION**

The recent progress with respect to the biochemistry of GAX biosynthesis in wheat has enhanced our understanding of the biosynthetic process of xylans. The picture that emerges here is that plant cells appear to use a cooperative mechanism (through multienzyme complexes) to synthesize many hemicellulosic polymers found in primary cell walls, namely GAXs, GXs, and XyGs. Thus, the “arabinosyl-Xyl” backbone of GAX is synthesized in a manner similar to the “xylosyl-Glc” backbone of XyGs in dicots. Furthermore, as a result of this cooperative mechanism, the newly synthesized polymers have regular structures. However, many challenges are still before us. For example, direct proof of the biochemical functions of the GT43, GT47, and GT61 members is still lacking. Whether or not primary and secondary cell walls require xylan synthases with different protein compositions, as was demonstrated for cellulose synthesis (for review, see Somerville, 2006), is not known. Clearly, progress in the xylan biosynthesis field is limited by the lack of enzymological studies.

A thorough understanding of any regulating mechanism, including the identification of transcription factors (Zhong and Ye, 2007; Aohara et al., 2009), would also certainly increase our understanding of xylan biosynthesis in the context of primary and secondary cell wall elaboration and would offer o-
portunities to genetically manipulate cell wall compo-
sition and structure to better fit human needs.

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LITERATURE CITED


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